levels of O_2 and CO_2 dissolved in water, some organisms would not be able to carry out net carbon assimilation if the glycolate formed through the oxygenase function of Rubisco were lost from the cells. However, there exist a number of mechanisms that allow algal cells to recoup at least part of the carbon in phosphoglycolate.

The most common of these mechanisms is shown in Fig. 1 and is based on the oxidation of glycolate via glycolate dehydrogenase and the subsequent recovery of some of the carbon into triose phosphate. This pathway by which the product of glycolate oxidation, glyoxylate, is metabolized, is known as the photorespiratory carbon oxidation cycle (PCOC).

Glycolate dehydrogenase occurs in many members of the Chlorophyta and in the class Bacillariophyceae of the Heterokontophyta, as well as in the Cryptophyta, Dinophyta and Euglenophyta and in the Cyanobacteria (Table 2). Not all of these organisms, however, possess a complete PCOC.

Glycolate dehydrogenase is found in mitochondria but does not lead to direct oxygen reduction. However, the enzyme is membrane bound and is associated with the thylakoid membranes in Cyanobacteria (Codd and Sellal, 1978) and with the inner mitochondrial membrane in eukaryotes (Paul et al., 1975; Paul and Volcani, 1976). As a result, oxidation of glycolate via glycolate dehydrogenase can be coupled to the respiratory redox chain and ATP generation (Raven and Beardall, 1981; Beardall and Raven,1990). Thus, oxygen uptake in photorespiration is a result of both Rubisco oxygenase activity and the coupling of glycolate dehydrogenase to oxidative phosphorylation. CO_2 release is associated with the subsequent metabolism of glyoxylate.

Glyoxylate is metabolized within the mitochondria as shown in Fig. 1. The net outcome of this process is the release of one CO_2 and the synthesis of one molecule of the three carbon compound glycerate for every two glycolate molecules oxidized. The glycerate is transported to the chloroplasts where it can be used in the synthesis of RuBP to allow the continuing operation of the Calvin cycle (Fig. 1). Ammonia released during the glycine to serine conversion is rapidly re-assimilated by the GS-GOGAT system in chloroplasts (Buchanan et al., 2000).

With the PCOC operating, as represented in Fig. 1, the mechanism for recovery of glycolate involves the release of 0.5 CO_2 for each phosphoglycolate converted to 0.5 triose phosphate (see Appendix 1 of Raven et al., 2000). Net carbon assimilation, relative to that in the absence of oxygenase activity, is then given by

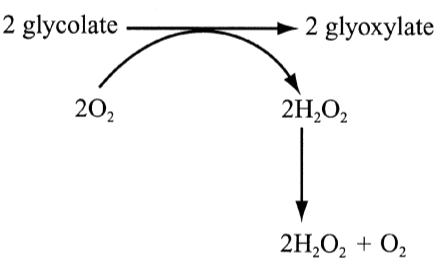
$$\text{Net carbon acquisition} = (v_c - 0.5v_o)/v_c \quad (6)$$

$$= 1 - (0.5\ v_o/v_c) \quad (7)$$

Values of v_o/v_c at given $[O_2]$ and $[CO_2]$ can be determined from Eq (4).

Applying this analysis to a range of organisms (Table 1) shows that given an active PCOC, net carbon acquisition at air equilibration levels of O_2 and CO_2 is possible, even with Rubiscos with low S_{rel} values.

In some species of algae (those in the Rhodophyta, the classes Eustigmatophyceae, Phaeophyceae, Raphidophyceae and Tribophyceae of the Heterokontophyta, and in the classes Prasinophyceae and Charophyceae of the Chlorophyta), as in higher plants, glycolate oxidation is brought about by glycolate oxidase rather than glycolate dehydrogenase (Raven, 1997a; Marin and Melkonian 1999; Iwamato and Ikawa 2000; Raven et al., 2000; Wingler et al., 2000). This peroxisomal enzyme couples glycolate oxidation to the reduction of oxygen to form H_20_2. Catalase subsequently breaks down the H_2O_2 to water and 0.5 O_2(Eq 8). For the Phaeophyceae at least, the resulting glyoxylate is converted to glycerate through the same sequence of reactions shown in Fig. 1.



(8)

However, in this case only the glycine-serine conversion is located in mitochondria, and the rest of the sequence from glycolate to glycerate is found in peroxisomes. Unlike the glycolate dehydrogenase based pathway, a PCOC based on glycolate oxidase will not generate NADH and ATP via oxidative phosphorylation.

There are variants to the PCOC pathway shown in Fig 1, other than the use of glycolate oxidase instead of glycolate dehydrogenase. For instance, Moroney

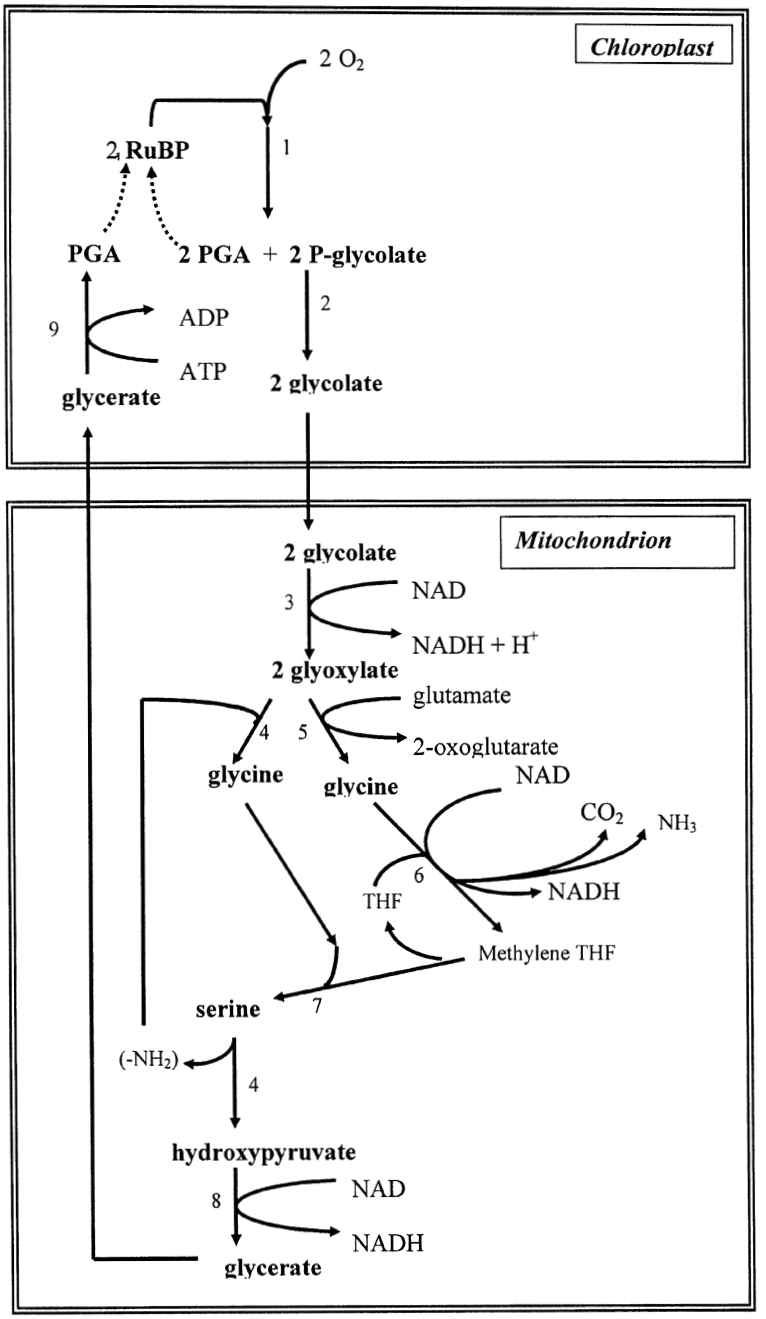


Fig. 1. A 'standard' photorespiratory carbon oxidation cycle (PCOC), based on glyoxylate metabolism via glycine and serine. Enzymes are: 1) oxygenase activity of Rubisco; 2) phosphoglycolate phosphatase; 3) glycolate dehydrogenase; 4) serine:glyoxylate aminotransferase; 5) glutamate:glyoxylate aminotransferase; 6) glycine decarboxylase; 7) serine hydroxymethyl transferase; 8) NAD^+- hydroxypyruvate reductase; 9) glycerate kinase. In some algae, glycolate oxidation is catalysed by glycolate oxidase rather than glycolate dehydrogenase. In this case only the glycine-serine conversion is located in mitochondria, and the rest of the sequence from glycolate to glycerate is found in peroxisomes.

Table 2. Occurrence of glycolate oxidase or glycolate dehydrogenase and mode of glyoxylate metabolism in different classes of algae.

	Glycolate oxidase	Glycolate dehydrogenase	glyoxylate metabolism
Charophyceae	+		PCOC
Prasinophyceae	+	+	PCOC
Chlorophyceae	+		PCOC
Ulvophyceae		+	PCOC
Euglenophyceae	+		PCOC/ glyoxylate decarboxylase yields the C1 unit for serine synthesis
Phacophyceae	+		PCOC
Tribophyceae	+		malate synthase
Raphidophyceae	+		PCOC
Eustigmatophyceae	+		PCOC
Rhodophyceae	+		PCOC
Dinophyceae		+	PCOC
Bacillariophyceae	+		malate synthase
Cryptophyceae	+		PCOC
Cyanophyceae	+		Incomplete PCOC; tartronic semialdehyde pathway

and Chen (1998) suggest a role for alanine: α-ketoglutarate aminotransferase rather than glutamate:glyoxylate aminotransferase in the glyoxylate-glycine transformation. However, this probably reflects the lack of specificity of aminotransferases, and in higher plants alanine and asparagine have both been shown capable of supplying amino nitrogen for glycine synthesis (Betsche, 1983; Ta and Joy, 1986). Furthermore, some Euglenophyceae have been shown to use the decarboxylation of glyoxylate rather than glycine as a source of the C1 moiety used to generate serine (Stabenau, 1992).

Another variation of the PCOC (Fig. 2) is found in the Heterokontophyta (other than Phaeophyceae and Rhodophyceae). These diatoms and tribophytes and their close allies, dinoflagellates, have glycolate dehydrogenase and all the enzymes of the PCOC as far as serine formation (Raven, 1984, 1997a). However, these organisms also contain malate synthase which catalyses the formation of malate from glyoxylate and acetyl coenzyme A (step 4 in Fig 2). As depicted in Fig 2, this pathway leads to the complete oxidation of glyoxylate and generation of NADH, which can be used in the formation of ATP via the mitochondrial electron transport chain. Alternatively, the malate formed from glyoxylate can be completely oxidized in the reactions of the Tricarboxylic Acid Cycle. Since continued operation of the latter version of glyoxylate metabolism would require a continued input of acetyl Co-A, this could be provided by the decarboxylation of triose phosphate, thus leading to the production of five molecules of CO_2 for every glyoxylate entering the pathway (Raven et al., 2000). Although this process would be costly in terms of carbon loss from the cell, it would lead to the generation of large amounts of ATP via oxidative phosphorylation (Raven et al., 2000)

On the basis of labeling studies and enzyme activities, the Cyanobacteria do not appear to contain a complete PCOC. There is little evidence, for instance, for glycolate metabolism to serine in these organisms (Colman and Norman, 1997; Raven, 1997a). These and some eukaryotes (Badour and Waygood, 1971; Codd and Stewart, 1973; Raven and Beardall, 1981; Beardall, 1989) may possess a mechanism for glycolate metabolism based on the conversion of glyoxylate to glycerate via tartronic semialdehyde (Fig. 3). Since this pathway does not invoke the transamination reactions of the 'standard' PCOC, it does not involve the release and re-assimilation of NH_3. However, due to the very high CCM activities found in Cyanobacteria and the consequent inhibition of Rubisco oxygenase activity, photorespiratory carbon flow through such a mechanism in Cyanobacteria is unlikely to be significant (Raven, 1997 a,b). Although the genome of the cyanobacterium *Synechocystis* PCC 6803 has been sequenced (Kaneko et al., 1996), Raven (1997a) has pointed out that while 55% of the proteins encoded are of unknown function, the genome appears depauperate in sequences encoding enzymes of the PCOC.

There appears to be some taxonomic relationship

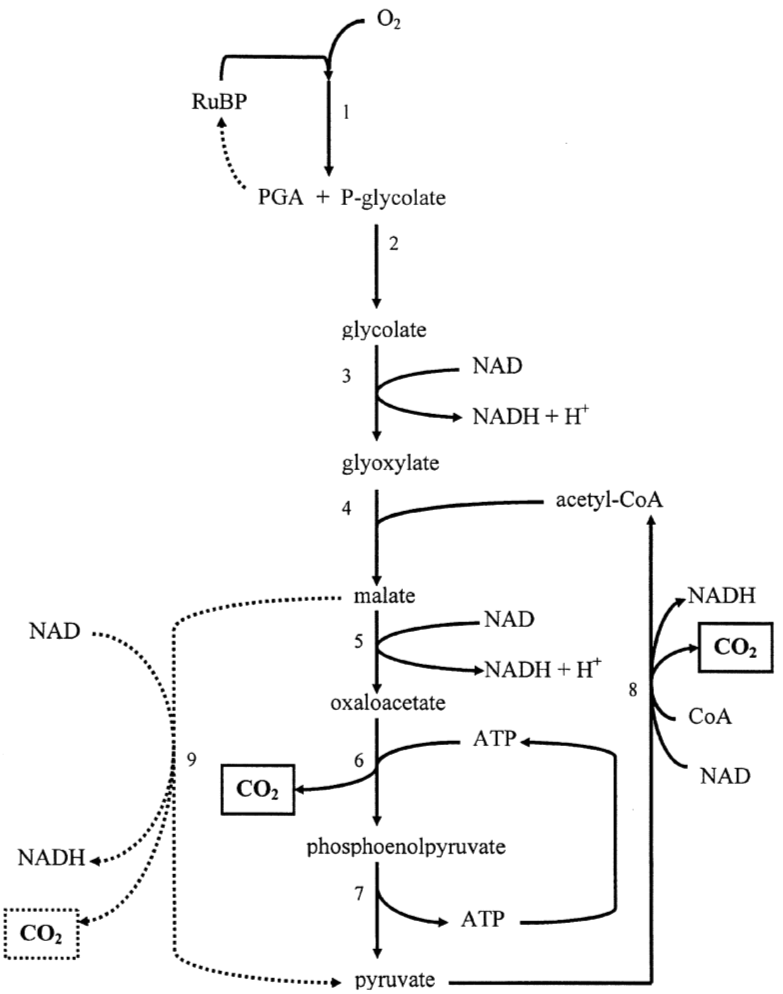


Fig. 2. Two possible alternative pathways for the metabolism of glyoxylate in the PCOC, based on malate synthase activity. Enzymes are: 1) oxygenase activity of Rubisco; 2) phosphoglycolate phosphatase; 3) glycolate dehydrogenase; 4) malate synthase; 5) malate dehydrogenase; 6) phospho*enol*pyruvate carboxykinase; 7) pyruvate kinase; 8) pyruvate dehydrogenase; 9) NAD malic enzyme.

in the use of glycolate dehydrogenase or glycolate oxidase. These alternative mechanisms for dealing with glycolate are never found together in the same species and most classes have either the oxidase or dehydrogenase. One exception is the prasinophyte *Mesostigma viride* which possesses glycolate oxidase whereas most other members of the Prasinophyceae have the dehydrogenase (Iwamoto and Ikawa 2000). This may, however, reflect the taxonomy of *Mesostigma*, which Marin and Melkonian (1999) and Qiu and Liu (2000) include as the simplest member of a streptophyte clade (charophycean green algae and their embryophyte descendants; Chapter 2, Larkum and Vesk).

It must be recognized, however, that the above discussions of the variations of photorespiratory metabolism and their taxonomic distribution are based on a limited number of studies using a relatively small range of species. There is thus considerable scope for improvements in our understanding of the phylogenetic significance of the different pathways for glycolate metabolism. Possession of glycolate oxidase vs glycolate dehydrogenase is presumably unrelated to evolutionary origins of the different algal classes as glycolate dehydrogenase is found in both Cyanobacteria and diatoms, groups whose evolutionary origins are separated by >2000 $\times$ 10^6 years. Similarly glycolate oxidase is found in the

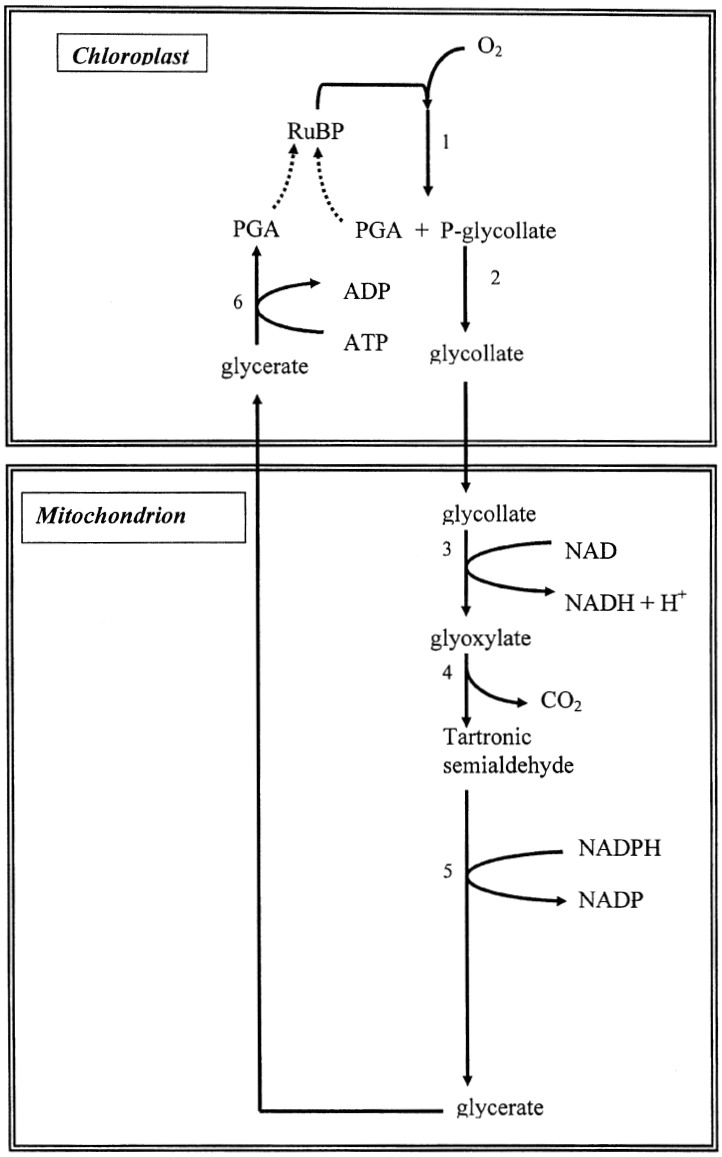


Fig. 3. A pathway for the PCOC in which glycolate metabolism is based on the conversion of glyoxylate to glycerate via tartronic semialdehyde (see text for details). Enzymes are: 1) oxygenase activity of Rubisco; 2) phosphoglycolate phosphatase; 3) glycolate dehydrogenase; 4) glyoxylate carboligase; 5) tartronic semialdehyde reductase; 6) glycerate kinase

Rhodophyta and, among the heterokonts, the Phaeophyceae, Tribophyceae, Raphidophyceae and Eustigmatophyceae, as well as in the 'prasinophyte' *Mesostigma*, the Charophyceae and the Embryophyta (higher plants), the three last-mentioned comprising the streptophyta, whose origins are also well separated in geological time. Furthermore, while the taxon 'diatom' only evolved ~ 250 × 10^6 years ago (or perhaps less), the ancestors of their plastids (a red alga) presumably had a glycolate metabolizing enzyme (most likely a glycolate oxidase) in its nuclear genome which it could have 'donated' to the 'host' genome. This offers a possible explanation for the occurrence of glycolate oxidase in the brown algae. It is less likely that the heterotrophic heterokont host had a glycolate dehydrogenase, though a proteobacterial (*E. coli*)-like glycolate dehydrogenase has been reported in some Cyanobacteria, while the

Synechocystis PCC 6803 genome encodes a three-subunit glycolate oxidase (Badger et al., 2002)! Clearly the phylogeny of the glycolate metabolic machinery is complex and requires further study.

C. Energetics of Photorespiratory Metabolism

Raven and colleagues have analyzed the energetic costs and benefits of photorespiration and elevation of intracellular CO_2 levels (and hence decreased oxygenase activity) via CCM activity (Beardall and Raven, 1990; Raven, 1997 a,b; Raven et al., 2000,). The costs of phosphoglycolate production and photorespiration via the various pathways above, as estimated by Raven et al. (2000), are summarized in Table 3. There is a small energetic advantage (1.5 ATP/glycolate) to cells to use the PCOC with glycolate dehydrogenase rather than glycolate oxidase. The variant PCOC in which C1 moiety used in serine synthesis comes from glyoxylate decarboxylation to formaldehyde rather than from glycine decarboxylation (Stabenau, 1992) also shows a slightly improved ATP balance. This is because this variant avoids the generation of NH_3 associated with the decarboxylation of glycine and, hence, 0.5 ATP is not required (by the GS-GOGAT system) to re-assimilate the NH_3 released. The large energetic advantages to cells come from using malate synthase, either with oxidation of glyoxylate as shown in Fig 2 or, especially, with complete oxidation of malate. Clearly though, this is at a major cost to the C economy of the cell.

These values for the energetic cost of glycolate metabolism can be combined with estimates of the cost of carbon assimilation by the carboxylase activity of Rubisco and with estimated v_o/v_c values (at given CO_2 and oxygen concentrations and the species-specific values of S_{rel}), to determine the theoretical cost of net carbon assimilation. Such analysis shows that for organisms with low S_{rel} Rubiscos and diffusive entry of CO_2, only the PCOC is energetically feasible, albeit with a high energy cost (4.25 NADPH and 4.25–6 ATP per net C assimilated). Similar cells using the malate synthase based pathway(s), or glycolate excretion, would be unable to carry out net carbon assimilation (see Appendix 2 in Raven et al., 2000). In contrast, with the highest S_{rel} Rubiscos, approximately 2.3 NADPH and 3.2 ATP are required per CO_2 fixed if cells use the PCOC, the values for NADPH requirements increasing to ~3–5.5 for cells using the malate synthase pathways. With malate completely oxidized, the ATP requirement per CO_2 assimilated into carbohydrate drops to 2.3 (Raven et al., 2000).

The assimilation of CO_2 via Rubisco and subsequent operation of the photosynthetic carbon reduction cycle (PCRC = Calvin cycle) requires an input of six NADPH and nine ATP for every three CO_2 molecules reduced to triose phosphate in the absence of oxygenase activity. When oxygenase activity is present, as given air equilibrium concentrations of CO_2 and oxygen it inevitably will, the energy cost of net carbon assimilation is increased.

D. Role of Photorespiration in Algal Cells

The phosphoglycolate formed as a result of the oxygenase activity of Rubisco can be dephosphorylated and potentially be excreted, as glycolate, from the cell. Glycolate excretion can occur, to some extent at least, from all algal cells. When it does occur, excretion thus represents a major loss of carbon and of the energy involved in phosphoglycolate synthesis. Leboulanger et al. (1998) for example estimated glycolate loss by cultures of the chlorophyte alga *Dunaliella* to be 16–20% of total C fixed. Moroney et al. (1986) showed that in *C. reinhardtii,* glycolate excretion was ~24% of C fixed in cells transferred to air after they were grown under high CO_2 to suppress CCM activity. This figure increased to 55%, if the capacity of cells for re-assimilation of glycolate was blocked by the inhibitor amino-oxyacetate. When cells were grown with air-levels of CO_2, glycolate formation was largely suppressed following induction of a CCM and glycolate excretion was only a maximum of ~7% of C fixed even when its metabolism was blocked. This value was even lower (0.1%) under conditions in which the further metabolism of glycolate was not inhibited. Possession of a CCM and a capacity for glycolate metabolism can thus be seen as complementary approaches to avoiding the inefficiencies of carbon acquisition *via* Rubisco and to recoup some of the carbon and energy that would otherwise be lost to the cell as excreted glycolate.

Photorespiration is also seen as a mechanism to protect cells against damage associated with high light and high O_2, i.e. it acts as a mechanism of energy dissipation. Under conditions, such as low CO_2 levels, where the photosynthetic assimilation of CO_2 is decreased, exposure to high light can lead to chronic photoinhibition (Osmond and Grace, 1995).

Table 3: Energetic costs of photorespiration (phosphoglycolate synthesis and metabolism) in algae with different pathways of glycolate metabolism (after Raven et al., 2000). Values are based on 2 CO_2 being assimilated to phosphoglycolate via Rubisco.

Pathway	NADH balance	ATP balance	C atoms converted to triose phosphate
Phosphoglycolate synthesis	–5	–7	0
Phosphoglycolate metabolism via PCOC and glycolate oxidase	–1	–1.5	1.5
Total	–6	–8.5	1.5
Phosphoglycolate synthesis plus phosphoglycolate metabolism via PCOC and glycolate dehydrogenase	–6	–7	1.5
Phosphoglycolate synthesis plus phosphoglycolate metabolism via PCOC and glycolate dehydrogenase, but with the C1 moiety for serine synthesis produced by glyoxylate decarboxylation	–6	–6.5	1.5
Phosphoglycolate synthesis plus phosphoglycolate metabolism via glycolate dehydrogenase and malate synthase as per Fig 2	–5	+0.5	0
Phosphoglycolate synthesis plus phosphoglycolate metabolism via glycolate dehydrogenase and malate synthase but with malate completely oxidised	–11	+6.5	0
Phosphoglycolate synthesis plus phosphoglycolate metabolism via glycolate dehydrogenase and tartronic semialdehyde	–6	–7	1.5

By promoting 'non-assimilatory electron transport,' photorespiration dissipates excess harvested light energy and mitigates the potential effects of chronic photoinhibition (Osmond and Grace, 1995; Badger et al., 2000; Wingler et al., 2000). A similar function has been ascribed to the Mehler reaction, which in fact may be more important than Rubisco oxygenase and the PCOC in light dependent oxygen consumption in algae (Badger et al., 2000).

E. Effects of Environmental Factors on Photorespiration

At a fundamental level, the flux of carbon through glycolate and the PCOC is determined by the ratio between the oxygenase and carboxylase activities of Rubisco. Thus any environmental parameter that affects this ratio will influence the rate of photorespiration.

1. Carbon Dioxide Supply

High levels of CO_2 will directly enhance rates of carboxylase relative to oxygenase due to the competitive nature of these two activities (Eq 4). Elevated CO_2 will cause suppression of the enzymes involved in the PCOC. These changes occur in parallel with induction/repression of the CCM in species in which the latter is present (Chapter 11, Raven and Beardall). In *Chlamydomonas* for instance, levels of phosphoglycolate phosphatase are repressed in cells grown under elevated CO_2 and induced when cells are switched to air equilibrium CO_2 levels where the oxygenase activity of Rubisco would be more significant (Spalding et al., 1991). The consequent increase in phosphoglycolate phosphatase activity is, however, transient and within five hours levels drop again. Similar data have been reported for alanine α-ketoglutarate aminotransferase (Chen et al., 1996; Moroney and Chen, 1998). The transient rise in enzyme activity corresponds to the time for induction of the CCM in this species (Spalding et al., 1991) and illustrates the roles of the CCM and PCOC in minimizing C loss consequent on Rubisco oxygenase.

2. Temperature

Although temperature affects the solubility of both oxygen and CO_2 in water, the effect on both gases is fairly similar so, for instance, the ratio of O_2:CO_2 solubility is only about 15% lower at 5 °C than it is at 25 °C. However, high temperature decreases S_{rel} values of higher plant Rubiscos (Brooks and Farquhar, 1985; Sherlock and Raven, 2001) and hence high temperature leads to higher oxygenase activity and higher energy costs associated with the increase in glycolate metabolism. In addition, some Rubiscos

exhibit a higher affinity for CO_2 at low temperatures (Raven and Geider, 1988; Beardall and Roberts, 1999; Chapter 11, Raven and Beardall). The activity of Rubisco oxygenase is thus favored at higher temperatures. It is not known, however, whether the levels of photorespiratory enzymes are modulated in response to changes in temperature.

3. Light

The high energy requirements for photorespiration and glycolate metabolism (and/or CCM) mean that in low light environments algae with high S_{rel} Rubisco would be at an advantage, and in fact a number of deep water red algae that rely on diffusive CO_2 entry do have Rubisco with this characteristic (Raven, 1997 a,b; Raven et al., 2000; 2002a). Increasing photon flux also causes an increase in the level of the enzymes of photorespiratory metabolism and Parker and Armbrust (2002) have reported increases in mRNA transcripts for glycine decarboxylase of the diatom *Thalassiosira weisflogii* in response to increasing photon flux and in cells undergoing dark-light transitions.

III. Chlororespiration: A Mechanism to Maintain Thylakoid Membrane Energization in the Dark?

A. Evidence for and Current Models of Chlororespiration

Prokaryotic organisms have photosynthetic and respiratory chains located in the same membranes, intersecting at the site of the plastoquinone pool, and sharing both electron transport components and coupling factors (Raven and Beardall, 1981; Scherer et al., 1988; Chapter 11, Raven and Beardall). Photosynthesis and respiratory processes based on the tricarboxylic acid (TCA) cycle and oxidative phosphorylation occur in the chloroplast and mitochondria, respectively, of eukaryotic cells (Raven and Beardall, 1981; Bennoun, 1982; Fork and Herbert, 1993; Chapter 11, Raven and Beardall). However, all these organisms are believed to possess an additional respiratory pathway, leading to consumption of oxygen, which is distinct from those of mitochondrial respiration, photorespiration or the Mehler reaction. It was Bennoun in 1982, who first coined the term chlororespiration in an attempt to understand the process of oxygen consumption in the thylakoid membranes of photoautotrophs that only occurs in the dark (Bennoun, 1982). In his original model, Bennoun proposed that the plastoquinone pool could be reduced by the action of NAD(P)H dehydrogenase, oxidized by oxygen via an oxidase, and at the same time generate an electrochemical gradient across the thylakoid membrane. Recent papers which have significantly contributed to our understanding of this process in phytoplankton include, but are not limited to: Peltier et al. (1987); Caron et al. (1987); Büchel and Wilhelm (1990); Scherer (1990); Bennoun (1994); Field et al. (1998); Cournac et al. (2000a,b).

Indications of what we now term chlororespiration were first provided in 1949 by Bessel Kok from his examination of photosynthesis versus photon flux curves (Kok, 1949). Slight deviations from linearity at very low photon flux (<15 μmol m^{-2} s^{-1}) were attributed to light-induced changes in the rate of respiration and referred to as the 'Kok effect.' In 1963, Goedheer was the first to postulate the existence of a chloroplastic respiratory system in *Chlorella*. The contemporary view is that the Kok effect is not considered to be a consequence of inhibition of mitochondrial respiration by light, but due to the inhibition of chlororespiration induced by Photosystem I activity (Peltier and Sarrey, 1988).

Bennoun (1982) interpreted measurements of chlorophyll fluorescence in terms of a putative respiratory electron transfer chain after showing that the redox-state of the PQ pool in *Chlamydomonas reinhardtii* could be modified in response to oxygen concentration and to some respiratory inhibitors (Bennoun, 1982, 1994). More recently, direct evidence for a chlororespiratory oxygen uptake has been obtained using flash illumination and a combination of amperometric and mass-spectrometric techniques (Peltier et al., 1987; Cournac et al., 2000 a,b), studies using inhibitors (Caron et al., 1987), mutants (Ravenel and Peltier, 1992), flash-induced electrochromic absorption-changes (Field et al., 1998), and changes in fluorescence induction curves (Bennoun, 1994) and fluorescence parameters such as photochemical quenching (qP) and non-photochemical quenching (qN) (Ting and Owens, 1993).

Few studies have been able to provide measurements of the absolute rate of chlororespiration. Rates of 2.4 μmol O_2 mg Chl^{-1} h^{-1} have been reported in *Chlamydomonas reinhardtii* (Peltier et al., 1987) and of 9.6 μmol O_2 mg Chl^{-1} h^{-1} in potato chloroplasts

(Corneille et al., 1998). Estimates of the chlororespiratory rate as a function of the total respiration rate are more common, and have been made for *Chlorella vulgaris* (20%) and *Chlamydomonas reinhardtii* (10%) by Diner and Mauzerall (1973) and Peltier et al. (1987) respectively. Using the data of Diner and Mauzerall (1973), Bennoun (1982) calculated a rate of oxygen uptake due to chlororespiration of ~10 μmol O_2 mg Chl^{-1} h^{-1} in *Chlorella vulgaris*.

This lack of absolute values for chlororespiration reflects the experimental difficulty in separating chlororespiration from other O_2-consuming processes such as photorespiration and the Mehler reaction (Myers, 1986). In particular, O_2 uptake of whole algal cells is not a suitable system from which to obtain any information on chlororespiration because mitochondrial respiration, which accounts for the majority of respiratory O_2 uptake, will be highly variable depending on the energy status of the cell. Wilhelm and coworkers (C. Wilhelm, personal communication) have tried measuring electron flow rates from NADPH to plastoquinone by blocking the terminal oxidase and measuring the changes in the re-reduction kinetics of P-700. From this data, they calculated a minimum chlororespiratory activity of ~8–10 μmol electrons mg Chl a^{-1} h^{-1} in *Phaeodactylum tricornutum*, which would be equivalent to 5% of dark respiration under non-induced conditions, and ~25 μmol electrons mg Chl a^{-1} h^{-1} under conditions where chlororespiration is active. However, this is a very rough calculation because the contribution of cyclic electron flow, which might be changed under the influence of cyanide (used to inhibit mitorespiration), has to be neglected.

While there is no clear consensus on the pathway of electron flow in chlororespiration, the most generally accepted alternatives are shown in Fig. 4, with electron transport from plastoquinone directed to either a terminal oxidase in the lumen side of the thylakoid or to a protein of similar function on the stromal side of the membrane. The model in Fig. 4 is based on studies with *Chlamydomonas* mutants (Table 4) which indicate that the chlororespiratory pathway functions without the involvement of cytochrome *f*, the Rieske-center protein, cytochrome b_6f complex or the chloroplast ATP synthase (Bennoun, 1982, 1983, 1994; Cournac et al., 2000b and references therein). On the other hand, in Cyanobacteria it is well established that the cytochrome b_6f complex works as a common transducer of electrons from PQH_2 to Photosystem I in light dependent electron flow and to a terminal oxidase during respiratory electron transport (Scherer, 1990; Schmetterer, 1994).

Plastoquinone is a redox carrier of the electron transport chain, common to photosynthetic and chlororespiratory pathways in microalgae (Diner and Mauzerall, 1973; Bennoun, 1982; Falkowski and Raven, 1997), Cyanobacteria (Hirano et al., 1980; Scherer et al., 1988) and higher plants (Caron et al., 1987). The PQ pool of the chloroplast is functionally located between the two photosystems, being reduced by electrons from Photosystem II, and oxidized by donation of electrons to Photosystem I. Therefore, it is plausible that chlororespiratory reactions may poise reduced and oxidized forms of the intermediates of cyclic electron transport (Diner and Mauzerall, 1973), under highly fluctuating light intensities, via the PQ pool (Casano et al., 2000). This was shown most elegantly by Bennoun (1982), using inhibitors (e.g. CO, NO, KCN, DCCD) to block oxidation of the PQ pool in the dark in *Chlamydomonas reinhardtii*. Bennoun (1982) showed that the fluorescence rise induced by inhibitors of chlororespiration was totally different from that induced by DCMU. Further, we now know the response to inhibitors is species-specific (Table 4).

NADH-specific dehydrogenase activity, located on the thylakoid membranes (Godde and Trebst, 1980), was thought to be the primary entry point for electrons generated from the non-photochemical reduction of the PQ pool (Fig 4). Using open-cell preparations of *Chlamydomonas reinhardtii* wild-type 137C, Bennoun (1982) found that NADH or NAD(P)H were able to reduce the PQ pool in darkness, in agreement with the previous work of Godde and Trebst (1980) and Shahak et al. (1981). However, these findings may be contentious, due to contamination by mitochondrial respiratory chain components in the above preparations (Atteia et al., 1992; Rich et al., 1998). Metabolically, NADPH is likely to be the most important physiological donor to plastoquinone because several photosynthetic metabolites in the chloroplast stroma can be coupled to NADPH formation (e.g. triose phosphates, malate, etc). Starch breakdown via the oxidative pentose phosphate (OPP) pathway (Raven and Beardall, 2002a) would produce a significant flux of NADPH that could enter the plastoquinone pool through NADPH oxidation in chlororespiration (Bennoun, 1982). Inhibition of mitorespiration, which would lead to lowered levels of cellular ATP, may stimulate

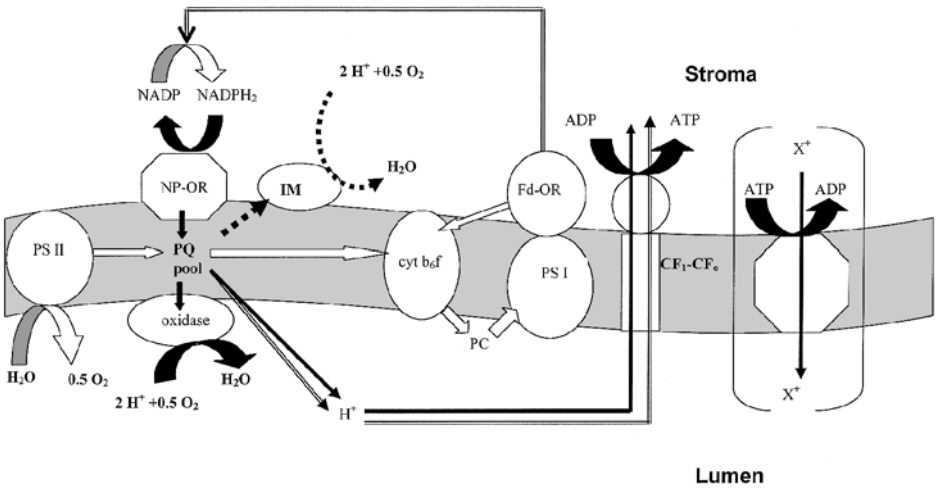


Fig. 4. A model showing various alternative pathways of chlororespiratory electron flow, given by the black arrows. Electron transport during photosynthesis, in the light, is shown by the white arrows. In the chlororespiratory pathway, NAPDH is oxidised via NADP-dependent plastoquinone oxidoreductase (NP-OR) which passes electrons and protons to the plastoquinone pool. Electron transport from plastoquinone (PQ) can lead to proton re-translocation to the intrathylakoid space and maintenance of a proton gradient in the dark (but see text for the evidence for this process). In one alternative, electrons are passed to a terminal oxidase in the lumen side of the thylakoid, where molecular oxygen is reduced to water. In Cyanobacteria, with shared respiratory and photosynthetic electron transport chains, this oxidase may be cytochrome *c* oxidase. An analogous enzyme presumably operates in eukaryotes, but has yet to be identified. Also shown (dashed black line) is the path of electrons proposed for chlororespiration in the higher plant, *Arabidopsis*, where electrons are passed directly from PQ to a terminal acceptor, IMMUTANS (IM), located on the stromal side of the thylakoid. There is some evidence for an analogous protein in *Chlamydomonas*, but not in Cyanobacteria. Protons can potentially cross the thylakoid membrane through the CF_1-CF_0 ATP synthetase. In brackets on the right hand side is shown a putative cation transporting ATPase as proposed by Bennoun (1994) (see text for details). Based on Bennoun (1994), Casano et al. (2000) and Nixon (2000). (PC=plastocyanin, Fd-OR=ferredoxin oxidoreductase).

glycolysis in the chloroplast (at least in those species possessing a glycolytic sequence in the plastids; Chapter 10, Raven and Beardall), resulting in enhanced levels of NAD(P)H and an increase in the rate of reduction of the plastoquinone pool through the action of NAD(P)H dehydrogenase(s) (Gans and Rebeillé, 1990). If one considers that chloroplasts and mitochondria are indeed in 'redox communication' (Nixon, 2000), the alternative possibility is that mitochondrial oxidases are in principle able to drive the oxidation of the plastoquinone pool through reverse electron flow from plastoquinol to $NAD(P)^+$ at a proton pumping NAD(P)H dehydrogenase, with the reaction driven by the proton electrochemical gradient across the membrane.

Corneille et al. (1998), using osmotically lysed potato chloroplasts, presented evidence for the existence of a NADH-plastoquinone-oxidoreductase. These authors found that the NADH-PQ-oxidoreductase reaction was inhibited by diphenylene iodonium, N-ethylmaleimide and dicoumarol, but was insensitive to rotenone, antimycin A and piericidin A (Table 4). By comparing the substrate specificity and the inhibitor sensitivity of this reaction, Corneille et al. (1998) concluded that ferredoxin-$NADP^+$-reductase is not involved in the NADH-PQ-oxidoreductase activity, but that a rotenone-insensitive NADH-PQ-oxidoreductase may participate. NAD(P)H-PQ-oxidoreductase is referred to as the Ndh complex, and is analogous to the eubacterial and mitochondrial NADH-ubiquinone-oxidoreductase (complex I) of mitochondria. However, this enzyme shows specificity for plastoquinone rather than ubiquinone (Kofer et al., 1998). It consists of at least eleven subunits (A-K) which are all encoded by plastid *ndh* genes. The Ndh-complex has been found in higher plant chloroplasts and Cyanobacteria, but not yet in the plastid genomes of *Chlamydomonas* and various other green algae (Nixon, 2000). It is uncertain whether this reflects a loss of the genes or their transfer to the nuclear genome.

On the basis of mutagenesis studies on genes

encoding various subunits, Kofer et al. (1998) showed that the Ndh complex of higher plants could act as a valve to dissipate excess reducing equivalents in the chloroplast, thereby supporting its role in chlororespiration. Recently, Casano et al. (2000) presented evidence which suggests chlororespiratory processes in higher plants consist of a sequence of reactions catalysed by the Ndh complex, hydroquinone peroxidase (acting to reduce PQ with H_2O_2), superoxide dismutase, and a non-enzymic one-electron transfer from a reduced Fe-S protein (FeSP) to O_2 (Fig. 4). If FeSP is a component of the cytochrome b_6f complex or of the same Ndh complex, then O_2 may be reduced, by NAD(P)H, in the dark. It has further been suggested that the Ndh complex is involved in cyclic electron flow around PS I.

Although it is now clear that there is a terminal oxidase involved in plastoquinol oxidation during chlororespiration, postulated on the basis of its sensitivity to cyanide in eukaryotes (Peltier et al., 1987) and to CO in Cyanobacteria (Schmetterer, 1994) (Table 4), the exact nature of the oxidase remains a subject of controversy and it is thought to vary between species (Bennoun, 1982; Peltier et al., 1987; Ravenel and Peltier, 1991; Cournac et al., 2000b; Nixon, 2000). In eukaryotes and higher plants, it may be a cytochrome oxidase or a cytochrome *b-c* complex (Peltier and Schmidt, 1991) or quinol oxidase (Cournac et al., 2000 a,b). In *Chlorella vulgaris*, chlororespiration is sensitive to SHAM (Bennoun, 1982), an inhibitor of the alternative oxidase of plant mitochondria. Myxothiazol (Peltier et al., 1987; Ravenel and Peltier, 1991) and antimycin A (Ravenel and Peltier, 1991) inhibit chlororespiration in *Chlamydomonas reinhardtii* (Table 4). These inhibitors act on the various oxidases involved in the alternative pathways of mitochondrial respiration, but have no effect on CO_2-dependent photosynthetic O_2 evolution or the 515 nm electrochromic absorbance change (Fork and Herbert, 1993). Peltier (1991) suggested that the unknown component(s) in the chlororespiration pathway of *Chlamydomonas* may have similarities to the bacterial and mitochondrial cytochrome *b-c* complexes but must be different since much higher concentrations of myxothiazol and antimycin A are required to inhibit chlororespiration compared to mitochondrial respiration. An alternative suggestion is that in higher plants, the low potential form of cytochrome b_{559} (Cyt b_{559}LP) may be responsible for the PQ-oxidase activity observed during chlororespiration of spinach thylakoids (Kruk and Strzalka, 1999).

From the inhibitor studies of Büchel and Garab (1995), it is clear that the oxidase of *Pleurochloris meiringensis* competes with cytochromes for electrons. The KCN concentration needed to re-reduce the cytochrome f/c_{533} electron pool following photooxidation by repetitive single-turnover flashes was 0.25 mM at half-saturation, whereas mitochondrial respiration was completely blocked at 0.1 mM. Büchel and Garab (1995) concluded that the oxidase in *Pleurochloris meiringensis* could not be identical to the cytochrome aa_3-oxidase of mitochondria and was most likely located in the chloroplast. Similarly, Lajko et al. (1997) found an enhancement of the activity of the cytochrome b_6f complex in the presence of KCN, occurring with the same mechanism as is found in *Chlamydomonas reinhardtii and Nicotiana tabacum.* The dependence of this effect on the concentration of KCN in these eukaryotes rules out an origin in mitochondrial respiration, superoxide dismutase or plastocyanin, strongly suggesting that a cyanide-sensitive terminal oxidase competes with the photosynthetic electron transport chain for reducing equivalents from the PQ pool (Lajko et al., 1997).

More recently, Cournac et al. (2000 a,b) have suggested that electron flow between Photosystem II and oxygen in chloroplasts of *Chlamydomonas reinhardtii* is mediated by a quinol oxidase involved in chlororespiration. Specifically, from inhibitor effects, electron requirements of the O_2 uptake process and the sensitivity of flash induced O_2 exchange to propyl gallate, the authors concluded that the oxidase must be present in the thylakoid membranes. From recent pharmacological and immunological studies (Carol et al., 1999; Wu et al., 1999), the quinol oxidase seen in *Chlamydomonas reinhardtii* (Cournac et al., 2000a,b) shows similarities to a protein identified in chloroplasts of *Arabidopsis thaliana.* This enzyme, which is encoded by the nuclear gene, *immutans*, is assumed to be the plastid terminal oxidase (PTOX) with the plastoquinol oxidizing activity and, therefore, involved in chlororespiration.

In Cyanobacteria, the PQ pool and the cytochrome b_6f complex are remarkably similar to the corresponding components in eukaryotes and higher plants (Scherer et al., 1988) but there appears to be no obvious Cyanobacterial homologue of *immutans*. Rather, the genome of *Synechocystis* sp. PCC 6803 contains three sets of genes for terminal oxidases: a cytochrome aa_3-type cytochrome *c* oxidase (*Cta*I),

Table 4. Inhibitor studies used to elucidate the pathway/mechanism of chlororespiration. (+) indicates that chlororespiration was inhibited, (-) = no effect

Organism	Inhibitor	Inhibition of oxidation of the PQ pool	Reference
Chlamydomonas reinhardtii	Dicyclohexyl-18-crown-6	–	Bennoun, 1983
	Antimycin A	+/–	Peltier and Sarrey, 1988/ Peltier et al., 1987
	SHAM	+/–	Peltier and Sarrey, 1988/ Peltier et al., 1987
	10 μM DCMU	–	Peltier et al., 1987
	1 mM Acetate	–	
	High $[O_2]$	–	
	0.5 mM Cyanide	+	
Chlamydomonas mutant (lacks PS I activity)	10 μM DCMU	–	
	1 mM Acetate	+/–	
Chlamydomonas reinhardtii mutant F15+ (lacks PS I)	DCMU	–	Ravenel and Peltier, 1992
	DBMIB	+	
Chlamydomonas reinhardtii mutant FuD6 (lacks cytochrome b_6f complex)	Antimycin A	–	
	Myxothiazol	–	
Chlamydomonas reinhardtii wild-type 137C (open cell preparations)	1 mM NADH	–	Bennoun, 1982
	1 mM NADPH	–	
	Dicyclohexyl-18-crown-6	–	
	CO	–	
Chlamydomonas mutant F14 (lacks PS I RCs)	NO	–	
	10 mM KCN	–	
	10 mM NaN_3	–	
	4 mM SHAM	–	
	Bu_3Sn	+	
	20 μM DCCD	–	
Chlamydomonas mutant F54.F14 (lacks PS I RCs and chloroplast ATPases)	Bu_3Sn	–	
Chlamydomonas mutant *FUD50* (lacks chloroplast ATPases)	CO	–	
	10 mM SHAM	–	
	CO	+	
	10 mM KCN	+	
	2 μM Bu_3Sn	+	
	2 μM Bu_3Sn + 10 mM	–	
Chlorella pyrenoidosa	SHAM	+	
	anaerobiosis		
Chlamydomonas reinhardtii wild-type, *137C* and *Chlamydomonas reinhardtii* mutant F15 (lacks PS I)	5 μM Antimycin A	+	Ravenel and Peltier, 1991
	5 μM Myxothiazol	+	
	5 μM Antimycin A	+	
	5 μM Myxothiazol	+	
Chlorella sp.	NaN_3	–	Maison-Peteri and Etienne, 1977)
Dunaliella tertioleca	Benzoquinone	+	Meunier and Popovic, 1990
Lysed potato chloroplasts	diphenylene iodonium	–	Corneille et al., 1998
	N-ethylmaleimide dicoumarol	–	
	rotenone	–	
	antimycin A	+	
	piericidin A	+	

Table 4. Continued

Mantoniella squamata	50 μM DCCD	+	Wilhelm and Duval, 1990
	5 μM Antimycin A	+	
	DCMU + 5 mM NH_4Cl	–	
	DCMU + FCCP	+	
	Anaerobiosis + DCCD	+	
	Anaerobiosis + Antimycin A	+	
Nicotiana tabacum L. (leaves, protoplasts and open cell preparations), and *Pisum sativum* L. (intact chloroplasts)	0.5–5 mM KCN	+	Garab et al., 1989
Phaeodactylum tricornutum	Antimycin + DCMU	+	Caron et al., 1987
	2–20 mM NH_4Cl+DCMU	+	
Phaeodactylum tricornutum	Antimycin A	+/–	Ting and Owens, 1993; Geel, 1997; Jakob et al., 1999
	CCCP	+	
	Nigericin	+/–	
	DCMU	–	
	Anaerobiosis	+	
	NH_4Cl	–	
	KCN	–	
Pleurochloris meiringensis	0.3 μM Antimycin A	+	Buchel and Wilhelm, 1990; Buechel and Garab, 1995
	20 mM NH_4Cl	–	
	0.01 mM DCCD	+	
	Antimycin A + NH_4Cl	–	
	SHAM	+	
	KCN	–	
Synechococcus sp.	5 μM DCMU	–	Hirano et al., 1980
	5 μM DBMIB	+	
	25 μM HOQNO	+	
	1 mM KCN	–	
Synechococcus sp. PCC 6301	1 mM KCN	–	Lajko et al., 1997
Chlamydomonas reinhardtii		–	
Nicotiana tabacum L. cv. Petit Havana SR1 protoplasts		–	

The apparent discrepancy between Peltier et al. (1987) and Ravenel and Peltier (1991) in relation to the effect of antimycin A on chlororespiration is explained by a lower antimycin A concentration used in the earlier experiments. Antimycin A – (blocks cyclic electron flow around PS I); Bu_3Sn – tri-*n*-butyltin (an inhibitor of ATPases); CCCP – carbonylcyanide *m*-chlorophenylhydrazone; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (blocks electron transport between the PQ pool and the cytochrome b_6f complex); DC – dicyclohexyl 18-crown-6 (an ionophore); DCCD – dicyclohexylcarbodiimide (acts on the membrane sector of ATPase inhibiting ATP synthase and an inhibitor of mitochondrial cytochrome *c* oxidase); DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea (blocks electron transfer between the primary quinone Q of system II and the PQ pool); FCCP – carbonyl cyanamidetrifluormethy-oxyphenylhydrazone; HOQNO – 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; SHAM – salicylhydroxamic acid (an inhibitor of the alternative oxidase in mitochondrial respiration)

which has been shown to be the major oxidase in thylakoid membranes; a possible *bo*-type quinol oxidase (*Cta*II) and a putative cytochrome *bd* quinol oxidase (*Cyd*) both of which play more minor roles (Howitt and Vermaas, 1998). The aa_3-type terminal cytochrome *c* oxidase has been well-characterized in Cyanobacteria. Earlier reports for *Synechocystis* sp. strain PCC 6803 suggested this organism may contain a KCN/CO-sensitive oxidase other than the aa_3-type (Schmetterer, 1994). Reduction of cytochrome *f* in the dark (NADPH as electron donor) was found to be dependent on PQ in Cyanobacteria whereas oxidation of cytochrome *f* by oxygen in the dark was found to occur by a cyanide sensitive reaction. Additional

evidence was also provided for a dual function of the cytochrome b_6f complex in respiratory electron transport and photosynthetic electron transport of Cyanobacteria (Scherer et al., 1988) but not in eukaryotes (Bennoun, 1982). Furthermore, in Cyanobacteria, the effect of cyanide on cytochrome reduction and photoinhibition of respiration can be explained in terms of competition at the level of PQ. This is because the photosynthetic and respiratory electron transport chains are localized in the same thylakoid membranes, so that a direct interaction between the two chains is possible. Secondly, Cyanobacteria contain plastoquinone but no ubiquinone (Hirano et al., 1980). Therefore, it may be reasonable to assume that the respiratory chain utilizes plastoquinone as the functional quinone.

Direct study of the chloroplast oxidase in all photosynthetic eukaryotes is, however, hindered by an inability to reconstitute the oxidase activity in isolated thylakoids (Field et al., 1998), as the oxidase is either removed or inactivated during thylakoid isolation. To complicate matters further, some Cyanobacteria and eukaryotic algae have alternative respiratory oxidases, which are not sensitive to KCN/CO but rather to substituted hydroxamic acids (references in Table 4; Schmetterer, 1994; Chapter 11, Raven and Beardall). From the recent studies of Casano et al. (2000) and Cournac et al. (2000a, b), it may be more plausible to suggest that there appear to be multiple pathways for the oxidation of plastoquinols, one involving a quinol oxidase and the other a peroxidase. These different pathways might be differentially regulated depending on the environmental conditions.

Until very recently, chlororespiration was thought to play a role in the formation of a transthylakoid proton gradient (ΔpH) in darkness (Nixon, 2000 and references therein). This was demonstrated in the early work of Bennoun (1982) in *Chlamydomonas reinhardtii*, by Wilhelm and Duval (1990) in *Mantoniella squamata*, and by Caron et al. (1987) and Ting and Owens (1993) in *Phaeodactylum tricornutum*. The membrane-bound NAD(P)H-PQ-oxidoreductase was thought to donate electrons to the plastoquinone pool (Wilhelm and Duval, 1990), in a proton translocating step leading to a ΔpH and membrane energization (Ting and Owens, 1993). It was considered that an electrochemical gradient could be built up across the thylakoid membranes in dark-adapted algae by two different processes: (i) reverse functioning of the chloroplast ATPases that hydrolyze a pool of ATP present in the chloroplast and (ii) transfer of electrons in the chlororespiratory chains that oxidizes a pool of NAD(P)H present in the chloroplast (Bennoun, 1982). In *Phaeodactylum tricornutum,* the ΔpH generated by chlororespiration was thought to be large enough to maintain a catalytically active chloroplast ATP synthase through generation of a $\Delta\mu_{H+}$ (Field et al., 1998 and references therein).

In *Phaeodactylum tricornutum*, this electron flow and build up of an electrochemical gradient ($\Delta\mu_{H+}$) was shown to lead to a decrease in the magnitude of the dark-adapted fluorescence parameters F_o and F_m, as a consequence of non-photochemical quenching (Ting and Owens, 1993). A decrease, induced by inhibitors of chlororespiration, in the chlorophyll fluorescence yield of dark-adapted cells confirmed that inhibition of chlororespiration decreases the electrochemical gradient across the thylakoid membranes (Bennoun, 1982; Ting and Owens, 1993). This membrane potential is known to inactivate some of the PS II centers into a non-quenching state (Wilhelm and Duval, 1990). In *Euglena gracilis*, non-photochemical quenching in the dark has been found to be induced partly by chlororespiration and partly by changes in the distribution of excitation energy between the photosystems (Doege, 2000). PS II centers are reactivated by lowering of the membrane potential through inhibition of ATPases or of the chlororespiratory chain or both (Bennoun, 1982). If the flow of electrons is used not only to maintain an energized state of the membrane but also to produce ATP, then the subsequent pathway of these electrons demands an acceptor system that has a relatively high oxidation capacity in the dark. The enzyme that corresponds with these features is a cytochrome *c* oxidase, usually present in the inner membranes of mitochondria (Wilhelm and Duval, 1990).

However, chlororespiration, at least in *Chlamydomonas reinhardtii,* is now no longer thought to contribute to the thylakoid membrane potential in the dark. Instead a novel ATP-driven ion pump quite distinct from the ATPase may fulfill this role (Bennoun, 1994; Rappaport et al., 1999). The electron transfer is thought to be electrogenic and responsible for the electrochemical gradient across the thylakoid membranes when CF_0-CF_1-ATPase activity is absent (Bennoun, 1994).

Evidence for chlororespiratory electron flow and

the establishment of a proton gradient that activates diadinoxanthin de-epoxidase in the dark has been presented by Jakob et al. (1999, 2001) for *Phaeodactylum tricornutum* and *Cyclotella meneghiana* grown under prolonged dark periods (light:dark cycle 8:40 h). First, uncoupling of electron transport with NH_4Cl at the beginning of a dark period prevented non-photochemical quenching of chlorophyll fluorescence and the formation of diatoxanthin during the dark period. Secondly, inhibition of the electron- and proton-consuming terminal redox component of the chlororespiratory electron transport oxidase by addition of KCN induced a stronger non-photochemical quenching and a higher de-epoxidation state of the xanthophyll cycle. These results strongly indicate that the activation of diadinoxanthin de-epoxidase in the dark is the consequence of a chlororespiratory proton gradient. Further evidence was presented which showed that the diatoxanthin formed by the chlororespiratory proton gradient has the same efficiency in the mechanism of enhanced heat dissipation as diatoxanthin induced by a light-driven ΔpH (Jakob et al., 1999, 2001).

B. Physiological role of Chlororespiration

The physiological function of chlororespiration remains an enigma. However, examining the participation of chlororespiration in various cellular events may elucidate its role in photosynthetic organisms. Many hypotheses have been proposed including the several possibilities discussed below.

1. Carbohydrate Breakdown

Evidence is mounting that the initial stages of carbohydrate breakdown in some algae are mediated by chlororespiration and that this process is inhibited by light (Peltier and Sarrey, 1988). In this case the role for chlororespiration could be that of supplying ATP for maintenance and synthetic processes in chloroplasts in the dark, supplementing or replacing ATP in the plastids, derived from glycolysis in the cytosol entering via the dihydroxyacetone phosphate phosphoglycerate exchanger, or the adenylate transporter in the plastid envelope membrane (Raven and Beardall, 2002a). Lewitus and Kana (1995) propose that the capacity for chlororespiration may be more highly expressed in an energy-sufficient cellular environment than during energy limitation. These authors found that glucose treatments of six species of microalgae brought on a Kok effect. This suggested that the glucose enrichment stimulated chlororespiratory rates, which in turn promoted the metabolism of glucose in the dark. Sustained starch degradation and mobilization from the chloroplast in the dark would require a regenerative cycling of adenine nucleotides and phosphate and possibly maintenance of transmembrane pH differences which would regulate the activity of enzymes in the pathway(s) (Falkowski and Raven, 1997; Field et al., 1998 and references therein). Such a role for chlororespiration may be appropriate for Chlorophyta, which have storage of polysaccharides in the plastid, but may be less likely in other algal divisions, with storage polysaccharides in the cytosol. There is some evidence for a glycolytic sequence in certain members of the Chlorophyta, but little is known of the occurrence of glycolytic enzymes in different cell compartments in other algal divisions (Raven and Beardall, 2002a).

2. Energy Transduction

When algae are exposed to light/dark transitions, chlororespiratory energization of the membrane might maintain the ATP synthase in an active state in the dark and facilitate ATP synthesis upon illumination (Peltier et al., 1987; Jakob et al., 1999). This would require the formation and maintenance of a sizable $\Delta\mu_H^+$. Whether chlororespiratory activity can achieve this, however, remains uncertain. As discussed above, the evidence is now against this occurring in *Chlamydomonas reinhardtii*, although it may be the case in other species. Chlororespiratory activity in sunflower leaves (*Helianthus annuus* L.) was found to be sufficient to maintain an activated chloroplast ATP synthase/ATPase during prolonged dark periods (Field et al., 1998). For *Chlamydomonas reinhardtii*, where there is little evidence for the Ndh complex, the chlororespiratory chain may be poorly electrogenic and so contribute little to ATP synthesis in the dark (Cournac, 2000a). However, even small levels (about 0.3% of light-saturated photosynthetic electron flow) are still considered to be physiologically important. For example, Nixon (2000), suggests a role for chlororespiration in the dark recovery of plants from photoinhibition through *de novo* protein synthesis. Clearly there are marked differences between species and the potential role of chlororespiration in membrane energization in the dark requires further investigation.

3. Photoprotection

Chlororespiration could aid in protection against photoinhibition by serving as a sink for photosynthetically-generated reducing equivalents (NAD(P)H) and thereby attenuate the generation of damaging superoxide and hydroxyl radicals by reduced ferredoxin via the Mehler reaction (Peltier and Schmidt, 1991). Evidence for the latter role has been recently provided by Casano et al. (2000). Chlororespiration is thought to poise cyclic electron transport in the dark and in doing so, consume reactive species of O_2 and, eventually, decrease their production by lowering the O_2 concentration in chloroplasts. It appears that the Ndh complex may be a catalyst capable of acting as this type of emergency electron sink (Nixon, 2000). The red alga *Porphyra* has a specialized Photosystem I, in that one third of the total PS I units have been reported to be engaged in linear electron transfer from Photosystem II with the remaining two thirds being specialized for cyclic electron flow (Fork and Herbert, 1993). In this organism, when chlororespiration is inhibited by excitation of PS I, electrons may be diverted away from the chlororespiratory chain into the photosynthetic electron transport chain. Therefore, in photoacclimation of *Porphyra*, the interaction of chlororespiration and photosynthesis may be considered as a mechanism optimizing ATP-synthesis under light-limitation and NADPH-formation when illumination is saturating (Wilhelm et al., 1990).

4. Regulation

State transitions are well characterized in algae (Dominy and Williams, 1987; Fork and Herbert, 1993) and higher plants (Williams and Allen, 1987) and are associated with migration of chlorophyll-protein complexes between PS II and PSI (Chapter 13, Larkum). Strong light induces a decrease of PS II antenna size, whereas in the dark or in weak light the antenna size is increased. Analysis of fluorescence induction kinetics of light-adapted and dark/weak-light-adapted cells provides evidence that chlororespiration and light-driven electron flow are interacting in the regulation of these state transitions. When the light reactions contribute to total electron flow, the activity of chlororespiration becomes down-regulated (Büchel and Wilhelm, 1990). Chlororespiratory activity leads to the formation of a pH gradient in the dark and functions as an additional photon pumping force in the thylakoid membrane. In the case of higher plants, it has been shown that the pH-gradient regulates the apparent quantum yield of PS II by changing the rates of heat dissipation under higher quantum fluxes (Genty et al., 1989) and that the redox level of the PQ pool changes.

Chlororespiration has been examined in *Mantoniella squamata* at light intensities that were limiting for growth and at those in which growth was saturated (Wilhelm et al., 1990). The enhancement of PS II antenna in low light conditions relative to high light was considered an adaptation by this organism to switch off chlororespiration at the lowest photon flux densities possible. In *Pleurochloris meiringensis*, it was shown that with increasing light intensities, the electron flux through the PQ pool generated by chlororespiration was successively replaced by enhanced input from PS II (Büchel and Wilhelm, 1990). If photosynthetic electron transport exceeds chlororespiratory activity and ATP synthesis becomes saturated, NADPH production is enhanced by changing the energy distribution in favor of PS I (low light/dark-adapted) (Büchel and Wilhelm, 1990). Therefore in high light, when light is superabundant, NADPH-production can be optimized by a stimulation of PS I activity. Assuming that neither the pH gradient nor the redox level of the PQ pool can be the signal for low light/dark to light transitions in *Pleurochloris meiringensis* (Büchel and Wilhelm, 1990), then the ATP/NADPH ratio seems to be the decisive factor.

Bulté et al. (1990) suggested that the main role of chlororespiration is to control these state transitions. Thus, when ATP levels are low (e.g. during starvation or under prolonged anaerobic conditions), cells will pass into a condition favoring light energy input into PS I and, upon illumination, there is balanced excitation energy to both systems, electron transport and ATP synthesis.

Cells therefore regulate their metabolic needs via modulation of respiratory complexes with photosynthetic electron transport. The Ndh complex appears to have a role in recycling $NAD(P)^+$ during starch mobilization, and IMMUTANS may be required to reoxidize plastoquinol for carotenoid biosynthesis and possibly other biosynthetic steps (Nixon, 2000). In this way, the pyridine nucleotide pool and plastoquinone (which act as coenzymes in a number of metabolic steps) are regenerated (Bennoun, 1982; Fork and Herbert, 1993).

C. Effects of Environmental Factors on Chlororespiration

1. Light

The light-induced inhibition of chlororespiration has been studied in organisms such as *Chlamydomonas reinhardtii* (Peltier et al., 1987), *Pleurochloris meiringensis* (Buechel and Wilhelm, 1990), *Mantoniella squamata* (Wilhelm et al., 1990) and *Dunaliella tertiolecta* (Meunier and Popovic, 1990). Flash-induced changes in O_2 concentration can be related to the inhibition of a respiratory process. If the rather fast inhibition of respiration is insensitive to antimycin A plus salicyl hydroxamic acid (inhibitors of mitochondrial respiration) and occurs on a single flash illumination, then the related respiratory activity must occur inside the chloroplast, that is, be attributable to chlororespiration. Indeed, chlororespiration was thought to be responsible for the reduction of the PQ pool in the dark interval between flashes fired at 0.5 Hz in *Dunaliella tertiolecta* (Meunier and Popovic, 1990).

In *Chlamydomonas reinhardtii* mutants lacking Photosystem I activity and those deficient in the cytochrome b_6f complex (FuD6: Table 4) no photoinhibition of respiration was observed in the O_2 uptake signals (Peltier et al., 1987; Ravenel and Peltier, 1992). This suggests that when the photosynthetic electron transport is impaired after the plastoquinones, flash illumination induces electron transport from PS II to O_2; this transfer involves the quinones of the PQ pool but not the cytochrome b_6f complex (Ravenel and Peltier, 1991; 1992; Wilhelm and Duval, 1990). In contrast, in wild-type cells, the light-induced inhibition of chlororespiration is thought to be due to the oxidation, by PS I activity, of electron carriers common to both photosynthetic and chlororespiratory chains (Peltier et al., 1987). In addition, in wild-type *Chlamydomonas* cells, the stimulation of chlororespiration by PS II also occurs when both photosystems are active (Ravenel and Peltier, 1992), but develops slower than its inhibition by Photosystem I.

In Cyanobacteria, light also causes the inhibition of chlororespiration (Brown and Webster, 1953). However, in these organisms, no evidence has been found for a cytochrome complex involved solely in respiration, and it is generally assumed that the cytochrome b_6f complex has a dual function and is involved in both photosynthetic and respiratory chains (Hirano et al., 1980; Scherer et al., 1988).

2. Anaerobic Conditions

The non-linearity, observed at low photon flux, in photosynthesis versus irradiance curves of *Chlamydomonas*, *Chlorella vulgaris* and *Phormidium luridium* cannot be ascribed to respiration effects as they also occur under anaerobic conditions (Diner and Mauzerall, 1973). Rather, the non-linear behavior observed under anaerobic conditions was attributed to a reduction of photogenerated oxidizing equivalents, occurring between the two sequential photoreactions of photosynthesis, which in turn resulted in a reversible decrease of active oxygen-producing centers at low light intensities (Diner and Mauzerall, 1973). In addition, the effects of DCCD and antimycin A on chlororespiration were abolished only under anaerobic conditions, when all components of the electron transport chain were reduced (Table 4). Anaerobiosis prevents the oxidation of the plastoquinone pool in the light by activity of PS I in the presence of antimycin A (Wilhelm and Duval, 1990; Ting and Owens, 1993).

3. Nitrogen Limitation

When grown under nitrogen limitation, *Chlamydomonas reinhardtii* developed pronounced (ten-fold increase) chlororespiratory activity (Peltier and Schmidt, 1991) and a thylakoid plastoquinone pool that was extensively reduced when N-limited cells were dark-adapted. Also augmented was the light-sensitive respiratory activity responsible for the Kok effect, reflecting competitive inhibition of chlororespiratory electron transport by PS I (Peltier and Schmidt, 1991). In addition, thylakoids of N-limited *Chlamydomonas reinhardtii* cells were found to have reduced amounts of cytochrome b_6, cytochrome f, and light-harvesting complexes. However, the thylakoid-bound NADPH-plastoquinone-oxidoreductase (with major subunits of 51 and 17 kDa) increased seven-fold and two novel cytochromes of 34 and 12.5 kDa became highly abundant (Peltier and Schmidt, 1991). This altered thylakoid membrane composition shows that the components of photosynthetic and chlororespiratory electron transport pathways are differently regulated by N availability. Cells that are N deficient are known to accumulate large amounts of carbohydrates and lipids. Enhanced chlororespiratory activity may serve to oxidize these

carbohydrates and/or NAD(P)H and in the process synthesize ATP (Fork and Herbert, 1993) as suggested above. Therefore, enhanced capacity for chlororespiration appears to be an adaptation to nitrogen deficiency in at least *Chlamydomonas reinhardtii* (Peltier and Schmidt, 1991).

4. Temperature

Competition between the (chloro)respiratory and the photosynthetic electron transport systems in cyanobacterial and algal cells incubated at elevated temperatures (30–50 °C) has shown that the respiratory control over photosynthesis becomes significant in cells exposed to heat-stress (Lajko et al., 1997). The exact mechanism of this phenomenon is species-specific. In potato and spinach, heat stress results in an increase in F_o due to the reduction of Q_A through PQ in the dark (Yamane et al., 2000). This also depends on the light conditions during the treatment, and therefore probably on the redox level of the cells.

A lowering of temperatures may also inhibit chlororespiration. Geel (1997) showed that F_v/F_m increased in *P. tricornutum* with decreasing temperature from 20 °C to 10 °C (caused by an increase in F_m) and 20 °C to 5 °C (caused by an increase in both F_o and F_m). The increase of F_o and F_m at 5 °C could not be explained by the removal of energy dependent quenching, rather it was thought to be due to a state transition from state I to state II.

D. Evolutionary Significance

The Kok effect is a common photosynthetic trait; observed for the prokaryotic alga, *Phormidium luridium*, and for the eukaryotic alga, *Chlorella vulgaris*, which are separated by 1–2 billion years on the evolutionary time-scale (Diner and Mauzerall, 1973). Hence, chlororespiration may be regarded as a residual respiratory activity of a primitive prokaryote, presumably *Prochloron*-like organism, which is thought to be at the origin of the chloroplasts of green algae (Lockhart et al., 1999; but see Moreira et al., 2000). The absence of *ndh* genes from sequenced plastomes other than those of higher plants and Cyanobacteria (and some green algae) suggests the lateral transfer of *ndh* genes to the nucleus or deletion from the organism. Specific gene losses have occurred throughout evolution, notably the transfer of the small subunit of Rubisco from the plastid to the host nucleus of green algae, their embryophytic descendants, and the euglenoids and chlororachniophytes. All other plastids code for both subunits. This lateral gene transfer may confer some fitness to a species (Allen and Raven, 1996; Race et al, 1999; Rujan and Martin, 2001).

The chlororespiratory pathway represents only 10% of the electron flow rate involved in mitochondrial respiration of photosynthetic organisms. However, its ability to maintain the acceptors of PS II (principally the PQ pool) in an appropriate redox state in the dark and/or by producing a sufficient pH gradient to maintain the ATPases in a functional form, may make it an important step which allows cells to initiate photosynthesis efficiently during a dark-to-light transition (Peltier et al., 1987).

E. Conclusions

The original data on chlororespiration can no longer be interpreted unambiguously. Analysis of chlororespiration has been and will continue to be hindered by the low abundance of the respiratory complexes in chloroplasts and the difficulty of excluding direct and indirect effects of mitorespiration. Despite this, there are now sufficient biochemical and genetic data to support the concept of chlororespiration found in Cyanobacteria and in the plastids of algae and higher plants. The variability of the redox state of the plastoquinone pool depends on the capacities and activities of the different sources and sinks in response to the incident light (Wilhelm and Duval, 1990), and is sensitive to various environmental stimuli (Cournac et al., 2000b). The pathways for both non-photochemical plastoquinone reduction and chloroplastic O_2 uptake are diverse. Understanding the molecular basis of these and their physiological significance will be the challenge of the future.

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Chapter 9

The Water-Water Cycle in Algae

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Summary

Photoreduction of O_2 to O_2^- at PS I of thylakoids in Cyanobacteria and algae is stimulated when photon energy is in excess of photosynthesis by environmental stress such as drought, chilling, high temperature and high light intensity. Active oxygen so produced is detoxified by the scavenging enzymes including superoxide dismutase, ascorbate peroxidase, ferredoxin, monodehydroascorbate radical reductase, dehydroascorbate reductase and glutathione reductase. In these redox reactions of oxygen, the electrons generated by the photooxidation of H_2O in PS II flow to O_2 in PS I of thylakoid membranes to H_2O, which is referred to as the water-water cycle. Here, the operation of the water-water cycle in algae is reviewed and the characteristics of the scavenging enzymes of active oxygen are summarized. In contrast to higher plant chloroplasts, the algal scavenging system of H_2O_2 comes in four types. The physiological functions of the water-water cycle in algae are described.

I. Introduction

In contrast to terrestrial plants, algae are usually exposed to aquatic environments. In respect of the photosynthetic process, the most remarkable difference between terrestrial plants and algae is an availability of CO_2 (Badger and Spalding 2000). The diffusion coefficients of CO_2 and HCO_3^- in water (1.9×10^{-9} m^{-2} s^{-1} at 25 °C) are around 10^{-4} that of CO_2 in air (1.9×10^{-5} m^{-2} s^{-1} at 25 °C). When the stomata open, a high diffusion rate of CO_2 in the gas phase from the atmosphere to chloroplasts via the stomata and the intercellular space in leaf tissues allows supply of CO_2 so as not to be a limiting step of photosynthesis. When the intercellular space of leaf tissues is filled with water, for example by vacuum infiltration, CO_2 supply to chloroplasts is largely suppressed (Evans and von Caemmerer, 1996). Increased photoinhibition by either rain or fog (Ishibashi et al., 1997) also indicates suppressed diffusion of CO_2 through liquid water to chloroplasts. In an aquatic environment the hydration of CO_2 from air to water and the diffusion of CO_2 to algal cells are both very slow as compared with those in the atmosphere (Adams, 1985; Talling, 1985), and lead to deficiency of CO_2 for algal photosynthesis, especially when streaming and mixing do not occur. To adapt to such an environment algae acquired evolutionarily the CO_2-concentrating mechanism (CO_2- and HCO_3^--pumps), which is capable of taking up low inorganic carbon (Ci) in water and to accumulate it in the cells (Badger et al., 1980, 1985). By the operation of these pumps, a Ci-pool is formed in algal cells and its concentration becomes higher by 10^2–10^4 than that in aqueous environments. Consequently, the concentration of CO_2 at the carboxylation site of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) becomes saturated for the carboxylase reaction of Rubisco, which suppresses the oxygenase reaction of Rubisco. Indeed, the O_2 at the concentration equilibrated with air does not reduce the net CO_2 assimilation rate of algae, even though the relative specificity for CO_2, an indicator showing the ability to function as a carboxylase versus as an oxygenase, of algal Rubisco is lower than that of terrestrial C_3-plants (Kerby and Raven, 1985; Burns and Beardall, 1987; Beardall, 1989; Spalding, 1989; Raven et al, 1990; Johnston, 1991; Chapter 11, Raven and Beardall). Carbonic anhydrase in the algal plasmalemma, in addition to that in the cytoplasm and chloroplasts, is also the adaptation mechanism to facilitate a rapid hydration of CO_2 to form H_2CO_3 and vice versa, and a rapid uptake of Ci (Badger and Price, 1994).

As described above, the suppression of the oxygenase reaction of Rubisco by high, cellular concentrations of Ci prevents the loss of photosynthetic products via photorespiration (Roy and Andrews, 2000). In terrestrial plants, the production of active oxygen in chloroplasts is suppressed at a

Abbreviations: Asc – ascorbate; APX – Asc peroxidase; Chl – chlorophyll; Cat-Per – catalase-peroxidase; Ci – inorganic carbon; DCMU – 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; DHA – dehydroascorbate; DHAR – DHA reductase; FAD – flavin-adenine dinucleotide; FBPase – fructose 1,6-bisphosphatase; Fd – ferredoxin; FNR – Fd-$NADP^+$ oxidoreductase; GAPDH – $NADP^+$-glycelaldehyde 3-phosphate dehydrogenase; GSH – reduced glutathione; GSSG – oxidized glutathione; GR – glutathione reductase; MDA – monodehydroascorbate radical; MDAR – MDA reductase; PQ – plastoquinone; PRK – ribulose 5-phosphate kinase; PS – photosystem; Q_p – photochemical quenching of Chl fluorescence; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; SBPase – sedoheptulose 1,7-bisphosphatase; SOD – superoxide dismutase; Thio – thioredoxin; TPX – thioredoxin peroxidase

high CO_2 (Asada, 1996, 1999). When plants suffer from photon excess stress, photosynthesis is limited by CO_2 supply to chloroplasts, resulting in the overreduction of the intersystem electron carriers of thylakoids. The excess electron flux reduces O_2 at PS I to produce active oxygen (Asada, 1996; Biehler and Fock, 1996; Lovelock and Winter, 1996; Miyake and Yokota, 2000; Makinao et al., 2002; Miyake et al., 2002). Conversely, an increase in the intercellular partial pressure of CO_2 in leaves suppresses the production of active oxygen (Miyake and Yokota, 2000), which is inferred by the suppression of the electron flow to O_2 by the stimulated regeneration of $NADP^+$ for the Calvin cycle. $NADP^+$ competes with O_2 for the electrons at PS I (Furbank and Badger, 1983).

Unless O_2^- and H_2O_2 photoproduced in the thylakoids are immediately scavenged, several enzymes in the Calvin cycle are oxidatively inactivated, resulting in a decrease in CO_2-fixation (Kaiser, 1976, 1979; Fucci et al., 1983; Ishida et al., 1997, 1998). Furthermore, the H_2O_2-scavenging peroxidase ascorbate peroxidase (APX) also is inactivated by H_2O_2 (Nakano and Asada, 1987; Miyake and Asada, 1996; Mano et al., 2001), which further enhances the accumulation of H_2O_2 in chloroplasts. Thus, a high CO_2 condition protects plants from their oxidative damage by active oxygen.

Surprisingly, in algae, even though they have CO_2-concentrating mechanism, O_2^- is photoproduced in PS I at a rate comparable to that of photosynthesis (Bunt and Heeb, 1971; Radmer and Kok, 1976; Sültemeyer et al., 1987; Li and Canvin, 1997a). Although algae show high production rates of active oxygen, they do not suffer from the oxidative damage, suggesting that the high rate of O_2-dependent electron flow has a specific function in algae, different from that of plants.

This chapter deals with the photoproduction and scavenging of active oxygen in algal cells. For elucidation of the mechanism and physiological significance of active oxygen metabolism in algae, the mechanism in higher plants is useful, which is referred to as the water-water cycle (Asada, 1999). We describe here, 1) a brief overview of the water-water cycle in higher plants, 2) the operation and characteristics of the water-water cycle in algae, 3) the molecular mechanism of the algal water-water cycle, 4) four types of algal water-water cycle, and 5) physiological functions of the algal water-water cycle.

II. The Water-Water Cycle in Plant Chloroplasts

The study of active oxygen in chloroplasts started in 1951, with the finding that thylakoid membranes photoreduce O_2 to H_2O_2 (Mehler, 1951). In the 1970s the primary photoreduced product of O_2 was identified to be O_2^- (a review, Asada and Takahashi, 1987) and the following enzymes for scavenging of active oxygen have been found in chloroplasts: superoxide dismutase (SOD) (Asada et al., 1973), dehydroascorbate (DHA) reductase (DHAR) (Foyer and Halliwell, 1976), glutathione (GSH) reductase (GR) (Halliwell and Foyer, 1978), ascorbate (Asc) peroxidase (APX) (Groden and Beck, 1979; Kelly and Latzko, 1979), monodehydroascorbate radical (MDA) reductase (MDAR) (Hossain et al., 1984) and ferredoxin (Fd) for the reduction of MDA (Miyake and Asada 1992a,b, 1994). For catalyzing the photoreduction of O_2 the following mediators have been found: Fe/S center of PS I (Takahashi and Asada, 1982), Fd (Furbank and Badger, 1983), FNR (Goetze and Carpentier, 1994) and MDAR (Miyake et al., 1998).

A. Molecular Mechanism of the Water-Water Cycle

In the water-water cycle (Asada, 2000)(Fig. 1A), the electrons produced by the photooxidation of water in PS II flow to O_2 in PS I of thylakoid membranes to water. First, O_2 is reduced to O_2^- and the O_2^- disproportionates to H_2O_2 and O_2 catalyzed by SOD. The H_2O_2 then is reduced to water by APX using Asc as the electron donor. Chloroplasts have four routes for the reduction of oxidized forms of Asc produced in the APX reaction. 1) Photoreduced Fd at PS I reduces the primary oxidation product of Asc, MDA, to Asc, 2) MDAR reduces MDA to Asc using NAD(P)H as the electron donor, 3) Asc is regenerated from the spontaneous disproportionation of MDA to Asc and DHA, and 4) DHAR reduces the two-electron-oxidation product of Asc-DHA, to Asc using GSH as the electron donor. GSH required for the DHAR reaction is regenerated by GR, and NAD(P)H for MDAR and GR reactions by FNR in PS I.

B. Limitation of the Flux of the Water-Water Cycle by the Photoreduction of O_2 at Photosystem I

The rate of light-dependent $^{18}O_2$-uptake which does

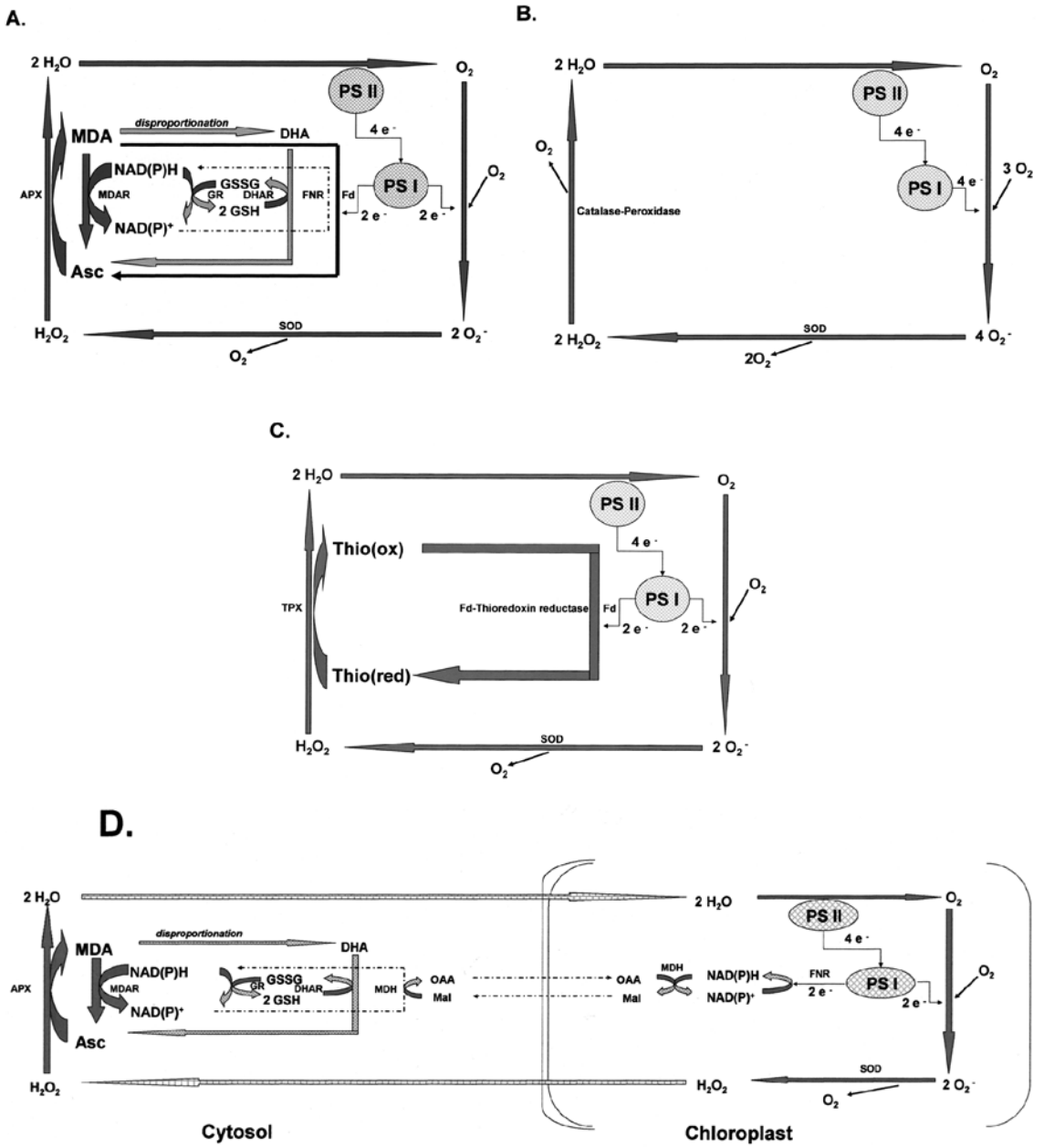


Fig. 1. Classification of the Water-Water Cycle in algae and Cyanobacteria. (A) Chloroplast APX-type water-water cycle, which consists of: photooxidation of water producing 4 electrons, the 2 electrons flow to 2 molecules of O_2 producing 2 molecules of O_2^-, superoxide dismutase (SOD) catalyzes the disproportionation of the O_2^- to H_2O_2 and O_2, ascorbate peroxidase (APX) reduces H_2O_2 to water using ascorbate (Asc) as an electron donor, ferredoxin and monodehydroascorbate radical reductase (MDAR) reduce most monodehydroascorbate radical (MDA) produced in the APX reaction to Asc, the MDA not reduced by the above reactions spontaneously disproportionates to Asc and dehydroascorbate (DHA), DHA reductase (DHAR) reduces DHA to Asc using reduced glutathione (GSH) using as an electron donor, GSH reductase (GR) reduces the oxidized glutathione (GSSG) produced in the DHAR reaction using NAD(P)H as an electron donor, Fd-$NADP^+$ oxidoreductase (FNR) regenerates NAD(P)H at PS I. All enzymes are localized in the chloroplasts. (B) Catalase-peroxidase (Cat-Per)-type water-water cycle in Cyanobacteria, where all electrons photoproduced in PS II flow to O_2 at PS I-producing O_2^-. The O_2^- disproportionates to O_2 and H_2O_2 in the SOD reaction and Cat-Per catalyzes the disproportionation of H_2O_2. SOD localizes

not depend on the oxygenase reaction of Rubisco has been evaluated in isolated chloroplasts and intact leaves (Marrsho et al., 1979; Furbank et al., 1982; Furbank and Badger, 1983). The $^{18}O_2$-uptake shows the apparent K_m for O_2 of 60–80 μM or 7–8 kPa and its maximum rate is about 80 μmol O_2 (mg Chl)$^{-1}$ h^{-1}. In watermelon leaves, the simultaneous determination of both the electron flux of PS II, as estimated by Chl fluorescence, and the electron flux needed to maintain the Calvin-Benson cycle, as calculated from the net CO_2 exchange rate, revealed the existence of a O_2-dependent electron flux, i.e. a water-water cycle. Its maximum rate is about 60 μmol O_2^- (mg Chl)$^{-1}$ h^{-1} with an apparent K_m for O_2 of about 7–8 kPa (Miyake and Yokota, 2000). These results indicate that the photoproduction of O_2^- is accounted for only by either Fd or MDAR-mediated reactions, but not by the thylakoid-bound Fe-S centers in PS I only (Furbank and Badger, 1983; Miyake et al., 1998). Further, the dependence of the activity of the water-water cycle on O_2 in the atmospheric condition shows that the limiting step of the water-water cycle is the photoreduction of O_2 at PS I (Asada, 1996; Miyake and Yokota, 2000).

C. Necessity of Scavenging of Active Oxygens in the Water-Water Cycle

O_2^- and H_2O_2 oxidize the chloroplast enzymes and inactivate their functions. Even low concentrations of H_2O_2 at around 10 μM inactivate photosynthesis to a half (Kaiser, 1976). The photoproduction rate of H_2O_2 in chloroplasts reaches 330–440 μM s^{-1}, as estimated from the production rate of O_2^- (Section II.B), therefore, unless H_2O_2 is scavenged, photosynthesis loses its activity within 20–30 ms. The enzymes of CO_2 fixation inhibited by H_2O_2 are fructose 1,6-bisphosphatase (FBPase), NADP$^+$-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribulose 5-phosphate kinase (PRK) and sedoheptulose 1,7-bisphosphatase (SBPase) (Kaiser, 1979; Tanaka et al., 1982). These enzymes have thiol groups participating in the catalytic reactions, and their oxidation to the disulfide forms by H_2O_2 converts the enzymes to the inactive forms. The oxidized form of these enzymes would be reduced to the active form by the Fd-thioredoxin system (Buchanan, 1991; Leegood et al., 1985). Under illumination, the activities of these enzymes would be determined by a balance between their oxidizing rate by photoproduced H_2O_2 and their reducing rate by the Fd-thioredoxin system.

The accumulated H_2O_2 in chloroplasts inactivates also APX. As the concentration ratio of H_2O_2 to Asc increases, the catalytic cycle of APX stops at the step for the reduction by Asc of the reaction intermediate Compound-I, and the Compound-I is irreversibly inactivated by H_2O_2 (Miyake et al., 1993; Miyake and Asada, 1996). In fact, the addition of MV to intact leaves and chloroplasts inactivates chloroplastic APX, even in the presence of Asc (Miyagawa et al., 2000; Mano et al., 2001). The inactivation of APX suppresses the turnover of the water-water cycle and increases H_2O_2 in chloroplasts.

Accumulation of both O_2^- and H_2O_2 photoproduced in chloroplasts induces further oxidative damage by generation of hydroxyl radical (·OH) via the transition metal-catalyzed Harber-Weiss reaction. ·OH has a higher redox potential than either O_2^- or H_2O_2, and can fragment DNA, proteins and lipids (Asada and Takahashi, 1987). Illumination of chloroplasts in the presence of MV produces ·OH, and inactivates and fragments Rubisco and glutamine synthetase (Fucci et al., 1983; Ishida et al., 1998).

D. Scavenging of Active Oxygens and Regeneration of Ascorbate in the Water-Water Cycle

1. Superoxide Dismutase

Plants have three isoforms of SOD: Cu,Zn-SOD, Fe-SOD and Mn-SOD (Asada and Takahashi, 1987). Cu,Zn-SOD and Fe-SOD are localized in chloroplasts (Asada et al., 1973; Jackson et al., 1978). If O_2^- is produced in chloroplasts at 660–890 μM s^{-1} ($v_p(O_2^-)$),

(*Fig. 1. Continued*) both on thylakoids and in cytosol. (C) Thioredoxin peroxidase (TPX)-type water-water cycle in Cyanobacteria, which consists of: photooxidation of water producing 4 electrons, the 2 electrons flow to 2 molecules of O_2 producing 2 molecules of O_2^-, superoxide dismutase (SOD) catalyzes the disproportionation of the O_2^- to H_2O_2, TPX reduces H_2O_2 to water using reduced thioredoxin [Thio(red)] as an electron donor, Fd-thioredoxin reductase reduces the oxidized thioredoxin [Thio(ox)] to regenerate Thio(red) at PS I using 2 electrons from water. TPX localizes in cytosol. (D) Cytosolic APX-type water-water cycle, which differs from (A) in the localization of both APX and the enzymes for the regeneration of Asc. APX, MDAR, DHAR and GR localize in the cytosol, but not in chloroplasts. Then, NAD(P)H is supplied from chloroplasts by the reaction of malate dehydrogenase (MDH) through the malate (Mal)/oxaloacetate (OAA) shuttle.

Section II.B), in the absence of SOD, O_2^- spontaneously disproportionates to H_2O_2 and O_2 with a k_{dis} of about 10^5 M^{-1} s^{-1} at pH 8.0, and the steady-state concentration of O_2^- is estimated to be 80–95 μM from $v_p(O_2^-) = k_{dis}[O_2^-]^2$. Since SOD is compartmented at 1 mM on the PS I complex (Ogawa et al., 1995), the steady-state concentration of O_2^- decreases to 330–450 pM, as estimated from $v_p(O_2^-) = k_{SOD}[O_2^-][SOD]$, where k_{SOD} is 2×10^9 M^{-1} s^{-1}. Thus, the chloroplastic SODs lower the steady-state concentration of O_2^- by 10^{-6}, which suppresses the oxidation of chloroplast components by O_2^- and reduces the production of ·OH.

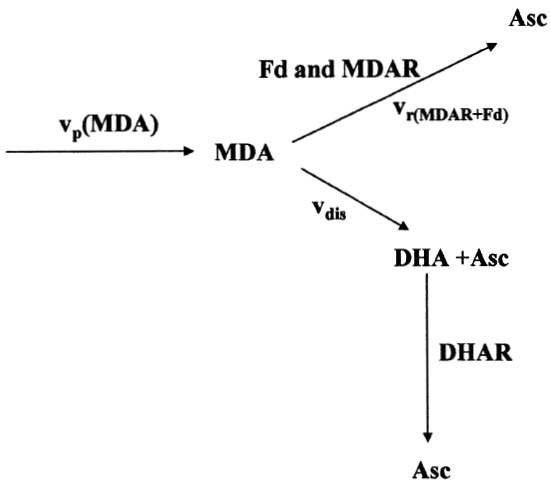
2. Ascorbate Peroxidase

In chloroplasts, APX is found in the stroma (Kelly and Latzko, 1979) and also binds to the thylakoid membranes (Groden and Beck, 1979; Miyake and Asada, 1992a,b). The thylakoid-bound APX is localized at 1 mM on the stroma thylakoids where the PS I complex exists (Asada, 1996), and reduces the H_2O_2 produced by the SOD reaction immediately to H_2O (Miyake and Asada, 1992a). The steady-state concentration of H_2O_2 in chloroplasts is estimated to be 30–40 nM from $v_p(H_2O_2) = k_1[APX][H_2O_2]$, where k_1 is 1.2×10^7 M^{-1} s^{-1} (Miyake and Asada, 1993) and Asc is assumed to be over 10 mM which is the saturated concentration for the APX reaction. Since chloroplasts usually contain Asc at 10–30 mM, APX lowers the concentration of H_2O_2 to lessen the inactivation of FBPase, PRK, GAPDH, and APX itself (Section II.C).

3. Ferredoxin, Monodehydroascorbate Reductase, Dehydroascorbate Reductase and Glutathione Reductase

For operation of the water-water cycle, Asc should be regenerated from either MDA or DHA for the APX reaction (Fig. 1A). Fd and MDAR participate in the reduction of MDA, and DHAR and GR for DHA. The relationship among the production rates of MDA [v_p(MDA)], reduction of MDA to Asc (v_r) by both Fd and MDAR and disproportionation of DHA (v_{dis}) at steady state is shown in Scheme (1).

v_p(MDA) is equal to $v_p(O_2^-)$ as expected from the stoichiometry of the water-water cycle (Fig. 1A). The reduction rates of MDA to Asc by MDAR at a saturated concentration of NAD(P)H and by the photoreduced Fd are k_{MDAR}[MDA][MDAR] and k_{Fd}[MDA][Fd], respectively, where $k_{MDAR} = 10^8$ M^{-1} s^{-1} (Sano et al., 1995) and $k_{Fd} = 10^7$ M^{-1} s^{-1} (Miyake and Asada, 1994). The spontaneous disproportionation rate of MDA, (v_{dis}), is k_{dis}[MDA]^2, where k_{dis} is 10^5 M^{-1} s^{-1} at pH 8.0 (Bielski et al., 1982). The reduction rates of MDA to Asc by Fd and MDAR, are expressed as follows:



Scheme 1. The relationship among the production rates of MDA [V_p(MDA)], reduction of MDA to Asc (V_r) by both Fd and MDAR and disproportionation of DHA (V_{dis}) at steady state. See text for details.

$$v_{r(Fd)} = [k_{Fd}[Fd]/(2k_{dis})]\{(k_{Fd}[Fd])^2 + (k_{MDAR}[MDAR])^2 + 4[v_p(MDA)k_{dis} + (k_{Fd}[Fd] + k_{MDAR}[MDAR])/2]^{1/2} - (k_{Fd}[Fd] + k_{MDAR}[MDAR])\},$$

$$v_{r(MDAR)} = [k_{MDAR}[MDAR]/(2k_{dis})]\{(k_{Fd}[Fd])^2 + (k_{MDAR}[MDAR])^2 + 4[v_p(MDA)k_{dis} + (k_{Fd}[Fd] + k_{MDAR}[MDAR])/2]^{1/2} - (k_{Fd}[Fd] + k_{MDAR}[MDAR])\}.$$

Assuming that the concentrations of MDAR and Fd in chloroplasts are about 10 and 3,000 μM, respectively, in chloroplasts (Hossain et al., 1984; Asada, 1996), $v_{r(Fd)}$, $v_{r(MDAR)}$ and v_{dis} are calculated against v_p(MDA)(Fig. 2). Over a range of v_p(MDA) estimated from both the in vivo production rate and the stoichiometry of the water-water cycle, $v_{r(Fd)}$ largely exceeds $v_{r(MDAR)}$ and v_{dis}. At the steady state, the reduction rate of MDA by MDAR and the production rate of DHA via the disproportionation of MDA is 30^{-1} and only 10^{-7} of $v_{r(Fd)}$, respectively, over the range of v_p(MDA). Thus, Fd functions for the main

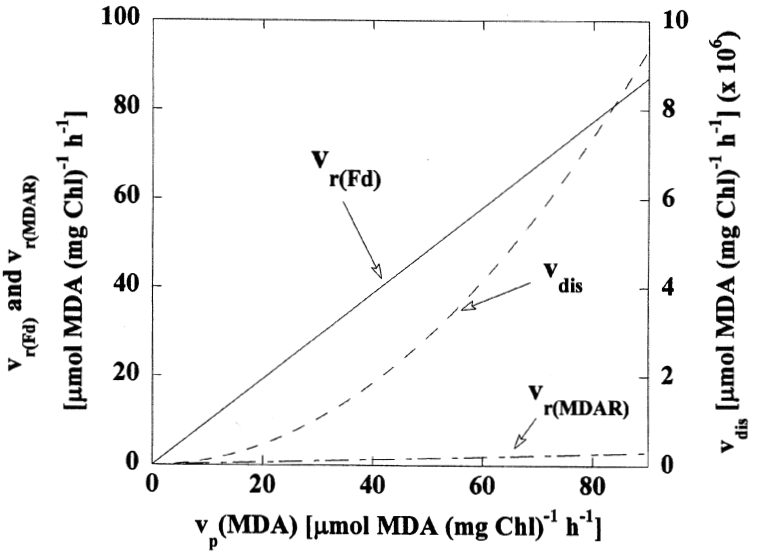


Fig. 2. The dependence of both the reduction rates of MDA by both MDAR and Fd and the disproportionation rate of MDA on the production rate of MDA [v_p(MDA)] in plant chloroplasts. The reduction rates of MDA by Fd and MDAR, $v_{r(Fd)}$ and $v_{r(MDAR)}$, and the disproportionation rate of MDA, v_{dis} are calculated as described in Section II.D.3. The figure shows that Fd functions as the main route for the regeneration of Asc over the range of v_p(MDA).

route to regenerate Asc from MDA while reduced Fd is available for MDA.

As simulated above, the production rate of DHA is low in the stroma as far as the water-water cycle effectively operates, but DHA is produced in the thylakoid lumen in the de-epoxidase reaction of violaxanthin in the xanthophyll cycle. The xanthophyll cycle is indispensable for dissipation of excess photon energy as a heat, and MDA is generated in the de-epoxidase reaction (Smirnoff 2000). Because no reducing system of either MDA or DHA is available and a low pH exists in the lumen, the MDA is rapidly disproportionated to DHA and AsA, and the DHA diffuses to the stroma (Mano et al, 1997). Thus, DHAR in the stroma would contribute to the regeneration of Asc from DHA produced in the lumen.

III. Operation of the Water-Water Cycle in Cyanobacteria and Eukaryotic Algae

Similar to plant chloroplasts, the water-water cycle operates in Cyanobacteria and eukaryotic algae, but several species of Cyanobacteria scavenge hydrogen peroxide only via its disproportionation by the catalase reaction of catalase-peroxidase.

A. Light-Dependent Uptake of O_2

When the green alga *Scenedesmus* and the cyanobacterium *Anacystis* are illuminated, they show the light-dependent uptake of $^{18}O_2$ prior to the start of CO_2-fixation, and its rate decreases accompanying an increase in CO_2-fixation (Radmer and Kok, 1976; Radmer and Ollinger, 1980). These algae take up $^{18}O_2$ even at high concentrations of CO_2 to suppress the oxygenase reaction of Rubisco (Glidewell and Raven, 1975; Radmer and Kok, 1976; Radmer and Ollinger, 1980; Peltier and Thiobault, 1985a,b). Furthermore, the addition of bicarbonate to the illuminated *Chlamydomonas reinhardtii* increases $^{18}O_2$ uptake with the stimulation of the carboxylase reaction of Rubisco (Sültemeyer et al., 1987). The uptake of $^{18}O_2$ by these algae does not saturate even at the light intensity for the maximum photosynthesis and increases further at higher intensities (Miller et al., 1988a,b, 1991; Sültemeyer et al., 1986; Li and Canvin, 1997a,b). The uptake of $^{18}O_2$ by these algae is not affected by cyanide, iodoacetamide or glycolaldehyde which inhibits photosynthesis (Glidewell and Raven, 1975; Radmer and Kok, 1976; Radmer et al., 1978; Li and Canvin, 1997b,c). Thus, the light-dependent uptake of O_2 cannot be accounted for by either photorespiration or respiration.

Algae take up O_2 via photorespiration only transiently within hours, when transferred from high to low CO_2 conditions, as observed by glycolate excretion (Nelson and Tolbert, 1969). During this period, cellular Ci increases by induction of Ci-transporter, and the ratio of Ci in cell to that in medium reaches more than 100 (Kaplan et al., 1980; Shelp and Canvin, 1984; Espie and Canvin, 1987; Espie et al., 1988; Miller et al., 1990; Badger and Price, 1992; Amoroso et al., 1998; Sültemeyer et al., 1998). Under these conditions, the rate of the carboxylase reaction of Rubisco saturates against CO_2 and the oxygenase reaction decreases (Badger, 1985). Therefore, in algae and Cyanobacteria grown even in air or low CO_2, the oxygenase reaction of Rubisco does not participate in the light-dependent uptake of O_2.

B. Photoproduction of O_2^- at Photosystem I

As discussed above, the light-dependent uptake of O_2 in algae reflects the photoreduction of O_2 at PS I of thylakoids. Actually this is inhibited by 3-(3,4-dichlorophenyl)–1,1-dimethylurea (DCMU) (Patterson and Meyers, 1973; Shiraiwa et al., 1988; Sültemeyer et al., 1993), and is not found in *Scenedesmus* which lacks PS I (Radmer and Ollinger, 1980). In Cyanobacteria, azide causes a leak of H_2O_2 from the cells (Morales et al., 1992) due to the inhibition of the catalase reaction of catalase-peroxidase (Mutsuda et al., 1996). The excreted H_2O_2 would derive from the O_2^- produced in PS I via the SOD reaction. The photoinhibition in SOD-less mutants of Cyanobacteria indicates the scavenging of O_2^- by SOD in the cells (Herbert et al., 1992; Campbell and Laudenbach, 1995).

Algae show an apparent K_m for O_2 in its photoreduction of 50–100 μM (Radmer et al., 1978; Brechignal and Andre, 1985; Canvin et al 1990; Miller et al., 1991; Li and Canvin, 1997b). These values are similar to those mediated by Fd and MDAR (Furbank and Badger, 1983; Robinson, 1988; Miyake et al., 1998), but are lower than that of the oxygenase reaction of Rubisco (1 mM)(Badger, 1980; Andrews and Abel, 1981), higher compared to those of the reduction of O_2 by cytochrome *c* oxidase (0.1 μM)(Rawsthorne and Larue, 1986) and alternative oxidase functioning in chlororespiration (0.5 μM)(Bendall and Bonner, 1971). The maximum rate of the photoreduction of O_2 in algae is 240–260 μmol O_2 (mg Chl)$^{-1}$ h^{-1} (Miller et al., 1988b; Sültemeyer et al., 1993; Mir et al., 1995a,b; Goosney and Miller, 1997). Fd and MDAR are reduced in PS I and the reduced mediators produce O_2^- by donating the electrons to O_2 (Section II). As Cyanobacteria do not show any activity of MDAR, Fd would mediate the photoreduction of O_2 to O_2^-.

C. Operation of the Water-Water Cycle in Algae

In algal and cyanobacterial cells, the light-dependent uptake of $^{18}O_2$ proceeds at the same rate as the evolution of $^{16}O_2$, under the following conditions where CO_2-fixation does not occur; a) before the photoactivation of the Calvin-Benson cycle enzymes from dark to light transition (Radmer and Kok, 1976; Radmer and Ollinger, 1980), b) at CO_2-concentrations of the compensation point, and c) in the presence of the inhibitors of the Calvin-Benson cycle (Miller and Canvin, 1987; Miller et al., 1988b; Li and Canvin, 1997b,c). This is the same stoichiometry for $^{18}O_2$ uptake and $^{16}O_2$ evolution as that observed in intact chloroplasts (Asada and Badger, 1984), where the water-water cycle operates. That is, when the water-water cycle operates in algal cells, the activity of the cycle is detectable only by using $^{18}O_2$, since it shows no net O_2-exchange.

IV. Scavenging System of O_2^- and H_2O_2 in the Algal Water-Water Cycle

As described below, the water-water cycle in algae is classified into four H_2O_2-scavenging types. Cyanobacteria have the catalase-peroxidase and thioredoxin peroxidase-types. Eukaryotic algae are grouped into two types; chloroplastic-APX and cytosolic-APX.

A. Scavenging of O_2^- by Superoxide Dismutase in Cyanobacteria

In algae, the O_2^- photoproduced in PS I is scavenged by SOD similar to higher plant chloroplasts (Asada and Takahashi, 1987; Obinger et al., 1998). The cyanobacteria *Plectonema boryanum, Nostoc verrucosum, Anabaena cylindrica, Anabaena variabilis, Spirulina platensis, Anacystis nidulans, Synechococcus 7942, Chroococciidiopsis* and *Gloeocapsa* contain either Fe-SOD and/or Mn-SOD at 10–50 μM (Abeliovich et al., 1974; Asada et al., 1975, 1977; Mishra and Keele, 1975; Lumsden et al.,

1976; Csèke et al., 1979; Okada et al., 1979; Laudenbach et al., 1989; Asada, 1994a,b; Hammouda, 1994; Caiola et al., 1996). The presence of SOD in Cyanobacteria is not surprising since even the anaerobic photosynthetic bacteria *Chromatium* and *Chlorobium* contain Fe-SOD (Kanematsu and Asada, 1978a,b), suggesting that ultra low concentrations of O_2 in the atmosphere prior to appearance of Cyanobacteria (Berkner and Marshall, 1965) was dangerous for these anaerobic bacteria and they required the effective scavenging of O_2^- for survival.

The amino acid sequence of Mn-SOD shows a high homology with that of Fe-SOD, indicating the evolution of Mn-SOD from Fe-SOD (Bannister et al., 1987; Asada, 1988, 1994a,b; Campbell and Laudenbach, 1995). Both isoforms of SOD have the same catalytic mechanism and similar molecular activity for the disproportionation of O_2^-, and complement each other (Carliotz and Touati, 1986; Laudenbach et al., 1989; Haas and Göbel, 1992; Purdy and Park, 1994; Takeshima et al., 1994). Expression of Fe-SOD encoded by *sodB* is suppressed by a limited supply of iron (Campbell and Laudenbach, 1995; Obinger et al., 1998). Mn-SOD is encoded in the gene family *sodA1*, *sodA2* and *sodA3*. The shortage of iron induces the expression of *sodA2*, which compensates the loss of Fe-SOD.

In spite of both high homology and similar reaction mechanism for scavenging of O_2^-, the molecular properties and cellular localization of Fe- and Mn-SODs are different from each other. Fe-SOD is a soluble enzyme, and a homodimer or homotetramer (Asada et al., 1975; Caiola et al., 1996; Thomas et al., 1998). Fe-SOD is found also in heterocysts of filamentous *A. variabilis* and *Anabaena cylindrica* (Bagchi et al., 1991; Canini et al., 1992). H_2O_2 inactivates Fe-SOD, but not Mn-SOD, and neither SOD is inhibited by cyanide, which is different from CuZn-SOD (Asada et al., 1975).

When cyanobacteria are illuminated with high light, the biosynthesis of SOD is induced, and such cells show a tolerance to high light (Abeliovich et al., 1974). Mn-SOD-enriched *P. boryanum* shows a resistance against light stress (Steinitz et al., 1979). These results suggest that SOD scavenges photoproduced O_2^- and protects from photoinhibition. Furthermore, the enhanced photoproduction of O_2^- by MV induces the expression of *sodA2* in *P. boryanum* (Campbell and Laudenbach, 1995). In the MV-resistant strain of *P. boryanum*, *sodA3* gene is expressed (Campbell and Laudenbach, 1995).

When cyanobacteria in the Baltic Sea *Nodularia*, *Aphanizomenon* and *Anabaena* ascend to the shallow regions and are exposed to strong light, the biosynthesis of Fe-SOD is induced, but when they are distributed in deep regions, Fe-SOD decreases (Canini et al., 1998). Thus, these cyanobacteria appear to regulate the biosynthesis of Fe-SOD in response to the photoproduction of O_2^-. *Gloeocapsa* exposed to a high temperature induces the biosynthesis of SOD (Hammouda, 1994). The addition of Cu ions to *Anabaena* cells enhances its SOD activity (Mallick and Rai, 1999), which would suppress the production of ·OH via the Cu-catalyzed Harber-Weiss reaction. Desiccation causes *Nostoc* to accumulate mRNA encoding Fe-SOD, and on rehydration, the accumulated mRNA is translated to Fe-SOD (Shirkey et al., 2000). Under desiccation stress, the cells were oxidatively damaged by active oxygen generated through both the Maillard- and Harber-Weiss reactions (Potts, 1985; Berlett and Stadtman, 1997; Henle and Linn, 1997). For terrestrial cyanobacteria, the desiccation and rehydration cycle is a daily event, and the scavenging of O_2^- by Fe-SOD would be essential to avoid photooxidative damage.

Mn-SOD binds to thylakoids and Fe-SOD occurs in the cytosol in *Plectonema* (Okada et al., 1979). Further, in *Chroococcidiopsis* Fe-SOD is compartmented on the thylakoid membranes as observed by immuno-electron microscopy (Caiola et al., 1996), which is similar to the attachment of soluble Cu,Zn-SOD on thylakoids in plant chloroplasts (Ogawa et al., 1995).

Cellular compartmentation of Fe-SOD suggests its function in the vicinity of the production site of O_2^- at PS I. The growth of *sodB*-less mutant of *Synechococcus* PCC 7942 is suppressed and the mutant suffers from photoinhibition under high O_2 or in the presence of MV. Under these stress conditions, although PS II in both the wild type and the mutant maintains its activity, only PS I is inactivated. The primary damaging site of PS I is [4Fe-4S], F_A/F_B center and Cyt *f* (Herbert et al., 1992; Thomas et al., 1998), and these components are degraded by O_2^- and ·OH (Fujii et al., 1990; Thomas et al., 1999). PS I is also inactivated in the *sodB*-less mutant at a low temperature (Thomas et al., 1999).

These results show that Fe-SOD is indispensable for the scavenging of O_2^- produced at PS I, in spite of the binding of Mn-SOD to thylakoid membranes. That is, the lowering of the steady-state concentration of O_2^- at its production site is important for protection

from oxidative damage. Photoinhibition of *sodB*-less mutant of *Synechococcus* PCC 7942 indicates that the localization of Fe-SOD at the site of O_2^- production lowers the chance of O_2^- to interact with H_2O_2.

B. Scavenging of O_2^- by Superoxide Dismutase in Eukaryotic Algae

Most eukaryotic algae contain Fe-SOD and Mn-SOD and lack Cu,Zn-SOD (Asada et al., 1977), but the green algae in which phragmoplasts appear in cell division as in vascular plants (*Chara, Nitella* and *Spirogyra*) contain Cu,Zn-SOD (Henry and Hall, 1977, Kanematsu and Asada 1989). Thus, photosynthetic organisms acquired Cu,Zn-SOD at a later evolutionary step in eukaryotic algae (Asada, 1994a; Asada and Takahashi 1987). The green alga *C. reinhardtii* contains three isoforms of Mn-SOD and a Fe-SOD (Sakurai et al., 1993; Chen et al., 1996), but not Cu,Zn-SOD (Asada et al., 1977; de Jesus et al., 1989; Egashira et al., 1989). *Euglena gracilis* also contains only Fe- and Mn-SOD (Kanematsu and Asada, 1979).

In eukaryotic algae, as in Cyanobacteria, the expression of SOD is regulated in response to environmental stress. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* increase SOD on exposure to chilling stress (Clare et al., 1984; Cho et al., 1994), and *Chlorella* shows the positive relationship between SOD activity and the tolerance to chilling stress. Sulfite or MV induces the biosynthesis of SOD in *Chlorella* (Rabinowitch et al., 1983, Clare et al., 1984; Rabinowitch and Fridovich, 1985). In the diatom *Ditylum brightwellii* grown with Cu ions, the activity of photosynthesis is low and the content of GSH decreases, whereas the activity of SOD is increased (Rijistenbil et al., 1994).

C. Scavenging Mechanism of H_2O_2 in Cyanobacteria

In the water-water cycle of algae and Cyanobacteria, the H_2O_2 produced from O_2^- by SOD is scavenged by the peroxidase and catalase reactions (Miyake et al., 1991). On addition of $H_2{}^{18}O_2$ *A. cylindrica* and *Synechocystis* PCC 6803 cells evolve $^{18}O_2$ and $^{16}O_2$ under illumination, but only $^{18}O_2$ in the dark (Fig. 3). On the other hand, *P. boryanum* and *A. nidulans* evolve only $^{18}O_2$ both in the light and dark (Fig. 3), which indicates that $H_2{}^{18}O_2$ is disproportionated via the catalase reaction ($2\ H_2{}^{18}O_2 \rightarrow 2\ H_2{}^{18}O + {}^{18}O_2$) (Miyake et al., 1991). The H_2O_2-dependent $^{16}O_2$ evolution in the light shows the reduction of H_2O_2 by a peroxidase reaction using the electron donor generated by photosynthetic electron transport, similar to plant and algal chloroplasts (Miyake et al., 1991);

$$H_2{}^{16}O \rightarrow 1/2\ {}^{16}O_2 + 2\ H^+ + 2\ e^- \quad \text{(PS II)}$$

$$H_2{}^{18}O_2 + 2\ D_{red}(e^-) \rightarrow 2\ D_{ox} + 2\ H_2{}^{18}O \quad \text{(peroxidase reaction)}$$

$$2\ D_{ox} + 2\ e^- \rightarrow 2\ D_{red}(e^-) \quad \text{(photoregeneration of electron donor at PS I)}$$

$$H_2{}^{16}O + H_2{}^{18}O_2 \rightarrow 1/2\ {}^{16}O_2 + 2\ H_2{}^{18}O$$

where, $D_{red}(e^-)$ and D_{ox} are the reduced and oxidized forms of the electron donor in the peroxidase reaction. The coupling of the reduction of H_2O_2 with the photosynthetic electron transport is also supported by both the H_2O_2-dependent photochemical quenching (Q_p) of Chl fluorescence and an increase in the quantum yield of PS II (Badger and Schreiber, 1993; Miller et al., 2000; Miyake et al., 1991).

This light-dependent peroxidase reaction is induced by low CO_2 conditions. *A. nidulans* grown in 1% CO_2 did not show any evolution of $^{16}O_2$ and H_2O_2-dependent Q_p on addition of $H_2{}^{18}O_2$ (Miyake et al., 1991), but, when grown in air, the cells show a large H_2O_2-dependent Q_p, compared to the cells grown in 5% CO_2 (Badger and Schreiber, 1993).

Thus far, it has remained a question whether or not Cyanobacteria have APX (Miyake et al., 1991; Tel-Or et al., 1985, 1986). The activity of APX detected is non-enzymatic and no Asc is found in Cyanobacteria. The genome from *Synechocystis* PCC 6803 does not encode the gene for APX (Tichy and Vermaas, 1999). No activity of GSH peroxidase is detected though the genome encodes its gene (Tel-Or et al., 1985; Miyake et al., 1991). GRs are purified from *Anabaena* PCC 7120, *Spirulina maxima* and *Nostoc muscorum* (Serrano et al., 1984; Bhunia et al., 1993; Jiang et al., 1995; Rendon et al., 1995; Jiang and Mannerik, 1999). Since Cyanobacteria have neither APX or DHAR activity, and the genome of *Synechocystis* PCC 6803 does not have the gene showing the homology with that of DHAR (Tichy and Vermaas, 1999), GR probably functions for the

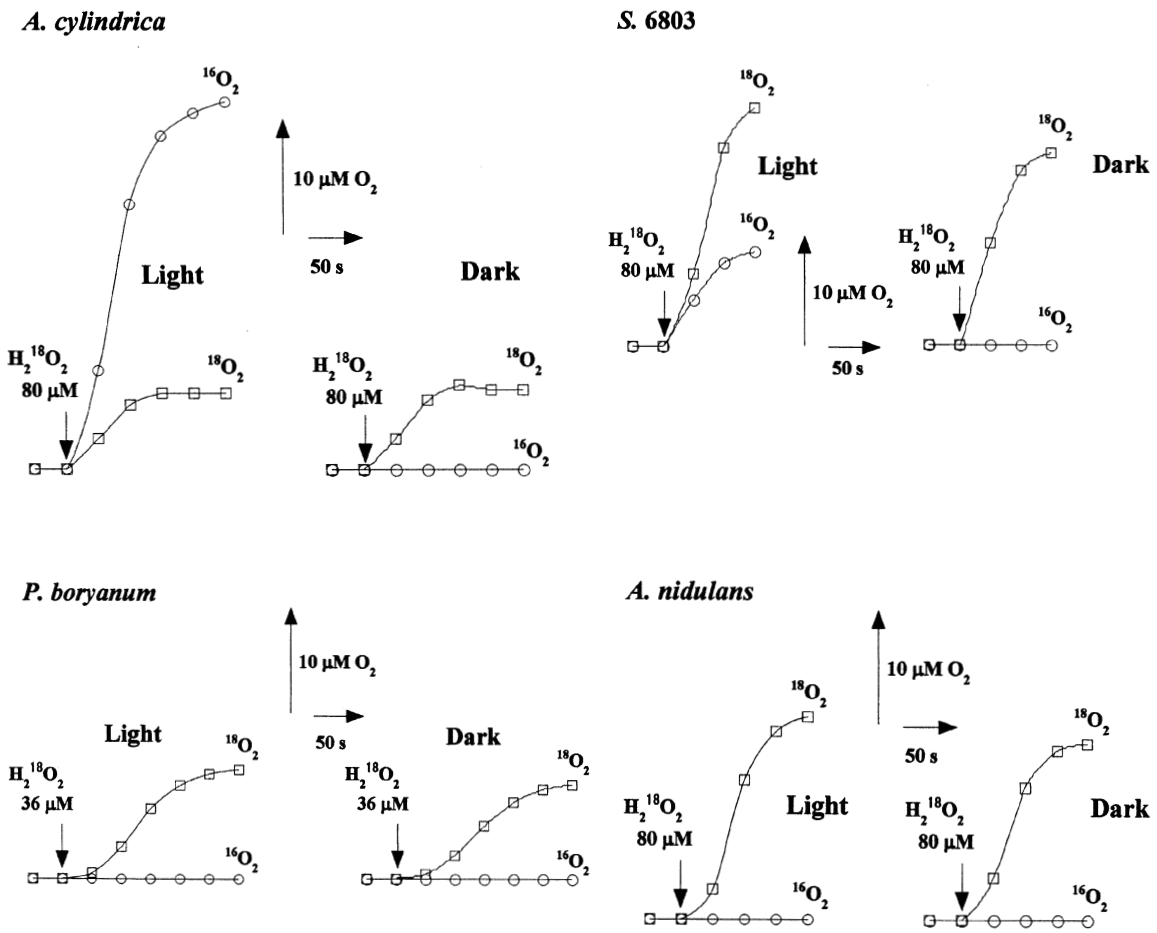


Fig. 3. $H_2{}^{18}O_2$–dependent evolution of $^{16}O_2$ and $^{18}O_2$ in *Anabaena cylindrica*, *Synechocystis* 6803, *Anacystis nidulans* and *Plectonema boryanum*. $H_2{}^{18}O_2$ (*A. cylindrica*, *S.* 6803, *A. nidulans*, 80 μM; *P. boryanum*, 36 μM) was added at indicated times in the light or dark. With the disappearance of 80 μM $H_2{}^{18}O_2$, 33 μM $^{16}O_2$ (*A. cylindrica*) and 9.5 μM $^{16}O_2$ (*S.* 6803) were generated in the light. Thus, 83% (*A. cylindrica*) and 24% (*S.* 6803) of the decomposition of hydrogen peroxide was accounted for by the peroxidase-catalyzed reaction linked to the photosynthetic electron transport. The amount of $^{18}O_2$ evolved was 7 μM (*A. cylindrica*) and 22 μM (*S.* 6803) in both the light and dark. Thus, 83% (*A. cylindrica*) and 24% (*S.* 6803) of hydrogen peroxide was decomposed by the catalase-catalyzed reaction. On the other hand, in *A. nidulans* and *P. boryanum* $H_2{}^{18}O_2$–dependent evolution of $^{16}O_2$ in the light was not observed, but only the evolution of $^{18}O_2$ was done. The decomposition of $H_2{}^{18}O_2$ with the evolution of $^{18}O_2$ was also detected in the dark. Thus, both *A. nidulans* and *P. boryanum* scavenge hydrogen peroxide by the catalase-catalyzed reaction. All data were revised from Miyake et al. (1991).

antioxidative action of GSH, not in the regeneration of Asc.

1. Thioredoxin Peroxidase

In addition to the H_2O_2-scavenging peroxidase in Cyanobacteria, thioredoxin peroxidase has recently been identified. In *Synechocystis* PCC 6803 the gene homologous to that of 2-cysteine-peroxidase (2-CysPX) in animal and fungi (Chae et al., 1993, 1994a,b, c) has been found (Klughammer et al., 1998). Thiol groups in 2-CysPX (28 kDa) reduce H_2O_2 to water.

$$H_2O_2 + 2\ [(2\text{-CysPX})\text{-SH}] \rightarrow 2\ H_2O + [(2\text{-CysPx})\text{-S-S-}(2\text{-CysPx})]$$

The oxidized 2-CysPX, [(2-CysPx)-S-S-(2-CysPx)], is reduced by reduced thioredoxin which is generated by reduced Fd-thioredoxin reductase. Thus,

2-CysPX can be regarded as thioredoxin peroxidase (TPX) and the scavenging of H_2O_2 by TPX is coupled with photosynthetic electron transport (Yamamoto et al., 1999). TPX from *Synechocystis* PCC 6803 reduces not only H_2O_2, but also alkyl hydroperoxide, *t*-butyl hydroperoxide, similar to GSH peroxidase. The scavenging activity of H_2O_2 by TPX is high; a k_{cat}/K_m for H_2O_2 of 1.4 x 10^7 M^{-1} s^{-1}. TPX-less strain of *Synechocystis* PCC 6803 does not show any H_2O_2-dependent q_P of Chl fluorescence and light-dependent scavenging of H_2O_2 (Yamamoto et al., 1999). These results show that TPX is the H_2O_2-scavenging peroxidase in Cyanobacteria, similar to APX in higher plant chloroplasts.

When the TPX-less mutant of *Synechocystis* PCC 6803 is exposed to high light, the activity of photosynthetic electron transport and viability is lost (Klughammer et al., 1998). This shows that TPX is requisite for the scavenging of H_2O_2 and that alkyl hydroperoxide is produced under strong light.

2. Catalase-Peroxidase

Catalase-peroxidase (Cat-Per) is a bifunctional, heme enzyme catalyzing both the catalase and peroxidase reactions (Hochman and Shemesh, 1987). The enzyme from *S.* PCC 7942 is a homodimer consisting of a 79-kDa subunit and each subunit has one proto-heme IX, and is inhibited by cyanide and azide. The N-terminal amino acid-sequence of Cat-Per from *S.* PCC 7942 shows a high homology with those of both hydroperoxidases from *Escherichia coli* and *S. typhimurium*, and Cat-Per from *Mycobacterium intracellular, M. tuberculosis* and *Rhodobacter capsulatus* (Mutsuda et al., 1996; Obinger et al., 1997). However, the domain of the distal His of Cat-Per from *S.* PCC 7942 does not have any homology with that of Cat-Per from other organisms, and rather has a homology with that of APX from higher plants and cytochrome *c* peroxidase from yeast (Mutsuda et al., 1996), suggesting that Cat-Per of Cyanobacteria might be the ancestor for APX and cytochrome *c* peroxidase.

Differing from Cat-Per from other organisms, the enzyme from Cyanobacteria prefers aromatic pyrogallol and *o*-dianisidine as the electron donor, but not NAD(P)H (Mutsuda et al. 1996). Its catalase reaction has a K_m for H_2O_2 of 4.2 mM and a k_{cat} of 2.6×10^4 s^{-1} (Tichy and Vermaas, 1999), which are similar to those of catalase from tobacco and pumpkin (Yamaguchi et al., 1986; Havir and McHale, 1990; Mutsuda et al., 1996).

Thus, in Cyanobacteria, H_2O_2 photoproduced at PS I is scavenged by either TPX or catalase reaction. The TPX mainly functions in the light-dependent reduction of H_2O_2, and Cat-Per would catalyze the catalase reaction. For the peroxidase reaction of Cat-Per in the cells, the physiological electron donor has not been identified.

D. Scavenging Mechanism of H_2O_2 in Eukaryotic Algae

Similar to Cyanobacteria, Chlorophyta scavenge H_2O_2 by the reactions of both catalase and peroxidase. In illuminated *Chlamydomonas*, almost all of H_2O_2 was scavenged by the peroxidase reaction (Miyake et al., 1991), in which APX and related enzymes are localized in the chloroplasts (Yokota et al., 1988; Miyake et al., 1991; Shigeoka et al., 1991; Serrano and Llobell, 1993; Sültemeyer et al., 1993; Takeda et al., 1993, 1997). This is also the case for halotolerant *Chlamydomonas* W80 (Takeda et al., 2000). These enzymes are induced on the transition of *Chlamydomonas* from high CO_2 to low CO_2 conditions (Sültemeyer et al., 1993). As described below, the enhancement of their activities reflects that the water-water cycle supplies ATP for concentrating Ci in the low-CO_2-grown cell. APX disappears on addition of selenite to the cells and GSH peroxidase increases (Yokota et al., 1988; Takeda et al., 1997). GSH peroxidase contains selenocysteine residue in its reaction center (Rotruck et al., 1973; Yokota et al., 1988; Takeda et al., 1997). However, since GSH peroxidase is localized in cytosol (Takeda et al., 1997), it remains to be solved how the cells growing in the selenite medium scavenge H_2O_2 in chloroplasts. Similar to Cyanobacteria, TPX would scavenge H_2O_2 in chloroplasts of *Chlamydomonas*.

APX from *C. reinhardtii* is a monomer of 34 kDa and has K_ms for H_2O_2 of 25 μM and for Asc of 5 μM, and its k_{cat} is 9.64 s^{-1} (Takeda et al., 1997). It can reduce *t*-butyl hydroperoxide and cumene hydroperoxide, similar to that from *E. gracilis*. Its N-terminal amino acid-sequence does not have any homology with those of APXs from higher plants and *E. gracilis*, but it loses the activity in the absence of Asc, similar to that from higher plant chloroplasts (Takeda et al., 1997). *C. reinhardtii* has two isoforms of GR; one is localized in the cytosol and the other in the chloroplasts (Serrano and Llobell, 1993; Takeda et al., 1993). Both isoforms have FAD and its molecular weight is 127 kDa (Takeda et al., 1993).

The red alga *Galdieria partita* from acidic hot

springs has two isoforms (APX-A and APX-B) and both are monomeric hemoproteins of about 28 kDa (Sano et al., 2001). The K_ms for H_2O_2 is 100 μM for both isoforms and 355 μM Asc for APX-A, 174 μM for APX-B. They can reduce also t-butyl hydroperoxide using Asc as the electron donor. The amino acid sequences of N-terminal of both the isoforms have a homology with each other, but not with those from other organisms. In contrast to APXs from *C. reinhardtii* and higher plant chloroplasts, *G. partita* APXs show a tolerance to H_2O_2 in the absence of Asc.

E. Scavenging Mechanism of H_2O_2 in Euglena

E. gracilis does not show any catalase activity, but the light-dependent scavenging of H_2O_2, similar to plant chloroplasts, is deduced from the fact that no $^{18}O_2$ is evolved from $H_2{}^{18}O_2$ and the stoichiometric evolution of $^{16}O_2$ on addition of $H_2{}^{18}O_2$ (Miyake et al., 1991). APX is localized in the cytosol, but not in chloroplasts (Shigeoka et al., 1987; Ishikawa et al., 1993). Then, H_2O_2 appears to leak from the chloroplasts to the cytosol and then is scavenged (Ishikawa et al., 1993), since MDAR, DHAR and GR are also compartmented in the cytosol (Shigeoka et al., 1980, 1987; Montrichard et al., 1999). NAD(P)H for the regeneration of Asc by MDAR and GR would be transported from the chloroplasts via the malate-oxaloacetate shuttle (Heineke et al., 1991; Backhausen et al., 1994). Otherwise, as in Cyanobacteria, TPX might scavenge H_2O_2 in *Euglena* chloroplsasts.

Euglena APX is a monomer of 58 kDa and contains protoheme IX (Ishikawa et al., 1996a). Its amino acid-sequence in the N-terminal does not have any homology with those from plant APXs, but the sequence around the distal heme domain shows a high similarity. The activity is lost in the absence of Asc, and the stability is similar to that of the plant enzymes (Miyake et al., 1991; Ishikawa et al., 1996b). In addition to H_2O_2, *Euglena* APX could reduce lipid hydroperoxide. Actually, lipid peroxidation is enhanced in the APX-less *E. gracilis* grown under Fe-deficient conditions (Ishikawa et al., 1993).

F. Four Types of the Water-Water Cycle

In Cyanobacteria, SOD catalyzes the disproportionation of the O_2^- produced in the thylakoids to O_2 and H_2O_2. Cat-Per disproportionates the H_2O_2 to H_2O and O_2, and TPX reduces the H_2O_2 to H_2O. In eukaryotic algae, SOD in chloroplasts scavenges O_2^- as in Cyanobacteria, and either chloroplastic and/or cytosolic APXs reduce H_2O_2 to H_2O by Asc. Then, the water-water cycle in Cyanobacteria and algae is classified into four types, from the point of H_2O_2-scavenging: 1) catalase-type water-water cycle where Cat-Per scavenges H_2O_2; 2) TPX-type water-water cycle where TPX reduces H_2O_2; 3) cytosolic APX-type water-water cycle where cytosolic APX reduces H_2O_2; and 4) chloroplastic APX-type water-water cycle where chloroplastic APX reduces H_2O_2, similar to higher plant chloroplasts.

1. Cat-Per-*type water-water cycle* (Fig. 1B). In the cyanobacteria *A. nidulans* and *P. boryanum*, Cat-Per scavenges H_2O_2 produced in the water-water cycle (Section IV.C).

$$2\ H_2{}^{16}O \rightarrow {}^{16}O_2 + 4\ H^+ + 4\ e^- \qquad \text{(PS II)}$$

$$4\ {}^{18}O_2 + 4\ e^- \rightarrow 4\ {}^{18}O_2^- \qquad \text{(Photoreduction of } O_2 \text{ at PS I)}$$

$$4\ {}^{18}O_2^- + 4\ H^+ \rightarrow 2\ H_2{}^{18}O_2 + 2\ {}^{18}O_2 \qquad \text{(SOD)}$$

$$2\ H_2{}^{18}O_2 \rightarrow 2\ H_2{}^{18}O + {}^{18}O_2 \qquad \text{(Cat-Per)}$$

$$2\ H_2{}^{16}O + {}^{18}O_2 \rightarrow 2\ H_2{}^{18}O + {}^{16}O_2$$

2. TPX-type water-water cycle (Fig. 1C). In the cyanobacterium *A. nidulans* grown at low CO_2, *A. cylindrica* and *S. 6803*, TPX scavenges H_2O_2 produced in the water-water cycle (Section IV.C).

$$2\ H_2{}^{16}O \rightarrow {}^{16}O_2 + 4\ H^+ + 4\ e^- \qquad \text{(PS II)}$$

$$2\ {}^{18}O_2 + 2\ e^- \rightarrow 2\ {}^{18}O_2^- \qquad \text{(Photoreduction of } O_2 \text{ at PS I)}$$

$$2\ {}^{18}O_2^- + 2\ H^+ \rightarrow H_2{}^{18}O_2 + {}^{18}O_2 \qquad \text{(SOD)}$$

$$H_2{}^{18}O_2 + 2\ \text{Thio(red)} \rightarrow 2\ \text{Thio(ox)} + 2\ H_2{}^{18}O \qquad \text{(TPX)}$$

$$2\ \text{Thio(ox)} + 2\ e^- + 2\ H^+ \rightarrow 2\ \text{Thio(red)} \qquad \text{(Regeneration of Thioredoxin)}$$

$$2\ H_2{}^{16}O + {}^{18}O_2 \rightarrow 2\ H_2{}^{18}O + {}^{16}O_2$$

Where, Thio(red) and Thio(ox) represent the reduced and oxidized thioredoxins.

3 and 4. Chloroplast and cytosolic APX-type water-water cycle (Fig. 1A and 1D). Chloroplast APX-type is observed in green alga *C. reinhardtii* and plant chloroplasts, and Cytosolic APX-type in *E. gracilis* (Section IV.C).

$$2\ H_2{}^{16}O \rightarrow {}^{16}O_2 + 4\ H^+ + 4\ e^- \qquad \text{(PS II)}$$

$$2\ {}^{18}O_2 + 2\ e^- \rightarrow 2\ {}^{18}O_2^- \qquad \text{(Photoreduction of } O_2 \text{ at PS I)}$$

$$2\ {}^{18}O_2^- + 2\ H^+ \rightarrow H_2{}^{18}O_2 + {}^{18}O_2 \qquad \text{(SOD)}$$

$$H_2{}^{18}O_2 + 2\ \text{Asc} \rightarrow 2\ \text{MDA} + 2\ H_2{}^{18}O \qquad \text{(Chloroplast or cytosolic APX)}$$

$$2\ \text{MDA} + 2\ e^- + 2\ H^+ \rightarrow 2\ \text{Asc} \qquad \text{(Regeneration of Asc)}$$

$$2\ H_2{}^{16}O + {}^{18}O_2 \rightarrow 2\ H_2{}^{18}O + {}^{16}O_2$$

V. Physiological Functions of the Water-Water Cycle in Cyanobacteria and Eukaryotic Algae

Four physiological functions of the water-water cycle in higher plant chloroplasts have been proposed; i) supply of ATP for the operation of both photosynthesis and photorespiration, ii) generation of ΔpH for the down-regulation of PS II, iii) dissipation of excess photon energy under the limited CO_2-assimilating conditions, and iv) the scavenging of active oxygen produced in chloroplasts (Asada, 1999, 2000). In algae the water-water cycle has, at least, one other physiological function: additional production of ATP required for the formation of Ci-pool in the cells.

A. Formation of Inorganic Carbon-Pool in Cells

The electron flux generated through the water-water cycle could supply ATP for the formation of Ci-pool in algal cells. The uptake of Ci in Cyanobacteria and green algae accompanies the photoreduction of O_2 (Miller et al., 1988b; Canvin et al., 1990; Sültemeyer et al., 1993). In the presence of the photosynthesis inhibitor iodoacetamide or glycolaldehyde Ci addition stimulates the uptakes of both Ci and $^{18}O_2$ in the light, with simultaneous evolution of $^{16}O_2$ from $H_2{}^{16}O$ and photochemical quenching of Chl fluorescence (Sültemeyer et al., 1987; Miller et al., 1988b; Li and Canvin, 1997a,b,c). These results show that Ci is coupled to the ATP generated via the water-water cycle. In *Hydrodictyon africanum* light-dependent uptake of O_2 accompanies the production of ATP (Raven and Glidewell, 1975).

In the cyanobacterium *Synechococcus* UTEX 625, when photosynthesis is inhibited, the maximum rate of CO_2 uptake for the Ci-pool is about 560 μmol CO_2 (mg Chl)$^{-1}$ h^{-1} (Miller et al., 1988a,b, 1989, 1991). In order to support this rate, ATP should be produced at 560 μmol (mg Chl)$^{-1}$ h^{-1}, since the uptake of one molecule of CO_2 requires one molecule of ATP (Gimmler et al., 1990; Spalding and Portis, 1985; Sültemeyer et al., 1993). During the formation of the Ci-pool, the O_2-uptake rate for the water-water cycle is 240 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, corresponding to the electron flux of 960 μmol e^- (mg Chl)$^{-1}$ h^{-1}. If the Q-cycle functions at a H^+/e ratio of 3 and 4, the production rate of ATP through the water-water cycle is 960 and 720 μmol (mg Chl)$^{-1}$ h^{-1}, respectively. In either case, enough ATP is produced for the formation of the Ci-pool through the water-water cycle.

C. reinhardtii also induces the Ci-transporter under low CO_2 conditions. During the induction of its transporter, the activities of enzymes in the water-water cycle increase to 4–20-fold higher than those under high CO_2 conditions, suggesting the production of ATP via the water-water cycle for the formation of Ci-pool (Sültemeyer et al., 1993).

B. Maintenance of Photosynthesis

In Cyanobacteria the presence of air-O_2 enhances CO_2 assimilation as compared with that under lower O_2 concentrations (Miyachi and Okabe, 1976; Li and Canvin, 1997b). As the concentration of CO_2 in cyanobacterial and algal cells saturates for the carboxylase reaction of Rubisco, the increase in the net rate of CO_2-uptake suggests that the rate of photosynthesis is limited by the regeneration of RuBP for Rubisco. In the Calvin-Benson-cycle of photosynthesis, the fixation of one molecule of CO_2 requires two molecules of NADPH and three molecules of ATP (Farquhar et al., 1980; von Caemmerer and Farquhar, 1981). Unless cyclic photophosphorylation operates, ATP produced by the photosynthetic electron transport is insufficient for the regeneration of RuBP to the saturated concentration against Rubisco. The requirement of

O_2 for the full activity of photosynthesis suggests that the shortage of ATP is compensated by the water-water cycle.

C. Dissipation of Excess Photon Energy

When plants are exposed to more photon energy than that required for CO_2-assimilation, PS II suffers from photoinhibition (Demmig-Adams and Adams III, 1992; Osmond and Grace, 1995; Chapter 16, Franklin et al.). The molecular species giving damage to PS II is ^{3}P680*, which reacts with O_2 to produce singlet oxygen, $^1O_2$ (Asada 1996). In fact, under these conditions, the $^1O_2$ produced in PS II degrades D1 protein (Hideg et al., 1994a,b; Chapter 13, Larkum). In algae also a similar photoinhibition has been observed (Kok, 1956; Kyle et al., 1984; Roudyk et al., 1996; He et al., 1997; Zhang et al., 1999), which is due to the production of ^{3}P680* by limitation of the usage of photon energy.

The presence of O_2 makes possible the extra electron flux through the water-water cycle. Enhancement of photoinhibition under anaerobiosis (Asada and Takahashi 1987) indicates that the presence of O_2 contributes to suppression of the production of ^{3}P680* by stimulating the electron flux through the water-water cycle and dissipation of the excess photon energy.

D. Scavenging of Active Oxygen: Protection of Target Molecules

As discussed above, the electron flux to oxygen in thylakoids is required for algal photosynthesis, but active oxygen is inevitably produced, even under moderate light intensity. The physiological functions listed above are operative only when the active oxygen is effectively scavenged to protect the target molecules from oxidative attack. To elucidate the algal target molecules of active oxygen resulting in photoinhibition, mutants lacking SOD and H_2O_2-scavenging enzymes have been employed. In the Fe-SOD-less mutant of *Synechococcus* PCC 7942, [4Fe-4S], the F_A/F_B center of PS I and Cyt *f* is degraded under light and its photosynthetic activity is lost (Fuji et al., 1990; Herbert et al., 1992; Thomas et al., 1999). In the Cat-Per/TPX-less mutant of *Synechocystis* PCC 6803, de novo synthesis of D1 protein in PS II is suppressed. It has been claimed that the H_2O_2 accumulated in the mutant, inactivates the translation of *psbA* mRNA, and PS II activity cannot recover (Nishiyama et al, 2001). Thus, prompt scavenging of O_2^- and H_2O_2 is indispensable for the maintenance of algal photosynthesis.

In contrast to the Calvin-Benson-cycle enzymes in higher plants FBPase, GAPDH, PRK and SBPase (Section II.C), the algal enzymes are tolerant to oxidative attack by H_2O_2 because they lack the H_2O_2-sensitive Cys residue (Takeda et al., 1995; Tamoi et al., 1996a,b; Tamoi et al., 1998). The plant enzymes contain H_2O_2-sensitive Cys-residues and the inactivate enzymes recover their activities through the Fd-thioredoxin system (Section II.C). In addition to the Calvin-Benson-cycle enzymes, algal APX also is tolerant of H_2O_2 as compared with plant APX (Sano et al., 2001). Thus, in algae, the Calvin-Benson-cycle enzymes and APX would not be the targets of active oxygen.

VI. Concluding Remarks

The study of the production and scavenging of active oxygen in higher plant chloroplasts was initiated by the discovery of the Mehler reaction. It has extended to the elucidation of its molecular mechanisms and established the water-water cycle (Asada 1999). During this period, the molecular mechanism in Cyanobacteria and algae has been also clarified. Although the operation of the water-water cycle in algae has been shown, as described in this chapter, differences between the water-water cycle of higher plant chloroplasts and algae have appeared. First, the activity of the water-water cycle in algae is higher than that in higher plant chloroplasts (Section III), which is due to the difference of the growth environments between the terrestrial and aquatic photosynthetic organisms. Because algae live in aquatic habitats, the diffusion of Ci dissolved in water to the cells limits photosynthesis, in contrast to the acquisition of CO_2 through mainly gas phases, for the carboxylation of ribulose-1,5-bisphosphate by Rubisco. To adapt to such environments, algae evolved the CO_2-concentrating mechanism. The concentrating of CO_2 in the algal cells requires ATP, but the linear electron transport to $NADP^+$ for photosynthesis cannot supply enough. The O_2-dependent large electron flux in the water-water cycle is a prerequisite for supporting the extra supply of ATP to algal photosynthesis (Sections III and IV). Second, algal water-water cycles have diverse methods for the scavenging system of H_2O_2

(Section III). In higher plants, the major SOD, including that in chloroplasts, is the CuZn-containing enzyme, but in algae Fe- and/or Mn-SOD are the major scavenging enzymes of O_2^-. In higher plant chloroplasts, H_2O_2 is scavenged by APX. On the other hand, in Cyanobacteria Cat-Per and TPX scavenge H_2O_2, and in eukaryotic algae this is done by chloroplastic and cytosolic APXs. The scavenging of H_2O_2 by both TPX and APX is coupled with photosynthetic electron transport to regenerate the electron donors for both the peroxidases. On the basis of these scavenging mechanisms of H_2O_2, the cyanobacterial and algal water-water cycle is classified into four types (Section III). The reason why algae have diversity in the scavenging of H_2O_2 has not been clarified. During the evolution of photosynthetic organisms from Cyanobacteria, APX was acquired and incorporated in the water-water cycle for the scavenging of H_2O_2. The activity of APX in plant chloroplasts is sensitive to H_2O_2 and the APX loses its activity in the absence of the electron donor Asc (Section II). It remains unclear why higher plant chloroplasts acquired such unstable APX in the water-water cycle.

Although the photoreduction of O_2 in the water-water cycle is indispensable for its physiological function (Section V), it produces active oxygen. Therefore, the scavenging system of active oxygen protects chloroplasts from oxidative injury and at the same time supports the physiological functions of the water-water cycle (Section III). On exposure to high light or low CO_2 conditions, the photon energy exceeds utilization by photosynthesis and the requirement for the acquisition of CO_2 increases. Under these conditions, the rate of the photoreduction of O_2 through the water-water cycle is enhanced, resulting in the stimulation of the production of O_2^- and H_2O_2. However, the induced expression of scavenging enzymes suppresses the oxidative damage (Sections IV and V). In the future, the molecular mechanism signaling expression of these active oxygen-scavenging enzymes under oxidative stress needs to be solved for further understanding of the water-water cycle in algae.

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Chapter 10

Carbohydrate Metabolism and Respiration in Algae

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Summary

Carbohydrates and their immediate derivatives have a number of roles related to photosynthesis in algae (including Cyanobacteria). In the form of phosphorylated sugars, carbohydrates are major intermediates in the photosynthetic carbon reduction cycle and the photorespiratory carbon oxidation cycle. Carbohydrates are common energy and carbon storage products in algae, permitting imbalances between the rate of reduced carbon production in photosynthesis and the rate of reduced carbon consumption in growth. Stored carbohydrates also allow dark survival for species-specific time periods. Sugars and sugar alcohols are compatible solutes in many algae, and also function, like the sugar oxidation product ascorbate, as free radical scanvengers. Structural polysaccharides in algal cell walls are important in containing turgor, and in permitting multicellularity.

Algal respiration, encompassing the very widespread glycolytic and oxidative pentose phosphate pathways,

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the tricarboxylic acid cycle and oxidative phosphorylation, has been less comprehensively studied than has algal photosynthesis. The available information suggests that algal 'dark respiration' processes resemble those in many other organisms and are essential for growth and maintenance processes. However, many algae have an alternative oxidase as well as cytochrome oxidase, as do higher plants and many other non-metazoans, and some algae have an incomplete tricarboxylic acid cycle. All of the 'dark respiration' pathways function in growth in the absence of light. In the light some of the functions of respiration, i.e. the provision of ATP and NADPH for growth processes, can be partly replaced by photosynthetic production of these cofactors. However, the 'dark respiration' pathways have an essential role in the light in providing a number of carbon skeletons (e.g. 2-oxoglutarate) required for growth.

I. Introduction

In selecting the topics to cover in a contribution on carbohydrate metabolism and respiration to a book on algal photosynthesis our guiding principles were as follows.

In considering carbohydrate metabolism we deal with compounds other than those in the photosynthetic carbon reduction cycle, i.e. intermediates between photosynthesis and respiration (including storage compounds) compatible solutes, protective agents, and structural components. We especially deal with those carbohydrates that interact fairly directly with photosynthesis other than acting as end-products of photosynthesis.

Respiration is, in gross chemical terms, the reverse of photosynthesis, and thus decreases the organic carbon gain in gross photosynthesis. The rationale for considering respiratory processes in a book on photosynthesis can be stated briefly. For photorespiration, i.e. the reactions necessary to convert the products of Rubisco oxygenase activity into carbohydrates or carbon dioxide (or excreted glycolate) it is clear that the removal of phosphoglycolate is essential in the short-term (minutes) to prevent inhibition of carbon dioxide fixation by phosphoglycolate even if the sequestration of phosphate can be overcome (Raven, 1997c). Accordingly, it is essential to consider the means of dealing with phosphoglycolate production when considering photosynthesis, even in organisms which have minimal phosphoglycolate production as a result of very high external CO_2 concentrations, very high CO_2/O_2 selectivity factors for Rubisco, or very high CO_2 accumulation ratios (internal/external), or a combination of these (Raven, 1997b,c; Raven, Kübler and Beardall, 2000).

Dark respiratory processes, i.e. glycolysis, the tricarboxylic acid cycle, oxidative phosphorylation and the oxidative pentose phosphate pathway, are all essential for photolithotrophic growth, at least when the conversion of photosynthate (carbohydrate or, less commonly, lipid) into the full range of cellular components (nucleic acids, proteins, lipids, structural polysaccharides) goes on in the dark phase of the diel light-dark cycle as well as in the light phase (Raven and Beardall, 1981; Raven, 1984a; Beardall and Raven, 1990). The organic carbon skeletons produced in the oxidative pentose phosphate cycle can also be produced in the light from the photosynthetic carbon reduction cycle. Furthermore, the NADPH and ATP from the oxidative pentose phosphate cycle and oxidative phosphorylation pathway can be produced in the light from thylakoid reactions, thus supplying energy not only for growth of cells from photosynthate but also, via the photosynthetic carbon reduction cycle, for the production of photosynthate. This leaves only the tricarboxylic acid cycle as a means of generating some essential components for growth, i.e. certain C skeletons, as a necessary function of dark respiration in photolithotrophic growth if the photolithotrophs are grown in continuous light (Raven and Beardall, 1981; Raven, 1984a). As we shall see, most algae are between these two extremes in terms of the qualitative and quantitative roles of dark respiratory processes. A further role of certain dark

Abbreviations: CF_1 – adenylate-binding component of the chloroplast or cyanobacterial CF_o-CF_1 ATP synthetase; CF_o – H^+-conducting transmembrane component of the chloroplast or cyanobacterial CF_o-CF_1 ATP synthetase; CF_o-CF_1 – chloroplast or cyanobacterial ATP synthetase; F_1 – adenylate-binding component of the mitochondrial or bacterial F_o-F_1 ATP synthetase; F_o – H^+-conducting transmembrane component of the mitochondrial or bacterial F_o-F_1 ATP synthetase; F_o-F_1 – mitochondrial or bacterial ATP synthetase; NAD^+ – nicotinamide adenine dinucleotide (oxidized); NADH – nicotinamide adenine dinucleotide (reduced); NADPH – nicotinamide adenine dinucleotide phosphate (reduced); PFK – phosphofructokinase; PFP – pyrophosphate-dependent fructose-6-phosphate-1-kinase; PQ – plastoquinone; PQH_2 – plastoquinol; UQ – ubiquinone; UQH_2 – ubiquinol

respiratory processes in photosynthetic organisms is in maintenance processes in the dark. Under anoxia (e.g. during ice encasement of benthic high-latitude macroalgae in the winter) the respiratory pathway involved in maintenance is fermentation (sometimes with H_2 evolution) (Beardall and Raven, 1990; Raven and Scrimgeour, 1997; Raven et al., 1998). In the presence of O_2, maintenance in the dark involves glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation, and directly relates to photosynthesis in (for example) repairing photoinhibitory damage incurred in the preceding photoperiod.

II. Carbohydrate Metabolism: Low M_r Compounds

Algae synthesize and accumulate a very wide range of low M_r carbohydrates including monosaccharides and di- and oligo-saccharides, more reduced derivatives such as sugar alcohols (linear and cyclic) and more oxidized derivatives such as ascorbate. While some of these compounds (e.g. ascorbate) are ubiquitous among algae (van den Hoek et al., 1995) many are much more restricted in their phylogenetic distribution and there is the possibility of a polyphyletic origin of the synthetic and regulatory pathways for those compounds which occur in a number of phylogenetically diverse taxa (Smirnoff, 1996). It is known that the pathways of synthesis of ubiquitous compounds such as ascorbate differ among the algae, although the complete biosynthetic sequence is not known for any alga; the occurrence of different biosynthetic pathways is inferred from differences in the distribution in ascorbate of carbon from specifically labeled positions of glucose supplied to the algae (Grün and Loewus, 1984; Wheeler et al., 1998; Smirnoff et al., 2001). The fact that a single ubiquitous carbohydrate derivative has different synthetic pathways in the algae suggests that the occurrence of low M_r carbohydrates of more restricted distribution may have even less phylogenetic information than is currently thought on the basis of their occurrence (e.g. the hexitol altritol in the Fucales (Phaeophyceae); de Reviers and Rousseau, 1999; Rousseau and de Reviers, 1999; Raven et al., 2001).

The low M_r carbohydrates can be synthesized from intermediates of the photosynthetic carbon reduction cycle in the light, or (in light or dark) from polysaccharides or other low M_r carbohydrates. The site (plastid or elsewhere) of synthesis of these compounds is not, in general, established.

Low M_r carbohydrates and their derivatives can function, to varying extents for different molecular species, in organic carbon and energy storage, as compatible solutes, and in removing free radicals or their precursors. These roles are not mutually incompatible. Thus, mannitol in the brown alga *Laminaria* functions as a compatible solute and also, seasonally, as an organic carbon and energy store, with accumulation of mannitol in the summer, when the upper mixed layer in temperate and polar waters is nutrient-depleted, but light energy is available, and depletion of mannitol in the winter when light availability is lower but nitrate and phosphate are more readily available due to deeper mixing of the surface ocean. This significant replacement of mannitol by potassium nitrate and vice versa on a seasonal basis maintains osmolarity of the protoplast, and hence maintains turgor, but unless the residual mannitol in winter is predominantly in compartments with a high volume density, and diversity, of catalytic proteins (cytosol, stroma, matrix) rather than in vacuoles then its role as a compatible solute is compromised (Davison and Reed, 1985). Mannitol also serves as a free radical (e.g. $^{\bullet}OH$) scavenger, presumably undergoing some chemical modification that terminates rather than expedites free-radical chain reactions. Whether such 'sacrificial' modifications render the altered molecule unavailable as a carbon and energy store, or non-functional as a compatible solute, is not clear.

At the other extreme is ascorbate, which has no documented function as a carbon and energy store, or as a compatible solute (although ascorbate plus dehydroascorbate can occur at up to 300 mol m^{-3} in higher plant chloroplasts, and is thus not an *in*-compatible solute), but which has clear functions in removing free radicals and their precursors (Smirnoff, 1996; Streb et al., 1997). Thus, ascorbate acts as a means of removing H_2O_2 (a precursor, via the Fenton reaction, of $^{\bullet}OH$) via the ascorbate peroxidase reaction, which occurs in the chloroplasts of most algae (and all higher plants) (Raven, Evans and Korb, 1999). At least under some circumstances the photosynthetic apparatus is the major generator of $O_2^{\bullet-}$ in the algal cell, and thence, via superoxide dismutase, H_2O_2, so that the non-enzymic scavenging and enzymic H_2O_2-consuming roles of ascorbate clearly relate to photosynthesis.

A further role of ascorbate in photosynthesis is as a cofactor in the de-epoxidation steps of the

xanthophyll cycles that convert epoxidized xanthophylls, which do not remove excess excitation energy, into de-epoxidized forms, which are functional in removing excess excitation energy (Smirnoff, 1996). This mechanism occurs in at least some representatives of all investigated algal classes, and all higher plants, with the exception of Cyanobacteria and red algae (Falkowski and Raven, 1997; Chapter 17, Raven and Geider). This absence of the xanthophyll de-epoxidation role of ascorbate in Cyanobacteria may be related to the low ascorbate levels in, and even the absence of ascorbate from many cyanobacteria, although this does not explain why red algae have 'typical' algal ascorbate concentrations (Raven, 1995a; Tel-Or et al., 1995; Asada, 2000).

A question which has been debated for a considerable time, and is now perhaps amenable to molecular genetic approaches, is that of whether the accumulation of low M_r carbohydrates represses photosynthesis at the gene expression or the post-transcriptional level. There are clear osmotic constraints on the role of photosynthetically generated carbohydrates of low M_r. However, it has long been known that sugars useable for growth can repress photosynthetic structures (and activity) in both light and darkness.

Having dealt with phylogeny and with function at the cell level, does the occurrence of particular low M_r carbohydrates relate to ecology (and especially the photosynthetic ecophysiology) of algae? Among red algae the occurrence of (straight-chain) hexitols rather than floridoside, isofloridoside and digeneaside as low M_r carbohydrate derivatives correlates polyphyletically, with high intertidal/subaerial habitats in *Bostrychia* (+ *Stictosiphonia*) and *Caloglossa* (but not *Catanella*): Karsten et al., 1990, 1992, 1995, 1998. The production of sugar alcohols is also a feature of those members of the green algae classes Trebouxiophyceae and Ulvophyceae (*Prasiola*, *Trebouxia*, *Trentepohlia*) which inhabit high intertidal and marine terrestrial habitats, and which are frequently symbiotic (as lichens) (Raven and Johnston, 1991). Aside from these conditions it is not easy to ascribe ecological functional significance to the occurrence of sugar alcohols rather than other low M_r carbohydrates (or, indeed, other compatible solutes, such as proline, betaines, or dimethylsulfoniopropionate) in these organisms.

III. Carbohydrate Metabolism: Storage Polysaccharides

The structural range of storage polysaccharides in algae is much smaller than that of the major low M_r carbohydrates, and is much more closely aligned to presently accepted phylogenies of the algae. Thus, α-1,4 glucan (with, usually, α-1,6 branches) is found in the same compartment as Rubisco in the Cyanobacteria *sensu lato* (unsurprisingly, as this is the only compartment of high protein density and diversity in these organisms) and in the Chlorophyta (i.e. in the stroma of the plastid). A similar polysaccharide occurs outside the plastids (i.e. in the cytosol) of the Cryptophyta, Dinophyta and Rhodophyta (Viola et al., 2001). Other divisions of algae (Chlorarachniophyta (probably), Euglenophyta and Heterokontophyta) have β-1,3 glucan in the cytosol (van den Hoek et al., 1995). The only other major storage polysaccharide in algae is polyfructan in some members of the Ulvophyceae (Dasycladales); the fructans are generally of lower degrees of polymerization, and are more water-soluble and osmotically active, than the glucans. A number of algal taxa predominantly store oils rather than polysaccharides, e.g. many diatoms (Heterokontophyta, Bacillariophyceae; van den Hoek et al., 1995).

Aside from the obvious source (photosynthesis)-sink (albeit intermediate, storage polysaccharide) relationship between photosynthesis and starch, β-1,3 glucan and fructan, is there any evidence for a feedback effect of polysaccharide accumulation in restricting photosynthesis? In chemical terms the notion of allosteric interactions with synthetic enzymes is much less likely or impossible for essentially insoluble materials. However, there is evidence for some feedback effects, predominantly for green algae where mechanical distortion in the plastids has been advanced as a reason for decreased rates of photosynthesis as starch builds up. However, the environmental conditions applied to establish high levels of starch accumulation as well as the achieved levels of starch need to be examined in considering explanations for the phenomenon, and there is clearly an opportunity for genetic manipulation to increase starch (or other storage polysaccharide) levels in disentangling the factors involved. Such genetic manipulations might also help to interpret the genotypic and phenotypic variations in the fraction of the organic carbon in an alga which is allocated to storage polysaccharides.

A further possible interaction of storage polysaccharides in, or near, the plastid and photosynthesis concerns the CO_2 concentrating mechanism(s). It has long been known that, in algae whose plastids contain a pyrenoid, there is frequently a spatial relationship between storage polysaccharide and the pyrenoid. This can occur even when the polysaccharide is in the cytosol and the pyrenoid is, as always, in the plastid; if the pyrenoid (surrounded by the plastid envelope membranes) is exerted, then the cytosolic polysaccharide can (and frequently does) surround all of the pyrenoid with exception of the isthmus connecting it to the rest of the plastid. This spatial association was originally tentatively 'explained' in terms of the pyrenoid as a site of polysaccharide synthesis (albeit with unexplained effluxes of pyrenoid-synthesized polysaccharide when polysaccharide stores occur outside the plastid). However, it is now clear that the major component of pyrenoids is Rubisco (ribulose-1,5-bisphosphate carboxylase-oxygenase) and there is little or no evidence for polysaccharide synthesis in pyrenoids.

Interest now focuses on pyrenoids as components of the CO_2 concentrating mechanism, with CO_2 generation from HCO_3^- in the pyrenoid or in the thylakoid transversing it. A suggestion as to the role of the polysaccharide sheaths is that they act as barriers to the efflux of CO_2 from the pyrenoid, thereby limiting CO_2 leakage from the site of Rubisco activity at (or near) which HCO_3^- is converted to CO_2 (Ramazanov et al., 1995) setting aside any questions of how HCO_3^- could have access to the pyrenoid while CO_2 loss is restricted. Villarejo et al. (1996) showed that a mutant of *Chlamydomonas reinhardtii* with a much lower starch content than the wild type did not show a significant impairment of the CO_2 concentrating mechanism. In this context it is of interest that the actual presence of a pyrenoid is not a *sine quo non* of the occurrence of a CO_2 concentrating mechanism (Raven, 1997a,b,c). Subsequently, very significant work has recently been performed on *Chlamydomonas* and the closely related *Chloromonas*. This work shows that, of five *Chloromonas* strains lacking pyrenoids, three strains lacked a CO_2 concentrating mechanism while the other two had a CO_2 concentrating mechanism (Morita et al., 1998). The work of Morita et al. (2000) suggests that, within the genera *Chlamydomonas* and *Chloromonas*, there were two losses of pyrenoids in the genus *Chloromonas* and that the absence of a pyrenoid makes for a lower intercellular to extracellular inorganic carbon ratio during steady-state photosynthesis in organisms expressing a CO_2 concentrating mechanism than is the case of closely related organisms with a CO_2 concentrating mechanism.

IV. Carbohydrate Metabolism: Structural Polysaccharides

The economic importance of the structural polysaccharides of some brown algae (alginates) and red algae (agars, carragheenans) has motivated a very considerable amount of research on the structural polysaccharides of marine algae. However, there are still very significant lacunae in our understanding of the biosynthesis, structure and function of algal cell walls. In this brief account of these end products of photosynthetic carbohydrate production we focus on these aspects of their structure and function which potentially feed back on photosynthesis.

The cell wall of algae *sensu lato* (i.e. including Cyanobacteria) is polyphyletic (van den Hoek et al., 1995; Raven, 1997d). The Cyanobacteria have the typical negibacterial peptidoglycan cell wall (which is thus not a polysaccharide wall) but frequently have a polysaccharide sheath. This distinction between turgor-containing (in Cyanobacteria, the peptidoglycan layer between the plasma membrane and the negibacterial outer membrane) and more highly hydrated components of the wall (in Cyanobacteria, the polysaccharide sheath) is reflected in many eukaryotic algae. The walls, as turgor-resisting entities, of algae are polyphyletic, assuming that chloroplasts arose by the phagotrophic uptake by a wall-less non-photosynthetic eukaryote (with mitochondria) of a cyanobacterium to yield glaucocystophytes, rhodophytes and chlorophytes (see Chapter 1, Douglas et al.) and that other algal divisions arose by phagotrophy of red algal unicells (cryptophytes, haptophytes, heterokontophytes and (?) dinophytes) or of green algal unicells (chlorarachniophytes and euglenophytes) with cells of up to seven different taxa of wall-less eukaryotes, depending on perceptions of the phylogeny of algae (van den Hoek et al., 1995; Raven, 1997d; Moreira et al., 2000; Palmer, 2000; Stiller et al., 2000).

The chemical monomer, and larger-scale structural, diversity of the structural (microfibrillar) and of the highly hydrated (non turgor-resisting) polysaccharides of cell walls is greater than the number of likely independent origins of turgor-resisting cell

walls. The quantity of wall material needed to resist a given turgor pressure depends on the structural properties of the polysaccharide, and the effective cell radius which, via Laplace's theorem, dictates the wall thickness as being directly proportional to the cell radius (Raven, 1982, 1984a).

In many macroalgae the turgor-resisting (microfibrillar) wall is surrounded by (as well as being interpenetrated by) highly hydrated polysaccharides, often with fixed negative charges (carboxyl, sulphate ester) with pK_a values between one and five (Boney, 1981; Raven, 1984a). The deformability of the thallus of such macroalgae largely reflects properties of this highly hydrated component flowing around the microfibrillar component in their turgor-resistant walls, albeit with some deformation of the cells and with perceived problems with maintaining the integrity of plasmodesmata in very deformable thalli (Raven, 1989, 1997d).

There are a number of implications of the occurrence of these predominantly polysaccharide walls for photosynthesis. One involvement of cell walls in photosynthesis concerns the supply of inorganic carbon from the medium to the photosynthetic machinery. The cell wall obviously has a lower water content than an equivalent thickness of water; this water is unstirred, so that any solute flux through the cell wall is by diffusion, unless deformation by current or wave action on macrophytes causes net water plus solute movement within the cell walls. The diffusion coefficient of solutes in the cell wall is lower than that in (unstirred) free solution, even allowing for the non-water fraction of cell wall volume as a result of tortuosity and surface factors (Raven, 1989; Hurd, 2000). The polyanionic nature of the cell walls means that the effective pH and the HCO_3^- concentration in water in the cell wall are lower than in an equilibrium free solution, but their product (and hence the CO_2 concentration) is the same as in equilibrium water. This means that the effective diffusivity of HCO_3^- is decreased more in the cell wall in comparison to water than is the case for CO_2, with implications for HCO_3^- use in photosynthesis. The decreased diffusion coefficient for solutes in the cell wall is a factor favoring acidification of the cell wall in acid bands of (freshwater) characeans, which is important for the mechanism of HCO_3^- use in photosynthesis based on HCO_3^- conversion to CO_2 in acid zones where high H^+ concentrations kinetically favor HCO_3^- and CO_2 interconversion and a high equilibrium CO_2 to HCO_3^- ratio (Raven, 1997a,b,c). Kraemer and Chapman (1991a,b) showed that variations in the mechanical stress to which thalli of the laminarian brown alga *Egregia menziesii* are subjected alters the allocation of photosynthate to wall polysaccharides, with possible implications for inorganic carbon diffusion through the wall.

A feature of the cell walls of some marine macroalgae (e.g. the fucoid brown alga *Ascophyllum*) is that of periodic sloughing of the outer layers of the epidermal (meristodermal) cell walls which abut on the medium. One result of this is that superficial epiphytic metazoa, algae and microorganisms may be removed. Epiphytes can absorb photons, and increase the thickness of diffusion boundary layers as well as (in the case of autotrophic epiphytes) acting as sinks for inorganic nutrients, including inorganic carbon. Consequently, removing epiphytes can improve access of photons and of inorganic carbon to the photosynthetic machinery. Chemoorganotrophic epiphytes can provide inorganic carbon (as CO_2) and other inorganic nutrients, so that removal of such epiphytes can reduce the inorganic nutrient supply to the phorophyte, although it increases photon supply. Loss of superficial epiphytes can, on balance, increase resource supply to phorophytes, at the cost of the energy and carbon lost in the sloughed cell wall material. Even *Ascophyllum* cannot rid itself of all epiphytes in this way; in particular, the rhodomelacean red alga *Polysiphonia lanosa*, which always grows as an epiphyte on *Ascophyllum* with a holdfast that penetrates between phorophyte cells, cannot be removed once it has grown beyond the sporeling stage (Moss, 1982 and references therein). The same argument may apply to the epiphyte *Pleurostichidium* (a close relative of *Polysiphonia*) on New Zealand populations of the Australasian fucoid *Xiphophora chondrophylla* (Phillips and Kraft, 2000)

While structural polysaccharides themselves do not attenuate photosynthetically active or UV-B radiation, they can act as anchors for UV-B-absorbing pigments (e.g. in many cyanobacteria, with UV-B absorbers on their polysaccharide sheaths; Bohm et al., 1995), and as anchors for proteins which cause thin film birefringence. They also act as templates for $CaCO_3$ and (?) SiO_2 deposition, with implications for solute diffusion and light scattering (Martin-Jézéquel et al., 2000).

V. Respiration: Carbon Pathways

A. Introduction

From the carbon perspective, respiration includes all reactions which produce CO_2, and the metabolic conversions related to them which do not themselves produce CO_2. Strictly, respiration occurs under aerobic conditions and involves O_2 uptake, although conventionally respiration also considers the oxidative pentose phosphate pathway, which occurs in aerobic conditions usually coupled to reductive synthesis rather than O_2 reduction, and fermentation which can occur in the absence of O_2 and may not (e.g. lactic acid fermentation) produce CO_2 (Stal, 1995).

The 'dark' respiratory carbon pathways are glycolysis (including fermentation), the oxidative pentose phosphate pathway and the tricarboxylic acid cycle. By 'dark' is meant 'absent in the light' or, indeed, 'uninfluenced by light.' By contrast the photorespiratory pathway(s) in which CO_2 is produced as a result of the oxidative metabolism of the glycolate produced from the phosphoglycolate generated by Rubisco oxygenase, is dependent on both light (to energize, and activate enzymes of, the photorespiratory carbon oxidation cycle) and O_2 (a substrate for Rubisco oxygenase).

Recent molecular genetic evidence (Schnarrenberger and Martin, 2002) has shown that the genes for many respiratory enzymes of higher plants, and hence of most if not all of the eukaryotic algae (Rotte et al., 2001), such as those of the citric acid cycle, were derived from the δ-proteobacterial ancestor of mitochondria. Krepinsky et al. (2001), however, showed that the nuclear genes for both chloroplast and the cytosolic isoenzymes of 6-phosphogluconate dehydrogenase, the second enzyme of the oxidative pentose phosphate pathway, were of cyanobacterial origin in spinach and hence were derived from the plastid ancestor. Finally, Nowitzki et al. (1998) showed that while the nuclear gene for tha chloroplast isoform of the glycolytic enzyme glucose-6-phosphate dehydrogenase of spinach was derived from the plastid ancestor, the gene for the cytosolic isoform came from the mitochondrial ancestor. These results suggest that the genes for the respiratory enzymes of algae were generally derived from the mitochondrial or the plastid ancestor, not from the ancestor of the host cell.

B. Glycolysis

This pathway links the sugars produced in the photosynthetic carbon reduction cycle, derived from reserves within the cells, imported from other cells of multicellular organisms, or imported from the medium, to the tricarboxylic cycle. A small segment of the glycolytic sequence runs in parallel with the oxidative pentose phosphate pathway. What biochemical details are known of glycolysis in algae shows that it resembles the process in higher plants more closely than that in metazoa, with the presence of both ATP-dependent (PFK or phosphofructokinase) and the PPi-dependent (PFP) enzymes capable of converting fructose-6-phosphate to fructose-1,6-bisphosphate. The pathway beyond phosphoenolpyruvate in the chlorophycean (trebouxophycean?) green microalga *Selenastrum* is capable of phenotypic modification when phosphate availability is low, with the pyruvate kinase reaction circumvented by the production of pyruvate resulting from phosphoenolpyruvate phosphatase, or from the sequential action of phosphoenolpyruvate carboxylase, malic dehydrogenase and malic enzyme (Raven, 1984a; Falkowski and Raven, 1997; Turpin et al., 1997).

In higher plants there is a complete set of glycolytic enzymes in the cytosol, with a less complete set of glycolytic enzymes in chloroplasts than in amyloplasts or leucoplasts (Buchanan et al., 2000). For green algae there is some variability in the occurrence of glycolytic enzymes in the cytosol and in plastids, with some having a complete set of glycolytic enzymes. The work of Smillie, Evans and Lyman (1963) on chloroplasts of *Euglena,* separated using non-aqueous techniques, shows that these plastids (derived by secondary endosymbiosis) lack glycolytic enzymes other than those common to the photosynthetic carbon reduction cycle. However, molecular genetic evidence suggests that isoforms of enolase are targeted to the stroma as well as to the cytosol of *Euglena* (Hannaert et al., 2000). Little is known of glycolytic enzyme occurrence in different cell compartments in other algal divisions.

C. Oxidative Pentose Phosphate Pathway

Green algae, like higher plants, have oxidative pentose phosphate pathway enzymes in both cytosol and plastids, based on aqueous extraction and separation media (Buchanan et al., 2000). The data obtained by

Smillie et al. (1963) using non-aqueous methodology shows that the plastids of *Euglena* lack the two dehydrogenases of the oxidative pentose phosphate pathway (and hence the CO_2-producing reaction) and also lack transaldolase, which is needed for the non-oxidative reactions of the oxidative pentose phosphate pathway but not the non-reductive reactions of the sedoheptulose-1,7-bisphosphate-1-phosphatase-dependent variant of the reductive pentose phosphate pathway (photosynthetic carbon reduction cycle). Raven (1984) speculated that the absence of the specific enzymes of glycolysis and of the oxidative pentose phosphate pathway in plastids of *Euglena* might be related to the absence of polysaccharides from the plastids of *Euglena*. Since *Euglena* is unable to use nitrate as a nitrogen source, a further correlation with the absence of specific oxidative pentose phosphate pathway enzymes from *Euglena* plastids could be the absence of need for reductant (NADPH) in the dark for nitrite reduction which occurs in plastids of green algae and higher plants. While there seem to be no reports on the location of oxidative pentose phosphate cycle enzymes in other algal divisions, it has recently been shown that isolated diatom (Heterokontophyta) plastids can show light-dependent O_2 evolution in the presence of nitrite (Wittpoth et al., 1998), so these plastids must have nitrite reductase activity.

D. Tricarboxylic Acid Cycle

This pathway can catalyze the complete oxidation to CO_2 under aerobic conditions of the pyruvate generated by glycolysis, with reoxidation of the NADH and reduced flavoprotein generated in the cycle via the oxidative phosphorylation sequence (Buchanan et al., 2000). The tricarboxylic acid cycle is also required to provide C skeletons for biosynthesis, because intermediates of the tricarboxylic acid include 2-oxoglutarate, which is an essential precursor of glutamate and thus of the glutamate family of amino acids and amides, and of algal tetrapyrroles (except for mitochondrial tetrapyrroles in *Euglena*, which are synthesized using another tricarboxylic acid intermediate, i.e. succinate, together with glycine), and which is uniquely synthesized by this pathway. The tricarboxylic acid cycle is the unique pathway for synthesis of 2-oxoglutarate. Removal of 2-oxoglutarate (and/or succinate) from the cycle cannot be compensated by inputs of pyruvate alone, and also requires the input of the C_4 dicarboxylates malate and/or oxaloacetate. In the absence of the glyoxylate cycle (i.e. in essentially all photolithotrophically growing algae) the dicarboxylates are produced by (C_3 + C_1) carboxylations based on pyruvate (pyruvate carboxylase) or phosphoenolpyruvate (phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase). All three of these carboxylases occur in algae, with pyruvate carboxylase having the most restricted distribution (Falkowski and Raven, 1997).

The role of the tricarboxylic acid cycle as a means of completely oxidizing pyruvate is absent from organisms with an incomplete cycle due to lack of expression of 2-oxoglutarate dehydrogenase, although its role in C skeleton biosynthesis can still be performed. The presence of only an incomplete tricarboxylic acid cycle has been suggested as a reason for obligate autotrophy (i.e. photolithotrophy in algae). Cyanobacteria are widely held to have such an incomplete tricarboxylic acid cycle, yet the complete genome of *Synechocystis* PCC 6803 has the gene for 2-oxoglutarate dehydrogenase (Kaneko et al., 1996).

The tricarboxylic acid cycle of eukaryotes is in the mitochondrial matrix. How the pyruvate and C_4 dicarboxylates get into the mitochondrion has been investigated in higher plants and metazoa (Buchanan et al., 2000), but the situation in algae is unclear. In some higher plants the main input of carbon from glycolysis for complete conversion to CO_2 or, after combination with oxaloacetate, the synthesis of 2-oxoglutarate for biosynthesis, is not as pyruvate but rather as malate (C_3 + C_1 carboxylation, then malate dehydrogenase), with NAD malic enzyme in the matrix generating pyruvate. The enzyme which converts pyruvate to acetyl CoA is usually pyruvate dehydrogenase, although *Euglena* has the usually O_2-sensitive pyruvate NADP$^+$:pyruvate oxidoreductase (Rotte et al., 2001).

Studies on 'higher eukaryotes' (Harris, 1995; Buchanan et al., 2000) show that the entry of the main ionic form of these organic acid substrates which occurs in the cytosol at pH 7.4, i.e. pyruvate$^-$ and malate^{2-}, requires entry of a single H^+ with each pyruvate$^-$ or malate^{2-}. For pyruvate$^-$ this directly involves H^+:pyruvate$^-$ symport. For malate^{2-} entry there is malate^{2-}:HPO_4^{2-} antiport, with HPO_4^{2-}:H^+ symport (despite the matrix being electrically negative relative to the cytosol) as the means of recycling the HPO_4^{2-} into the matrix. This requirement for recycling H^+ from the cytosol to the matrix in supplying

tricarboxylic acid cycle substrates to the mitochondrion will be taken up again in the next section. There we consider the coupling of electron transport from reduced NAD^+ and flavoproteins (produced in the dehydrogenase reactions of the tricarboxylic acid cycle) to O_2 to active H^+ efflux from the mitochondrial matrix to cytosol, and the involvement of the downhill H^+ influx to the matrix in the phosphorylation of ADP to ATP in the matrix, to the exchange of internal ATP^{4-} for external ADP^{3-} and HPO_4^{2-}, and in the entry of tricarboxylic acid cycle substrates.

VI. Respiration: Redox Reactions and Energy Conservation

A. Introduction

Here we consider the redox reactions associated with membranes of mitochondria and plastids involved in respiration *sensu lato*.

Mitochondrial electron transport, H^+ active efflux, and the use of H^+ re-entry to the mitochondrion in ADP phosphorylation, adenylate and phosphate fluxes, and entry of tricarboxylic acid cycle substrates

Eukaryotes appear, with very few exceptions, to have very great commonality of the integral and peripheral membrane proteins of the inner mitochondrial membrane (Harris, 1995; Falkowski and Raven, 1997; Hippler et al., 1998; Turmel et al., 1999; Buchanan et al., 2000).

Four main integral protein complexes are involved in electron transport. These are complex I (NADH dehydrogenase; NADH-UQ oxidoreductase), complex II (succinate dehydrogenase; succinate-UQ oxidoreductase), complex III (cytochrome bc_1 complex; UQH_2-cytochrome *c* oxidoreductase) and complex IV (cytochrome oxidase; cytochrome *c*-O_2 oxidoreductase). In Cyanobacteria these complexes are in the thylakoid membranes and, in some cases, the plasma membrane (*faute de mieux* in thylakoidless cyanobacteria), and the UQ/UQH_2 is replaced by PQ/PQH_2 (Raven, 1984). In eukaryotes the complexes are in the inner mitochondrial membrane. More subunits of these complexes are encoded in the mitochondrial genome in algae than in higher plants, with what appears to be an irreducible minimum of genes encoding redox components in the higher plant mitochondria (Allen and Raven, 1986; Burger et al., 1999; Race et al., 1999; Turmel et al., 1999; Adams et al., 2000; Gray, 2000). The coupling of redox reactions to active H^+ movement from the matrix to the cytosol has been clearly demonstrated for complexes I, III and IV; energetically and observationally complex II does not pump H^+. The H^+/electron stoichiometry in the three H^+-pumping complexes is not yet entirely clear. There is evidence for 2 H^+ per electron in the NADH-UQ (complex I) segment, although there is also evidence favoring 1 H^+ per electron (Falkowski and Raven, 1997; Buchanan et al., 2000). The UQH_2-cytochrome *c* segment (complex III) is widely accepted to have a H^+/electron ratio of 2, while the cytochrome *c*-O_2 segment (complex IV) is commonly held to pump 1 H^+ per electron. Accepted wisdom thus has it that transfer of one electron from NADH (in the matrix) to O_2 pumps 5 (or less likely 4) H^+ using complex I, III and IV, and that the transfer of one electron from succinate (in the matrix) to O_2 using complexes II, III and IV pumps 3 H^+ (Ferguson, 2000).

Some eukaryotes, and especially those which are functionally, if not always phylogenetically, similar to algae, have redox components in their inner mitochondrial membrane which catalyze reactions in parallel to those catalyzed by complexes I, III and IV. These parallel pathways do not pump H^+ (Buchanan et al., 2000). For complex I, there can be a parallel electron pathway to UQ from NADH in the matrix which does not pump H^+; there is also an external NADH dehydrogenase which oxidizes NADH derived from (for example) glycolysis. For complexes II and III, the 'alternate oxidase' provides a more direct link from UQH_2 to O_2 than do complexes II and III; as with the bypasses of complex I, there is no H^+ pumping. This possibility of bypasses means that the H^+/electron ratio of 5 (or less likely 4) for NADH-O_2 and of 3 for succinate-O_2, using respectively complexes I, III and IV, and complexes II, III and IV, are upper limits and can be significantly lower.

The major use of the energy released when H^+ recycles from the cytosol to the matrix is the phosphorylation of matrix ADP with matrix $H_2PO_4^-$ to form matrix ATP. The stoichiometry of the F_0-F_1 ATP synthetase has not been firmly established and may well be variable (Seelent et al., 2000), but 3 H^+ per ATP is widely held to be the value (Ferguson, 2000). Indeed, the value is probably not always an integer, since three adenine nucleotide-binding sites per F_1 and 10 H^+-conducting integral membrane *c* subunits per F_0 suggests 3.3 H^+ per ATP in *Saccharomyces cerevisiae* mitochondria, and 12 such

Table 1. P/O ratios for mitochondrial ATP synthesis with NADH and succinate as electron donors, with varying assumptions about the H^+/electron ratio in complex I, the H^+/ATP ratio of F_0-F_1 ATP synthetase, and of the final use of the ATP in the mitochondrial matrix or the cytosol. These values do not take into account the H^+ recycling involved in the entry of the organic anion substrate (pyruvate for NADH as electron donor, or succinate).

Electron donor	Assumed H^+/electron of complex I	Assumed H^+/ATP of F_0-F_1	ATP sink	P/O ratio
NADH (matrix)	2	3	matrix	3.33
NADH (matrix)	1	3	matrix	2.67
succinate (matrix)	–	3	matrix	2.00
NADH (matrix)	2	4	matrix	2.5
NADH (matrix)	1	4	matrix	2.0
succinate (matrix)	–	4	matrix	1.5
NADH (matrix)	2	3	cytosol	2.5
NADH (matrix)	1	3	cytosol	2.0
succinate (matrix)	–	3	cytosol	1.5
NADH (matrix)	2	4	cytosol	2.0
NADH (matrix)	1	4	cytosol	1.6
succinate (matrix)	–	4	cytosol	1.2

subunits in the *Escherichia coli* F_0-F_1 suggesting a H^+:ATP ratio of 4 (Ferguson, 2000; Seelent et al., 2000). Assuming 3 H^+ per ATP, the ratio of matrix ADP phosphorylated to electrons transferred from NADH to O_2 is 5 (or 4)/3 or 1.67 (or 1.33), while the ratio of matrix ADP phosphorylated to electrons transferred from succinate to O_2 is 3/3 or 1.0. This corresponds to P/two electrons or P/O ratios of 3.33 (or 2.67) (NADH) or 2.0 (succinate). Current views on the thylakoid CF_0-CF_1 ATP synthetase are that the H^+/ATP is 4; if this also applies to F_0-F_1, then the P/O ratios are 2.5 (or 2.0) for NADH, and 1.5 for succinate (Table 1) (Falkowski and Raven, 1997).

The great majority of the ATP generated in the matrix is consumed in the cytosol or in organelles other than mitochondria, and a significant use of H^+ re-entry to the matrix is in energizing (electrically) ATP^{4-} efflux coupled to ADP^{3-} influx, and H^+ influx coupled to HPO_4^{2-} influx (Harris, 1995; Buchanan et al., 2000). Overall the entry of 1 H^+ energizes the efflux of 1 ATP from, and influx of 1 ADP and 1 HPO_4^{2-} to, the matrix. Table 1 shows that this further reduces the ATP/O ratio so that the highest value (NADH in the matrix as electron donor; H^+/electron of 2 for complex I; H^+/ATP of 3) for P/O is 2.5, while the lowest value (succinate in the matrix as electron donor; H^+/ATP of 4) for P/O is 1.2.

Another component of H^+ re-entry is the entry of $pyruvate^-$ and $malate^{2-}$. $Pyruvate^-$ enters with H^+ (or in exchange for OH^-, which is equivalent from the point of view of acid-base regulation and energetics: Buchanan et al., 2000). $Malate^{2-}$ enters in exchange for HPO_4^{2-}, while $H_2PO_4^-$ enters in exchange for OH^-; charge and acid-base balance is maintained by the conversion of $HPO_4^{2-} + H^+$ to $H_2PO_4^-$ in the cytosol and by the conversion of $H_2PO_4^-$ to $HPO_4^{2-} + H^+$ in the matrix (Buchanan et al., 2000). The overall stoichiometry is $1H^+$ entering with 1 $pyruvate^-$ and $2H^+$ entering with 1 $malate^{2-}$. Before concluding that maintaining charge and acid-base balance related to H^+ entry with $pyruvate^-$ and $malate^{2-}$ involves redox-driven H^+ efflux from the matrix which could otherwise be coupled to ADP phosphorylation, and thereby lowers the effective ADP:O ratio, we must examine the fate of the organic anion substrates. For complete oxidation to CO_2, the H^+ deposited in the matrix with the organic anion substrate is consumed in the production of CO_2, and efflux of CO_2 from the matrix (Raven, 2001) would not lead to further acid-base imbalance. This means that the use of the tricarboxylic acid cycle to generate ATP for maintenance or growth processes involving complete oxidation of substrate to CO_2 has no energy cost for organic anion substrate uptake into mitochondria. The energy cost of transport across the inner mitochondrial membrane when the tricarboxylic acid cycle is used in C skeleton biosynthesis depends on the nature of the organic product exported to the cytosol (Fig. 14.40 in Buchanan et al., 2000), but the energy cost is less than the equivalent of half an ATP

exported to the cytosol for each malate^{2-} or pyruvate^{-} (or malate^{2-} plus pyruvate^{-}) entering the cytosol and used in C skeleton biosynthesis.

There are relatively few data sets for the P/O for mitochondria from photosynthetically grown algae. Eriksson et al. (1995) found ADP/O ratios, in terms of external ADP, of 1.8 for pyruvate as the external substrate (i.e. internal NADH) for *Chlamydomonas reinhardtii*. This is less than the expected value of 2.5 for an H^+/electron of 2 for complex I and a H^+/ATP of 3, assuming the ATP sink is in the cytosol, but closer to the value (2.0) expected with the assumptions made above and an H^+/ATP of 4 (Table 1; Pfeiffer et al., 2001).

A further pathway for H^+ re-entry across the inner mitochondrial membrane is via H^+ uniport channels. In this case the H^+ entry is not coupled to any endergonic process and the energy released in H^+ re-entry appears as heat. Such 'uncoupling channels' were discovered in metazoan mitochondria and have subsequently been found in higher plants (Laloi et al., 1997; Echtay, Winkler and Klingenberg, 2000). Their overall effect is similar to that of the non-H^+-pumping bypasses of complexes I, III and IV, in that all of the energy released when electrons from NADH are transferred to O_2 is lost as heat. Dissipation of energy as heat is, as Breidenback et al. (1997) point out, a necessary part of fully coupled mitochondrial electron transport if the rate of useful energy output is to reach an evolutionary compromise with the energetic efficiency of the energy output (Raven, 1984). Thus the difference in heat output between uncoupled (H^+ channel) or non-coupled (bypass of complexes I, III and III) electron transport and fully coupled electron transport is quantitative rather than qualitative (Breidenback et al., 1997). The thermogenic role of naturally uncoupled and non-coupled electron transport seem to be restricted, among photosynthetic organisms, to specified organs in higher land plants.

Maintaining an aquatic photosynthetic organism at a temperature significantly different from the surrounding water by respiratory processes is impossible, in view of the maximum respiratory rate per unit volume of the organism, the large surface area per unit volume of most structures in aquatic algae and the combination of boundary layer thickness around algae, the thermal conductivity of water and the specific heat of water (Denny, 1993; Raven et al., 2002). Even the energy dissipated between light absorption by photosynthetic pigments and the production of stable photosynthetic end products (e.g. carbohydrates), which is one or two orders of magnitude more than can be produced in uncoupled or non-coupled respiration, does not significantly raise the temperature of the organism relative to its aqueous environment (Denny, 1993; Raven, 2002). Similarly, the energy transferred from waves to benthic macro-algae, stretching the thalli with subsequent release of the energy as heat when the thallus relaxes before the next wave breaks, does not significantly increase thallus temperature above the temperature of the surrounding water although the energy dissipation rate can exceed that related to the absorption of photosynthetically active radiation (Raven, 2002).

A role for alternative oxidase respiration in acid-base regulation has been suggested by Sakarno (1998). The argument here is that alternative oxidase activity permits the oxidation of organic anions, with generation of OH^- and thus the capacity to neutralize excess H^+, unconstrained by coupling to ADP phosphorylation. Such metabolism could be important in shoots or land plants in neutralizing excess H^+, although the organic anions required would have to be produced in roots with excretion of H^+ to the root medium (an option not open to shoots in air) (Raven, 1986). Since growing algae are generally submerged for at least part of each day such a need for H^+-neutralizing processes not constrained by the demand by ATP is much less than in the case considered by Sakarno (1998).

More generally, Pfeiffer et al. (2001) discuss the possibility of an evolutionary trade-off between the rate of energy transformed and the yield of ATP per unit organic carbon oxidized (Månsson and McGlade, 1993; Pattern, 1993). Pfeiffer et al. (2001) specifically consider microorganisms with an essentially unlimited supply of organic substrate, which in at least some cases correlates with rapid but inefficient substrate use (ATP synthesis via fermentation or oxidative phosphorylation with a low P/O ratio), and contrast these with organisms on a limited supply of respiratory substrate where the ATP generation rate is slower but the P/O ratio is higher. Photosynthetic organisms generally only have access to intraorganismal stores of respiratory substrates, and so might be expected to follow the 'high yield' strategy.

B. Chlororespiration

Controversy still surrounds the extent of the

pathway(s), its function and even the occurrence of chlororespiration in algae (Raven et al., 1999; Cournac et al., 2000; Nixon, 2000; Chapter 8, Beardall et al.). Chlororespiration encompasses the redox processes that occur in vivo, or in isolated intact chloroplasts, in the dark. Gas exchange, fluorescence, absorption spectroscopy and inhibition studies have been used to indicate the oxidation of carbon reserves, with O_2 as terminal electron acceptor and probably using NAD(P)H dehydrogenase, plastoquinone, the cytochrome b_6f complex, and an unknown terminal oxidase, with the potential for coupling of this electron transport to H^+ active transport into the lumen and (if the CF_0-CF_1 ATP synthetase is activated in the dark) ADP phosphorylation. However, in no case has this complete pathway been demonstrated in any species of alga, and there are contra-indications for the occurrence of one or more of the components listed above for one or more algae (Raven et al., 1999). A further complication (see below) is that at low photon flux densities, it is difficult to distinguish chlororespiration from cyclic electron transport unless labeled oxygen is employed. It is tempting to use cyanobacterial respiration, with its long-known interactions with photosynthesis, as a template for chlororespiration in eukaryotes, but this may not be legitimate.

If chlororespiration does occur, then its role could be in supplying ATP for maintenance and synthetic processes in chloroplasts in the dark, supplementing or replacing ATP from glycolysis in the plastids, and from the cytosol entering on the dihydroxyacetone phosphate phosphoglycerate exchanger, or the adenylate transporter in the plastid envelope membrane. Chlororespiration represents a relatively minor flux compared with light-saturated photosynthesis and is probably significantly less than mitochondrial respiration (Raven et al., 1999; Cournac et al., 2000; Nixon, 2000).

A more detailed discussion of chlororespiration is given elsewhere in this volume (Chapter 8, Beardall et al.).

C. Photorespiration

By contrast with chlororespiration, which occurs (if at all) predominantly in the dark, photorespiration occurs only in the light (or immediately after the light has been extinguished), and, while it begins (in eukaryotes) in chloroplasts with the production of phosphoglycolate and then glycolate, other cellular compartments are also involved (see Badger et al., 2000; Raven et al., 2000; Chapter 8, Beardall et al.).

Photorespiration is based on the oxygenase activity of ribulose bisphosphate carboxylase-oxygenase (Rubisco). This enzyme is only active in illuminated photosynthetic cells, and the extent of oxygenase activity relative to carboxylase activity is determined by the intrinsic carbon dioxide-oxygen selectivity factor of the Rubisco of the organism, and the carbon dioxide and oxygen concentrations at the site of Rubisco. The majority of algae either grow naturally in habitats with CO_2/O_2 ratios in their cell walls which are higher than the value in air-equilibrium solution, or have an inorganic carbon concentrating mechanism, or both, so that the oxygenase activity as a fraction of the carboxylase activity is less than expected at a given temperature on the assumption that Rubisco is exposed to air-equilibrium CO_2 and O_2 concentrations. Despite these naturally-occurring means of suppressing Rubisco oxygenase activity, all algae have a means of metabolizing phosphoglycolate to produce glycolate which, unlike phosphoglycolate, is neither as intrinsically inhibitory to photosynthesis as is phosphoglycolate, nor is it a phosphate sink, i.e. there is always a means of metabolizing the products of Rubisco oxygenase activity in algae (Raven, 1984a, 1997b,c).

Glycolate was recognized early in the use of $^{14}CO_2$ in photosynthesis studies as a product of algal photosynthesis which, from chemical and radiochemical assay, appeared in the medium. Clearly, even this process could be considered, in very general terms, respiration, since it involves O_2 uptake in the Rubisco oxygenase reaction. However, it appears that all algae have a means of oxidizing glycolate to glyoxylate, and for further metabolism of glyoxylate (Raven, 1997b,c; Raven et al., 2000; Chapter 8, Beardall et al.).

In some eukaryotic algae (and in all cyanobacteria) glycolate is oxidized using a glycolate dehydrogenase associated with thylakoid (cyanobacteria) or inner mitochondrial (eukaryotic algae) membrane, feeding electrons from glycolate dehydrogenation to the electron transport chain at the ubiquinone level and thence to the terminal oxidase, generating H_2O. The algae with glycolate dehydrogenase include all members of the Chlorophyta except the Charophyceae, the Euglenophyta, and the Bacillariophyceae in the Heterokontophyta (Raven et al., 2000; Chapter 8, Beardall et al.). The alternative enzyme is glycolate oxidase, with a direct interaction with O_2 to produce

H_2O_2 which is then acted on by catalase with the possibility of some H_2O_2 efflux to the aqueous external medium (Raven et al., 1999; Antunes and Cadenas, 2000). Glycolate oxidase is (with catalase) found in peroxisomes, and occurs in the class Charophyceae in the Chlorophyta, the Rhodophyta and, in the Heterokontophyta, the Phaeophyceae (Fucophyceae) and Xanthophyceae (Tribophyceae) (Raven et al., 2000; Chapter 8, Beardall et al.). All of these reactions overall involve net uptake of 0.5 O_2 per glycolate oxidized, but only glycolate dehydrogenase permits the phosphorylation of ADP associated with glycolate oxidation, at the presumed expense of having additional electron transport capacity in complexes III and IV of mitochondria to accommodate the electron flux associated with glycolate dehydrogenase in the light as well as that linked to tricarboxylic acid cycle activity in the light.

After the conversion of glycolate to glyoxylate, the higher plant pathway (photorespiratory carbon oxidation cycle) converts two glycolate successively to two glycine, and then to serine plus CO_2, hydroxypyruvate, glycerate and phosphoglycerate, which can then be treated as part of the photosynthetic carbon reduction cycle or of glycolysis. This pathway of glyoxylate metabolism is found in the Chlorophyta, Rhodophyta and, in the Heterokontophyta, the Phaeophyceae, as well as (with some modification) the Euglenophyta (Raven et al., 2000; Chapter 8, Beardall et al.).

While some of the reactions of the photorespiratory carbon oxidation cycle occur in other algae (and in Cyanobacteria), the complete pathway does not seem to be present, and the presence of the glycine-serine interconversion reactions could be rationalized in terms of the biosynthetic relationship of these two protein amino acids regardless of the occurrence of a complete photorespiratory carbon oxidation cycle in the organism. While in some cases the organisms which lack a complete photorespiratory carbon oxidation cycle have malate synthase activity (e.g. Xanthophyceae, Bacillariophyceae), other organisms (Cyanobacteria) do not express malate synthase, at least under photolithotrophic growth conditions (Raven et al., 2000; Chapter 8, Beardall et al.; but see Parker and Armbrust, 2000 for the occurrence of glycine decarboxylase in diatoms). Malate synthase permits glyoxylate to produce succinate, provided that acetyl CoA is stoichiometrically available. What happens to any malate synthesized in this way is not clear (Raven et al., 2000). Gluconeogenesis from malate involves the loss of one CO_2 per triose unit produced which, with the CO_2 produced from conversion of each triose to acetyl CoA, effectively catalyzes the conversion of triose plus glyoxylate to triose plus two CO_2 with, of course, ATP generation (Raven et al., 2000). The alternative seems to be complete oxidation of malate to CO_2, so that here not only the glyoxylate, but effectively one triose phosphate (which generates acetyl CoA for malate synthase), are oxidized to CO_2. While ATP synthesis occurs, perhaps more ATP is generated than is used in a growing photolithotroph, at least above a certain ratio of oxygenase to carboxylase activities of Rubisco (Raven et al., 2000).

It is clear that a lot of work is still needed to determine the pathway of glyoxylate metabolism in many algae, and especially those with malate synthase, and the extent to which mitochondrial carbon metabolism and redox reactions are involved. What is clear is that the mitochondrial redox chain is involved in the photorespiratory pathway, regardless of how glyoxylate is metabolized. Furthermore, any of the likely mechanisms of glyoxylate metabolism involve CO_2 production in the mitochondrial matrix (Raven et al., 2000). This supplements any CO_2 produced in the matrix of the mitochondrion by the tricarboxylic acid cycle reactions in the light (see below). We note that all known decarboxylases produce inorganic carbon as CO_2 rather than as HCO_3^-. Even those CO_2 concentrating mechanisms which accumulate CO_2 to high levels around Rubisco in eukaryotes do not seem to completely suppress Rubisco oxygenase activity, and thus permit some phosphoglycolate production, so that *all* eukaryotes with a CO_2 concentrating mechanism produce CO_2 in their mitochondria in the light in addition to what is produced by the tricarboxylic acid cycle reactions.

Eriksson et al. (1996) have found a carbonic anhydrase in *Chlamydomonas reinhardtii* which is expressed only in the mitochondrial matrix, and which is much more greatly expressed in cells growing photolithotrophically at low CO_2 concentrations than in cells growing under other trophic conditions. A rationale for this carbonic anhydrase in mitochondria when a CO_2 concentrating mechanism is expressed has been suggested by Raven (2001). The background is that the CO_2 from mitochondrial decarboxylations (Raven 1992a, 1995b; Smith and Ferry, 2000) effluxes, in the absence of a mitochondrial carbonic anhydrase, into the cytosol as CO_2. Most evidence suggests that the CO_2 concentrating mechanism

delivers HCO_3^- to the cytosol and removes HCO_3^- from the cytosol into the plastids, and that the cytosol lacks carbonic anhydrase activity. This means that the CO_2 from mitochondria is not converted to HCO_3^- at a rate which competes well with CO_2 efflux across the plasmalemma, and is thus lost from the cell rather than being recycled to Rubisco. However, carbonic anhydrase in the mitochondrial matrix, together with a (hypothetical) bicarbonate channel in the inner mitochondrial membrane, could convert essentially all of the CO_2 produced in the mitochondrial matrix to HCO_3^- and move essentially all this HCO_3^- to the cytosol. In the absence of cytosolic carbonic anhydrase the HCO_3^- arriving in the cytosol is readily cycled into the plastids and (with plastidial carbonic anhydrase) converted to CO_2 and fixed by Rubisco. As Raven (2001) points out, such a set of reactions based on carbonic anhydrase activity in the mitochondrial matrix can increase CO_2 fixation by Rubisco by at least 10% in an organism with an appropriate CO_2 concentrating mechanism relative to an organism lacking the reactions delivering inorganic carbon from the mitochondria to the cytosol as HCO_3^-. Such a mechanism is only likely to function in eukaryotes; in Cyanobacteria CO_2 produced in any respiration in the light is directly delivered to the cytosol in which Rubisco (in carboxysomes) is located.

VII. Respiration: Spatial and Temporal Aspects

A. Spatial Aspects of Respiration

All living cells of algae, including cyanobacteria, have respiratory machinery which is functional under most normal growth conditions. However, whole cells or organisms may at times be subjected to anoxic conditions in their normal growth habitat. Examples are the lowermost algal cells in a microbial mat at night where community respiration can render essentially the whole mat O_2-free, and ice-encased macroalgae in the winter, as well as algae which overwinter in anoxic mud (e.g. *Enteromorpha* spp. in estuaries) (Raven, 1984a; Raven and Scrimgeour, 1997; Raven et al., 1998; Stal, 1995). While these situations are normal for the algae mentioned, essentially all of their growth (photosynthesis, and net synthesis of all cell components from photosynthate) occurs under oxic conditions. This may not be the case for the rhizoids of those rhizophytic macroalgae with 'shoots' in oxygenated water and rhizoids in anoxic sediments; the capacity of cytoplasmic streaming in the rhizoid to carry O_2 to the growing apex is questionable in view of the O_2 sinks (biotic and abiotic) in the anoxic sediment and the high O_2 permeability of cell membranes (Raven, 1984a, 1989).

B. Temporal Aspects of Respiration

Respiration rates of algae vary with time. The quantitative aspects of involvement of respiration in growth and maintenance processes is considered in Section VIII, but for the moment we note that the rate of respiration varies as a function of the time for which a photolithotrophically growing alga has been deprived of light and, in synchronous cultures of unicellular algae, with the time in the cycle for either respiration measured in the dark phase or in dark interruptions of the normal light phase (Raven and Beardall, 1981; Raven, 1984a; Falkowski and Raven, 1997). As we shall see, the 'steady' (if such there is) rate of respiration in the 'normal' dark phase of algae growing in a diel light-dark cycle can reflect growth plus maintenance processes if the alga converts photosynthate into cell material in the dark, or maintenance alone if the alga does not perform growth-related metabolism in the dark phase. In the first mentioned case the longer the 'normal' dark phase is prolonged the (generally) lower respiration rate and the more that the rate approximates to that required for maintenance alone. The content of organic carbon reserves, the rate at which energy (ATP) is used for essential maintenance process; and the efficiency of coupling of organic carbon oxidation to maintenance processes via respiration, are presumably determinants of how long algae can stay alive in the dark. We have seen (above) that some algae can stay alive for prolonged periods in anoxic conditions, relying on fermentation to power such maintenance processes as occur.

In the context of the relation of respiration to photosynthesis a crucial question is the extent of occurrence in the light of respiratory processes, including those which use the pathways of classic dark respiration and of chlororespiration. Three techniques have been used to approach this problem (Raven, 1972a,b, 1976a,b, 1984a,b; Raven and Beardall, 1981; Krömer, 1995). One is to quantify the metabolites whose synthesis can only occur via

'dark' respiratory pathways; essentially this means the C skeletons which are produced by the tricarboxylic acid cycle. A second is to measure net gas exchange immediately after cessation of illumination. The third is to measure exchanges of tracers ($^{13}CO_2$,$^{14}CO_2$,$^{18}O_2$).

All of these methods have their limitations. Dealing first with the quantitation of metabolites whose synthesis can only occur via 'dark' respiratory pathways, we have seen that this only relates to the tricarboxylic acid cycle and the 'lower' part of glycolysis. The major categories of compounds here are derivatives of oxaloacetic acid (produced by a $C_3 + C_1$ *carboxylation* of pyruvate phosphoenolpyruvate for each C_4 unit), derivatives of 2-oxyglutarate (produced by a $C_3 + C_1$ *carboxylation* of pyruvate or phosphoenolpyruvate, and two *decarboxylations* in converting pyruvate plus oxaloacetate to 2-oxoglutarate for each C_5 unit), and fatty acids (derivatives of acetyl CoA produced by one decarboxylation of pyruvate for each C_2 unit). The C_5 pathway includes synthesis of tetrapyrroles, with only mitochondrial tetrapyrroles of *Euglena* produced by the succinate/glycine pathway. The acetyl CoA pathway was formerly thought to include the synthesis of terpenoids as well as of fatty acids, but it is now known that most algal terpenoids are produced via 1-deoxy-D-xylulose-5-phosphate, produced from triose phosphate and pyruvate with one carboxylation per C_5 unit produced from two C_3 as opposed to the 4 decarboxylations to produce one C_5 from three C_3 via the classic (acetyl CoA) pathway (Lichtenthaler, 1999). Quantitation of compounds produced by this pathway in the light phase involves destructive harvesting and analysis, but does give a precise (and, accurate) estimate of net CO_2 produced in metabolism of C_3 compounds. Thus it is a good minimum estimate of the CO_2 side of necessary dark respiration (minimum because other dark respiratory processes could occur, e.g. tricarboxylic acid cycle ATP synthesis, and the oxidative pentose phosphate pathway). However, it does not tell of the fate of NADH generated in the C skeleton synthesis and so does not give O_2 uptake via mitochondrial terminal oxidases.

The second method to determine the rate of respiration in the light is to measure net gas exchange immediately after the cessation of illumination. By respiration is meant the net O_2 uptake and CO_2 evolution immediately (seconds-minutes) after the light-dark transition. O_2 uptake and CO_2 evolution have relatively rarely been measured in the same experiment; furthermore, there are many O_2 and CO_2 exchanges other than 'dark' respiration and chlororespiration in these light-dark transients. These other gas exchange processes include continuation of photosynthetic CO_2 fixation and O_2 evolution after cessation of illumination, the Mehler peroxidase reaction (water-water cycle), the continuation of the photorespiratory carbon oxidation cycle, and inorganic C efflux from an intracellular inorganic C pool maintained by light-dependent pumping of extracellular inorganic C.

We can immediately rule out the Mehler reaction; this has equal net O_2 uptake and net O_2 evolution, with the minimal proviso that more electrons are 'stored' at the reducing side of PS I than of positive (electronic) charge stored at the oxidizing side of PS II (Asada, 1999). Any post-illumination inorganic C leakage from the intracellular inorganic C pool, generated by light-driven inorganic C influx in excess of inorganic C fixation plus inorganic C efflux, occurs when the light-dependent inorganic C pump and the inorganic C assimilation processes are turned off when the light ceases, but the inorganic C efflux can continue in the dark. This inorganic C efflux is not matched by an O_2 uptake. Photorespiration can continue in the dark due to the pool of intermediates of the photorespiratory carbon oxidation cycle left after the production of phosphoglycolate has been shut off by cessation of illumination and, *inter alia*, prevention of Rubisco-catalyzed production of phosphoglycerate and phosphoglycolate. The cessation of the O_2-consuming production of phosphoglycolate from RUBP, and the continuance of O_2-consuming conversion of glycolate to glyoxylate, and of glycine to serine, and CO_2 production in converting glycine to serine, means that continuing photorespiration involves O_2 uptake *and* CO_2 evolution. The likelihood of inorganic release from the inorganic carbon concentrating mechanism, and of CO_2 production and O_2 uptake from the photorespiratory carbon oxidation cycle, are inversely related in an air-equilibrated aquatic medium; the inorganic C concentrating mechanism restricts the oxygenase activity of Rubisco, and hence restricts the production of phosphoglycolate, and hence of O_2 uptake and CO_2 production in the photorespiratory carbon oxidation cycle.

These complications mean that the use of the immediate (seconds-minutes) post-illumination analysis of the gas exchange to infer the rates of dark respiratory processes during illuminations needs

considerable care. However, in a number of cases the post-illumination enhancement of O_2 uptake and (less frequently measured) CO_2 evolution can be related to enhanced rates of the dark respiratory processes in the post-illumination period relative to the steady-rate level in the dark. One suggestion as to the reason for this enhancement is that the rate of processes consuming NADPH and ATP in converting photosynthate plus external inorganic nutrients into cell material proceed faster in the light than in the dark, and that the deceleration of the rate of these processes upon darkening accounts, via respiratory control, for the decreasing rate of dark respiratory processes (Raven, 1976a,b). This argument does not necessarily mean that dark respiratory reactions were supplying the additional NADPH and ATP for these growth-related processes in the light, since NADPH and ATP from thylakoid reactions could supplement the respiratory supply. The alternative possibility of stimulation of dark respiration immediately after illumination directly by increased photosynthetic supply of organic carbon substrate, rather than by increased availability of ADP, P_i and $NADP^+$, fits less well with the respiratory control paradigm, and suggests that the higher rate of dark respiration immediately after illumination is a reflection of the rate during illumination (Beardall et al., 1991).

The third method for measuring respiratory (O_2 uptake, CO_2 production) during photosynthesis is to use tracer gases such as $^{18}O_2$, $^{13}CO_2$ and $^{14}CO_2$. The O_2 isotopes are generally preferred because of less complication due to internal recycling in experiments lasting tens of seconds or more.

In an experiment on algae in the light with labeled O_2 (^{18}O-enriched) and unlabeled H_2O (^{18}O at the natural abundance level) the increase in $^{16}O_2$ is a measure of gross photosynthetic O_2 production (Badger, 1985; Badger et al., 2000). However, the decrease in $^{18}O_2$ can be attributed not only to dark (mitochondrial) respiration via cytochrome oxidase and the alternate oxidase with tricarboxylic acid cycle activity plus any photorespiratory component of electron flow in mitochondria, but also to O_2 uptake in the Mehler peroxidase reaction, Rubisco oxygenase and, in those algae with glycolate oxidase, peroxisomal O_2 uptake in glycolate oxidation. We note that both the Mehler peroxidase reaction and the glycolate oxidase reaction involve the uptake of more O_2 than eventually appears in water, with O_2 evolution involving superoxide dismutase and catalase respectively. However, the O_2 comes from ^{18}O-labeled superoxide and hydrogen peroxide generated from the uptake of $^{18}O_2$, so that the O_2 regenerated in this way is ^{18}O-labeled and does not vitiate measurement of net O_2 uptake at the reducing end of the water-water cycle and in glycolate oxidase.

Just as with the O_2 uptake (and CO_2 evolution) at a light-dark transient, the uptake of labeled O_2 cannot be apportioned into fractions corresponding to the different possible respiratory processes (*sensu lato*) without a comparison of different experimental conditions, e.g. variations in O_2 or inorganic C concentration. The method of Laisk and Loreto (1996) for delimiting mitochondrial CO_2 evolution in the light does not yet seem to have been applied to algae.

The methodological difficulties that have just been discussed mean that there is still doubt as to the extent of dark respiratory processes during photosynthesis in any alga or cyanobacterium, but it appears unlikely that the rate is substantially larger than the dark rate measured after several minutes of darkness.

VIII. Quantifying Carbohydrate Metabolism and Respiration in Relation to Growth and Maintenance

Attempts to quantify the role of respiration in the growth and maintenance processes in algae include those of Geider and Osborne (1989), Raven (1976a,b, 1982, 1984a), Raven and Beardall, (1981) and Raven et al. (2000) while recent analyzes emphasizing vascular land plants are those of Cannell and Thornley (2000) and Thornley and Cannell (2000).

One approach to quantification of respiration (oxidative phosphorylation, tricarboxylic acid cycle, oxidative pentose phosphate pathway, glycolysis) is to estimate the growth and maintenance costs of the alga from known pathways of biochemical synthesis, solute transport and cell motility and the extent of the pathways during growth and maintenance. These latter estimates require that the (bio)chemical constituents of the algae be known. Furthermore, in order to estimate maintenance costs, the turnover of organic components in the absence of growth, the leakage and reaccumulation (inorganic and organic) or resynthesis (organic) of solutes and (for wall-less freshwater cells) active water efflux must be known. Such analyzes have been performed for algae by Raven (1976a,b, 1982, 1984a,b), and need updating in the context of more recent knowledge of the stoichiometry of ATP synthesis in respiration (Section

VI) and of the energy costs of protein synthesis (considered in the context of maintenance processes by Raven et al., 2000). A problem with such analyzes is that they can only strictly be applied to growth and maintenance processes in the dark phase of a diel light-dark cycle (Raven, 1976a,b, 1982, 1984a,b; Raven and Beardall, 1981). This problem results from the potential for the substitution of photosynthetic partial reactions in substituting for all the growth (photosynthate use) and maintenance requirements for dark respiration except for C skeleton synthesis using the tricarboxylic acid cycle.

The other approach is the (apparently) simple one of estimating the rate of dark respiration for growing algae. For the reasons discussed in Section VII the estimation of the rate of 'dark' respiratory processes during photosynthesis is difficult, especially if an additional constraint is imposed, i.e. that the estimate should be under the conditions in which steady-state growth is measured. Such estimates during growth give the rates of growth plus maintenance respiration; maintenance respiration is estimated by extrapolating to zero growth rate the respiratory rates determined under conditions which give a range of growth rates (Geider and Osborne, 1989; Cannell and Thornley, 2000; Thornley and Cannell, 2000). Of course, this assumes that the rates of protein turnover and of solute leakage are independent of growth rate, an assumption which is clearly not true for variations in growth rate imposed by varying photon flux density in the case of turnover of the D1 polypeptide of Photosystem II (Raven et al., 2000).

Comparison of the two methods generally shows that there is an excess of the measured rate of 'dark' respiratory processes over the rate estimated from mechanistic considerations. However, there are exceptions, e.g. protein turnover in *Dunaliella* and *Phaeodactylum* in the dark has a higher estimated energy requirement than can be provided by respiration (Quigg, 1999).

The difficulties in estimating the role of dark respiration processes in algal growth and maintenance mean that there are still unresolved problems at the biochemical and cell physiological level. However, the level of quantitative analysis which is possible is probably adequate for many ecological and biogeochemical purposes.

Turning to quantitative aspects of carbohydrate metabolism, it is less easy to draw generalizations, granted the multiplicity of functions served by carbohydrates. Thus, algae living in high-osmolarity habitats generally have more soluble low-M_r per unit biomass than do algae in low-osmolarity environments, although in some cases proline, betaines and dimethylsulphoniopropionate are used as the compatible solutes (Raven, 1984). The extent of storage of polysaccharides, and (vacuolar) low M_r carbohydrates, relates to adaptive 'expectations' of the organism, as well as to its recent (phenotypic) history (Raven, 1984). Finally, the extent of production of extracellular mechanical polysaccharides varies with the mechanisms of volume regulation in the organism, with a turgor-resisting wall in cells which are hyperosmotic to their environment and which lack contractile vacuoles, or their functional equivalent, and to other mechanical, and non-mechanical, functions of extracellular polysaccharides (Raven, 1982, 1984, 1989).

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Chapter 11

Carbon Acquisition Mechanisms of Algae: Carbon Dioxide Diffusion and Carbon Dioxide Concentrating Mechanisms

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Summary

At least 95% of the organic carbon in cyanobacteria and algae has been fixed as CO_2 by Rubisco. The kinetic properties of Rubisco are such that even the genetic variants with the highest CO_2:O_2 selectivity would show limited CO_2 fixation and significant oxygenase activity if CO_2 and O_2 fluxes into cells are driven solely by diffusion. This is especially the case for submerged algae with low gas diffusion coefficients relative to diffusion boundary layer thicknesses. There is evidence from gas exchange properties and intracellular inorganic concentration that inorganic carbon concentrating mechanisms (CCMs) function in all cyanobacteria and many algae. CCMs can, in theory, operate through three categories of mechanism: the first mechanism is active transport of HCO_3^- and/or CO_2 across membranes, the second is CO_2 pumping by a biochemical mechanism analogous to C_4 and CAM (Crassulacean Acid Metabolism) pathways in higher land plants, and the third mechanism involves active transport of H^+ producing an acid compartment supplied with HCO_3^-, which generates a high (equilibrium) CO_2 concentration that supplies CO_2 to Rubisco in a nearby more alkaline compartment. Most evidence favors the first mechanism as being responsible for CCM activity in algae. The CO_2 (and HCO_3^-) permeability of membranes is crucial in defining the extent of inorganic C leakage from CCMs, and the functioning of diffusive CO_2 supply to Rubisco from the medium. Like carbonic anhydrases, CCMs, are probably polyphyletic.

I. Introduction

It is now widely held that most algae, and all cyanobacteria, express inorganic carbon concentrating mechanisms (CCMs) when growing in their natural habitats (reviewed by Raven 1997a,b,c; Badger et al., 1998; Kaplan and Reinhold, 1999; Badger and Spalding, 2000). CCMs give rise to steady-state CO_2 concentration around ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) in excess of the external CO_2 concentration. Since at least 90% of the organic carbon in photolithotrophically growing algae is fixed by Rubisco, CCMs in cyanobacteria and algae are involved in much of the inorganic carbon assimilation in oceans and perhaps one-third of the 100 Pg or so of organic carbon produced in net primary productivity on Earth each year (Raven, 1997b,c). However, a significant fraction of algal photosynthesis involves diffusive supply of CO_2 to Rubisco, so that it is important to consider algae which lack CCMs as contributors to local and global productivity and not just as a contrast to algae which express CCMs.

Abbreviations: CAM – Crassulacean acid metabolism; CCM – carbon concentrating mechanism; k_{cat} – substrate-saturated rate of catalysis on a unit enzyme basis; $K_{1/2}$ – concentration of substrate at which the reaction rate is half of the substrate-saturated rate; L_2 – form II Rubisco with two large subunits; L_8S_8 – form I Rubisco with eight large and eight small subunits; PCOC – photosynthetic carbon oxidation cycle; PCRC – photosynthetic carbon reduction cycle; Rubisco – ribulose bisphosphate carboxylase-oxygenase; S_{rel} – selectivity factor of Rubisco for CO_2 relative to that for O_2

The ensuing discussion of CCMs and of diffusive CO_2 entry begins (Section II) with an outline of the properties of Rubisco and the range of inorganic C and O_2 concentrations in the environment(s) of algae. These two aspects are combined in considering the extent of mismatch between CO_2 concentration, and CO_2/O_2 concentration ratio, that would be found at the Rubisco site if CO_2 and O_2 fluxes were diffusive from and to the natural environment, and the CO_2 and O_2 concentrations needed for maximal Rubisco carboxylase activity. We then briefly consider (Section III) the various lines of evidence used to distinguish algae and cyanobacteria relying on diffusive CO_2 supply to Rubisco from those using CCMs. There then follows a consideration of the extent to which the presence of CCMs can be correlated with taxonomy, structure and ecology of algae and cyanobacteria as a prelude to a brief discussion of the mechanism(s) of CCMs (Section IV). Finally, the present taxonomic and ecological distribution of CCMs is considered in relation to the likely time of evolution of cyanobacteria and algae and environmental conditions in the past (Section V) and, in Section VI, conclusions and prospects are presented.

II. Rubisco Kinetic Properties in Relation to the CO_2 and O_2 Concentrations in Cyanobacterial and Algal Habitats

Much has been written about the properties of Rubisco and their phylogenetic variability (reviews by Badger

et al., 1998; Raven, 2000; Tortell, 2000) and only a brief summary will be given here.

A. Rubiscos from Different Taxonomic Groups have Different Characteristics

All known Rubiscos have competitive carboxylase and oxygenase functions and have relatively low substrate-saturated carboxylase activities on a protein mass basis. The selectivity factor defining the relative rates of carboxylase and oxygenase reactions is given by Eq. (1), where $k_{cat}(CO_2) = CO_2$ saturated specific rate of carboxylase activity of Rubisco (mol CO_2 mol^{-1} active site s^{-1}), $K_{1/2}(CO_2)$ = concentration of CO_2 at which the CO_2 fixation rate is half of $k_{cat}(CO_2)$, $k_{cat}(O_2) = O_2$ saturated specific rate of oxygenase activity of Rubisco (mol O_2 mol^{-1} active site s^{-1}) and $K_{1/2}(O_2)$ = concentration of O_2 at which the O_2 fixation rate is half of $k_{cat}(O_2)$.

$$S_{rel} = \frac{K_{1/2\,1/2}(O_2) \cdot k_{cat}(CO_2)}{K_{1/2\,1/2}(CO_2) \cdot k_{cat}(O_2)} \quad (1)$$

The half-saturation concentration of substrate ($K_{1/2}$) for, and the substrate-saturated rates (k_{cat}) of, carboxylation and oxygenation both show significant phylogenetic variability (Table 1). The general correlation is that a low $K_{1/2}(CO_2)$, and a high S_{rel} are correlated with a low $k_{cat}(CO_2)$, and vice versa (Table 1).

Among cyanobacteria and algae, a number of molecular phylogenetic subfamilies of Rubisco are represented. Almost all O_2-evolvers have L_8S_8 Rubiscos, i.e. comprising eight large (catalytic) subunits and eight small subunits in each enzyme molecule. It is likely that the original Rubisco of O_2 evolvers, as found in almost all cyanobacteria and in all eukaryotes with chlorophyll *b*, was the variant found in ancestral cyanobacteria and which was transferred to the eukaryotes by endosymbiosis, remaining in Chlorophyta and Embryophyta as well as Euglenophyta and (presumably) Chlorarachniophyta (Raven, 1997b,c, 2000; Badger et al., 1998). Another form, β-proteobacterial Rubisco, can be accounted for by lateral gene transfer of this variant of Rubisco to several cyanobacteria and, among eukaryotes, to Rhodophyta, Cryptophyta, Haptophyta and Heterokontophyta (Raven, 1997b,c, 2000; Badger et al., 1998). The exception to the occurrence of L_8S_8 Rubiscos in eukaryotes is the peridinin-containing members of the Dinophyta which have an L_2 (two large subunits) Rubisco, obtained by lateral gene transfer from a δ-proteobacterium (Badger et al., 1998; Raven, 1997b,c, 2000).

These different Rubiscos have a range of S_{rel}, $K_{1/2}(CO_2)$ and $k_{cat}(CO_2)$ values, with some red algal Rubiscos having the highest S_{rel} but relatively low $k_{cat}(CO_2)$, and dinoflagellate L_2 and cyanobacterial L_8S_8 Rubiscos having the lowest S_{rel} and, where tested, highest $K_{1/2}(CO_2)$ and $k_{cat}(CO_2)$(Badger et al., 1998; Raven, 2000). Regardless of the absolute values for $k_{cat}CO_2$, Rubiscos as a family have low specific reaction rates for the carboxylase at CO_2 saturation. This low specific reaction rate means that, even at substrate saturation, the rate of fixation per unit time per unit N or energy used in constructing Rubisco is low relative to that of many other enzymes. However, the significant kinetic variation among Rubiscos means that those with low S_{rel} fix CO_2 fastest per unit N or per unit energy used in Rubisco synthesis at CO_2 saturation, but perform less well per unit N or energy at low CO_2 levels due to their low S_{rel}. Since Rubisco accounts for several percent at least of the total N in a cyanobacterium or alga, such kinetic considerations have significant impacts on the N budget of the organism as a function of the Rubisco properties and CO_2 level at which the enzyme operates in vivo.

B. The Potential for Net CO_2 Fixation in Air-Equilibrated Solution Depends on Rubisco Characteristics and the Mechanism of Glycolate Metabolism

We now consider the potential for net CO_2 fixation by Rubisco with the appropriate photosynthetic carbon reduction cycle (PCRC) and phosphoglycolate metabolism enzymes and means of generating ATP and NADPH from light in air-equilibrated solutions. Such estimates of the potential for net CO_2 fixation require assumptions about the fate of the phosphoglycolate generated by Rubisco oxygenase (Chapter 8, Beardall et al.). At one extreme is complete oxidation, (Raven et al., 2000a) so that for every O_2 taken up by the oxygenase function two CO_2 are produced; in this case net CO_2 fixation requires that $v_o < 2\,v_c$, where lower case v represents the achieved rate of carboxylation (c) or oxygenation (o). The absence of glycolate metabolism (i.e. glycolate excretion) may at first sight seem to permit net CO_2

Table 1. Some kinetic properties of Rubisco from O_2-evolving organisms *(modified from Badger et al., 1998, Raven 1997, Raven et al. 2000a and references therein).*

Rubisco source	S_{rel}	$K_{0.5}(CO_2)$ (mmol m^{-3})	$k_{cat}(CO_2)$ (mol CO_2 fixed mol^{-1} active site s^{-1})	$k_{cat}(CO_2)/K_{0.5}(CO_2)$ (mol CO_2 fixed mol^{-1} active site s^{-1} $mmol^{-1}$ m^3)	Approximate range of CO_2 accumulation relative to external concentration
Cyanobacteria (L_8S_8)					
5 species with CO_2 concentrating mechanisms	35–56	105–185	11.4–12	0.061–0.072	~800–900
Dinophyta (L_2)					
1 species *(Amphidinium carterae)* with a CO_2 concentrating mechanism	37	–	–	–	2–26
Prorocentrum micans	–	–	–	–	10
Symbiodinium	–	–	–	–	2–24
Chlorophyta (L_8S_8)					
2 species with CO_2 concentrating mechanisms	61–63	29–38	–	–	5–180
1 species (*Coccomyxa* sp) with diffusive CO_2 entry	83	12	–	–	~1
Embryophyta (L_8S_8)					
21 species (*Zea mays*) with a CO_2 concentrating mechanism	78	32	4.2	0.134	–
2 species with diffusive CO_2 entry	82–90	10–11	2.9–3.0	0.276–0.286	–
Rhodophyta (L_8S_8)					
Porphyridium cruentum	128	22	1.6	0.072	–
Porphyridium purpureum	–	–	–	–	3
Cyanidium, 2 species	224–238	6.6–6.7	1.3–1.6	0.194–0.242	–
Cyanidioschyzon merolae	–	–	–	–	20–80
Heterokontophyta (L_8S_8) Bacillariophyceae					
3 species with CO_2 concentrating mechanisms	106–114	31–36	0.78–5.7	0.054–0.14	3.5–20
Chloromonadophyceae					-
1 species (*Olisthodiscus luteus*) with a CO_2 concentrating mechanism	101	59	0.83	0.014	–

fixation in air-equilibrated solution with Rubisco having S_{rel} values lower than those of any known Rubisco. However, the absence of glycolate metabolism to produce C_3 compounds would restrict, or even abolish, regeneration of RuBP as substrate for Rubisco, and thus of any surplus organic C which can be used for cell growth and maintenance. The absence of glycolate metabolism thus also gives a requirement of $v_o < 2\ v_c$ for net CO_2 fixation. For the most effective known way of converting glycolate into compounds which can be used for regenerating RuBP, i.e. the photorespiratory carbon oxidation cycle (PCOC), three carbons in triose (i.e. at the carbohydrate redox level) and one CO_2 are produced for every two glycolate metabolized. Here the requirement for net CO_2 fixation in air is that S_{rel} shall not be so low that $2\ v_o < v_c$.

For air-equilibrated solutions, it can be seen that glycolate metabolism via the PCOC permits net CO_2 fixation with a S_{rel} for Rubisco only one-quarter of that needed to permit net CO_2 fixation with no glycolate metabolism or complete glycolate oxidation. Increasing S_{rel} increases the net CO_2 fixation in air-equilibrated solution with a decreasing relative influence of PCOC activity as opposed to complete oxidation of glycolate or no glycolate metabolism in determining net CO_2 fixation.

These considerations show that algal and cyanobacterial Rubiscos with the lowest S_{rel} values do not permit net CO_2 fixation in air-equilibrated solutions regardless of the fate of glycolate. By contrast, the Rubiscos with the highest S_{rel} values permit very considerable net CO_2 fixation in air-equilibrated solution, again regardless of the fate of glycolate, although quantitatively the net CO_2 fixation rate for a given S_{rel} is increased if the PCOC is used rather than other fates of glycolate. These arguments based on air-equilibrated solutions cannot be directly applied to intact cells in their natural environment. Diffusive CO_2 entry (and O_2 efflux) means that internal CO_2 is lower, and O_2 is higher, than that in the bulk medium during steady state photosynthesis. Furthermore, the CO_2 concentration in natural waters is very generally not in equilibrium with the atmosphere (Duarte and Agusti, 1998; Raven and Falkowski, 1999). The effect of diffusion on internal CO_2 and O_2 during photosynthesis when there is a diffusive CO_2 flux to Rubisco accentuates the need for CCMs when CO_2 in the bulk medium is less than that at air equilibrium, and diminishes the possibility of diffusive CO_2 supply to Rubisco when bulk phase CO_2 is in excess of air equilibrium.

III. Lines of Evidence Used in Distinguishing Organisms Relying on Diffusive CO_2 Entry from Those Using Carbon Concentrating Mechanisms (CCMs)

A. Indirect Evidence

CCMs result in a higher whole-organism photosynthetic CO_2 affinity, and lower O_2 inhibition of whole-organism photosynthesis, than would be predicted from (1) the CO_2-saturated Rubisco activity in vivo (measured in the same units as in vivo photosynthesis), (2) the $K_{1/2}CO_2$ and S_{rel} of Rubisco, and (3) the CO_2 diffusion pathlength relative to Rubisco activity on an organism surface area basis (Raven, 1997b). These physiological indicators of the occurrence of a CCM are based on the presence of a higher concentration of CO_2 around Rubisco in steady state photosynthesis than in the bulk medium, assuming that the latter concentration is not extremely high (at least ten times the air equilibrium value). This higher CO_2 level around Rubisco, and the concomitant increase in CO_2/O_2 concentration ratio at the Rubisco active site, means that Rubisco can function at much closer to its maximum carboxylase activity, with minimal oxygenase activity, than would be the case with diffusive CO_2 supply. A third category of indicator is that of the natural abundance $^{13}C/^{12}C$ ratios in the organisms relative to source CO_2 $^{13}C/^{12}C$; very high values (approaching those for Rubisco in vitro) are best accommodated by diffusive CO_2 entry, with lower values indicating either operation of a CCM or diffusive CO_2 entry associated with a low diffusive conductance relative to the potential for CO_2 fixation. How do these two categories of indicators of CCMs translate into the methodology used to determine if a CCM occurs in an organism? The gas exchange, and Rubisco enzyme quantity and kinetics, which form the basis of the first set of attributes of CCMs, have not been widely performed *in toto* for cyanobacteria and algae. Generally, in vivo gas exchange characteristics are measured and compared with the values for Rubisco $K_{1/2}(CO_2)$ and S_{rel} from (usually) related organisms, with the assumption made that the maximum in vivo activity of Rubisco (at CO_2 saturation) is not greatly in excess

of the in vivo rate of CO_2 fixation (at CO_2 and light saturation). Such measurements are the most commonly reported means of investigating the occurrence of CCMs.

B. Direct Measurements of Dissolved Inorganic Carbon (DIC) Accumulation

The second set of methods of investigating the presence of CCMs involve measurements of internal CO_2 levels around Rubisco during steady-state photosynthesis (Raven, 1997b). Clearly CO_2 concentrations around Rubisco are not readily (indeed, at all) measured, and global intracellular inorganic carbon and pH values are more commonly substituted. pH is generally determined by the distribution of ^{14}C-labeled weak organic acids, and inorganic carbon from the acid-labile fraction of intracellular ^{14}C at various times after the addition of ^{14}C-labeled inorganic C. Determination of intracellular acid-labile ^{14}C inorganic C, and of ^{14}C-labeled weak acid (plus its anion) for pH estimations, requires rapid separation techniques (silicone oil centrifugation; filtration) and also methods of determining extracellular volume (^{14}C-labeled non-permeant solutes). While fluorescence microscopy of weak acids can give spatial resolution of pH within algal cells, it cannot thus far give spatial resolution of inorganic C.

Another method of determining the inorganic C pool is to measure the inorganic C pool in cells as the O_2 evolution in light after removal of external inorganic C, assuming that one mole O_2 evolved is equivalent to one mole inorganic C consumed (Raven, 1997b).

The natural abundance $^{13}C/^{12}C$ measurements are indicative rather than definitive, although the correlation of very high discriminations with diffusive CO_2 entry is very good (Raven et al., 1994, 2000b, 2002a,b; Raven, 1997b; Burkhardt et al., 1999; Keller and Morel, 1999).

These methods can give estimates of mean internal CO_2 concentration. Where direct comparisons are possible with the gas exchange studies it is generally found that indications of a CCM from gas exchange agree with indications of a CCM from inorganic C accumulation measurements. In some cases gas exchange evidence indicates the presence of a CCM but the evidence from measurements of intracellular inorganic C and pH is less conclusive. For marine *Synechococcus,* for instance, the absence of accumulation of inorganic C has been shown to be an artifact of photoinhibition during the experimental procedures for determining intracellular inorganic C (Raven, 1997b). However, we shall see that there are means of explaining (or, at least, rationalizing) gas exchange that indicate the presence of a CCM despite measurements that suggest that bulk intracellular inorganic C is not higher than that in the medium. Thus, while most hypotheses of CCM operation require inorganic C accumulation within the bulk cytoplasm (= chloroplast plus all other organelles apart from the vacuole) or, at least, the chloroplast, one suggested mechanism does not necessarily involve inorganic C accumulation at the cytoplasm or chloroplast level (Section IV.B).

IV. Occurrence and Mechanism of CCMs

A. Occurrence of CCMs

1. Taxonomic Distribution of CCMs

While relatively few of the ~1500 described species of Cyanobacteria or ~53,000 described species of eukaryotic algae (van den Hoek et al., 1995; Falkowski and Raven, 1997) have been investigated by the methods mentioned in Section III, some taxonomic patterns in the occurrence of CCMs can be discerned. All Cyanobacteria and peridinin-containing dinoflagellates have CCMs, as would be expected from the low S_{rel} of Rubisco in these organisms (Raven, 1997b,c; Badger et al., 1998; Leggat, Badger and Yellowlees, 1999). At the other extreme, freshwater representatives of the Chrysophyceae *sensu lato* and of the Batrachospermales (Rhodophyceae) all rely on a diffusive CO_2 flux of CO_2 to Rubisco, as do several marine macroscopic red algae (Raven, 1997b; Saxby-Rouen et al., 1997, 1998; Ball et al., 2000). This again is consistent with the high S_{rel} values for Rubisco in these organisms. However, while a high S_{rel} of Rubisco permits diffusive CO_2 entry to be an option for algae, it is clear that most algae expressing Rubiscos with high S_{rel} values do have CCMs, e.g. members of the Bacillariophyceae and Phaeophyceae and, with somewhat lower S_{rel} values, most members of the Chlorophyta (Raven, 1997b,c; Tortell et al., 1997; Badger et al., 1998; Tortell et al., 2000). The extent of CCM activity (as the range of CO_2 accumulation above that expected from diffusion) in various taxonomic groups is summarized in Table 1.

2. Structural Correlates of CCMs

At the subcellular level, the Cyanobacteria all have carboxysomes. These sites of most, or all, of the cellular compartment of Rubisco and (probably) all of the intracellular carbonic anhydrase have crucial roles in the currently most widely accepted hypotheses for the operation of cyanobacterial CCMs (Kaplan and Reinhold, 1999; Ludwig et al., 2000).

Subcellular correlates of CCMs in eukaryotes are much less consistent. Pyrenoids, regions of the plastid stroma where most, or essentially all, of the cellular complement of Rubisco is localized, are only found in cells which are expressing (or can, under appropriate conditions, express) a CCM (Raven, 1997b,c; Badger et al., 1998). However, pyrenoids are not found in all algae capable of expressing CCMs (Raven, 1997b,c; Badger et al., 1998; Morita et al., 1998, 2000). Where pyrenoids do occur, they are frequently associated with the insoluble polysaccharide reserve, even when the polysaccharide is outside the plastids. However, it has been shown for *Chlamydomonas,* with the pyrenoid surrounded by (intraplastidial) starch, that a mutant with much less starch has an unimpaired CCM (Villarejo et al., 1996). This finding showed that the starch sheath around the pyrenoid seems to have no particular role in the CCM (e.g. in decreasing CO_2 leakage; Section IV.B).

The endoplasmic reticulum component of the chloroplast envelope, which occurs in many chromophyte algae (Chapter 2, Larkum and Vesk) has been proposed (Lee and Krugens, 1998, 2000) to play a role in CCMs (Section IV.B). While not denying this possibility, it is clear that a division (Heterokontophyta), which invariably has chloroplast endoplasmic reticulum, contains classes which lack a CCM, at least in their freshwater representatives (Chrysophyceae *sensu lato*; Saxby-Rouen et al., 1997, 1998; Ball et al., 2000), as well as classes which seem to invariably have CCMs (Bacillariophyceae; Phaeophyceae).

3. Ecological Distribution of CCMs

We can distinguish three categories of habitat of cyanobacteria and algae in terms of CO_2 supply. Firstly, the ocean, as the largest algal habitat, has CO_2 concentrations in the surface waters which are usually between half and twice the air equilibrium value at the sea surface temperature and salinity, although wider variations can occur in areas of very high productivity and in upwellings (Raven and Falkowski, 1999). The high buffering capacity of seawater also means that the bicarbonate/carbonate concentration is high (approximately 2 mM). Secondly, inland waters are more variable in CO_2 concentrations, with very significant supersaturation in smaller water bodies as a result of CO_2 and organic C inputs in groundwater and rivers (Duarte and Agusti, 1998). Thirdly, algae which are exposed to the atmosphere permanently (free-living and lichenized terrestrial algae) or intermittently (intertidal algae) have perhaps the most constant, over an algal life-span, CO_2 concentration in the bulk medium although supply to the organisms' surface is modulated by the extent of surface water films.

In addition to these variations in bulk phase CO_2 concentrations, the supply of CO_2 to the algal surface is a function of the thickness of the diffusion boundary layer round the organisms and diffusion coefficient in the medium (10^4 greater in air than water). The diffusion boundary layer is thicker around larger than smaller planktonic cells, and is thicker around larger than smaller benthic algae (provided that even the smaller algae project through the substratum boundary layer) in rapidly flowing water. A further environmental variable is temperature which, in addition to influences on CO_2 solubility, influences S_{rel} of Rubisco such that it decreases with decreasing temperature (Sherlock and Raven, 2001). A final set of environmental variables which could influence the selective advantage of diffusive CO_2 supply relative to that of CCMs is the limitation of photosynthesis by the availability of resources other than inorganic C. Thus, low light and low N, Fe or Zn availability could have differential effects on CO_2 fixation rate per unit named resource (mol photon absorbed per second, or per mol N, Fe or Zn in the plant) as a function of diffusive CO_2 entry rather than occurrence of a CCM (Falkowski and Raven, 1997; Beardall et al., 1998). These considerations suggest a very complicated set of ecological interactions, which could determine the relative evolutionary advantage of CO_2 diffusion or of CCMs. However, it is clear that the Cyanobacteria and dinoflagellates require a CCM under the entire range of environmental conditions; diffusive CO_2 entry does not seem to be an option (Badger et al., 1998; Raven, 1997b,c, 2000). Rather, we must examine organisms with Rubiscos with higher S_{rel} values which could permit diffusive CO_2 use by Rubisco in at least some

conditions. A few examples only can be analyzed in the context of the range of variables.

One set of organisms is those marine red algae which live at low light at depth in the ocean, often in kelp beds, and usually at low (annual mean 18 °C or less) temperatures (Raven, 1997b; Sherlock and Raven, 2001; Raven et al., 2002a,b). Low light means that, for a given morphology, a smaller CO_2 flux is needed through the diffusion boundary layer, while the relatively low temperature means a high S_{rel}, yielding a high ratio of carboxylase to oxygenase activity. However, while this argument can rationalize the diffusive CO_2 entry in these shade red algae and even such intertidal shade species as *Lomentaria*, it is less readily applied to red algae in the very high intertidal which also use diffusive supply of CO_2 (Raven, 1999).

A further example of an ecologically (and taxonomically) defined group of red algae which seem to rely entirely on diffusive CO_2 flux to Rubisco are the Batrachospermales (Florideophyceae), freshwater macroalgae with a semi-erect gametophyte phase (Raven and Beardall, 1981; Raven et al., 1982; MacFarlane and Raven, 1990; Vis and Entwisle, 2000). Here the macroalgae live in freshwaters with CO_2 levels which are usually several times the air equilibrium value, and often live in flowing water as well, of course, as having a Rubisco with a high S_{rel}. However, one characteristic of most species of *Batrachospermum* is the occurrence of a gelatinous layer ten to hundreds of μm thick outside the cell wall proper which increases the thickness of the diffusion boundary layer. Furthermore, these algae share their habitat with green macroalgae, mosses and angiosperms of a similar semi-erect life form (e.g. in fast flowing streams; Raven et al., 1994, 2000b). While all mosses and some angiosperms rely on CO_2 diffusion to Rubisco, all the green algae and most angiosperms have CCMs. Can the lower upper limit on S_{rel} for chlorophyte and embryophyte Rubiscos, rather than for red algal Rubiscos, account for this pattern?

A final example of algae relying on a diffusive CO_2 flux to Rubisco is that of some terrestrial free-living and lichenized algae (Palmqvist, 2000). Here the CO_2 supply to Rubisco resembles that of C_3 higher plants, albeit without the stomata and, in some cases, the capacity to keep the distance from the gas-water interface to Rubisco constant by unwettable cell walls. However, what data are available (for *Coccomyxa*) show that the S_{rel} of Rubisco is essentially identical to that of C_3 land plants, and is higher than that of green algae with CCMs (Badger et al., 1998; Palmqvist, 2000; Raven, 2000). It is also relevant, as with immersed intertidal algae, that the organisms using diffusive CO_2 flux to Rubisco probably lose more CO_2 per unit water lost in a wetting-drying cycle than those with CCMs (Raven, 1994).

These three examples show that it is not easy to predict when diffusive flux of CO_2 from the medium to Rubisco occurs as the option favored by the selectivity of the enzyme. Nevertheless, there are broad indicators of when genotypes lacking CCMs are likely to be competitive with those expressing CCMs, taking into account phylogenetic variations in S_{rel}.

Further ecological influences on the occurrence of CCMs as opposed to diffusive flux of CO_2 from the bulk medium to Rubisco are seen in phenotypic effects on expression of CCMs. Within the ecologically relevant range of photon flux densities and of CO_2 concentrations there is evidence that the expression of CCMs is greatest at high photon flux densities (Beardall, 1991) and at low CO_2 and, when both CO_2 and light are varied, at high light and low CO_2 (Kübler and Raven, 1995). Thus, for CCMs based on the active transport of inorganic C species (see below), inorganic carbon transport and accumulation has been shown to be an active process, with evidence from several groups suggesting that the ATP necessary for carbon transport is derived from electron flow associated with PSI (Spalding et al,. 1984; Ogawa and Ogren, 1985; Ogawa et al., 1985; Palmqvist et al., 1990). Given that CO_2 fixation coupled to operation of a CCM is energetically costly (Beardall et al., 1998), it might be expected, *a priori,* that limitations on energy supply (i.e. photon flux) could have effects on the activity of CCMs. Acclimation to low photon flux results in a decreased *capacity* for DIC transport in a number of algae (Shiraiwa and Miyachi, 1985; see Beardall et al., 1998 for a recent review). However, in *Anabaena variabilis*, a decrease in CCM capacity was only found under severe light-limitation (Beardall 1991). Nonetheless, the immediate *activity*, rather than the capacity of CCMs does appear to be significantly influenced by photon flux. Beardall (1991) showed that in *Anabaena* DIC transport rates as a function of incident photon flux followed Michaelis-Menten kinetics with half maximal rates being achieved at 70–80 μmol photons.m^{-2}.s^{-1}

There appears to be a continuum in the degree to which the CCM is expressed in response to external DIC concentration, with higher CO_2 concentrations leading to a greater degree of suppression of CCM activity (Miller et al., 1984; Shiraiwa and Miyachi, 1985; Mayo et al., 1986; Badger and Gallagher, 1987). Mayo et al. (1986) suggested that for cyanobacteria the controlling factor governing CCM expression is the HCO_3^- concentration in the external medium. In some eukaryotic algae though, e.g. *Peridinium gatunense* (Berman-Frank et al., 1995) and *Chlorella ellipsoidea* (Matsuda and Colman, 1995) and *Chlorella kessleri* (Bozzo et al., 2000), CO_2 appears to be the Ci species that is the regulating factor. Any environmental factor that affects inorganic carbon levels in the bulk medium around algal cells and the speciation of inorganic carbon can thus affect the phenotypic expression of CCMs. Factors such as pH, temperature and salinity have thus been shown to alter CCM activity (Beardall et al., 1998; Beardall and Giordano, 2002).

Clearly, there are significant implications for an organism, in possessing a CCM. However, possession of the capacity for inorganic carbon accumulation does not necessarily lead to expression of that capacity as environmental constraints serve to regulation CCM capacity. Only the development of techniques sensitive enough to measure the extent of CCM activity in natural populations, preferably in situ, will allow us to accurately define the degree to which CCMs are operative in the natural environment.

B. Mechanism of CCMs and of Diffusive CO_2 Fluxes

1. Remit

Only a brief description and analysis will be provided for the 'conventional' models of CCMs based on active transport of HCO_3^- and/or CO_2 with compartmentation of carbonic anhydrase. There then follows a concise account of CCMs based on spatially or temporally separated carboxylation of C_3 acids and decarboxylation of C_4 acids. An outline will then be given of proposed mechanisms based on HCO_3^- conversion to CO_2 in (an) acidic compartment(s) with diffusive CO_2 supply to Rubisco in the neighboring, more alkaline stroma (or, in cyanobacteria, cytosol). Finally, a brief account is given of CO_2 diffusion as it relates to CCMs and to photosynthesis dependent on diffusive flux of CO_2 to Rubisco.

2. CCMs Based on Active Transport of Inorganic C Species

One major group of models of CCMs depends on active transport of HCO_3^- and/or CO_2 across one of more of the membranes separating the bulk medium from Rubisco (Fig. 1). A further requirement is that the membrane across which active transport occurs shall have a low permeability to the inorganic species delivered to the side of the membrane closest to Rubisco, otherwise active transport is short-circuited. This rules out the outer of the two plastid envelope membranes as well as the gram-negative outer membrane of cyanobacteria since these have high densities of porins with negligible selectivity for molecules of M_r less than ~800. This requirement would also rule out the chloroplast endoplasmic reticulum membrane of plastids derived by secondary endosymbiosis in the unlikely event that it had porin-type molecules of the type found in the outer plastid envelope and gram-negative outer membranes.

For Cyanobacteria the only membrane which could be involved in active transport of inorganic C is the plasmalemma. Here the active transport mechanism (Ritchie et al., 1996; Kaplan and Reinhold, 1999; Klughammer et al., 1999; Omata et al., 1999) appears to deliver HCO_3^- to the cytosol, regardless of the species (CO_2 or HCO_3^-) removed from the periplasm. The HCO_3^- then diffuses into the carboxysomes which exhibit the only carbonic anhydrase activity (Süllemeyer, 1998; Lane and Morel, 2000; Smith and Ferry, 2000) in the cytosol compartment. The CO_2 generated by this carbonic anhydrase builds up to a higher steady state concentration in the carboxysomes than in the bulk medium, thus very substantially abolishing, by competition between CO_2 and O_2, the oxygenase activity of Rubisco (Kaplan and Reinhold, 1999; Ludwig et al., 2000). Finally, some of the accumulated CO_2 and HCO_3^- leaks out of the cell, although some of the CO_2 is recouped by a light-dependent mechanism (Kaplan and Reinhold, 1999).

These mechanisms have been investigated in β-cyanobacteria (Badger, Hanson and Price, 2002; Badger and Price, 2003) such as the *Synechocystis* and some strains of *Synechcoccus* for which complete gene sequences are available. Certain α-cyanobacteria (e.g. two strains of *Prochlorococcus*) have also been completely sequenced, and the annotated genomes lack many of the genes involved in the CCMs of β-cyanobacteria (Badger et al, 2002; Badger and Price, 2003).

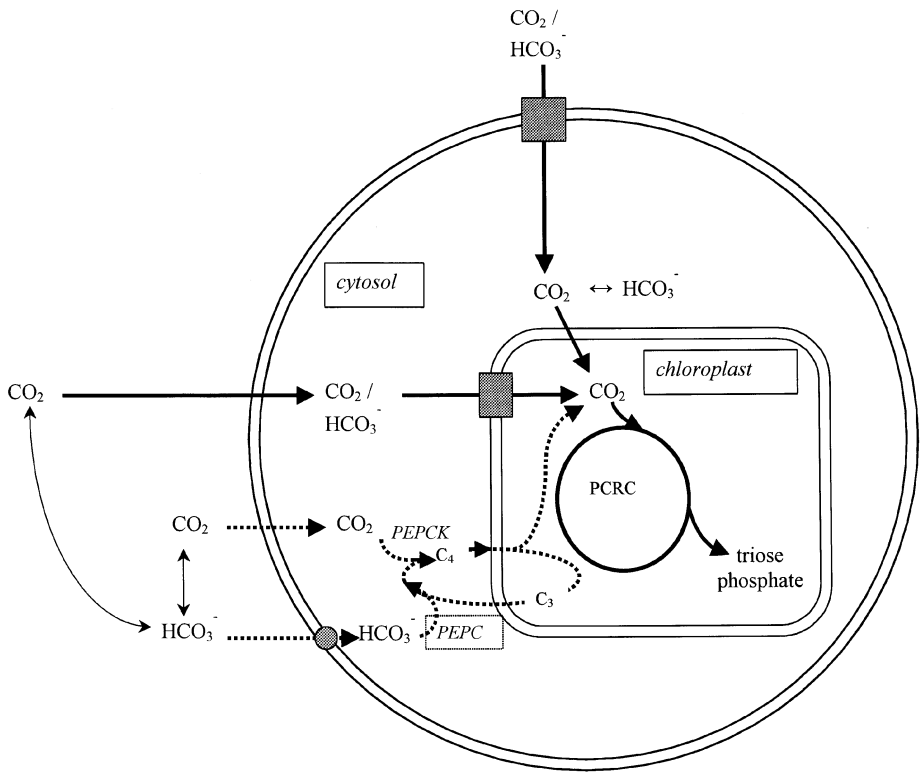

Fig. 1. A simplified scheme for transport of inorganic carbon into eukaryotic algal cells via active transport of CO_2 and/or bicarbonate. As explained in the text, CO_2 will cross membranes by diffusion, whereas active transport (shown by the shaded boxes) can be of CO_2 or HCO_3^-. Active transport can occur at the plasmalemma or at the chloroplast envelope or at both membranes. Carbonic anhydrases in the periplasmic space, cytosol and chloroplast maintain equilibrium between CO_2 and HCO_3^-. Also shown (dotted line) is a putative role for C_4-like metabolism in CO_2 concentration (see text for details). PCRC – photosynthetic carbon reduction cycle; PEPC – phosphoenolpyruvate cartboxylase; PEPCK – phosphoenolpyruvate carboxykinase. Redrawn after Sültemeyer (1998).

The same mechanism could, in principle, function in eukaryotes with CCMs taking up HCO_3^- or CO_2 from the medium (Sültemeyer, 1998; Axelsson et al., 1999; Kaplan and Reinhold, 1999; Van and Spalding, 1999; Lane and Morel, 2000; Young, Beardall and Giordano, 2001). The involvement of extracellular carbonic anhydrase in converting the non-transported into the transported species of inorganic C may be called into question by the studies of Van and Spalding (1999). Here a *Chlamydomonas reinhardtii* mutant lacking extracellular carbonic anhydrase showed essentially no phenotypic effects except a slight reduction in photosynthetic rate at very low inorganic C levels (Van and Spalding, 1999). While it could be objected that very considerable down-regulation of chloroplast carbonic anhydrase activity only has very small effects on photosynthesis in C_3 higher plants (Majeau et al., 1994; Price et al., 1994; Williams et al., 1996) the *Chlamydomonas* work involved modification of the structural gene of *Cah1* so that there was no expression of extracellular carbonic anhydrase, as far as we know (Van and Spalding, 1999).

For green and red algae with CCMs the plasma membrane, or the inner plastid envelope membrane, or both, could be the location of this active transport mechanism (Amoroso et al., 1998; Moroney and Chen, 1998; Kaplan and Reinhold, 1999; Villarejo et al., 2001; Young et al., 2001). Other algae have one (dinophyte, euglenoid) or two (chlorarachniophyte, cryptophyte, haptophyte and heterokont) additional chloroplast envelope membranes which are frequently, but incorrectly, termed chloroplast endoplasmic reticulum (Cavalier-Smith, 2000). The involvement of these additional envelope membranes in the sort of CCM discussed above (Fig. 1) has

apparently not been examined. Heterokont plastids can function photosynthetically as kleptoplastids in sacoglossan gastropods (Rumpho et al., 2000) and in vitro (Wittpoth et al., 1998) in the absence of the chloroplast endoplasmic reticulum; however, this does not necessarily tell us about the essentiality or otherwise of chloroplast endoplasmic reticulum for CCMs. As we have seen (Section IV.A), pyrenoids are not universal in the plastids of algae with CCMs, so that mechanisms analogous to that of the carboxysome of prokaryotes cannot occur in all eukaryotes with CCMs (Morita et al., 1998, 2000; Arancibia-Avila et al., 2001).

For some endosymbioses involving algae it is known that the endosymbiont expresses a CCM, at least when cultured independently of the host (Leggatt et al., 1999). The invertebrate-alga symbioses are also known to show characteristics of organisms with CCMs, but the contribution the animal makes relative to the algal partner to active inorganic C transport and carbonic anhydrase activity is not entirely clear (Raven, 1997b; Allemand et al., 1998; Furia et al., 1998; Weis and Reynolds, 1999).

It is clear that much more work is needed, especially on eukaryotes, before we have a completely convincing model of the CCM in any organism. This is especially the case for considerations of leakage (see later), the location of carbonic anhydrases (Arancibia-Avila et al., 2001; Moroney et al., 2001; Villarejo et al., 2001), and the location and mechanism of active inorganic C transport mechanisms.

3. CCMs Involving C_4 Dicarboxylic Acids as Obligatory Intermediates between External Inorganic C and CO_2 Fixation by Rubisco

The consideration of CCMs above, and much of the literature ever since 1980, has been predicated on the (general) absence of an obligatory role for C_3 acid carboxylation to produce a C_4 dicarboxylic acid (and its subsequent decarboxylation to regenerate a C_3 acid) between exogenous inorganic C and CO_2 fixation by Rubisco (Raven, 1997b,c). The role of such a C_4 dicarboxylic acid intermediate can be as a transporter of inorganic C from a site with access to exogenous inorganic C to a site (where Rubisco is active) with limited access by inorganic C, with a turnover time of the dicarboxylate of tens of seconds (Fig. 1). This is C_4 photosynthesis. In crassulacean acid metabolism (CAM) the dicarboxylate residence time is about 12 h, so that inorganic C fixed by (C_3 + C_1) carboxylation in the night can, at least in part, be refixed in the day.

C_4-like metabolism, in at least some algae, has been suggested at intervals since the early 1970s (Raven, 1997b; Busch and Schmid, 2001; Hillrichs and Schmid, 2001). The evidence here comes from the time course of ^{14}C-inorganic C incorporation into acid-stable compounds and the activity and location of ($C_3 + C_1$) carboxylases and ($C_4 - C_1$) decarboxylases. C_4-like metabolism is indicated if the first acid-stable product of ^{14}C-inorganic C assimilation is a C_4 dicarboxylic acid rather than 3-phosphoglycerate, and especially if replacing ^{14}C- with ^{12}C-inorganic C (chase) after a short (1–5 s) inorganic ^{14}C labeling time (pulse) shows transfer of label from a C_4 acid to phosphoglycerate. In terms of enzyme activity and enzyme compartmentation, C_4 metabolism requires a ($C_3 + C_1$) carboxylase in a compartment accessible to external inorganic C and a ($C_4 - C_1$) decarboxylase in the compartment containing Rubisco. In individual algal cells the cytosol is the potential (C_3 + C_1) carboxylation site and the plastid stroma the (C_4 – C_1) decarboxylation site. As for the carboxylase and decarboxylase, the cytosolic carboxylase could be phosphoenolpyruvate carboxylase (PEPc) or phosphoenolpyruvate carboxykinase (PEPck), while the chloroplastic decarboxylase could be PEPck, or NAD^+ (or $NADP^+$) malic enzyme (ME). If PEPck is to be used as both carboxylase and decarboxylase then significantly different concentrations of substrates are required for the decarboxylase and carboxylase reactions (other than CO_2), such that the carboxylase activity is favored in the cytosol where the CO_2 level is, *ex hypothesis*, low, yet there is to be net carboxylation, and so that the decarboxylase activity is favored in the plastid where the CO_2 level is, *ex hypothesis*, higher than in the cytosol.

There are two organisms for which the best cases have been made for C_4 photosynthesis in algae. One is the green ulvophycean benthic macroalga *Udotea flabellum* (Reiskind and Bowes, 1991; Raven, 1997b). The other is the planktonic diatom *Thalassiosira weissflogii* grown under inorganic C-limited conditions, i.e. low inorganic C levels, or low Zn availability and hence low carbonic anhydrase expression (Reinfelder et al., 2000). In both cases there are tracer kinetic, as well as enzyme activity and localization, data. *Udotea* uses a cytosolic PEPck as the ($C_3 + C_1$) carboxylase, and, possibly, NAD ME as the stromal decarboxylase (Reiskind and Bowes, 1991; Raven, 1997b). The suggestion for *Thalassiosira* is the use

of cytosolic PEPc as the (C_3 + C_1) carboxylase and stromal PEPck as the (C_4 – C_1) decarboxylase (Reinfelder et al., 2000). The tracer kinetic data shows, in both cases, transfer of ^{14}C from malate to sugar phosphates during the chase period, and the ^{14}C label in malate relative to phosphoglycerate during the pulse labeling is higher than expected for C_3 biochemistry (Reiskind et al., 1988; Reiskind and Bowes, 1991; Reinfelder et al., 2000). However, the shortest pulse labeling times were 10 s (Reiskind et al., 1988; Reiskind and Bowes, 1991) and 5 s (Reinfelder et al., 2000), and Johnston (1991) showed that labeling times as short as 1 s were needed to show that phosphoglycerate rather than a C_4 acid was the initial product of photosynthetic inorganic C fixation in the brown macroalga *Ascophyllum*. Evidence for true C_4 biochemistry in *Thalassiosira* at least is equivocal (Johnston et al., 2001). It would be especially useful to have more very short-term pulse-label data, not only for *Udotea* and *Thalassiosira*, but also for other algae which are presently believed to have C_3 biochemistry. It must be remembered that CCMs based on active inorganic C (above) or H^+ (below) transport would, like C_4-like metabolism, account for C_4-like gas exchange physiology (high CO_2 affinity; O_2 insensitivity), and that active inorganic C transport could make pulse-chase experiments more difficult to interpret since any $^{14}CO_2$ released from ^{14}C-dicarboxylates would be less likely to be 'chased' out of the cell and more likely to be refixed by Rubisco even if this is not an obligate pathway from external inorganic C to Rubisco. A low $^{13}C/^{12}C$ ratio in the organic C of the organism relative to source CO_2 can be explained by a CCM based on inorganic C or H^+ active transport as well as by CCMs based on C_4-like metabolism using PEPc as the (C_3 + C_1) carboxylase, and more readily than if PEPck is the carboxylase (Raven, 1997b).

CAM has also been proposed as a contributor to photosynthetic inorganic C assimilation in brown macroalgae, albeit providing less than 10% of the total organic C (Johnston, 1991; Raven, 1997b). The evidence here is the high PEPck activity in the brown algae, and the increase in titratable acidity and malate measurable in algal homogenates overnight and a decrease during the day (Johnston, 1991; Raven, 1997b). However, Keeley (1996) found no evidence for ^{14}C transfer from dicarboxylates labeled at night into sugars in the day in *Ascophyllum nodosum*, the alga in which the titratable acidity and malate change was originally found. Kawamitsu and Boyer (1999) found evidence for CO_2 storage in an organic form in the intertidal brown alga *Fucus vesiculosus,* which can be used to supply photosynthesis in low CO_2 conditions and which is replenished maximally in high light and high CO_2 conditions; this adds to earlier work reviewed by Raven (1997b).

These discussions of C_4-like and CAM-like metabolism in algae show that there is significant evidence for C_4-like metabolism in *Udotea* and in CO_2-deprived *Thalassiosira*, and there are some suggestions of CAM-like processes in many fucalean brown macroalgae. However, more data are needed to further test these suggestions.

4. CCMs Involving Active Transport of H^+, with HCO_3^- to CO_2 Conversion in the Acid Compartment Adjacent to the Compartment Containing Rubisco

The basic premise here is that HCO_3^- from an alkaline medium/compartment is transported to a compartment which is maintained at a low pH by an H^+ pump (Figs. 2, 3). Here the equilibrium CO_2: HCO_3^- ratio is much higher than that in the first medium. Details of how this equilibrium may be maintained are given by Raven (1997b). The CO_2 produced in this acidic compartment then diffuses to Rubisco in an adjacent, more alkaline (pH 7.5–8.0) compartment. Such mechanisms work most effectively if there is no carbonic anhydrase in the compartment containing Rubisco, at least in the cases where the Rubisco compartment is closer to the medium than is the acidic compartment (Raven, 1997b).

This model was first suggested, and quantified, by Walker et al. (1980) for the acid zones on the surface of internodal cells of characean freshwater macroalgae (Allemand et al., 1998; Furia et al., 1998), and by Pronina and Semenenko (Pronina and Semenenko, 1992) for the thylakoid lumen as the acidic compartment. The thylakoid model (Fig. 2) was quantitatively modeled by Raven (1997a,b), based on the occurrence of an α-carbonic anhydrase on the inner side of the thylakoid membrane which is needed for growth of *Chlamydomonas reinhardtii* in ambient CO_2 (Karlsson et al., 1998; van Hunnik et al, 2001). An involvement of this carbonic anhydrase in CO_2 generation from HCO_3^- as part of the reaction sequence between exogenous inorganic C and Rubisco does not preclude a role for the enzyme in photoactivation

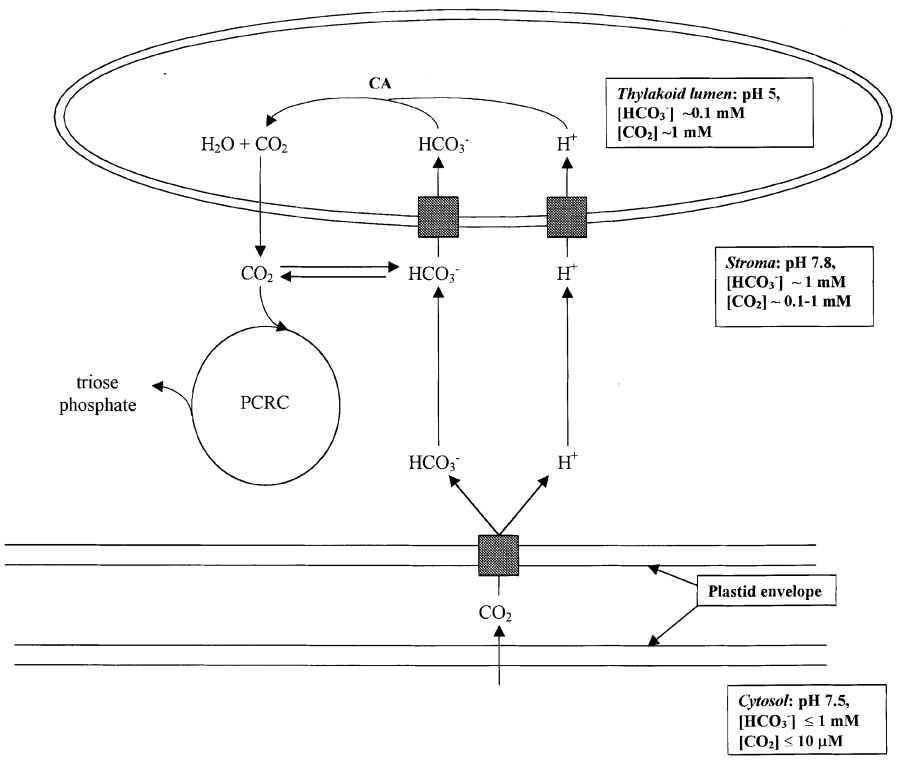

Fig. 2. A model for accumulation of CO_2, based on inorganic carbon transport into the thylakoid lumen. As a result of the lower pH in this compartment, brought about by active H^+ transport, CO_2 levels and supply to Rubisco in the stroma are enhanced. See text for details. PCRC – photosynthetic carbon reduction cycle. Redrawn after Raven (1997a,b).

and function of the water oxidizing complex of Photosystem II (reviewed by Ananyev et al., 2001).

For the variants of this hypothesis which involve intracellular acid compartments rather than an extracellular acid zone as in Walker et al. (1980) there is a requirement for HCO_3^- transport from an adjacent alkaline (pH 7.0–8.0) compartment to the acid compartment. The alkaline compartment from which bicarbonate is moved (by, *ex hypothesis*, a bicarbonate channel) into the acid compartment is supplied with bicarbonate from the medium via the plasmalemma and, when the acid compartment is the thylakoid, the chloroplast envelope membranes (Raven 1997a,b,c, and Figs. 2 and 3).The compartments into which HCO_3^- is, *ex hypothesis*, transported are the thylakoid lumen (Fig. 2) (Pronina and Semenenko, 1992; Raven 1997a,b,c), the vacuole (Fig. 3) (Raven, 1997a,b,c), an aqueous compartment(s) bounded by additional chloroplast envelope membrane(s) in algae other than chlorophytes and rhodophytes (Lee and Kugrens, 1998, 2000) and, for algae endosymbiotic in invertebrates, the perisymbiont space (although this compartment is not very acid, at least in the case of the *Anemonia-Symbiodinium* symbiosis: Rands et al., 1993). There is little evidence thus far as to the occurrence or nature of these hypothesized HCO_3^- channels. Indeed, Villarejo et al. (2001) found no evidence for uptake of HCO_3^- and its conversion to CO_2 using mass spectrometric methods on isolated thylakoids, competent in electron transport and ATP synthesis, of *Chlamydomonas reinhardtii*. However, any HCO_3^- channels might have been inhibited by some component of the assay medium (Berecki et al., 1999), or require a signal related to carbon metabolism before they have a high likelihood of being open. Undeterred, Raven (2001) has produced a model for the recycling of respiratory and photorespiratory CO_2 from mitochondria to plastids as HCO_3^-, using the matrix β-carbonic anhydrase of *Chlamydomonas* mitochondria (Eriksson et al., 1996), and requiring an absence of carbonic anhydrase activity in the

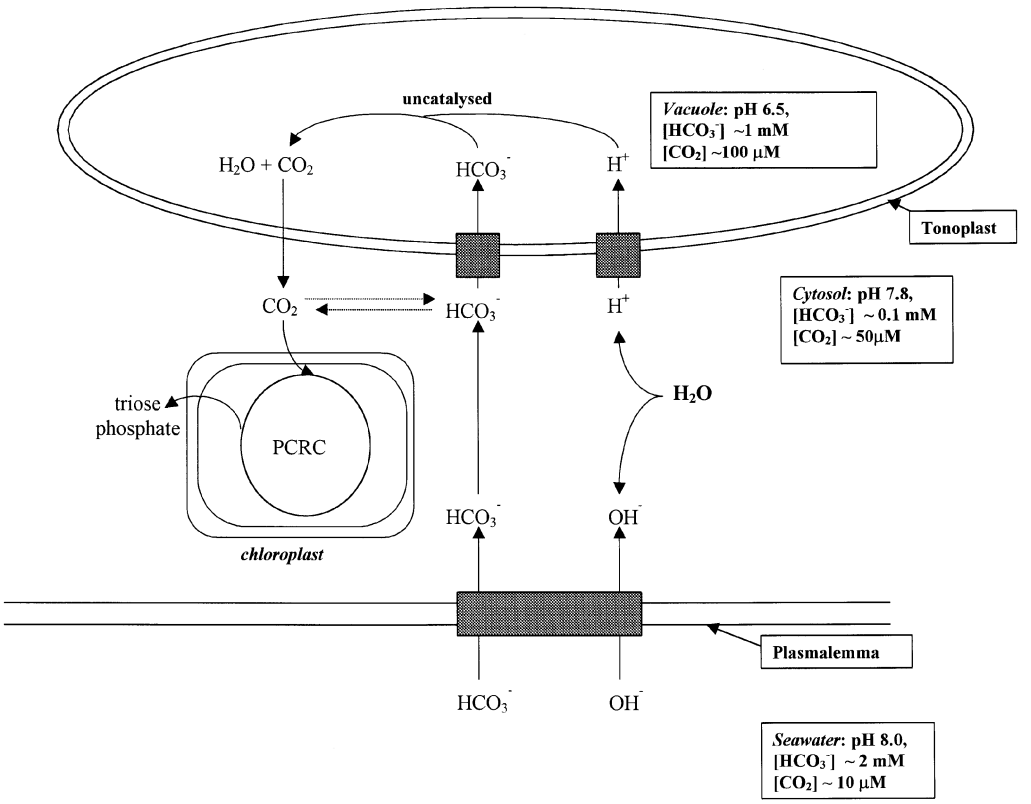

Fig. 3. A model for the accumulation of CO_2 based on inorganic carbon transport into the vacuole. As for the thylakoid model, lower pH in the vacuole, associated with active H^+ transport across the tonoplast, results in elevated levels and enhanced supply of CO_2 to Rubisco in the chloroplast. See text for details. Redrawn after Raven (1997b)

cytosol. It is known that higher plant mitochondria have a pH-regulated anion channel in their inner membrane (Beavis and Vercesi, 1992), although the capacity of this channel to transport HCO_3^- is not established.

Clearly a closer examination of the distribution of carbonic anhydrase within algal cells, and a search for bicarbonate channels or their functional equivalents, are needed to evaluate the hypothesis suggested here. However, it is clear that such a mechanism can account for cases where gas exchange physiology suggests the presence of a CCM yet the average inorganic C concentration in the cells is not greater than that in the medium. Furthermore, the compartmentation of well-established carbonic anhydrases in *Chlamydomonas reinhardtii* is consistent with the hypotheses of Raven (1997a,b,c) and Raven (2001).

5. The CO_2 Permeability of Algal Membranes has Implications for CCMs and for Diffusive Flux of Inorganic C from the Bulk Medium to Rubisco

The CO_2 permeability of membranes is an important aspect of the diffusive CO_2 supply to Rubisco, where high CO_2 permeability of the membranes maximizes the flux. The same applies to the membrane(s) separating the acid compartment, in which HCO_3^- is converted to CO_2, from the slightly alkaline compartment containing Rubisco in the hypothetical mechanism for CCMs with CO_2/HCO_3^- disequilibrium in the compartment containing Rubisco. By contrast, the CCMs based on inorganic C active transport involve a higher CO_2 concentration around Rubisco than in the medium, and the energetic cost of the net pumping is minimized if the membranes between

Rubisco and the medium have low CO_2 permeability. The same applies to membranes between Rubisco and the medium when HCO_3^- to CO_2 conversion occurs in an acid compartment centripetal to Rubisco.

The technically most adept measurement of the CO_2 permeability coefficient of an algal cell membrane is that by Sültemeyer and Rinast (1996) on the plasmalemma of *Chlamydomonas reinhardtii*. Similar values, of 1.5 and $1.8 \cdot 10^{-5}$ m s^{-1} respectively, were obtained for the plasmalemma from cells grown at high CO_2 (mainly diffusive CO_2 influx) and from those at low CO_2 (CCM). These values are very considerably lower than those found for phospholipid plus sterol bilayers, presumably because the plasma membrane contains different phospholipids than those in the model bilayer, as well as containing proteins and carbohydrates. The absence of sterols from internal membranes of algae suggests that P_{CO_2} values for these membranes are likely to be higher than those for the plasmalemma. The presence of galactolipids in the plastid membranes may also have effects on P_{CO_2}. Using methods which are more likely than those of Sültemeyer and Rinast (1996) to underestimate P_{CO_2}, Baier et al. (1990) and Gimmler et al. (1990) found P_{CO_2} values for plasmalemmas of a range of photosynthetic organisms which were below 10^{-6} m s^{-1}. Baier et al. (1990) and Gimmler et al. (1990) attributed higher P_{CO2} values to proteinaceous pores.

It is likely that the P_{CO_2} of the *Chlamydomonas* plasmalemma is at the low end of the range for algal plasmalemmas. The argument here involves the findings that at least some aquaporins (Murata et al., 2000) facilitate the transport of CO_2 (Nakhoul et al., 1998; Prasad et al., 1998), and that cells like *Chlamydomonas* which regulate their volume with contractile vacuoles have such low water permeabilities of their plasmalemma that they appear to have no functional aquaporins (Raven, 1995). It is possible that other algal plasmalemmas could have higher P_{CO_2} as a result of aquaporins, e.g. *Chara* (Henzler and Steudle, 2000), acting as suggested by Baier et al. (1990) and Gimmler et al. (1990) as CO_2 channels. However, the work of Sun et al. (2001) on the same aquaporin (aqp1) as was used to demonstrate carbon dioxide permeation failed to confirm the earlier work. A further role of proteinaceous channels in permitting CO_2 transport across membranes is in the porins of the outer plastid envelope membrane (and the outer membrane of cyanobacterial cells); we assume that, relative to other membranes, the impedance to CO_2 diffusion at this membrane is negligible.

What implications do these estimates have for diffusive CO_2 flux from the medium to Rubisco and for CCMs? For algae relying on diffusive CO_2 entry there are computations of the overall conductance for transfer of CO_2 from the bulk medium to Rubisco using photosynthetic rates on an area basis, external CO_2 concentrations, and the $^{13}C/^{12}C$ of external CO_2 and of algal organic C. For the marine shade red macroalga *Delesseria sanguinea* a conductance value of $6.6 \cdot 10^{-6}$ m s^{-1} has been calculated (Johnston et al., 1992; Maberly et al., 1992). With a P_{CO_2} of $1.5 \cdot 10^{-5}$ m s^{-1} (Sültemeyer and Rinast, 1996) for the plasmalemma and for the inner plastid envelope membrane, these two membranes in series would (by addition of their reciprocals, i.e. resistances) have a conductance of only $7.5 \cdot 10^{-6}$ m s^{-1}. Ignoring the resistance to CO_2 diffusion across these membranes, permitted Maberly et al. (1992) to compute a diffusion pathlength from the bulk medium to Rubisco in *Delesseria* of 20 μm. Taking into account the membrane conductance of $7.5 \cdot 10^{-6}$ m s^{-1} the residual conductance must be $5.5 \cdot 10^{-5}$ m s^{-1}, allowing only 2.4 μm of aqueous phase diffusion of CO_2, which is unbelievably small (Maberly et al., 1992). Even allowing for the assumption, it does seem that one or the other of the conductances for *Delesseria* is underestimated. Accordingly, the CO_2 permeability of the plasmalemma and/or the inner envelope membrane in *Delesseria* must be higher than that of the *Chlamydomonas* plasmalemma (Raven et al., 2002a,b). Similar considerations apply to the computations of MacFarlane and Raven (1990) for the freshwater red macroalga *Lemanea mamillosa*. However, the computations of Raven (1997a) on the hypothesis of HCO_3^- conversion to CO_2 in thylakoid lumen with CO_2 diffusion to the stroma as components of a CCM show that the mechanism can function readily when the lower P_{CO_2} value ($1.5 \cdot 10^{-5}$ m s^{-1}) is assumed for the thylakoid membrane.

Turning to the effect of the value of $1.5–1.8 \cdot 10^{-5}$ m s^{-1} for P_{CO_2} of the plasmalemma of *Chlamydomonas* on algae with CCMs, Sültemeyer and Rinast (1996) point out that this P_{CO_2} suggests that the CO_2 gradient (higher around Rubisco than in the medium) is not maintained across the plasmalemma. However, the suggestion made above is that the inner envelope membrane of the plastid is unlikely to have a lower P_{CO_2} than the plasmalemma. This accords with the computations of Raven and Johnston (1991), who showed that, for a spherical unicellular green alga of

radius 5 μm, with a CO_2 permeability of 10^{-5} m s^{-1} for the sum of the membranes between Rubisco and the medium, the CO_2 leakage imposes a lower limit of 3 μmol m^{-2} s^{-1} for the photon flux density (400–700 nm) for growth (Raven et al., 2000a). Even allowing that there is a lower phenotypic (acclimatory) or genotypic (adaptive) likelihood of CCMs at low photon flux densities (Beardall, 1991; Kübler and Raven, 1995) there is evidence suggesting that the decrease in photon yield predicted from the assumption of P_{CO_2} of 10^{-5} m s^{-1} in algae with CCMs are not invariably present (Raven et al., 2000b). However, while there are possible explanations of decreased leakage of CO_2 despite an effective P_{CO_2} of the membranes between Rubisco and the medium of 10^{-5} m s^{-1} (Kaplan and Reinhold, 1999), these have not thus far been quantified in a mechanistic way.

While not wishing to trivialize measurements of larger inorganic C effluxes (CO_2 and HCO_3^-) from cells with CCMs than can be accounted for by the P_{CO_2} and (lipid solute) $P_{HCO_3^-}$ of biological membranes (Raven, 1984; Sültemeyer and Rinast, 1996), we reiterate (Raven, 1984, 1997b,c) that the unidirectional efflux of CO_2 or HCO_3^- could be energetically neutral if it represented the slippage operation of a CO_2 or HCO_3^- pump (Sanders, 1990). This 'efflux-energizing influx' mechanism seems less plausible, however, for cases in which active CO_2 influx is paralleled by HCO_3^- efflux or vice versa (Kaplan and Reinhold, 1999).

Clearly there are still issues of contention surrounding the permeability of algal membranes to CO_2 and the consequences of this for CO_2 diffusion and CCM operation.

V. Evolution of CCMs

This topic has been considered by, *inter alia*, Raven (1997b,c), Badger et al. (1998) and Tortell (2000); see also Chen et al. (2000). The general long-term trend of decreased availability of inorganic C with time since O_2-evolving photolithotrophs evolved at least 2.4 Ga ago (and probably 3.5 Ga ago) (Falkowski and Raven, 1997; Berner and Kothavala, 2001) underpins the increasing evolutionary requirement for CCMs as inorganic C limitation increases, despite evolutionary increases in S_{rel} and decreased $K_{1/2}CO_2$ of Rubisco, in taxa which evolved at later times. This does not give us any direct evidence as to monophyly or polyphyly of CCMs in prokaryotes or eukaryotes, though Raven (1997b) argues for a polyphyletic origin. At least carboxysomes have molecular genetic markers (Ludwig et al., 2000) which may help to decide on monophyly or polyphyly of these structures; this is not the case for pyrenoids or, indeed, any component of the eukaryotic CCM. However, the possibility of lateral gene transfer in the origin of CCMs and associated structures cannot be ignored.

It is possible that there were significant CO_2 decreases at intervals in the Proterozoic when natural selection may have favored the evolution of CCMs. These low CO_2 intervals could have coincided with low-latitude sea-level glaciations (Hoffman et al., 1998; Williams et al., 1998). Could these low CO_2, cool events have created a genetic bottleneck in the evolution of photolithotrophs and provided conditions for the selective usefulness of CCMs? In terms of the evolution of CCMs in eukaryotes the Neoproterozoic glaciation (2.4–2.2 Ga) would have more relevance than the Palaeoproterozoic glaciation (0.82–0.55 Ga) (Falkowski and Raven, 1997; Raven 1997b,c, 2000). However, such bottlenecks could not explain the overall distribution of CCMs (e.g. the absence of CCMs in freshwater chrysophytes *sensu lato*).

VI. Conclusions and Prospects

CCMs have been experimentally established, as opposed to being hypothetical constructs, for rather more than 40 years. Over that time there has been very significant progress in determining the mechanism(s) involved, especially for the β-cyanobacteria where there has been a large contribution from molecular genetics to complement biochemical, biophysical and physiological data and interpretations. There has also been much study of the diversity of algae expressing CCMs, the variety of mechanisms involved in different CCMs, and the relationship of CCMs to Rubisco kinetics. While this work has shown that most algae express CCM activity when growing under near-natural conditions, it has also shown that there are significant variations in the details and, probably, more fundamental aspects of the mechanisms of the CCMs, and that a significant minority of algal species which do not express CCM activity.

As to prospects for advances in the near future, genomics are clearly a very important tool. It is already known from complete genome sequences of cyanobacteria that α-cyanobacteria (e.g. *Prochloro-*

coccus) must have significantly different CCM mechanisms than those of the better investigated β-cyanobacteria. Complete sequences of diatom genomes should soon be available, and will help in analysis of the diatom CCM(s). The varied phylogenetic origins of eukaryotic algae mean that the diatom sequences may not be very helpful in determining the mechanism(s) of the CCM(s) in dinoflagellates. Improved experimental techniques, and models, will also contribute to our understanding of algal CCMs. Finally, further genomic and other studies are needed to determine the magnitude, and determinants, of the CO_2 permeability of algal membranes.

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Chapter 12

Modeling the Excitation Energy Capture in Thylakoid Membranes

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Summary

The thylakoid membrane of organisms carrying out oxygenic photosynthesis is composed of a great variety of extrinsic and intrinsic peripheral light-harvesting complexes together with two types of core complexes that bind the cofactors of the reaction centers. In a complex interplay, the macromolecular supercomplexes perform an optimized conversion of light into chemical free energy. In the past decades much knowledge has accumulated on the structure and dynamics of the individual pigment-protein supercomplexes: This can be advantageously used to model light-harvesting in various kinds of thylakoid membranes and the distribution of excitation energy to the photosystems.

This chapter focuses on the experimental dissection and theoretical reassembly of the components of thylakoid membranes. By means of three examples it will be demonstrated how known structural building blocks are related to so-called states can be put together in reaction schemes. The mathematical treatment of such self-consistent reaction schemes which predicts unique solutions for all measurable quantities will be explicitly outlined. The solutions can be used to analyze experimental data (target analysis), to test their consistency with experimental data, and to predict experimentally difficult accessible quantities like: (i) quantum yields and (ii) the proportion in which the two photosystems are excited. Fluorescence induction, lateral energy transfer, distribution and redistribution of excitation energy between the photosystems (spillover), uphill energy transfer and low temperature data will also be discussed.

I. Introduction

All photosynthetic **r**eaction **c**enters (**RC**s) are boosted by antenna pigments which deliver excitation energy to them with the sole purpose of increasing their turn-over rates. These photochemically inactive pigments are bound, depending on the class, covalently or by van der Waals forces, to polypeptides (or **l**ight-**h**arvesting **c**omplexes, **LHC**s) and these in turn to the RC. The binding of the pigments to the polypeptides modulates to some extent the photophysical properties (wavelengths of maximal absorption, molar absorption coefficients) compared to the same pigments in organic solvents.

In oxygenic photosynthesis two different RCs (RC-PS I and RC-PS II) with significantly different functions and properties act together. In some cases these are supplied by the same, but in other cases by very distinct LHCs.

To describe the functional aspects of thylakoid membranes, structural and kinetic models are required which finally lead to reaction schemes being composed of states. The term (excited) 'state' is used here for an exciton residing on a group of pigments that can be approximated as homogeneous regarding its kinetic relations with the environment. These reaction schemes are then open to a self-consistent quantitative analysis. Such an analysis predicts the kinetics and the yields of the involved states. The theoretical simulations deliver valuable criteria to test an assumed model for agreement or disagreement with experimental data (Holzwarth, 1996). They can also be used to determine physical meaningful parameters from fits to experimental data obtained from isolated pigment-protein complexes, chloroplasts or whole algal cells.

The concept of this chapter is first to review structural entities of the thylakoid membrane and their spectral properties. Second, absorption and fluorescence spectra of these entities will be mathematically defined, so that they become suited for storage in a data base. Third, the structural entities will be reassembled theoretically for the purpose of quantitative modeling of the thylakoid membrane. Any envisaged structural model can then be converted into a reaction scheme consisting of states that are functionally coupled by rate constants.

We will describe the mathematical formulation of

Abbreviations: APC – allophycocyanin; DAS – decay associated spectra; FCP – fucoxanthin-Chl *a*/*c*-binding proteins; IUPAC – International Union of Pure and Applied Chemistry; LHC – light-harvesting complex; MCA – metabolic control analysis; sPSU – stoichiometric photosynthetic unit; PBP – phycobiliprotein; PBS – phycobilisome; PC – phycocyanin; PCP – peridinin-Chl *a*-binding protein; PE – phycoerythrin; PSE – photosynthetic entity; PS I – Photosystem I; PS II – Photosystem II; PSU – photosynthetic unit; RC – reaction center; RT – room/ambient temperature; SAAS – species associated absorption spectrum; SAES – species associated emission spectrum; sPCP – soluble peridinin-Chl *a*/*c*-binding protein; SRCM – spectrum-reconstitution method; TE – transfer equilibrium; TRES – time-resolved emission spectra

such systems by means of rate matrices, which—with a given initial condition—determine the following quantities: photochemical and fluorescence quantum yields, fluorescence spectra, time-resolved fluorescence, and distribution of the excitation energy to the two photosystems.

The efficiency of such an analysis will be demonstrated by treating in detail three examples: (i) the thylakoid membrane of the siphonal green alga *Ostreobium* sp. which contains a large number of strongly red-shifted Chl *a* forms, (ii) the thylakoid membrane of cyanobacteria/rhodophytes which contain phycobilisomes as the main antenna system and (iii) PS II in *Acaryochloris marina* which possesses Chl *d* absorbing at 705 nm for light-harvesting.

In addition, a brief introduction to some basic formulas of fluorescence induction will be given. This will provide information on the antenna size of PS II and the separation distance between **p**hotosynthetic **u**nits (**PSU**). Furthermore, the Kennard-Stepanov relation (Kennard, 1918; Stepanov, 1957), which allows the transformation of the absorption spectrum into a fluorescence spectrum and vice versa, will be discussed by comparing the thermally equilibrated case with a kinetic treatment in a model system.

Whenever feasible emphasis is placed on algal systems. However, the principles of modeling outlined in the chapter are the same for thylakoid membranes of plant chloroplasts.

II. Structural Composition of the Thylakoid Membrane

Because of the great complexity of the thylakoid membrane and its proteineous constituents it is nearly impractical to perform a modeling that accounts for all individual pigments, their distances and mutual orientations within each protein complex. However, such an ambitious modeling has been carried out for PS I by Byrdin et al. (2002). Advantageously, the complexity of a model should be kept at such a minimum that experimental information allows the determination of the parameters of the model.

In the following, the decomposition of the pigmented part of the thylakoid membrane into appropriate entities will be briefly discussed. The entities will be characterized with respect to the number of pigments bound to them, the spectral properties, and the energy transfer kinetics between the pigments. This results in the definition of building blocks suited for modeling the photosynthetic membrane.

A. Antenna Pigments

A variety of compounds serve the photosynthetic light-harvesting function. First of all, there are the closed-chain tetrapyrroles, chlorophylls (Chl), of which Chl *a*, Chl *b*, various types of Chl *c*, and Chl *d* are the most abundant (Hoff and Amesz, 1991; Chapter 13, Larkum). Photophysical properties like oscillator strengths and radiative rate constants for fluorescence emission (k_{rad}) do not differ much between organic solvents and the LHC bound forms. Typical values for Chl *a*, *b*, *c*, and *d* are molar absorption coefficients of the Q_y-bands of $\varepsilon \approx 80\,000$, 50 000, 10 000, 70 000 M^{-1} cm^{-1} and radiative rate constants of $k_{rad} \approx (15, 10, 2, 15\ ns)^{-1}$, respectively.

Binding to polypeptides causes red-shifts of up to 70 nm. Chl *b* has lesser red shifts than Chl *a* and Chl *c*. Since all binding sites of the chromophores within a LHC differ, the absorption bands of the LHCs are heterogeneously broadened (spectral forms). Assuming invariable oscillator strengths this leads to apparent molar absorption coefficients that are smaller (10–30%) than those in organic solvents.

Secondly, there are open-chain tetrapyrrole chromophores, the bilins with different numbers of conjugated double bonds: phycocyanobilin, phycoerythrobilin, phycourobilin, phycoviolobilin or phycobiliviolin, and cryptobilin. The photophysical properties of the bilins, like the molar absorption coefficients in organic solvents and their radiative lifetimes, differ strongly from those bound in the protein (Scheer, 1982).

Finally, there are the carotenoids which also serve light-harvesting, though not uniquely (Hiller, 1999; Chapter 15, Mimuro and Akimoto). In vivo the main absorption bands lie between 450 and 500 nm. They consist of three sub-bands which are often observed only as shoulders. Their molar absorption coefficients are only slightly dependent on the solvent polarity (Lichtenthaler, 1987) and are all on the order of $\varepsilon \approx 100\,000\ M^{-1}\ cm^{-1}$ (Britton, 1995). The singlet excited state lifetimes (S_2 and S_1) are extremely short and their light-harvesting function rests on positioning them at van der Waals distances to chlorophyllous acceptors in the LHCs. In the context of this chapter they contribute to absorption but not to fluorescence.

The solvent absorption spectra of the chromophores can be described mathematically as outlined in Section III.A, stored in a database, and then can be used either for a spectral decomposition of isolated pigment-protein complexes, for constructing assumed model complexes or even a whole thylakoid membrane.

B. Structural Entities and their Exciton Dynamics

The building blocks (structural entities) of a thylakoid membrane can be roughly classified into the RC-core complexes and various peripheral LHCs. In this section the currently known complexes are briefly discussed with respect to some structural details, pigment content, stoichiometric ratios, and exciton transfer dynamics. The latter have been extensively reviewed by van Grondelle (1985), Holzwarth (1986), Renger (1992), van Grondelle et al. (1994), Jennings et al., (1996), and Owens (1996).

In most instances these building blocks can be used for defining a state in a reaction scheme. In other instances part of the pigments in a building block may be pooled into states if this is relevant for modeling.

1. PS I-Core Complex

The essential cofactors involved in the primary charge separation of PS I are the primary donor P700 absorbing maximally at 700 nm and the chlorophyllous acceptors A and A_0 as well as the phylloquinone A_1 (reviewed by Sétif, 1992; Nechushtai et al., 1996; Brettel, 1997). The molecular rate constant for the first step of the primary charge separation in the RC is not explicitly known. This is due to the fact the PS I-core complex cannot be physically separated into a naked RC and a core antenna complex. Thus, only indirect estimates, based on modeling, exist which suggest a primary charge separation of $\leq$1 ps (Byrdin et al., 2002).

The PS I-core consists of 12 subunits which bind 96 Chl *a* molecules and 22 carotenoids. The distances between neighboring Chl *a* molecules range between 9–16 Å (Fromme et al., 1996; Jordan et al., 2001). The Förster theory predicts pigment-to-pigment hopping times of less than a few 100 fs for these small distances (see Section IV.D.1), which means that on the statistical average an exciton needs $\approx$10 ps to visit once all 100 Chl *a* molecules. This time agrees with experimentally determined equilibration times of $\approx$5 ps (Hastings et al., 1994) or $\approx$15 ps (Holzwarth et al., 1993; Croce et al. 2000). However, the equilibration does not involve the primary donor P700 and the 4 Chl *a* cofactors (Croce et al., 1996). Therefore the trapping mechanism is probably of the transfer-to-the-trap limited type (Valkunas et al., 1995).

Fluorescence spectra at low temperatures often show intense peaks at >700 nm (up to 760 nm) demonstrating that the core-antenna can contain long wavelength absorbing Chl *a* molecules (Fork and Mohanty, 1986; Govindjee and Satoh, 1986). This spectral heterogeneity has to be considered in model calculations if the precise shape of the fluorescence spectrum is of interest.

The overall trapping time is in the order of 20–30 ps (Hastings et al., 1995). However, it may vary considerably with the absence or presence of long-wavelength absorbing Chl *a* forms, their abundance, and their energy level(s). Extreme trapping times for cyanobacterial PS I-core complexes are from a *Synechocystis* PS II-deficient mutant (with no traceable amounts of red forms) of $\approx$22 ps (Hecks et al., 1994) and from *Spirulina platensis* (with red forms up to 737 nm) of $\approx$70 ps (Karapetyan et al., 1998; Koehne and Trissl, 1998). In this latter case, the modeling of PS I requires at least two spectral states.

The core complexes of PS I in the thylakoid membrane of Cyanobacteria show a tendency to trimerize (Kruip et al., 1994) as is the case in *Prochlorococcus* (Garczarek et al., 1998). The trimeric state serves as a possible docking station for PBSs (Bald et al., 1996). It can only be speculated whether in red algae trimerization of PS I-cores also occurs. In thylakoids of green plants and green algae the core complexes of PS I are monomeric.

The primary donor of PS I of nearly all prokaryotes and eukaryotes is a Chl *a* dimer with a Q_y band close to 700 nm (P700). An exception is the cyanobacterial prokaryote *Acaryochloris marina* in which the primary donor consists of Chl *d* absorbing at 740 nm (P740) (Hu et al., 1998; Chapter 3, Partensky and Garczarek).

Depending on how detailed thylakoid membranes are to be modeled, the PS I-core building block may be taken as just one state or divided into proximate, bulk, and red-form chlorophylls, each forming a state.

2. PS II-Core Complex

The minimal size of a functional core complex of

PS II is often considered to be the D1-D2-Cyt b_{559} complex which binds 6 Chl *a* molecules, 2 pheophytins and 2 β-carotenes. However, for the purpose of modeling, it is more convenient to include the closely associated Chl *a*-bearing polypeptides CP43, CP47 and the minor polypeptides CP26 and CP29. If each of them binds ≈11 Chl *a* and ≈5 β-carotenes (Alfonso et al., 1994) the number of Chl *a*-molecules within this building block is then 50. Chl *b* is not known to bind to any appreciable amount. The arrangement of the polypeptides within the D1-D2-Cyt b_{559}/CP43/CP47/CP26/CP29 supercomplex has been reviewed by Barber et al. (1999) and a crystal structure of the core complex of PS II at 3.8 Å has been obtained for *Synechococcus elongatus* (Zouni et al., 2001). This core complex of PS II may be taken as a building block for modeling PS II.

Equilibration of excitons within this PS II-core requires ≈15 ps (van Grondelle et al., 1994; Dau and Sauer, 1996) and is thought to include the primary donor P680. Trapping in this photosystem is therefore RC-controlled. This has led to a description of the energy conversion in PS II by the exciton-radical-pair-equilibrium model (Fig. 1) in which the recombination of the primary radical pair $P680^+Phe^-$ into the excited state contributes to fluorescence by a clearly discernible kinetic phase, even if the RC is in the open Q_A-state (Schatz et al., 1988). Upon reduction of Q_A (closed state) charge separation becomes slower and recombination faster so that the closure of the PS II RC leads to a several fold increase in fluorescence (i.e. fluorescence induction; Section IV.B). It is worth mentioning that the radiationless decay path of the radical pair state into the ground state (k_d) largely controls the fluorescence yield in closed RCs but to a lesser extent than in open RCs, and therefore also controls the ratio of maximal to minimal fluorescence.

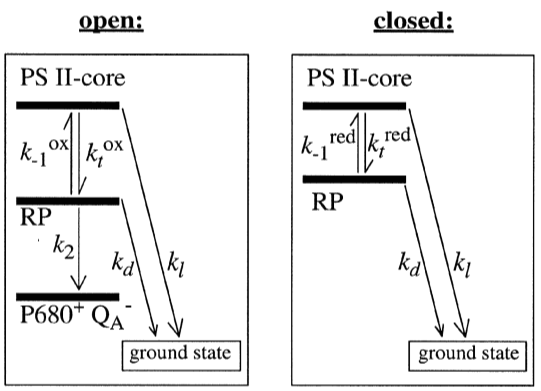


Fig. 1. Exciton-radical-pair equilibrium model for the PS II core in the open (Q_A) and closed (Q_A^-) state. k_f stands for non-photochemical losses in the antenna. The following rate constants are suggested for modeling of PS II_α: k_t^{ox} = (0.1 ns)$^{-1}$; k_{-1}^{ox} = (2 ns)$^{-1}$; k_2 = (0.4 ns)$^{-1}$, k_t^{red} = (0.3 ns)$^{-1}$; k_{-1}^{red} = (1 ns)$^{-1}$; k_d = (2 ns)$^{-1}$. For k_f = (4 ns)$^{-1}$ these latter values yield F_m/F_o = 5.3. The rate constant k_d can be assumed to be the same in open and closed RCs because of the low influence of this parameter on the properties of open RCs.

Published values of rate constants for the exciton-radical-pair mechanism diverge considerably and even the most sophisticated studies quote wide ranges for the parameters (Roelofs et al., 1992). Reasonable estimates that can be taken for modeling are given in the legend of Fig. 1. Given thermal equilibration in the core complex the molecular rate constant of the primary charge separation in the RC ($P680^* \rightarrow P680^+Pheo^-$) can be estimated to be of the order of (2 ps)$^{-1}$, assuming a 100 ps trapping time, 50 680 nm-antenna pigments, and P680* to be one state.

In not time-resolved experiments, like steady state fluorescence assays, the molecular rate constants of the exciton-radical-pair mechanism may be combined in effective overall rate constants k_o and k_c for the open and closed state, respectively:

$$k_o = \frac{k_t^{ox}(k_2 + k_d)}{k_{-1}^{ox} + k_2 + k_d}, \tag{1a}$$

$$k_c = \frac{k_t^{red} k_d}{k_{-1}^{red} + k_d}, \tag{1b}$$

which account specifically for the overall deactivation paths at the RC (Trissl, 1999).

For detailed modeling the PS II-core building block requires several states. If the biphasic fluorescence decay kinetics are to be accounted for, it is necessary to assign a state to the radical pair ($P680^+Pheo^-$). If the spectral heterogeneity that is apparent by fluorescence peaks at 685 nm and 695 nm at low temperatures (Cho and Govindjee, 1970a,b; Gasanov et al., 1979; Rijgersberg et al., 1979) is to be analyzed, additional red shifted states need to be introduced.

3. Peripheral Light-Harvesting Complexes

More than ten different biochemically well characterized chlorophyll *a/b*-binding LHCs are known for

plants (Jansson, 1994). They are commonly divided into two classes, LHCI- and LHCII-type, according to the photosystem to which they preferentially bind. However, this is not a strict rule. In general the LHCI polypeptides aggregate to dimers (possibly heterodimers) whereas the LHCII polypeptides, particularly Lhcb1, Lhcb2 and Lhcb3, aggregate to trimers (eventually heterotrimers). The other polypeptides Lhcb4–6 (CP29, CP26, CP24) are intimately bound to the PS II-core complex in one copy per complex (for reviews see Pichersky and Jansson, 1996; Simpson and Knoetzel, 1996; Sandona et al. 1998).

The three *trans*-membrane α-helix chlorophyll-binding proteins derive from an extended gene family (Green and Durnford, 1996; Chapter 4, Durnford). A range of these polypeptides with very different pigment binding capacities and specificities exist in plants and algae. The binding capacity for chlorophylls is 8–14 per monomer and that for carotenoids (xanthophylls) 1–8. There is wide range in the stoichiometries for Chl *a*, Chl *b*, and Chl *c* binding. Chl *a*/*b* ratios in chloroplasts of green algae can range from 0.64 to 5, whereas in higher plants the range is between 1 and 1.4 (Thompson, 1986). For an LHCII monomer from higher plants the following pigment numbers have been determined: Chl *a*/Chl *b*/carotenoids 7/6/3.4 (Naqvi et al., 1997).

Although the absorption maxima of the LHCI- and LHCII-type polypeptides lie around 680 nm, the Q_y-bands display considerable spectral heterogeneity. In particular the Lhca1,4 heterodimer carries significantly red shifted forms as deduced from the 77 K fluorescence peaking at 730 nm (Schmid et al., 1997). Interestingly, the occurrence of 705 nm absorbing red forms which leads to a clearly discernible shoulder in the absorption spectrum of an *Ostreobium* species is correlated with the overproduction of Lhca1 (Koehne et al., 1999). The authors concluded that this particular Lhca1 is able to accommodate up to four red-shifted (705 nm) Chl *a* forms per monomer.

An atomic model of the LHCII-trimer shows that the distances between chlorophyll molecules are of the order of 8–15 Å (Kühlbrandt et al., 1994). These short distances suggest pigment to pigment hopping times between nearest neighbors of less than a few 100 fs. Such short hopping times have been demonstrated experimentally by transient absorption measurements with femtosecond time resolution (van Grondelle et al., 1994) as well as by exciton-exciton annihilation studies (Wulf and Trissl, 1996). The energy transfer from Chl *b* to Chl *a* can be as fast as 350 fs (Gradinaru et al., 1998). Therefore, the LHCII-trimer forms a building block in which Chl *b* contributes fully to absorption but negligibly to fluorescence.

Diatoms, dinoflagellates and chrysophytes possess LHCs highly loaded with xanthophylls (Wilhelm, 1990). The **f**ucoxanthin-**C**hl *a*/*c* light-harvesting **p**rotein complexes (**FCP**) found in diatoms and brown algae show some homology to the LHC of higher plants. They bind fucoxanthin, Chl *c* and Chl *a* in an approximately 2:1:1 ratio (Grossman et al., 1990). As shown by fluorescence excitation spectra, both fucoxanthin and Chl *c* transfer excitation energy efficiently to Chl *a* (see references in Grossman et al., 1990; Chapter 15, Mimuro and Akimoto). For a historical perspective, see Govindjee (1999). If a monomeric FCP complex should bind in total 12 chlorophyll molecules in analogy to the LHC proteins, the absolute pigment content is 6 Chl *a*, 6 Chl *c* and 12 fucoxanthin molecules.

In dinoflagellates a water **s**oluble **p**eridinin-**C**hl *a* light-harvesting **p**rotein (**sPCP**) is found (Haidak et al., 1966; Song et al., 1976), in addition to intrinsic peridinin-binding proteins related to the chlorophyll *a*/*b* light-harvesting proteins. This is the rare case of a water soluble light-harvesting protein containing chlorophyll. The sPCP is encased within the lumen of the thylakoids.

All sPCPs occur in various isoforms. One of them (32 kDa) has been reported to bind eight peridinin and two Chl *a* molecules. Crystals of an sPCP from *Amphidinium* show a trimeric arrangement of subunits (Hofmann et al., 1996; Chapter 15, Mimuro and Akimoto), which suggests that the building block of sPCP consists of 24 peridinin and six Chl *a* molecules.

The peridinins are in van der Waals contact (3–4 Å) with the tetrapyrrole rings of Chl *a*, allowing for fast energy transfer and effective light-harvesting function of the carotenoids. In contrast the Chl *a* molecules in adjacent monomers within a trimer are rather distant (≈50 Å) and the rate constant for Chl *a*-Chl *a* transfer is on the 100 ps time scale (Kleima et al., 1998; see also Fig. 6). This slow rate determines the equilibration kinetics within the trimer but the exciton transfer from each Chl *a* of the trimer to a receiver pigment in the membrane might be much faster. In this case the chlorophylls transfer in a parallel manner and the sPCP trimer can be taken as a building block or a state.

Cryptophycean algae possess, in addition to a xanthophyll-rich Chl a/c_2 LHC, water-soluble light-harvesting proteins which are either **p**hycoc**y**anin (**PC**) or **p**hycoe**r**ythrin (**PE**). PC as well as PE accommodate covalently bound linear tetrapyrrole chromophores, the bilins. PCs or PEs of cryptophytes are located in the lumen without forming phycobilisomes. However, electron micrographs sometimes show small lumenal particles and the existence of linker polypeptides seems likely (Lichtlé et al., 1987). A structural unit of these peripheral columns of LHCs may be defined by a dimeric aggregate $(\alpha\beta)_2$ which, in the case of phycocyanin PC–645 from *Chroomonas* binds eight tetrapyrrole molecules. Each α-polypetide binds one bilin and each β-polypeptide three bilins, one cryptoviolin and two phyco-cyanobilins (MacColl et al., 1994). A similar arrangement is apparent in the recently determined structure of PE from *Rhodomonas* (Wilke at al., 1999).

The covalent binding of bile pigments to apoproteins increases the excited state lifetime from <100 ps to 2–3 ns (Holzwarth, 1986, 1991). In the protein the radiative lifetime k_{rad} is $(4–6\ \text{ns})^{-1}$. Consequently, PBPs are more efficient fluorescence emitters than Chl-containing LHCs. Molar absorption coefficients depend on the molecular environment of the binding pocket and may vary between the different complexes. However, $\varepsilon \approx 100\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ indicates the right order of magnitude for one tetrapyrrole molecule bound in an α- or β-polypeptide (Füglistaller et al., 1987). A guiding value for the radiative rate constant of bilins in phycobiliproteins is that of phycocyanin $k_{rad}(\text{PC}) \approx (5\ \text{ns})^{-1}$.

In red algae and Cyanobacteria the phycobiliproteins are organized in supramolecular structures, the **p**hyco**b**ili**s**omes (**PBS**), as has been comprehensively reviewed by Sidler (1994) and basic photophysical properties have been published by Grabowski and Gantt (1978; see also Chapter 14, Toole and Allnutt). These extrinsic structures consist of several so-called rods and several core-rods. The attachment of the PBS to the membrane occurs by means of the core-rods (refer to Fig. 9). Earlier it was thought that the PBSs in cyanobacteria are only connected to (dimeric) PS II-cores but there is increasing evidence that they also bind to (trimeric) PS I-cores (Mullineaux 1992; Bald et al., 1996).

A small structural element of most phycobilisomes is a $\alpha\beta$-hetero-dimer (sometimes called monomer) which binds via thioether linkages 2–5 tetrapyrrole chromophores. The exact stoichiometry depends on the type of $\alpha\beta$-units and the organism. Three $\alpha\beta$-hetero-dimers aggregate to form a rather stable $(\alpha\beta)_3$-trimer. By face-to-face dimerization of trimers less stable $(\alpha\beta)_6$-hexamers are formed which are called discs. If a $(\alpha\beta)$-monomer binds three bile pigments as in C-phycocyanin a disc contains 18 chromophores ($N = 18$). If a $(\alpha\beta)$-unit binds five bile pigments as in **p**hycoe**r**ythro**c**yanin (**PEC**) a disc contains 30 chromophores ($N = 30$). The cyanobacterial CU-phycoerythrins can bind even six bile pigments per $(\alpha\beta)$-unit, resulting in 36 chromophores per disc (Ong and Glazer, 1991).

Phycoerythrins often contain phycourobilin in addition to phycoerythrin. In red algae linker polypeptides called γ-subunits occur, that have binding capacities of 3 or even 5 phycobilins (Stadnichuk et al., 1997).

An exception to the $\alpha\beta$-hetero-dimer rule is found in the red algae *Rhodella reticulata* R6 where a unique β-type subunit assembles to hexameric or dodecameric elements by means of an unusual linker (Thomas and Passaquet, 1999). One of this β-polypeptide binds one phycoerythrobilin and two phycocyanobilins.

By means of mostly colorless linker polypeptides a variable number of discs are glued together to form a rod. The rods may be viewed as a one-way avenue for exciton flow toward the PBS core-rods, since discs containing blue-absorbing chromophores lie more distant from the rod-core attachment site than those containing the more red-absorbing chromophores.

Energy transfer in isolated trimers and hexamers has been extensively investigated by time-resolved fluorescence (reviewed by Holzwarth, 1991). Multiphasic decays ranging from 10 ps to 3 ns are commonly observed. The phases depend on the preparation, the aggregation state, and the presence or absence of linker polypeptides. The energy transfer within intact rods may be noticeably different from that in various isolated structural entities as discussed by Holzwarth (1991). Despite this difficulty and spectral heterogeneity it seems reasonable to take hexameric disks as the building blocks of phycobilisome rods.

In the hexamer of C-phycocyanin ($N = 18$) the energy flows from the short-wavelength S-chromophores (β-155) to the long-wavelength F-chromophores (β-84) within ≈10 ps. Hence the contribution of the S-chromophores to the total fluorescence yield is small.

The $\alpha\beta$-units of the core-rods **allo**phyco**cy**anins (**APC**) bind two phycocyanobilins. If each core-rod consists of 4 $(\alpha\beta)_3$-trimers and one linker polypeptide with one bile pigment bound, a tricylindrical core has an antenna size of 75 chromophores. Equilibration within the tricylindrical APC-core of cyanobacteria has been suggested to be 6 ps (Glazer et al., 1985).

Reasonable building blocks for PBS modeling may be trimers or hexamers for the rods whereas the APC-core elements may be treated as one state on the basis of the fast internal equilibration. All bound chromophores are then assumed to contribute to absorption but only the one absorbing farthest to the red contributes to fluorescence.

A detailed kinetic model for the energy transfer in phycobilisomes based on time-resolved fluorescence decay kinetics has been proposed by Suter and Holzwarth (1987).

C. Photosynthetic Unit (PSU) and Photosynthetic Entity (PSE)

In the past the definition of the photosynthetic unit (PSU) underwent different meanings (Wilhelm, 1993). The early definition of the PSU by Emerson and Arnold (1932a,b) was a functionally defined stoichiometry based on oxygen yield measurements, with no evidence that this was reflecting a structural entity. When the RC concept was clarified and single electrons counted, the notion evolved to refer the PSU to the number chlorophylls per RC. Although the distinction was made between PS I and PS II, often the PSU was understood to comprise both photosystems.

The situation has much changed during the past decades as many structural details of the RCs, the LHCs and their assembly became known. Currently the term PSU is mostly used for the structural entity composed of one type of RC-core complex, no matter whether it is mono- di- or trimeric, together with its physically associated LHCs (Fig. 2).

Because of the rather complicated stoichiometries prevailing in the photosynthetic membrane of oxygenic organisms where different light-harvesting super-complexes can serve different types of RCs, the concept of the PSU has been considered of limited use (Larkum and Barret, 1983). However, by introducing the concept of the **p**hotos**y**nthetic **e**ntity (**PSE**) the above difficulty can be overcome, as outlined below.

Proper quantitative modeling requires knowledge

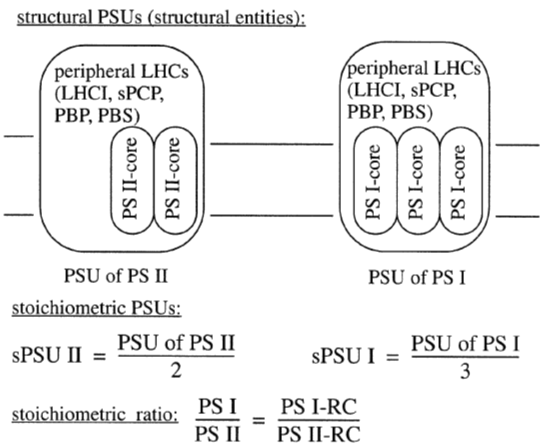


Fig. 2. Conceptual model of the thylakoid membrane and its constituent complexes performing light-harvesting and primary photochemistry. Introduction to the stoichiometries and the terminology used to define the photosynthetic entity PSE in Eq. (2).

of the various stoichiometries involved. This can be achieved by introducing the term '**s**toichiometric' **PSU** (**sPSU**), which is characterized by the stoichiometric number N of antenna pigments excitonically connected to one monomeric core complex. N is independent of the chemical identity and of the position of the long-wavelength maxima of the antenna pigments.

To characterize the photosynthetic electron transport chain which consists of two PSUs in variable stoichiometries (Fig. 2), a PSE is defined by:

$$\mathrm{PSE} = \mathrm{sPSU\,II} + \frac{\mathrm{PS\,I}}{\mathrm{PS\,II}}\,\mathrm{sPSU\,I}. \qquad (2)$$

This definition uses the stoichiometric ratio PS I:PS II because in algae the abundance of PS I is mostly subject to adaptive regulation processes rather than the abundance of PS II. In plants the reverse appears to be true (Chow et al., 1990). It is obvious then how a PSE can be combined from building blocks. The above definition of a PSE takes precisely into account the balance of the electron flow between the two photosystems under light-limiting condition (Section IV).

D. Whole Thylakoid Membranes

Possible assemblies of building blocks in thylakoids of plants and algae have been reviewed by many

authors (Jennings et al., 1996; Melis, 1996; Simpson and Knoetzel, 1996; Staehelin and van der Stay, 1996). For the assembly of phycobilisomes and the association with the core complexes of both photosystems several models have been proposed as discussed by Bald et al. (1996) and MacColl (1998). Only a brief survey of the basic designs found in the different oxygenic organisms will be given here (Fig. 3).

Particularly interesting cases are membrane systems in which only one type of peripheral LHC seems to be associated with both photosystems. Possible examples are the Chl *c*-containing LHC of *Mantoniella squamata* (Schmitt et al., 1993), Chl *a*/*b* type LHC of the prochlorophytes (Post et al., 1993; Partensky et al., 1999; Chapter 3, Partensky and Garczarek) and the water-soluble PBPs in Cryptophyceae.

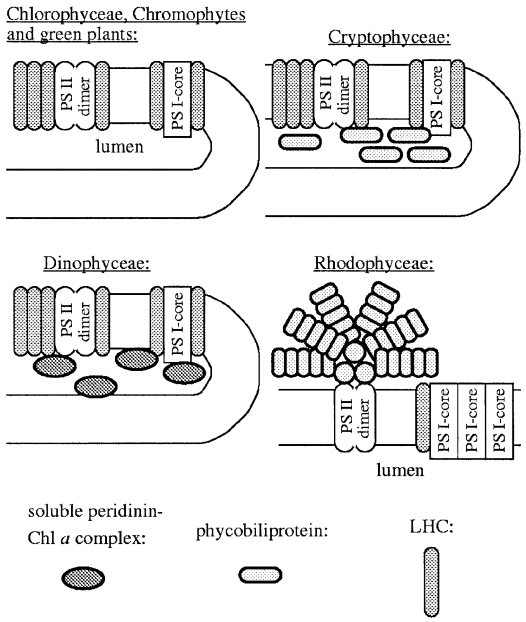


Fig. 3. Association of the different peripheral LHCs with the RCs of PS I and PS II in the thylakoid membranes of different groups of algae.

III. Experimental Approaches

The mathematical treatment of models allows the simulation of experimental data emerging from different experimental assays. Numerical values for the model parameters can be obtained from fitting the experimental data. In the following some of the spectroscopic approaches will be discussed with respect to the information content relevant for modeling.

A. Absorption Spectra

The pigment composition of the building blocks can potentially be obtained from a spectral decomposition of the absorption spectrum. The frequently applied decomposition of absorption spectra by Gauss-functions (named after C. F. Gauß but mostly designated as Gaussians) is convenient, because most laboratory data analysis programs offer this tool as a standard routine. This has the significant drawback of not allowing a direct correlation to pigment stoichiometries, since neither the whole spectra nor the Q_y-bands of the chromophores have the shape of a simple Gauss-function. The inappropriateness of the method becomes obvious by considering a Chl *a*/*b* system. If the Q_y-band of Chl *a* is described by a Gauss-function at 680 nm (width 9 nm) the contribution of the Chl *a*-absorption at the position of the Q_y-band of Chl *b* at 650 nm is negligible. This is in contrast to real spectra in which Chl *a* contributes to the absorption at 650 nm with the Q_x-band and a vibrational shoulder of the Q_y-band of about 20% amplitude (referred to the Q_y-maximum).

Two alternatives exist to avoid the drawback of simple Gauss-function use. One is the **s**pectrum-**re**constitution **m**ethod (**SRCM**) introduced by Naqvi et al. (1997). In this method all chromophores of a pigmented complex are extracted into a common organic solvent and the absorption spectrum is recorded. Then, the absorption spectra of the separate constituent pigments (chlorophylls, carotenoids, etc.) in the same solvent are taken and multiplied with factors (= fit parameters) to fit the spectrum of the pigment mixture. These factors yield the stoichiometries of the pigments if their molar absorption coefficients in the solvent are known (Table 1).

The other way, though not as precise as the former, is a decomposition of an absorption spectrum of a thylakoid membrane or an isolated complex by means of solvent spectra of the constituent pigments (Emerson and Lewis, 1942; Emerson and Lewis, 1943). However, since these latter may not agree with their protein bound spectra, red-shifts and line broadenings have to be taken into account appropriately.

This can be achieved by describing mathematically

Table 1. Molar absorption coefficients of some chromophores and LHC-subunits

chromophore	λ_{max} / nm	ε / mM^{-1} cm^{-1}
Chl *a*	662	90[a]
Chl *b*	644	56[a]
Chl *c*	≈630	27
phycoerythrocyanin-trimer (*N*=15)	568	≈1 500
C-phycocyanin-trimer (*N*=9)	614	≈900
APC-trimer	673	≈1,000[b]
carotenoids	440–500	80 –130

[a] Dissolved in di-ethylether (Porra, 1991)

[b] Füglistaller et al., 1987.

experimental solvent absorption spectra by a set of coupled Gauss-functions (Trissl et al., 1999):

$$A^p\left(\lambda,\lambda_{max},\Delta\lambda\right)=\sum\nolimits_m \frac{a_m}{\sqrt{2\pi}\Delta\lambda\beta_m} e^{-\left(\frac{\lambda_{max}\alpha_m-\lambda}{\sqrt{2}\left(\Delta\lambda\beta_m\right)}\right)^2}. \tag{3a}$$

For a Chl-spectrum in the range 400–750 nm at least eight sub-bands are needed. The parameters involved can be obtained by standard fit programs using Gauss-functions. The coupling refers to the location and width of a master Gauss-function (m = 1; $\alpha_1 = \beta_1 = 1$) of width $\Delta\lambda$ at λ_{max} that describes most of the Q_y-band. Note, that $\Delta\lambda$ = 0.425 FWHM. All other Gauss-bands needed for fitting the whole solvent spectrum are derived from this master band by relative shifts $\lambda_{max}\cdot\alpha_m$, relative widths ($\Delta\lambda\cdot\beta_m$) and relative amplitudes (a_m). For proper quantification, the molar absorption coefficients $\varepsilon^p(\lambda_{max})$ have to be taken into consideration which are known for most photosynthetic pigments with sufficient precision (Table 1):

$$\varepsilon^p\left(\lambda,\lambda_{max},\Delta\lambda\right)=\frac{\varepsilon^p\left(\lambda_{max}\right)}{A^p\left(\lambda_{max},\lambda_{max},\Delta\lambda\right)}A^p\left(\lambda,\lambda_{max},\Delta\lambda\right). \tag{3b}$$

The spectral shifts and the change of the band width when the pigment binds to the protein can be easily emulated with Eq. (3a) by choosing a new λ_{max} and a new $\Delta\lambda$. An important property of this function is a constant area which does not vary with the shift and the band width. In other words, the oscillator strengths of the Q_y- or Soret-bands remain the same. Furthermore, moderate changes of the width maintain the characteristic band shape. The procedure may not be appropriate in the case of strong pigment-pigment interactions.

Once the solvent spectra of the chromophores are mathematically generated they can be stored in a data bank and later used for the spectral decomposition of absorption spectra of isolated complexes. Although awkward, the suggested procedure has the definite advantage of delivering good estimates for the pigment stoichiometries, provided that the experimental spectrum, e.g. of an alga, is not too much distorted by a flattening artifact (Duysens, 1956).

In this way the absorption spectrum of a building block can be described by the sum of the absorption spectra of all its constituent pigments. If there are j constituent pigments this would result in j spectral species and in an overwhelmingly voluminous analysis. However, most data allow only a few spectral components to be resolved, thus reducing considerably the number of identifiable species in a building block.

To simplify kinetic modeling, it is convenient to pool in a building block all species that are kinetically well connected. These pooled species then enter the analysis as a state (new collective species). Several states are usually separated from each other by rate-limiting steps (bottlenecks). (The terms 'states,' 'collective species', or simply species may be used synonymously.) Such a state may have a very heterogeneous absorption spectrum (like PE, PC, APC, or Chl *a*/*b* in LHCII). In the case of the PBP-trimers or PBP-hexamers the master band has to be the red-most one.

The absorption spectra of such collective species (indexed by s) are called **s**pecies-**a**ssociated **a**bsorption **s**pectra (***SAAS***). They are either given mathematically by the spectral decomposition:

$$SAAS_s\left(\lambda\right)=\sum\nolimits_i N_i\cdot\varepsilon_i^p\left(\lambda,\lambda_{max},\Delta\lambda\right), \tag{4}$$

or directly by an experimental absorption spectrum of a species fitted by Eq. (3a).

The absorption spectrum of a PSU is then the sum of the $SAAS_s$ multiplied by the stoichiometric ratio of species present in the photosystem:

$$A^{\mathrm{PSU}}(\lambda) = \Sigma_s N_s \cdot SAAS_s(\lambda), \tag{5}$$

and the total absorption spectrum of the PSEs the sum of the absorption spectra of the two photosystems weighted by their stoichiometric ratio:

$$A^{tot}(\lambda) = A^{\mathrm{sPS\,II}}(\lambda) + \frac{\mathrm{PS\,I}}{\mathrm{PS\,II}} A^{\mathrm{sPS\,I}}(\lambda). \tag{6}$$

B. Fluorescence Spectra

The above defined species are often also structural entities for which experimental fluorescence spectra are available. These so-called **s**pecies **a**ssociated **e**mission **s**pectra (**SAES**) can also be fitted by coupled Gauss-functions. For the fluorescent chlorophyllous systems and PBPs the master Gauss-function for fluorescence can be coupled to the master Gauss-function for absorption by assuming the same width and an appropriate Stokes shift:

$$SAES_s(\lambda) = \frac{1}{\Sigma_m a_m} \left\{ \Sigma_m \frac{a_m}{\sqrt{2\pi}\Delta\lambda\beta_m} e^{-\left(\frac{\lambda_{em}\alpha_m - \lambda}{\sqrt{2}\Delta\lambda\beta_m}\right)^2} \right\}. \tag{7}$$

In contrast to Eq. (3a) the master Gauss-function of Eq. (7) describes the blue-most emission band. It is displaced from λ_{max} by the Stokes shift. To achieve versatility of the computation, λ_{em} may be coupled to the position and the width of the master Q_y-absorption band (Eq. (3a)) according to:

$$\lambda_{em} = \left[\frac{1}{\lambda_{max}} - \frac{10^{-7}}{k_B T} \left(\frac{10^7}{\lambda_{max}} - \frac{10^7}{\lambda_{max} + \Delta\lambda} \right)^2 \right]^{-1}. \tag{8}$$

In this equation λ is to be expressed in units of nm and $k_B T$ in cm^{-1}. The equation derives from an approximation which assumes that the Stokes shift is the squared band width divided by $k_B T$ (Kazachenko, 1965).

Also the $SAES_s$ invariably have the area of one. Stationary fluorescence spectra result from summation of these normalized fluorescence spectra multiplied by the fluorescence quantum yields of the species (Section IV.D.2; Eqs 37a–c).

It is worth noting that two stationary fluorescence spectra are experimentally available: first under F_o-(open RCs) and second under F_m-conditions (closed RCs). Their difference yields the variable fluorescence spectrum ($F_v(\lambda)$) which is solely a PS II emission spectrum, since PS I fluorescence is redox insensitive. This spectrum is helpful in separating the fluorescence of both photosystems (Lavorel, 1963).

An alternative way is to calculate the fluorescence spectrum from the absorption spectrum by means of the Kennard-Stepanov-relation. However, this may work properly in only a few special cases as will be discussed in Section IV.E.6.

C. Fluorescence Excitation and Action Spectra

Fluorescence excitation spectra provide information on the efficiency of energy transfer from sensitizing to emitting species (Goedheer, 1965; Fork and Amesz, 1969; Govindjee 1999). In a perfectly coupled system the fluorescence excitation spectra agree with the absorption spectra if the sample is highly diluted. In concentrated samples it would agree with the absorptance spectrum (see Eq. (10a)). A quantitative interpretation of fluorescence excitation spectra has rarely been performed. Such an analysis becomes feasible with the formalisms presented in Section IV.

Other action spectra provide similar information but they are more specific to the photosystems, if the product yield of one of the photosystems is the measure for the action. This is most obvious for action spectra of oxygen production which unambiguously reflect the spectral properties of PS II, given that PS I-activity is not limiting (Joliot, 1965).

According to the IUPAC recommendations, a plot of a response per number of *incident* photons against wavelength is called *action spectrum*, whereas a plot per number of *absorbed* photons against wavelength is called *efficiency spectrum*. The quantitative treatment of fluorescence excitation or efficiency spectra requires model calculations as will be discussed in Section IV.A.

D. Time-resolved Fluorescence Decay Kinetics

Time-resolved fluorescence decay measurements at different wavelengths bear information on the dynamics of the system as well as on the spectral components involved. The data are commonly

Table 2. Fluorescence decay kinetics of some building blocks

Building block	exponential time constants
PS I-core (monophasic)	30–80 ps
open PS II-core (biphasic)	100–500 ps
LHCII	3–4 ns
PC	≈2 ns

analyzed by a sum of exponential components. In the global data analysis the provable assumption is made that the same set of time constants holds at all emission wavelengths, and only the amplitudes vary.

The wavelength dependence of the amplitudes of the kinetic decay components yields are called **d**ecay **a**ssociated **s**pectra (**DAS**). These spectra give an idea of the species involved. If s kinetic phases can be resolved it can be concluded that the system investigated contains at least s species. However, from the phases it is difficult to derive a physical model. Alternatively, in the target analysis one starts from a physical model and uses the parameters of the model as fit parameters (Section IV.E.7).

Table 2 summarizes some characteristic fluorescence decay time constants of building blocks.

IV. Kinetic Modeling of the Thylakoid Membrane

In this section the structural entities (building blocks) discussed in Section II.B. are functionally assembled to mimic the thylakoid membrane and to quantify the exciton transfer processes and the distribution of excitation energy to the photosystems.

Kinetic modeling requires the introduction of mathematically defined states or species. Both terms are used interchangeably. This means that the PSE has to be dissected into a number of states. The number and the definition of these states depend on the purpose of the modeling and need care as discussed by three examples.

(i) If several identical building blocks are working in parallel (like the rods in PBS or LHCII-trimers connected to a PS II-core) it is not necessary to consider the energy flow in each building block separately. Rather they can be conveniently pooled into one state, because energy transfer in each building block is the same.

(ii) If significant bottlenecks for energy transfer between different complexes are known, as for instance between PE and PC discs (Fig. 9), all PE hexamers and all PC discs may be combined in one state. Such a spectrally heterogeneous state is characterized by its absorption and fluorescence spectrum.

(iii) If, however, the details of the energy transfer within a building block (like a PC hexamer or a core complex) are to be studied one has to dissect the complex into more states.

A. The Excitation

In this section procedures are described that allow us to quantify the relative excitation of the species into which the PSE is decomposed. Two factors play a role: the spectral distribution of the excitation light and the absorption of the antenna.

Let the absorption spectrum of the thylakoid membrane or a whole cell be $A^{tot}(\lambda)$. In general, this spectrum is composed not only of the absorption spectra of PS II and PS I and light-harvesting complexes connected to them but also of pigments or pigment complexes, that are physically separated from the photosynthetic membrane. For instance, violaxanthin, protochlorophyllide or chlorophyllide are contained in the envelope membranes of chloroplasts (Douce and Joyard, 1990), or carotenoids in the plasma membrane of cyanobacteria (Yamamoto and Bassi, 1996) and in the chromoplasts of plant cells (Vishnevetsky et al., 1999). In algae photosynthetically inactive carotenoids can be found in the plastide or in droplets. These unconnected pigments should be treated separately in modeling. If they are non-fluorescent (like the carotenoids), their absorption spectrum $A^{uc}(\lambda)$ can be subtracted from $A^{tot}(\lambda)$ to obtain the spectrum relevant for photosynthesis:

$$A(\lambda) = A^{tot}(\lambda) - A^{uc}(\lambda). \tag{9}$$

Neglecting this preparatory step may lead to the misleading conclusion that the photosynthetic quantum yield of carotenoids is, say 0.5, although in reality there could be a 50% of perfectly transferring and another 50% of unconnected carotenoids present. Although in practice it may be difficult to distinguish

between the two cases, the two possibilities should be considered (Emerson and Lewis, 1942).

The quanta absorbed in a sample are given by its absorptance or the one minus transmission:

$$1-T(\lambda)=1-10^{-A(\lambda)}. \tag{10a}$$

If the PSE consists of s species the excitation of each species $z_s(\lambda)$ is calculated according to its fractional contribution to the absorption (see Eqs. (4) and (5)):

$$z_s(\lambda)=\frac{SAAS_s(\lambda)}{A(\lambda)}\left(1-10^{-A(\lambda)}\right) \tag{10b}$$

It is also a function of the spectral characteristics of the light source.

The exciting light is quantified by the wavelength dependence of the intensity per wavelength interval $I(\lambda)$ given by a particular light climate or by the wavelength of monochromatic light mostly used in laboratory experiments. For the latter case $I(\lambda)$ is conveniently defined as a δ-function:

$$I(\lambda_{ex})=\delta(\lambda_{ex}), \tag{11}$$

the integral of which is 1. Generally, $I(\lambda)$ has to be normalized such that $\int I(\lambda)\,d\lambda = 1$.

The absolute fractional excitation rate of a species z_s is then given by the overlap integral:

$$z_S=\int z_s(\lambda)I(\lambda)d\lambda, \tag{12a}$$

or, when efficiency spectra are to be simulated, it has to be calculated for each wavelength λ_{ex}:

$$z_s(\lambda_{ex})=\int z_s(\lambda)\delta(\lambda_{ex})d\lambda. \tag{12b}$$

To achieve quantification of the yields in model calculations of the PSE it is necessary to use the normalized fractional excitation (Eq. (12a)):

$$Z_s=\frac{z_s}{\sum_s z_s}, \tag{12c},$$

for which $\Sigma Z_s = 1$. This means that one exciton is created in a PSE (Eq. (2)). Consequently, the calculated photochemical yields of PS I and PS II reflect their turn-over rates and also the distribution of excitation energy to the photosystems under light-limiting conditions.

For calculating efficiency spectra this normalization must not be used but the un-normalized term (Eq. (12b)).

B. Fluorescence Induction

PS II is known for its redox-sensitive fluorescence changes. This provides a valuable tool for identifying its spectral properties, since fluorescence changes of PS I are negligibly small at RT (Trissl, 1997).

Measurements of the transition kinetics from the low fluorescing to the high fluorescing state by continuous illumination and under single turn-over conditions (fluorescence induction curves) allows one to gain information on the connectivity, i.e. the lateral exciton transfer between the PS II units as reviewed recently (Dau, 1994; Govindjee, 1995). A formalism with rate constants has been introduced by Lavergne and Trissl (1995). Here a brief summary of the current models used for analysis will be given.

1. Connected Units Model

In this model a PSU can adopt two distinct redox states, open and closed. The PSUs in the open state are quantified by the normalized concentration q and the closed ones by 1-q (Fig. 4), leaving out of the account structural entities and topological details. PSUs containing an open RC are quenched by the RC with an effective rate constant k_o (Eq. (1a)) and those containing a closed RC are quenched by the

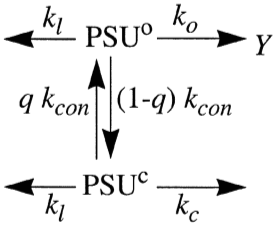


Fig. 4. Connected units model used for fluorescence induction analysis. For correlating the rate constants k_o and k_c with the rate constants of the exciton radical pair model (Fig. 1 and Eq. (1a) and Eq. (1b); see also Trissl (1999)). The photochemical product is only formed in PSUs with open RCs.

RC with an effective rate constant k_c (Eq. (1b)). Exciton transfer between the units is described by an overall rate constant k_{con}.

Two special cases follow straightforwardly. If exciton transfer between the units is infinitely slow ($k_{con} = 0$), the units are isolated (separate units or puddle model; Robinson, 1966). If exciton transfer between the units is infinitely fast ($k_{con} = \infty$) the units are perfectly connected (lake or matrix model).

Under conditions of blocked electron transport (single turn-over conditions) the connected units model (Fig. 4) allows one to calculate theoretical fluorescence induction curves by analytical equations (Lavergne and Trissl, 1995). The two basic equations needed are the dependence of the fluorescence yield on the fraction of open RCs, q:

$$F(q) = \frac{F_m - q[F_m - F_o(1+J)]}{1+Jq}, \tag{13}$$

and the relation between the time and the fraction of open RCs:

$$z \cdot t \cdot \Phi_p(1) = \frac{J(1-q) - \ln q}{1+J}, \tag{14}$$

in which z stands for the exciton creation rate in an PSU (Eq. (12a)) and J is a parameter discussed below. For optically thin suspensions and monochromatic light, z is related to the antenna size N of a 'stoichiometric' PSU, the absorption cross-section σ of a single antenna pigment at the given excitation wavelength and the light intensity I (photons per cm^2 and s) by:

$$z = N \cdot \sigma \cdot I. \tag{15}$$

The absorption cross-section σ (dimension cm^2) is related to the molar absorption coefficient ε by:

$$\sigma = \frac{\varepsilon \cdot \ln 10}{6.02 \cdot 10^{20} \mathrm{M}^{-1} cm^{-3}}. \tag{16}$$

Theoretical fluorescence induction curves can be calculated by combination of Eqs. (14) and (13), using q as parameter. A fit of experimental curves with this scaling allows the determination of the antenna size of PS II.

The minimal and maximal fluorescence are:

$$F_o = \frac{k_{rad}}{k_o + k_1}, \tag{17a}$$

$$F_m = \frac{k_{rad}}{k_c + k_l}, \tag{17b}$$

and the ratio of maximal to minimal fluorescence follows either by division of these latter equations or from Eq. (13) with q=1 and q=0, respectively:

$$\frac{F_m}{F_o} = \frac{k_o + k_l}{k_c + k_l}. \tag{17c}$$

Of particular interest is the parameter J, since this parameter determines the degree of sigmoidicity of the induction curve and contains the information on the physical connectivity between PSUs.

The parameter J, which is a fitting parameter in analyzing experimental traces, depends on the rate constants of the connected units model:

$$J = \frac{(k_o - k_c)k_{con}}{(k_c + k_l)(k_{con} + k_o + k_l)}. \tag{18}$$

Solving this equation for the rate constant of the inter-unit exciton transfer between PSUs, k_{con}, and using the relation of the other rate constants to the extreme fluorescence yields F_o and F_m results in:

$$k_{con} = \frac{(k_o + k_l)J}{F_m / F_o - 1 - J}. \tag{19}$$

By means of this equation, k_{con} can be determined easily from measurable quantities. Numerical values for $k_o + k_l$ are known for most photosystems by time-resolved fluorescence measurements, whereas J and F_m/F_o-ratios follow from fits of experimental fluorescence induction curves. Values for k_{con} in PS II are on the order of k_{con}=(300–800 ps)$^{-1}$ (Lavergne and Trissl, 1995; Trissl and Lavergne, 1995).

The rate constant k_{con} was introduced as an overall rate constant which does not take into account any structural details of the antenna system (Fig. 4). However, assuming thermal equilibration within

photosynthetic units, one can derive equations that estimate the rate constant of the energy transfer at a theoretical single contact site (i.e. hopping time) k_h.

If N isoenergetic antenna pigments are within a thermally equilibrated PSU which is surrounded by cn nearest neighbor PSUs (cn=coordination number) one can estimate the rate constant between two PSUs at a contact site by:

$$k_h = k_{con} \cdot \frac{N}{cn}. \qquad (20)$$

Assuming $cn = 6$ this yields inter-unit hopping times between two PS II-units of 9–27 ps, compared to 4–5 ps between two LH1-rings of LH1-only purple bacteria (Trissl, 1996).

In turn these inter-unit transfer times allows one to deduce average distances between neighboring PSUs. Using the Förster law (see Fig. 6), the value of k_h estimated above (about $(9–27\ ps)^{-1}$) would imply a distance in the range 30–36 Å. Hence, whole PSUs in thylakoid membranes are on the average more separated than single pigments within a photosystem (compare Section IV.C).

Such an estimation of the inter-unit exciton transfer by means of fluorescence induction and the rate constant k_{con} yields a lower limit since, if the PSUs display a distribution of antenna sizes the sigmoidicity is less pronounced than in a homogeneous system as can easily be modeled with the above formalism. Evidence for this situation has come from the observation of different types of PS II-mega-complexes (Boekema et al., 1999). Hence, the actual distances between the PSUs might be smaller.

2. Domain Model (Dimers)

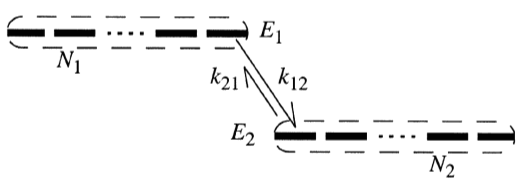
In the so-called domain model an integer number of identical PSUs are pooled under the assumption of infinitely fast exciton transfer within the pool (= domain), so that the domain can be considered as a mini-lake. The general physical relevance of this model may be left undecided here, but the special case of two connected PSUs is most likely given for PS II (Dainese and Bassi, 1991; Hankamer et al., 1997). Also a tetrameric aggregation of PS II-core complexes has been suggested for higher plants (Boekema et al., 1999) as well as for the cyanobacterium *Phormidium* (Westermann and Wehrmeyer, 1995).

Theoretical treatments of domains that include the transition from open to the closed state are rather complicated. The most comprehensive one has been published by Den Hollander et al. (1983). Recently a simplified treatment for fluorescence induction in dimers was published by Bernhardt and Trissl (1999), who showed that the fluorescence induction kinetics for dimers can be calculated by rather simple analytical equations.

The sigmoidicity of fluorescence induction curves calculated by the domain theory for dimers matches closely experimental curves from thylakoids lacking grana/stroma differentiation, like in *Mantoniella squamata* (Hecks et al., 1996) or intermittent light grown chloroplasts of peas (Jahns and Trissl, 1997). It seems likely that a composite of both models—connected dimers—is the most appropriate one to describe the real situation.

C. Thermal Equilibrium Approach

For introduction it is helpful to consider a simple case of two different LHCs at close distance, which possess pigments (N_1 and N_2) with Q_y absorption bands at λ_1 and λ_2. (Only these are responsible for energy transfer.) The due energy levels are labeled E_1 and E_2.



If each pigment system is in thermal equilibrium it forms a degenerate state. Then the overall rate constant of the uphill energy transfer is related to the overall rate constant of the downhill transfer according to the population weighted Boltzmann's law (van Grondelle, 1985):

$$k_{21} = k_{12} \cdot \frac{N_1}{N_2} \cdot e^{-\frac{E_1 - E_2}{k_B T}}. \qquad (21)$$

The two rate constants in Eq. (21) may be decomposed by introducing a rate constant k_{con} with which the degenerate states are connected:

$$k_{12} = k_{con}\frac{1}{N_1}, \tag{22a}$$

$$k_{21} = k_{con}\frac{1}{N_2}e^{-\frac{E_1-E_2}{k_BT}}. \tag{22b}$$

The rate constant k_{con} controls formally the coupling strength between the two states at the bottleneck site. Its physical interpretation in terms of single hopping steps requires the assumption of the number of contact sites, i.e. the coordination number *cn*. Because the degeneracy of the states is already considered, Eq. (20) simplifies to:

$$k_h = \frac{k_{con}}{cn}. \tag{23}$$

Numerical values for k_{con} in chlorophyllous systems fall in the range $(50\ \text{fs})^{-1}$ to $(100\ \text{fs})^{-1}$ if experimentally determined single step hopping times of 200–400 fs (Wulf and Trissl, 1996; and references therein) and a coordination number $cn = 4$ are assumed.

As discussed before, in LHCs the pigments are arranged in fixed positions and at close distances, so that the exciton hopping between neighboring pigments is on the sub-picosecond time range and even the time for visiting all pigments in the complex does not exceed 10–20 ps. This is much faster than all non-photochemical losses of the excited state (1–4 ns) and, therefore, the isoenergetic pigments within one LHC may be approximated by one, though degenerate, state.

The fundamental nature of Eq. (21), Eq. (22a) and Eq. (22b) is often not sufficiently appreciated. The relation offers an effective constraint and reduces the number of adjustable parameters considerably, because, once the coupling strength is chosen, the forward and backward rate constants follow immediately from the number of pigments and their absorption maxima. Furthermore, Eq. (21) establishes the temperature dependence of the excited state distribution. Obviously, the lower the temperature the higher the occupation of the lowest energy level.

In real systems both states (surrounded by dashed lines) are subject to deactivation processes and are part of a highly dynamic antenna system. Then the two states are no longer in a thermal equilibrium which will be approached only for $k_h \rightarrow \infty$. To what extent the thermal equilibrium approach (Eq. (21)) is valid will be discussed in Section IV.E.6.

A less simple case is given when the LHCs are spectrally heterogeneous. Then the various isochromic pigments may be pooled to form different states. In a general system there may be *s* states. If thermal equilibrium can establish the probability for an exciton to reside in one of the states, p_s is given by:

$$p_s = \frac{N_s}{N_{eff}}\cdot e^{-\frac{E_s-E_{min}}{k_BT}}, \tag{24a}$$

with

$$N_{eff} = \sum\nolimits_s N_s \cdot e^{-\frac{E_s-E_{min}}{k_BT}}. \tag{24b}$$

The denominator in Eq. (24a) is the well known partition function, also termed 'effective antenna size'. It can be understood to represent the number of pigments on the lowest state energy E_{min} in a hypothetical antenna system in which all pigments are isoenergetic at E_{min}. Note that the energy level of the primary donor has also been used as reference for the definition of N_{eff} (Trissl, 1993).

In the general case the Gibbs Energy ($\Delta G = -RT \ln K$) between two states *s* and *s*+1 follows from the corresponding Boltzmann equilibrium constant:

$$K_{s,s+1}^{Boltz} = \frac{N_{s+1}}{N_s}e^{\frac{E_s-E_{s+1}}{k_BT}}. \tag{25}$$

The fluorescence spectrum $F(\lambda)$ of such an equilibrated antenna system follows from the normalized fluorescence spectrum of the states (Eq. (7)) weighted with the states' probability according to:

$$F_s(\lambda) = \sum\nolimits_s p_s \cdot SAES_s(\lambda). \tag{26}$$

In principle such spectra agree with those calculated from the absorption spectra by means of the Kennard-Stepanov relation (Section IV.E.6).

The application of the thermal equilibrium approach requires considerable care, since it represents an approximation of a basically dynamic

system. It may be justified for those cases, for which the quantity of interest is only slightly dependent on the detailed kinetics of energy transfer. The quality of the approach will be discussed in Section IV.E.6 where the Kennard-Stepanov relation is presented.

D. Kinetic Approach

1. Basic Aspects of Energy Transfer

The photosynthetic conversion of electromagnetic energy into free chemical energy is generally assumed to have a quantum efficiency of about 1. It is informative to plot the quantum yield as a function of the trapping time in a small, thermally equilibrated PSU (Fig. 5). The quantum yield of photochemistry is given by:

$$\Phi_p = \frac{k_t}{k_l + k_t}, \tag{27}$$

if trapping occurs with k_t and k_l comprising all non-photochemical losses. Obviously, a high yield (>80%) can still be achieved with trapping times up to 1 ns, provided that the time constant of non-photochemical losses is ≥4 ns.

Energy transfer (rate constant k_{DA}) between two molecules D and A by dipole-dipole interaction (Förster mechanism) depends on the inverse 6th power on the distance d (Förster, 1948):

$$k_{DA}(d) = k_{rad}\left(\frac{R_0}{d}\right)^6. \tag{28}$$

R_0 is the so-called Förster radius which is a measure for a 50% efficiency for energy transfer. R_0 is proportional to the spectral overlap integral, the square of the orientation factor, and inversely proportional to the 4th power of the refractive index. As has been pointed out by Knox and Gülen (1993) the Förster mechanism is still applicable in the case of rather strong coupling, provided that dipole-dipole interaction dominates the electronic coupling and after exciton coherence is lost. This seems to be the case in chlorophyllous systems. The strong dependence of the transfer rate on the distance is illustrated in Fig. 6 for two typical antenna pigments, Chl *a* (R_0 = 8 nm) and phycocyanin (R_0 = 5 nm).

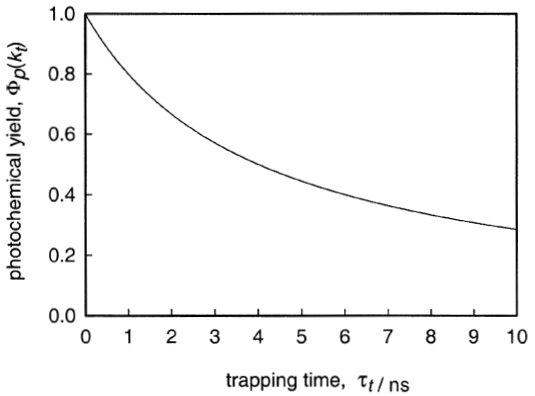


Fig. 5. Dependence of the photochemical quantum yield Φ_p on the trapping time using a simple competitive model of the PSU with $k_l = (4\ \text{ns})^{-1}$.

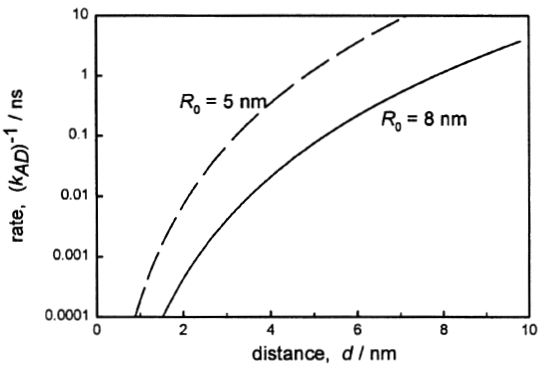


Fig. 6. Dependence of the energy transfer kinetics between two chromophore molecules separated by d (Eq. (28)) assuming R_0 = 8 nm for Chl *a* and R_0 = 5 nm for phycocyanin, respectively. The orientation factor has been set at $\kappa^2 = 2/3$ (random orientation of transition moments) and the refractive index is included in R_0. Note that κ can have a maximal value of 2 (for parallel orientation) and that the maximal transfer rate may then be 6-times faster than depicted, since the transfer rate is proportional to the square of κ.

2. Modeling Long-Wavelength Absorbing Chlorophylls *in* Ostreobium

In this section the mathematics necessary to analyze a given model will be explicitly described. The procedure for the (i) derivation of a rate matrix from a reaction scheme (= model), (ii) solution of the latter for a given excitation condition, and (iii) the resulting equations for the measurable quantities will be explained by means of a specific example: the

thylakoid membrane of the alga *Ostreobium*. Some computed experimental observables will be illustrated.

The genus *Ostreobium* is a boring green alga probably distributed over all oceans. This coral and chalky substrate-penetrating alga has been classified as a member of the caulerpales (Chlorophyceae). It can adapt to extremely low-light climates that prevail around 200 m depths (Littler et al., 1985). A species that has been described by Halldal (1968) was isolated from a coral (*favia*) covered by thick layers of dinoflagellates which absorb most of the visible light but let through far red light beyond the Q_y maximum of Chl *a*. To survive under these extremely low-light and red-light enriched conditions *Ostreobium* must have evolved a more efficient method of harvesting light than high-light organisms. Interestingly, some but not all *Ostreobium* species have adapted their light collection apparatus to capture photons >700 nm.

As has been reported by Halldall (1968) and Koehne et al. (1999) light of 715 nm is fully competent in oxygen evolution proving that PS II is supplied with long-wavelength absorbing Chl *a* forms. These red forms have an absorption maximum at 706 nm and are bound to a Lhca1 type polypeptide (Koehne et al., 1999). It is a rare case in which the association of a LHCI-type with PS II has been demonstrated, whereas the reverse, the association of a LHCII-type with PS I, is well known (Melis, 1996).

From an energetic view, efficient photosynthesis of a PS II with many red forms appears intuitively unrealistic, since the energy gap between 715 nm excitation light and 680 nm, the absorption maximum of P680, is 3.5 k_BT at ambient temperatures. By means of the following model calculation it can be shown that such a system is nonetheless capable of operating with high photochemical yield.

The rate matrix (transfer matrix) T (Eq. (29)) for the scheme depicted in Fig. 7 considers only coupled states. Irreversibly formed states (like the products) must not be included. The matrix is squared having the dimension *dim*.

T is solved for the eigenvalues γ_i ($-\gamma_i^{-1}$ = apparent time constants, τ_i) and the eigenvector matrix *ev*. A coefficient matrix ($a_{i,s}$) is then calculated by solving the linear system

$$ev \cdot U_s = Z_s \tag{30}$$

for U_s, in which Z_s is the initial condition given by the normalized fractional excitation of the states (Eq. (12c)):

$$Z_s = [1\,0\,0\,0\,0\,0\,0\,0], \tag{31}$$

which, in this particular example, has been chosen for selective excitation of LHCII (state #1). The index s goes from 1 ... *dim*. If quantum yield action spectra are to be calculated, Eq. (12b) has to be used instead of Eq. (31).

The coefficient matrix which contains the characteristic weighting factors is then given by:

$$a_{i,s} = ev^T \cdot U_s. \tag{32}$$

By means of the eigenvalues γ_i and the coefficient matrix $a_{i,s}$, all measurable quantities can be simulated. In the following, relevant examples are explicitly assigned.

The time-courses of the states s are sums of exponentials (Holzwarth, 1996):

$$L_s(t) = \sum_i a_{i,s} \cdot e^{\gamma_i \cdot t}. \tag{33}$$

The irreversibly formed states (= products), i.e. the charge stabilized states of PS II, $Y^{PS\,II}$, and PS I, $Y^{PS\,I}$, are derived from their predecessors (#8) and (#7), respectively:

$$Y^{PS\,II}(t) = k_2 \cdot \sum_i \left[\left(e^{\gamma_i \cdot t} - 1 \right) \cdot \frac{a_{i,8}}{\gamma_i} \right] \tag{34a}$$

$$Y^{PS\,I}(t) = k_t^{PS\,I} \cdot \sum_i \left[\left(e^{\gamma_i \cdot t} - 1 \right) \cdot \frac{a_{i,7}}{\gamma_i} \right]. \tag{34b}$$

The final product yields (photochemical yields) follow from Eqs. (34a) and (34b) for $t \to \infty$:

$$\Phi_p^{PS\,II} = \lim_{t \to \infty} Y_{PS\,II}(t) = -k_2 \cdot \sum_i \frac{a_{i,8}}{\gamma_i} \tag{35a}$$

$$\Phi_p^{PS\,I} = \lim_{t \to \infty} Y_{PS\,I}(t) = -k_t^{PS\,I} \cdot \sum_i \frac{a_{i,7}}{\gamma_i}. \tag{35b}$$

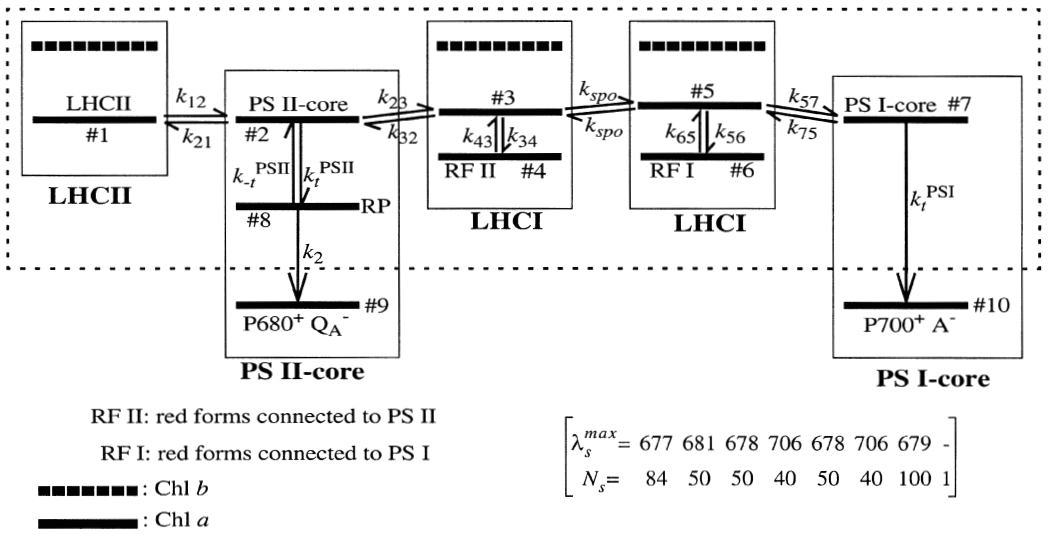


Fig. 7. Model for the thylakoid membrane of *Ostreobium* sp. containing LHCI with red Chl *a* forms. For the purpose of reducing the size of this presentation some obvious states (like the Chl *b* and carotenoids) have been omitted in the state numbering, but they were used in the model calculation. To reduce the size of the matrix the columns for states #4 and #5 are left void. The structural entities considered are boxed by solid lines whereas the states considered are represented by the thick solid horizontal bars. The dashed box encircles the coupled states. All excited states are assumed to decay non-photochemically with $k_f = (4\ \text{ns})^{-1}$. Spillover between the photosystems was assumed to proceed via the bulk chlorophylls of LHCI ($k_{35} = k_{53} = k_{spo}$). The rate constants for the exciton radical pair equilibrium model for the PS II core are listed in the legend of Fig. 1. Trapping in the PS I-core was assumed to occur with $k_t^{PS\,I} = (30\ \text{ps})^{-1}$. The other parameters used for the model calculation have been adapted from Koehne et al. (1999). An equal coupling strength of $k_{con} = (0.1\ \text{ps})^{-1}$ was assumed, which yields the following rate constants for the overall inter-state exciton transfer: $k_{12} = (8\ \text{ps})^{-1}$; $k_{21} = (7\ \text{ps})^{-1}$; $k_{43} = (71\ \text{ps})^{-1}$; $k_{34} = (5\ \text{ps})^{-1}$, just to quote a few. The quoted values yield $F_m/F_o = 2.2$ for the whole PSE upon 715 nm-excitation. The rate matrix for the above reaction scheme reads:

$$T = \begin{bmatrix} -(k_f + k_{12}) & k_{21} & 0 & \dots\ \dots & 0 & 0 & 0 \\ k_{12} & -(k_f + k_t^{PS\,II} + k_{21} + k_{23}) & k_{32} & \dots\ \dots & 0 & 0 & k_{-t}^{PSII} \\ 0 & k_{23} & -(k_f + k_{32} + k_{34} + k_{spo}) & \dots\ \dots & 0 & 0 & 0 \\ 0 & 0 & k_{34} & \dots\ \dots & 0 & 0 & 0 \\ 0 & 0 & k_{spo} & \dots\ \dots & k_{65} & k_{75} & 0 \\ 0 & 0 & 0 & \dots\ \dots & -(k_f + k_{65}) & 0 & 0 \\ 0 & 0 & 0 & \dots\ \dots & 0 & -(k_f + k_{75} + k_t^{PS\,I}) & 0 \\ 0 & k_t^{PS\,II} & 0 & \dots\ \dots & 0 & 0 & -(k_2 + k_d + k_{-t}^{PS\,II}) \end{bmatrix} \tag{29}$$

Under light-limiting conditions the turnover rates of the photosystems are proportional to these two yields. The normalization used here in connection with the definition of the PSE (Eq. (2)) implies that, in the absence of losses, $\Phi_p^{PS\,I} + \Phi_p^{PS\,II} = 1$. Therefore, the ratio $\Phi_p^{PS\,I}/\Phi_p^{PS\,II}$ yields the measure for the balance of electron flow through PS I and PS II.

The fluorescence quantum yields of the individual states are given by:

$$\Phi_f^s = -k_s^{rad} \cdot \sum_i \frac{a_{i,s}}{\gamma_i}. \tag{36}$$

This equation takes into account the different radiative lifetimes of the chromophores (Section II.A).

Stationary fluorescence spectra follow by summation of the fluorescence yields (Eq. (36)) multiplied by the species associated emission spectra (Eq. (7)):

$$F(\lambda) = \sum_s \Phi_f^s \cdot SAES_s(\lambda). \tag{37a}$$

If the fluorescence spectra of the two photosystems are to be computed a partial summation is required:

$$F^{\mathrm{PS\,II}}(\lambda) = \sum_{s=1}^{4} \Phi_f^s \cdot SAES_s(\lambda), \tag{37b}$$

$$F^{\mathrm{PS\,I}}(\lambda) = \sum_{s=5}^{7} \Phi_f^s \cdot SAES_s(\lambda). \tag{37c}$$

It is worth noting that the spectrum of the variable fluorescence is also given by Eq. (37b). Spectra of delayed fluorescence (Lavorel, 1975; Jursinic, 1986) emanating from PS I or PS II can be simulated by solving the rate matrix for the initial condition either $Z_7 = 1$ or $Z_8 = 1$, respectively.

The time courses of the species associated absorption spectra (SAAS) are simply given by the product of Eq. (4) and Eq. (33):

$$SAAS_s(\lambda, t) = SAAS(\lambda)_s \cdot L_s(t). \tag{38}$$

The **d**ecay-**a**ssociated **s**pectra (**DAS**) are given by the product:

$$DAS_i(\lambda) = \sum_s a_{i,s} \cdot SAES_s(\lambda). \tag{39}$$

The **t**ime-**r**esolved **e**mission **s**pectra (**TRES**) are calculated by multiplying the kinetics of the states (Eq. (32)) with the species associated emission spectra (Eq. 4) and summation over the species:

$$TRES(t, \lambda) = \sum_s L_s(t) \cdot SAES_s(\lambda). \tag{40}$$

The time-resolved emission kinetics at a given wavelength (detection wavelength) corresponds to the measured fluorescence decay kinetics at the detection wavelength.

A very descriptive quantity is the total fluorescence decay which reflects for the most part the dynamics of the excited state depopulation by the primary photochemistry (Croce et al., 2000). It is given by the time-dependent area of the *TRES*:

$$F(t) = \int TRES(t, \lambda) d\lambda. \tag{41}$$

Finally, the temporal establishment of the **t**ransfer **e**quilibrium (**TE**) between two states (s and $s+1$) can be calculated straightforwardly:

$$TE_{s,s+1}(t) = \frac{L_{s+1}(t)}{L_s(t)}. \tag{42}$$

This function approaches asymptotically $TE(t \rightarrow \infty)$ a constant value, which may be called the transfer equilibrium constant:

$$K_{s,s+1}^{TE} = \frac{a_{dim,s+1}}{a_{dim,s}}. \tag{43}$$

This constant may then be compared to the Boltzmann equilibrium constant (Eq. (25)). The difference between both is a sensible indicator for the goodness of the thermal equilibrium approach (Section IV.C and Fig. 10a; inset). The definition of *TE* (Eq. (42)) used here deviates from the one introduced by Laible et al. (1994) by a difference in the normalization procedure.

There are twice as many solutions when considering open (Q_A) and closed (Q_A^-) PS II-RCs. The corresponding labeling of symbols has been omitted here. However, when this is done one obtains two stationary fluorescence spectra (Eq. (37a)), the difference of which is the variable fluorescence spectrum, solely due to PS II. As expected the shape of this variable fluorescence spectrum then agrees with the computed PS II fluorescence spectrum (Eq. (37b)).

Next we shall illustrate some computed results using the numerical values given in the legend of Fig. 7. Both photosystems are assumed to be present in equal amounts (PS I/PS II=1).

First, we compute the time courses of product formation of the two photosystems in the absence of exciton exchange, i.e. no spillover (Fig. 8). White light (a) and 715 nm excitation (b) are compared. The figures illustrate the much faster trapping in PS I

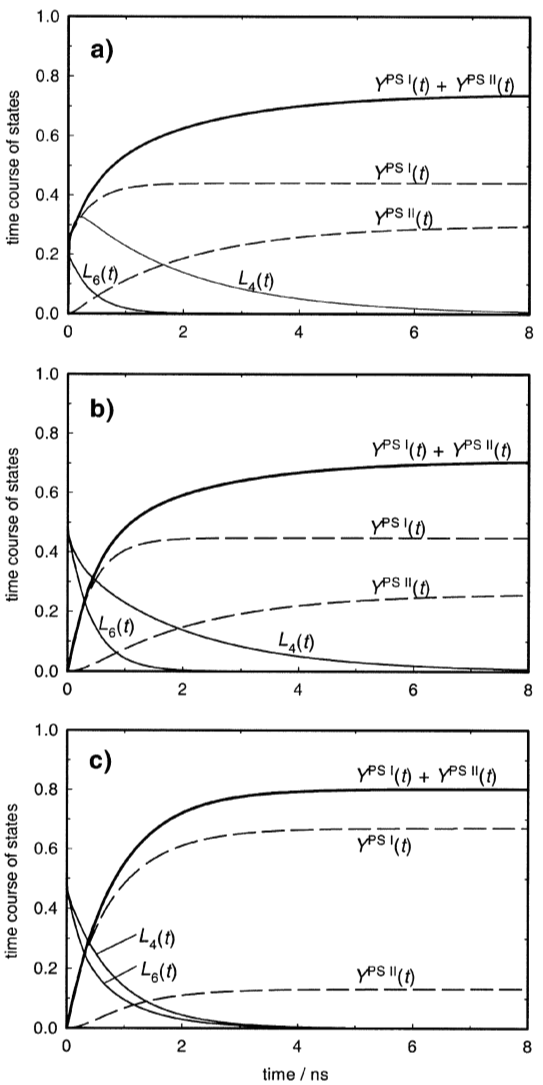


Fig. 8. Time courses of selected states (L_s) defined in Fig. 7. Product formation $Y(t)$ calculated by Eq. (34a) and Eq. (34b). a) Excitation with white light and no spillover between the photosystems. b) Excitation of red forms and no spillover. c) Excitation of red forms and spillover (discussed in section IV.E.3).

than in PS II. There are only minor differences between the two excitation wavelengths, which means that red light is equally effective in driving oxygen production as normal visible light. Furthermore, PS I turns over at a slightly higher rate than PS II ($\Phi_p^{PS\,I}/\Phi_p^{PS\,II}$=1.2). This latter number changes to $\Phi_p^{PS\,I}/\Phi_p^{PS\,II}$=1.5 for 715 nm excitation.

Second, the photochemical quantum yields of the separate photosystems are calculated. This is done by selective excitation of LHCII or PS I-core (Eq. (30)) again in the absence of spillover. The resulting quantum yields (Eq. (35a) and Eq. (35b)) are $\Phi_p^{PS\,I} = 0.95$ and $\Phi_p^{PS\,II} = 0.63$. This calculation shows that PS II can operate rather effectively even with a large number of long wavelength absorbing pigments in its peripheral antenna. The determining factor for the quantum yield is the non-photochemical loss, which have been set to $k_l = (4\ \text{ns})^{-1}$. Any values larger than this lead to a dramatic drop of the yield.

The most informative illustrations of the dynamics of the system are three-dimensional graphs of *TRES*. These are depicted for 670 nm and 715 nm excitation in Color Plate 3. Cuts at a given wavelength reflect the fluorescence decay kinetics at that wavelength. Marked differences in the kinetics are clearly recognizable if the two emission wavelengths of 680 nm and 740 nm are compared. The rising kinetics at 740 nm upon 670 nm excitation are due to the downhill energy transfer from bulk Chl *a* to the red forms.

3. Modeling Thylakoid Membranes with Phycobilisomes

As a further example for modeling we have chosen a thylakoid membrane with peripheral PBS light-harvesting complexes (Fig. 9). The model membrane was built up by the following structural entities. A PBS has six rods and a three-cylindrical APC-core. Each rod consists of three PC hexamers (630 nm) and two PE hexamers (590 nm). The stoichiometric ratio of the photosystems is given by the assumption of one PS I-core trimer and one PS II-core dimer (PS I/PS II = 3:2). One PBS is attached to one PS II-core dimer.

To limit the number of states entering the mathematical formulation the spectroscopically distinct hexamers were pooled into states as indicated by the numbering in Fig. 9 (top). This takes into account that the main bottlenecks of energy transfer in PBSs are at the coupling sites of different PBPs in a rod (Suter and Holzwarth, 1987). Then the first state #1 is the sum of four PE trimers. Each trimer is assumed to possess one lowest energy pigment from which the fluorescence is emitted. The degeneracy of state #1 is therefore 4 (two disks made up of four trimers) and that of state #2 is 6 (three disks made up of six trimers). The degeneracy of the terminal emitters of APC tricylindrical core (state #3) was

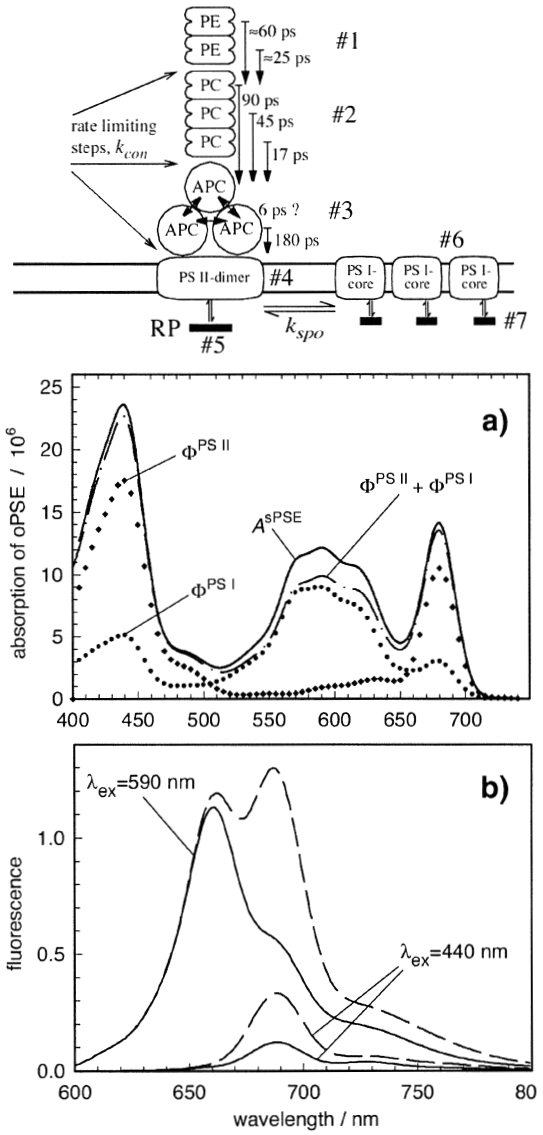


Fig. 9. Top: Energy flow at RT in thylakoid membranes with hemidiscoidal phycobilisomes as suggested by Suter and Holzwarth (1987) and Holzwarth (1991) in the absence of spillover. Only one of the 6 rods of the phycobilisome is shown. a) Absorption spectrum (solid) and efficiency spectra for total photochemistry (dash-dot), PS II-photochemistry (filled circles) and PS I-photochemistry (diamonds). b) Stationary F_o (solid line) and F_m (dashed) fluorescence spectra upon PBS-excitation (λ_{ex} = 590 nm) and selective Chl *a*-excitation (λ_{ex}=440 nm).

assumed to be 3 at 670 nm. Chromophores on the core-membrane linker polypeptide (L^{CM}) were not considered.

PS II was modeled with two states as shown in Fig. 7 and PS I was supplied with one long wavelength absorbing Chl *a* at 720 nm (state #7). The parameters mentioned above together with those quoted in the legend of Fig. 9 are sufficient to define the system uniquely.

Two solutions of the system will be provided: quantum yield action spectra of photochemistry and stationary fluorescence spectra.

The action spectra for the photochemical yields of PS II, PS I and the sum of both are shown in Fig. 9a. They were calculated by using the absolute fractional excitation (Eq. (12b)) as the initial condition. A quantum yield of 1 at all wavelengths would lead to a perfect match of the action spectrum with the absorptance spectrum (or the absorption spectrum if $A(\lambda)<<1$). Hence, Fig. 9a illustrates a high (>80%) quantum yield of the PSE at all wavelengths. Around the position of the Q_y band of Chl *a* the yield is higher. This can be ascribed to the higher efficiency of PS I compared to PS II and the dominance of PS I absorption at this wavelength (Fig. 9).

If the system is excited with white light, the photochemical yields of PS II and PS I adopt values of approximately 50% each. These are optimal conditions for linear electron transport. If, however, light of 590 nm is predominant the PS I activity becomes negligible (Fig. 9a) because all energy coming from the PBS is funneled to PS II in the model of Fig. 9. This unfavorable situation can be overcome by either photophysical spillover to PS I or attachment of PBSs to PS I, as discussed by van Thor et al. (1998).

In PBS systems fluorescence from PBP dominates the fluorescence from Chl *a*. This is clearly seen in Fig. 9b in which the F_o and F_m flourescence spectra are computed for 590 nm and 440 nm excitation. These RT fluorescence spectra agree qualitatively with experimental ones (Krey and Govindjee, 1966; Koehne and Trissl, 1998).

4. *Modeling Photosystem II in* Acaryochloris marina

The recently discovered cyanobacterial prokaryote *Acaryochloris marina* contains Chl *d* as the major antenna pigment which absorbs with its Q_y-band at 715 nm (Miyashita et al., 1996). It may be surprising that such a strongly red-shifted pigment system allows for a high photosynthetic activity in both photosystems.

In the case of PS I it has been shown that the

primary donor consists also of Chl *d* and absorbs maximally at 740 nm (Hu et al., 1998); exciton transfer occurs in a downward direction and is therefore efficient.

In the case of PS II there is no evidence for a modified RC. Fluorescence induction measurements display a variable fluorescence that is very similar to that of green plants and algae (Schiller et al, 1997). In accordance with this observation, a model calculation based on the principles outlined above shows that a PS II-core complex consisting of 46 715 nm-pigments and a primary donor absorbing at 680 nm (P680) can indeed exhibit a high photochemical yield and a large F_m/F_o. Specifically, if we assume P680 to consist of four Chl *a* (λ_{max} = 680 nm), insert a $(2\ ps)^{-1}$ rate constant for the primary charge separation in the isolated RC (Section II.B.2), and for the other rate constants adapt the values given in the legend of Fig. 7, the simulation predicts a photochemical quantum yield of 66% and F_m/F_o = 3.1.

E. Applications

1. Low Temperature Spectra

In the present modeling formalism the temperature dependence of the yields is inherently covered due to the coupling of rate constants by means of the population weighted Boltzmann terms (Eq. (21)).

For example, the simulated 77 K stationary fluorescence spectrum of the PBS system in Fig. 9 shows peaks at 660, 686, and 730 nm which can be assigned to PBS, PS II and PS I, respectively. However, at low temperature PS II is known to emit at 686 and 695 nm. To simulate this particular detail would require an extension of the model by splitting state #4 (PS II-core) into two spectrally distinct states.

2. Quantum Yields

The present formalism allows computation of photochemical quantum yields for various special cases using an extended model such as that in Fig. 7. For example, (i) the yields (i.e. turnover rates) of the two photosystems in the PSE upon non-selective excitation with white light using a constant $I(\lambda)$ in Eq. (12a), or (ii) the quantum yield of each of the photosystems in the PSE by selective excitation of one of them using Eq. (31), or (iii) the quantum yield of the photosystems without energy transfer between them ($k_{spo} = 0$), again by selective excitation of one of them. The selection of all these cases is controlled by Eqs. (12a)–(12c) or Eq. (31).

The total photosynthetic quantum yield of the PSE, $\Phi_p^{PS\,I} + \Phi_p^{PS\,II}$, is then given by Eqs. (35a) and (35b) and the relative contribution of the photosystems by $\Phi_p^{PS\,I}/\Phi_p^{PS\,II}$. Under light-limiting conditions the latter quantity is a measure of the fraction of linear and cyclic electron flow in the electron transport chain in the thylakoid membranes.

3. Spillover

In all oxygenic photosynthetic organisms the balance of electron flow between the two photosystems is subject to different regulatory processes according to the biochemical demands (Fujita et al., 1994).

One of the mechanisms is the photophysical distribution and redistribution of excitation energy, frequently called spillover (Myers and Graham, 1963; Satoh et al., 1976; Chapter 13, Larkum). As discussed before, trapping in PS I is about 3–5 times faster than in PS II. Also, the Gibbs energy of the PS I antenna system lies below that of PS II due to a general slight red-shift of the bulk Chl *a* molecules and the presence of some few strongly red-shifted Chl *a* molecules in PS I. Therefore, any close approach of the two photosystems leads to efficient energy transfer from PS II to PS I and concomitantly to a diminished yield of PS II. Because this appears detrimental to an optimized photosynthetic machinery, it has been argued that nature had to develop strategies to avoid the close approach of the dissimilar photosystems (Trissl and Wilhelm, 1993).

In fact, many observations support this idea. One strategy is the grana-stroma differentiation which is a prominent feature of the thylakoid ultrastructure in most, but not all, Chl *b*-containing chloroplasts. It keeps most PS II apart from PS I by collecting the former in grana membranes and leaving the latter in stroma lamellae (Albertsson, 1995). Another strategy, which may be realized in Cyanobacteria and red algae, is the collection of PS II beneath PBS thereby keeping PS I at a 'safe' distance.

In algal chloroplasts that show a homogeneous thylakoid membrane system, i.e. without grana-stroma differentiation (like *Mantoniella* or *Ostreobium*), both photosystems may mix and allow for spillover. This leads to a diminished ratio of maximal to minimal fluorescence as can be simulated in an approximate manner by an increased rate constant

for non-photochemical losses ($k_f^{PS\ II}$) in Eq. (17c). Such a tendency correlates with experimental findings as discussed by Trissl and Wilhelm (1993).

By means of model calculations for the PSE this photophysical argument can be addressed more exactly. We inspect the effect of spillover for the *Ostreobium* membrane (Fig. 7) by choosing $k_{spo} = (10\ \text{ps})^{-1}$, which yields $k_{35} = k_{53} = (0.5\ \text{ns})^{-1}$. This latter value corresponds to the connectivity between PS II units as revealed by fluorescence induction (Section III.B). Fig. 8c (dashed lines) shows that both photosystems are strongly imbalanced. PS II loses excitation energy toward PS I. Its photochemical yield in the PSE drops below 15% and the quantum yield below 30%. Cyclic electron transport would then be the main source to supply the cell with chemical energy. The same tendency is simulated when long-wavelength absorbing spectral forms are absent. Nevertheless, the total photosynthetic quantum yield of the PSE remains high (Fig. 8c).

On the one hand, to prevent a strong over-excitation of PS I at the expense of PS II the physical approach of the photosystems should not be too close. On the other hand, a moderate approach can serve fine tuning of linear versus cyclic electron transport. From the rate constant of spillover at which the imbalance begins one can estimate a distance between the two photosystems that is optimal for regulation. Again assuming a coordination number of 6, according to Eq. (20) the hopping time would be 60 ps which corresponds to a separation of >40–50 nm (Fig. 6) between PS I-PS II units. A similar picture has emerged for the thylakoid membrane of *Mantoniella squamata* (Hecks et al., 1996).

In thylakoid membrane systems that appear homogeneous in electron micrographs, any excitonic separation of PS I and PS II cannot be assigned to grana-stroma differentiation. Rather the binding of the peripheral LHCs to the photosystems must be significantly stronger than binding between PSUs. The attractive van der Waals forces that fasten the peripheral LHCs to the core complexes are strong enough ($>k_BT$) to hold the PSU-supercomplexes together. However, to allow for large distances between PSUs, the interaction at their periphery must be weaker than k_BT or even repulsive. This requires asymmetrically distributed amino acid residues at the periphery of the LHCs or specific interactions of the hydrophilic N-terminal domains, so that the LHC core complex binding is strong and once the binding has occurred the outer part prevents further interaction. This aspect deserves more attention in future.

The photophysical PS II → PS I spillover in PBS-containing membranes will be discussed for the case in which PBSs are connected only to PS II but not to PS I. Cyanobacteria or red algae which adapt to large depths in the ocean where only light of 400–500 nm is available, suffer from overexcitation of PS II (Fig. 9) and need the photophysical spillover mechanism to achieve a balanced electron flow between the two photosystems. This situation can be modeled by a $k_{spo} \approx (5\ \text{ps})^{-1}$ corresponding to an estimated distance of 25 nm (Fig. 6) between PS II-dimers and PS I-trimers. The total photochemical quantum yield (referring to the PSE) still remains above 85%.

4. State Transitions

Another basic mechanism for regulating the electron flow between the two photosystems, is the physical detachment of peripheral LHCs from one and the attachment to the other photosystem. This type of mechanism is known for LHCII and also for PBS which binds to the photosystems reversibly (van Thor et al., 1998; Chapter 13, Larkum).

In the Chl *a/b*-containing organisms LHCII can be moved physically from PS II to PS I by a phosphatase/kinase regulation system (Allen and Nilsson, 1997). The modeling of this transition requires the fractionation of LHCII into a PS II and PS I associated part.

A corresponding mechanism has been suggested for PBS-containing systems which resulted in the formulation of the mobile PBS model for the regulation of energy distribution. In this mechanism PBSs detach from PS II and attach to PS I (Mullineaux et al., 1997; Sarcina et al., 2001). The model calculation for this mechanism (Fig. 9) reveals that, in order to achieve the same activity of both photosystems at 590 nm excitation, more than 50% of the PBSs have to be relocated. The rearrangement is accompanied by a predicted fluorescence change of less than a factor of 2 which is difficult to test experimentally.

A related mechanism consists of the sole detachment of PBSs from the membrane. This can be modeled by a decrease of k_{34} (Fig. 9). The occurrence of this mechanism leads to a strong increase of the PBS-fluorescence yield if a noteworthy attenuation of the PS II photochemistry is to be achieved. Therefore, the mechanism is easy to test experimentally.

A long-term regulation mechanism available to all organisms is a change of the PS I:PS II ratio. The quantitative treatment of this adaptation process in the present modeling is taken into account by the definition of the PSE (Fig. 2 and Eq. (2)).

5. Uphill Energy Transfer

Uphill energy transfer, i.e. the energy transfer from energetically low to higher energy levels, has been reported to occur in various photosynthetic systems (Zankel and Clayton, 1969; Jennings and Forti, 1975; Wang and Myers, 1977; Trissl et al., 1999). This process has significance for the interpretation of the emission spectrum of recombination luminescence (or delayed fluorescence) and also for studies in which long wavelength absorbing forms are investigated by excitation in the red wing of the absorption spectrum.

Also, this special case is covered by the present formalism. In the example of *Ostreobium* either the excitation vector Z_s is set to one for the radical pair of PS II ($Z_8 = 1$ in Fig. 7 and performing the calculation for the reduced case) or the excitation wavelength λ_{ex} is shifted to the extreme red (Eq. (11)).

In this example, as well as in the case of *Acaryochloris marina* excitation at 735 nm leads to substantial uphill energy transfer as evidenced by 685 nm shoulders in the stationary fluorescence spectra which are only slightly smaller than the ones for downhill energy transfer. This simulated result has been experimentally demonstrated for *Acaryochloris marina* by Mimuro et al. (2000).

The extent of uphill energy transfer is theoretically limited by the Boltzmann equilibrium (Eq. (24a)). How close this is reached in a particular three state system which imitates PS I is shown in Fig. 10a (inset). Here, the kinetics of the transfer equilibrium $TE(t)$ (Eq. (42)) is depicted for excitation into two different states. It is seen that $TE(t)$ reaches the same constant value after about 50 ps for both downhill and uphill transfer, demonstrating the efficiency of the latter. The $TE(\infty)$ value (Fig. 10; inset) differs significantly from the Boltzmann equilibrium constant (Eq. (25)).

6. Kennard-Stepanov Relation

The Kennard-Stepanov equation (Kennard, 1918; Stepanov, 1957) allows the transformation of the absorption spectrum of a chromophore into a fluorescence spectrum and vice versa under the assumptions of a similar configuration of the ground and excited states and thermal equilibration among the sublevels before the emission. An extension of the theory has been formulated by Knox et al. (1999). The relation is also applicable to pigment clusters as has been derived by Dau (1996). Basically, in PSUs the Kennard-Stepanov theory assumes perfect thermal

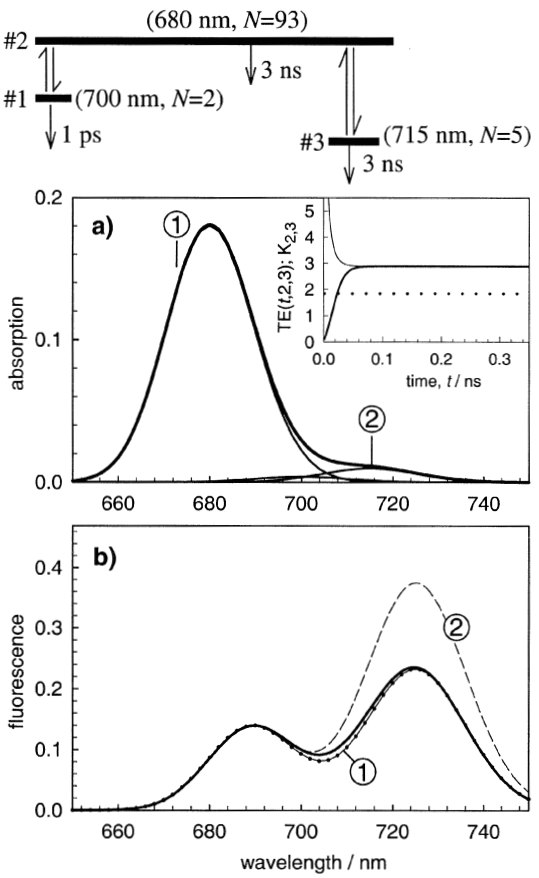


Fig. 10. Comparison of the thermal equilibrium approach and a kinetic treatment by means of a model calculation for a three state pigment system at 20 °C. a) Absorption spectra of the system and its sub-bands. The shape of the absorption spectra was modeled by Gauss-functions centered at λ_s^{max} and widths of k_BT. The encircled numbers indicate two different excitation wavelengths. Inset: Kinetics of the transfer equilibrium $TE(t)$ upon blue and red excitation (Eq. (42)). The dots indicate the thermodynamic equilibrium constant $K_{2,3}^{Boltz}$ (Eq. (25)). b) Fluorescence spectra according to thermal equilibrium (solid line), blue excitation (curve 1) and red excitation (curve 2). (The inter-state rate constants resulting from this particular example calculated for a coupling rate constant of $k_{con} = (0.1\ \text{ps})^{-1}$ are $k_{23} = k_{21} = (9.3\ \text{ps})^{-1}$, $k_{12} = (1.6\ \text{ps})^{-1}$, and $k_{32} = (17\ \text{ps})^{-1}$.)

equilibrium between all members of the pigment cluster (for instance Chl *b*, bulk Chl *a* and Chl *a* red forms in an LHC). However, as discussed before, time-resolved fluorescence and transient absorption studies resolved various kinetic phases in LHCs as well as in core complexes with positive and negative DAS being typical of equilibration processes. The detection of equilibration kinetics proves that in real systems the prerequisite of the Kennard-Stepanov-relation is not strictly met. Nonetheless, the relation has already been used in photosynthesis research in 1967 (Szalay et al. 1967) and later found to hold with good accuracy for PS II (Dau and Sauer, 1996) and PS I preparations (Croce et al., 1996).

The quantitative treatment of a PS I-like model system can clarify this apparent contradiction. Let us assume 3 states with λ_{max} = 700, 680, and 715 nm made up of $N_1 = 2$, $N_2 = 93$, and $N_3 = 5$ pigments (Fig. 10). Antenna losses occur with $k_l = (3\ \text{ns})^{-1}$. The first state, performs photochemistry with $k_t = (1\ \text{ps})^{-1}$ in order to simulate primary photochemistry from this state (Fig. 10). Without limiting the generality of the conclusions one can assume the absorption bands to be Gaussians in wavenumbers. The fluorescence spectra of the states are calculated with the Kennard-Stepanov-relation which reads for the fluorescence yield per wavenumber interval (Dau and Sauer, 1996):

$$F_s(\tilde{\nu}) \sim \tilde{\nu}^2 \cdot A_s(\tilde{\nu}) \cdot e^{-\frac{h \cdot c \cdot \tilde{\nu}}{k_B T}}. \qquad (44a)$$

These spectra are then normalized to the area of one. The fluorescence spectrum for the perfectly equilibrated system was computed in two ways, firstly by the Kennard-Stepanov-transformation of the total absorption spectrum (Eq. (5)) according to:

$$F^{PS}(\tilde{\nu}) \sim \tilde{\nu}^2 \cdot A^{PS}(\tilde{\nu}) \cdot e^{-\frac{h \cdot c \cdot \tilde{\nu}}{k_B T}}, \qquad (44b)$$

and secondly from the normalized fluorescence spectra of the species (Eq. (7)) and Boltzmann's law (Eq. (24a)). Both spectra agree within less than 10^{-4} in amplitude (data not shown).

To compare the thermally equilibrated system with the solution from a kinetic treatment, we make use of the laws holding between rate constants (Eqs. (22a) and (22b)) and are left with only one rate constant, k_{con}, to define the system unambiguously. Assuming a common coupling strength of $k_{con} = (0.1\ \text{ps})^{-1}$ the predicted equilibration time between state #2 and #3 for example is $k_{23} + k_{32} = (12\ \text{ps})^{-1}$, which is of the order of measured values.

If the excitation occurs hypsochromically there is a reasonable agreement of the 'kinetic' with the 'Kennard-Stepanov' fluorescence spectrum (Fig. 10b, curve 1). The small deviations are due to the effective quenching of the first state by the fast photochemical reaction. This deviation is equivalent to the term equilibrium perturbation introduced by Jennings et al. (1997). When this decay path is omitted in the model (>10 ps) the two fluorescence spectra match within the line thickness.

However, if the excitation is very bathochromic the emission spectrum deviates strikingly from the equilibrium spectrum (Fig. 10 b, curve 2). Correspondingly, K^{TE} (Eq. (43)) is markedly different from the thermodynamic equilibrium constant K^{Boltz} (Fig. 10, inset, dotted line). This model calculation illustrates the relevance of fluorescence photons emitted before the establishment of a transfer equilibrium. This outcome agrees qualitatively with a 4-state model calculation for PS I (Jennings et al., 1997) and also with data reported for a cyanobacterial PS I-core preparation (Gill and Wittmershaus, 1999).

It is worth noting that the photochemical quantum yield in this system remains >96% at all excitation-wavelengths, including 730 nm-excitation.

In conclusion, the coupling of pigments in chlorophyll-containing complexes is so efficient that intrinsic kinetics (equilibration times), which definitely exist, can hardly be resolved from an analysis of the shape of the fluorescence spectrum with the Kennard-Stepanov-relation when the excitation occurs in the Q_y-absorption maximum or on its hypsochromic side. Excitation on the bathochromic side is much more sensitive for finite equilibration kinetics. Generally, any deviation from the Kennard-Stepanov relation indicates the necessity of kinetic modeling.

7. Target Analysis

A widely applied method of data analysis is to fit a set of experimental curves by a number of mathematical parameters, adhering to the principle of parsimony (minimal number of parameters necessary to fit the data). However, these parameters—like amplitudes and time constants of kinetic phases or DAS—may not have an obvious physical meaning. An alternative method is the target analysis in which the experimental data are fitted

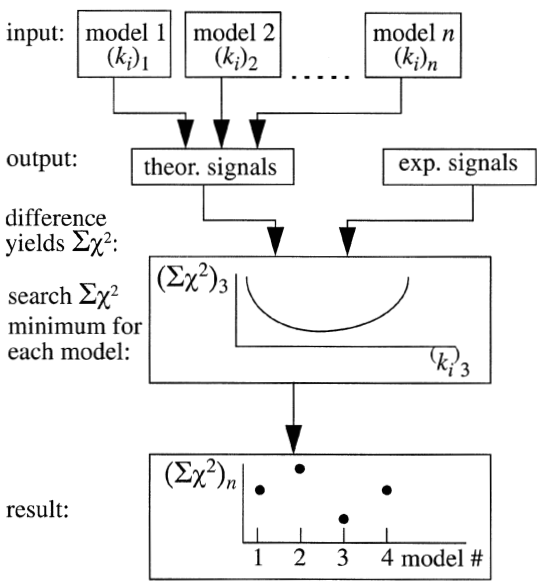


Fig. 11. Flow diagram of a target analysis. In this example model 3 describes the experimental data best.

with the parameters of a physical model (Roelofs et al., 1992; Holzwarth, 1996). The principle of the method is illustrated in Fig. 11. Although often comprising more parameters than according to the parsimony principle, it has the advantage of obtaining fit parameters that have direct physical meaning. Furthermore, it offers the possibility to test different models for compatibility with the experimental data.

The most important advantage of the target analysis (or kinetic modeling) is however the facility to fit the results of very different experimental assays at one time. 'In this sense the physical model testing represents a truly holistic approach' (Holzwarth, 1996).

V. Concluding Remarks

The mathematical formalism described in this article requires extensive programming which may be a prohibitive task for many readers. The author is presently developing an interactive, user-friendly modeling program for the Internet, which will contain all relevant subroutines outlined above (access via http://www.biologie.uni-osnabrueck.de/biophys/trissl/modeling.htm).

It can be asserted that model calculations serve fruitful purposes:

- Different models can be tested for compatibility with experimental data.
- The parameters of a model may be determined directly by global fitting of experimental data (= target analysis).
- Experimentally accessible as well as inaccessible quantities are predicted by the simulations.
- Model calculations can be used to test in advance whether planned experiments can potentially distinguish between alternative hypotheses.

One should be aware of the simplifications involved in such calculations when modeling the thylakoid membrane with few states. However, this is the current state of the art for many researchers when they are confronted with the task of evaluating their data with the aim of a physical interpretation. The more experimental data a model shall comprise the higher the number of states and therewith the complexity. It can be foreseen that this is the trend for the coming years of research in the field.

For a given model of the PSE the way of analyzing the energy capture and the distribution of excitation energy to the photosystems by differential equations predicts uniquely all kinetic parameters and all yields. However, the thylakoid membrane contains many more enzymes, a great deal of them are interacting and being regulated (e.g. by the redox state of the plastoquinone pool). They are involved in a complicated network of substrate flows (Pfannenschmidt et al., 1999). If such an adaptable system is to be analyzed for optimal efficiencies, the **m**etabolic **c**ontrol **a**nalysis (**MCA**) is the method of choice (Fell, 1992; Cornish-Bowden, 1995; Heinrich and Schuster, 1996; Internet, 1999). This method, though commonly utilized in enzyme kinetics, has not yet been applied to the early light-driven reactions and the regulated LHC systems in thylakoid membranes.

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Chapter 13

Light-Harvesting Systems in Algae

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Summary

Light harvesting in algae is very diverse and reflects the broad spectrum of organisms involved and their long history of evolution compared to higher plants. This chapter concentrates on the chlorophylls (Chls) and their binding proteins, as they are the major photosynthetic pigments. Three chlorophylls occur in the algae, Chl *a*, Chl *b* and Chl *c*, attached to light-harvesting proteins, mainly in the CAB/CAC family. In classical Cyanobacteria typical CAB/CAC proteins are not found and the only member of the Chl *c* family present is Mg divinyl-2,4-pheoporphyrin methyl ester (MgDVP). In Cyanobacteria the light-harvesting proteins are typically phycobiliproteins (under nitrogen sufficient conditions) and isiA proteins (under nitrogen limiting conditions). In prochlorophytes and *Acaryochoris marina*, which are Cyanobacteria, a prochlorophyte chlorophyll binding protein (pcb protein) binds Chl *a* and Chl *b*, and sometimes MgDVP, or Chl *d*. The binding of Chl in these proteins and in other antenna proteins is discussed. These proteins serve to optimize energy distribution to the two photosystems, with controls at several levels of organization. A major problem in all oxygenic photosynthetic organisms (Cyanobacteria, algae and higher plants) is the generation of oxygen free radicals, particularly by Photosystem II. This leads to photoinhibitory damage, which is partially offset by mechanisms which down-regulate photosynthesis, particularly Photosystem II, and dissipate incoming energy as heat. The xanthophyll cycle is found in all algae, with the possible exception of red algae and cryptophytes, and, by processes which are only partially known, diverts light energy to heat energy when switched on. Algae can control their uptake of light energy in a variety of ways: by physiological mechanisms and by regulation of transcription and translation of proteins. These responses can be to both light quality and light quantity. Algae show a wide range of rearrangements of the light harvesting apparatus in relation to the photosystems, known as state transitions, which alter the optical cross-sectional areas of PS I and PS II.

I. Introduction

Photosynthesis supports the majority of the life on the Earth and therefore underpins our planet's biodiversity. Yet old as oxygenic photosynthesis is, possibly dating back to 3.5 billion years ago (BYA) but at least to 2.7 BYA (Brocks et al., 1999; Summons et al., 1999), the photosynthetic pigments and the major light-harvesting systems are surprisingly uniform. There are only two major primary photosynthetic pigments, chlorophyll and bacteriochlorophyll (Scheer, 1991) and there are only eight or so major light-harvesting systems (see *Light-Harvesting Antennas*, volume 13 in this series; Green and Parson, 2003). The photosynthetic systems of Cyanobacteria and plants (photosynthetic protists and higher plants) are built around chlorophyll. This is remarkable because the photosynthetic bacteria employ bacteriochlorophyll; although Heliobacteria employ γ-bacteriochlorophyll which, while it is a bacteriochlorophyll i.e. possesses a bacteriochlorin ring, it is close to chlorophyll in structure (Scheer, 1991). It must be concluded therefore that the oxygenic photosynthesis adopted by Cyanobacteria and plants can only function with chlorophyll and not bacteriochlorophyll. Perhaps even more remarkable is the fact that these two great realms of the photosynthetic world do not share any similar light-harvesting systems, despite the fact that both groups have developed a wide, but not fully comprehensive, set of such pigment systems, to absorb energy from sunlight. In the Cyanobacteria and plants the light-harvesting systems are based mainly on the chlorophylls, with the notable exception of the phycobiliproteins (Chapter 14, Toole and Allnutt). The proteins interacting with the chlorophylls are surprisingly few: the CAB proteins, the relatives of the inner antennae complex (CP43 and CP47), and the novel peridinin chlorophyll complex (PCP). In addition to this, Cyanobacteria, red algae and cryptophyte algae possess phycobiliproteins. For other reviews in this area the reader is referred to Larkum and Barrett, 1983; Falkowski and Raven, 1997; Larkum and Howe; 1997. Light harvesting in

Abbreviations: BYA – billion years ago; CAB – chlorophyll *a/b* binding protein; CAC – chlorophyll *a/c* binding protein; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ELIP – early light induced protein; HLIP – high light-induced protein; isiA – iron stress-induced protein A; LHC – light-harvesting chlorophyll complex; LHP – light-harvesting protein; MgDVP – Mg-2, 4-divinyl pheoporphyrin methyl ester; MSH – membrane spanning helix; pcb – prochlorophyte chlorophyll binding protein; PQ – plastoquinone; PS I – Photosystem I; PS II – Photosystem II; qE – fluorescence quenching due to energization of the thylakoid membrane

terms of adaptation, acclimation and regulation is further discussed in Chapter 17, Raven and Geider.

II. Chlorophylls

The chlorophylls are formed of two basic pigments, the magnesium protoporphyrin ring (Chl *c*) and the magnesium chlorin ring (Chl *a*, Chl *b* and Chl *d*; Table 1; Figs. 1 and 2). While magnesium is the only metal now known to function in chlorophyll, it is possible that other metals substituted for this role in the early Earth (Larkum and Barrett, 1983). Recently it has been shown that zinc can substitute for magnesium in bacteriochlorophyll (Wakao et al., 1996). Further details on the chlorophylls and their biosynthesis are given in Chapter 6, Cahoon and Timko.

A. Chlorophyll a

The major chlorophyll is Chl *a* (Fig. 1) which is found in both the reaction centers of all Cyanobacteria with the exception of *Acaryochloris marina* (see below) and all algae and higher plants (Table 1). It must therefore be assumed that this structure plays a unique role in the photochemical conversion of energy in these systems (Scheer, 1991). The only exception to this rule is in the case of Chl *d*. Chl *d* has a formyl group in ring I at position C3 (Fig 1) and occurs in one characterized cyanobacterium, *Acaryochloris marina* (Miyashita et al., 1996) and possibly in one other (Murakami et al., 2001). Chl *d* is present as the major photosynthetic pigment, while Chl *a*, the only other Chl, occurs in amounts no greater than 5%. Chl *d* has been shown to be the photo-reactive pigment in reaction center (RC) I of *Acaryochloris* (Hu et al.,

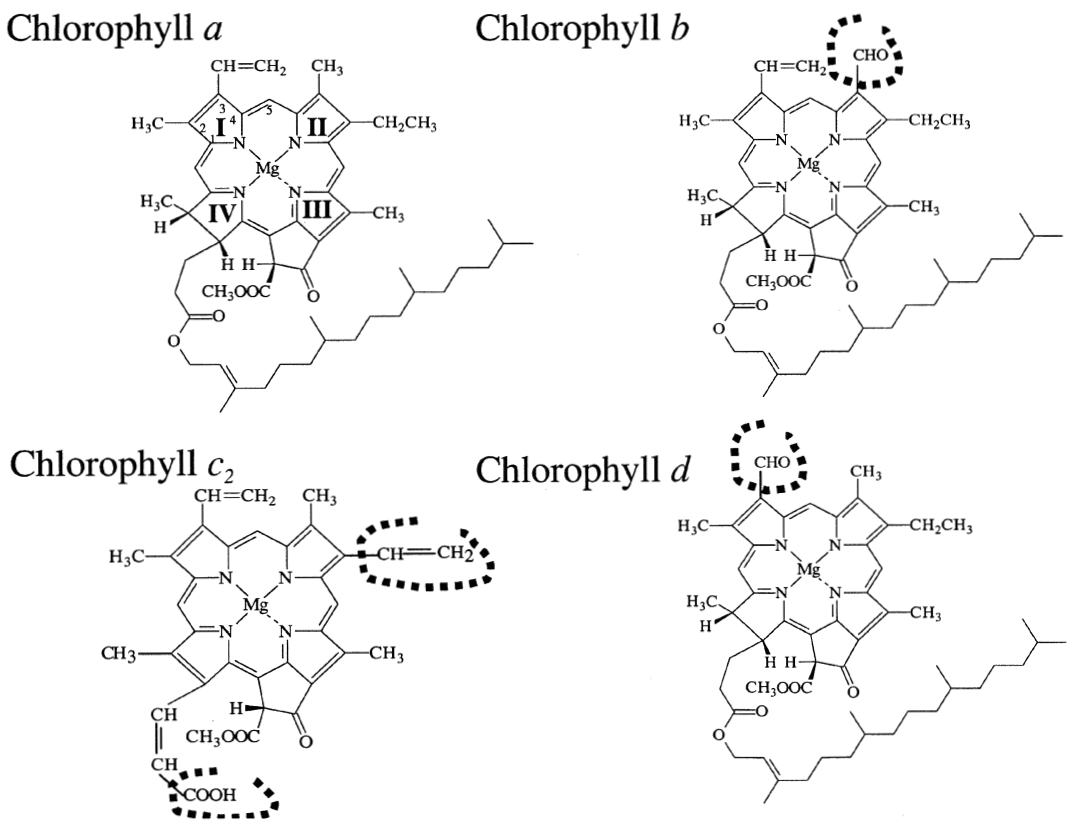

Fig. 1. The structure of the chlorophylls. Characteristic groups of Chl *b*, Chl c_2 and Chl *d* are indicated. Note that Chl c_1 has a ethyl group in the place of the vinyl group of Chl c_2. Note also that the only difference between Chl *b* and Chl *d* is that the formyl group is on Ring I in Chl *d* and on Ring II in Chl *b*.

1998) and may play a similar role in RC II (see below).

Chlorophyll *a* has typically a hydrocarbon tail formed of a phytol molecule linked by an ester bond to ring IV. Other hydrocarbons are known to substitute for the phytyl unit. There is no known case of Chl *a* existing without such a tail. However, Chl *c*'s typically have no hydrocarbon tail, but as reported below, some Chl *c's* have recently been found with a hydrocarbon tail (see below). The phytol tail makes both Chl *a* and Chl *b* very lipophilic, as demonstrated by the position at which they run under chromatographic separation (Scheer, 1991).

While there are many potential isomers and substituent forms of Chl *a* there are very few found in Nature. Divinyl Chl *a* ([8-vinyl]-Chl *a*) has been found in *Prochlorococcus marinus* (Chapter 3, Partensky and Garczarek). The 13^2S-epimer of Chl *a* (Chl *a'*) (Scheer, 1991) has been implicated to have a function in PS I, where it occurs in the proportion of 1:1 with Chl *a* in P700 (Scheer, 1991); recently this has been confirmed (Jordan et al., 2001).

Chl *a* occurs as the primary donor (P700 and P680) in RCI and RCII, respectively (Chapter 7, Nugent et al.). It occurs as the only chlorophyll in CP43 and CP 47 of the inner antennae of PS II and as the four Chls on the acceptor side of PS I. Chl *a* occurs together with Chl *b* in the CAB proteins of green algae, euglenophyte algae, chloraracniophytes and higher plants. It occurs together with Chl *c* (either c_2 or $c_1 + c_2$; Chapter 2, Larkum and Vesk) in the CAB proteins of chromophyte algae (with the exception of eustigmatophytes, which have only Chl *a*). Red algae also possess a CAB protein attached to PS I, but this complex bears only Chl *a*. Chl *a* also occurs alone in various proteins which seem to be involved in transport of Chl to the thylakoid membrane such as ELIP and HLIP proteins (Green and Durnford, 1996). It also occurs in the isiA (iron stress induced chlorophyll-binding) protein which is now known to form a ring of light-harvesting polypeptides around PS I in Cyanobacteria (Bibby et al., 2001a; Boekema et al., 2001). It is also possibly present in psbS which has a function in down-regulation of PS II (Li et al., 2000). In prochlorophytes (Chapter 3, Partensky and Garczarek) Chl *a* occurs together with Chl *b* in Pcb (prochlorophyte chlorophyll binding) proteins which bear some similarity to isiA and CP 43 proteins (La Roche et al., 1996). Pcb has recently been shown to form a ring around PS I in *Prochlorococcus*, in a similar way to isiA (Bibby et al., 2001). Chl *a* occurs together with peridinin in the peridinin chlorophyll complex (PCP) of dinoflagellates which is a unique protein (Section III.F).

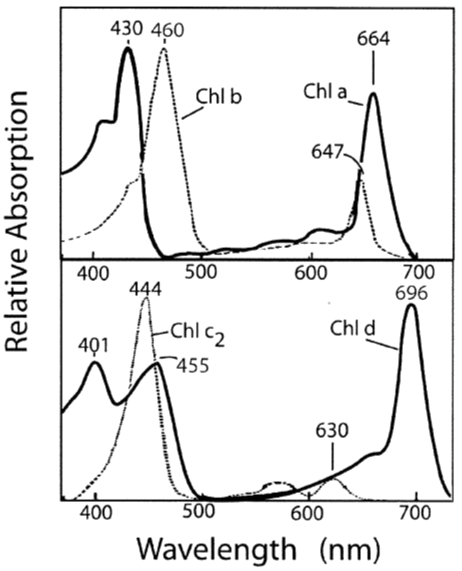

Fig. 2. The absorption spectra of Chl *a*, Chl *b*, Chl c_2 and Chl *d* in 90% acetone-water. The peak wavelengths are indicated: note that the peak wavelengths are affected by the solvent used and the concentration of that solvent if diluted with water; in the case of Chl *c* only the wavelengths for Chl c_2 are shown: for Chl c_1 in 90% acetone-water the peaks are 442 nm and 630 nm. Note that the chlorins (Chl *a*, *b* and *d*) have strong bands in the red region of the spectrum as well as for the Soret (blue) bands, whereas the porphyrins (Chl *c*'s) have a weak band in the red region and only a strong Soret band.

In most of these chlorophyll protein complexes, Chl *a* occurs not only with other chlorophylls but also with carotenoids (Chapter 15, Mimuro and Akimoto).

B. Chlorophyll b

Chl *b* differs from Chl *a* in having the 7-methyl group in ring II replaced by a 7-formyl group (Fig. 1). This confers a slightly lower lipophilic character and shifts the major absorption bands in the red and blue towards the green. Since Chl *b* is found in the light harvesting CAB and pcb proteins, together with Chl *a*, it is clear that the light harvesting properties are important. This is also shown by the increased amounts of such light-harvesting proteins occurring under shade conditions (Larkum and Barrett, 1983). In extreme shade in some green algae, such as *Dunaliella*, *Codium* spp. and deep-water green algae

Table 1. Occurrence, distribution, type and spectral characteristics of photosynthetic pigments from cyanobacteria and algae

Pigment	Algal/cyanobacterial group	Chemical type	Major forms	Absorption bands (nm)	Fluorescence emission (nm)*
Chlorophyll *a*	All organisms	Tetrapyrrol ring, phytol tail	Chl *a* (minor form Chl *a'*)	430, 670–690	680 (RT) 680–730 (LT)
Chlorophyll *b*	Prochlorophytes Green algae Chlorarachniophytes Euglenophytes	Tetrapyrrol ring, phytol tail	Chl *b* (minor form Chl *b'*)	455, 650–660	660–670
Chlorophyll *c*	Chromophytes (except eustigmatophytes)	Tetrapyrrol ring, generally no phytol tail	Chl c_1 Chl c_2 Chl c_3	442–444, 630	633 (640 LT) 635
Chlorophyll *d*	Acaryochloris marina	Tetrapyrrol ring, phytol tail	Only one form known	380, 440, 700–720	720
MgDVP	Prochlorophytes (some) Acaryochloris ? Micromonadophytes	Tetrapyrrol ring	Only one form known	438, 625	634
Phycobili-proteins	Prochlorophytes Acaryochloris Cyanobacteria Reds Cryptophytes Glaucocystophytes	Open chain tetrapyrrol	Allophycocyanin (APC) Phycocyanin (PC) Phycoerythrin (PE)	625–634 (APC) 610–635 (PC) 495–560 (PE)	570–650
Carotenes	All	carotenoid	α, β, γ	420–470	425–490
Xanthophyll	All	carotenoid	Many forms	410–500 (540)	415–505

For more details on the pigments, refer to Scheer, 1990; Glazer, 1999; Rowan, 1990; Chapter 14, Toole and Allnutt. MgDVP, Magnesium-2,4-divinyl pheoporphyrin methyl ester; RT, room temperature; LT, low temperature (77K). *Fluorescence emission from the intact pigment-protein complex

of the phytoplankton, the ratio of Chl *b* in proportion to Chl *a*, increases and there may also be enhanced levels of the light-harvesting xanthophyll, siphonaxanthin (Hiller et al., 1991; Ohki and Honjho, 1997). The extent to which the ratio also changes in the several CAB proteins of higher plants is still under investigation. While the most abundant CAB protein (LHCII) is associated with PS II there are a number of CAB proteins attached to PS I (Green and Durnford, 1996; Chapter 4, Durnford).

Chl *b* occurs in green algae, euglenophyte algae, chlorarachniophytes and higher plants (Table 1). It also occurs in the pcb's of prochlorophyte algae; and the biosynthetic pathway seems to be similar to higher plants, involving Chl *b* synthase (Tomitani et al., 1999). In *Prochorococcus marinus* (a prochlorophyte) Chl *b* occurs also as the divinyl Chl *b* form (Chapter 3, Partensky and Garczarek).

Since Chl *b* biosynthesis has a step involving molecular oxygen it has been suggested that Chl *b* could not have evolved when the Earth's atmosphere was anoxic (Larkum and Barrett, 1983). However, since oxygenic photosynthesis evolved at least 2.7 BYA (Summons et al., 1999) and localized pockets of oxygen existed from an early stage (Canfield, 1997) Chl *b* could have been a fairly early arrival on the light-harvesting scene.

C. Chlorophyll c

Chl *c* should correctly be called chlorophyllide *c* since it generally lacks a phytyl tail. However phytylated Chl *c's* have recently been characterized (Garrido et al., 1995). Chl *c* is also based on a porphyrin ring rather than a chlorin ring. This changes the absorption spectrum to produce a strong Soret (blue) absorption band (approx 450 nm) in comparison with a weak band in the red at approx 630 nm.

There are two major Chl *c's*, Chl c_1 and Chl c_2. Chl c_2 (Fig. 1) can be formed from Mg-2,4-divinyl pheoporphyrin methyl ester (MgDVP; sometimes

called Mg-protoporphyrin) by a single reductive step. Chl c_1 can be formed from Chl c_2 by a further reductive step. However the biosynthetic pathways of the Chl *c's* have not been established.

In addition to these two major Chl *c*'s, the structure of at least one other — Chl c_3 (Fookes and Jeffrey, 1989) — has been documented and others have been proposed (Jeffrey, 1989).

Chl c_2 occurs alone in two chromophyte algal groups, the dinoflagellates and the cryptophytes (Chapter 2, Larkum and Vesk). In all the other chromophyte groups which possess Chl *c*, Chl c_1 and Chl c_2 occur in approximately equal proportions. In all cases Chl *c* is bound in vivo to a CAC protein, where it acts like Chl *b* as a light harvesting pigment. It clearly extends the light harvesting capacity of these algae. However the presence of a number of xanthophylls in the CAC protein adds additional capabilities of harvesting light up to approximately 540 nm in the green region of the visible spectrum. In brown algae, diatoms, chrysophytes and haptophytes this pigment is fucoxanthin (or a near relative); in dinoflagellates it is peridinin (Chapter 15, Mimuro and Akimoto).

The role of Chl c_3 and other forms of Chl *c* has not been established. Chl c_3 occurs in varying proportions with Chl c_1 and Chl c_2 in several groups of chromophyte algae (Jeffrey, 1989). However, it is not present in every species within any one group and this has prevented its use for taxonomic purposes.

MgDVP has been shown to act in a light harvesting capacity in certain prasinophyte green algae, where it is bound to a CAB protein (e.g. in *Mantoniella* and *Micromonas*; Wilhelm and Lennartzt-Weiler, 1987) and in the prochlorophyte, *Prochloron didemni* (Larkum et al., 1994), where it is bound to a pcb protein (La Roche et al., 1996). The structure of MgDVP has recently been confirmed (Helfrich et al., 1999). Although it is less reduced than the Chl *c's* it has a similar absorption spectrum and can therefore act as a light-harvesting pigment (Raven, 1996).

D. Chlorophyll d

Chl *d* differs from Chl *a* in the presence of a 3-formyl group which replaces the 3-divinyl group in ring I. Chl *d* was first isolated in minor amounts from certain red algae (Manning and Strain, 1943). Its existence in red algae has never been firmly established and it is therefore possible that its presence was the result of an artifact of some kind. However the way in which it would be formed as a product of isolation has never been clear although it can be formed from Chl *a* (Scheer, 1991). Thus it is possible that its presence in extracts from red algae was due to a contaminant of some kind; the recent report of a cyanobacterium containing Chl *d* on the frond bases of some red algae (Murakami, 2001) suggests that this may have been the case. Whatever the explanation, it is now clear that it occurs as the major photosynthetic pigment in *Acaryochloris marina*, which is a photosynthetic prokaryote, lying within the cyanobacterial evolutionary radiation (Miyashita et al., 1996). Since Chl *a* occurs in amounts up to only 5%, Chl *d* must take on the role of the major photosynthetic pigment in the inner antennae proteins and in the light-harvesting proteins. As mentioned earlier, Chl *d* is the functional Chl in P700 in *A. marina* (Hu et al., 1998) and, possibly, in P680. Since in comparison with Chl *a*, Chl *d* has a red-shifted peak in the red/far-red region, its role in energy transfer and redox chemistry has been a matter of some interest. Blankenship and Hartman (1998) suggested that Chl *d* may have evolved to take advantage of far-red light environments. Since there are a number of forms of Chl *d* which absorb well into the far-red region the question of energy transfer to the reaction center has been studied. Mimuro et al. (2000) concluded that this would require a special 'uphill' transfer mechanism, within the Chl *d* pigment bed. However, Reimers et al. (unpublished) have studied the absorption properties of Chl *d* in various solvents and have concluded that energy transfer can occur with reasonable efficiency even in an 'uphill' direction, if the various quantum states are taken into account (see also Chapter 12, Trissl). The occurrence of Chl *d* in place of Chl *a* in the equivalent of P700, P740, is also of interest in that this would suggest generation of a smaller reducing power of P740*: 1.68 V compared with 1.77 V for P700*. However due to the lower midpoint redox potential of P740/ $P740^+$ (Hu et al., 1998), the reducing power is approximately equal to that for P700*.

E. Specific Binding of Chlorophylls

Some evidence suggests that the binding of chlorophylls to chlorophyll-proteins is not absolutely specific. Scheer and coworkers have carried out a large amount of work on the removal of (B)Chl *a* from bacterial and PS II RCs and light-harvesting antennas and replacement with a variety of substituted

(B)Chls (Fraser et al., 2001; Zehetner et al., 2002). Synthetic proteins and small polypeptides which specifically bind only one or two cofactors (maquettes) are now being used to gain a better understanding of the principles involved (Rau et al., 2001).

In the case of algae it has been shown that a number of chlorophylls can be substituted in the case of the CAB protein of PS I in red algae (Grabowski et al., 2001) and in the peridinin-chlorophyll *a* protein of dinoflagellates (Hiller et al., 2001). In Cyanobacteria, Chl *b* genes can be introduced and Chl *b* can be expressed and substituted in cyanobacterial LHC systems (He et al., 1999). These examples may be taken to imply that when there are evolutionary changes which result in new chlorophylls, such as Chl *b* or Chl *d,* these may find a ready attachment on existing chlorophyll proteins, which subsequently may be optimized to the new chlorophylls. However the studies are at an early stage. We do know that in the reaction centers of PS I and PS II there are specifically bound chlorophylls (e.g. phaeophytin *a* and Chl *a*′) which presumably need a very specific binding mechanism for these rare molecules to become attached at the right site and in the correct orientation. Eggink et al. (2001) have recently put forward the suggestion that the chemical properties of Chl *b* (and possibly Chl *c*) in contrast to those of Chl *a* enhance binding of chlorophyll to the CAB/CAC light harvesting proteins (see below).

III. Light-Harvesting Proteins

There are six major light-harvesting proteins of algae (including Cyanobacteria). These are:

A. Inner antennae proteins (Chapter 4, Durnford)

B. IsiA and Pcb proteins (Chapter 3, Partensky and Garczarek)

C. CAB/CAC proteins (Chapter 4, Durnford)

D. PsbS

E. Phycobiliproteins (Chapter 14, Toole and Allnutt)

F. Peridinin chlorophyll protein

Since these proteins are dealt with extensively in other chapters only a brief overview will be given here.

A. Inner Antenna Proteins—CP 43, CP 47 Chlorophyll Proteins

The six groups of LH proteins have been placed in that order to reflect an evolutionary sequence. The inner antennae almost certainly developed closely upon the 'invention' of RCI and RCII. There is now good evidence that RCI and RCII are homologous, although now very different (Mulkidjanian and Junge, 1997; Jordan et al., 2001; Zouni et al., 2001). A series of gene duplications and fragmentations appear to have given rise to the RCs of PS I and PS II. However the exact evolutionary sequence of events is still being debated. One view supports an early 11 membrane-spanning helix (MSH) polypeptide, which later fragmented to give the 5 MSH RC + 6 MSH inner antennae (CP43 + CP47) situation in PS II (Xiong et al., 1998; Baymann et al., 2001). The other view supports an archetypal RC of 5 MSHs and an archetypal light harvesting polypeptide of 6 MSHs which fused to form the 11 MSH RCs of photosynthetic bacteria and PS I (Fromme, 1999; Fyfe et al., 2002). The present roles of CP 43 and CP47 which bind 12 and 14 Chl *a*'s, respectively (Zouni et al., 2001) are clearly to funnel excitation energy to P680 from the outer antennae. However initially they could have acted in a light-harvesting role and there could have been several copies of each per RC. In PS I subunits A and B (which share homologies with CP43 and CP 47; Jordan et al., 2001) act in a similar role, although the structure is more complex, with 64 Chl *a*'s arranged in a bowl shape (Jordan et al., 2001). This arrangement of chromophores is very different from that in PS II or the photosystems of photosynthetic bacteria.

B. IsiA and Pcb Proteins

IsiA proteins are known to be present in many Cyanobacteria under oligotrophic conditions of low nitrogen supply and low iron. It was previously thought that the role of these Chl *a*-bearing polypeptides was in transporting Chl to the thylakoid membranes (Green and Durnford, 1996). However it is now clear that they form a multi-unit light-harvesting ring structure around PS I (Bibby et al., 2001a; Boekema et al., 2001).

The pcb proteins of prochlorophytes are in the same family as CP43 , CP47 and isiA (La Roche et al., 1996; van der Staay et al., 1998; Chapter 3, Partensky and Garczarek), and here the role is definitely light-harvesting. In pcb Chl *b* and MgDVP (*Prochloron* and *Prochloroccus* but not *Prochlorothrix;* Helfrich et al., 1999) are bound as well as Chl *a* (Chapter 3, Partensky and Garczarek).

In the Chl *d*-containing cyanobacterium, *Acaryochloris marina* there is also a pcb-like protein (Chen et al., 2002) which binds Chl *d* and a small amount of Chl *a*. This protein, which occurs under all conditions, forms a layer on either side of PS II (Chen et al., unpublished). In addition it appears that there is also an isiA-type protein, which may carry out light harvesting for PS I or PS II.

In evolutionary terms it is unclear at present whether the family of CP43, CP47, isiA and pcb proteins is ancestral to or derived from the photosynthetic RCs. Previously it was concluded that the isiA and pcb proteins evolved from CP43 and CP 47 (van der Staay et al., 1998) but the recent results showing that these proteins form a ring around PS I opens up the possibility that the whole family is indeed an ancient light-harvesting complex, which in plastids has been replaced by CAB/CAC proteins.

C. CAB/CAC Proteins

The evolutionary experiment with Chl *b* and Chl *c*-type pigment binding to pcb in prochlorophytes cannot be judged a success, based on their low abundance in the modern world. In contrast, the use of CAB/CAC proteins for this purpose is now the basis for photosynthesis in the majority of plastids—and for all land plants (tracheophytes); the exception being most of the extant Cyanobacteria (excluding prochlorophytes). The enigma then is why CAB-type proteins are not used for light-harvesting in Cyanobacteria. In fact a putative precursor to the family of CAB proteins occurs in some Cyanobacteria. Several proteins in Cyanobacteria have been found with homology to CAB proteins (Dolganov and Grossman, 1995; Funk and Vermaas, 1999). The first of these is in the form of an HLIP (High Light Induced Protein) coded by an *hli*A gene (Dolganov and Grossman, 1995); for which a homologue has recently been found in higher plants, Jansson et al., 2000). This protein which is induced by high light or UV radiation is of 72 amino acid residues and is homologous with CAB. However this length is only sufficient for one membrane-spanning region whereas typical CAB/CAC proteins have three. Green and Pichersky (1994) have speculated that CAB proteins evolved by two serial gene duplications, followed by a loss of the fourth MSR. Support for this hypothesis comes not only from the HLIP protein but also from the presence of a 2-MSR homologue (Heddad and Adamska, 2000) and a 4-MSR homologue, psbS, a protein which probably binds chlorophyll and is crucial for non-photochemical quenching (Li et al., 2000; Nield et al., 2000; see below).

While the CAB/CAC protein has its origins in Cyanobacteria it is not known to be present, in any form, as a light-harvesting protein in any extant cyanobacterium. It is possible that a light harvesting CAB/CAC protein occurred at one time in Cyanobacteria. Alternatively light-harvesting CAB/CAC proteins could have evolved independently in the line of Cyanobacteria giving rise to plastids or at the level of plastids themselves. Since 3 MSR CAB/CACs occur in chromophyte algae, green algae and red algae (Chapter 4, Durnford) any evolution at the stage of plastids must have occurred at the base of this radiation (unless lateral gene transfer is invoked).

The role of CAB/CACs in light harvesting may seem obvious, in augmenting the absorption capacity of Chl *a* alone. In the case of Chl *b* the case is well supported since the spectral peaks of Chl *b* in vivo (465 and 652 nm) are well separated from those of Chl *a* (436 and 675–695 nm). In the case of Chl *c* the advantage is less clear since the spectral peaks are at 442 and 630 nm, and the peak at 630 nm is very small. It is true that CAC proteins bind xanthophylls very efficiently and these may extend the absorption out to the green region (540 nm) as in the case of siphonaxanthin, peridinin and fucoxanthin (Chapter 14, Toole and Allnutt). However this hardly explains why Chl *c* has been retained in so many chromophyte CACs. It is possible that the chemical or biophysical properties of Chl *c* are necessary for the functioning of this light harvesting protein. Eggink et al. (2001) have recently suggested that both Chl *b* and Chl *c* enhance the binding of chlorophylls to the CAB/CAC proteins because of their oxidized properties.

D. PsbS

PsbS protein is thought to be a chlorophyll-binding protein and is homologous to CAB proteins. However it does not have a light-harvesting role. In preparations of PS II it is more often associated with the core

complex than the LHCs (Nield et al., 2000), which suggests that it is not an integral part of the light-harvesting process. Li et al. (2000) have suggested that it may be in an intermediate position between the LHC and RCII. They provide evidence that it is essential for regulation of light-harvesting and suggest, from mutant studies of *Arabidopsis*, that it regulates non-photochemical quenching by sensing the pH of the lumen and triggering heat dissipation through zeaxanthin at low pH. This is supported by the work of Gilmore et al. (2000). As suggested above psbS has four MSHs and is likely to be the result of two serial gene duplications from an ancestral gene coding for a protein homologous to the HLIP of Cyanobacteria (Dolganov et al., 1995)

E. Phycobiliproteins

The development of phycobiliproteins (PBP) provided those algae possessing such proteins with a powerful light-harvesting system in a region of the spectrum where few other light-harvesting proteins are active (Chapter 14, Toole and Allnutt), i.e. in the green to orange region of the visible spectrum. It is interesting that this is a spectral region not covered well by any other photosynthetically active pigment. While other pigment systems such as rhodopsin (Kockendoerfer et al., 1999) and the green fluorescent protein family (Wall et al., 2000) have been able to cover a greater part of the visible spectrum by tuning of the chromophore, this has not occurred in any photosynthetic pigment. The reason for this may be the special needs of RCII in relation to a suitable redox potential for water splitting (Barber and Archer, 2001). Furthermore it is relevant that the light-harvesting systems of photosynthetic bacteria are quite different from those of Cyanobacteria, algae and higher plants. It is also surprising that the PBPs are not more widely distributed: they occur in Cyanobacteria, red algae, glaucocytophytes and cryptophyte algae (Chapter 1, Douglas et al.; Chapter 14, Toole and Allnutt). They occur in conjunction with light-harvesting CAB proteins only in cryptophyte algae and in PS I of red algae (Chapter 4, Durnford). Of interest too is the fact that the prochlorophytes which possess a light-harvesting pcb system have either no or a very primitive (in some *Prochlorococcus* strains) PBP system (Chapter 3, Partensky and Garczarek), while *Acaryochloris marina* with a pcb system that binds Chl *d* has a well defined PBP system. In all these instances the typical phycobilisome stromal light-harvesting system (Chapter 14, Toole and Allnutt) is absent. These facts suggest that there is some basic weakness in the phycobilisome system. The phycobilisome system is not only an 'extraneous' protein system but is very 'expensive ' in terms of nitrogen (Table 2). In fact in a number of Cyanobacteria it is known that under conditions of iron stress (a proxy for low nitrogen) phycobilisomes are lost. Under these conditions isiA proteins are induced (see above) and form a light-harvesting ring around PS I (Bibby et al., 2001a; Boekema et al., 2001). This should be a fruitful area for future research.

Nevertheless where it is present the phycobilisome system of light harvesting is very effective and red algae are shade algae par excellence in many marine waters (Larkum and Barrett, 1983).

F. Peridinin Chlorophyll Protein

The last light-harvesting protein is the peridinin chlorophyll protein (PCP). This water-soluble protein occurs in many, but not all dinoflagellates (Larkum and Barrett, 1983). It is the second site of peridinin in such organisms, since peridinin also occurs in integral membrane light-harvesting chlorophyll *a/c* (CAC) proteins (Hiller, 1999; Chapter 4, Durnford). The gene sequence for PCP was established by Hofmann et al. (1996) and has no homology to any other known protein. The crystal structure indicates a protein with the monomer binding two Chl *a* molecules and eight peridinin molecules (Hofmann et al., 1996; Chapter 15, Mimuro and Akimoto), essentially as predicted from CD spectra by Song et al. (1976). Peridinin has an in vivo absorption spectrum extending up to 540 nm and adds greatly to the absorption capacity of dinoflagellates in the blue-green region of the spectrum. Clearly PCP significantly augments the light-harvesting capacity of the dinoflagellate CAC proteins and does this at low nitrogen cost (Table 2).

IV. Optimizing Light-Harvesting Architecture

That a plant has a satisfactory array of light-harvesting pigments (LHPs) is a necessary but not sufficient condition for efficient photosynthesis. Oxygenic photosynthesis relies on the cooperation of two photosystems (PSs) which operate in series to produce

Table 2. The efficiency of light capture by various light-harvesting proteins based on four criteria, i) the proportion of molecular mass per chromophore, ii) the photon cost (of synthesis of LH apparatus per mol of chromophore)(Raven, 1984), iii) nitrogen cost (of synthesis of chromophore, protein and bilayer forming lipids)(Raven, 1984), and the absorbance (max) per mg DW protein.

Light-harvesting Protein	Max A/mg	kDa/Pigment	Photon Cost	Nitrogen Cost
APC	2	16	10200	197
PC	4	13	8600	166
R-PE	8	7	4000	75
Crypto PE	8	7	4000	75
PCP	22	4		35
FMO Protein	15	6		34
LHCII	~40	1	2150	29
Pavlova LHC	~24	2	2400	34
Amphidinium LHC	~60	0.9	2500	35

Columns 2 and 3: unpublished data of R.G. Hiller, with permission. FMO protein, Fenna-Matthews-Olson protein of *Prosthecochloris aestuari.* (Adapted from Larkum and Howe, 1998)

photosynthetic electron flow, to power the 'dark' reactions. The minimum reduction time by the PS I reaction center (RCI) as measured by P-700 bleaching (Junge, 1977) is 50–100 times faster than the minimum turnover time for O_2 production as measured in benthic macroalgae (Mishkind and Mauzerall, 1980); also evidence from unicellular algae suggests a faster turnover of RCI compared with RCII. All this means that there can be no simple, equal apportionment of LHPs. The ratio of PS I:PS II is known to vary widely, especially in algae (Larkum and Barrett, 1983). Thus a primary requirement is to apportion the LHPs to effect equal activity of the photosystems. However this is not a simple matter since there may be different LHCs, for each PS, and in response to different light conditions, and also differential connectivity of phycobiliproteins for the two photosystems (Abe et al., 1994; Sarcina et al., 2001). Finally, as discussed in Chapter 2 (Larkum and Vesk), from the streptophyte line of green algae up to higher plants, there has developed a complex system of lateral heterogeneity, whereby the two photosystems, in these plants, are physically separated and electron flow between the two PSs is effected by diffusion of electron transport intermediates (Kirchhoff et al., 2000). The competitive advantage of lateral heterogeneity in streptophyte plastids is discussed by, among others, Larkum and Barrett (1983), Trissl and Wilhelm (1993) and Anderson (1999). In all other algae including the Cyanobacteria there appears to be no lateral heterogeneity, i.e. the two photosystems lie close to one another and effect electron transport by direct flow between nearest neighbors. Nevertheless the constraints outlined above mean that the arrangement of LHCs and PSs can be complex in order to satisfy the requirement for a reasonable light-harvesting activity (cross-sectional area) for each PS unit (for further discussion of cross-sectional area and photosynthetic unit size, see Chapter 17, Raven and Geider). Furthermore the system is dynamic with 'State Transitions' allowing for fast rearrangement of cross-sectional areas of the two PSs. State Transitions are discussed in Section VII.F.2: they partially fulfill a need to react to rapid changes in light intensity and/or light quality (see also Chapter 17, Raven and Geider). Longer term changes in these parameters can be accommodated by control of gene expression (Section VII.B–D).

Rapid light changes occur during a diurnal cycle due to i) the procession of the sun, ii) changes in cloud cover and, in the sea, iii) changes in sea surface conditions, tidal changes, changes in depth and changes in turbidity and dissolved materials such as 'yellow substance' (Chapter 16, Franklin et al.). Such changes make it very difficult to optimize any single light harvesting architecture. State Transitions are not able to change the ratio of energy between the PSs by more than a few percent (Section VII.F.2), so any light harvesting architecture must be regarded as a compromise between the highest and lowest photon fluxes that a plant may encounter in any one day. For terrestrial plants this generally means that they are

optimized either as shade plants, at one extreme, and 'sun' plants, at the other. Aquatic plants are often regarded as shade plants (Reiskind et al., 1991; Krause-Jensen and Sand-Jensen, 1998). However there is a great difference between plants that are, for instance intertidal and subtidal and between phytoplankton which is near the surface of the ocean and that near the compensation depth (Chapter 18, Hanelt et al.). Thus in aquatic plants, especially algae, the light-harvesting systems are highly regulated and controlled.

Finally some mention should be made of other strategies that plants may adapt in terms of light harvesting, some of which are counter-intuitive. Plastid movement is clearly used in some benthic macroalgae to change their light absorption (Chapter 18, Hanelt et al.). However plants may simply overcome the lack of suitable light-harvesting pigments by over-producing pigments which they have. This leads, overall, to a black-body absorber but at the local level may mean that different chloroplasts receive a very different light climate, red and blue light being absorbed mainly in the upper layer and green light in lower layers. *Codium* species are a good example of this condition, where massive amounts of chlorophyll may be produced to create a black body surface (Ramus, 1978) enhanced by light-fiber effects.

In a high light environment the strategy for plants in general is to reduce the LHPs and thereby the cross-sectional areas of each PS. This is because even 'sun' plants appear to optimize their PS cross-sectional areas to provide maximum photosynthetic electron flow at intermediate levels of photon flux (~ 800–1000 μmol photons $m^{-2}s^{-1}$; Larkum and Barrett, 1983). However there appear to be exceptions to this rule. In the green macroalga *Chlorodesmis,* which grows over a wide depth range on coral reefs, those plants near the surface, which may be exposed to full sunlight at low tide, are just as green as deeper plants. Here the strategy seems to be to utilize the high light conditions to produce a rapid turn over of the photosynthetic machinery and to replace it at a rapid rate, notwithstanding the damaged by high light conditions. Perhaps the cyclosis of plastids which can occur in these plants allows a rapid replacement of the PS machinery by replacing not only damaged proteins but whole chloroplasts (Franklin and Larkum, 1997).

V. Problems with Photosystem II

It has become clear over the last decade that the gains of oxygenic photosynthesis in utilizing water as a source of electrons are offset to some extent by the conditions under which PS II is forced to operate in order to be able to draw electrons to $P680^+$. The redox potential of $P680/P680^+$ must be in the region of ca. 1.2V and at this potential it is not possible to place a carotenoid close to the special pair, because at this Eh, the carotenoid would be chemically oxidized (Cogdell et al., 2000)—in sharp contrast to all other photosynthetic systems, where, in both light harvesting and reaction center complexes, a carotenoid lies close to the active Chl(s). The reason for the more common situation (of a close proximity of a carotenoid) is that Chl (and BChl) in an excited state has the potential to a form triplet state and this triplet state can react with oxygen molecules in the vicinity to form singlet oxygen:

$$^3\text{Chl}^* + O_2 \rightarrow \text{Chl} + {}^1O_2^*$$

Singlet oxygen is highly chemically reactive and damage can arise quickly. However the so-called **'triplet valve'** usually occurs whereby the triplet state of Chl (BChl), when it arises, is passed on to the adjacent carotenoid:

$$^3\text{Chl}^* + \text{car} \rightarrow \text{Chl} + {}^3\text{car}^*$$

The ^{3}car* then decays by various mechanisms releasing heat (Frank and Cogdell, 1996).

In PS II, although carotenoids are present they are more distant from the special pair (Zouni et al., 2001) in order to prevent oxidation. Under these conditions there is much greater production of singlet oxygen and a higher level of damage. The occurrence of singlet oxygen in PS II preparations has been demonstrated (Hideg et al., 1994; Telfer et al., 1999) and in leaves under photoinhibitory conditions (Hideg et al., 1998). The damage that occurs is probably general but in particular it is known that there is a high level of D1 turnover (Barbato et al., 1999; Andersson and Aro, 2001), presumably as a result of this damage. It is known that there is an elaborate system of removing damaged D1, hydrolyzing it and replacing it with new D1 (Andersson and Aro, 2001; Silva et al., 2002): a process which takes several hours (Andersson and Aro, 2001). In Cyanobacteria

the situation is even more complex with several forms of D1 (Campbell et al., 1996).

It should be noted that singlet oxygen production mechanism proposed for damage to D1. Anderson et al(1998) have argued that, since damage to PS II is apparently a single process, the damage itself is brought about by the concentration of $P680^+$.

The damage process described above is often referred to as **photoinhibition**, or chronic photoinhibition (Chapter 16, Franklin et al.). Since photoinhibition is a term that has been used to describe not only the damage process but down-regulation as well (next paragraph), the two processes have therefore been described as 'chronic photoinhibition' and 'dynamic photoinhibition,' respectively, but these terms are no longer recommended, see Chapter 16 (Franklin et al.).

VI. Off-Loading Excess Light Energy: Xanthophyll Cycle and Reaction Center Sinks

A. General Principles

PS II is at risk of damage under any circumstances in the light because photoinhibition is a dose-dependent response (Park et al., 1995) but plants and algae are particularly susceptible at high light intensities. It is for this reason that plants have evolved mechanisms for dissipating as much as possible of the excess light energy that is absorbed by the LHCs during the middle part of the day (**down-regulation of photosynthesis**). The most thoroughly investigated process of down-regulation is the xanthophyll cycle, which is present in higher plants and all eukaryotic algae, except, perhaps, rhodophytes, glaucocystophytes and cryptophytes (Table 3). The operation of the xanthophylls in down-regulation in Cyanobacteria is less understood (and a conventional xanthophyll cycle as described below does not occur in these organisms). A second process of down-regulation may involve the core of PS II itself. It is quite possible that excitation can be dissipated as heat within RCII and the inner antennae. However much less is known of such a process (Larkum and Howe, 1997). Gilmore et al., 2000 have demonstrated that most of the energy dissipated by down regulation is through the xanthophyll cycle. However some energy dissipation is proposed to take place through light-inactivated PS II centers (Anderson and Aro, 1994).

B. The Xanthophyll Cycle

The Xanthophyll Cycle in its generally recognized form occurs in most eukaryotic algae and in higher plants. The xanthophylls involved are violaxanthin, antheraxanthin and zeaxanthin or diatoxanthin and diadinoxanthin (Table 3). While much work has been carried out on higher plants and green algae (Demmig-Adams and Adams, 1993) much less work has been carried out on algae other than some green algae such as *Chlamydomonas* (Dwivedi et al., 1995; Baroli and Melis, 1996). However, there is good evidence to believe that a similar cycle exists in many chromophyte algae (Arsalene et al., 1994; Olaizola et al., 1994; Lichtlé et al., 1995; Goss et al., 1998; Brown et al., 1999; Lohr and Wilhelm, 1999).

In the Xanthophyll Cycle in the light violaxanthin or diatoxanthin are converted by de-epoxidation to zeaxanthin or diadinoxanthin (Fig. 3). The details of this de-epoxidation and subsequent epoxidation in the dark have been documented in recent times by Yamamoto (1979); Mohanty et al. (1995); Demmig-Adams and Adams (1993). Thus one molecule of oxygen is liberated (de-epoxidation) or taken up (epoxidation) for a complete transition. In algae these changes were worked out in detail by Stransky and Hager (1970) and their general conclusions, shown in Table 3, were as follows: Group 1 (Rhodophyceae, Cryptophyceae, Cyanobacteria and, presumably, Glaucystophyceae), no epoxide cycle takes place although changes in levels of zeaxanthin occur; Group 2 (Bacillariophyceae, Chrysophyceae,

Table 3. The three groupings of algal phyla according to the xanthophylls present and the operation of the xanthophyll cycle. Group 1 does not show reversible epoxidation reaction and it is thought that thermal dissipation occurs by RC down-regulation (see text). Groups 2 and 3 have the xanthophyll cycle. (Based on Stransky and Hager, 1970.)

Group 1	Group 2	Group 3
zeaxanthin	zeaxanthin/ violaxanthin	diadinoxanthin/ diatoxanthin
Cyanophyceae	Phaeophyceae	Diatoms
Rhodophyceae	Chlorophyceae	Chrysophyceae
Cryptophyceae (?)	Chrysophyceae	Xanthophyceae
Glaucocystophyceae	Xanthophyceae	Chloromonads
	Mosses	Dinoflagellates
	Ferns	Euglenophyceae
	Gymnosperms	
	Angiosperms	

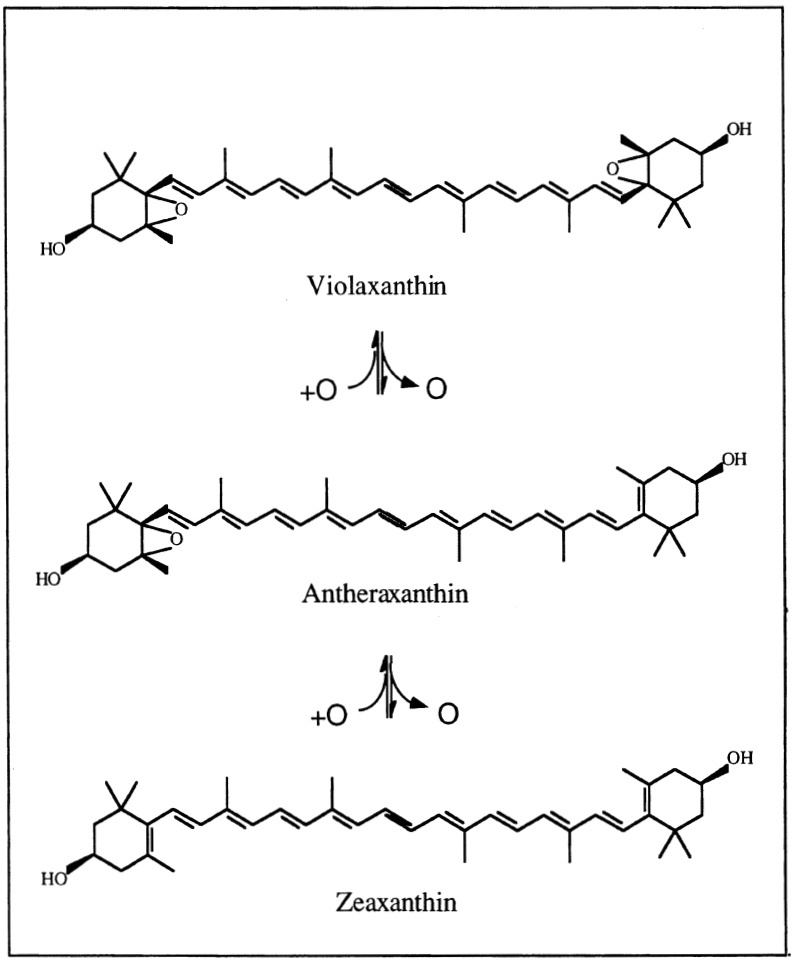

Fig. 3. The key xanthophylls which take part in the xanthophyll cycle of green algae and land plants. In chromophyte algae these xanthophylls are replaced by diatoxanthin and diadinoxanthin which take part in a similar cycle (see Table 3). The downward pathway (de-epoxidation) occurs in light: the upward pathway (epoxidation) occurs in darkness.

Xanthophyceae, Chloromonadophyceae, Dinophyceae and Euglenophyceae), diadinoxanthin is the oxygenated carotenoid and diatoxanthin is the de-epoxidated carotenoid; Group 3 (Phaeophyceae and Chlorophyceae and odd species of some other Classes), the conventional Xanthophyll Cycle is present. In micromonad algae only a part of the conventional Xanthophyll Cycle is present—that converting violaxanthin to antheraxanthin (Goss et al., 1998); furthermore, Lohr and Wilhelm, 1999 have shown that some algae displaying the diadinoxanthin type of Xanthophyll Cycle also display features of the violaxanthin-based cycle.

In general as light levels increase so the level of violaxanthin/diadinoxanthin decreases, reaching a steady level, and, conversely, the level of zeaxanthin/diatoxanthin increases to an asymptote (Yamamoto, 1979; Demmig-Adams and Adams, 1993; Brown et al., 1999)).

The function and operation of the Xanthophyll Cycle are still matters for debate but evidence is accumulating to indicate strongly that zeaxanthin (and diatoxanthin) stimulate a non-photochemical quenching of energy in the PS II/LHCII assemblage. A number of workers earlier implicated LHCII in this process (Jennings et al., 1996; Horton et al., 1996). However more recently a specific role for the chlorophyll-binding, 22 kDa protein, psbS, has been

shown (Li et al., 2000). This protein may lie in an intermediate position between LHCII and the inner antennae of RCII (Nield et al., 2000). The evidence suggests that energy-dependent quenching, qE (which is defined as that component of the total non-photochemical quenching, qN, directly attributable to the energization of the thylakoid membrane and therefore the rapidly entrained component of qN) occurs when i) there is a ΔpH across the thylakoid membrane and ii) zeaxanthin (or diadinoxanthin) is at a high concentration (and violaxanthin/diatoxanthin at a low concentration) as a result of de-epoxidation of violaxanthin (diatoxanthin).

A number of specific details are known concerning the reactions involved in quenching by zeaxanthin. For instance, dibucaine stimulates the quenching and antimycin A, dithiothreitol (DTT) and the protein carboxyl-modifying agent dicyclohexylcarbodiimide (DCCD) inhibit the quenching. Horton and Ruban (1994) suggested that there is a pocket extending from the intrathylakoid lumen into the membrane by which low pH in the thylakoid lumen can influence a critical site in the thylakoid membrane. Since psbS is essential for qE to occur it may be the protein which senses the low pH and the binds zeaxanthin or it may play a crucial structural role in energy transfer/dissipation (Li et al., 2000). The mechanistic details of energy quenching have yet to be fully worked out. Clearly if the mechanism is to work zeaxanthin has to be able to change its molecular excitation states, which would then allow it to dissipate excitation energy as heat when triggered by low pH. Recent work in Frank's group (Frank et al., 2000) and others (Chapter 15, Mimuro and Akimoto) suggest that the S1 state of carotenoids is important for this kind of down-regulation. In an interesting speculation, Crofts and Yerkes (1994) suggested that under conditions of energy dissipation, Chl is displaced from glutamine binding sites; it is possible that under these conditions certain Chl *a* molecules are brought into closer contact with zeaxanthin, leading to a 'triplet valve' situation of energy dissipation.

The situation in algae is more complex than in higher plants. First, chlororespiration may make up a larger component of electron flow than in higher plant plastids (Chapter 8, Beardall et al.) and chlororespiration may induce a pH gradient even in the dark (e.g. in *Euglena*; Doege et al., 2000). Secondly, as shown by Doege et al. (2000), qE seems to be independent of the xanthophyll cycle in *Euglena*. Thirdly, Cyanobacteria, admittedly oxygenic photosynthetic bacteria and not algae, but nevertheless with similar photosynthetic mechanisms, sharing a common origin, do not have the conventional xanthophyll cycle yet carry out down-regulation of photosynthesis (Schreiber et al., 1995)

C. Down Regulation of RCII

A number of workers (Falkowski et al., 1988; Ting and Owens, 1994; Olaizola et al., 1994) have argued for some time that in addition to energy dissipation via the Xanthophyll Cycle there is another system involving the core of PS II by which excess energy is dissipated as heat (and resulting in down regulation of PS II activity). The work of Franklin and Badger (2000) and Longstaff et al. (2002) also supports such a conclusion for algae at high light intensities. Some resolution of the conflicting data has emerged recently. Mohanty et al. (1995) presented evidence, based on fluorescence characteristics, for two processes of energy dissipation in intact spinach chloroplasts, a classical xanthophyll-type process and a reaction center related process. The RC process was i) independent of F_0 quenching, ii) associated with acidification of the thylakoid lumen and inhibited by dibucaine, iii) unrelated to de-epoxidation of violaxanthin, iv) strongly inhibited by ascorbate (indicative of donor side inhibition). On the other hand Gilmore et al. (2000) present convincing evidence that the Xanthophyll Cycle accounts for the predominant energy dissipation as heat in higher plants.

Jennings et al. (1996) have presented an analysis of exciton movement in PS II in which they show that energy transfer is via a shallow energy funnel and that trapping by P680 is very slow (ca. 300 ps vs. 50–100 ps for PS I) allowing excitons to revisit each center 3–7 times between trapping. As a result of this, and the large antenna size, thermal dissipation can be seen as taking place both in the antenna and in RCII, by down regulation of the reaction center. Evidence also suggests that the state of Q_A reduction may control the activity of the antenna-based non-photochemical quenching (Jennings et al., 1996).

It is therefore possible that PS II has evolved to take advantage of the inherent difficulties of splitting water (as discussed in Section V) to incorporate two non-photochemical quenching mechanisms, i) the Xanthophyll Cycle in the peripheral antenna and ii) a down-regulation mechanism in RCII, possibly involving β-carotene or cyclic electron transport. In Cyanobacteria and red algae (and possibly Crypto-

phyceae) only the reaction center process is apparently present and it possibly for this reason that Cyanobacteria (and possibly red algae) have evolved a system with at least two forms of D1 and a related quenching system to allow for sensitive down-regulation (photoinhibition) of PS II (Campbell et al., 1996). Delphin et al. (1995) supported a major role of down-regulation of RCII in red algae without spillover to PS I. Somewhat similar results were also obtained by Rouag and Dominy (1994) for the cyanobacterium *Synechococcus* 6301, although the role of down-regulation was less clearly identified.

In other algae (chromophytes and chlorophycean green algae) there is a strong Xanthophyll Cycle but there may also be remnants of the RCII down-regulation system. While in streptophyte green algae (Chapter 2, Larkum and Vesk) we possibly see the full development of the Xanthophyll Cycle and loss of the RCII down-regulation system—with the possible exception of energy dissipation by damaged, non-functional PS II units (Andersson and Aro, 1994). If this is true then we can see that these developments go hand in hand with the evolution of CAB/CAC proteins in chromophyte and green algae (Chapter 4, Durnford) and the evolution of grana in streptophyte green algae (Chapter 2, Larkum and Vesk)

D. Other Mechanisms of Energy Quenching

The Xanthophyll Cycle involves only five carotenoids out of many 100s of carotenoids found in the 12 or so phyla of algae and land plants. Many of these carotenoids occur in thylakoid membranes (Chapter 15, Mimuro and Akimoto). Many perform a light-harvesting role but the function of others is not well known. There is much interest at the present time in the possibility that there are other energy quenching mechanisms involving carotenoids (Niyogi, 1999). Future studies with carotenoid mutants should resolve some of the issues involved. The role of the water-water cycle in photoprotection in algae is dealt with in Chapter 9 (Miyake and Asada).

VII. Control of Light Harvesting

A. Phylogenetic Adaptation

This term refers to control of light-harvesting by the inherent genetic characteristics of algae: genes, which are constitutively expressed and form the typical state of the mature plant (Larkum and Barrett, 1983). Indeed it was the photosynthetic coloration of algae that led in large part to the origins of current systematics of the algae at the phylum level. Thus, the red algae are characterized by the presence of only Chl *a* (with the possible but now unlikely exception of Chl *d*, above) and the presence of a phycobilisome LH system, which is similar to but differs significantly from that in Cyanobacteria; the green algae are characterized by the presence of Chl *a* and Chl *b* and a suite of characteristic carotenoids; chromophytes are characterized by the presence of Chl *a* and Chl *c* and the presence of one or more diagnostic carotenoids in many phyla.

Ontogenetic adaptation also occurs in algae in addition to phylogenetic changes. These are the changes which occur during the life-time of an individual in response to environmental and other factors (disease and parasitism). Perhaps the most important for an alga is a high or low light climate (see below).

In addition to the obvious phylogenetic and ontogenetic changes outlined above are a suite of responses to rapid changes in environmental conditions such a rapid changes in light climate. Algae, like tracheophytes, have mechanisms to help adjust quickly to such changes. These may be regarded as phylogenetic adaptive mechanisms (e.g. short-term control of light-harvesting), since, because of the speed of response, they result from machinery which must be 'in place' at the time i.e. a constitutive expression of proteins of an alga, rather than the result of regulation of proteins at the level of gene transcription and translation.

There were early attempts to correlate the present ecological niche of algal phyla with their depth or some other light-related factor. However these attempts have generally not been successful and thus the 19th Century theory of '**phylogenetic complementary chromatic adaptation**' is not widely upheld today (Larkum and Barrett, 1983). Nevertheless there still exists a need for an explanation for the current phylogenetic diversity. One fruitful approach is to consider the origin and the selection pressures, which produced the different light-harvesting systems in the first place. This is a speculative endeavor which is not helped by the lack of current information on the origin of plastids (Chapter 2, Larkum and Vesk)! If the plastids evolved monophyletically then the selection pressures giving rise to the present genera operated in the rather recent history of the Earth,

since say 1 BYA. If they evolved by polyphyletic means then the origin of many of the systems was at the stage of Cyanobacteria (*sensu lato*) and an origin of the pigment systems may go back to near the origin of Cyanobacteria. Larkum (1991) suggested that selection pressure gave rise to pigment systems in the following order: first Chl *b*, then Chl *c* (after a very early stage in which Mg-2, 4 -divinyl pheoporphyrin methyl ester was replaced by Chl *a*) and finally the PBS system. If this were true then the Cyanobacteria (sensu stricto) would be a relatively recent group—and would be the result of selection pressure to develop a light harvesting system that could use green light, in a world where red and blue light were absorbed largely by organisms with Chl *a*+*b* or Chl *a*+*c* and their associated carotenoids.

B. Ontogenetic Adaptation

Classically the ontogenetic response is the control of expression of light-harvesting genes induced by environmental cues. It was exemplified best by the complementary chromatic adaptation shown by many but not all Cyanobacteria (*sensu stricto*) to green light (expression of phycoerythrins) and to orange/red light (expression of phycocyanins).

C. Ontogenetic Complementary Chromatic Adaptation in Cyanobacteria

The subject of chromatic adaptation has been elucidated at exquisite molecular detail (Tandeau de Marsac and Houmard, 1993). Clearly light-climate conditions, which trigger this adaptive response, have been common in many aquatic environments. Such changes in light quality can be brought about by changes in concentration of 'yellow substance' (Chapter 16, Franklin et al.), a common occurrence in freshwater and in shore environments, and by algal blooms in the overlying water column. In red algae such ontogenetic complementary changes do not occur (but see Abe et al., 1994, and Chapter 14, Toole and Allnutt). It has therefore been asked what triggers the increases that occur in LH pigments in red algae with increase in depth or shading (in caves and overhangs)? Both light quality and light quantity have been suggested in the past. Talarico and Maranzana (2000) have recently reviewed this subject and conclude that both factors are important.

D. Responses of LHC to Changes in Light Quantity and Quality

Increases in LHC in response to decreased light and to changes in light quality at depth have been well-documented (Larkum and Barrett, 1983). Such changes generally accompany increases in the levels of Chl *a*, carotenoids, number of thylakoids per chloroplast and number of chloroplasts, except where there is only one as in *Chlamydomonas*. The response is similar for CAB and CAC light-harvesting systems. An important question is whether the response is triggered by light quantity or light quality. It is generally contended that the response is to light quantity changes (Larkum and Barrett, 1983; Falkowski and Raven 1997) and the response to light quality has been little explored. A number of different LHCs may be induced under varying conditions (Garczarek et al., 2000, Nishigaki et al., 2000). The light intensity effect is widely regarded as being triggered by the redox state of plastoquinone influencing protein phosphorylation in higher plants (Allen, 1992). There are indications that this process also applies in algae (Durnford and Falkowski, 1997; Chapter 4, Durnford) although the evidence is minimal at present. Evidence from the green alga *Dunaliella*, indicates that light intensity controls the level of LHCII and that it does this through a mechanism controlled by the redox state of the plastoquinone pool (Escoubas et al., 1996; Durnford and Falkowski, 1997). A similar system may also exist in dinoflagellates (ten Lohuis and Miller, 1998).

Two particular instances concerning light-harvesting chlorophyll protein changes in algae should be noted. Both are responses to low light and a change in spectral quality from a broad spectrum to a band centered on blue-green light at increasing depth in the oceans. Ohki and Honjho (1997) showed that a number of CAB proteins were induced in deep-water chlorophytes. These were members of the picophytoplankton collected between 75 and 150 m; some were flagellated and all contained a single chloroplast which filled a large part of the cell. LHCs, with a single polypeptide between 22 and 27 kDa, were isolated from three clones and all were shown to have more Chl *b* than Chl *a*, and none cross-reacted with antibody to LHCII from higher plants. A similar situation occurs in the prochlorophyte *Prochlorococcus*. Two ecotypes of the prokaryote *Prochlorococcus* adapted to distinct light niches in the ocean have been described: one a high-light form

and the other an extreme shade form (Ting et al., 2002). These ecotypes are characterized by their different divinyl-Chl *a* to Chl *b* ratios and 16S rRNA gene signatures, as well as by their significantly distinct irradiance optima for growth and photosynthesis. Garczarek et al. (2000) show that the low-light-adapted *Prochlorococcus* strain SS120 possesses a gene family of seven transcribed genes encoding different Chl *a/b*-binding proteins (pcb's). In contrast, *Prochlorococcus* sp. MED4, a high-light-adapted ecotype, possessed a single pcb gene. Thus, the multiplication of pcb genes appears as a key factor in the capacity of deep *Prochlorococcus* populations to survive at extremely low photon fluxes (Ting et al., 2002). It will be interesting to see how these changes in *Prochlorococcus* are related to the recently discovered ring of pcb proteins around PS I (Bibby et al., 2001b)

E. Thylakoid Appression and Lateral Heterogeneity

Thylakoid appression is defined as a very close proximity of thylakoid membranes, much closer than would be expected generally in the cell (Larkum and Barrett, 1983). The typical number of appressed thylakoids varies from two in Cryptophyta, three in many chromophytes (and euglenophytes) to many in green algae and to a typical granal structure in land plants (Larkum and Barrett, 1983; Staehelin, 1986). A scheme to illustrate lateral heterogeneity between granal (inner) thylakoids and stromal thylakoids is shown in Fig. 4.

It is not known for certain whether thylakoid appression is accompanied by lateral heterogeneity in any alga, although this seems certain for streptophytes (Chlorophyta)(Gunning and Schwartz, 1999; Chapter 2, Larkum and Vesk). For chromophyte algae earlier evidence from freeze fracture particle distribution (Dwarte and Vesk, 1982) suggested that lateral heterogeneity occurred. There is also recent support from the xanthophyte *Pleurochloris*, but one recent investigation on the diatom *Phaeodactylum* (Pyszniak and Gibbs, 1992) is less supportive. Two investigations involving cryptophytes, where the nature of the appression between the thylakoids (in pairs) is still unclear, indicated a lack of lateral heterogeneity (Lichtlé et al., 1992; Vesk et al., 1992). Lack of lateral heterogeneity has also been reported in the micromonadophyte *Mantoniella* (Hecks et al., 1996). Interestingly, some form of microheterogeneity has been suggested in prochlorophytes (Chapter 3, Partensky and Garczarek).

A number of hypotheses were put forward by Larkum and Barrett (1983) to explain thylakoid appression, i.e.. the close proximity of thylakoid membranes in most eukaryotic algae with the exception of red algae. The theoretical advantages of lateral heterogeneity—cooperativity of PS II units and control of spillover, put forward by Andersson

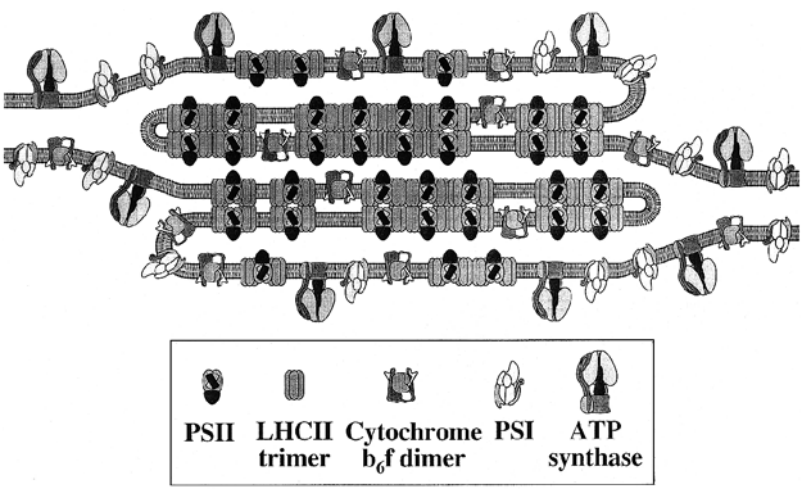

Fig. 4. Artist's impression of lateral heterogeneity between granal (inner) thylakoids and stromal thylakoids. Redrawn with permission from Forsberg and Allen (2001a).

Table 4. Features of PS I/PS II separation and energy distribution in various thylakoids (based on Trissl and Wilhelm, 1993).

Alga	Excitonic separation	Efficiency of energy distribution	Degree of PS separation	F_m/F_o	PS II/PS I
Higher plants	high	State 1-2	High	~5	1.2–1.6
Chlorella	high	State 1-2	High	4–5	1.3–1.5
*Pleurococcus**	low	(State 1-2)*	Medium	2.5–4	1.8–2.9
Mantoniella	low	Absent	Low	1.5–4	2.5–3.9
Cryptophyta		?	Low	~4	
Cyanobacteria	?	State 1-2	Low	~5	0.5–0.9
Prochlorophytes	low	?	Low		1.5

**Pleurococcus* showed somewhat anomalous results which correlate with evidence for lateral heterogeneity.

and Anderson (1980) are not the only possible advantages of thylakoid appression. Other advantages may be,

(i) more efficient packing of light-harvesting complexes.

(ii) more efficient maintenance of the light-driven proton pump at low irradiance.

(iii) increased light scattering with enhanced absorption of green light.

(iv) more efficient harvesting of light enriched in green-yellow light deep inside chloroplast stacks by light-harvesting complexes, which absorb such light more efficiently and are preferentially located in appressed regions.

The major disadvantage of the appressed thylakoid system as it exists in algae today appears to be the lack of a suitable light-harvesting complex that is both located in the membrane and harvests light efficiently throughout the 500–630 nm region.

A recent and interesting proposal concerning stacking was put forward by Trissl and Wilhelm (1993)(Table 4) based on the proposition that the two photosystems process photons at very different rates (PS I, fast and PS II, slow)—see also Jennings et al. (1996) and Chapter 12, Trissl. The result of such a difference in efficiency, in a homogeneous system with an equal ratio of PS I and PS II, would be a process in which photons would be funneled into PS I with consequent loss of overall efficiency. In homogeneous systems (Cyanobacteria and most eukaryote algae) this is counteracted by unequal PS I:PS II ratios. In some green algae (streptophycean green algae) with many appressed thylakoids and land plants with grana (Chapter 2, Lakum and Vesk), this is counteracted by the adaptation of lateral heterogeneity with the photosystems separated and the ratio of PS II/PS I able to rise well above one. Furthermore LHC is differentiated into LHCI and LHCII and controlled so that the optical cross-sections of the photosystems can be controlled more closely. Short-term control is afforded by the mechanism of **state transitions** (see below) by which it is possible to rearrange the optical cross-sections of PS I and PS II to some degree.

F. Control of Energy Supply to PS I and PS II: Absorption Cross-Section and State Transitions

1. Absorption Cross-Section

As discussed above, for maximum efficiency of non-cyclic electron transport the activity of PS I and PS II must be equal. One way of effecting this, when the flux of electrons for PS I and PS II is not equal, is to reduce the excitation energy of one PS and to increase it to the other, i.e. to reduce the absorption cross-section of one PS and possibly increase the cross-section of the other. Initially it was supposed that there was a mechanism (‘**spillover**’) which simply diverted energy from one photosystem to the other—predominantly from PS II to PS I (see e.g. Larkum and Barrett, 1983). However spillover is now generally discounted. The mechanism is now generally proposed in terms of mobile light-harvesting units which change the optical cross-section of one or both photosystems (Forsberg and Allen, 2001a).

A general mechanism for changes in absorption cross-section in higher plants has been available for over 20 years (Allen et al., 1981; Allen 1992). As summarized in Fig. 5 the mechanism is thought, in

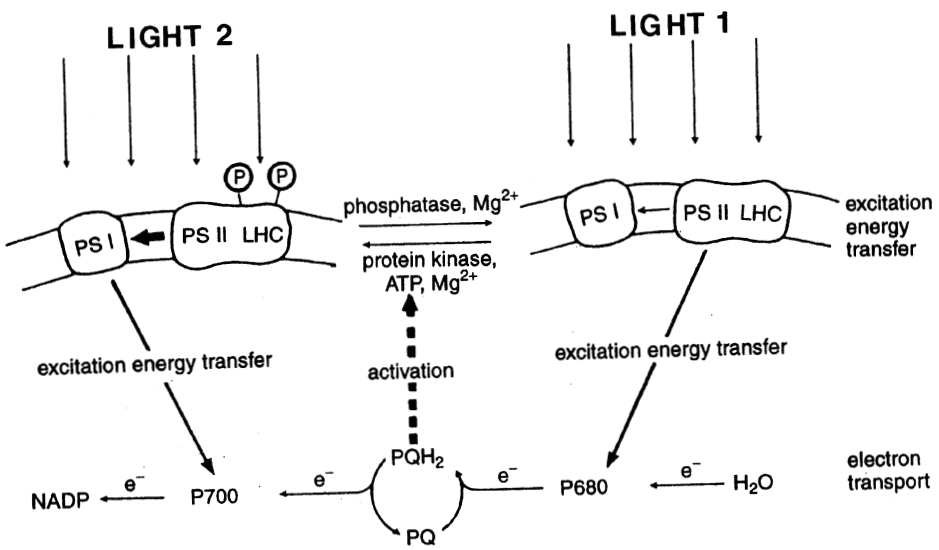

Fig. 5. Scheme for control of energy sharing between the two photosystems based on Allen et al., 1981, with permission. Light 1 is preferentially absorbed by Photosystem I and induces State I in which there is an excess of light available to and absorbed by PS I and a decrease in the amount of excitation energy distributed from the light-harvesting pigments to PS I. Light 2 which is preferentially absorbed by Photosystem II induces State II in which there is an excess of light available to and absorbed by PS II and an increase in the amount of excitation energy distributed from the light-harvesting pigments and PS II to PS I.

higher plants, to be as follows. Preferential illumination of PS II (**Light 2**) leads to reduction of the plastoquinone (PQ) pool, between the two PSs. Under these conditions, and through the mediation of the Q_o site of the Cyt b_6f complex, at least one, and possibly more than one, membrane-bound protein kinase becomes activated leading to the phosphorylation of mobile LHCII and other polypeptides. Phosphorylated LHCII then moves away from the appressed thylakoid regions, to unappressed thylakoids on the outside of grana or in the stroma, where it associates with PS I (Fig. 6). The membrane bound kinase is deactivated in the dark or in **Light 1** (light which preferentially activates PS I and when PQ is oxidized) and a latent phosphatase continually reverses the action of the kinase (Allen, 1992). Evidence for a similar mechanism in the green algae *Dunaliella* has been presented by Escoubas et al., 1996 and there is some evidence that it may also exist in dinoflagellates (ten Lohuis and Miller, 1996).

While the general principles of this proposed mechanism have been supported, in the interval there has been much progress in many areas—both in higher plant and in algal studies. In higher plants it has been shown that here is a specific LHCI, which acts to harvest light specifically for PS I (Green and Durnford, 1996). Thus changes in cross-sectional area of PS I and PS II due to re-association of mobile LHCII can only contribute a small fraction of change in cross-sectional areas (usually < 20%). In higher plants too it has been shown that the PS I subunit H polypeptide is essential for docking of phosphorylated LHCII and in mutants lacking the H subunit phosphorylated LHCII stays associated with PS II—with no changes in cross-sectional area (Lunde et al., 2000; Haldrup et al., 2001). Furthermore it has been shown that phosphorylation of LHCII is not linearly dependent on the reduction of PQ (Haldrup et al., 2001): the degree of phosphorylation reaches a peak at rather low light intensities (of light which preferentially activates PS II). At high light intensities there is a decrease in phosphorylation. From these results Haldrup et al. (2001) argue for a mechanism that involves other control systems, as well as that of phosphorylation.

Another significant finding has been that of a PQ-redox controlled transcription of the genes for the photosystems (Pflannschmidt et al., 1999). Shade algae show large increases in chlorophyll content (and other light harvesting pigments), in comparison with algae in well-lit habitats (Larkum and Barrettt, 1983; Section VII.D). A large part of this increase is

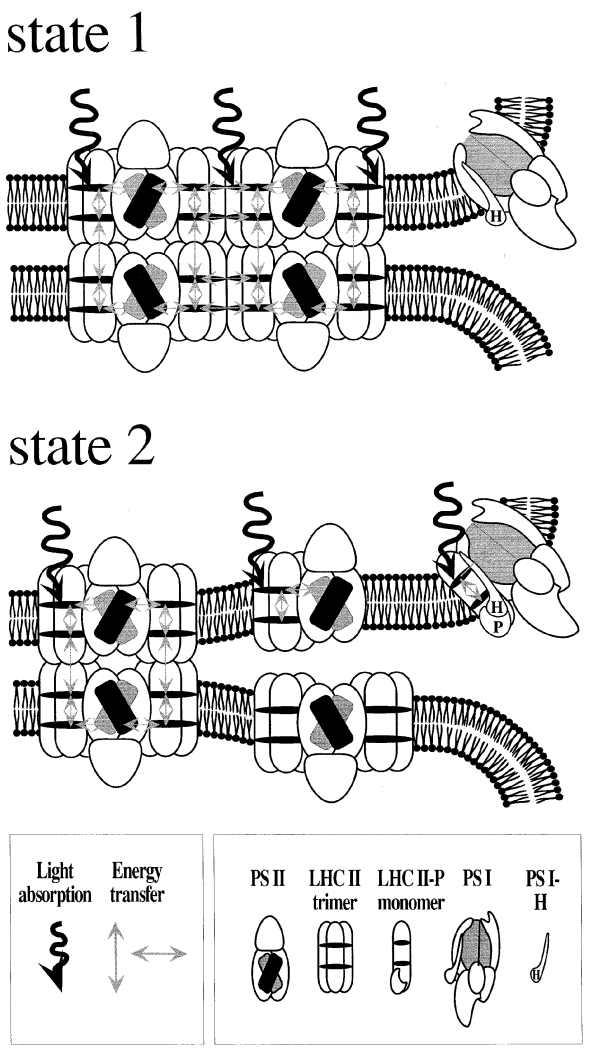

Fig 6. Artist's impression of the difference between State 1 and State 2 organization in appressed and non-appressed thylakoids in green plants. Redrawn with permission from Forsberg and Allen (2001a).

due to increases in optical cross-sectional area of PS I and PSII (photosynthetic unit size of PS I and PS II). The PQ-redox control of transcription may be responsible for much of this increase (Chapter 17, Raven and Geider). Allen and Raven (1996) have argued that the plastid redox system is the main cause that all genes from the plastid DNA have not moved to the nucleus. However it known that LHC is also under redox control (Maxwell et al., 1995; Pursiheimo et al., 2001) and LHC genes are nuclear-encoded and thus there must be feedback systems to the nucleus as well as to the plastid DNA.

In algae (other than streptophyte green algae) the distribution of PS I and PS II appears to be much more homogeneous (Chapter 2, Larkum and Vesk). Here there is less evidence for phosphorylation of LHCs driven by Light 1 and Light 2 (and the involvement of a PQ-driven mechanism). Gibbs and Biggins (1989, 1991) argued against such a phosphorylation mechanism in the Chl *c*-containing chrysophyte alga, *Ochromonas*. Allen (1992) gives a review of this early work on eukaryotic algae, which apart from some work on *Chlamydomonas* (Fleischmann et al., 1999; Kruse et al., 1999) and red algae

(Delphin et al., 1995) has not received much attention recently.

In Cyanobacteria and in red algal and glaucocystophyte plastids, the main light-harvesting system is the phycobilisome (PBS)(Chapter 14, Toole and Allnutt) and there has been much debate over whether the attachment of PBS to PS II or PS I is driven by the redox state of PQ involving phosphorylated proteins (for a detailed review see Allen, 1992). Certainly there is good evidence that the absorption cross-sections of PS I and PS II change in response to Light 1 and Light 2. Many proteins are also phosphorylated in the light. However there has been much debate as to whether the phosphorylation responds in a predictable way to Light 1 and Light 2 and to redox changes in PQ. Allen (1992) argued that despite the differing results a case could be made for a redox-driven phosphorylation of one or more proteins. The most promising protein was a 18.6 kDa protein (in *Synechococcus* 63101) where tyrosine was phosphorylated in Light 2 but not in Light 1. This protein is probably β-phycocyanin. Other proteins of 13 and 15 kDA were also found to be phosphorylated under reducing conditions or in DCMU-sensitive reactions. Thus there could be a channeling of energy from an immobile set of PBS (possibly attached to PS II) or there could be a movement of PBS between PS II and PS I attachment sites. Recent evidence seems to suggest that the PBS physically move between PS II and PS I sites (Sarcina et al., 2001; Mullineaux and Sarcina, 2002).

Almost certainly there are distinct differences in the mechanisms by which short-term accommodation of Light 1 and Light 2 effects changes in the cross-sectional areas of PS I and PS II in Cyanobacteria, algae and land plants. Pursiheimo et al. (1998) proposed three categories: **Group 1**, Cyanobacteria and red algae, which did not show phosphorylation of any of the Photosystem II (PS II) proteins; **Group 2** consisting of a moss, a liverwort and a fern, which phosphorylated both the light-harvesting chlorophyll a/b proteins (LHCII) and the PS II core proteins D2 and CP43, but not the D1 protein, and **Group 3** where reversible phosphorylation of the D1 protein of PS II was found only in seed plants and was seen as the most recent evolutionary event in the series. In terms of phosphorylation of LHCII they found that Groups 2 and 3 were similar with maximal phosphorylation of LHCII at low light and nearly complete dephosphorylation at high light. Clearly this survey did not include any algae dependent on CAB/CAC light-harvesting systems. The few studies in this area (see above) suggest that it is an important area for elucidating the evolutionary aspects of short-term control of light-harvesting.

2. State Transitions

Evidence for state transitions first came from the work of Murata (1969, 1970) who showed in red algae and in spinach chloroplasts that the amount of variable fluorescence (assigned to PS II) was affected by the previous conditions of illumination. Bonaventura and Myers (1969) first defined the phenomenon, albeit in a spillover model, which may be restated, as follows: **State I** in which there is an excess of light available to and absorbed by PS I (light I) and a decrease in the amount of excitation energy distributed from the light-harvesting pigments to PS I; and **State II** in which there is an excess of light available to and absorbed by PS II (light II) and an increase in the amount of excitation energy distributed from the light-harvesting pigments and PS II to PS I (Fork and Satoh, 1986). In darkness a State I condition is usually found.

Satoh and Fork (1983) proposed a putative third state (State III) from evidence using the red alga *Porphyra perforata*. Illumination of this alga either in State I or State II with light II produced State III in which light energy reaching PS II was decreased with no attendant increase in the energy supply to PS I. Although there was no change in the distribution of energy between the two photosystems there was a decrease in the overall amount of excitation energy migrating to RCII from the light-harvesting pigments. This phenomenon is probably a photoinhibitory response whereby some phycobilisomes are decoupled under high light to protect the RCs from overactivity.

The first physical model of the fluorescence changes in State Transitions came from Butler and coworkers (Butler, 1978) again in terms of a spillover model. It now seems more likely that the changes are effected in terms of reassociation of light harvesting complexes between the two PSs (rather than rechanneling of energy absorbed by one PS to the other), as discussed above. In higher plants there is strong evidence that this occurs and so one must expect a component of fluorescence change in situations which over-excite one photosystem against the other. However it is now known that down-regulation of PS II occurs, which is brought about,

mainly, by the xanthophyll cycle (see above). Thus a rigid definition of State Transitions in terms of fluorescence changes is fraught with difficulty. The maximal fluorescence is determined in Light 1/State 1 (F'_{m1}) and in Light 2 /State 2 (F'_{m2}) -assuming that these changes do not affect down regulation of PS II. The relative changes in fluorescence (Ft) when Light 1 is switched on in State 2 and is turned off in State 1 is calculated as

$$Ft = [(F'_1 - F_1) - (F'_2 - F_2] / (F'_1 - F_1)$$

where F_1 and F_2 are the new levels of fluorescence yield following the change in light (Haldrup et al., 2001).

State Transitions can then be expressed either as the difference in maximum fluorescence yield in Light 1 and Light 2

$$F_{m1} + F_{m2}$$

or as

$$F_\tau$$

Note that these changes which are short-term do not take account of changes in down regulation of PS II.

State Transitions have been observed in all groups of oxygenic photosynthetic organisms and in most algal groups (see Table 2, Chapter 17). They are largest in Cyanobacteria and red algae and lowest in tracheophytes (Schreiber et al., 1995). The mechanism of State Transitions is likely to be similar to that for changes in optical cross-section of the PSs. However as recent work indicates the two mechanisms may not be identical (Haldrup et al., 2001). State transitions have been a simple way to study short-term changes in energy distribution to PS I and PS II and have been used extensively for this purpose (Haldrup et al., 2001; Kruse, 2001; Wollman, 2001). Recently there have been a number of attempts to identify 'state transition' mutants in various oxygenic photosynthetic organisms (Kruse et al., 1999; Lunde et al., 2000). PsaH of PS I has been identified as a vital component of State Transitions in *Arabidopsis thaliana* and mutants deficient in this polypeptide do not show State Transitions (Lunde et al., 2000). Since psaH is only found in land plants it can only have a role in these plants. The mechanism for the involvement of PS I subunit H has been discussed by Haldrup et al. (2001) and Forsberg and Allen (2001a,b). In the absence of subunit H it appears that mobile LHCII cannot dock with PS I. Under these conditions it also appears that mobile LHCII remains attached to PS II units since the absorption cross sections remain the same. Thus these results support a re-association of mobile LHCII from stacked thylakoid regions to unstacked thylakoids (to outer granal thylakoid membranes or stromal thylakoids)(Fig. 6). However Haldrup et al. (2001) present further evidence on redox levels and degree of phosphorylation which suggest that other factors besides phosphorylation regulates State Transitions and especially that phosphorylation of LHCII cannot explain the dissociation of LHCII from PS II. However as explained by Fosberg and Allen (2001a,b) more than one site of phosphorylation of LHCII may account for differential effects of high light and redox control.

State Transitions have been investigated in a number of other algae since the early work, which was mainly directed to Cyanobacteria, green algae and higher plants: the groups investigated include cryptophytes (Bruce et al., 1986); chrysophytes (Gibbs and Biggins, 1989, 1991); brown algae (Fork et al., 1991); and *Pleurochloris* (Chromophyta) (Büchel and Wilhelm, 1990, 1993). In *Pleurochloris* the State Transitions were wavelength-independent. As mentioned above, the extent of State Transitions is much more pronounced in algae than in plastids of higher plants. Furthermore, apart form the streptophyte algae, it appears that there is little lateral heterogeneity in the thylakoids of algae (Chapter 2, Larkum and Vesk) and there is the possibility of energy transfer between the PSs. Thus further scrutiny of light energy distribution to the PSs in algal plastids is more than justified.

Chlamydomonas, which can be transformed, is clearly a very useful organism for investigating absorption cross-section changes and State Transitions. In this context, the correlation of State Transitions with changes designed to bring about equal activity of the PSs was challenged by Bulté et al. (1990) who suggested that the main function of State Transitions might be to balance the production of ATP and $NADPH_2$. They showed that inhibition of ATP production in intact cells in *Chlamydomonas reinhardtii* led to a transition to State II while an increase of ATP production caused a change to State I. Delosme et al. (1996) showed, in a study using photoacoustics, that in a mutant lacking PS II cores more than 80% of light absorbed by LHCII could be

transferred to PS I. In another mutation State Transitions are blocked, but by a different mechanism than that involved in the PS I subunit H (which does not exist in algae) and such mutants were unable to phosphorylate LHCII (Kruse et al., 1999; Fleischmann et al., 1999).

Delphin et al. (1995) presented evidence to show that in the unicellular red alga *Rhodella violacea,* in contrast to *Chlamydomonas'* State Transitions were not accompanied by phosphorylation of thylakoid proteins. However, Delphin et al. (1996) have shown that state transitions (in the phenomenological sense) occur in *Rhodella* under conditions where the activity of PS I does not change (Rouag and Dominy, 1994). Delphin et al. suggest that ΔpH changes across the thylakoid membrane trigger 'State II' quenching possibly through a down-regulation process of RCII.

These studies indicate the flux of ideas and conflicting results which exist in the algal field at present.

3. Conclusion

In higher plants there seems to be a good case for the existence of small changes in the cross-sectional areas of the PSs, in order to balance the distribution of light energy between the two systems. State Transitions are an important indicator of these distributional changes but they may not signal exactly the same mechanism. In higher plants and streptophyte algae there is lateral heterogeneity of PS I and PS II. In algae other than streptophytes the distribution of PS I and PS II appears to be homogeneous. Here there is the possibility of 'spillover' from one PS to the other. However these algae have received very little attention and so the operation of mechanisms for distribution of light energy between the PSs is still an open question. In Cyanobacteria whose forbears gave rise to the plastids of algae there is clear evidence for mechanisms of control of excitation energy between the PSs and for large State Transitions. In those organisms however the main light harvesting system is the extrinsic phycobilisome, which exists only in the plastids of red algae and glaucocystophytes. All other algae have a CAB/CAC intrinsic light-harvesting system. It is therefore of great importance that future studies concentrate on these algae (and not only in *Chlamydomonas*) to elucidate the mechanisms for energy sharing which surely exist.

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Chapter 14

Red, Cryptomonad and Glaucocystophyte Algal Phycobiliproteins

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Summary

A significant amount of research has been done on phycobiliproteins over the past ten years. A much fuller description of the cryptomonad bilins has expanded the number of known chromophores in phycobiliproteins. A number of new phycobiliproteins have been identified from red algal strains. Comparative genomics has become possible with the sequencing of the vast majority of phycobiliprotein and linker polypeptides in Cyanobacteria and in red, glaucocystophyte and cryptomonad algae. Structural studies have elucidated most of the major phycobiliprotein subunits and now some of the linker polypeptides to more fully understand the energy transfer within phycobiliproteins. While the majority of the research still focuses on cyanobacterial systems, red and cryptomonad algal phycobiliproteins enjoyed a renewed emphasis during this decade. The glaucocystophytes have also been studied, as much for their significance for plastid evolution as for their phycobiliprotein structure and function, which is very much like that in Cyanobacteria. Most of the research on these eukaryotic organisms focused on crystal structure and gene comparisons to cyanobacterial systems, with a liberal amount of new methodology for phycobiliprotein isolation from red algal strains thrown in. The focus has mostly been on comparison of the red and cryptomonad algal systems to what has been demonstrated in cyanobacterial systems when it comes to structure/function relationships. The lack of useful genetic systems in red and cryptomonad algae is a current major inhibitor of direct structure/function studies in the red and cryptomonad algal systems; allowing cyanobacterial research to take the lead so far.

I. Introduction

Phycobiliproteins are protein pigments produced by Cyanobacteria and certain algae (red, cryptomonad and glaucocystophyte algae) which occupy photosynthesis' spectral 'green valley,' largely unoccupied by chlorophyll or carotenoid absorption. The phycobiliprotein spectra span from about 450 to 660 nm in a series of overlapping absorbance/emission peaks, such that they affect an efficient and seamless transfer of energy to Photosystem II (PS II) and Photosystem I (PS I). There are three major phycobiliprotein classes based upon different spectral characteristics, phycoerythrin and phycoerythrocyanin (PE; having the shortest absorbance wavelength with λ_{Max} = 545–575 nm and PEC λ_{Max} = 635–595 nm); phycocyanin (PC, λ_{Max} = 575–645); and allophycocyanin (APC, λ_{Max} = 590–654) (Glazer, 1982). Phycobiliproteins are primarily PS II photosynthetic antennae complexes of Cyanobacteria and red, glaucocystophyte and cryptomonad algae (Grossman et al., 1993). In Cyanobacteria and red algae, phycobiliproteins are found in large, supramolecular complexes called phycobilisomes. These structures reside on the stromal surface of the thylakoid membrane and have molecular weights of 4.5×10^6 to 1.5×10^7 Daltons (Gantt and Lipschultz, 1974; Gantt et al., 1979; Bryant, 1991). Phycobiliproteins are oriented in the phycobilisome for efficient energy transfer from higher to lower energy absorbing chromophores (bilins) until the energy is funneled directly into chlorophylls via two specialized terminal acceptor proteins, allophycocyanin B and the large core membrane protein ApcE (also designated L_{CM}) (Glazer and Bryant, 1975; Mullineaux and Ashby, 1999). As a consequence of phycobilisomes being a major component of the cell's total protein content (often greater than 15% and can be up to 40%), an inducible system regulates its degradation when the cell is under nutrient stress (Grossman et al., 1993; Dolganov and Grossman, 1999; Sauer et al., 1999). The *nbl*B and *nbl*A genes are at least a portion of this system and help to coordinate phycobilisome degradation, where *nbl*A is induced under nutrient limitation and *nbl*B is constitutively expressed, but both are required for phycobilisome degradation (Dolganov and Grossman, 1999).

Cryptomonads are complex evolutionary chimeras that appear to be the product of an eukaryotic organism

Abbreviations: Abs – absorbance; APB – allophycocyanin-B; APC – allophycocyanin (also sometimes abbreviated at AP); C – core; CM – subscript for L saying location of linker as the core/membrane interface; Cr-PC – cryptomonad phycocyanin; Cr-PE – cryptomonad phycoerythrin; CV – cryptoviolin (same as phycobiliviolin); Fluor – Fluorescence; L_{RC}^{MW} – linker polypeptide, superscripted molecular weight, subscript CM, RC, and R for location of liner in phycobilisome; PC – phycocyanin; PCB – phycocyanobilin; PE – phycoerythrin; PEB – phycoerythrobilin; PEC – phycoerythrocyanin; PS I – Photosystem I; PS II – Photosystem II; PUB – phycourobilin; PXB – phycobiliviolin; R – subscript used to indicate location of linker in rod; RC – subscript used to indicate location of linker at the rod/core interface; R-PC – R-phycocyanin

and an engulfed red alga (Glazer and Wedemayer, 1995; Moreira et al., 2000; Chapter 1, Douglas et al.). Cryptomonads contain four different genomes, nuclear, mitochondrial, chloroplastic and a fourth DNA-containing organelle called the nucleomorph (Wastl et al., 1999; Chapter 2, Larkum and Vesk). There is still controversy on the taxonomic classification of these organisms (Clay et al., 1999). The unique cryptomonad phycobiliproteins have been used as phylogenetic tools to sort out this complex group of organisms (Marin et al., 1998; Kugrens et al., 1999; MacColl et al., 1999a).

Cryptomonad algae do not form phycobilisomes (Dodge, 1969; Gantt et al., 1971), but instead form looser rod structures inside the lumen of the thylakoids (Hill and Rowan, 1989). Little information is available on the cryptomonad multimeric structures. In addition, cryptomonads have only a single phycoerythrin or phycocyanin in each organism. Although several early reports had indicated two types of phycobiliproteins could be present, this phenomenon has not been confirmed (MacColl and Guard-Friar, 1987).

Red algal photosynthetic systems hold similarities to both higher plants and Cyanobacteria. Red algae generally lack the chlorophyll *a/b*-binding proteins characterized by higher plants as antennae complexes, instead relying on phycobilisomes of similar structure to those found in Cyanobacteria (Gantt and Lipschultz, 1972; Bryant et al., 1976). Therefore, granal stacks seen in higher plants are missing in red algae. Recent studies on PS I complex, isolated from the unicellular red alga *Porphyridium cruentum* (*P. cruentum*), demonstrated strong structural homology to PS I from higher plants and the presence of a CAB-type protein (Wolfe et al., 1994; Chapter 4, Dunford). In contrast, purified PS II complexes recently obtained from another red alga, *Cyanidium caldarium*, contain cytochrome *c*-550 and the 12 kDa extrinsic polypeptide of PS II, more similar to Cyanobacterial systems (Enami et al., 1995).

To perform light-harvesting activities, the phycobiliproteins of Cyanobacteria, red algae and cryptomonads have linear tetrapyrrole chromophores, called bilins, covalently attached through one or two thioether bond(s) to specific cysteine residues on the protein moiety (Lundell et al., 1981; Redlinger and Gantt, 1981; Wedemayer et al., 1991, 1992). The protein scaffold surrounding the bilins assists in stabilizing them in response to pH, thermal and chemical challenges (Ducret et al., 1994). Because the different bilin molecules are held in rigid, extended conformations by interactions with specific amino acid residues found on the phycobiliprotein backbone, different spectral properties are found even for the same bilin (e.g. phycocyanobilin in APC and C-PC).

In this chapter, we will concentrate on recent advances in red and cryptomonad algal phycobiliproteins. An exhaustive review of the literature up to 1986 was done by MacColl and Guard-Friar (MacColl and Guard-Friar, 1987) and another by Rowan (Rowan, 1989). For more detailed background information please refer to these excellent resources. Recent reviews on the cyanobacterial phycobiliproteins, where a large majority of the recent work has been concentrated, provide exquisite detail on the cyanobacterial phycobiliproteins (Ducret et al., 1994; Glazer, 1999). Therefore, we will focus on contrasting the current status of the red and cryptomonad algal phycobiliproteins with the existing cyanobacterial research in this area. Discussion of recent advances in bilins, energy transfer and other phycobiliprotein-associated phenomenon will include cyanobacterial information that complements the recent red and cryptomonad algal research findings or points out areas requiring more research effort. Phycobiliproteins, bilins and phycobilisomes are finding increasing in vitro utility, with application in a variety of different uses important to the research of many non-photosynthesis researchers (e.g., specific binding assays, drug discovery and cell biology). For this reason, phycobiliprotein in vitro applications will also be briefly covered in this chapter.

II. Structure and Components of Phycobilisomes

Phycobilisomes are the macromolecular structures that are the antennae complexes of Cyanobacteria, glaucocystophytes and red algae. They come in two general morphological types, hemidiscoidal (Bryant et al., 1979; Glazer, 1984) and hemiellipsoidal (Gantt, 1980; Lange et al., 1990). However, other types have also been described: bundle shaped (Guglielmi et al., 1981), cord-like (Dibbayawan et al., 1990) and block-shaped (Gantt and Lipschultz, 1980). The reliance on transmission electron microscopy has led to the aforementioned structural models. Recent advances in scanning probe microscopy and atomic force microscopy are providing additional tools to confirm and refine these structures (Zhang et al., 1999).

Zhang and colleagues' results indicate that some of the previous ideas about hemidiscoidal phycobilisome structure might be incomplete, as they provided a model for a cyanobacterial phycobilisome that was more space filling. The structure of cyanobacterial phycobilisomes has been recently reviewed (Anderson and Toole, 1998; Apt and Grossman, 1993; MacColl, 1998). For this reason, we will focus here on an update of the red algal phycobilisome structure and assembly using the cyanobacterial work as a reference.

Both hemidiscoidal (Morschel et al., 1977) and hemiellipsoidal (Lichtle and Thomas, 1976; Gantt, 1981) phycobilisomes occur in Cyanobacteria and red algae. Both types of phycobilisomes are divided into two domains, the central core and peripheral rods. The more common cyanobacterial phycobilisome is the hemidiscoidal structure as schematically depicted in Fig. 1. The rod-substructures, comprised of phycocyanin, are located proximal to the core and, when present, phycoerythrin is located at the rod periphery. These rod-substructures radiate outward from the core in two dimensions in a structure resembling a fan. The core contains several types of allophycocyanin molecules, including α and β allophycocyanin subunits and specialized allophycocyanin B trimers, as well as specific linker polypeptides. The linker polypeptides are mostly chromophore-free and assist in orienting the components of the antennae complexes properly with regards to the thylakoid membrane and PS II, but they also function in attachment of the phycobilisome to the thylakoid membrane and energy transfer optimization. The hemiellipsoidal phycobilisome structure is classically represented by the work done by Gantt and colleagues with *P. cruentum* (Gantt and Lipschultz, 1972, 1974; Gantt, 1988). The hemiellipsoidal phycobilisome can be envisioned as half of a ball with the three-cylinder core subassembly in the half-ball center and peripheral rods radiating out from the center to form the rounded surface of the half-ball. The APC-rich core and peripheral rods have structures similar to those described for the hemidiscoidal structure.

In 1983, the two terminal acceptors, allophycocyanin B (APC-B) and the anchor or large core membrane linker polypeptide ApcE, were first described in the literature for the cyanobacterium *Synechocystis* PCC6701 (Gingrich et al., 1983). Two copies each of these polypeptides were found per phycobilisome, which function to bridge the spectral

Fig. 1. Schematic representation of a hemidiscoidal phycobilisome of *Synechocystis* sp. PCC6701 (Syn6701). The Syn6701 hemidiscoidal phycobilisome contains six rod structures (rod) attached to the core comprised of allophycocyanin (APC). Two hexamers of phycocyanin (PC) are located proximal to the core and two hexamers of phycoerythrin (PE) are located at the rod periphery.

gap between the chlorophylls of the photosystem and APC. ApcE has a single phycocyanobilin attached at amino acid residue Cys186, in the cyanobacterium *Synechococcus* sp. PCC7002 (Gindt et al., 1992), and is a multifunctional protein (Bryant, 1991). ApcE has three primary functions: (1) to assemble allophycocyanin into the core substructure (Isono and Katoh, 1987); (2) to assist in funneling energy from the shorter emitting phycobiliproteins of the core to PS II (Redlinger and Gantt, 1981, 1982); and (3) to help in attachment of the phycobilisome to the thylakoid membrane (Gantt, 1988). The ApcE chromopeptide has several distinct domains necessary for accomplishing such diverse functions in the phycobilisome. These include the BP domain (phycobiliprotein-like domain), the LOOP domain, two to four 'repeat' or REP domains (comprised of approximately 120 residues each) and two to five domains forming the connections between the BP and REP domains, called ARMS (comprised of approximately 50 residues each). Sequence analysis of the REP domains shows similarity to the conserved domains of the linker polypeptides and they are believed to play a similar role (i.e., providing the binding domains that function in connecting the APC trimers of the core). The amino-terminal PB domains show approximately 35% identity to α and β subunit families of the phycobiliproteins, although one apparent difference is that this domain of ApcE is approximately 65 amino acid residues longer. This

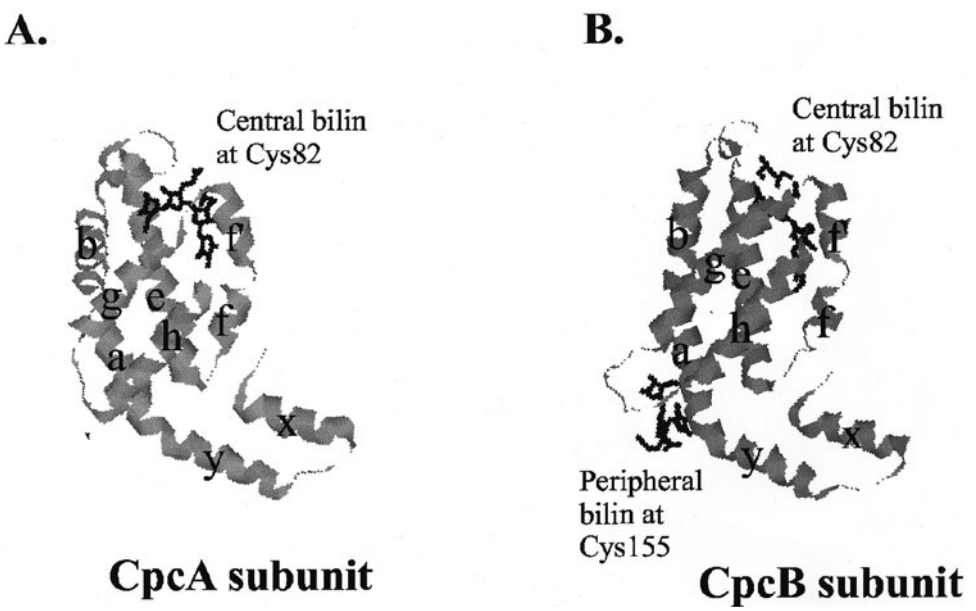

Fig. 2. Structural similarity of CpcA and CpcB subunits. The three-dimensional biliprotein structures for CpcB and CpcA where nine α-helices are depicted as ribbons and the phycocyanobilins are modeled as space-filled. These nine α-helices are labeled x-y-a-b-e-f-f'-g and h. The globular domain is formed by α-helices a through h, and helices x and y run antiparallel to each other and extend outward from the globular portion of each subunit. Phycocyanobilin chromophores are shown as sticks. Structures were generated using Rasmol and the PDB coordinates for phycocyanin from *Fremyella diplosiphon* (Deurring et al., 1991).

extra length corresponds to a small insertion in the loop region located between α-helices B and E of the phycobiliproteins (i.e., the LOOP domain) (see Fig. 2 for the location of the B-E loop domain in a phycobiliprotein subunit). This LOOP domain was proposed to function in attachment of the phycobilisome to the thylakoid membrane (Capuano et al., 1991). However, recently 54 amino acids of the LOOP domain were deleted in *Synechocystis* sp. PCC6714 (replacing the endogenous wild-type ApcE). The resulting transformants formed fully functional phycobilisomes attached to the thylakoid membranes, clearly demonstrating that at least those 54 amino acids were not responsible for phycobilisome attachment to the thylakoid membrane (Ajlani and Vernotte, 1998).

Allophycocyanin B is the other terminal energy acceptor of the core substructures and was first purified as a trimeric complex comprised of $(\alpha^{AP\text{-}B}\alpha_2^{APC}\beta_3^{APC})\cdot L_C^{8.9}$, where the structurally similar $\alpha^{AP\text{-}B}$ subunit replaces an α^{APC} subunit in the trimer complex of the core (Glazer and Bryant, 1975). This subunit ($\alpha^{AP\text{-}B}$) is encoded by the *apc*D gene and is 161 amino acid residues with a molecular weight of 17,800 Daltons. This is similar to other known α and β phycobiliprotein subunits. Only 25–28% identity is found between $\alpha^{AP\text{-}B}$ subunits and β^{APC}, yet a much higher degree of identity exists between the $\alpha^{AP\text{-}B}$ and α^{APC} subunits (52–55% identity). However, one major difference between these two subunits, $\alpha^{AP\text{-}B}$ and α^{APC}, is the presence of two tryptophan residues, Trp59 and Trp87, found in close proximity to the central bilin attachment site, Cys82, in the $\alpha^{AP\text{-}B}$ subunit (Suter et al., 1987; Houmard et al., 1988). In APC α subunits, amino acid Phe59 replaces Trp59, while Tyr residues are found at this position in phycocyanin α subunits. All of these subunits $\alpha^{AP\text{-}B}$, β^{APC}, α^{APC}, α^{PC} and β^{PC} have the same bilin attached (phycocyanobilin). Because of the location of these tryptophan residues in $\alpha^{AP\text{-}B}$ (i.e., close to the central bilin attachment site), it was proposed that they could be responsible for the red-shifted absorbance and fluorescence emission spectra found in allophycocyanin B. The dependence on electron microscopy and the lack of specific genetic systems available in red algae and most phycoerythrin-containing Cyanobacteria has hampered determination of the exact structural or functional contribution of these

residues to the spectral differences found among these phycobiliprotein subunits, $\alpha^{AP\text{-}B}$, β^{APC}, α^{APC}, α^{PC} and β^{PC}.

III. Molecular Biology of Red Algal, Glaucocystophyte and Cryptomonad Phycobiliproteins

The phycobiliproteins of red algae are encoded on the chloroplast genome (Goff and Coleman, 1988; Roell and Morse, 1991; Shivji, 1991; Apt et al., 1995). The conserved nature of the deduced amino acids for both Cyanobacteria and red algal plastid-encoded phycobiliprotein genes (65–85%) led to the theory that all phycobiliproteins arise from a single ancestral gene (Zuber et al., 1987; Wolfe et al., 1994). It is proposed that the red algal genes originated from a cyanobacterial endosymbiont (Gray, 1989; Moreira et al., 2000; Chapter 1, Douglas et al.).

Many Cyanobacteria have multiple copies of the phycobiliprotein α and β subunit genes that are differentially expressed under different environmental conditions and stress (Grossman et al., 1988). However, in the red alga *Aglaothamnion neglectum* (*A. neglectum*), single copies of *apc*AB and *cpc*BA gene sets were found located on the plastid genome with a weakly homologous *cpc*BA set tentatively identified (Apt and Grossman, 1993). Two copies of the *cpc*BA genes were found in *Porphyridium cruentum* (Shivji, 1991).

The genes encoding α and β subunits of a given phycobiliprotein are present as a single operon in both red algae (Apt et al., 1993) and Cyanobacteria (Bryant, 1991). A cryptomonad species, *Cryptomonas* [PHI], was found to have unlinked α and β subunit genes that were independently transcribed (Reith and Douglas, 1990). Another cryptomonad, *Chroomonas* CS24, was found to have a nuclear encoded α subunit gene with no β-subunit gene identified in the 3′ and 5′ flanking regions (Jenkins et al., 1990). In *A. neglectum*, α and β phycobiliprotein subunits are cotranscribed (Apt and Grossman, 1993). The linker polypeptides of red algae (Apt and Grossman, 1993) and Cyanobacteria (Bryant, 1991) are normally found as part of the same operon as the phycobiliproteins. The *apc*E gene in Cyanobacteria can normally be found either cotranscribed with the *apc*AB operon or as a monocistronic transcript (Apt et al., 1993). In the filamentous red *A. neglectum*, only a cotranscribed transcript was present (Apt et al., 1993).

The PC linker polypeptides in Cyanobacteria are usually 3′ to the *apc*AB operon and cotranscribed (Bryant, 1991). In the filamentous red, *A. neglectum*, this does not hold true. The *cpc*G rod core linker (L_{RC}) is actually 5′ to the *cpe*BA operon and transcribed on the opposite DNA strand (Apt and Grossman, 1993). A high AT codon bias was also found in *cpc*G, as expected for a plastid-encoded gene (Maid et al., 1990). The *cpe*BA genes are not closely linked to the PE linker polypeptide genes in Cyanobacteria (Federspiel and Grossman, 1990) or red algae (Apt and Grossman, 1993). These linkers are nuclear encoded in red algae (Federspiel and Grossman, 1990).

The α^{PE} and β^{PE} subunits (encoded by *rpe*A and *rpe*B genes, respectively), isolated from the unicellular red alga *Rhodella violaceae* (*R. violaceae*) show strong homology to α subunits isolated from the unicellular red alga *P. cruentum* (85 and 83% identity, respectively) (Ducret et al., 1994). This contrasts with the β^{PE} from *R. violaceae,* which demonstrates homology to the β^{PE} isolated from the cryptomonad, *Cryptomonas* Φ (81% identity) (Bernard et al., 1992). The homology of the *R. violaceae* α^{PE} and β^{PE} subunits decreased when compared to Cyanobacterial α^{PE} and β^{PE} subunits (about 70% identity) (Bernard et al., 1992). These authors identified a type II-like intron in *rpe*B, which is the first intron reported from red algal phycobiliprotein genes. Cyanobacterial systems are only known to have type I introns (Kuhsel et al., 1990; Xu et al., 1990). Highly regulated expression in *R. violaceae* was indicated by the presence of Shine-Delgano sequences five and six base pairs upstream from the initiation codons for the *rpe*B and *rpe*A genes, respectively (Bernard et al., 1992). Secondary structures were identified downstream that could be termination sequences for this operon.

The large, core-membrane linker polypeptide (L_{CM}), is encoded by the *apcE* gene (Apt and Grossman, 1993; Bryant and Tandeau de Marsac, 1988). The *apc*E gene was found 5′ to the genes encoding the α and β subunits of allophycocyanin in *A. neglectum* (Apt et al., 1993). This gene is cotranscribed with the *apc*AB genes (encoding α^{APC} and β^{APC} subunits) as a monocistronic unit (Apt et al., 1993) and is 50% homologous to Cyanobacterial and the unicellular glaucocystophyte *Cyanophora paradoxa apc*E genes. The *apc*E gene in *A. neglectum* is located on the plastid genome and its protein product has an A/T codon bias in the third position.

Regulatory sequences were also identified. The ApcE polypeptide (*A. neglectum*) has an N-terminal domain homologous to the subunits of phycobiliproteins and three linker-like domains (REPS) homologous to PC and PE linker polypeptides. These domains have >60% homology to those of *Cyanophora paradoxa* (Glaucocystophyta).

The phycobiliproteins identified thus far have not changed appreciably since the late 1980s as reviewed by MacColl and Guard-Friar (1987) and by Rowan (1989). A compilation of the known phycobiliproteins is provided in Table 1. An exception to this is the work by Waterbury in marine Cyanobacteria describing new high phycourobilin-containing phycoerythrins (Waterbury et al., 1989; Swanson et al., 1991). These high urobilin phycoerythrins were designated CU-PE and have extraordinary levels of phycourobilin compared to R-PE, such that the level of absorbance at 498 nm (the phycourobilin absorbance maximum) exceeded that at 565 nm (phycoerythrobilin absorbance maximum) in some cases. A new phycocyanobilin-containing phycoerythrin was also recently described by Glazer and colleagues from a red alga and will be discussed in the red algal phycobiliprotein section (Glazer et al., 1997). Another recent paper described a red algal phycoerythrin isolated from *R. reticulata* strain R6 that has no α subunits (Thomas and Passaquet, 1999).

Isolated phycobiliproteins are basically artifacts of the purification process used. In nature they exist as oligomeric structures (in contrast to the one described above) composed of the basic $(\alpha\beta)_3$ assembly with colorless linker polypeptides intimately associated with each. Few isolated phycobiliproteins (B-PE and R-PE being the major exceptions) retain their linker polypeptides on isolation. The following discussion must, by necessity, focus on spectral characteristics of the isolated phycobiliproteins, but be aware that the colorless linkers markedly affect the absorbance and fluorescence characteristics of phycobiliproteins in their native environment.

IV. Phycobiliprotein Structure

The most stable protein complex found in phycobilisomes is the biliprotein monomer (actually a heterodimer comprised of α and β subunits) the basic building block for all phycobiliprotein classes (Zhou et al., 1990). All phycobiliproteins have a very similar subunit structure comprised of 9 α-helices (x-y-a-b-e-f-f′-g and h) connected by irregular loops (Schirmer et al., 1985). Ribbon structures for β and α subunits of PC are shown in Figure 2, with the nine α-helices labeled in the sequence, x-y-a-b-e-f-f′-g and h. Both proteins have identical topologies. The phycobiliprotein subunit structure consists of a carboxyl-terminal globular domain (α-helices a through h) and two short anti-parallel α-helices (x and y), located at the N-terminal that extend out from the globular domain (Schirmer et al., 1985). The monomer is formed by symmetrical associations between α and β subunits and is dominated by hydrophobic residues that are highly conserved in the phycobiliprotein sequence database. Figure 3 represents the monomer where α-helices X and Y of one subunit interact with the globular domain of its partner subunit. In addition to contributing to the $\alpha\beta$ interface, the globular domain also serves as a scaffold for the enzymatic addition of specific chromophores to the apoprotein subunit, yielding spectral characteristics unique to each phycobiliprotein class. Residues that are conserved between the different classes of phycobiliprotein in their aligned sequences presumably have crucial structural roles. In fact, structurally constrained alignment of all phycobiliprotein subunits in the sequence database reveals residues that demonstrate a strong pattern of conservation within a specific phycobiliprotein class, yet show variation between the classes (Apt et al., 1995). One of the most extensive areas of amino acid identity, both between and among the phycobiliprotein subunits, is found in the β subunits on α-helices e and f. The central chromophore attachment site (βCys82) is isologous in most of the phycobiliproteins (both α and β subunits) and located on α-helix e. At this location, the bilin extends down into a pocket formed by amino acid residues of α-helices e and f. The additional chromophore on the phycocyanin β subunit is situated on the periphery of every phycobiliprotein aggregate (monomer, trimer and hexamer), reflecting a consistent theme of peripheral location for additional chromophore attachments seen in the phycoerythrins and phycoerythrocyanins (Deurring et al., 1991; Ficner et al., 1992). The conservation of amino acid residues in α-helices e and f is possibly due to the importance of the central bilin for unidirectional energy transfer in the rod. Interactions with amino acid sidechain residues located in α-helices e and f assist in maintaining the central bilin in an extended conformation and create the protein-chromophore environment important for affecting

Table 1. Phycobiliprotein types and specific parameters of each phycobiliprotein. (for primary references see tables from MacColl and Guard-Friar (MacColl and Guard-Friar, 1987) and Rowan (Rowan, 1989) or the specific references provided). For Glaucocystophyta, see Section V.D.

Phycobiliprotein	Alternative Name(s)	Abs. Peak(s) (nm)	Fluor. Emission (nm)	Subunit/ (#) Bilin	Distribution (Refs)
Allophycocyanin	APC	598(s) 629 (s) 650	660	α / (1) PCB β / (1) PCB	Red algae Glaucocystophytes Cyanobacteria (A)
Type I	APC I	654	678		
Type II	APC II	590 620 650	660		
Type III	APC III	625 650	660		
Allophycocyanin A	APC A	595 623 654			
Allophycocyanin B	APC-B, APC-680	610 650 667(s)	680		Red algae Glaucocystophytes Cyanobacteria
C-Phycocyanin	C-PC	615–625	637–647	α / (1) PCB β / (1)PCB	Red algae Glaucocystophytes Cyanobacteria
Type II	PC II	617–618	642–648		(A)
PC 623	PC 623	623	642		
PC 637	PC 637	637	653		
Phycocyanin 569	Cr-PC569			α / (1) PCB β / (1) PCB (2) 584B	Cryptomonads
Phycocyanin 612	Cr-PC612, CryptoFluor-1,	575 612	634	α / (1) PCB β / (2) PCB (1) DBV	Cryptomonads
Phycocyanin 617	Cr-PC617			α / (1) PCB β / (2) PCB (1) CV	Cryptomonads
Phycocyanin 630	Cr-PC630	583 630			Cryptomonads
Phycocyanin 645	Cr-PC645, CryptoFluor-2	585 625(s) 645	660	α / (1) 697B β / (2) PCB (1) CV α / (1) MBV β / (2) PCB (1) DBV	Cryptomonads Cryptomonads

Table 1. (Continued)

Phycobiliprotein	Alternative Name(s)	Abs. Peak(s) (nm)	Fluor. Emission (nm)	Subunit/ (#) Bilin	Distribution (Refs)
R-phycocyanin	R-PC, R-PC I	555 614–620	636–650	α / (1) PCB β / (1) PCB (1) PEB	Red algae Cyanobacteria (B)
	R-PC II	533 554 615	646		Red algae
Phycoerythrin 545	Cr-PE545, CryptoFluor-3	545 560(s)	585	α / (1) DBV β / (3) PEB	Cryptomonads
Phycoerythrin 555	Cr-PE555, CryptoFluor-4	555	578		Cryptomonads
Phycoerythrin 566	Cr-PE566, CryptoFluor-5	566 620(s)	617	α / (1) 585B β / (1) PEB (2) 584B	Cryptomonads
		565–566 604	617		
Phycoerythrin 570	Cr-PE570	569 630	650–656		Cryptomonads
B-phycoerythrin	B-PE	498(s) 545–546 563–565	575–578	α / (2) PEB β / (3) PEB γ / (2) PEB (2) PUB	Red algae (C,D)
Type I	B-PE I	496–503 538–550 564–570	574–577		Red algae
Type II	B-PE II	498–499 529–537 566–568	574–575		Red algae
b-phycoerythrin	b-PE	545 563	570–575	α / (2) PEB β / (3) PEB	Red algae
C-phycoerythrin	C-PE	565–562	576–577	α / (2) PEB β / (3–4) PEB	Cyanobacteria (B, E)
Type I	C-PE I	540 560			Cyanobacteria
Type II	C-PE II	540 563			Cyanobacteria
CU-phycoerythrin	CU-PE	495 547 562	573	α / (3) PEB β / (3) PEB (1) PUB	Cyanobacteria (F, G)
Type I	CU-PE I	498–501 540 564–567	573		Cyanobacteria

Table 1. (Continued)

Phycobiliprotein	Alternative Name(s)	Abs. Peak(s) (nm)	Fluor. Emission (nm)	Subunit/ (#) Bilin	Distribution (Refs)
Type II	CU-PE II	496–500 536–547 558–565	573		Cyanobacteria
Type III	CU-PE III	492 543	565		Cyanobacteria
R-phycoerythrin Type I	R-PE	493–498 534–545 564–568	572–578	α / (2) PEB β / (2-3) PEB (1) PUB γ1/(3) PEB (2) PUB γ2/(1-2) PEB (1) PUB	Red algae (H, I)
Type II		496–497 538–551 565–566	574–577		Red algae
Type III		496 539–541 564–567	572		Red algae
r-phycoerythrin	r-PE	498 542 560	575		Red algae
Phycoerythrin	PE	495 563 605	626	α / (2) PEB β / (1) PUB (1) PCB (1) PEB γ / (1) PUB (2) PEB	Red algae Audouinella (J)
$\beta\beta$-Phycoerythrin	87 PE-LR $\beta\beta$PE	562 604	630		Red algae *Rhodella reticulata* R6 (K)
Phycoerythrocyanin	PEC	535(s) 570–575 590–595(s)	625	α / (1) CV β / (2) PCB	Cyanobacteria

References: A – (Glazer and Fang, 1973) , B – (Glazer and Hixson, 1977), C – (Redlinger and Gantt, 1981), D – (Glazer and Hixson, 1977), E – (Klotz and Glazer, 1985), F – (Bryant et al., 1976), G – (Kursar et al., 1981), H – (Stadnichuk et al., 1984), I – (Glazer and Hixson, 1977), J – (Glazer et al., 1997), K – (Thomas and Passaquet, 1999)

spectral differences among the phycobiliprotein classes (Schirmer et al., 1987). Although some preliminary work demonstrated the importance of individual residues in β^{PC} for subunit folding and assembly (*i.e.*, amino acid residues β75/β76 for trimer assembly) (Toole, 1998), information obtained using correlations between structure and function (using molecular biology) is lacking for these similar proteins.

A new model for phycobilisome assembly in Cyanobacteria was recently proposed. In this assembly model, subunit translation and initial folding is followed by lyase-mediated attachment of bilins (Anderson and Toole, 1998). The genes encoding α and β subunits for the different phycobiliprotein classes have been isolated and cloned from numerous Cyanobacteria (and some red algae) and for all the

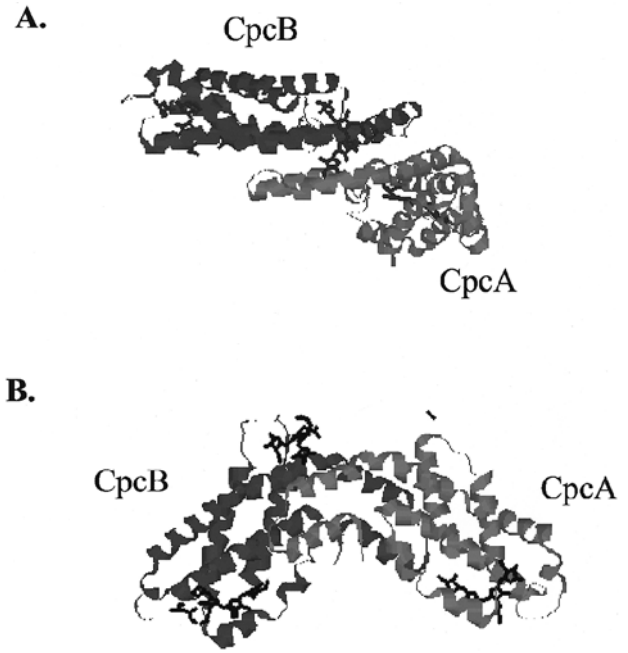

Fig. 3. Structure of a phycocyanin monomer. Panel A) Structure of the phycocyanin monomer consisting of the α subunit and the β subunit depicted as ribbon structures. The three covalently attached chromophores are shown as stick figures. The β and α subunits have one central bilin attached to Cys82 and the β subunit has an additional bilin attached to βCys155. The monomer interface is shown. Structures were generated using Rasmol and the PDB coordinates for PC from *Fremyella diplosiphon* (Deurring et al., 1991). Panel B) The structure of the monomer in Figure 2 (a) has been rotated approximately 90° about the X-axis showing the x-y domain of one subunit interacting with the globular domain of its partner subunit and visa versa. CpcB and CpcA are shown as ribbon structures and the covalently attached chromophores are depicted as stick figures.

major phycobiliproteins these genes are contiguous and co-transcribed. Proximal translation of the phycobiliprotein subunits would provide short-term stability by allowing the rapid association of the nascent α and β subunits, thereby forming a transient monomer species. The importance of monomer formation in the early steps of phycobiliprotein biosynthesis and assembly is suggested by the characterization of a PC-minus strain of *Synechococcus* sp. PC6803, strain 4R, where a lesion in *cpc*B is responsible for a null-PC phenotype (Plank et al., 1995). Introduction and expression of the *cpc*BA operon from a closely related cyanobacterium *Synechococcus* sp. PCC6701 could rescue the endogenous PC α subunit. It was concluded that subunits that do not have a partner subunit available with which to bind and form the protected monomeric state, are subject to rapid degradation possibly by exposure of protease sensitive domains. Other mutations that alter only one subunit (α or β) have also resulted in a significant reduction of its corresponding partner subunit. Su et al. (1992) found that a subunit truncation in β^{APC} also eliminates α^{APC}. Indirect deletions of the central bilin in α^{PC} by interruption of the lyase genes *cpc*E and/or *cpc*F result in an 80 to 100% reduction in both subunits (Zhou et al., 1990; Swanson et al., 1992). These assembly mutants emphasize the importance of monomer formation in phycobilisome assembly and suggest that if one subunit is unable to bind with its partner subunit both subunits are rapidly degraded.

V. Phycobiliprotein Types

A. Red Algal Phycobiliproteins

Red algae range from unicellular organisms that can barely be seen with the naked eye (e.g., *P. cruentum*, a single celled red alga) to macroalgae grown on nets

to be used for the wrappers of your favorite sushi (e.g., *Porphyra yezoensis*). This diverse algal Division produces phycobiliproteins that belong to the phycoerythrin, phycocyanin and allophycocyanin classes and which are organized into phycobilisomes (Table 1).

1. Allophycocyanins

Red algae have both APC (trimer assemblies comprised of α^{APC} and β^{APC} subunits) and a specialized APC-B trimer that serves as a terminal acceptor for the phycobilisome, emitting at 680 nm due (in part) to the specialized $\alpha^{AP\text{-}B}$ subunit found in these trimeric core substructures. APC has the typical $(\alpha\beta)_3$ trimer assembly unit comprised of α and β APC phycobiliprotein subunits. However, the anchor protein (ApcE or L_{cm}), which also serves as a terminal acceptor in the phycobilisome, replaces the α subunit in some of the APC trimers within the phycobilisome core to provide a measure of diversity within each phycobilisome's APC constituents. This is very important to the structure of the phycobilisome and energy transfer within this structure (Zilinskas, 1982).

Red algae respond to light quality, but there is some disagreement whether this response is similar to the complementary chromatic adaptation seen in Cyanobacteria or compensatory chromatic adaptation, where light quality initiates changes in the overall amount of pigment produced leaving pigment ratios essentially unchanged (Chapter 13, Larkum; Chapter 17, Raven and Geider). Complementary chromatic adaptation has been widely studied in Cyanobacterial systems but is not believed to occur widely in red algae (Grossman et al., 1993). However, recent work in red algal systems indicates that complementary chromatic adaptation may be occurring (Lopez-Figueroa and Niell, 1990; Algarra et al., 1991; Sagert and Schubert, 1995; Torres et al., 1995). Work with *A. neglectum* studying the transcript analysis of the γ subunit of R-PE demonstrated that the level of message was regulated by light intensity (Apt et al., 1993). A decrease in the number of phycobilisomes correlated directly to an increase in light levels, while the phycobilisome structure remained constant. This contrasts with an earlier study on *Griffithsia pacifica* where high light affected a change in the ratio of PE to the other phycobiliproteins, indicative of a change in the phycobilisome structure (Waaland et al., 1974). A recent study suggests that the level of light does more than just decrease the number of phycobilisomes but indeed leads to a specific removal of the peripheral phycoerythrins from the phycobilisomes of *R. violaceae* (Bernard et al., 1996). These authors suggested that there are two possible response mechanisms in this alga. The first is that described by Apt and Grossman in *A. neglectum* where the level of transcript is reduced with a concomitant reduction in the number of phycobilisomes; this occurs at moderate levels of light (Apt and Grossman, 1993). The second response occurs at higher levels of light where a specific loss of peripheral B-phycoerythrins occurs (chromatic adaptation) (Apt and Grossman, 1993). It is still controversial whether this can be classified as classical chromatic adaptation, but it seems obvious that this is certainly a light quality driven event. The alternative strategy of compensatory chromatic adaptation, where adjustments in the amount of pigment produced to even out the energy production by the photosystems has been demonstrated in red algae (Chow et al., 1989; Cunninghan et al., 1990; Carmona et al., 1996). In the proposed system in *R. violacea* there would be two levels of control of PE and its associated linkers: 1) where an intermediate (proximal) PE and its linker are constitutively produced but reduced in number when irradiance is increased in equal ratios with the other phycobiliprotein components; versus 2) compared to the terminal PE hexamer and its linker that are specifically lost under specific irradiance conditions (Bernard et al., 1996).

2. Phycocyanins

Red algae produce both R-phycocyanin and C-phycocyanin (again confounding the old nomenclature scheme) (Table 1). The R-PC and C-PC have $(\alpha\beta)_6$ hexameric structures. R-PC has phycoerythrobilin in addition to phycocyanobilin chromophores resulting in slightly higher absorption in the high 500 nm range while C-PC contains entirely phycocyanobilin chromophores and a maximal absorbance around 615 nm with emission at 639 nm (MacColl et al., 2000).

3. Phycoerythrins

R-phycoerythrin (R-PE) has a classical $(\alpha\beta)_6$ structure but also contains an associated linker polypeptide called the γ subunit, giving a final structure of $(\alpha\beta)_6\gamma$ (Apt et al., 1993). The R prefix was provided to