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BIOCHEMISTRY OF DIATOM PHOTOSYNTHETIC MEMBRANES AND PIGMENT-PROTEIN COMPLEXES

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

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DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

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BIOCHEMISTRY OF DIATOM PHOTOSYNTHETIC MEMBRANES AND PIGMENT-PROTEIN COMPLEXES

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Abstract

Diatoms are an ecologically important group of algae in both marine and freshwater systems, but in spite of their significance little is known about the structure of their photosynthetic apparatus. This is due in part to the lack of a highly purified, oxygen-evolving thylakoid membrane preparation. Since thylakoid membranes purified from diatoms using methods developed for green plants did not evolve oxygen, a new procedure was developed for use with diatoms. An oxygen-evolving thylakoid membrane preparation is crucial for the study of photosynthetic pigment-protein complexes from these algae because the stability of the Photosystem I (PS I) and Photosystem II reaction centers was shown to be greatly reduced in thylakoid preparations that did not retain electron transport activity. As a result of the instability of PS I in some thylakoid preparations, a novel chlorophyll-binding complex was isolated that contained only the PsaA polypeptide. The isolation of this complex should prove useful in elucidating the structure of the PS I reaction center in all plants. Immunological and N-terminal protein sequencing methods were used to identify several photosynthetic proteins in the purified thylakoid preparation. These results provided evidence for posttranslational modification of two light-harvesting polypeptides (LHCPs) as well as of the PsaB subunit of the PS I reaction center core. Posttranslational modification of LHCPs and/or of PsaB has not been observed in green plants. In contrast to green plants, PS I in diatoms has been shown to be located in the inner thylakoid membranes. It was hypothesized that proteolytic processing of the C-terminus of PsaB in diatoms may be necessary for the PS I holocomplex to be present in the inner membranes, and that this processing may be responsible for the instability of PS I in purified diatom thylakoids. The existence of a functional, highly purified, and extensively characterized thylakoid preparation from diatoms will promote our understanding of the photosynthetic apparatus in these algae.

Table of Contents

List of Figures

List of Tables

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Chapter 1

General Introduction

1.1 Photosynthesis

Photosynthesis uses the energy contained in sunlight to convert inorganic compounds into organic compounds that can be used by living organisms for growth. There are two main types of photosynthesis: oxygenic and non-oxygenic. Non-oxygenic photosynthesis occurs in photosynthetic bacteria, while oxygenic photosynthesis occurs in cyanobacteria and eukaryotic plants and algae. As a group, oxygenic photosynthesizers are much more diverse than the anaerobic photosynthetic bacteria, and are found in a wide variety of habitats.

In oxygen-evolving photosynthesis, water is used as the electron donor, and the process is often summarized by the equation: $6CO_2 + 6\ H_2O$ + light energy = $C_6H_{12}O_6 + 6\ O_2$. The simplicity of this equation belies the complexity of the overall process, which actually involves two distinct sets of events. The first set of reactions is known as the "light reactions" because they require light energy. During the light reactions, energy from sunlight is harnessed and stored in the form of ATP and NADPH. Water is split to provide some of the protons necessary for ATP formation, and O_2 is produced as a by-product. The ATP and NADPH produced during the light reactions are subsequently used in what are called the "dark reactions", which do not have a direct requirement for light. In the dark reactions, the energy stored in ATP and the reducing power stored in NADPH are used to fix CO_2 into carbohydrate.

1.2 Components of the photosynthetic apparatus

The majority of our knowledge concerning the structure of the photosynthetic apparatus in oxygen-evolving plants comes from work done with higher plants, green algae, and cyanobacteria. The processes involved in oxygenic photosynthesis appear to be the same in all organisms, and it is widely accepted that the major protein complexes involved in these processes are similar in all plants (e.g. Thornber, 1986, Curtis, 1988). These major complexes include the reaction centers (Photosystem I and Photosystem II), the cytochrome

b_6/f complex, and ATP synthase. Although sequence homology from one organism to another may differ substantially for a particular protein subunit, the function of each of these major protein complexes appears to be the same in all oxygenic plants. In addition to the complexes mentioned above, specialized pigment-binding proteins that enhance the absorption of light by the reaction centers are also present. While their overall role in photosynthesis is similar in all organisms, these complexes are extremely diverse in terms of their structure and pigment composition (see 1.2.3).

1.2.1 Photosystem I

Photosystem I (PS I) is comprised of at least 12 subunits, products of the genes *psa*A-O (Ikeuchi, 1992). The PsaA, PsaB, and PsaC subunits form the core of the reaction center, as discussed below. The stromally-exposed extrinsic protein, PsaD, seems to be required for the proper orientation of PsaC (Li et al., 1991), and is also the proposed docking site for ferredoxin based on cross-linking studies (Wynn et al., 1989). The precise function of PsaE is still not understood, however, it appears to be necessary for stabilizing the interaction of PsaC with the PsaA/PsaB dimer (Weber and Strotmann, 1993) and also for maintaining the proper structural conformation of the site of ferredoxin reduction (Rousseau et al., 1993). The function of many of the remaining subunits of the PS I holocomplex is unclear at this time (reviewed in Scheller and Møiler, 1990; Ikeuchi, 1992).

The PsaA and PsaB polypeptides (Fish et al., 1985) bind the electron transfer components P700, A_0, A_1, and F_X (Parrett et al., 1989), while the iron-sulfur centers F_A and F_B are bound on the PsaC polypeptide (Høj et al., 1987). It is generally accepted that the order of the electron transfer components is P700, A_0, A_1, F_X, and F_A/F_B (Golbeck, 1988), however this pathway has not been proven. A_0 has been identified as a specialized monomeric chlorophyll (Chl) *a* molecule that absorbs at 690 nm (Mathis et al., 1988). A_1 is

believed to be phylloquinone (Vitamin K_1), and it has bean shown that there are two phylloquinone molecules present per PS I reaction center (Schoeder and Lockau, 1986; Malkin, 1986; Biggins and Mathis, 1988). One of these is easily extracted, while the other is tightly bound (Malkin, 1986). It has been established that F_X is a [4Fe-4S] center (Scheller et al., 1989; Guigliarelli et al., 1993), although it has been thought to be composed of two [2Fe-2S] centers (Golbeck et al., 1987; McDermott et al., 1988).

The PS I reaction center from a thermophilic *Synechococcus* sp. has been crystallized, and its atomic structure determined to 6Å (Krauss et al. 1993). The electron density maps indicate the locations of the heme groups of the P700 reaction center and A_0 Chls, but the polypeptide boundaries have not been established. Hence, at this time it is not known whether P700 is bound between PsaA and PsaB, with each polypeptide binding one of the two Chls in the dimer, or if some other arrangement exists. Likewise, the precise locations of A_0 and the two molecules of phylloquinone (A_1) have not yet been determined. The existence of one or two long-wavelength (e.g. 702-705 nm) absorbing Chls near P700 has been predicted on the basis of computer simulation studies (Jia et al.,1992; Werst et al., 1992). The precise location of these "red" Chls is not known at this time, however, work presented in this dissertation suggests that at least one "red" Chl is bound to the PsaA subunit (Martinson and Plumley, in prep.; Chapter 5).

1.2.2 Photosystem II and the Oxygen-Evolving Complex

Like PS I, Photosystem II (PS II) is a large complex, containing as many as 22 subunits (Masojidek et al., 1987), which are products of the genes *psb*A-*psb*V. The smallest PS II complexes that are capable of charge-separation contain five polypeptides: PsbA, PsbD, PsbE, PsbF, and PsbI (Nanba and Satoh, 1987). The PsbA and PsbD polypeptides are often referred to as D_1 and D_2, respectively, and the PsbE and PsbF gene products correspond to the α- and

β-subunits of the cytochrome b_{559} complex. The D_1 and D_2 polypeptides share sequence homology with the L and M subunits of the purple bacterial reaction center (Rochaix et al., 1984; Hearst, 1986). In the bacterial reaction center, the electron transport components are arranged symmetrically such that there are two possible pathways for electron transport (Michel et al., 1986). However, only one pathway is active. A similar arrangement seems to exist for PS II (Govindjee and Wasielewski, 1989) although no experimental evidence is available to support this hypothesis (Hansson and Wydrzynski, 1990).

The D_1 and D_2 polypeptides bind the electron transport components that are active in charge separation. These components include two tyrosine residues, designated Y_Z and Y_D; the reaction center Chls, P680; two molecules of phaeophytin; two plastoquinone molecules, Q_A and Q_B; and a nonheme iron. By analogy with the bacterial reaction center, P680 and the nonheme iron are assumed to be bound between the D_1 and D_2 polypeptides (Govindjee and Wasielewski, 1989). The photochemically active tyrosine, Y_Z, is bound by the D_1 polypeptide (Debus et al., 1988b), as are one of the phaeophytin molecules and Q_B (Govindjee and Wasielewski, 1989). Thus, both the reducing side (Y_Z) and the oxidizing side (Q_B) of PS II are located on the same polypeptide, D_1 (Metz et al., 1986; Ikeuchi and Inoue, 1987). The D_2 polypeptide binds Y_D (Barry and Babcock, 1987; Debus et al., 1988a; Vermaas et al., 1988), the second phaeophytin, and Q_A (Govindjee and Wasielewski, 1989). The roles of the cytochrome b_{559} complex and of PsbI, the other two complexes necessary for photochemical activity, are not clear at this time (Debus, 1992), however, recent evidence suggests that cytochrome b_{559} may play a role in protecting PS II against photoinhibition (Poulson et al., 1995).

PS II particles that are capable of evolving oxygen at high rates *in vitro* contain at least one additional polypeptide, the 33 kD product of the *psb*O gene (Ikeuchi et al., 1985; Tang and Satoh, 1985). The PsbO polypeptide is believed to protect or stabilize the cluster of four

manganese ions that are involved in the oxidation of water (Kuwabara et al., 1987; Tyagi et al., 1987; Philbrick and Zilinskas, 1988). Removal of PsbO can result in the loss of 50% (2) of the Mn ions from the cluster (Mohanty et al., 1993). However, deletion mutagenesis experiments have shown that PsbO is not an absolute requirement for oxygen evolution, although the rate of oxygen evolution is much reduced in mutants lacking the protein (Burnap and Sherman, 1991; Philbrick et al., 1991). In addition to Mn ions, which play a role in extracting electrons from water (Debus, 1992), calcium and chloride are also necessary cofactors in the process of water oxidation (Critchley, 1985; Homann, 1987; Boussac and Rutherford, 1988). Higher plants and green algae contain two additional polypeptides of 23 and 17 kD (PsbP and PsbQ, respectively), which are thought to regulate the binding of Ca^{+2} and Cl^-, respectively, although the nature of these interactions is still unclear (Debus, 1992).

In cyanobacteria, the oxygen-evolving complex contains the 33 kD polypeptide, cytochrome c_{550} (17 kD) and a 12 kD polypeptide (Shen et al., 1992; Shen and Inoue, 1993). The PsbP and PsbQ polypeptides appear to be absent in these algae (Debus, 1992). Recently, the complete chloropiast genome from the diatom *Odontella sinensis* was sequenced and found to contain the gene (*psb*V) encoding cytochrome c_{550} (Kowallik, 1996). This suggests that the photosynthetic apparatus of diatoms may be more similar to that of cyanobacteria than to that of green plants. In accordance with this, we found that antibodies to the PsbP and PsbQ polypeptides in *Chlamydomonas* did not cross-react with any proteins in the thylakoid membranes of the diatom *Cylindrotheca fusiformis* (not shown). The photosynthetic components of this diatom are described in more detail in Chapter 4 (Martinson et al., in prep.).

1.2.3 Light-Harvesting Complexes

The capture of light energy by photosynthetic reaction centers is enhanced by the presence of light-harvesting complexes (LHCs). These complexes contain specialized pigments that permit the absorption of wavelengths of light not absorbed by Chl *a*, which is the primary pigment present in the reaction centers. These pigments are bound by polypeptides (LHCPs), and the pigment-protein complex (PPC) as a whole interacts with the reaction center or with other LHCs.

In plants and algae, including cyanobacteria and diatoms, each reaction center has an internal antenna. In PS II, the internal antennae consist of two Chl *a*-binding complexes, CP47 and CP43 (Camm and Green, 1983), which are products of the *psb*B and *psb*C genes, respectively (Morris and Herrmann, 1984; Holschuh et al., 1984; Alt et al., 1984). The Chls that constitute the internal antenna of PS I are located on the PsaA and PsaB core polypeptides (Mullet, 1980).

In green plants and all eukaryotic algae, each reaction center has one or more peripheral antennae. The peripheral antennae of green plants and green algae have been characterized in detail, while comparatively little work has been done on the antenna systems of Chl *c*-containing algae such as diatoms. In higher plants, there are five Chl *a*/*b*-binding peripheral antennae: CP29, CP26, CP24, LHCII, and LHCI (Camm and Green, 1980; Bassi et al., 1987; Dunahay and Staehelin, 1986). CP29, CP26, and CP24 are minor antennae associated with PS II, while LHCII and LHCI are the major antennae associated with PS II and PS I, respectively. In higher plants the peripheral antenna of PS I can be divided into two subtypes, known as LHCI680 and LHCI730 (Lam et al., 1984; Bassi et al., 1985) based on their fluorescence emission at 77K.

As noted above, very little is known about the LHCs of diatoms and other Chl *c*-containing algae. In part, this is due to the lack of a highly purified, functional (e.g. oxygen-

evolving) thylakoid preparation. Work presented in this dissertation reveals the importance of using such a preparation for purifying and characterizing LHCs in diatoms (Martinson et al., in prep.; Chapter 4; Chapter 6). Six genes encoding LHCPs have been found in the diatom *Phaeodactylum tricornutum*, referred to as *fcp*A-*fcp*F (Bhaya and Grossman, 1993), which share some sequence homology with the Chl *a*/*b*-binding LHCPs (Cabs; Grossman et al., 1990). In a previous communication, we documented the immunological similarity of the Fcp polypeptides (encoded by the *Fcp* genes) to Cab polypeptides from a diverse array of algae and plants (Plumley et al., 1993). Nevertheless, very little is known about the organization of Fcps within LHCs, nor about the interactions of these LHCs with the reaction centers of diatoms. In the work reported here, three proteins homologous to FcpE and FcpF were identified in *Cylindrotheca*, and were shown to be part of an LHC in this alga (Martinson et al., in prep.; Chapter 4). In addition, we identified two additional LHCPs in *Cylindrotheca* whose N-terminal sequences are not like those of any photosynthetic polypeptides sequenced to date, including the Fcps. Interestingly, these LHCPs were part of a PS I complex, implying that they are specifically associated with PS I. This is the first conclusive identification of a PS I-associated LHC in diatoms. Finally, the lack of sequence homology with known Fcps suggests that there may be additional *Fcp* genes present in this diatom, and perhaps in others as well.

1.3 Structure of the chloroplast

Free-living eukaryotic algae and plants have chloroplasts, but the arrangement of the thylakoid membranes within the chloroplast differs between groups. Three types of arrangements can be distinguished. In the first group, which includes cyanobacteria and red algae, thylakoids are not grouped together as they are in all other eukaryotic plants and algae (see below). This is presumably because a tight appression would not leave sufficient room

for the phycobilisomes to associate with PS II and PS I at the surface of the thylakoid. The second group includes green algae and higher plants, whose thylakoids are arranged in stacks called grana. Grana typically contain two to six thylakoids, although as many as 100 thylakoids have been observed in the grana of plants adapted to extreme shade (Anderson et al., 1973). Grana are often connected by a single thylakoid, which is referred to as a stromal lamella; thylakoids that are stacked into grana are called granal lamellae. In the third group, which includes the chromophytes (e.g. Chl *c*-containing algae such as diatoms) and euglenoids, the thylakoids are arranged in groups of three and are not segregated into granal and stromal lamellae (Lee, 1980).

The variation in the ultrastructure of the chloroplast among different groups of algae and plants suggests the possibility of there being fundamental differences in the organization of the photosynthetic protein complexes within the thylakoid membranes. Immunocytochemistry and electron microscopy of freeze-fractured thylakoids from higher plants has shown that PS I and the ATP synthase are localized almost exclusively in the stromal lamellae, while PS II, LHCII, and the oxygen-evolving complex are confined largely to the granal lamellae (Andersson and Anderson, 1980; Vallon et al., 1985). In contrast, immunocytochemistry of freeze-fractured thylakoids from the brown alga *Fucus serratus* (Lichtlé et al., 1992) and the diatom *Phaeodactylum tricornutum* (Pyszniak and Gibbs, 1992) showed that the LHCs were distributed equally among external and internal (appressed) thylakoid membranes. In addition, as many as 60% of the PS I reaction centers in *Phaeodactylum* appear to be localized in appressed regions (Pyszniak and Gibbs, 1992). These results show that the segregation of LHC and PS I as observed in higher plants and green algae does not occur to the same extent in diatoms and brown algae. Moreover, the localization of PS I in appressed regions of diatom thylakoid membranes suggests that some modification of the PS I complex may be required in order for it to be in this type of environment. Results presented in this dissertation suggest

that posttranslational proteolytic processing of the C-terminus of the PsaB polypeptide may be a means of modifying the structure of PS I in diatoms (Martinson and Plumley, in prep.; Chapter 5).

1.4 The importance of studying the photosynthetic apparatus of chromophytes

The immunocytochemical studies described above strongly suggest that the organization of the components of the photosynthetic apparatus within the thylakoid membranes of chromophytes differs significantly from that found in higher plants and green algae. Such differences are likely to have implications for the regulation of photosynthesis in chromophytes, and indeed, some data are available which show that the LHCs of brown algae (Berkaloff et al., 1984) and diatoms (Owens, 1986a) transfer excitation energy equally to both reaction centers. Moreover, the distribution of excitation energy appears to be controlled by adjusting the rate at which excitation energy is transferred to PS II, and is independent of the redox state of the plastoquinone pool (Owens, 1986a). This is in sharp contrast to the situation in cyanobacteria, red algae, green algae, and higher plants, where the phycobilisome or mobile LHC II can alter the distribution of light energy between PS II and PS I by preferentially associating with one reaction center or the other, a process which has been shown to be dependent on the redox state of the plastoquinone pool.

As mentioned previously, much of what is known about the structure of the photosynthetic apparatus comes from studies on cyanobacteria, green algae, and higher plants, yet these groups represent only two of the three possible arrangements of thylakoid membranes within the chloroplast or cell. In addition, the regulation of photosynthesis in chromophytes has been found to be significantly different from that seen in cyanobacteria, green algae, and higher plants. In light of this, the relative paucity of studies done with

chromophytes has potentially left a gap in our knowledge of photosynthesis, as well as in our understanding of the evolution of photosynthesis.

1.5 Pigment-protein complexes of chromophytes

With regard to pigment composition, chromophytes differ from green plants in that they contain Chl *c* rather than Chl *b*. Since Chls *b* and *c* are accessory pigments, it is logical to hypothesize that the major differences between the photosynthetic proteins of chromophytes and those of green plants would be in the structure of the LHCs. As such, most of the work done with chromophytes has dealt with the isolation and characterization of LHCs (Ingram and Hiller, 1983; Friedman and Alberte, 1984; Owens and Wold, 1986; Caron et al., 1988; Passaquet et al., 1991; Wilhelm and Wiedemann, 1991; Sukenik et al., 1992; Büchel and Wilhelm, 1993; Hiller et al., 1993; Durnford and Green, 1994) with very little emphasis placed on the reaction centers (Holdsworth and Arshad, 1977; Lichtlé et al., 1987; Douady et al., 1993).

One of the major obstacles to understanding the structure of the photosynthetic apparatus in chromophytes seems to be the lack of well-defined, highly-purified, oxygen-evolving thylakoid preparations. Oxygen-evolving chloroplasts have been prepared from brown algae (Popovic et al.1983; Strbac et al., 1994), but have not been extensively characterized with regard to their biochemical and biophysical properties. For studies on the polypeptide composition of PPCs, the importance of a thylakoid preparation that is free from contamination by both non-chloroplast proteins as well as by stromal components cannot be underestimated. Moreover, biochemical and biophysical evidence presented in this dissertation shows that the PS II and PS I reaction centers of diatoms are easily dissociated (Martinson et al., in prep.; Chapter 4; Martinson and Plumley, in prep.; Chapter 5; Chapter 6). This provides an additional constraint when attempting to purify PPCs from diatoms in that

the thylakoid preparation used must also be functional. This differs significantly from work with higher plants, where PPCs can be isolated from thylakoids regardless of their functionality.

Many of the studies with diatoms done to date have utilized only crude membrane preparations, making the determination of the precise polypeptide composition of an isolated PPC difficult. In addition, the 77K fluorescence properties of the thylakoid preparations used in many studies have not been characterized. This is a serious problem in that dramatic alterations in 77K fluorescence emission spectra have been observed upon disruption of cells of *Phaeodactylum tricornutum* (Chrystal and Larkum, 1988). Alterations in the low-temperature fluorescence emission spectrum are indicative of a disruption in excitation energy transfer between PPCs within the membranes, and may in some cases be caused by alterations in the structure of the PPCs themselves. Finally, some of the more interesting phenomena observed in chromophytes, including the regulation of excitation energy transfer (Berkaloff et al., 1984; Owens, 1986a), the nature of the rapid fluorescence quenching in diatoms (Owens, 1986a; Caron et al., 1987), and the heterogeneity exhibited by PS II (Owens, 1986b) could be more extensively studied in purified thylakoid preparations.

1.6 Purpose of this work

The purpose of this work was to determine some of the organizational features of the photosynthetic apparatus of one group of chromophytic algae, the diatoms (Bacillariophyceae). Diatoms were chosen over other chromophytes as the experimental organism because of their ecological importance and because they are comparatively easy to culture and can be grown to sufficiently high densities in a short period of time. The first part of the work (Martinson et al., in prep.; Chapter 4) describes a method for purifying thylakoid membranes from a marine diatom, *Cylindrotheca fusiformis*, and outlines the conditions

necessary for maintaining oxygen-evolving capabilities. The thylakoid membranes were characterized with respect to their polypeptide composition, spectral characteristics, and pigment composition. Oxygen-evolving thylakoid membranes have not previously been purified from diatoms, and moreover, the thylakoid preparations used by others have been poorly characterized with respect to their biochemical and biophysical properties.

In the second part of the work, several pigment-protein complexes were isolated using mildly denaturing LDS-PAGE. These studies, in addition to underscoring the importance of using thylakoid preparations of high integrity when purifying PPCs, have revealed fundamental differences in the stability of the PS I reaction center in diatoms relative to that found in a variety of other algae and higher plants (Martinson and Plumley, in prep.; Chapter 5). In conducting this research, a method for extracting pigments from PPCs contained in polyacrylamide was developed and demonstrated to be superior to commonly used methods (Martinson and Plumley, 1995; Chapter 3).

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Chapter 2

Isolation and Characterization of Plant and Algal Pigment-Protein Complexes[1]

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2.1. Introduction

Pigments such as chlorophyll (Chl) and carotenoids enable oxygen-evolving photosynthetic organisms to harvest the light energy needed for photosynthesis. Although pigment-binding protein complexes were isolated from higher plants in the mid-1960s,[1,2] it wasn't until a decade later that Markwell et al.[3] provided convincing evidence that all the Chl present in photosynthetic membranes was bound to protein. Since then numerous methodological advancements have permitted us to greatly increase our understanding of plant and algal pigment-protein complexes (PPCs). This review will cover some of the more important methods routinely used in our lab to isolate and characterize PPCs.

The numerous PPCs in plants and algae can be divided into two main groups based on their role in photosynthesis: the reaction centers and light-harvesting complexes. The reaction centers, Photosystem I (PS I) and Photosystem II (PS II) convert light energy into chemical energy, while the light-harvesting complexes (e.g. LHC I and LHC II) enlarge the cross-sectional absorption area of the reaction centers. The reaction centers have been highly conserved throughout evolution, with little variation in their structure and function.[4] In contrast, LHCs from different types of photosynthetic organisms can be quite different, both in structure and pigment composition. An exhaustive discussion of all the different types of PPCs is beyond the scope of this paper, and the reader is directed to recent reviews.[4,5] Specific information on PS II,[6,7] PS I,[8,9] higher plant/green algal LHCs,[5,10] phycobilisomes,[11] and LHCs from Chl *c*-containing algae[4,12] is also available.

PPCs are technically difficult to isolate because the majority of them are hydrophobic, membrane-embedded structures (exceptions include phycobilisomes and the peridinin-Chl *a* complex). The first step involved in the isolation of membrane-embedded PPCs is the purification and recovery of photosynthetic membranes. In the next step, detergents are used to disrupt the lipid matrix of the membranes, thereby solubilizing the hydrophobic PPCs.

However, the proteins and pigments in PPCs are bound to one another through weak noncovalent interactions and are easily dissociated by many detergents. Over the past decade a number of detergents have been found which will solubilize PPCs without dissociating them. The solubilized complexes can be isolated using a variety of methods (e.g., sucrose density gradient centrifugation, nondenaturing PAGE) and characterized in terms of their biochemical and biophysical properties. In this chapter, we will begin by describing the methods for purifying thylakoid membranes from various types of samples (e.g., higher plant leaves, algal cells), followed by a brief discussion of detergent solubilization protocols. Next we will present methods for resolution and recovery of PPCs and the subsequent determination of their pigment and polypeptide compositions. An overview of methods for the biophysical characterization of PPCs will conclude the chapter.

2.2. Starting material

PPCs can be obtained from unfractionated samples such as leaves or algal cells, or from isolated chloroplasts, thylakoid membranes, or sub-thylakoid preparations (e.g. granal and stromal lamellae). The choice of starting material may be based on a number of factors, including ease of preparation, the type of plant/alga being studied, and whether the PPCs that one is attempting to purify are membrane-bound or soluble.

Algal cells and leaf tissue homogenates are the simplest type of starting material in that they are readily prepared from plants and most algae. These homogenates are crude, however, and contain large amounts of non-PPC protein. This will necessitate the use of higher concentrations of detergent that may lead to the dissociation of PPCs. In addition, PPCs are more likely to be contaminated with non-PPC proteins, making accurate determinations of their polypeptide composition impossible. Cell and tissue homogenates therefore have a limited use as a starting material for purifying membrane-bound PPCs.

However, they are the starting point for purifying water-soluble PPCs such as phycobilisomes[13] and the peridinin-containing complexes of dinoflagellates.[14]

Chloroplasts, thylakoid membranes, and sub-thylakoid preparations are considerably cleaner than cell or tissue homogenates, although chloroplasts still contain large amounts of non-PPC protein (e.g. chloroplast envelope proteins, stromal proteins such as RUBISCO). For most types of work, it is preferable to use purified thylakoid membranes which contain fewer contaminating polypeptides. This reduces the amount of detergent required to solubilize the PPCs and decreases the likelihood that contaminating polypeptides will be misidentified as components of a given PPC. When isolating PPCs from green plants and algae, it may be useful to separate thylakoids into granal and stromal lamellae,[15,16] as PS II and PS I are preferentially localized in these two types of membranes, respectively.[17]

Here, we present methods for purifying thylakoid membranes from higher plants and a variety of algae. Since we do not routinely use chloroplasts to purify PPCs, procedures for isolating chloroplasts are not presented in detail. Higher plant chloroplasts can be isolated according to Joy and Mills,[18] Mishkind et al.,[19] and Walker et al.[20] Methods for isolating chloroplasts from green algae are discussed by Price et al.[21] Chloroplasts from brown algae,[22,23] a chrysophyte,[24] and a diatom[25] can be isolated as described.

2.2.1 Purification of thylakoid membranes

The protocols outlined below for purifying thylakoid membranes from higher plants and most types of algae are adapted from methods described by Chua and Bennoun.[26] All samples and solutions should be kept on ice or at 4°C throughout the procedures that follow.

2.2.1.1 Method for higher plants

Chop or cut 30 g of deribbed leaves using a razor blade or scissors. Note, 30 g of spinach will yield purified thylakoids containing about 5 mg Chl *a* + *b*, sufficient for several PPC purifications. Leaves with thick cuticles and/or leaves with few chloroplasts per unit weight will require more material to obtain these same yields. Alternatively, the protocol can be easily scaled down if tissue is scarce or if numerous samples are to be analyzed (e.g., if screening putative PPC mutants). Add 90 mL GR (prepare fresh; 0.33 M sorbitol, 2 mM (disodium) EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM Hepes-KOH, pH 8.2) to the chopped tissue. For plants containing high levels of phenolic compounds, polyvinylpyrrolidone (PVP-40) can be added to a final concentration of 4%.[22] Strbac et al.[23] suggest using 1.2% BSA and alkaline (pH 8) Tris-borate to prevent damage by polyphenols when isolating chloroplasts and thylakoids from brown algae. A more complete discussion of methods for dealing with phenolics is given by Loomis.[27]

Homogenize with a Polytron (Brinkmann Instruments) using three bursts of 5 s each or with an ice cold blender using three or four 10-15 s bursts at medium speed. A cooling period between bursts is recommended as longer bursts may cause heat dissociation of PPCs; larger amounts of leaf material and/or leaf material that is tougher will require more bursts. Filter through four layers of miracloth or 8 layers of cheesecloth to remove cellular debris. Centrifuge extract in three 50 mL tubes at 10,000 g for 5 min and discard supernatants (PPCs can be isolated from the crude chloroplast pellet if desired). Resuspend each pellet in 5 mL Buffer 2 (0.3 M sucrose, 5 mM Hepes-NaOH pH 7.5, 10 mM EDTA, 1 mM ACA, 40 μM BAM) and homogenize using a motor-driven Teflon pestle (MDTP) (it is expedient to use a pestle which fits tightly into 50 mL centrifuge tubes). Dilute each tube to 30 mL with Buffer 2 and mix. Centrifuge at 42,000 g for 10 min and discard the supernatant containing soluble proteins (PPCs can be purified from the crude chloroplast-membrane pellet if desired).

Resuspend each pellet in 5 mL Buffer 3 (1.8 M sucrose, 5 mM Hepes-NaOH pH 7.5, 10 mM EDTA, 1 mM ACA, 40 μM BAM), homogenize with MDTP, transfer each sample to a 13-mL ultracentrifuge tube (e.g. Beckman polyallomer), and purify thylakoids by flotation through sucrose gradients.

To purify thylakoids using sucrose step gradients,[26] overlay the crude thylakoids obtained above (in Buffer 3) with 4 mL of Buffer 4 (same composition as Buffer 3, but with 1.5 M sucrose). The remainder of each tube is then filled with Buffer 5 (same composition as Buffer 3, but with 0.5 M sucrose). Thylakoids are floated by ultracentrifugation of the step gradients in a swinging bucket rotor (e.g. Beckman SW40). For most plants and algae, centrifugation at 284,000 g for 1 h is sufficient. Centrifugation should be done at 4°C, and gradual acceleration/deceleration employed. After centrifugation, purified thylakoid membranes are collected at the Buffer 4/5 interface with a Pasteur pipet and placed in 50 mL centrifuge tubes. Dilute thylakoid membranes with 3 to 4 volumes of Buffer 6 (5 mM Hepes-NaOH pH 7.5, 10 mM EDTA, 1 mM ACA, 40 μM BAM) and mix well with vortex mixer. Centrifuge at 40,000 g for 15-30 min to pellet thylakoids. Discard supernatant and resuspend pellet in a minimal volume of: a) 100 mM sorbitol, 15 mM Tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$[28] for storage; b) Gel Sample Buffer (100 mM Tris-HCl, pH 8.5, 100 mM dithiothreitol (DTT), 5 mM ACA, 1 mM BAM) for quick preparation of samples for denaturing SDS-PAGE (see 5.1.2.3); or c) another buffered solution such as the one to be used for detergent solubilization (see 3.2). Determine the Chl concentration of the concentrated thylakoids (see 5.2.1) and dilute if desired to appropriate concentration; to store for SDS-PAGE, we dilute to 1.6 mg total Chl/mL, but a higher (2-4 mg total Chl/mL) concentration is often preferable for purification of PPCs. Thylakoids should be frozen in small (e.g. 0.060-1 mL) aliquots at -60°C.

2.2.1.2 Method for most unicellular algae

Harvest cells from a 300 mL mid- to late-log phase culture by centrifugation at 5000 g for 5 min. Resuspend pellet in 20-30 mL Buffer 1 (0.3 M sucrose, 5 mM Hepes-NaOH pH 7.5, 1 mM $MgCl_2$) and transfer to 50 mL centrifuge tube. Centrifuge at 5000 g for 5 min, resuspend pellet in 20 mL Buffer 1, and add ACA (10 mM) and BAM (2 mM). Break cells using either pressure (French or Yeda Press) or sonication. Since different species vary widely with respect to the ease with which they are broken, it is usually necessary to experiment to find the optimal conditions for breakage. Breakage can be monitored microscopically. Guenther and Melis[29] used a Yeda Press at 13.7 MPa (~2000 psi) to break cells of *Dunaliella salina*. We use a French Press at 5000-15000 psi (34-104 MPa) depending on the species. The chamber for the French press should be refrigerated overnight before use. For sonication, we use a sonicator (Bronwill Scientific) with a 1.25 cm diameter probe. For optimal results, the diameter of the container used for sonication should be no larger than 3 times that of the probe. Since different algae vary in the ease with which they are broken, the intensity, duration, and number of bursts necessary to obtain maximal cell breakage must be determined experimentally. Bursts lasting 5-30 s at intensities ranging from 75-120 W are typical. To avoid excessive heating, samples should be kept in an ice bath during the procedure and allowed to cool for 1-2 min between bursts.

After breaking cells, immediately add PMSF to 1 mM. Divide among two 50 mL tubes and homogenize with an MDTP. Centrifuge at 40,000 g for 20 min, discard supernatant, resuspend each pellet in 5 mL Buffer 2 (see 2.1.1), and homogenize with an MDTP. Dilute to 30 mL with Buffer 2 and mix. Centrifuge at 40,000 g for 20 min, discard supernatants, resuspend pellets in 5 mL Buffer 3 (see 2.1.1), homogenize with an MDTP, and transfer each sample to a 13-mL ultracentrifuge tube (e.g. Beckman polyallomer). Purify thylakoids by flotation through sucrose gradients as described above for higher plants (see 2.1.1).

2.2.1.3 Method for diatoms

Purification of thylakoids from diatoms and other Chl *c*-containing algae presents special problems. An extreme heterogeneity in the properties of thylakoids of different diatom species has been noted previously[30] and well documented in our lab. Protocols for purification of thylakoids from diatoms are only now being developed, and still require some minor modifications for each species, but the protocol outlined below appears to be broadly applicable. An important observation is that sorbitol is a better osmoticum than sucrose for purification of thylakoids and PPCs from diatoms.[31]

Harvest 3 L of culture by centrifugation at 3,000 g for 10 min in 500 mL centrifuge bottles. Culture volume, speed and time need to be optimized for each species. Resuspend the pellet in one bottle with 30 mL of Buffer A (1 M sorbitol, 20 mM MES pH 6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ACA, 1 mM BAM) and sequentially use this to resuspend cells in the remaining centrifuge bottles. Dilute to 60 mL with Buffer A. Break cells in 10 mL aliquots using a sonicator (Bronwill Scientific) with a 1.25 cm diameter probe. For optimal results, the diameter of the container used for sonication should not exceed three times that of the probe and samples should be kept in an ice bath during sonication. The intensity and duration of the bursts used to break the cells will depend on the species of diatom; breakage is monitored microscopically. We typically use one or two bursts of 15 s at 120 W (cooling the probe in ice for 30 s in between each burst) and then centrifuge at 1000 g for 5 min to pellet unbroken cells and debris. The supernatants are transferred to separate 50 mL centrifuge tubes and stored on ice in darkness. Pellets are resuspended in 10 mL Buffer A and sonicated as before. Typically, 3-4 cycles of sonication/centrifugation are necessary to achieve 95% breakage. After each centrifugation, the supernatants are combined.

After breakage is complete, centrifuge the supernatants at 40,000 g 15 min. Discard supernatants, resuspend each pellet in 10 mL Buffer B (1 M sorbitol, 20 mM MES pH 6, 5 mM

EDTA pH 7.5, 5 mM ACA, 1 mM BAM), homogenize with an MDTP, and dilute to 30 mL with Buffer B. Centrifuge at 40,000 g for 15 min, discard supernatants, resuspend each pellet in 5 mL Buffer C (4.4 M sorbitol, 5 mM MES-NaOH pH 6, 5 mM ACA, 1 mM BAM), homogenize with an MDTP, and transfer each sample to a 13-mL ultracentrifuge tube (e.g. Beckman polyallomer). Purify thylakoids by flotation through sorbitol gradients.[31] Overlay the crude thylakoids with 2 mL of Buffer D (same composition as Buffer C, but 3.8 M sorbitol), and 3 mL of Buffer E (same composition as Buffer C, but with 3.2 M sorbitol). The remainder of each tube is filled with Buffer F (same composition as Buffer C, but with 2.5 M sorbitol) and thylakoids are floated by ultracentrifugation in a swinging bucket rotor (e.g. Beckman SW40) at 284,000 g for 16-18 h at 4°C using gradual acceleration/deceleration. Collect purified thylakoids at the Buffer E/F interface with a Pasteur pipet and place in 50 mL centrifuge tubes. Dilute with an equal volume of Buffer G (5 mM MES pH 6, 5 mM ACA, 1 mM BAM) and mix well. Centrifuge at 40,000 g for 15-30 min to pellet thylakoids. Discard supernatant and resuspend pellet in a minimal volume of Buffer H (1 M sorbitol, 5 mM MES pH 6, 5 mM ACA, 1 mM BAM). Determine the Chl concentration of the concentrated thylakoids (see 5.2.1), dilute to appropriate concentration (2-3 mg total Chl/mL), and freeze in small (e.g. 0.060-1 mL) aliquots at -60°C. The integrity of the thylakoid membranes can be verified by performing a functional assay (e.g. O_2 evolution)[32]. Alternatively, the 77K fluorescence emission spectrum (section 6.2) of the crude membranes should be qualitatively unchanged relative to that of whole cells.

2.3. Detergent solubilization

As noted above, most PPCs are hydrophobic, intrinsic membrane complexes that require detergents for solubilization. A confounding issue, however, is that PPCs are easily dissociated by many detergents. Detergents are amphiphilic molecules which can either form

micelles or partition into a lipid bilayer. The critical micelle concentration (cmc) is the concentration at which the detergent molecules begin to coalesce into micelles. Above the cmc, an equilibrium exists between micelles and detergent monomers. When mixed with lipid membranes, monomeric detergent molecules partition into the lipid bilayer. As the concentration of detergent increases, more monomers partition into the bilayer, thereby destabilizing it. Solubilization of the lipid membrane occurs when the concentration of detergent is equal to or greater than the cmc. As the detergent to protein ratio is increased, micelles consisting of protein, lipid, and detergent are produced, while still higher ratios will produce detergent/protein and detergent/lipid micelles.[33] Detergent micelles containing proteins and/or lipids are generally considered to be "soluble" if they do not pellet when centrifuged at 105,000 g for 1 h,[33] however, some very large complexes (e.g. PPCs such as PS I) may pellet at these speeds.[34]

The exact ratio of detergent to protein at which a given type of membrane is solubilized depends on a number of factors, including the type of detergent and the nature of the proteins and lipids in the membrane. To date, no specific method exists for determining which detergent to use for a particular application. Determination of the type of detergent to use, as well as the detergent:protein ratio needed for solubilization, must be determined empirically for a particular system.[33-36]

2.3.1 Types of detergents

A wide variety of detergents are available, each differing in their charge, size, and critical micelle concentration (cmc). In general, detergents are divided into four main classes: non-ionic, anionic, cationic, and zwitterionic. Non-ionic detergents are generally considered to be less destructive, and are frequently employed when one wishes to retain the biological activity and/or native structure of the protein of interest. Findlay[34] and Neugebauer[35] provide

information on specific detergents. A successful strategy for isolation of PPCs is to use two detergents in combination, one being a stronger, (ionic) detergent for membrane disruption and the other a gentler (non-ionic) detergent which may help prevent dissociation of pigments from PPCs.

2.3.2 Detergent solubilization of thylakoid membranes

A large number of protocols for solubilizing thylakoid membranes exist in the literature, some of which are presented in Table 2.1. While this may be confusing to researchers unfamiliar with the field, there are a number of common features. As a rule of thumb, all solubilization solutions should contain the following: one or more detergents (discussed below), a buffer, and protease inhibitors. Typical buffers used in isolating PPCs include Tris, Tricine, MES, and Hepes, with final concentrations between 5 and 50 mM, and pH values ranging from 6 to 8.5. The protease inhibitors ACA (5 mM), BAM (1 mM), and PMSF (1 mM) are widely used.

The two most important factors to keep in mind when developing a solubilization procedure are the type of detergent and its concentration. Choice of detergent largely depends on the complex that one wishes to isolate; for instance, the PS I complex of green plants is very stable to most detergents whereas the PSII complex is considerably more labile. Some of the more commonly used detergents include digitonin (DIG), deoxycholate (DOC; precipitates at pH values less than 6.3), dodecylmaltoside (DM), octylglucoside (OG), Triton X-100 (TX-100), and lithium dodecyl sulfate (LDS). Sodium dodecyl sulfate (SDS), although less expensive than LDS, is insoluble at low temperature, thus limiting its use in purifying PPCs at 0-4°C. When choosing a concentration of detergent to use, it is important to keep in mind that since Chls and carotenoids are lipids, they may be removed by detergents. Use of high detergent:protein ratios may increase the loss of pigment molecules from the protein

complexes. Therefore, it may be more helpful to consider the ratio of detergent to Chl (rather than protein) when solubilizing photosynthetic membranes. We have found that detergent:Chl ratios of 2-20:1 are suitable for most detergents, except DIG, which can be used at higher concentrations (40-60:1) because it is relatively gentle. It is important to note that a single solubilization step may not be sufficient to purify a particular complex to homogeneity. In this case, treatment of a partially purified PPC crude complex with a second detergent may be necessary.

The protocols presented in Table 2.1 are divided into three groups, depending on the method used to resolve the PPCs after solubilization. The three methods are mild LDS-PAGE,[37] sucrose density gradient ultracentrifugation,[38-40] and column chromatography,[41,42] which are discussed in Section 4.

2.4. Resolution of pigment-protein complexes

After solubilization of the plant membranes with detergents, the liberated PPCs are routinely resolved by gel electrophoresis (Fig. 2.1), sucrose density gradient ultracentrifugation (Fig. 2.2), and/or column chromatography. Mild ("green gel") LDS-PAGE is primarily an analytical tool in that further fractionation of the PPCs is difficult, in part because they are embedded in a polyacrylamide matrix but also because they are usually in their simplest (i.e., smallest) form. In contrast, PPCs obtained via chromatography or sucrose density gradient centrifugation tend to be larger, are recovered without a solid matrix, and can be treated further, perhaps with a second detergent, to resolve additional complexes. For example, PS I-LHC I particles may be purified using one detergent and then treated with a second detergent to release LHC-I. Finally, it should be pointed out that co-migration (electrophoresis), co-sedimentation (sucrose density gradients), or co-elution (chromatography) of complexes may occur, leading to erroneous conclusions about the biochemical composition and biophysical

properties of a given complex. The best solution to this problem is to use a combination of methods: first resolving a complex on the basis of density (sucrose density gradients), and then by size (gel filtration) or charge (ion exchange chromatography).

2.4.1 Mild "Green gel" LDS-PAGE

A variety of PAGE systems have been used to resolve plant PPCs.[5] The different systems vary as to the amount of free pigment that is produced during electrophoresis, as well as with regard to the pattern of complexes on the gel. Some gel systems use either Deriphat[43] or DOC[30] to reduce the amount of pigment released from complexes, but LDS is still widely used. For resolving PPCs, it is possible to use either slab or tube gels. A benefit of the latter is that larger sample volumes can be loaded (e.g. 200 μl for a 5 mm (I.E.) tube compared to 80 μl for conventional slab gels). However, the resolution of PPCs on slab gels is generally higher than that obtained with tube gels.

For mild LDS-PAGE, we use a 0.1 cm thick gel with a 3.5 cm stacking gel and 20 cm resolving gel. Although these large format gels take longer (10-12 h) to run, we have found that they give better resolution of PPCs than do the shorter mini-gels. The stacking gel contains 5% acrylamide/0.13% bisacrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% TEMED, and 0.1% ammonium persulfate (AP). For the resolving portion of the gel, polyacrylamide concentrations ranging from 8 to 10% are suitable for separating most PPCs, although linear gradients of polyacrylamide (e.g. 7.5-15%) may provide greater resolving power. A good general-purpose gel contains 8% acrylamide/0.21% bisacrylamide, 375 mM Tris-HCl, pH 8.8, and 10% glycerol, with polymerization achieved by adding 0.03% TEMED and 0.02% AP. The reservoir buffers contain 383 mM glycine and 50 mM Tris Base with 0.1% SDS in the upper reservoir buffer. Gels and buffers should be refrigerated for 4-6 h prior to loading samples, and electrophoresis should be done in darkness at 4°C to enhance stability of the

PPCs.

2.4.2 Sucrose density gradient ultracentrifugation

Resolution of PPCs using sucrose density gradient ultracentrifugation may be done using either linear (continuous) or step (discontinuous) gradients. Linear gradients provide a higher resolving capacity, while step gradients are frequently useful when purifying a single PPC. Linear gradients of sucrose can be prepared using either a gradient maker (e.g., Bio-Rad) or by freezing and thawing. For the latter, centrifuge tubes (Beckman polyallomer or other tube suitable for ultracentrifugation) are filled with a solution containing 30% sucrose, 50 mM buffer, 5 mM ACA, 1 mM BAM, and 0.01-0.1% detergent and subjected to three cycles of freezing (-20°C for >6 h) and thawing (room temperature). This procedure will form gradients that are approximately 0-60% sucrose. For resolving PPCs that differ slightly in density, 0-30 and 0-40% gradients are frequently useful. Step gradients are formed by sequentially layering solutions of decreasing sucrose concentration in a centrifuge tube. Solutions differing in concentration by increments of 10% are easily layered; layering of solutions that differ in sucrose concentration by less than 10% is hampered by turbulent mixing. Centrifugation in a swinging bucket rotor at 280,000 g for 16-18 h is generally sufficient to attain equilibrium. Gradual acceleration and deceleration should be used to avoid disturbing the gradients. Gradients should be kept cold (0-4°C) at all times, and exposure of samples to light should be minimized. Pigmented fractions are collected from the gradient using a Pasteur pipet.

2.4.3 Column chromatography

Ion-exchange and size exclusion chromatography are useful techniques for isolating PPCs, particularly in the later stages of purification. Excellent discussions of these techniques are given by Scopes[44] and Stellwagen.[45] In general, these methods are not suitable for

resolving several PPCs at once, but are most frequently used to purify a single complex.

For purification of PPCs using ion-exchange chromatography, anion exchange (e.g. DEAE, QAE) is almost always used (as opposed to cation (e.g. CM) exchange). Empirical methods for choosing a suitable ion-exchange column material for a specific application are presented in Scopes.[44] PPCs are commonly eluted from an ion-exchange column using gradients of salts such as NaCl or KCl (see Table 2.1).

A variety of column packings are available for gel filtration, and numerous advances have been made in recent years with regard to matrix stability.[44] Packing materials such as AcA34 (LKB, 20-350 kiloDaltons; kD) and Sephadex G200 (Pharmacia; 10-600 kD) may be useful for separating small PPCs such as LHCs. For purification of larger complexes (e.g. trimeric PS I), it may be necessary to use materials such as Sephacryl S-400 (Pharmacia; 10-4000 kD) or HW-65 (Merck/Toyo Soda/Fractogel TSK/Toyopearl; 30-3000 kD).

2.5. Biochemical characterization of pigment-protein complexes

2.5.1 Determination of polypeptide composition

Once the PPCs have been resolved, their polypeptide composition can be determined using denaturing SDS-PAGE. Excellent discussions of the principles of PAGE can be found in Hames,[46] Garfin,[47] or in the text by Allen and Budowle.[48] The method below is suitable for analysis of PPCs in either polyacrylamide gel slices or sucrose density gradient fractions.

2.5.1.1 Composition of gels and running buffers

For optimal resolution of polypeptides using denaturing SDS-PAGE, it is once again preferable to use long (20L x 30W x 0.1T cm) gels as opposed to mini-gels. A linear gradient of 10-20% or 7.5-15% polyacrylamide (acrylamide:bisacrylamide = 37.5) resolves polypeptides from 5-200 kD, and is suitable for analyzing the polypeptide composition of most

plant PPCs. In addition to polyacrylamide, the resolving gel contains 375 mM Tris-HCl (pH 8.8), 0.03% TEMED, 0.02% AP, and a gradient of 5-12.5% sucrose (used to help hold the acrylamide gradient during polymerization), while the stacking gel consists of 5% acrylamide/0.13% bisacrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% TEMED, and 0.1% AP. The running buffers contain 383 mM glycine and 50 mM Tris Base, with 0.2% SDS present in the upper reservoir buffer.

2.5.1.2 Sample preparation

2.5.1.2.1 Gel slices from "green gels"

PPCs resolved using mild LDS-PAGE should be excised from the gel with a razor blade immediately after electrophoresis and equilibrated in 125 mM Tris-HCl (pH 6.8), 1 mM DTT, 5 mM ACA, 1 mM BAM, and 0.1% SDS for 30-60 min at room temperature. The polyacrylamide slices should not be heated, as this may cause irreversible aggregation of the proteins and/or damage due to acrylic acid. After equilibration, the gel slices can be inserted into the wells of a 10-20% or 7.5-15% gel, and sealed with a solution of 0.1% agarose, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, and 0.2% bromphenol blue. Gels should be run at 16 mA for 16-20 h, and at room temperature to insure denaturation of the proteins.

Alternatively, an entire gel lane can be subjected to second-dimensional denaturing SDS-PAGE.[49] This method can help identify contaminants which are comigrating with a PPC, because proteins that are denatured during the first dimensional run will form a single diagonal band of protein in the second dimension (Fig. 2.3), whereas any polypeptide above or below this main diagonal was part of a PPC in the first dimension. Electrophoresis of a gel lane is done much as for a gel slice, except that the flat edge of a gel comb is used to create a single well that runs nearly the entire length of the second-dimension gel. The well should be no more than 0.5 cm deep; deeper wells pose problems when positioning the gel lane. Pieces of

gel spacer ≈ 1 cm wide can be used to create wells in the stacking gel for molecular weight markers or thylakoid standards. Gel lanes, trimmed to ≈ 1 cm width, should be excised from the first-dimensional gel immediately after electrophoresis. Each lane is then carefully laid over the top of the second-dimension gel and gently pushed into the well before being sealed with agarose (see above). To ensure complete denaturation of the complexes, gels are run at room temperature at 16 mA for 20-24 h.

2.5.1.2.2 Sucrose density gradient fractions

To prepare sucrose density gradient fractions for denaturing SDS-PAGE, a solution of 10% LDS, 312.5 mM Tris-HCl pH 8.5, and 312.5 mM DTT (5X solubilization buffer) is added to a final concentration of 1X. For samples that are not heavily pigmented, bromphenol blue can be added to a final concentration of 0.3-0.5% to aid in visualization of the sample. Samples are then heated at 100°C for 1-2 min (or 70°C for 3 min if TX-100 is present) prior to loading. Since the proteins have been fully denatured by heating, gels can be run at 4°C to increase the sharpness of the polypeptide bands.

2.5.1.2.3 Preparation of standards

Polypeptides on denaturing SDS-PAGE gels are usually described in terms of their apparent molecular weight (M_r). Premixed solutions of proteins with known M_rs are available commercially (Sigma) and are prepared as described by the manufacturer. For analysis of PPCs, it is frequently useful to run a lane of denatured thylakoid membranes as a reference, particularly if the identity of some or all of the proteins is known (e.g. from immunological work).

To prepare thylakoid membranes for denaturing SDS-PAGE, an aliquot corresponding to 15 μg Chl is brought to 18 μl in Gel Sample Buffer (see 2.1.1), and 12 μl of a solution

containing 30% sucrose, 5% LDS is added. The sample is heated at 100°C for 1 min and placed on ice for a few minutes prior to loading. Loading 10 μg of Chl (20 μl) per lane will result in a polypeptide profile that is readily visible when stained with Coomassie Blue (5.1.3.1).

2.5.1.3 Visualization of polypeptides

After electrophoresis, proteins can be visualized by staining gels with either Coomassie Brilliant Blue or with silver. It is recommended that gels be stained with Coomassie first, destained to remove most of the Coomassie, and then stained with silver if necessary. Some photosynthetic proteins do not stain well with Coomassie (e.g. D1 and D2), and so silver must be used. In order to keep track of the orientation of the gel, it is helpful to cut off the lower right-hand corner.

2.5.1.3.1 High-sensitivity Coomassie Blue stain[50]

We have found this method to have superior sensitivity over other Coomassie protocols and it requires less methanol which is a possible health hazard. To prepare the stock solution, 100 g ammonium sulfate is dissolved in approximately 500 mL ddH_2O containing 12.5 mL 85% phosphoric acid. After bringing to 1 L with ddH_2O, 1 g Coomassie Brilliant Blue G-250 is added. The solution should be stored (unfiltered) in a sealed bottle at room temperature and mixed well before each use. Stain gels in 80% stock solution/20% methanol for 10-12 h at room temperature with gentle agitation (gels do not have to be fixed prior to staining). Destain in ddH_2O or briefly in 25% methanol. Gels can be stored in ddH_2O or in 30% methanol/7% acetic acid.

2.5.1.3.2 Silver-staining method[51]

If the gel has been stained previously with Coomassie Blue (5.1.3.1), it should be destained extensively (up to several days) in 25% methanol, and washed overnight in ddH_2O. Prepare the following solutions just prior to use: Solution A, 0.0625 g sodium dithionite in 250 mL ddH_2O; Solution B, 0.5 g silver nitrate, 19 μl 37% formaldehyde in 250 mL ddH_2O; Solution C, 15 g sodium carbonate, 112 μL 37% formaldehyde, 0.0016 g sodium thiosulfate-5-hydrate in 250 mL ddH_2O. To stain gels, sensitize gel 1 min in Solution A then rinse 3 X 20 s in ddH_2O. Add Solution B and agitate 20-30 min at room temperature. After staining, rinse 2 X 20 s in ddH_2O (Note: silver can be precipitated from waste Solution B with solid NaCl). Develop gel in Solution C for 5-20 min, depending on how much protein is present on the gel. Stop further development by addition of 9 mL acetic acid, and rinse 4 X 30 min in ddH_2O. It is best to photograph gels immediately, as they tend to yellow with time. Store gels in 30% methanol, 7% acetic acid.

2.5.1.4 Immunochemical identification of proteins

PPC proteins can also be visualized and, with some procedures, quantified by immunological methods. Both rocket- and crossed-immunoelectrophoretic conditions suitable for analysis of PPC proteins in 2% SDS have been developed.[39,52-55] These protocols provide extensive quantitative (RIE and CIE) and qualitative (CIE) information, but are limited in that they require large quantities of polyclonal antibodies. For this reason, they have been largely supplanted by western blots.[55 56] Details for western blotting are covered by XX in Chapter XX of this volume. One note, however, is that hydrophobic PPC proteins with high M_rs tend to be extremely difficult to transfer to membranes. For instance semi-dry techniques do not work well for PS I apoproteins and wet transfer of these proteins requires 24-48 h for complete transfer if quantitative data are required.

2.5.2 Determination of pigment composition

A variety of pigments are present in plant PPCs. In addition to Chls *a* and *b*, higher plant complexes also contain carotenoids such as ß-carotene, lutein, violaxanthin, and neoxanthin. Other carotenoids (e.g. zeaxanthin, antheraxanthin) are also present in minor quantities and are associated with various LHCs. Green algae generally contain the same major carotenoids as higher plants, but may also contain others such as loroxanthin, prasinoxanthin, or siphonaxanthin. Diatoms and other chromophytes contain Chl *a* and one or more of the three forms of Chl *c*. In addition, carotenoids such as ß-carotene, fucoxanthin, diadinoxanthin, diatoxanthin, peridinin, and dinoxanthin may also be present. A more complete review of algal carotenoids is available.[57]

Pigments are routinely extracted from PPCs using organic solvents (e.g. acetone, methanol) and analyzed by absorption and/or fluorescence spectroscopy. Resolution of pigments using thin-layer or high-performance liquid chromatography (e.g. TLC or HPLC) is also common, and the individual pigments that are resolved may subsequently be characterized by spectroscopy. Here, we present methods for extracting pigments from both thylakoid membranes and isolated PPCs, and for their subsequent quantification and/or identification using absorbance spectroscopy and chromatography.

2.5.2.1 Extraction of pigments using acetone

Acetone is used extensively to extract pigments, and although a number of technical problems arise when extracting pigments from PPCs (discussed in 5.2.2), it remains the solvent of choice for rapid quantification of pigments using absorption spectroscopy. When using acetone it is important to note that although chlorophylls can be extracted in 80% acetone, extraction of the more polar carotenoids such as ß-carotene requires use of ≥ 90% acetone.[58] Extractions should be done at 0°C and in dim light to prevent photochemical

damage to the extracted pigments. In addition, acetone should be supplemented with $MgCO_3$ (saturating) or NH_4OH (0.01 N) in order to prevent damage to pigments (e.g. pheophytinization/loss of Mg) which may occur due to acidification of the extract.

In general, no preparation of thylakoids is necessary prior to extraction of pigments with acetone. However, it is important to note that the formation of sucrose- or sorbitol-containing phases which trap pigments may occur upon addition of acetone to thylakoids that are in a buffer containing high concentrations (e.g. 1-2 M) of sucrose or sorbitol. As long as thylakoids are diluted in ddH_2O prior to addition of acetone (see below), this is generally not a problem. Problems will occur, however, if 80 or 90% acetone is added directly to the thylakoids or vice versa.

To prepare PPCs in aqueous solution (e.g. from a sucrose density gradient or column) for extraction of pigments using acetone, sucrose must first be removed if present at a high concentration. The fractions can either be diluted and centrifuged at high speeds (e.g. 200,000 - 400,000 g) for 3-24 h, or washed by centrifugation in a microconcentrator (Amicon, Whatman). Both of these treatments will also allow concentration of the PPC material prior to extraction. PPCs contained in polyacrylamide must be eluted from the gel slice prior to extraction of pigments because addition of 80-90% acetone causes dehydration of the gel slice, thereby trapping the pigments. PPCs can be eluted by grinding the gel slice to a fine slurry in ddH_2O using an MDTP. Elution generally takes several hours to a few days, and should be done at 4°C in darkness. Alternatively, an electro-elution device (Bio-Rad, Hoefer) can be used. After elution of the PPCs from the gel slice, a concentration step (as described above) may be necessary.

For extraction of pigments from pelleted PPCs or thylakoids using 80% acetone, resuspend PPC pellets or dilute 7.5-15 μL thylakoids in 300 μL ddH_2O, and slowly add 1.2 mL of 100% acetone. Resuspension of PPC pellets in water is done to prevent aggregation of

proteins and trapping of pigments, which are likely to occur if 80% acetone is added directly to the pellet; the reason for diluting thylakoids has already been discussed above. Alternatively, for complete extraction of ß-carotene[58] or for samples containing Chl *c*, dilute 7.5-15 µL thylakoids or resuspend pelleted PPCs in 150 µL ddH_2O and add 1.35 mL 100% acetone (90% final). For extraction of pigments from PPCs in aqueous solution (e.g. non-pelleted) using 80% acetone, slowly add 1.2 mL of 100% acetone to a 300 µL aliquot (for 90% acetone, use 1.35 mL and 150 µL, respectively).

Pigments are extracted in darkness for 10 min at 0°C, and the extract is clarified by centrifugation at 16,000 g for 5 min. The pigments can then be quantified by absorption spectroscopy (equations follow) or analyzed using chromatographic methods (see 5.2.3). If necessary, pigments can be concentrated by transferring to ether (see 5.2.1.1) and then resuspending in a smaller volume of 100% acetone (saturated with $MgCO_3$) or other solvent suitable for TLC or HPLC (see Table 2.2).

Pigments in acetone can be quantified using absorption spectroscopy and the composition of Chls and carotenoids determined using one of several published equations.[58-60] In the equations below, a concentration factor (F) must be used which accounts for the dilution of pigments in the extract and is determined by dividing the total volume of the extract by the volume of the sample extracted. For example, if 0.5 mL of a PPC-containing sucrose density gradient fraction is pelleted by centrifugation, resuspended in 300 µL ddH_2O, and then extracted in a final volume of 1.5 mL 80% acetone, then F would be 1.5 mL / 0.5 mL, or 3.

For samples extracted in 80% acetone, measure the OD in a 1 cm cell at 663.6, 646.6, and 750 nm. Subtract the OD value at 750 nm from the OD values obtained at 663.6 and 646.6 nm, and then use equations of Porra et al.[59] to determine the concentrations of Chl *a* and *b*:

$$(12.25\ OD_{663.6} - 2.55\ OD_{646.6}) \times F = \mu g\ Chl\ a/mL \quad (1)$$

$$(20.31\ OD_{646.6} - 4.91\ OD_{663.6}) \times F = \mu g\ Chl\ b/mL \quad (2)$$

To calculate the concentration of carotenoids (in 80% acetone), measure the OD at 470 nm then use the equation of Lichtenthaler[58]:

$$(1000\ OD_{470} - 1.82\ C_a - 85.02\ C_b)/\ 198 = \mu g\ carotenoids/mL \quad (3)$$

where C_a and C_b are the concentrations (in μg/mL) of Chl *a* and Chl *b*, respectively.

For samples containing Chl *c* in 90% acetone, measure the OD in a 1 cm cell at 664 and 630 nm. Determine pigment concentrations using the equations of Jeffrey and Humphrey[60]:

$$(11.47\ OD_{664} - 0.4\ OD_{630}) \times F = \mu g\ Chl\ a/mL \quad (4)$$

$$(24.36\ OD_{630} - 3.73\ OD_{664}) \times F = \mu g\ Chl\ c/l\ mL \quad (5)$$

2.5.2.1.1 Concentration of pigments by transferring to ether[61]

To concentrate pigments in dilute acetone extracts by transferring to ether, add 0.267 mL cold water-saturated ether and 0.267 mL cold 4 M NaCl to 1 mL of acetone extract and mix gently. Add 0.53 mL cold ddH_2O, mix well, and centrifuge (3000 g, 5-10 min) to separate the phases. Collect upper (ether) phase and transfer to a clean tube. If pigments remain in the acetone phase, more ether can be added and the sample centrifuged as before. Combined ether fractions are treated with anhydrous sodium sulfite (until the particles become flocculent indicating complete removal of water) and dried under a stream of nitrogen gas. The dried pigments are resuspended in a small volume (10-100 μL) of 100% acetone (saturated with $MgCO_3$) or another solvent compatible with the TLC or HPLC system being used.

2.5.2.2 Extraction of pigments using *sec*-butanol

As mentioned previously, acetone extraction of isolated PPCs is plagued by several technical difficulties. First, PPCs eluted from polyacrylamide gel slices as well as fractions from sucrose density gradients or columns are sometimes diffuse and/or of low abundance. Extraction in 80-90% acetone requires further dilution of the sample, often reducing pigment concentrations below levels that can be readily detected using absorption or even HPLC. Pigments can be concentrated by transferring to ether (see 5.2.1.1), but this may result in modification and/or loss of pigments, especially when pigments are present at the low concentrations typically encountered when working with PPCs. Second, when acetone is added to samples containing sucrose, a sucrose-containing phase is formed which traps pigments, thereby necessitating the removal of sucrose prior to extraction of pigments. The methods described above for eliminating sucrose are tedious, as are elution methods for PPCs contained in polyacrylamide. To overcome these problems, we developed a method using *sec*-butanol which simultaneously extracts and concentrates pigments from PPCs in either aqueous solution or polyacrylamide.[62] The method is as effective as acetone, and the pigments are not chemically modified during extraction (Fig. 2.4). One drawback of the *sec*-butanol method is that Chl concentrations cannot be determined using absorption spectroscopy because extinction coefficients for pigments in *sec*-butanol are not yet available. However, pigments extracted from low abundance PPCs with *sec*-butanol are readily assayed by HPLC, thus making this the solvent of choice for work with most PPC samples.

Pigments can be extracted directly from thylakoids using *sec*-butanol unless the Chl concentration exceeds 1 mg/mL in which case the sample will require dilution with ddH_2O. For PPCs in polyacrylamide, the gel slices must first be ground to a fine slurry in water. We accomplish this by placing the pigmented gel slice in the bottom of a 1.5 mL microfuge tube, barely covering it with water ($\approx 150\mu L$), and homogenizing for 10 to 30 s with an MDTP. In

some cases small gel fragments fall to the bottom of the tube and have to be resuspended by mixing before homogenization can continue; the entire process still requires less than 1 min per sample. Sucrose density gradient and column chromatography fractions can be extracted directly.

After preparation of samples, pigments are extracted from PPCs or thylakoids by adding 2/3 volume of *sec*-butanol and shaking to mix the phases. Extractions are done at 0°C and are generally complete in 5-10 min. Addition of 1/3 original volume 4 M NaCl and centrifugation at 16,000 g for 5-10 min partitions pigments into the *sec*-butanol phase with nearly 100% efficiency. Depending upon their concentration, the pigments may now be further concentrated by addition of water to the *sec*-butanol phase or diluted using water-saturated *sec*-butanol to obtain an optimal working concentration. The pigment-containing *sec*-butanol phase can be injected directly onto an HPLC column (see 5.2.3.2). While it is possible to spot the extract onto TLC plates (see 5.2.3.1), *sec*-butanol does not evaporate quickly and may result in sample origins that are too large. This can be prevented by concentrating the pigments (by adding water) into a very small volume of *sec*-butanol so that only a few μL need to be spotted.

2.5.2.3 Resolution/identification of pigments

Pigments are routinely resolved using either TLC or HPLC. The specific application of these procedures to the resolution of chlorophylls is discussed by Shioi[63] and details for carotenoids are provided by Britton and Goodwin.[64]

2.5.2.3.1 TLC

For TLC of pigments and lipids extracted from PPCs, glass plates coated with either silica gel or a reverse-phase (RP-18) matrix are commonly used. Methods using paper or

powdered sugar generally have lower resolving capacity, and are not particularly useful when analyzing the small amounts of pigments obtained from PPCs. It is important to note that pigment modification/damage frequently occurs on silica gels. Most of the problems can be eliminated by washing plates in chloroform:methanol (1:1) prior to use, and conducting all steps under an atmosphere of N_2. A number of solvent systems exist for separating chlorophylls using TLC.[63] In Table 2.2, we present two solvent systems for resolving Chls *a* and *b* and carotenoids simultaneously.[65-67]

2.5.2.3.2 HPLC

For HPLC of pigments extracted from PPCs, reversed-phase C-18 columns are widely used because of their superior stability, particularly when exposed to a variety of organic and aqueous solvents. In addition, virtually no decomposition of chlorophylls and carotenoids occurs on these type of columns. Occasionally specialty columns (e.g. polyaromatic hydrocarbon; PAH) may be used, but they are typically more expensive and more difficult to maintain relative to the C-18 columns. Several HPLC protocols are outlined in Table 2.2.[68-71]

For HPLC, the chlorophyll concentration of the extract should be at least 0.2 mg/mL if an absorbance detector is used, but may be as low as 0.5 μg/mL if pigments are detected by fluorescence. It is important to remember, however, that due to the fluorescence properties of carotenoids, these pigments are best detected by their absorption.

2.6. Biophysical characterization of pigment-protein complexes

PPCs are frequently identified by their spectroscopic properties. The most widely used spectroscopic techniques for studying plant PPCs include absorption, fluorescence, and circular dichroism (CD). For general reviews of spectroscopy, see Brown[72] and Tinoco.[73] Specific information on UV and visible spectroscopy,[74] fluorescence,[75] and circular dichroism[76,77] is

available.

A qualitative measure of the pigment composition can be determined from an absorption spectrum of an isolated PPC while information about the environment of the pigments and their interactions with other pigments or with the PPC protein can be obtained using data from fluorescence and circular dichroism spectroscopy. A comparison of the fluorescence excitation spectrum with the absorption spectrum of a given complex can provide information about the efficiency of transfer of energy between different pigments within the complex.[78]

An important aspect to consider when isolating PPCs is the possibility of an alteration in pigment-protein interactions by detergents. For example, insertion of a protein into a detergent micelle may cause conformational changes in the protein.[36] In PPCs, such conformational changes may cause changes in the relative orientations of bound pigment molecules, resulting in the disruption of energy migration within the PPC. Alterations in the fluorescence emission properties of PS I have been attributed to this phenomenon.[79] Similarly, alterations in the environment of a particular pigment molecule that occur during solubilization may result in changes in its optical properties. For this reason, correlations cannot always be made between the spectral properties of an isolated PPC and specific features of the spectra of intact thylakoid membranes or whole cells.

2.6.1 Absorbance

Absorbance spectra are readily obtained for PPCs in aqueous solution (e.g. sucrose density gradient or column fractions). Complexes contained in polyacrylamide can be inserted directly into a cuvette. For this type of sample, a piece of the gel that does not contain any protein should be used as a reference. A quartz cuvette with a 1 cm pathlength is generally sufficient for obtaining high quality spectra of isolated PPCs. For all types of samples, the best

results are obtained with samples having an optical density no greater than 1.0.

2.6.2 Fluorescence

Fluorescence spectra are readily obtained from isolated PPCs, either in aqueous solution or in polyacrylamide. These measurements are generally done at low temperature (77 K) to increase fluorescence of PPCs such as PS I (which does not fluoresce appreciably at room temperature), to sharpen the peaks of other PPCs, and to enhance small shoulders in the spectra. The Chl concentration should be in the range of 5-10 μg/mL. Above these concentrations, reabsorption of emitted light leads to artifactual enhancements in the emissions at longer wavelengths.[32] For aqueous samples, glycerol is added to a final concentration of 50% and the sample is mixed thoroughly and rapidly before freezing in liquid nitrogen. Gel slices are inserted into the sample cuvette (e.g. disposable borosilicate culture tubes (6 x 50 mm; Kimble) or NMR tubes), barely covered with 50% glycerol, and held in liquid nitrogen briefly to freeze the gel slice into the cuvette. The cuvette is then filled with 50% glycerol, and the sample is frozen thoroughly in liquid nitrogen. Pre-freezing the gel slice in a small amount of glycerol prevents it from floating up to the top when more glycerol is added.

For fluorescence measurements at 77K, a Dewar is fitted into the cuvette holder of the spectrofluorometer and filled with liquid nitrogen. We use a glass Dewar made in our local glass shop and borosilicate culture tubes (see above) as cuvettes. Fluorescence emission spectra are typically measured between 600 and 750 nm, using an excitation wavelength of 440 nm for Chl *a* or 470 nm for Chls *b* and *c* (excitation wavelengths can be chosen more precisely from an absorption spectrum of the isolated PPC prior to fluorescence measurements). Excitation spectra generally cover wavelengths between 400 and 500 nm for green plants, though longer wavelengths are appropriate for PPCs from algae whose absorption

peaks (e.g., from carotenoids or phycobilisomes) extend beyond 500 nm.

2.6.3 Circular dichroism

CD spectra measured in the visible range (400-750 nm) provide information on pigment-pigment and pigment-protein interactions.[80] For CD of PPCs in aqueous solution, we routinely use a PPC sample with an OD at 672 nm of 0.3 to 0.5 (in a 10 mm cuvette). The sample is placed in a cuvette with a 1 mm pathlength for CD measurements to reduce scattering. A blank spectrum obtained using the same buffer present in the sample is subtracted from the spectrum of the sample. For measurement of the CD spectra of PPCs in polyacrylamide, the gel slices are gently compressed between two microscope slides and inserted into the cuvette holder. Pieces of acrylamide that do not contain any proteins are used to measure a baseline, which is then subtracted from the spectrum of the sample. To avoid artifacts due to differences in the optical properties of different slides, it is important to use the same microscope slides when measuring the sample and its baseline.

CD measurements taken between 180-250 nm (UV-CD) provide information about the conformation of protein in PPCs. Since proteins have an intense UV-CD signal, it is generally possible to use very small amounts of PPCs for these assays. It is important to keep the OD below 1, preferably ≈ 0.3, for accurate CD measurements. This corresponds to a protein concentration of ≈ 1-2 mg/mL when using appropriate cuvettes (the protein:Chl ratio (w/w) of thylakoids is ≈10-20 while the ratio in PPCs ranges from ≈ 2.5 to 20, depending upon the PPC and its purity). We use a 0.02 mm cuvette which holds only 20 μl of sample; the thin cuvette is required to minimize the CD signal from the solvent, especially water, at lower wavelengths. Sucrose, as well as certain buffers (e.g. MES), also interfere with measurements in the UV range and must be removed. For this purpose, we dilute samples with ddH_2O or dilute phosphate buffer (pH of 6-8 depending upon the PPC) and wash them by centrifugation

using a 30 kD microconcentrator (Amicon, Whatman). Repeating the process of dilution and centrifugation several (7-10) times usually reduces sucrose and buffer concentrations down to levels that do not interfere with UV-CD measurements. PPCs in polyacrylamide must be eluted before UV-CD. This can be achieved by grinding the gel slice to a fine slurry in ddH_2O (see 5.2.1) followed by centrifugation to remove polyacrylamide fragments, or by using an electroelution apparatus (Bio-Rad, Hoefer). Detailed interpretation of UV-CD data is available.[76]

2.7. Literature cited

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Table 2.1. Protocols for Solubilization and Resolution of PPCs.

Plant/Alga	PPCs Isolated	Solubilization Conditions[§,¶,•]	Isolation Conditions[§]	Ref.
All species examined	PSI, PSII, LHCs	To 20-60μg Chl in 20-60 μL of 100 mM Tris-HCl pH 8.5, 100 mM DTT add LDS to 1 or 2% mix 30-60 sec	LDS-PAGE[••] DOC-PAGE	37
		To 20-60μg Chl in 20-60 μL of 100 mM Tris-HCl pH 8.5, 100 mM DTT add either 2% DM, OG, Nonidet, Triton X-100, or DOC mix for 10-15 min add LDS to 0.17%	LDS-PAGE DOC-PAGE	37
Pea	PSI	To 0.8 mg Chl in 1 mL ddH_2O add TX-100 to 0.8% mix at RT 30 min; 40,000 g 30 min	0.1-1.0 M SDG[•••] in ddH_2O w 0.02% TX-100; 100 K g for 15 h	38
Diatoms	LHCs, PSII, PSI	To 1 mg Chl in 1 mL 50 mM Tris-HCl pH 8.5 add DM to 1%, mix 15 min	0-60% SDG in same buffer; 284 K g 14-16 h	40
Pea, Spinach	LHCII, PSII, PSI-LHCI, PSI-LHCII	To 0.5 mg Chl in 1 mL 20 mM MES-NaOH pH 6, 10 mM NaCl add DM to 1.0%, mix 30 min	10-30% SDG in same buffer w 0.05% DM; 165 K g 5 hr	39
Pea, Spinach	LHCII, CP43, PSII, PSI-LHCI, PSII-LHCII	To 0.5 mg Chl in 1 mL 20 mM MES-NaOH pH 6, 10 mM NaCl add TX-100 and OG to 1% and 40 mM, mix 1-2 h	10-30% SDG in same buffer w 0.03% TX-100, 165 K g 10 hr	39
Pea	LHCII	To an LHCII fraction from TX-100 solubilized thylakoids, add KCl to precipitate; resolubilize in 50 mM OG at 3-5 mg Chl/mL	Fractogel TSK DEAE-650 S in 10 mM Na/K phosphate pH 7.4 w 30 mM OG; elute w same buffer with 200 mM NaCl	41
Barley	PSI	To 2 mg Chl in 1 mL in 20 mM MES pH 6.3, 5 mM $MgCl_2$, 15 mM NaCl add DM to 1.5%, mix 30 min, 48 K g 30 min, dilute sup 5x w 20 mM Tricine pH 7.5, 0.3% DM	DEAE Sepharose Fast Flow in 20 mM Tricine pH 7.5, 0.3% DM; elute w 60-500 mM NaCl	42

[§] Conducted at 0-4°C in darkness unless otherwise noted. [¶] We recommend including 5 mM ACA/1 mM BAM. [•] All samples are centrifuged at 16,000 g for 10 min following solubilization unless otherwise noted. [••] For PAGE, samples must contain sucrose or glycerol at ≈5% or they will float out of the well when loaded. [•••] Sucrose density gradient.

Table 2.2. Protocols for Chromatographic Analysis of PPC Pigments.

Method	Pigments Resolved*	Matrix	Solvent	Conditions	Ref.
TLC	ß-C, a, b, L, V, N	RP-18	ethyl acetate:MeOH:H_2O (50:40:10)	Standard TLC conditions	65
TLC	N, V, Lo, L, a, b, ß-C	Silica gel 60	benzene:isopropanol:water (100:10:0.25)	All steps must be under N_2 atmosphere	66 67
HPLC§	N, Lo, V, L, a, b, ß-C	RP-18	A=water B=acetone	75% B for 3 min, 15 min ramp to 100% B, 1 mL/min, RT	68
HPLC	N, V, A, L, Z, a, b, ß-C	RP-18	A=acetonitrile:MeOH (7:1) B=acetonitrile:MeOH:H_2O:ethyl acetate (7:0.96:0.04:2) C=as B except (7:0.96:0.04:8)	100% A for 2 min, 100% B for 1 min, 100% C for 7 min	69
HPLC	N, V, A, L, Z, a, b, ß-C, *α*-C	RP18 not endcapped	A= acetonitrile:MeOH (85:15) B=MeOH:ethyl acetate (68:32)	100% A for 14.5 min, 2 min ramp to 100% B; 30°C, 1 mL/min	70
HPLC	12 pigments including c_1, c_2, c_3	Poly-aromatic hydro-carbon	A=MeOH:acetonitrile:1 M ammonium acetate (5:3:2) B=acetonitrile:ethyl acetate (1:1)	100% A for 2 min, 26 min ramp to 100%B; 1 mL/min	71

* Pigments are: N, neoxanthin; Lo, loroxanthin; V, violaxanthin; L, lutein; a, Chl *a*; b, Chl *b*; *α*- and ß-C, *α*- and ß-carotene; c_1, c_2, c_3, Chl c_1, c_2, c_3, respectively.

§ Detection wavelength for HPLC are generally in the range of 440-450nm, though fluorescence detectors for Chls provide ≈1000 X sensitivity.

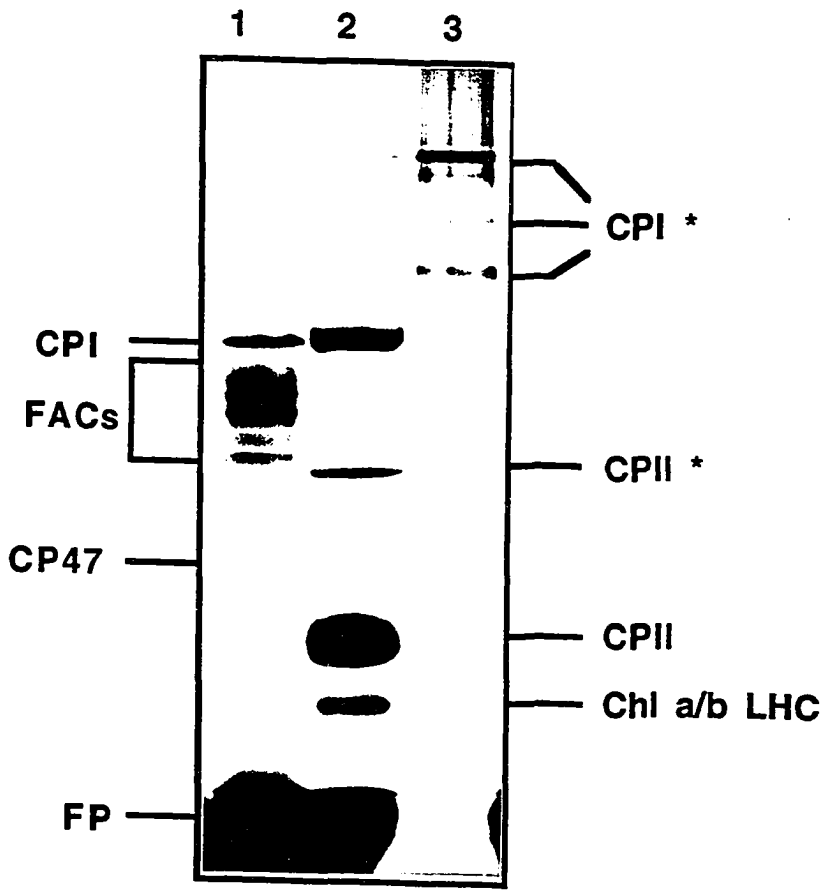

Figure 2.1. Mild LDS-PAGE of thylakoid membranes solubilized with LDS. The gel contained 8% polyacrylamide with 10% glycerol. Lane 1, thylakoids from the diatom *Cylindrotheca fusiformis* (LDS:Chl = 10, 60 μg Chl loaded); Lane 2, thylakoids from romaine lettuce (LDS:Chl = 10, 60 μg Chl loaded); Lane 3, membranes from *Synechococcus leopoliensis* (LDS:Chl = 20, 60 μg Chl loaded). CPI, core complex of Photosystem I; CPI*, oligomeric forms of CPI; FACS, fucoxanthin-Chl *a/c* light-harvesting complexes; CP47, internal antenna of Photosystem II; CPII, LHCII; CPII*, oligomeric form of CPII; FP, free pigment. The gel was not stained.

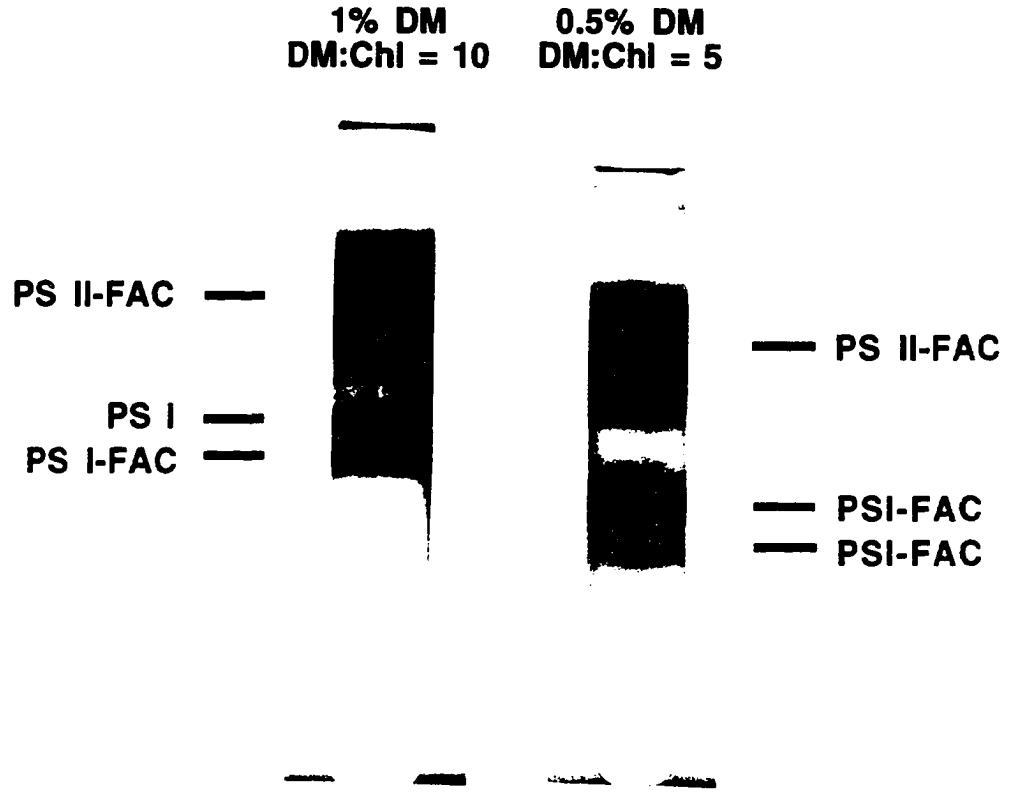

Figure 2.2. Sucrose density gradients (0-60%) of thylakoid membranes of *Cylindrotheca fusiformis* solubilized with dodecylmaltoside (DM) at different DM:Chl ratios. The PS II-FAC particle from DM:Chl = 5 is similar to the one obtained at DM:Chl = 10, but the PS I-enriched particles from DM:Chl = 5 retained more FACS as evidenced by their color (light brown) and by denaturing SDS-PAGE analysis of their polypeptide composition (not shown). PS II-FAC, dark brown particle containing Photosystem II and fucoxanthin-Chl *a/c* light-harvesting complexes; PS I, a green particle containing Photosystem I; PS I-FAC, particles containing Photosystem I and fucoxanthin-Chl *a/c* light-harvesting complexes (light brown in DM:Chl = 5; olive-green in DM:Chl = 10).

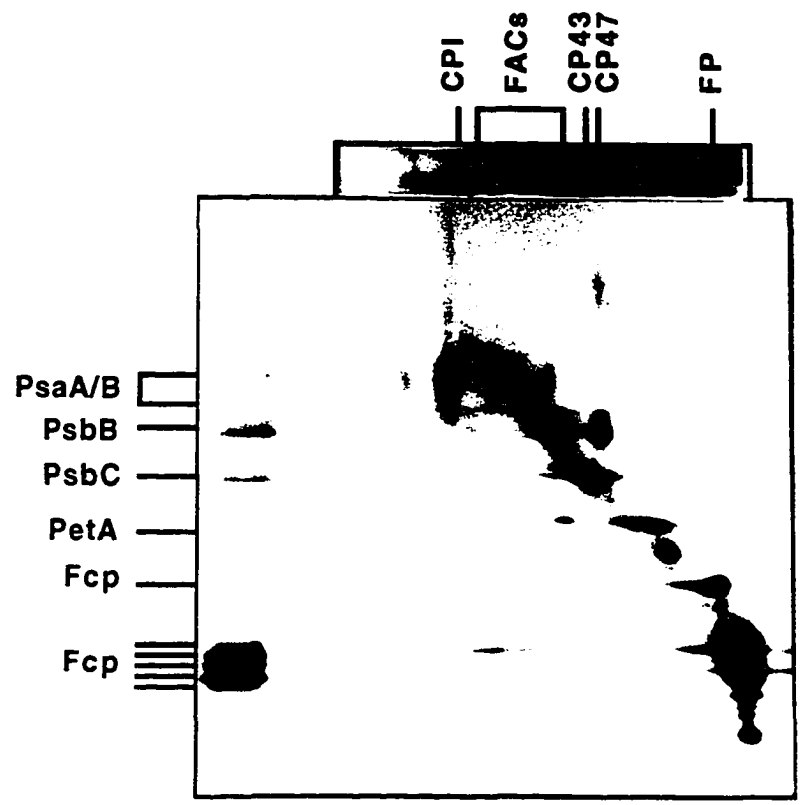

Figure 2.3. Two-dimensional SDS-PAGE of PPCs from *Cylindrotheca fusiformis*. PPCs from LDS-solubilized (LDS:Chl = 10) thylakoids were resolved using mild LDS-PAGE. The "green gel" lane (first dimension), stained with Coomassie Blue for presentation, is shown at the top of the figure. The second-dimension gel was 7.5-15% polyacrylamide, and was stained with Coomassie Blue. Note the diagonal line consisting of proteins that were denatured in the first dimension and proteins off the diagonal that were associated with pigments in PPCs during the first dimension. Abbreviations for the PPCs resolved in the first dimension are as in Fig. 2.1. Polypeptides present in denatured thylakoid membranes (shown on the left) are PsaA/B, core apoproteins of Photosystem I; PsbB, apoprotein of CP47; PsbC, apoprotein of CP43, PetA, cytochrome f; Fcp, apoproteins of fucoxanthin-Chl *a/c* light-harvesting complexes.

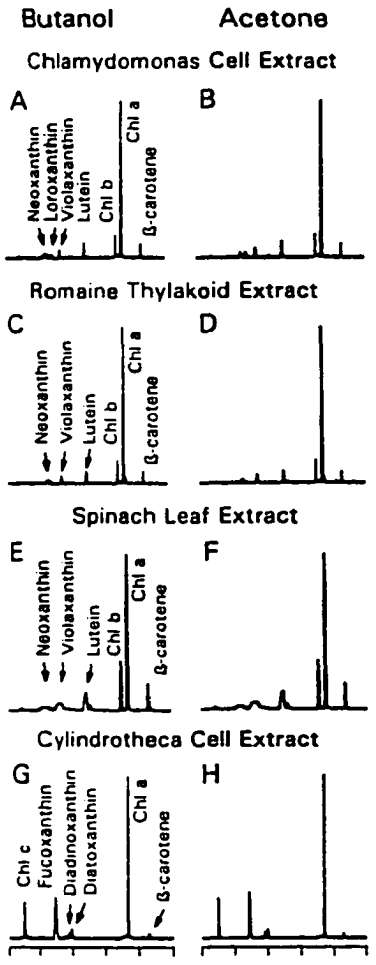

Figure 2.4. HPLC profiles of total pigments extracted from whole cells of *Chlamydomonas* (A,B), romaine thylakoid membranes (C,D), spinach leaf extract (E,F), or whole cells of *Cylindrotheca* (G,H) using *sec*-butanol (A,C,E,G) or 90% acetone (B,D,F,H). Extracts from equivalent samples were adjusted to equal volumes before injection. Each HPLC profile is shown full scale and was not normalized. Chromatograms were 25 min in duration. (From Martinson, T.A. and Plumley, F.G., Anal. Biochem. 228:123-130 (1995), with permission).

Chapter 3

One-step Extraction and Concentration of Pigments and Acyl Lipids by *sec*-Butanol from *In Vitro* and *In Vivo* Samples[1]

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[1] Analytical Biochemistry, 228: 123-130 (1995).

3.1 Abstract

Photosynthetic pigments and acyl lipids were simultaneously extracted and concentrated by *sec*-butanol. Pigments extracted with *sec*-butanol were indistinguishable from those extracted using acetone as determined by quantitative and qualitative HPLC. Use of *sec*-butanol has several advantages over conventional extraction solvents: 1) pigments are extracted directly from polyacrylamide gel slices without an elution step; 2) pigments in dilute, isolated pigment-protein complexes are extracted and concentrated without first concentrating the sample; 3) when necessary, the concentration factor is readily increased by addition of water; 4) *sec*-butanol extracts acyl lipids and Vitamin K_1 as effectively, but much quicker, than chloroform:methanol; 5) *sec*-butanol rapidly extracts and concentrates pigments from thylakoids of all plant species tested and even directly from many algal/higher plant cells, facilitating analysis of pigment biosynthetic pathways using radioactive substrates; and 6) pigments are stable in *sec*-butanol for several days at room temperature in the dark, or for many weeks if stored at -20°C in darkness. Finally, *sec*-butanol is preferable to ether for concentrating pigments extracted with acetone.

3.2 Introduction

Pigments are generally easily and effectively extracted from whole cells and thylakoid membranes using acetone, methanol, or ethanol at concentrations greater than 80% (1-4). In contrast, extraction and recovery of pigments from isolated pigment-protein complexes is wrought with technical difficulties. Often the complexes are obtained in low yields and may be present as a diffuse band when resolved on sucrose density gradients. Pigment-protein complexes that have been resolved by non-denaturing PAGE are particularly problematic because pigments cannot be efficiently extracted directly from polyacrylamide with the organic solvents routinely used for pigment extraction unless the complexes are first eluted

from the gel. Although protocols have been developed for eluting pigment-protein complexes from polyacrylamide gels (5,6), none work satisfactorily in our hands or else are extremely tedious. Moreover, once the pigment-protein complexes are recovered, the pigments still have to be extracted. Extraction of pigments from eluted pigment-protein complexes or dilute sucrose density gradient fractions using eight to nine volumes of acetone reduces their concentrations below levels which can be readily analyzed. Pigments that have been extracted in this manner can be concentrated by transferring them to ether (7), which can then be evaporated using a stream of N_2 gas. We have found that these steps frequently result in incomplete or differential recovery and/or chemical modification of pigments (e.g., allomerization, oxidation).

The purpose of this work was to determine if an alcohol with low solubility in water could be used to simultaneously extract and concentrate pigments from chlorophyll (Chl)-protein complexes resolved by either PAGE or sucrose density gradient ultracentrifugation. We tested the efficacy of a number of 4- and 5-carbon solvents in extracting pigments from detergent-solubilized complexes, thylakoids, and whole cells and found that *sec*-butanol consistently and effectively extracts and concentrates pigments from these samples.

3.3 Materials and Methods

3.3.1 Cultures and Growth Conditions

Cultures of wild type (WT137) and an azide-induced Chl *b*-deficient mutant (PA2.1; 8) of *Chlamydomonas reinhardtii* (WT137), *Cylindrotheca fusiformis* (Watson's strain 13), *Porphyridium* (Carolina Biological Supply) and *Phaeodactylum tricornutum* Bohlin *(UTEX 646)* were grown and harvested as described previously (9,10). *Plectonema boryanum* (UTEX 594) was grown in JM (11) and *Chlorella ellipsoidea* (UTEX 247) was grown in a minimal salts medium (12); standard growth conditions (9,10) were employed for these algae. Romaine

lettuce, broccoli, and spinach were obtained from local markets. Potato and tobacco were grown in an environmental chamber under standard conditions.

3.3.2 Purification of Thylakoid Membranes

Thylakoid membranes were purified by floatation through sucrose density gradients as described previously (7,10), and resuspended in gel sample buffer (100 mM Tris-HCl, pH 8.5, 100 mM DTT, 5 mM ϵ-aminocaproic acid, 1 mM benzamidine).

3.3.3 Isolation of Pigment-Protein Complexes

Thylakoid membranes were solubilized with either LDS (LDS:Chl = 10:1) or a mixture of SDS, nonylglucoside, octylglucoside, and Triton X-100 (S:N:O:T:Chl = 9:6:12:20:1) as described (13). Pigment-protein complexes were resolved using either non-denaturing SDS-PAGE in the buffer system of Laemmli at 50 mM Tris base, 383 mM glycine, and 0.1% SDS, or by sucrose density gradient ultracentrifugation (0-60% sucrose, 5 mM Tris-HCl, pH 8.5, 5 mM ϵ-aminocaproic acid, 1 mM benzamidine) at 284,000 x g for 16 hours.

3.3.4 Preparation of samples for extraction

Prior to extraction, PAGE samples had to be homogenized to a fine slurry for complete extraction. Homogenization was accomplished by placing the pigmented gel slice in the bottom of a 1.5 ml microfuge tube, covering it with water, and pulverizing for 10 to 30 s with a motor-driven Teflon pestle. In some cases, small gel fragments fell to the bottom of the tube and had to be resuspended by mixing before homogenization could proceed; the entire process still required less than 1 min per sample. Sucrose density gradient fractions isolated as described above were extracted without further treatment. Thylakoid membrane samples were generally too concentrated and required dilution to $\approx$ 1 mg Chl/ml in water before

addition of *sec*-butanol. Algal cultures were extracted directly in their liquid culture medium. If large volumes (i.e., liters) of algal cultures need to be extracted, cells can be concentrated (e.g., by centrifugation) before addition of *sec*-butanol but the final Chl concentration must remain below ≈0.25 mg/ml to insure complete extraction. Leaves of higher plants required grinding for complete extraction; 50-75 mg of leaf material was ground for 30-45 s in liquid nitrogen with a hand-held pestle and mortar followed by addition of 500 µl of $MgCO_3$-saturated water. Increasing the leaf material above ≈150 mg/500 µl resulted in incomplete extraction.

3.3.5 Extraction of pigments

Samples in aqueous solution (see above) were extracted by mixing with 2/3 volume of *sec*-butanol. Extractions were carried out at 0°C to prevent freezing of the aqueous phase, which interferes with extraction. For isolated pigment-protein complexes and thylakoids, pigment extraction was complete in 5-10 min. For algae and higher plant leaf tissue, extraction requires several hours; we routinely used overnight extractions for convenience. After extraction, complete transfer of pigments to the *sec*-butanol phase was effected by adding 1/3 original volume of 4M NaCl. Centrifugation at 16,000 x g for 10 min hastened phase separation and clarified the extract.

To summarize, sec-butanol and NaCl are used at a 3:2:1 (v/v/v) ratio of sample:*sec*-butanol:4M NaCl. With these ratios, the pigments are quantitatively recovered in the organic phase whose volume is almost identical to the volume of added butanol; this represents a concentration factor of 1.5. The sample can be further concentrated by adding more water or, if necessary, the *sec*-butanol phase can be diluted using water-saturated *sec*-butanol.

3.3.6 Pigment transfer from acetone to sec-butanol

Pigments in dilute acetone extracts can be concentrated using *sec*-butanol. For each volume of pigment extract in 90% acetone, 1 volume of *sec*-butanol, 4 volumes of 4M NaCl, and 2-4 volumes of water were added, depending on the desired degree of concentration. After mixing, samples were centrifuged as above to separate the phases.

3.3.7 Extraction of total lipids

Fifty ml of a *Chlamydomonas* WT137 culture at $\approx 10^4$ cells/ml in Tris-Acetate-Phosphate (TAP; 12) was centrifuged (5 min, 5000 x g), washed in 50 ml of MIN, and then transferred to 1 ml of MIN (MIN is the same as TAP but HCl replaces acetic acid). Cells were incubated for 60 min in the light to deplete intracellular acetate pools. [^{14}C]-acetate (New England Nuclear; 2 μCi/mmol) was added to 1.5 μCi/ml and cells were pulse labeled in the light for 60 min. Cultures were diluted 10-fold in TAP. Eight 1-ml aliquots were placed in separate centrifuge tubes and extracted in either *sec*-butanol as described above or with 4 volumes of methanol followed by 2 volumes of chloroform in a modified Bligh-Dyer extraction procedure (14). Five μl of each organic phase was counted in a liquid scintillation counter and corrections were made for differences in the volumes of the two types of organic phases.

3.3.8 Acetone extraction

Algal cells and thylakoids were extracted in 90% acetone/0.01N NH_4OH overnight at -20°C. Samples were centrifuged at 16,000 x g for 10 min prior to HPLC analysis. Higher plant leaves were extracted in $MgCO_3$-saturated 90% acetone overnight at -20°C.

3.3.9 HPLC Analysis

All samples were analyzed on a Rainin Microsorb MV C-18 column (4.6 mm x 25 cm). Pigment fractions were injected into a mobile phase of acetone:water (75:25 v/v) at a flow rate of 1 ml/min. After 3 min, the concentration of acetone was brought to 100% over 15 min and pigments detected by their absorption at 448 nm. Vitamin K_1 was detected using a mobile phase of methanol:isopropanol (80:20 v/v) at a flow rate of 1 ml/min, and detected at 270 nm (15); the standard (Sigma Chemical Co.; Cat. # V-3501) was diluted in acetone.

3.3.10 Other methods

The relative effectiveness of acetone and/or several four- and five-carbon alcohols for extracting pigments was compared by estimating Chl concentrations using a Turner Model 111 fluorometer equipped with a Wratten 47B blue excitation filter and a Wratten 25 long-wavelength cut-off filter (2).

3.4 Results

Several solvents were evaluated in terms of their ability to extract pigments from whole cells or from pigment-protein complexes isolated using PAGE or sucrose density gradient centrifugation. Commonly used solvents such as acetone and ethanol dehydrated acrylamide gel slices, releasing very little pigment to the organic phase (data not shown). Moreover, these solvents are completely miscible in water, and when used to extract pigments from sucrose density gradient fractions, often resulted in a pigmented phase that was too dilute for spectroscopic or HPLC analyses (data not shown). Other solvents, such as hexane and pentane, are so immiscible with water that they failed to extract any pigments, whether they were in an aqueous phase or in a gel slice (data not shown). In contrast, short-chain alcohols, which are only slightly miscible in water, were capable of extracting pigments

from whole cells and gel slices, and had the additional advantage of concentrating the pigments in the organic phase. However, preliminary analyses indicated that these short-chain alcohols extracted pigments to varying degrees, as evidenced by a pigment-rich interface between the organic and aqueous phases and/or a pigmented pellet (data not shown). These analyses, although only qualitative, indicated that *sec*-butanol was the most effective solvent because the interfaces/pellets of samples extracted in this alcohol were visibly devoid of pigments.

A more quantitative measure of the efficiency of several 4- and 5-carbon alcohols in extracting pigments from a variety of samples was obtained by comparing the Chl-induced fluorescence of each pigment extract. Since some alcohols gave fluorescence values higher than the *sec*-butanol extracts, even though they clearly did not extract all pigment (i.e. pigmented interfaces/pellets), a method for normalizing the data had to be devised (see Table 3.1 for details) which circumvented the well-known quantitative and qualitative effects of solvents on fluorescence yield (1). We found that Chls were readily extracted from isolated light-harvesting complexes (CPII; Table 3.1) by all alcohols tested, including *n*-butanol, which was otherwise largely ineffective in extracting Chl from the samples tested. In contrast, most alcohols tested extracted less than 80% of the Chl from the Photosystem (PS) I complex (CPI; Table 3.1) except *sec*-butanol which extracted 93% of the Chl. Surprisingly, we also found that these alcohols extracted Chl from whole cells of the green alga, *Chlamydomonas*, and the diatom, *Cylindrotheca*, though again, their efficacy was highly variable (Table 3.1). In general, the 5-carbon alcohols were more effective than *n*-butanol, but less so than either *sec*-butanol or *tert*-butanol. Although *tert*-butanol was almost as effective as *sec*-butanol for all sample types, it dissolved pipet tips and because it was substantially more miscible with water, samples frequently did not separate into two phases even after addition of NaCl.

Having verified that *sec*-butanol was the most effective long-chain alcohol for extracting pigments (Table 3.1), we next wanted to determine if it was as effective as solvents routinely used for pigment extraction (e.g., acetone). We compared the Chl extraction efficiencies of 90% acetone and *sec*-butanol with higher plant tissues and with algal species that are more recalcitrant than those in Table 3.1. Overall, we found *sec*-butanol to be as effective as 90% acetone in extracting Chl from most higher plants tested (Table 3.2). The somewhat lower values obtained with *sec*-butanol for broccoli and lettuce (Table 3.2) are likely due to problems in completely recovering the organic phase, free of the aqueous phase and/or plant material trapped at the interface. The efficacy of both 90% acetone and *sec*-butanol extraction was dependent upon the extent to which higher plant material was ground into a fine powder using a mortar and pestle and liquid N_2. *sec*-Butanol also effectively extracted Chl from several algal species (Table 3.2). *sec*-Butanol was superior to 90% acetone in extracting Chl from some algae while acetone was superior with other species. Neither 90% acetone or *sec*-butanol completely extracted all Chls from *Chlorella*, though *sec*-butanol was clearly superior. Poor extraction of *Chlorella* has been noted previously. Complete extraction of *Chlorella* with *sec*-butanol, but not 90% acetone, could be achieved if the extraction time was extended to ≈ 48 h, but Chls were converted to their ester-free Chlide derivatives (data not shown).

To determine if *sec*-butanol extracts carotenoids as well as Chl (Table 3.1) and if this solvent chemically alters pigments, we compared 90% acetone and *sec*-butanol extracts of several plants/algae/thylakoids by HPLC. The HPLC profiles of both Chls and carotenoids in the *sec*-butanol extracts were qualitatively and quantitatively identical to those in acetone extracts (Fig. 3.1). For both acetone and *sec*-butanol, it was necessary to extract whole cells (leaf tissue or algae) overnight to completely extract ß-carotene (data not shown). In cases where one solvent was superior to the other, the HPLC profiles were consistent with the Chl

extraction efficiencies (Table 3.2), except that there was a preferential loss of polar pigments (e.g., neoxanthin, violaxanthin) relative to Chls and ß-carotene in the *sec*-butanol extracts (data not shown). Overall, however, *sec*-butanol completely extracted all pigments, including polar xanthophylls and Chl *c* (Fig. 3.1), from all thylakoids and higher plants and from the majority of algal cells examined. Moreover, no changes were detected in the HPLC profiles when the *sec*-butanol samples were reassayed after several days in darkness at room temperature or several weeks at -20°C (data not shown).

The main goal of this work was to find a solvent suitable for extracting pigments from polyacrylamide gel slices and dilute sucrose density gradient fractions. The data in Table 3.1 indicate that *sec*-butanol extracts Chls from pigment-protein complexes contained in polyacrylamide. However, as was the case with higher plants, algal cells, and thylakoids, it was necessary to rule out the preferential extraction of some pigments relative to others. HPLC pigment profiles of two SDS-PAGE complexes, the PS I core complex, CPI, and the light-harvesting complex, CPII, are shown in Figure 3.2. The CPI complex from romaine lettuce contains only Chl *a* and ß-carotene (Fig. 3.2A) at a ratio typically found in the PSI core complex (16). CPII is a monomeric form of the light-harvesting complexes, which contains Chls *a* and *b*, in addition to the xanthophylls lutein, violaxanthin, and neoxanthin (5,7). Our HPLC profiles for CPII (Fig. 3.2B) show these pigments to be present in the *sec*-butanol extract, and at ratios that are typically found in these complexes (17). Equally important, *sec*-butanol does not cause detectable pigment alteration during extraction from polyacrylamide. We also verified the utility of *sec*-butanol for extraction of pigments from sucrose density gradient fractions. A PS I-enriched particle from *Chlamydomonas* and a PS II-enriched particle from romaine lettuce, both obtained by detergent solubilization and sucrose density gradient centrifugation, were extracted using *sec*-butanol. Again, the HPLC pigment profiles obtained for these particles (Figs. 3.2C and 3.2D) were typical of those routinely obtained using the

more laborious methods for extracting pigments from isolated Chl-protein particles (18). Importantly, there was no visible pigment, either Chl or carotenoid, present in the aqueous phase after *sec*-butanol extraction and, moreover, the HPLC profiles of acetone-extracted complexes, though too dilute to give high quality HPLC profiles, were indistinguishable from those shown in Fig. 3.2 (data not shown).

Since *sec*-butanol extracted a wide range of pigments, we were curious about its capacity to extract other lipids. Data in Table 3.3 compare the amount of radiolabelled lipids extracted from whole cells of *Chlamydomonas* using either *sec*-butanol or a chloroform/methanol procedure (14). Clearly, *sec*-butanol was at least as efficient as chloroform/methanol in extracting total lipids from whole cells. To specifically assay the extractability of one non-pigmented lipid, we examined phylloquinone (Vitamin K_1), which is found in the PS I complex of plants (15,19). A CPI complex was isolated from romaine lettuce using non-denaturing LDS-PAGE and extracted with *sec*-butanol. The HPLC profile of this extract (Fig. 3.3) shows that Vitamin K_1 is present at a level consistent with its abundance relative to Chl in the PS I reaction center core complex (i.e., two molecules of Vitamin K_1 per 140 Chl molecules).

3.4.1 Practical considerations

The protocol outlined so far utilized *sec*-butanol at a sample:*sec*-butanol:4M NaCl ratio of 3:2:1, a ratio that was suitable for most types of samples. Realizing that samples containing extremely low quantities of pigments might warrant use of less *sec*-butanol, we determined the minimum concentration of *sec*-butanol that could be used to extract pigments without compromising either the completeness of the extraction or the stability of the pigments. This is an important consideration, as concentrations of acetone less than 90% do not fully extract ß-carotene (3), while chlorophyllase activity is enhanced in low

concentrations of acetone (20). We reasoned that similar problems might be encountered with *sec*-butanol. By varying the amount of *sec*-butanol added to replicate samples, we found that the minimal amount that could be used was 20%, which corresponds to a sample:*sec*-butanol:4M NaCl ratio of 3:1:1 (data not shown). At concentrations <20%, pigments were incompletely extracted, while concentrations >33% were effective but unnecessary for samples with Chl concentrations as described in Materials and Methods.

Once pigments were extracted, if their concentration was too low, the final volume of the *sec*-butanol phase could be decreased by addition of water. Care had to be taken not to eliminate the *sec*-butanol phase, however, because irreversible chemical modifications occurred when the pigments were forced into the aqueous phase (data not shown).

Occasionally, dilution of the *sec*-butanol extract may be necessary, and we found that it was preferable to use water-saturated *sec*-butanol, particularly in samples that contained Chl *c* or fucoxanthin. Use of neat *sec*-butanol resulted in the dimerization of Chl *c* and chemical modification of fucoxanthin, as evidenced by split peaks in the HPLC pigment profile (data not shown). Similar reversible modifications have been noted previously with these pigments (21).

In some cases, it is desirable to know the Chl concentration of a particular fraction. Until extinction coefficients for pigments in *sec*-butanol are determined, other solvents (e.g., acetone) must be used to determine Chl spectrophotometrically. For acetone extracts too dilute for HPLC analysis, pigments were transferred directly to *sec*-butanol, using a ratio of sample:*sec*-butanol:4M NaCl:water of 1:1:4:3 (data not shown). The amount of water added was varied from 2-4 volumes, depending on the degree of concentration desired. The concentrated extracts could also be stored for long periods of time in *sec*-butanol without undergoing chemical modification (data not shown).

3.5 Discussion

Our understanding of the pigment composition of Chl-protein complexes has progressed at a slower pace than our knowledge of their protein composition. There are several reasons for this slow progress, but, overall, the largest problem may relate to the technical difficulties of recovering pigments from pigment-protein complexes, particularly those that have been resolved by non-denaturing PAGE. Many of the solvents that are currently used (e.g., acetone) are completely miscible in water and have to be used at such high concentrations (≥80%) that the resulting pigment extracts are frequently too dilute for most analyses. Furthermore, these solvents dehydrate polyacrylamide, making the direct extraction of pigments impossible. Our goal was to find a solvent that could simultaneously extract and concentrate pigments from polyacrylamide as well as from a variety of other material.

A number of solvents differing in their degree of miscibility in water were tested for their ability to extract pigments. Solvents such as hexane, which are essentially immiscible in water, proved unsatisfactory, and the difficulties encountered when working with completely miscible solvents such as acetone have already been discussed. It seemed logical to investigate the pigment extraction capacity of solvents with intermediate miscibility. We tested three 4-carbon alcohols and two 5-carbon alcohols and found each capable of at least partially extracting pigments (Table 3.1). Two 4-carbon alcohols, *sec*-butanol and *tert*-butanol, were the most effective (Table 3.1), however, there were problems with *tert*-butanol as described in the Results. *sec*-Butanol was clearly the solvent of choice.

The data in Tables 3.1 and 3.2 indicated that *sec*-butanol effectively extracted Chls while the HPLC profiles (Figs. 3.1 and 3.2) indicated that *sec*-butanol also extracted pigments whose polarities ranged from very hydrophobic (e.g., ß-carotene) to moderately hydrophilic (e.g., Chl *c*). Importantly, many of these pigments were not driven from the aqueous phase

into *sec*-butanol unless high concentrations of NaCl were added to the sample. Though not addressed in this report, it may be possible to use lower NaCl concentrations in order to achieve selective recovery of pigments in the organic phase, a strategy similar to other phase-separation protocols (1). It should also be noted that *sec*-butanol does not extract pigments from all samples. For instance, if samples are too concentrated, *sec*-butanol may not be 100% effective. This can be remedied by dilution of the sample before extraction. Another shortcoming of *sec*-butanol was noted with recalcitrant algae such as *Chlorella* (22). Interestingly, *sec*-butanol was more effective than 90% acetone with this alga. We conclude that, overall, *sec*-butanol extracts pigments as effectively as acetone.

Since *sec*-butanol proved to be highly efficient at extracting pigments, we reasoned that it might also be capable of extracting other kinds of lipids. As shown in Table 3.3, *sec*-butanol extracted total lipids from algal cells as efficiently as chloroform:methanol. Moreover, it extracted Vitamin K_1 from PS I reaction center preparations resolved by PAGE (Fig. 3.3) or on sucrose density gradients (data not shown). Although not addressed directly in this work, the ability of *sec*-butanol to completely extract lipids from whole cells suggests that this alcohol will also provide an easy method for extracting and concentrating tightly bound acyl-lipids from pigment-protein complexes (23). Finally, *sec*-butanol may prove useful in extracting other important cellular lipids (e.g., steroids; ubiquinone) from a variety of cells and tissues.

Although *sec*-butanol offers clear advantages over other solvents in total lipid extraction, some of the other alcohols tested may be more beneficial for certain tasks. For example, *n*-butanol and *sec*-pentanol extracted very little pigment from a mutant of *Chlamydomonas* deficient in light-harvesting complexes, but partially extracted pigments from the wild-type strain (Table 3.1). This implies that these two solvents extracted pigments from light-harvesting complexes, but inefficiently extracted pigments from reaction centers. This

hypothesis was supported by the observation that neither of these solvents extracted all the pigments from CPI, the PS I reaction center core, yet both effectively extracted pigment from CPII, the isolated light-harvesting complex (Table 3.1). This differential pigment extraction needs to be investigated further but may offer a simple means of selectively extracting pigments from certain pigment-protein complexes (e.g., during pulse-chase labeling studies).

In this paper, we presented several examples to demonstrate the utility of *sec*-butanol for extracting pigments (Figs. 3.1 and 3.2), and concluded that this alcohol is as effective as the commonly used solvent, acetone. However, in some instances, use of *sec*-butanol had clear advantages over acetone. The major advantage was undoubtedly its ability to extract pigments directly from pigment-protein complexes in polyacrylamide (Fig. 3.2A,B). However, it was also useful for circumventing another problem that occurred during acetone extraction of sucrose density gradient fractions. Upon addition of acetone to sucrose-containing samples, a sucrose-containing phase was often produced which trapped pigments. This phase could be eliminated by addition of acetone and water but this was generally undesirable. In contrast, sucrose did not form a separate phase during *sec*-butanol extraction and pigments were readily obtained from sucrose gradient samples that contained one or more detergents, including SDS, Triton X-100, nonylglucoside, octylglucoside (Fig. 3.2C,D), and dodecylmaltoside (data not shown).

Use of *sec*-butanol offers several advantages over conventional solvents for the manipulation and storage of pigments. For instance, pigments could be rapidly transferred from acetone to *sec*-butanol. This method of concentration is much easier than the conventional ether method. Additionally, pigments could be stored for at least several weeks in *sec*-butanol without modification, even when not stored under N_2. This contrasts with storage in hydrated solutions of acetone and methanol (1,3; data not shown), in peroxide-producing ether solutions (1), and in some neat solvents (24). Although we did not directly

address the question of longer-term stability of pigments in this study, we suggest that *sec*-butanol may be the solvent of choice for extracting, concentrating, and storing pigments.

3.6 References

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Table 3.1. Chlorophyll extraction efficiencies of several four- and five-carbon alcohols.

Plant/Alga	Sample	Chl Extracted (%)				
		sec-butanol[a]	*n*-butanol[b]	*t*-butanol[b]	*sec*-pentanol[b]	*t*-pentanol[b]
Chlamydomonas WT	whole cells	**97**[c]	**49**	**<95**[d]	**87**	**91**
Chlamydomonas PA2.1	whole cells	**98**	**37**	**<94**	**53**	**87**
Cylindrotheca	whole cells	**94**	**<80**	**97**	**<72**	**<88**
Romaine Lettuce	CPI, SDS-PAGE	**93**	**<60**	**80**	**<51**	**<70**
Romaine Lettuce	CPII, SDS-PAGE	**100**	**100**	**100**	**100**	**100**

[a] Samples were extracted in *sec*-butanol as described in Materials and Methods. Pellets and/or material at the solvent interfaces were collected and reextracted in *sec*-butanol. The amount of Chl in each *sec*-butanol extract was determined and the sum of these two set to 100%. The percent extraction by the first *sec*-butanol treatment was then calculated. [b] Samples were extracted in the solvent indicated using the same procedure as for *sec*-butanol. Pellets and/or any material at the solvent interfaces were collected, washed with water, and extracted using *sec*-butanol. The amount of Chl in each pellet/interface was determined by fluorescence. This value used to calculate the amount of Chl extracted by the first solvent as compared to the *sec*-butanol standard (see footnote a above). [c] Bold-face entries indicate the most effective solvent for each sample. [d] Underlined values indicate that the interface/pellet was not completely extracted by *sec*-butanol, presumably because the first alcohol caused aggregation or dehydration of the sample and this inhibited subsequent extraction. Therefore, the value given is the maximum amount of Chl that was extracted by the first alcohol.

Table 3.2. Chlorophyll extraction efficiencies of acetone and *sec*-butanol with various algal cells or higher plant leaves.

Plant/Alga	Chl Extracted (%)	
	Acetone	sec-Butanol
Broccoli	98[a]	95[b]
Lettuce	99[a]	92[b]
Potato	98[a]	99[b]
Spinach	98[a]	99[b]
Tobacco	98[a]	91[b]
Chlorella	80[a,c]	173[b,c]
Cylindrotheca	100[d]	98[e]
Phaeodactylum	96[d]	81[e]
Plectonema	86[d]	99[e]
Porphyridium	74[a]	86[b]

[a]Samples were extracted in 90% acetone and the pellet reextracted in 90% acetone. Each extract was diluted with an equal volume of H_2O-saturated *sec*-butanol and Chl fluorescence measured. The sum of the two fluorescence values was set to 100% and the percent extraction by the first acetone treatment calculated. [b] Sample was extracted in sec-butanol, the extract diluted with an equal volume of 90% acetone and Chl fluorescence measured. The percent extraction was calculated relative to that with two extractions in 90% acetone (see footnote a). [c] Pellet was visibly green after second extraction. [d] Extraction efficiencies were calculated as described in Table 3.1 (footnote a) but using 90% acetone as the standard.
[e] Sample was extracted with *sec*-butanol. The pellet and interface was extracted with 90% acetone and the Chl in this extract determined by fluorescence. The percent Chl extraction by *sec*-butanol was calculated relative the total Chl extracted with two acetone treatments (footnote d).

Table 3.3. Extraction of total cellular lipids using either *sec*-butanol or chloroform/methanol.

Chlamydomonas whole cells	*sec*-butanol (counts per minute)	$CHCl_3$:MeOH
1	984	883
2	943	878
3	989	981
4	918	876
$\bar{X}$	958±68	904±102
Range	890-1026	802-1006

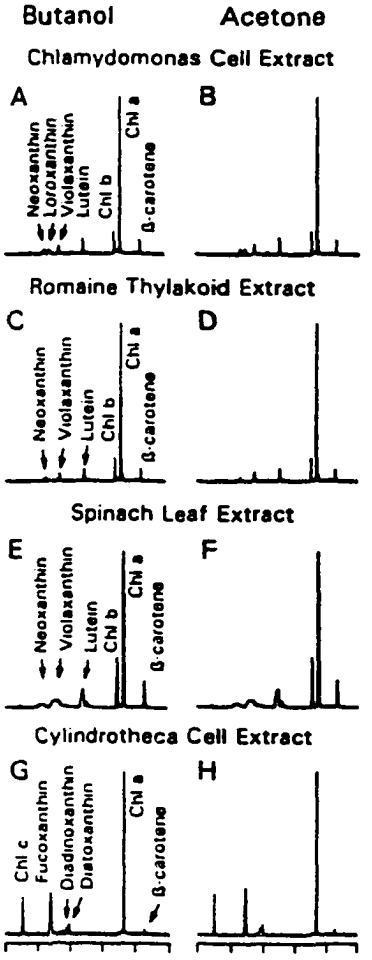

Figure 3.1. HPLC profiles of total pigments extracted from whole cells of *Chlamydomonas* (A,B), romaine thylakoid membranes (C,D), spinach leaf extract (E,F) or whole cells of *Cylindrotheca* (G,H) using sec-butanol (A,C,E,G) or 90% acetone (B,D,F,H). Extracts from equivalent samples were adjusted to equal volumes before injection. Each HPLC profile is shown full scale and was not normalized. Chromatograms were 25 min in duration.

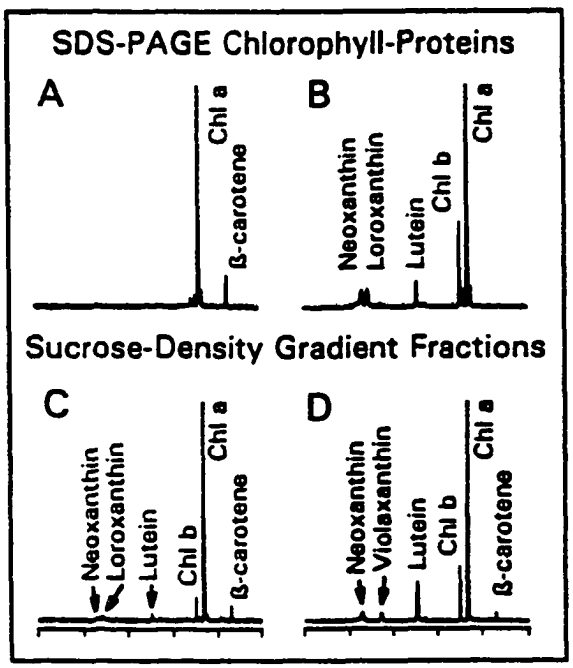

Figure 3.2. HPLC profiles of total pigments extracted from pigment-protein complexes in polyacrylamide (A,B) or in sucrose density gradient fractions (C,D) using *sec*-butanol. Romaine lettuce Photosystem I core complex, CPI (A); *Chlamydomonas* light-harvesting complex, CPII (B); *Chlamydomonas* Photosystem I-enriched fraction (C); and a Photosystem II-enriched particle from romaine lettuce (D). Chromatograms were 25 min in duration.

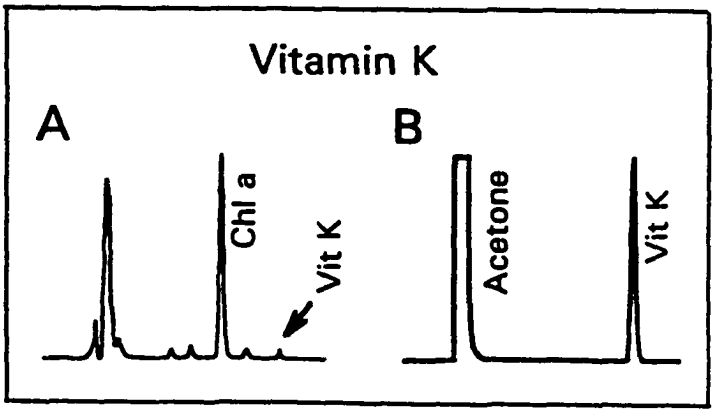

Figure 3.3. HPLC profile showing the presence of vitamin K_1 in a *sec*-butanol extract of romaine CPI (A) and 1.9 μg of the vitamin K_1 standard diluted in acetone (B). Chromatograms were 12.5 min in duration.

Chapter 4

Oxygen-evolving Thylakoid Membranes of a Marine Diatom: Biochemical and Biophysical Properties[1]

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4.1 Abstract

Thylakoid membranes purified from a marine diatom using sorbitol supported high rates of O_2 evolution with ferricyanide (FeCN) as electron acceptor; V_{max} and K_m were 72 μmoles O_2·mg Chl a^{-1}·h^{-1} and 184 μM, respectively. The V_{max} is comparable to rates *in vivo* with the diatom (without exogenous acceptors) and lettuce thylakoids (with FeCN). The K_m is ≈20-fold higher than observed with lettuce thylakoids. With *para*-benzoquinone (pBQ) as acceptor, V_{max} (27 μmoles O_2·mg Chl a^{-1}·h^{-1}) and K_m (1.13 mm) were ≈3-fold lower and ≈300-fold higher, respectively, than for lettuce thylakoids. DCMU abolished O_2 evolution with pBQ but reduced activity by only ≈50% with FeCN. These characteristics are similar to those observed for highly purified PS II reaction centers of green plants and cyanobacteria. The rate of PS I-dependent electron transport to methylviologen (19 μmoles O_2·mg Chl a^{-1}·h^{-1}) was reduced 2 to 5-fold relative to those of green plant thylakoids. Fluorescence spectra at 77K indicate that both Chl *c* and carotenoids were energetically coupled to Chl *a*. Thylakoids retained the emission at 715 nm but the 685 nm signal of whole cells was red-shifted 4 nm. Enhanced signals at 692 and 710 nm were present and tentatively ascribed to easily detached light-harvesting complexes (LHCs). One LHC contained a single polypeptide (LHCP) of 18 kD whereas the other contained predominantly the 18 kD protein and minor amounts of a 17 kD polypeptide. The purified thylakoids were free of contaminating stromal proteins such as RuBPCase. The major polypeptides of PS I, PS II, ATPase, and LHC are structurally similar to green plant homologs as determined by immunoblotting. Five proteins of PS I, PS II, and LHC were unequivocally identified by protein sequencing; the N-terminal sequences of five other proteins (including one that is immunologically related to LHCPs) were unlike sequenced photosynthetic proteins. The presence of a hydroxyproline residue at position 11 in the sequence of both the 18 and 17 kD LHCPs suggests functionally important post-translational modifications that are unknown in green plant LHCPs. This is the first report: 1) of O_2-

evolving thylakoids from any chromophyte; 2) of long wavelength fluorescence in purified thylakoids of any chomophyte; and 3) linking diatom photosynthetic proteins to the genes that encode them. The protocols and characterizations provided in this manuscript provide the foundation for more detailed analyses of photosynthetic structure-function relationships in diatoms and other Chl *c*-containing algae.

4.2 Introduction

Diatoms (Bacillariophyceae) are an ecologically important group of algae in both freshwater and marine systems. These algae are responsible for an estimated 25% of global primary production (Mann, 1982). In spite of their significance, comparatively little is known about the photosynthetic apparatus of diatoms. Spectroscopic data suggest that the organization of photosynthetic pigment-protein complexes (PPCs) in the thylakoid membranes of diatoms differs fundamentally from that found in membranes of higher plants and green algae (Owens, 1986; Chrystal and Larkum, 1988). Additional evidence comes from immunocytochemical studies with the diatom *Phaeodactylum tricornutum* (Pyszniak and Gibbs, 1992), showing that Photosystem I (PS I) and the fucoxanthin-Chl *a/c* light-harvesting complexes (Fcps) are largely present in both appressed and unappressed regions of the thylakoids. This is in sharp contrast to the lateral heterogeneity observed for thylakoids of higher plants and green algae (Andersson and Anderson, 1980), and suggests that the mechanisms for regulating photosynthetic electron flow (e.g. state transitions) in diatoms may differ from those documented in higher plants and green algae.

The lack of information on the properties of photosynthetic membranes in diatoms is due in part to difficulties in obtaining purified thylakoid membrane preparations in high yield. In addition, the long-wavelength fluorescence emission seen *in vivo* is frequently lost during the isolation of thylakoids (Chrystal and Larkum, 1988), suggesting changes in the organization

of PPCs within the thylakoid membranes. Although O_2-evolving chloroplasts have been isolated from several species of brown algae (Popovic et al., 1983; Strbac et al., 1994), a method for purifying O_2-evolving thylakoid membranes from diatoms or other Chl *c*-containing algae has not been reported. In this paper, we describe a method for obtaining highly purified thylakoid membranes from a marine diatom, *Cylindrotheca fusiformis*, that retain high O_2-evolving capacity as well as long-wavelength fluorescence emission characteristics.

4.3 Materials and Methods

4.3.1 Cultures and Growth Conditions

Cultures of *Cylindrotheca fusiformis* (Watson's strain 13), were grown in 3 L batches in artificial seawater (ASW) medium as described (Plumley et al., 1993). Cultures were illuminated using two cool-white fluorescent bulbs ($\approx$110 μEin m^{-2} s^{-1}) with continuous stirring and aeration using an air supply supplemented with CO_2.

4.3.2 Purification of Thylakoid Membranes

Cells in log phase (10^5-10^6 cells/mL) were harvested in 500 mL aliquots by centrifugation at 2,000 g for 10 min. All steps were carried out on ice or at 4°C. Each pellet was resuspended in 10 mL Buffer A (1 M sorbitol, 20 mM MES (2-[N-morpholino]ethanesulfonic acid)-NaOH (pH 6), 5 mM ε-aminocaproic acid (ACA), 1 mM benzamidine (BAM), 1 mM $MgCl_2$, 1 mM $MnCl_2$). Cells were broken by sonication (1.25 cm probe, Bronwill Scientific) in 10 mL aliquots using two 15 sec bursts at 120 W. Cells were kept in an ice water bath during sonication and were cooled for 3 min between bursts. After sonication, unbroken cells and debris were pelleted by centrifugation at 1000 g for 5 min. The supernatants were set aside and the pellets resuspended in Buffer A and subjected to sonication again. Three to four cycles of sonication/centrifugation were generally sufficient to achieve >95% breakage. The

supernatants were combined, centrifuged at 40,000 g for 15 min, and the crude membrane pellet resuspended and washed in 10 mL Buffer B containing EDTA (1 M sorbitol, 20 mM MES-NaOH (pH 6), 5 mM EDTA, 5 mM ACA, 1 mM BAM). After homogenization with a motor-driven Teflon pestle and dilution to 30 mL with Buffer B, samples were centrifuged at 40,000 g for 15 min. Pellets were resuspended in a total of 30 mL Buffer C (4.4 M sorbitol, 5 mM MES-NaOH (pH 6), 5 mM ACA, 1 mM BAM), homogenized using a motor-driven Teflon pestle, and divided among six 13-ml centrifuge tubes. The sample in each tube was successively overlaid with 2 ml of Buffer D (same as C, but with 3.8 M sorbitol), 3 ml of Buffer E (same as C but with 3.2 M sorbitol), and 3 ml of Buffer F (same as C but with 2.5 M sorbitol). After centrifugation in a Beckman SW-40 swinging bucket rotor at 284,000 g for 16-18 hours, thylakoid membranes were collected at the E/F interface, diluted with an equal volume of Buffer G (same as C, but without sorbitol), and centrifuged at 40,000 g for 30 min. Pellets were resuspended in a small volume of Buffer H (same as Buffer A, but without 1 mM $MnCl_2$). Chlorophyll concentrations were determined in 90% acetone using the equations of Jeffrey and Humphrey (1975). Yields of 20% (calculated on a Chl basis) were readily obtained using this procedure. Freshly prepared thylakoids were used for all experiments.

Thylakoid membranes from lettuce were purified by flotation through sucrose gradients as described previously (Martinson and Plumley, in press; Chapter 2), except that EDTA was eliminated from the buffers used for washing and flotation. Chlorophyll was determined in 80% acetone using the equations of Porra et al. (1989).

4.3.3 Oxygen Evolution

O_2 evolution was measured with a Clark-type electrode (Gilman), at 20°C and a total Chl concentration of 3 or 10 μg/ml (diatom cells and thylakoids, respectively) or 20 μg/ml (lettuce thylakoids). Unless otherwise noted, assays were done in 1 M sorbitol (diatoms) or 0.1 M sorbitol (lettuce), 5 mM MES-NaOH pH 6, 1 mM $MgCl_2$. O_2 evolution was measured using either potassium ferricyanide (FeCN) or freshly sublimed *para*-benzoquinone (pBQ) (stock concentrations were 100 mM and 37 mM, respectively, in ddH_2O). Saturating red light (180 μEin m^{-2} sec^{-1}, 660nm with 25nm spectral line half width; Hansatech) was used to avoid artifactual O_2 evolution that occurs with FeCN in white light. O_2 uptake (Mehler reaction) was measured with red light in 1 M sorbitol, 10 mM phosphate pH 7.0, 5 mM Tricine (N-tris[hydroxymethyl]-methylglycine)-NaOH pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, 3 mM ascorbate, 100 μM DCPIP (2,6-dichlorophenol-indophenol), 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), 10 mM methylamine, 1 mM KCN, and 100 μM methylviologen (MV). Other additions to the assay medium are described in Table 4.1. Kinetic constants were calculated using the curve-fitting function in SigmaPlot (Jandel Scientific).

4.3.4 Spectroscopic Analyses

Absorbance spectra of samples containing 6 μg Chl/ml were obtained with a Hewlett-Packard HP8452A diode array spectrophotometer. 77K fluorescence spectra of samples containing 1.6 μg Chl/ml were measured in 50% glycerol using an Aminco SLM500-C spectrofluorometer with a 4 nm bandpass for both emission and excitation. Emission spectra were corrected for the decrease in the detector efficiency using the correction factor supplied with the instrument.

4.3.5 Pigment Analysis

Pigments were extracted from whole cells or thylakoid membranes using *sec*-butanol (Martinson and Plumley, 1995) and resolved on a Spherisorb 15 cm x 4.6 mm PAH (Phase Sep Ltd.) column using highly buffered ion-pairing reagents (Garrido and Zapata 1993).

4.3.6 Isolation of pigment-protein complexes

To obtain enriched fractions for protein sequencing, thylakoids were solubilized with ß-dodecylmaltoside (DM; DM:Chl = 10:1) as described (Martinson and Plumley, in press; Chapter 2). PPCs were resolved by ultracentrifugation at 284,000 g, 16 h, 4°C, on 5-50% sucrose step (5% increments) gradients containing 10 mM Tris (Tris[hydroxymethyl]aminomethane)-HCl pH 8.5, 5 mM ACA, 1 mM BAM, 0.05% DM.

For isolation of long-wavelength LHCs, thylakoids were solubilized with either 1% DM (DM:Chl = 20:1) or 0.2% deoxycholate (DOC; DOC:Chl = 4.3:1) and resolved on 0-60% sucrose (continuous) gradients or 10-60% sucrose step (10% increments) gradients, respectively. After centrifugation at 284,000 g for 15-16 h, the dark brown fraction at the 40% sucrose layer in both types of samples was collected, Triton X-100 (TX-100) was added to 0.05%. DM/TX-100 samples were loaded on 0-60% sucrose (continuous) gradients; DOC/TX-100 samples were loaded on 10-30% sucrose step (10% increments) gradients. Fractionation was by centrifugation as described above. LHC-containing fractions were collected as a pellet in the DOC/TX-100 sample and at the 25-30% sucrose layer in the DM/TX-100 sample.

4.3.7 SDS-PAGE

For denaturing SDS-PAGE, whole cell pellets were solubilized in 100 mM Tris-HCl (pH 8.5), 2% LDS, 100 mM dithiothreitol (DTT), 5 mM ACA, 1 mM BAM, and 12% sucrose, at a concentration of 0.25 mg Chl/ml. Samples were heated at 100°C for 2 min, centrifuged at 16,000 g for 5 min, and 10 μg Chl loaded per lane. Thylakoids were solubilized at a concentration of 0.5 mg Chl/ml by diluting an aliquot (10 μg Chl) to 16 μl (total volume) with 10 mM Tris-HCl (pH 8.5) and adding 4 μl 5X solubilization buffer (375 mM Tris-HCl pH 8.5, 375 mM DTT, 10% LDS). For sucrose density gradient fractions, 12 μl 5X solubilization buffer was added to 60 μl of sample. Samples were heated at 100°C for 1-2 min (unless otherwise noted; e.g. for cytochrome staining). Electrophoresis was performed on 10-20% polyacrylamide gels with a 4 cm stacking gel of 5% polyacrylamide (Plumley et al., 1993; Martinson and Plumley, in press; Chapter 2). The reservoir buffer contained 50 mM Tris Base and 383 mM glycine, with 0.1% SDS in the upper buffer (Laemmli, 1970). Electrophoresis was carried out at 4°C for 16-18 h at 16 mA. Cytochrome staining with TMBZ (3,3',5,5'-tetramethylbenzidine dihydrochloride) was done as per Hoyer-Hansen (1980). Proteins were detected with Coomassie Brilliant Blue G-250 (Neuhoff et al., 1988).

4.3.8 Immunodetection and N-terminal amino acid sequencing

Immediately following denaturing SDS-PAGE, proteins were transferred to nitrocellulose (immunoblotting) or PVDF (protein sequencing; Millipore) in a semi-dry transfer apparatus (Bio-Rad) at 4°C for 1.5 h at 20 V. After transfer, the nitrocellulose was stained with Ponceau S (Mishkind et al., 1987) and air dried. PVDF membranes were stained with Amido Black.

For immunodetection, blots were reacted with antibodies to the PS I complex from cyanobacteria, the CP47, CP43, D1, D2, and 33 kD polypeptides from spinach, the large (Plumley et al., 1986) and small subunits of RuBPCase, the ß-ATPase subunit from romaine

lettuce (Plumley et al., 1989), or a *Chlamydomonas* 29 kD LHCP (Plumley et al., 1989; Plumley et al., 1993), and developed using alkaline phosphatase conjugated goat anti-rabbit IgG in conjunction with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Mishkind et al., 1987).

N-terminal amino acid sequences were obtained at the Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan, using an Applied Biosystems model 477A protein sequencer.

4.4 Results

Sucrose and sorbitol are widely used as osmotica in the purification of O_2-evolving thylakoids from cyanobacteria, red algae, and green plants, with typical concentrations ranging from 0.3 to 1 M. Previously published methods for purifying thylakoid membranes from diatoms using 0.3 M sucrose (Plumley et al., 1993) resulted in membranes that were no longer capable of O_2 evolution in the presence of exogenous electron acceptors (not shown). Moreover, the membranes lacked PS I activity (ascorbate/DCPIP to MV) and exhibited other characteristics typically associated with disrupted membranes (e.g. altered fluorescence emission properties, not shown; Chapter 5). Preliminary experiments indicated that the loss of O_2 evolution was related to the use of buffers with low osmotic strength in conjunction with harsh breaking conditions (e.g. passage through a French press at 10,000-18,000 psi, and/or freezing of cells at -60°C prior to breakage). These results suggested that the loss of Photosystem II (PS II) and PS I activity could be avoided through use of higher osmotic concentrations and milder breaking conditions.

4.4.1 Optimization of membrane isolation procedure

Two commonly used methods for disrupting algal cells, the French press and sonification, were compared. Preliminary results indicated that regardless of the method used to break cells, repeated and/or prolonged disruption of samples led to loss of O_2 evolution and resulted in small membrane fragments that were difficult to pellet without using high speeds (e.g. 360,000 g for 2 h; not shown). This problem was eliminated if cell fragments were removed by centrifugation (1000 g, 5 min) between each breaking cycle, and if breaking cycles were limited to no more than two bursts for sonification, or one pass through the French press (not shown). Several passages through the French press (10,000 psi) were required if cells had not been frozen; the whole process typically took ≈3 h to achieve 95% breakage. In contrast, sonication was a rapid and effective means of disrupting diatom cells, with 95% breakage attained in ≈1 h under the conditions employed here (two 15 sec bursts at 120 W). These studies showed that sonication was superior to the French press for achieving high breaking efficiencies without prior freezing of the cells, thereby reducing the amount of time required to break cells by almost 70%.

As mentioned above, another potentially important factor in maintaining O_2 evolution capabilities in diatom thylakoid membranes is the osmotic concentration of the buffers used for purification. Various concentrations of sorbitol and sucrose were tested for their ability to preserve O_2 evolution in thylakoid membranes from a marine diatom, *Cylindrotheca fusiformis* (Fig. 4.1A). For these experiments, O_2 evolution (H_2O to 2 mM FeCN) was measured in either the absence of osmoticum or in the same buffer used to purify the membranes. For thylakoids purified and assayed in either sorbitol or sucrose, there were distinct, but opposite, concentration-dependent effects; low concentrations of sorbitol and high concentrations of sucrose reduced O_2 evolution rates (Fig. 4.1A, closed symbols). When O_2 evolution was assayed in the absence of any osmoticum (Fig. 4.1A, open symbols), the

activity of the sucrose-purified thylakoids decreased dramatically, while the O_2-evolving capacity of sorbitol-purified thylakoids decreased only slightly. This suggests that sorbitol-purified thylakoids are more stable in the absence of an osmoticum than are sucrose-purified thylakoids. This is an important factor to consider when performing further biochemical analyses, which may require the removal of most or all osmoticum (e.g. detergent solubilization of thylakoids prior to sucrose density gradient centrifugation; Martinson and Plumley, in press; Chapter 2).

Using the breaking conditions described here (2 x 15 sec at 120 W), the highest rates of O_2 evolution (63 μmoles $O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$) were obtained from thylakoids prepared using 1 M sorbitol. Although 2 M sorbitol was also very effective in maintaining high rates of O_2 evolution, the efficiency with which cells were broken was significantly reduced (Fig. 4.1B). Breakage occurred most rapidly in 0.5 M sorbitol, however the functionality of these membranes was significantly reduced (Fig. 4.1A). Thus, in terms of preserving O_2-evolving capacity (Fig. 4.1A) and allowing for efficient breakage of cells (Fig. 4.1B), 1 M sorbitol appeared to be optimal for use with *Cylindrotheca*.

4.4.2 Electron transport characteristics of purified thylakoids

Once conditions for purifying active thylakoid membranes from *Cylindrotheca* had been identified, the electron transport activities of these membranes were characterized further (Table 4.1). No changes in O_2 levels were detected upon illumination of thylakoids in the absence of added electron acceptors/donors. Whole-chain electron transport, measured with MV) $H_2O \rightarrow$PS II$\rightarrow$Cyt $b_6f/c_{553} \rightarrow$PS I$\rightarrow$MV), was inactive as evidenced by the lack of O_2 uptake upon illumination. The possibility that O_2 evolution resulting from endogenous catalase or superoxide dismutase activity was masking O_2 uptake by MV (resulting in no net O_2 evolution or uptake) was ruled out by addition of KCN, an inhibitor of both catalase and superoxide

dismutase (Table 4.1). The absence of whole chain electron transport suggested either a loss of cytochrome c_{553} (the homolog of plastocyanin found in diatoms) during the purification process, or that MV was unable to accept electrons from PS I. To rule out the latter, PS I-dependent O_2 uptake (ascorbate/DCPIP to MV via P700) was measured and found to be 19.4 μmoles O_2·mg Chl a^{-1}·h^{-1}.

The activity of PS II was measured using either FeCN or pBQ (Table 4.1). The membranes were capable of high rates of O_2 evolution with 2 mM FeCN as electron acceptor; 1 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) or 20 μM DCMU inhibited only 36 or 48% of the rate, respectively. Rates of O_2 evolution in membranes were similar to those observed for whole cells in ASW without FeCN (72 μmoles O_2·mg Chl a^{-1}·h^{-1}), except that cells were completely sensitive to DBMIB and DCMU (Table 4.1). Rates of O_2 evolution with 0.5 mM pBQ were significantly lower (6 μmoles O_2·mg Chl a^{-1}·h^{-1}) than those observed with 2 mM FeCN, reached a maximum at ~3 mM pBQ (22 μmoles O_2·mg Chl a^{-1}·h^{-1}), and were completely abolished by DCMU. These data suggested that FeCN was accepting electrons from both Q_B (DCMU-sensitive) and Q_A (DCMU-insensitive), whereas the pBQ results were indicative of changes in the Q_B sites of some of the PS II reaction centers.

These possibilities were investigated further by comparing the kinetics of O_2 evolution in thylakoids of *Cylindrotheca* and lettuce (Fig. 4.2). Rates of O_2 evolution were measured for a range of FeCN and pBQ concentrations, data fitted using the Michaelis-Menten equation, and the parameters V_{max} and K_m determined. Using FeCN, V_{max} values for both diatom and lettuce thylakoids were 72 μmoles O_2·mg Chl a^{-1}·h^{-1} (Fig. 4.2A). However, the K_m value for diatom thylakoids (184 μM) was much higher than that observed for lettuce thylakoids (9 μM). Using pBQ, V_{max} was much lower in the diatom thylakoids than for lettuce (27 vs. 74 μmoles O_2·mg Chl a^{-1}·h^{-1}), and the K_m values were 1.13 mM and 4 μM for diatom and lettuce

thylakoids, respectively (Fig. 4.2B). These data suggest a reduced affinity for both FeCN and pBQ in the diatom thylakoids relative to that observed in lettuce.

4.4.3 Spectroscopic characterization of purified thylakoids

The room temperature absorption spectrum of purified thylakoid membranes exhibited major peaks at 438 and 672 nm (Fig. 4.3), due to chlorophyll *a*, and shoulders at 460 and 480 nm, due to chlorophyll *c* and fucoxanthin, respectively. There was a broad region of absorption between 500 and 560 nm that is likely due to fucoxanthin and other carotenoids such as diadinoxanthin and diatoxanthin (Anderson and Barrett, 1979). Since *in vivo* absorption spectra are frequently distorted due to scattering artifacts (Hipkins and Baker, 1986), the pigment composition of thylakoids and whole cells was compared using HPLC (Fig. 4.4); profiles were normalized to fucoxanthin and Chl c. There are two major forms of Chl *a* in the thylakoid extract, and only one major form in whole cells. The additional form observed in thylakoids is most likely the result of a minor chemical modification; at present the origin of this modified Chl *a* is unclear. Relative to whole cells, there was a 10% reduction in Chl *a* (all forms included) in thylakoids. Overall, the pigment composition of the purified thylakoids compared favorably with that in whole cells, and with that expected for diatoms (Goodwin, 1988).

The organization of Chls and carotenoids within PPCs was investigated using low temperature (77K) fluorescence spectroscopy (Fig. 4.5). When illuminated with 440, 470, or 490 nm light (preferentially exciting Chl *a*, Chl *c*, and carotenoids, respectively), whole cells exhibited emission maxima at 685 and 712 nm (Fig. 4.5A). The fluorescence yields at 685 nm with 470 and 490 nm excitation were ≈27% and 14% higher, respectively, than with 440 nm excitation. Similar values were obtained for the 717 nm emission peak. These data are consistent with the excitation spectra for the 685 and 717 nm emission peaks (Fig. 4.5C

and not shown), which were qualitatively identical and revealed substantial contributions by Chl c and fucoxanthin relative to Chl *a*. These data suggest the presence of a substantial light-harvesting component in the cells examined here, as would be expected in shade adapted cells such as those used in this work (i.e., cell density of 10^6/ml in 3 L).

In purified thylakoid membranes, there was little fluorescence emission between 600 and 660 nm (where free Chl emits) indicating that the major accessory pigments, Chl *c* and fucoxanthin, were energetically coupled to Chl *a* in the thylakoid preparation (Fig. 4.5B). Relative to whole cells, the major emission peak shifted to 689 nm, and there was a broadening of the fluorescence emission in the 690-720 nm range (Fig. 4.5D). In addition, the total fluorescence yield increased by a factor of ≈ 2 over that observed *in vivo* (Fig. 4.5D). The fluorescence yields of Chl *c* and carotenoids at 689 nm were 2% higher and 40% lower than that of Chl *a*, respectively (Fig. 4.5B). In accordance with this, the excitation spectrum for the 689 nm emission peak showed a reduction in the amount of Chl *c* and fucoxanthin relative to Chl *a* (Fig. 4.5C). These results imply that the abundant LHC coupled to PS II and PS I *in vivo* is uncoupled in the purified thylakoids, a conclusion that is supported by the overall increase in fluorescence yield relative to whole cells (Fig. 4.5D).

In order to analyze the putative detached LHC(s) in more detail difference emission spectra were calculated for all excitation wavelengths by subtracting the whole cell spectrum from that for thylakoids (Fig. 4.5E). The difference emission spectra for thylakoids and whole cells excited with 440 and 470 nm light revealed a major peak at 692 and 693 nm, respectively. The major emission peak in the difference spectrum for 490 nm excitation was at 696 nm, and the fluorescence yield was reduced relative to that with 440 and 470 nm light (Fig. 4.5E).

Earlier studies (Plumley and Martinson, in prep.) showed that *Cylindrotheca* thylakoids contain two LHCs with properties matching those of the putative detached LHC(s). One LHC, obtained from DM/TX-100 solubilized thylakoids contained a single 18 kD polypeptide (not

shown) and fluoresced at 690 nm at 77K when excited with 440 (Fig. 4.5F) and 470 (not shown) nm light. Another LHC obtained using DOC/TX-100 contained primarily an 18 kD polypeptide with minor amounts of a 17 kD polypeptide (not shown) and had a fluorescence emission peak at 698 nm with a shoulder at 680 nm when excited with 440 nm light (Fig. 4.5F). These data suggest that there are at least two long-wavelength antennae present in the thylakoids of *Cylindrotheca*. Since uncoupled LHCs typically have high fluorescence yields relative to the reaction centers, these results suggest that two uncoupled antennae emitting at 692-696 nm are responsible for both the increase in fluorescence yield relative to whole cells and the broadening of fluorescence emission between 690 and 720 nm seen in purified thylakoids.

4.4.4 Identification of photosynthetic polypeptides

The purity of the thylakoid preparation was assessed by denaturing SDS-PAGE and immunoblotting. A comparison of the polypeptide profiles of whole cell protein extracts and purified thylakoid membranes revealed the expected overall reduction in the amount of protein present in thylakoids relative to whole cells when gels were loaded on an equal Chl basis. Immunoblotting analysis showed the thylakoids to be free of the large subunit of the stromal enzyme, RuBPCase (Fig. 4.6A). An antibody to the small subunit of RuBPCase did not cross-react with any polypeptides present in purified thylakoids, but recognized two polypeptides in the whole cell extract, both at ≈13.5 kD (Fig. 4.6A). A doublet at 13.8 kD observed previously in *Cylindrotheca* using 2D electrophoresis was attributed to the small subunit of RuBPCase (Plumley et al., 1986). These results confirmed the purity of the thylakoid membrane preparation.

Immunochemical and histochemical procedures were used to conclusively identify the major photosynthetic proteins in purified thylakoids of *Cylindrotheca*. For immunochemical

studies, blots were probed using a number of antibodies against well-characterized photosynthetic proteins of green plants (Fig. 4.6B). An antibody to the PS I reaction center polypeptides (PsaA and PsaB) from cyanobacteria recognized one protein of ≈65 kD (lane 3), and a doublet at 55 kD corresponded to the ß-subunit of ATPase (AtpB; lane 4). The presence of the 33 kD polypeptide of the water oxidation complex (PsbO) was verified using an antibody to the spinach protein (lane 6). Antibodies to the PsbC (CP43) and PsbB (CP47) polypeptides recognized polypeptides of ≈45 kD and a doublet at ≈52 kD, respectively (lanes 5,6). Lanes 7 and 8 were probed using antibodies to the PS II reaction center polypeptides, D1 (PsbA) and D2 (PsbD), revealing polypeptides at 34 and 32 kD, respectively. Finally, when blots were challenged with an antibody against the 29 kD LHCP of *Chlamydomonas reinhardtii*, a number of bands from *Cylindrotheca* cross-reacted (lane 9). These bands have apparent molecular weights (M_rs) of 27, 20, 18, 17.5, 17, and 16.5 kD. Cytochromes were identified using TMBZ-staining, which revealed bands at 36 and 14 kD (Fig. 4.6B) in both heated and non-heated samples. Based on its M_r and stability when heated, we believe that the 36 kD polypeptide is cytochrome f (PetA); the identity of the band at 14 kD is unknown, but it may correspond to cytochrome c_{554} found in the diatom *Navicula* (Yamanaka et al., 1967). These results demonstrated the presence of the major photosynthetic proteins in purified thylakoids of *Cylindrotheca* and confirmed their overall homology to those of green plants and cyanobacteria.

The distribution of the photosynthetic proteins present in purified thylakoids was studied in more detail using detergent-solubilization and fractionation techniques (Martinson and Plumley, in press, Chapter 2). Sucrose-density gradient fractionation of PPCs from DM-solubilized thylakoids resulted in a number of pigmented fractions (not shown). The uppermost fraction on the gradient was yellow/orange in color, and contained several low M_r polypeptides in addition to a polypeptide at 33 kD (Fig. 4.7, lane 2). A dark brown-colored

fraction was enriched in LHCPs (Fig. 4.7, lane 3). Two green fractions were obtained, one enriched in PS II (Fig. 4.7, lane 4), and the other containing both PS II and PS I polypeptides (Fig. 4.7, lane 5). The heaviest fraction was olive-green, and was enriched in PS I (Fig. 4.7, lane 6). The biophysical and biochemical properties of these fractions will be characterized in a subsequent paper (Martinson et al., in prep.). Of importance for this manuscript is the presence of a number of polypeptides with identical M_rs in the purified thylakoids (Fig. 4.7). Most noteworthy are polypeptides at 27, 18, 17, and 14 kD, which are observed in two well-separated fractions (Fig. 4.7, lanes 3,6). In other experiments, we have observed a major 18 kD LHCP in two distinct sucrose density gradient fractions (not shown). The identity of many of these proteins is unknown, and moreover, it is unclear whether these polypeptides are products of the same or different genes.

Evidence to support the existence of multiple gene products with identical M_rs was provided by immunochemical studies of fractionated thylakoids. An antibody against the 29 kD LHCP of *Chlamydomonas* cross-reacted with a 27 kD polypeptide in thylakoids (Fig. 4.6B, lane 9), but did not cross-react (not shown) with the 27 kD polypeptide present in the PS I fraction (Fig. 4.7, lane 6); although the cross-reactivity of the 27 kD polypeptide present in the LHC fraction (Fig. 4.7, lane 3) has yet to be assessed, a positive reaction seems likely. We conclude from these results that there are at least two 27 kD polypeptides present in purified thylakoids of *Cylindrotheca*: one is immunologically related to the green alga LHC polypeptide, the other is not. It is clear from these results that polypeptides present in purified complexes cannot be assigned identities based solely on their M_r.

In order to unequivocally identify some of the proteins present in purified thylakoids of *Cylindrotheca*, several polypeptides present in the sucrose density gradient fractions shown in Fig. 4.7 were subjected to N-terminal sequencing (Table 4.2). The N-terminal sequence of the 33 kD protein present in the uppermost yellow-colored fraction (Fig. 4.7, lane 2) was

found to be homologous to the manganese-stabilizing protein (PsbO) from higher plants (Fig. 4.8). This confirms our immunological identification of this polypeptide (Fig. 4.6B), and moreover, indicates that this protein is easily dissociated from PS II by detergent solubilization.

N-terminal amino acid sequences were obtained for four proteins in the dark brown LHC fraction (Fig. 4.7, lane 3), with M_rs of 18, 17.5, 17, and 14 kD (Table 4.2). The 14 kD polypeptide was not homologous to sequences deposited in the database (GenBank, non-redundant), however, the 17, 17.5, and 18 kD polypeptides showed extensive homology to the deduced amino acid sequences from the *Phaeodactylum tricornutum* light-harvesting genes *fcp*F, and *fcp*E (Fig. 4.8), and slightly lower homology to fucoxanthin Chl *a/c* polypeptides from the diatom *Odontella sinensis* and the brown alga *Macrocystis pyrifera*. In addition, amino acid sequencing revealed the presence of hydroxy-proline residues at position 11 in both the 18 and 17 kD polypeptides (Table 4.2). Overall, these results confirm the relatedness of the *Cylindrotheca* LHCPs to those of other chromophytes, and also suggest the presence of post-translational modifications that are unknown in LHCPs of green plants.

Three bands from the PS I-enriched fraction (Fig. 4.7, lane 6) were sequenced. The 27 and 22 kD polypeptide sequences (Table 4.2) were not homologous to any sequenced proteins, however, a polypeptide at 14 kD was found to be 91% identical to PsaD from the diatom *Odontella sinensis* (Fig. 4.8). Interestingly, an antibody to the 29 kD LHCP of *Chlamydomonas* cross-reacted with the 17.5 and 22 kD polypeptides present in this fraction (not shown), making this the first unequivocal demonstration of PS I-associated LHCPs in diatoms. In addition, the lack of N-terminal homology between the 22 kD polypeptide (Table 4.2) and LHCP sequences currently available in the GenBank suggests that this may be a novel gene product. Although the 27 kD polypeptide did not cross-react with the LHCP antibody and did not exhibit homology to any known protein, we occasionally see this polypeptide as a

component of an orange-colored band on mild LDS-PAGE gels (T.A.M., unpublished data), suggesting that it may be a carotenoid-binding protein.

4.5 Discussion

Much of the work regarding the photosynthetic PPCs of diatoms and other chromophyte algae has been done with thylakoid membranes that have not been characterized with respect to their biophysical and biochemical properties (Caron and Brown, 1987; Büchel and Wilhelm, 1993; Hiller et al., 1993). A solid understanding of the biochemical and biophysical properties of thylakoid membranes is necessary before conclusive statements can be made regarding the characteristics of individual PPCs. Although highly purified thylakoids have been isolated from a diatom by us and characterized immunologically (Plumley et al., 1993), the biophysical properties of these membranes differed substantially from those observed *in vivo* (e.g. altered fluorescence emission, lack of electron transport activity). Our results were not surprising as other researchers have documented the ease with which *in vivo* fluorescence emission characteristics are lost upon breakage of diatom cells (Chrystal and Larkum, 1988). In addition, there have been no reports of O_2-evolving activity in purified thylakoids from any chromophytic alga, and, to the best of our knowledge, no documented PS I activity. Years of experimentation in our lab, however, revealed extensive damage to the PS II (Chapter 6) and PS I (Chapter 5) reaction centers in diatom membranes that lacked electron transport capabilities. The dissociation of the reaction center complexes made their isolation and subsequent characterization difficult, if not impossible, and led to additional difficulties in identifying the LHCs specifically associated with each reaction center. Thus, we determined that functionally intact thylakoid membranes were needed before we could continue our work on the PPCs of diatoms.

4.5.1 Optimization of thylakoid purification procedure

Preliminary experiments suggested that the loss of electron transport activity was due to a combination of two factors: harsh breaking conditions/freezing of cells and use of buffers with low osmotic strength. Breakage of cells in a French Press was a lengthy process unless cells had been frozen previously. Subsequent tests showed that cells were easily broken by sonification, and that freezing at -60°C prior to breakage was not required for rapid disruption of cells.

The importance of osmotic strength was investigated by testing different concentrations of sorbitol and sucrose with regard to their ability to preserve O_2 evolution in purified thylakoid membranes from the diatom *Cylindrotheca fusiformis* (Fig. 4.1A). We chose sucrose and sorbitol because of their widespread use as osmotica in purifying thylakoid membranes from a variety of plants and algae. The highest rates of O_2 evolution were observed in thylakoids purified and assayed in 1 M sorbitol (closed circles); thylakoids purified and assayed in either 1 or 0.5 M sucrose also exhibited high activity (closed squares). Marked differences were observed in the stability of electron transport in thylakoids purified in these two types of osmotica, however. The activity of the sucrose-purified thylakoids, regardless of the concentration used for purification, decreased dramatically when O_2 evolution was measured in the absence of sucrose (open squares). In contrast, the activity of sorbitol-purified thylakoids was only somewhat reduced when measurements were done in the absence of sorbitol (open circles). These results indicate that sorbitol-purified thylakoids are more stable in the absence of an osmoticum than are sucrose-purified thylakoids, and suggests that sorbitol is better at stabilizing the overall organization of photosynthetic proteins within the thylakoid membranes.

The procedure reported here has been successfully used with another diatom, *Cyclotella cryptica*, indicating that the method is generally applicable to a variety of diatoms. However,

it should be pointed out that the conditions described here were optimized specifically for *Cylindrotheca*. The intensity and duration of bursts used to break cells may need to be altered for other species of diatoms due to varying degrees of silicification (Werner, 1977) and/or other factors. Conditions must be optimized for each species of diatom such that maximal activity is obtained while at the same time minimizing the time required to achieve maximal breakage of cells. We have found that excessive sonication, either by using higher wattages, longer bursts, or repeated sonication of material already released from the cells (e.g. more bursts prior to each low-speed centrifugation), resulted in decreased O_2 evolution, severe decreases in yields, and in some instances a complete loss of activity (not shown). In addition, the stability of PS I (Martinson and Plumley, 1993; Martinson and Plumley, in prep.; Chapter 5) and PS II (Chapter 6) were severely compromised.

4.5.2 Characterization of electron transport activity

Purified diatom thylakoid membranes facilitated electron flow from ascorbate/DCPIP (artificial donors to PS I) to MV, but not from H_2O to MV (Table 4.1). There are three possible reasons for the lack of whole chain electron transport. First, cytochrome c_{553}, the functional equivalent of plastocyanin in diatoms (Sandman, 1986), could have been lost during purification; plastocyanin is also lost from thylakoids of cyanobacteria (Stewart and Kaethner 1983) and spinach (Takano et al., 1985) if sonication is used. Another alternative is that the donor side of PS I has been altered, perhaps at the level of cytochrome c_{553} binding. Although we cannot entirely rule out this possibility, it seems unlikely that extensive damage to the site of electron donation to P700 has occurred because the membranes exhibit O_2 uptake with MV when ascorbate and DCPIP were included in the assay as donors to P700 (Table 4.1). The observed O_2 uptake rates, however, are about 2- to 5-fold lower than those observed for purified thylakoids (F.G.P., in prep.) or broken cell extracts (Tan et al., 1995) of

green algae. A third explanation is that the Fe-S centers, F_A and F_B (located on the PsaC polypeptide; Høj et al., 1987), have been lost; these centers are the site of electron donation to MV in spinach (Fujii et al., 1990). The PsaC polypeptide is buried beneath the PsaD and PsaE subunits (Oh-Oka et al., 1989) and although we cannot rule out the absence of PsaE, PsaD is present in our thylakoid preparation (Figs. 4.7, 4.8). Hence, the complete loss of the PsaC polypeptide (and therefore of F_A and F_B) can largely be ruled out. Moreover, it is unlikely that both centers have been lost because some O_2 uptake is observed (Table 4.1). A number of studies have shown F_B to be more susceptible to denaturation than F_A (Golbeck and Warden, 1982; Malkin, 1984; Fujii et al., 1990). In addition, spinach PS I particles depleted of F_B exhibited reduced rates of ascorbate/DCPIP/MV-mediated O_2 uptake (He and Malkin, 1994). In light of these data, the most logical explanation for the low rates of O_2 uptake in our preparation is that one of the Fe-S centers, most likely F_B, has been lost or altered.

Thylakoid membranes purified using the procedure outlined in this report exhibit light-dependent electron flow from water to either FeCN or pBQ (Table 4.1). As a lipophilic molecule, pBQ readily partitions into the thylakoid membranes of green plants and cyanobacteria where it accepts electrons primarily from Q_B^{2-} (Tanaka-Kitatani et al., 1990). In unfragmented thylakoid membranes of green plants, FeCN accepts electrons primarily from PS I and only to some extent from PS II, with the relative proportions being dependent on the integrity of the preparation (Izawa, 1980). However, FeCN is capable of accepting electrons directly from PS II in subchloroplast preparations (Izawa, 1980), perhaps at a location between Q_A and Q_B (Yruela et al., 1991), or directly from Q_B (Bowlby and Yocum, 1993). Since our thylakoid preparation does not exhibit whole chain electron transport, it is unlikely that electron flow from H_2O to FeCN via PS I is occurring and we conclude that O_2 evolution in the presence of FeCN is exclusively due to electron flow from PS II to FeCN.

In the presence of 2 mM FeCN, thylakoids evolved O_2 at rates comparable to those of whole cells (Table 4.1; Fig. 4.2A), however, O_2 evolution in diatom thylakoids was only partially inhibited by 1 μM DBMIB or 20 μM DCMU (Table 4.1). The failure of DBMIB to completely inhibit O_2 evolution in thylakoids was not unexpected, since it has been shown that DBMIB does not fully inhibit FeCN reduction in fragmented systems (Trebst, 1980) or in sonicated chloroplasts (Izawa et al., 1973). In addition, DBMIB can accept electrons from plastoquinone and donate them to FeCN (Izawa et al., 1973), a situation that would complicate the assessment of inhibition of O_2 evolution by DBMIB.

The lack of complete inhibition of electron flow from H_2O to FeCN by DCMU suggests that FeCN is capable of accepting electrons from at least two sites, one of which is located prior to the Q_B site in PS II (e.g. Q_A). In higher plants and green algae, DCMU-insensitive electron flow from H_2O to FeCN has been documented primarily for purified PS II particles (Yamada et al., 1989; Bowlby and Yocum, 1993; Bumann and Oesterhelt, 1994) or trypsinized thylakoid membranes (Yruela et al., 1991). Furthermore, Bowlby et al. (1988) have shown that the DCMU-sensitivity of FeCN-supported O_2 evolution decreases with increasing purification of PS II preparations. They attributed this to an increased accessibility of FeCN to Q_A resulting from the removal of 28, 22, and 10 kD polypeptides from the PS II reaction center complex, with the largest decrease in sensitivity occurring in conjunction with the loss of the 28 kD polypeptide. Interestingly, the K_m value for FeCN reduction in our thylakoid preparation (184 μM) is similar to that reported for cholate-treated PS II reaction center complexes (181 μM) which were depleted in the 22 kD polypeptide (Bowlby and Yocum, 1993). In contrast, untreated PS II membranes (also called BBY membranes) exhibited a K_m value of 34 μM (Bowlby and Yocum, 1993). The K_m value obtained in this study for lettuce thylakoids (9 μM; Fig. 4.2A) was much lower, probably because the thylakoids used in this study were more intact than the BBY membranes. This is supported by the fact that electron

flow from H_2O to FeCN in lettuce thylakoids was completely inhibited by DCMU (not shown), whereas in BBY membranes, inhibition typically ranged from 70-90% (Bowlby et al., 1988). Together, the decreased sensitivity of FeCN-supported O_2 evolution to DCMU and the increased K_m value suggest that Q_A is more exposed in the PS II reaction centers of purified diatom thylakoids, and that the increased accessibility may be due to a loss of the 10, 22 and/or 28 kD polypeptides during purification.

Genes for the 22 kD (*psb*S) and 10 kD (*psb*H) polypeptides are present in the chloroplast genome of the diatom *Odontella sinensis* (Kowallik, 1996), however, the presence or absence of the 28 kD polypeptide has yet to be investigated in diatoms. Moreover, the identity of this protein in higher plants is unclear. It has been suggested that the 28 kD polypeptide, which binds Chl a (Ghanotakis et al., 1987), is an LHC referred to as CP29, but this has not been proven (Bowlby et al., 1988). A more intriguing hypothesis is that the 28 kD polypeptide is functionally analogous to the H subunit of the purple bacterial reaction center (Bowlby et al., 1988). If this is true, then one would expect to find the 28 kD polypeptide in the PS II reaction centers of diatoms, and the loss of this protein could explain our results described above.

Another means of measuring the functional attributes of PS II is to determine the kinetics of electron transport from H_2O to exogenous quinones (Fig. 4.2B), a reaction that can be inhibited with DCMU. Activity was completely abolished by 20 μM DCMU over the entire range of pBQ concentrations tested (0.1-5 mM), however, the maximum rate of O_2 evolution was only about 37.5% of the maximum rate obtained with FeCN (Table 4.1). The inefficiency of pBQ in accepting electrons from PS II (relative to substituted benzoquinones such as 2,6-dichloro-*p*-benzoquinone or phenyl-*p*-benzoquinone) has been documented for purified PS II reaction centers from a thermophilic cyanobacterium, and was attributed to the low hydrophobicity of pBQ relative to substituted quinones (Tanaka-Kitatani, et al., 1990).

Indeed, V_{max} values for O_2 evolution in purified PS II preparations from *Synechococcus* increased with increasing hydrophobicity for various benzoquinones, and were lowest for pBQ (Satoh et al., 1992). One could argue that this is the reason for the low V_{max} observed for diatom thylakoids, and that rates would be higher with a more hydrophobic benzoquinone. Preliminary results, however, indicate that O_2 evolution with diatom thylakoids is only moderately enhanced using a more hydrophobic benzoquinone. More work is needed to resolve this discrepancy. Another complicating issue is that pBQ supported O_2 evolution in lettuce thylakoids at rates equal to those measured with FeCN. Therefore, the low value of V_{max} in diatom thylakoids with pBQ cannot be attributed solely to an inherent inefficiency of pBQ. Instead, the low values are most likely due to either a reduction in the number of PS II reaction centers capable of donating electrons to pBQ (model 1), a decrease in rate of the final step in the reduction of pBQ (e.g. dissociation and diffusion away from the Q_B site; Satoh et al., 1992) due to alterations in the Q_B site (model 2), or a combination of the two.

The high K_m for pBQ in the diatom thylakoids (1,130 μM) implies that binding of pBQ to the Q_B site is impaired in diatom PS II reaction centers relative to those in lettuce thylakoids, (K_m of 4 μM; Fig. 4.2B), thus supporting the second model described above. Interestingly, the K_m value for pBQ in diatom thylakoids is similar to that observed with 2,6-dimethylbenzoquinone (1.35 mM; Satoh et al., 1992). The higher K_m values for substituted benzoquinones such as 2,6-dimethylbenzoquinone were attributed to reduced access to the Q_B site due to steric hindrance (Satoh et al., 1992). Compared to the K_m values for various benzoquinones in purified PS II reaction centers, the K_m value (4 μM) that we obtained for pBQ-supported O_2 evolution in lettuce thylakoids is extremely low relative to that reported for purified PS II reaction centers from *Synechococcus* (340 μM; Satoh et al., 1992). Once again, this is most likely due to the higher integrity of the PS II reaction centers in non-fractionated thylakoids. Together, these observations suggest that the low V_{max} for pBQ-dependent O_2 evolution in

diatoms is due to a poor interaction between pBQ and Q_B resulting from an alteration in the Q_B site such that pBQ is unable to bind as efficiently (model 2). We cannot at this time, however, rule out the possibility that a fraction of the PS II reaction centers in purified diatom thylakoids are completely incapable of reducing pBQ (model 1).

4.5.3 Spectroscopic characterization

Resolution of pigments by HPLC revealed the presence of Chls *a*, c_1, and c_2, as well as fucoxanthin, ß-carotene, diadinoxanthin, and diatoxanthin (Fig. 4.4). These results compare favorably with published data concerning the pigment composition of diatoms (Goodwin,1988). Although the major Chl *a* peak in the thylakoid pigment extract was somewhat reduced relative to that in the whole cell extract, the actual reduction was only about 10% when the other forms of Chl *a* were taken into account. These data suggest that some Chl *a* may have been lost during purification. The lack of fluorescence emission at wavelengths less than 680 nm (Fig. 4.5B) indicates that all the Chl *a* and *c* present in the thylakoids is bound to proteins. Furthermore, preferential excitation of chlorophyll *c* and carotenoids with 470 nm and 490 nm light, respectively, results in emission peaks at the same wavelengths as the emission peaks when chlorophyll *a* is preferentially excited (Fig. 4.5B). This shows that the carotenoids and chlorophyll *c* are energetically coupled to chlorophyll *a*, and verifies the integrity of the thylakoid preparation.

The purified thylakoids retained long-wavelength (>700 nm) fluorescence emission characteristics typical of whole cells. The low temperature emission spectrum is somewhat altered from that seen *in vivo* (Fig. 4.5D). The difference spectrum of whole cells and thylakoids excited with 440 nm light reveals a single component at 692 nm, with a shoulder at 710 nm (Fig. 4.5E). It is possible that the 692 nm peak arises from one or more antennae which are no longer transferring excitation energy to the reaction centers. This hypothesis

is supported by the increase in fluorescence yield seen in purified thylakoids relative to whole cells (Fig. 4.5D); uncoupled LHCs generally have a substantially higher fluorescence yield than reaction centers. These results suggest the presence of at least one long-wavelength antenna in *Cylindrotheca*.

Two long-wavelength LHCs have been isolated from *Cylindrotheca* (Plumley and Martinson, in prep). These antennae were dark brown in color, indicating the presence of fucoxanthin. One, purified using deoxycholate and Triton X-100 (DOC/TX-100), contained a single polypeptide of 18 kD and fluoresced at 690 nm (Fig. 4.5F). Dodecylmaltoside and Triton X-100 (DM/TX-100) were used to purify a second complex, fluorescing at 698 nm (Fig. 4.5F) and consisting primarily of an 18 kD protein with lesser amounts of a 17 kD polypeptide. The major 17 and 18 kD polypeptides present in *Cylindrotheca* have been unequivocally identified as LHCPs both immunologically (Fig. 4.6B; Plumley et al., 1993) and by N-terminal sequence analysis (Fig. 4.8). This, coupled with the obvious presence of fucoxanthin in the fractions, allows us to conclude that the 17 and 18 kD components of the long-wavelength antennae are indeed LHCPs.

At this time, it is unclear whether there are two distinct long-wavelength antennae in *Cylindrotheca* or only one. The polypeptide compositions of the two antennae are almost identical (not shown), and yet the fluorescence emission maxima are quite distinct. One could argue that the longer wavelength emission seen in the DM/TX-100 antenna arises from the 17 kD component. This polypeptide is of such low abundance relative to the 18 kD component, however, that it seems more likely that the 17 kD polypeptide is responsible for the shoulder at 680 nm. Further fractionation of this complex would be necessary to determine if this is the case.

Under the assumption that there are two distinct long-wavelength antennae present in thylakoids, then the presence of a single polypeptide of identical M_r in both antennae could

be explained in one of two ways: 1) the polypeptides are the products of different genes, and are therefore unique, or 2) there is only one gene product. Since all six mature fucoxanthin-Chl *a/c* LHCPs in *Phaeodactylum* are about the same length (197-200 amino acids; Bhaya and Grossman, 1993), it is essentially impossible to resolve them from one another on SDS-PAGE gels. Therefore, without N-terminal sequence data from each of the 18 kD polypeptides, we cannot rule out the possibility that there are two distinct gene products (e.g. the first explanation), and even then the high degree of homology between the six different gene sequences (Bhaya and Grossman, 1993) may make unequivocal identification of the two proteins impossible.

The second explanation is that the 18 kD polypeptide present in the DOC/TX-100 LHC is identical to that in the DM/TX-100 LHC (e.g. only one gene product), leading us to conclude that the differences in fluorescence emission properties between the two antennae are not due to fundamental differences in the primary sequence of the 18 kD apoprotein, but instead may be due to conformational differences. The longer fluorescence emission maximum of the DOC/TX-100 LHC suggests that some portion of the Chl *a* molecules in this complex have longer absorption maxima than the Chl *a* molecules present in the DM/TX-100 LHC. Since the absorption properties of pigments are dictated by the protein environment (Nobel, 1991), this implies that the binding of Chl *a* by the 18 kD apoprotein of the DOC/TX-100 LHC differs from that by the 18 kD apoprotein of the DM/TX-100 LHC. We suggest that these differences may be caused by a conformational change in the 18 kD polypeptide present in the DOC/TX-100 LHC due to either a) binding of the 17 kD polypeptide, b) post-translational modification (e.g. proline hydroxylation), or c) differences in the amount of bound carotenoids. In the first case (a), the association of the 17 kD polypeptide with the 18 kD polypeptide could either be stabilized by DOC against dissociation by TX-100, or be disrupted by DM. This hypothesis could be tested by further solubilization of the DOC/TX-100 fraction with DM in an attempt

to remove the 17 kD polypeptide. In the second case (b), a conformational change in the 18 kD polypeptide could be induced by post-translational modification(s). This hypothesis is supported by protein sequence data (Table 4.2), which revealed a hydroxyproline residue at position 11 in an 18 kD LHCP. Sequence data for the 18 kD polypeptides present in the DOC/TX-100 and DM/TX-100 LHCs are needed to address this possibility. The third possibility (c) is that the different conformation of the 18 kD polypeptide in the DOC/TX-100 LHC (relative to that in the DM/TX-100 LHC) is caused by the binding of different amounts of carotenoids, such as fucoxanthin, diadinoxanthin, or diatoxanthin. The role of carotenoids in maintaining Chls in their proper orientation has been documented for green plant LHCII (Plumley and Schmidt, 1987) and PS I (Öquist et al., 1980). Thus, it is not inconceivable that changes in the amount of carotenoids bound to the 18 kD polypeptide could alter its conformation. This possibility could be addressed by comparing the pigment compositions of the two types of LHCs.

Finally, it is interesting that only one long-wavelength antenna was isolated for each of the detergent-solubilization procedures. If there are two unique long-wavelength antennae (as discussed above), then these results suggest that one of the antennae is stable in the presence of DOC, and unstable in the presence of DM, while the converse is true for the other complex. On the other hand, if there is only one long-wavelength antenna, then the results suggest that the differences observed between the two types of detergent-solubilization procedures are due to either 1) removal of the 17 kD polypeptide as discussed (a, above), or removal of the carotenoids by some detergents (c, above). It is becoming increasingly evident that N-terminal sequence data for the *Cylindrotheca* LHCPs are needed to adequately characterize the LHCs in this alga, as well as in other chromophytic algae. Initial work toward this goal is discussed below.

4.5.4 Immunological and histochemical identification of photosynthetic polypeptides

The polypeptide composition of the purified thylakoid membranes was characterized using antibodies to an array of photosynthetic proteins. We were able to identify the PsaA and PsaB proteins using an antibody made to the PS I core complex of cyanobacteria. On 10-20% SDS-PAGE gels, this antibody recognized only "one" polypeptide, with an M_r of 60-70 kD. Since the PsaA and PsaB proteins are almost the same size, it is frequently difficult to resolve them. However, we were able to resolve two polypeptides on a 6-12% acrylamide gel, both of which reacted with the P700 antibody (Martinson and Plumley, in prep.; Chapter 5).

In this work, we found that the antibody against the 29 kD LHCP of *Chlamydomonas reinhardtii* recognized several polypeptides of 27, 20, 18, 17.5, 17, and 16.5 kD. Similar results were obtained previously (Plumley et al., 1993), with the notable exception of the 27 and 20 kD polypeptides. We attribute this to: 1) the use of a different batch of antibody: the batch used in this work contains antibodies to additional epitopes; and 2) the increased purity of these thylakoids.

Two cytochromes were identified in our thylakoid preparation, the active heme in both was stable to heating. Based on its apparent molecular weight, the 36 kD polypeptide is most likely cytochrome f. The identity of the cytochrome at 14 kD is unknown, although it is interesting to note that a 13 kD c-type cytochrome was isolated from the diatom *Navicula pelliculosa* (Yamanaka et al., 1967). This cytochrome, which the authors referred to as c_{554}, has spectral properties nearly identical to those of cytochrome c_{553} purified from *Phaeodactylum tricornutum* (Shimazaki et al., 1978). It remains to be seen whether cytochrome c_{554} is functionally analogous to cytochrome c_{553}. In addition to cytochrome c_{553}, diatoms contain cytochrome c_{550} (17 kD; Shimazaki et al., 1978), which has been shown to play a role in O_2 evolution in cyanobacteria (Shen et al., 1995). The gene encoding this component, *psb*V, was recently shown to be encoded in the chloroplast genome of the diatom

Odontella sinensis (Kowallik, 1996). We were unable to demonstrate the presence of cytochrome c_{550} in our thylakoids, however, in *Phaeodactylum*, cytochrome c_{550} is released from cells simply by freezing in distilled water. Therefore, it is possible that this protein has been washed off the membranes during the purification procedure.

In addition to the c-type cytochromes discussed above, one would expect to find cytochrome b_6 (18 kD) and cytochrome b_{559} (two subunits of 4 and 9 kD), which are seemingly ubiquitous among oxygenic photosynthesizers (Cramer and Knaff, 1991; Hoober 1984). No bands at these molecular weights could be detected using the TMBZ staining method, although b-type cytochromes are often difficult to detect following SDS-PAGE.

4.5.5 Identification of photosynthetic proteins by N-terminal amino acid sequencing

N-terminal amino acid sequences were obtained for several polypeptides present in purified thylakoid membranes (Fig. 4.7; Table 4.2). Two of the polypeptides sequenced, an 18 kD polypeptide present in PS I particles (Fig. 4.7, lane 6) and a 14 kD polypeptide present in a LHC fraction (Fig. 4.7, lane 3) showed no significant homology to sequenced photosynthetic proteins. These data suggest that diatoms may contain photosynthetic proteins with unique N-terminal sequences.

A sequenced polypeptide of 33 kD was found to be homologous to the 33 kD protein of the water oxidation complex from higher plants (Fig. 4.8), a finding which is in agreement with immunological results (Fig. 4.6B). Moreover, this protein copurified with a LHC fraction rather than with PS II particles (Fig. 4.7), suggesting that it is easily dissociated from the PS II reaction center by DM treatment. This finding is not unexpected, as this protein is readily washed off membranes by treatment of green plant and cyanobacterial thylakoids with 0.8 M Tris buffer, 1 M $CaCl_2$, 250 mM NaCl, or 10 mM EDTA (Vermaas et al., 1993).

The N-terminal sequence of a 14 kD polypeptide present in PS I particles (Fig. 4.7) was 91% identical to the deduced amino acid sequence of PsaD from *Odontella sinensis*, and somewhat less homologous to PsaD from *Chlamydomonas reinhardtii* (67%) and barley (58%) (Fig. 4.8). Interestingly, the N-terminus of the *Cylindrotheca* polypeptide aligns precisely with the beginning of the deduced amino acid sequence from the *Odontella psa*D gene (Fig. 4.8). In contrast, the region of homology in the deduced sequence from barley and *Chlamydomonas* begins at residues 69 and 57, respectively (Fig. 4.8), because the gene product contains a transit sequence which allows it to be imported into the chloroplast. This suggests that the *psa*D gene product in diatoms does not contain a transit peptide sequence, which is consistent with this gene being located in the chloroplast genome in diatoms (Kowallik, 1996).

N-terminal amino acid sequences were obtained for three polypeptides (Table 4.2) identified as LHCPs by immunoblotting (Fig. 4.6B). The major polypeptides at 18 and 17.5 show significant homology to the FcpF polypeptide of *Phaeodactylum tricornutum*, while the major polypeptide at 17 kD is 96% identical to the FcpE polypeptide (Fig. 4.8). Alignment of the 17.5 kD polypeptide was somewhat problematic in that the identity of the amino acid at position 3 could be either S or N (Table 4.2), making this protein most homologous to either FcpF or FcpE, respectively (Fig. 4.8). As noted above, the Fcp proteins in *Phaeodactylum* are all nearly identical in terms of their molecular weight (Bhaya and Grossman, 1993). Hence, the ambiguity of the third residue in the 17.5 kD polypeptide sequenced here (Table 4.2) could be due to comigration of two polypeptides, one homologous to FcpF and the other to FcpE.

Finally, the N-terminal sequences of the 27 and 22 kD polypeptides (Table 4.2) present in the PS I fraction (Fig. 4.7, lane 6) were not homologous to any known proteins. The cross-reactivity of the 22 kD protein with antibodies to the 29 kD LHCP from *Chlamydomonas* (not shown), coupled with the lack of N-terminal homology with other known LHCP sequences, implies that there is at least one uncharacterized LHC gene present in *Cylindrotheca*, and

perhaps in other diatoms as well. Studies are currently in progress to determine the number of LHCs present in *Cylindrotheca*, their biochemical composition, and their specific associations with the reaction centers.

4.5.6 Conclusions

The components of the photosynthetic apparatus of diatoms are largely homologous to those found in other oxygenic photosynthesizers, as determined by immunoblotting (this communication; Plumley et al., 1993) and N-terminal protein sequencing (this communication). Nevertheless, immunocytochemical studies have shown distinct differences in the organization of PS I and the LHCs within the thylakoid membranes of diatoms (Pyszniak and Gibbs, 1992), while spectroscopic analyses indicate that there are differences in the regulation of excitation energy transfer in these algae (Owens, 1986). The isolation and characterization of PS I and PS II from diatoms has suffered due to their extreme sensitivity to the methods used during purification of thylakoids (Martinson and Plumley, 1993; Martinson and Plumley, in prep.; Chapters 5, 6). This extreme sensitivity may reflect structural differences between the reaction centers of diatoms and those of green plants, which may have implications for the regulation of photosynthetic electron flow in diatoms. Now that a method exists for purifying O_2-evolving thylakoids from diatoms (this communication), more detailed investigations regarding the regulation of photosynthesis in these algae can begin.

4.6 References

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Table 4.1. Electron transport characteristics of *Cylindrotheca fusiformis*. All values are the mean of at least three determinations, ± the standard deviation.

Sample	Type of assay	Additions	$\frac{\mu moles\ O_2}{mg\ Chl \cdot h}$
Whole cells	PS II-PS I oxygen evolution	10 mM HCO_3^-	72 ± 1.6
		10 mM HCO_3^- + 1 μM DBMIB	0
		10 mM HCO_3^- + 20 μM DCMU	0
Purified thylakoids	PS II-PS I oxygen evolution	100 μM Methylviologen	0
		100 μM Methylviologen + 1 mM KCN	0
	PS II activity (O_2 evolution)	2 mM FeCN	70 ± 4.3
		2 mM FeCN + 1 μM DBMIB	44.5 ± 5
		2 mM FeCN + 20 μM DCMU	36.5 ± 2.3
		0.5 mM pBQ	5.7 ± 0.5
		0.5 mM pBQ + 20 μM DCMU	0
		3 mM pBQ	21.8 ± 2.8
		3 mM pBQ + 20 μM DCMU	0
	PS I activity (O_2 uptake)	Ascorbate/DCPIP and methylviologen	- 19.4±5.2

Table 4.2. Partial N-terminal sequences of various polypeptides present in thylakoids of *Cylindrotheca fusiformis*. Fractions were obtained by sucrose density gradient ultracentrifugation of dodecylmaltoside-solubilized thylakoids. Hydroxy-proline residues are denoted by P.

M_r Polypeptide	N-terminal Sequence	Putative Identity
33 kD from LHC fraction	EINALTYLQVKGTGLAN	33 kD oxygen-evolving complex polypeptide
27 kD from LHC fraction	(Ky)GM(PE)D(RL)(ME)XDQ (em)vDnXIX	Carotenoid-binding LHCP
22 kD from PS I fraction	AERTKqLPFMNRPtXt	?
18 kD from PS I fraction	(Ka)F(DM)(Nds)G(LV)V(Dpg) (Fp)(Le)(Gs)(YP)(Er)t(In)	PS I reaction center subunit
18 kD from LHC fraction	NAFESELGAQPPLGFFDPFGLLsGDATEER	Chl *a*/*c*-fucoxanthin LHCP
17.5 kD from LHC fraction	(NKSA)FE(NS)ELGAQDPLGFFDPLGXVe(dn)(ag)DQXKFD	Chl *a*/*c*-fucoxanthin LHCP
17 kD from LHC fraction	SFENELGAQPPLGFFDPLGLVADGDQEKFDRL	Chl *a*/*c*-fucoxanthin LHCP
12 kD from LHC fraction	(Nat)LFDDIFNMDLFAPVKDQ (DR)(TD)YGAY(Kt)(Kg)XXLt	?
12 kD from PS I fraction	KLNLQTPFPXFGgX	PS I reaction center subunit

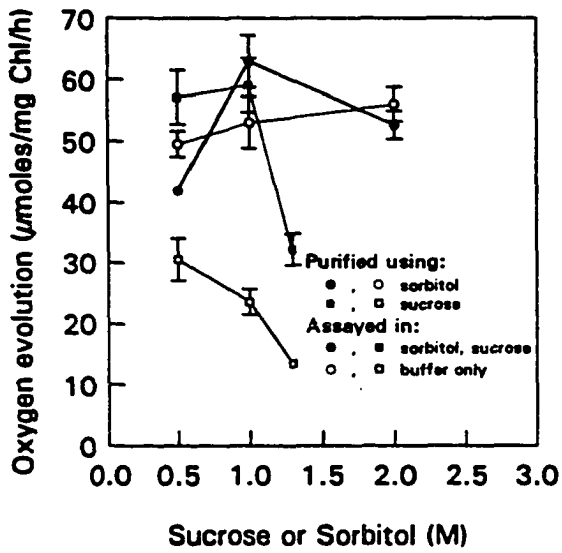


Figure 4.1(A). Oxygen-evolving capacities of thylakoid membranes purified from *Cylindrotheca* using different concentrations of sorbitol (circles) or sucrose (squares). Thylakoids were purified as described in Materials and Methods, except that the composition of Buffers A-F was modified: for sorbitol samples, the sorbitol in Buffers A and B was varied from 0.5-2 M; for sucrose samples, sucrose replaced sorbitol in all buffers and was used at concentrations from 0.5 to 1.5 M in Buffers A and B and 2.1, 1.8, 1.5, and 0.5 M in Buffers C-F, respectively. O_2 evolution (H_2O to 2 mM FeCN) was assayed in either the same Buffer A formulation used to purify thylakoids (solid symbols) or in 5 mM MES pH 6, 5 mM ACA, 1 mM BAM, 1 mM $MgCl_2$ (open symbols). Other assay conditions were as described in Materials and Methods. Error bars are one standard deviation.

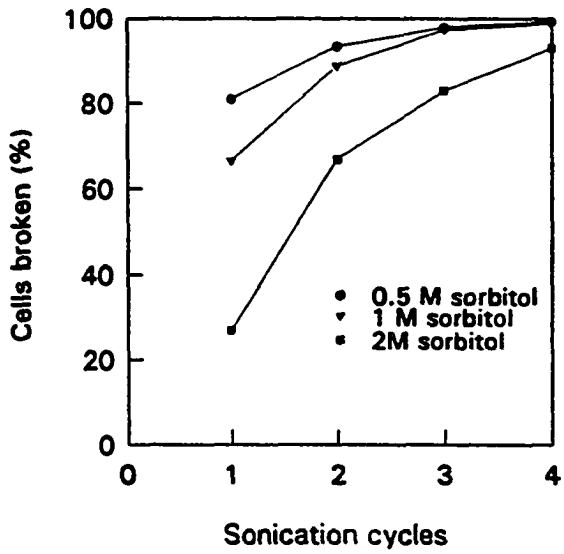


Figure 4.1(B). Cell breakage as a function of sorbitol concentration. Harvested cells were resuspended in modified Buffer A solutions as described in Fig. 4.1 (A), and aliquots (100 μl) removed for cell counts (t = 0). Cells were broken by sonication as described in Materials and Methods. After each cycle of sonication (2 x 15 sec at 120 W), samples were centrifuged at 1000 g for 5 min. Pellets were resuspended in the appropriate buffers, and aliquots (100 μl) were removed for counting. The procedure was repeated for 4 cycles of sonication. Cells were counted using a hemocytometer; percent breakage was calculated by subtracting the number of cells remaining in the pellet from the number of cells initially present in the sample (prior to any sonication), then dividing by the number of cells initially present in the sample.

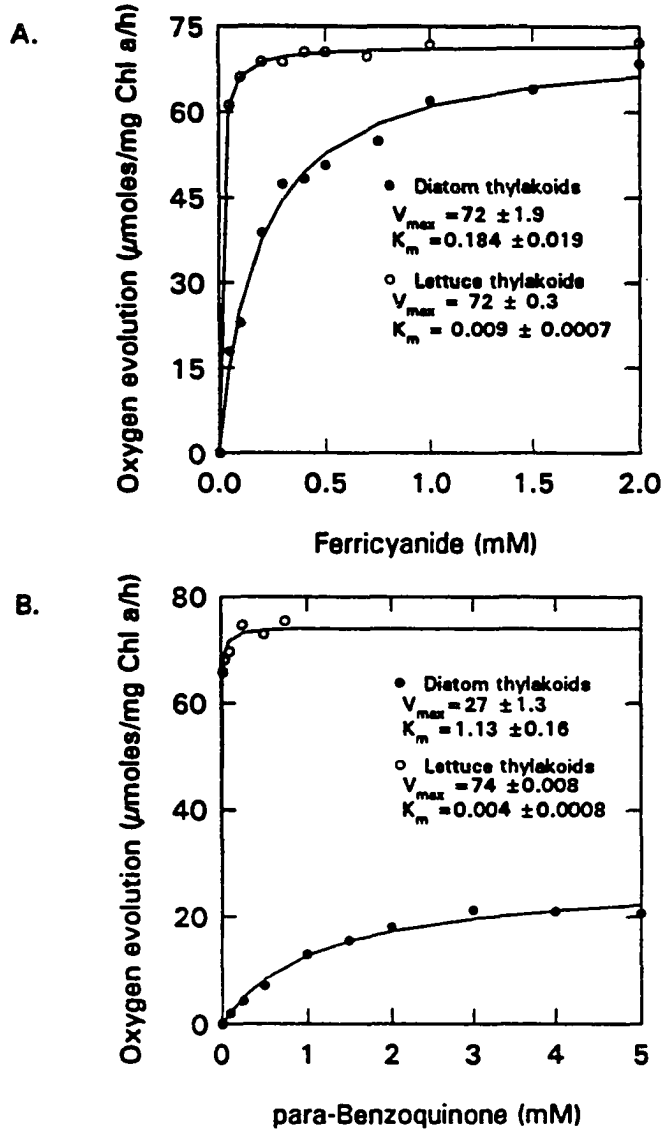


Figure 4.2. Rates of O_2 evolution in purified thylakoids from the diatom *Cylindrotheca* and lettuce as a function of ferricyanide (A), or *para*-benzoquinone (B) concentration. O_2 evolution was assayed as described in Materials and Methods.

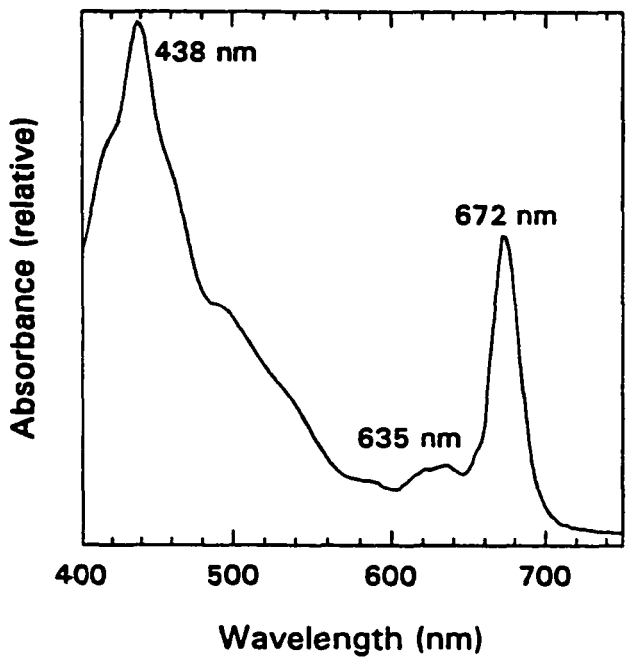


Figure 4.3. Absorption spectrum of purified thylakoid membranes from *Cylindrotheca*.

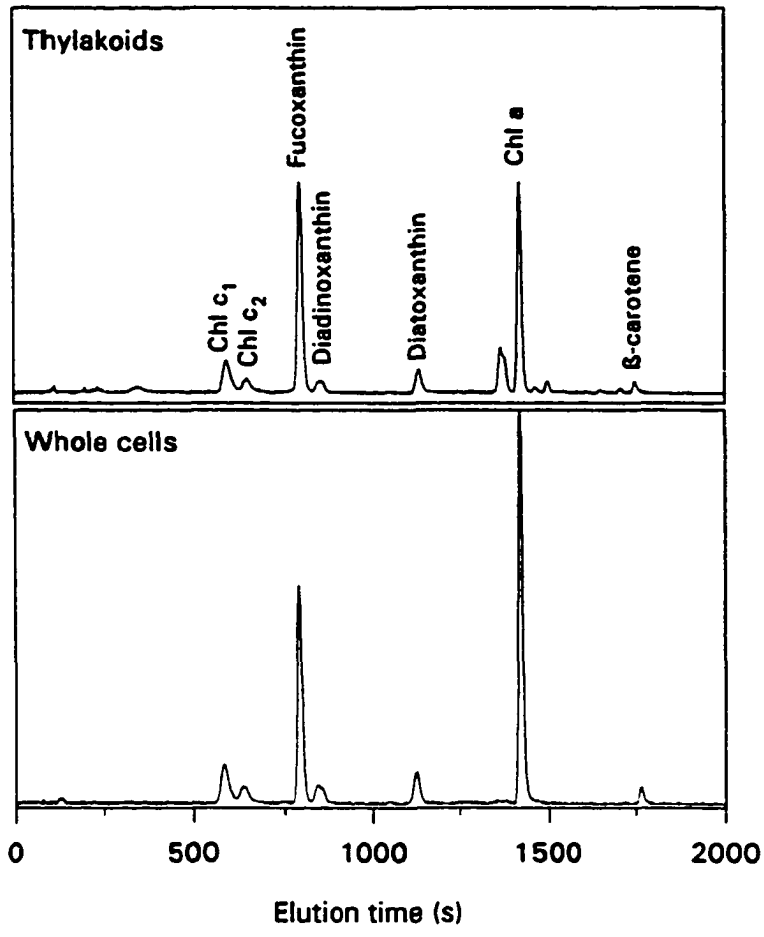


Figure 4.4. Pigment composition as determined by HPLC of whole cells and purified thylakoid membranes. Spectra were plotted to the same relative height of fucoxanthin and Chl c.

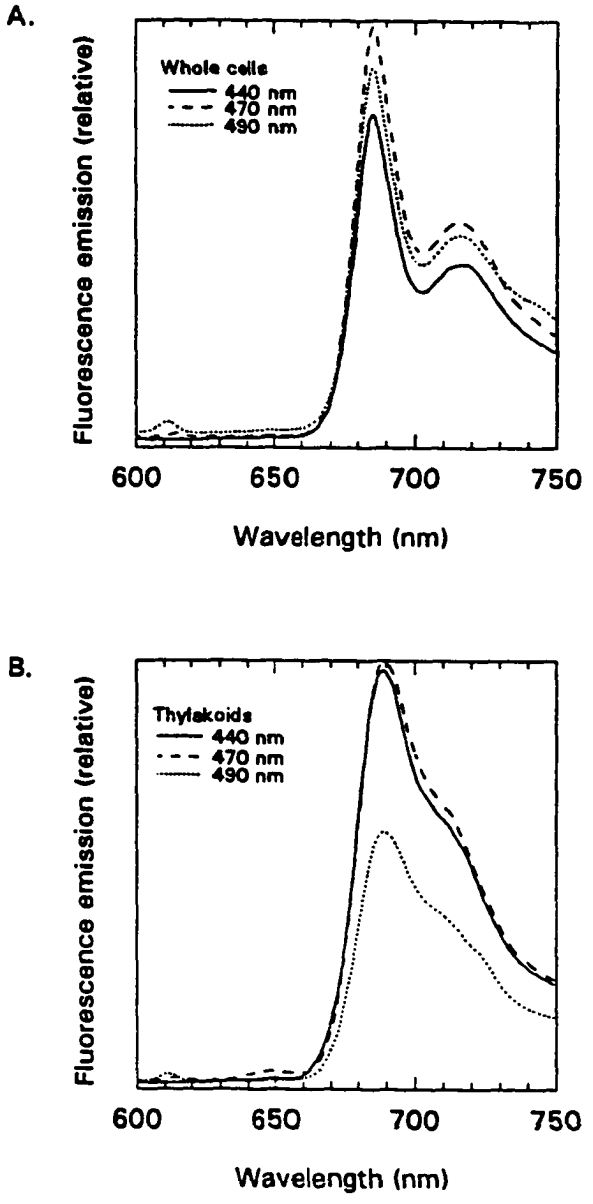


Figure 4.5. Low-temperature (77K) fluorescence properties of whole cells and purified thylakoids from *Cylindrotheca*. Emission spectra of whole cells (A) and purified thylakoid membranes (B) obtained with 440 , 470, and 490 nm excitation.

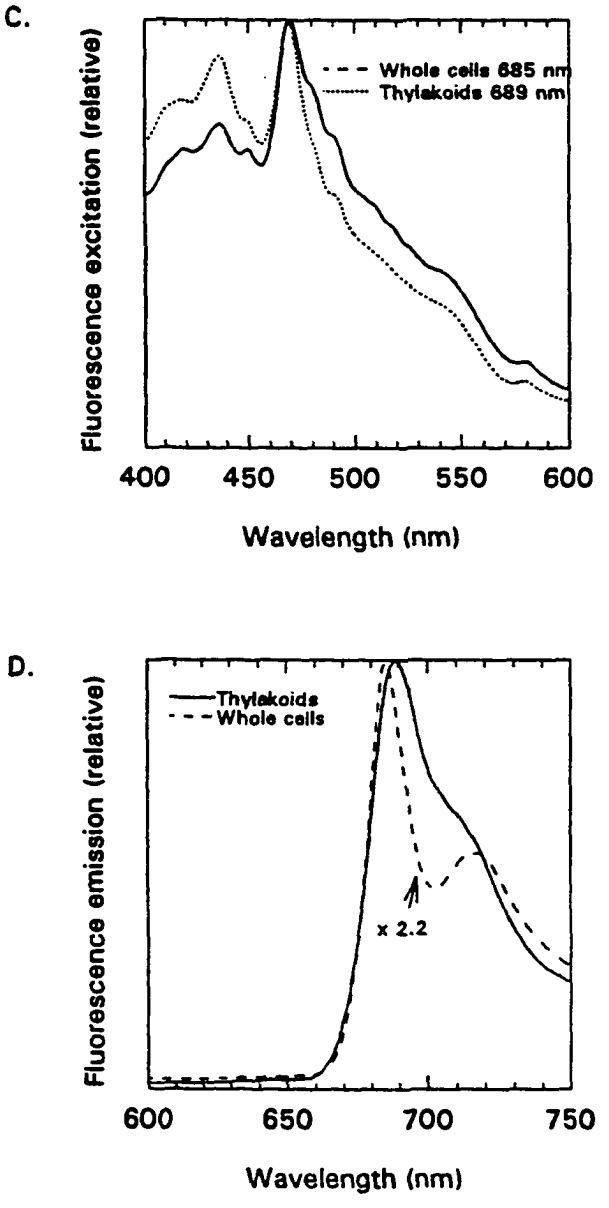


Figure 4.5 (cont.). Comparison of the excitation spectra of whole cells (685 nm emission) and purified thylakoids (689 nm emission) (C). Comparison of the emission spectra (440 nm excitation) of whole cells and purified thylakoids (D).

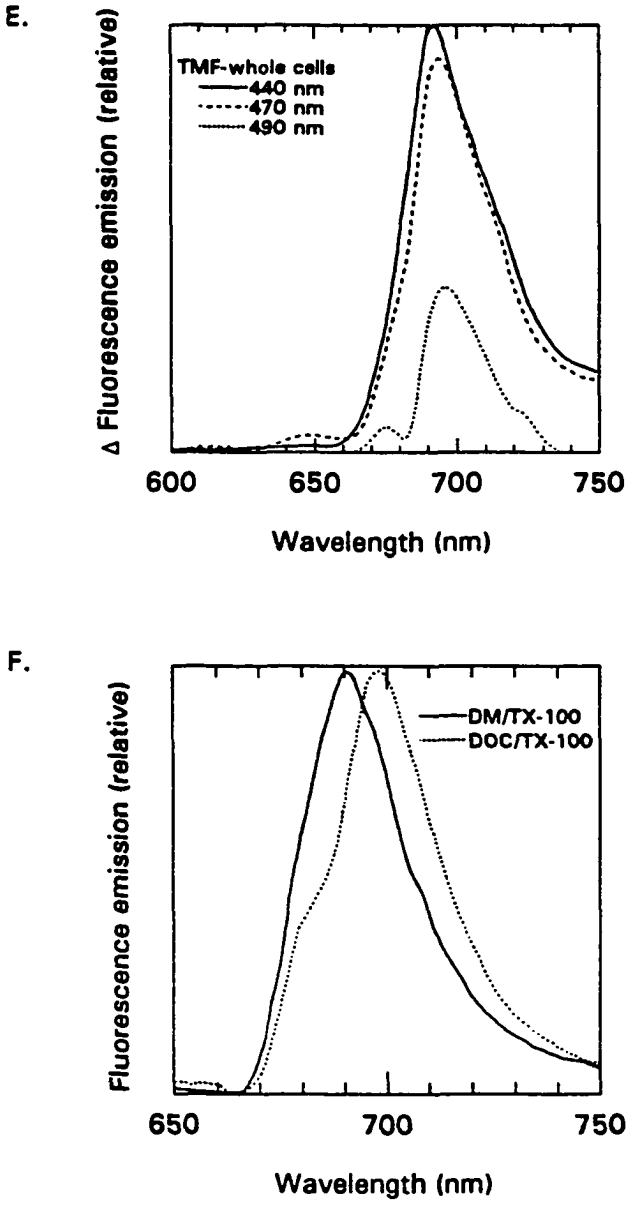


Figure 4.5 (cont.). Difference spectra (thylakoids minus whole cells, for all excitation wavelengths) (E). Emission spectra for DOC/TX-100 and DM/TX-100 LHCs (F).

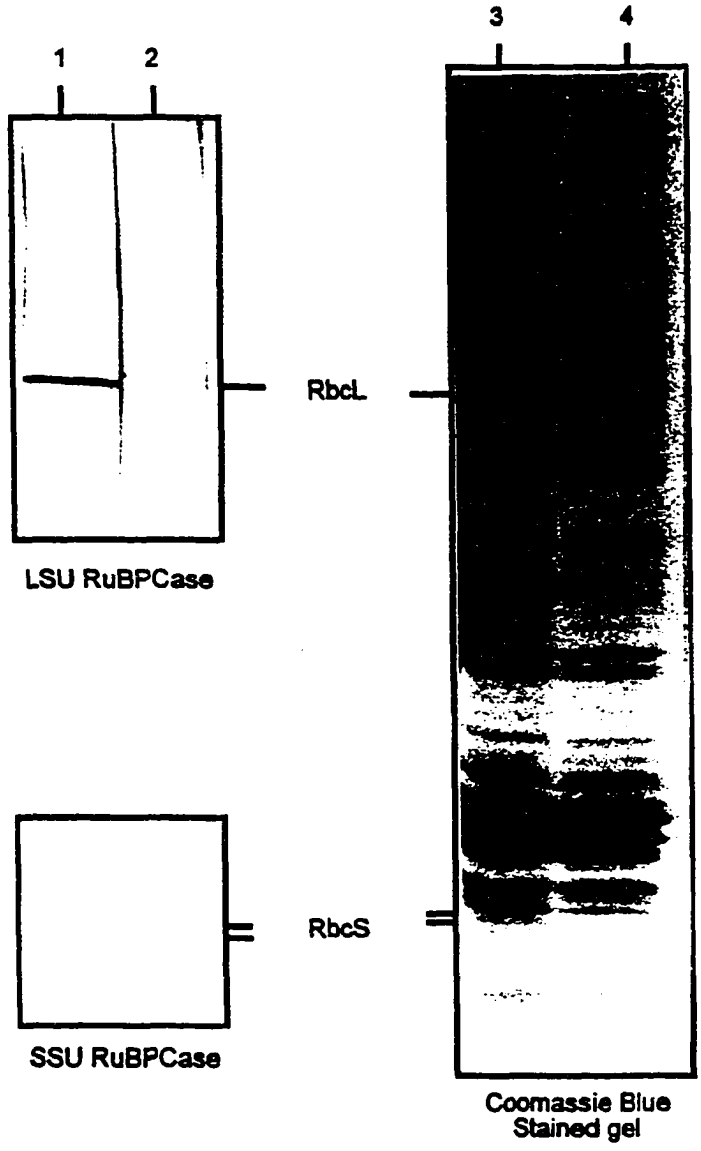
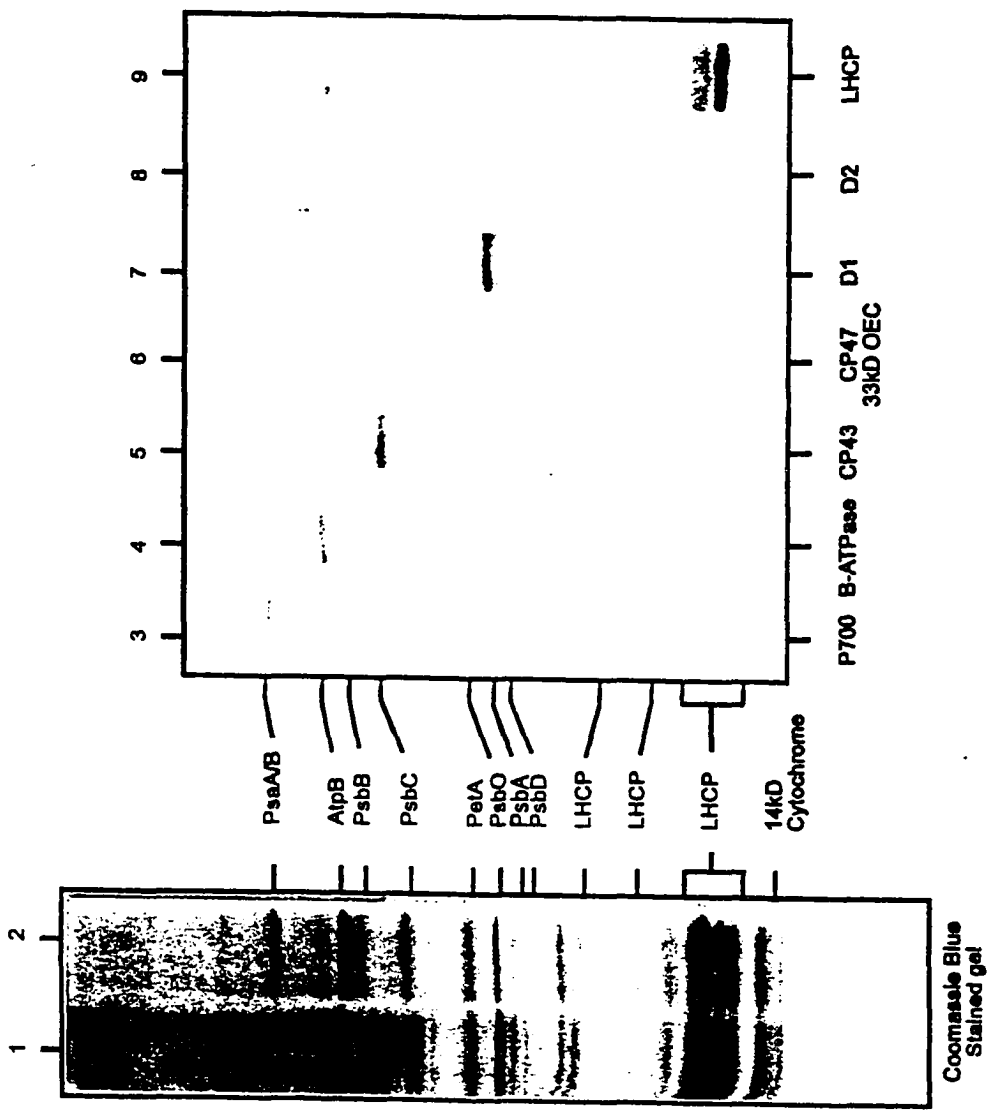


Figure 4.6(A). Denaturing SDS-PAGE of whole cells (10 μg Chl; lane 3) and purified thylakoids (10 μg Chl; lane 4). Immunoblots of whole cells and thylakoids showing the loss of the large and small subunits of RuBPCase in the purified thylakoids (lanes 1,2).



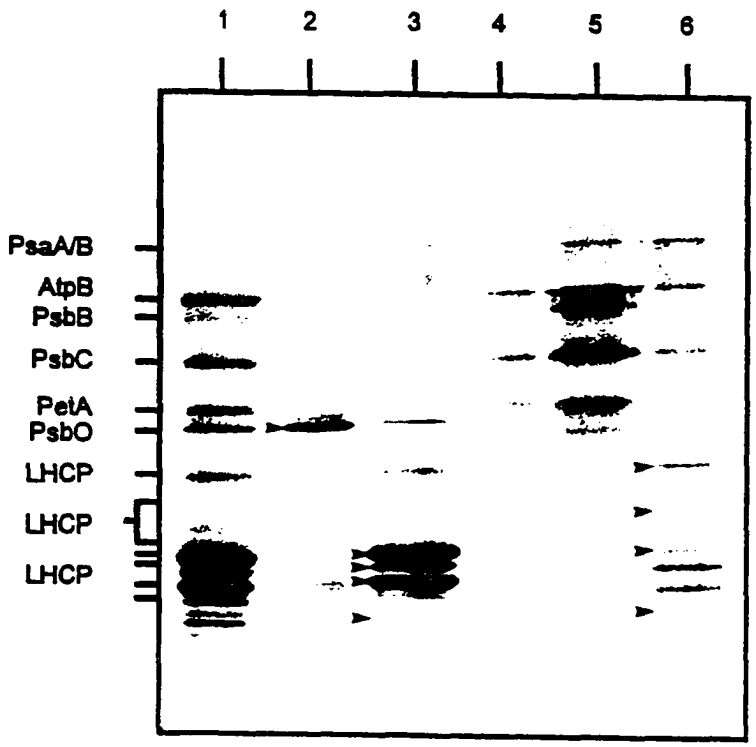


Figure 4.7. Denaturing SDS-PAGE of sucrose-density gradient fractions obtained from ß-dodecylmaltoside-solubilized thylakoids. Polypeptides for which N-terminal amino acid sequences were obtained are marked with arrows. Lane 1: thylakoids. Lane 2: LHC-containing fraction (yellow/orange). Lane 3: LHC-enriched fraction (dark brown). Lane 4: PS II-enriched fraction (green). Lane 5: PS II/PS I-containing fraction (dark green). Lane 6: PS I-enriched fraction (olive green).

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C. fusiformis 33 kD:                    3    EINALTYLQVKGTGLAN  19
[1]Spinach OEC 1:                      10      SK   E      T    26
[2]Wood tobacco OEC 1:                 11      QSK  ME     T    27
[3]Chlamydomonas OEE1 precursor:       56      QG          S I  72
[4]Pea OEE1 precursor:                 92      QSK  E      T   108
[5]Rice OEC 1:                         11      QSK  ME     T    27
[6]Wheat OEE1 precursor:               90      QSK  ME     T   106
[7]Tomato OEC precursor:               93      QSK  ME     T   109
[8]A. nidulans woxA:                   29    DLGT   D I D       45
[9]E. gracilis OEE 1 precursor:       100     LQS S  E  SS I     115

C. fusiformis 18 kD LHCP:    2   AFESELGAQPPLGFFDPFGLLSGDATEE  29
[10]P. tricornutum FcpF:    32                 L  VADGDQ K     59
[11]O. sinensis FCP:        31                 L M A           52
[12]P. tricornutum FcpE:    32     N           L  VADGDQ K     59
[13]P. tricornutum FcpC:    32     D           L  VADGDQ K     59
[14]P. tricornutum FcpD:    32     D           L  VADGDQ K     59
[15]P. tricornutum FcpB:    32         A   Y   L  VADGDQ K     59
[16]M. pyrifera FCP:        35   S   I   I  W   L  VA          56
[17]M. pyrifera FCP:        34   S   I   A  W   L   A          55

C. fusiformis 17.5 kD LHCP:    2   FEXELGAQDPLGFFDPLGXVEXXDQXKFD 30
[10]P. tricornutum FcpF:      33       S    P        L ADG  E   61
[13]P. tricornutum FcpC:      33       D    P        L ADG  E   61
[14]P. tricornutum FcpD:      33       D    P        L ADG  E   61
[12]P. tricornutum FcpE:      33       N    P        L ADG  E   61
[15]P. tricornutum FcpB:      33       S    A   Y    L ADG  E   61
[11]O. sinensis FCP:          32       S    P        MLADA  ER  60
[18]P. tricornutum FcpA:      33       N I  Q   YW   L ADG  E   61
[16]M. pyrifera FCP:          36       S I  P I W    L ADA  ER  64

C. fusiformis 17 kD LHCP:    1   SFENELGAQPPLGFFDPLGLVADGDQEKFDRL  32
[12]P. tricornutum FcpE:    32   A                                63
[13]P. tricornutum FcpC:    32   A  D                             63
[14]P. tricornutum FcpD:    32   A  D                             63
[10]P. tricornutum FcpF:    32   A  S                             63
[15]P. tricornutum FcpB:    32   A  S     A    Y                  63
[18]P. tricornutum FcpA:    32   A     I   Q   YW                 63
[11]O. sinensis FCP:        31   A  S                 ML  A   R   62
[16]M. pyrifera FCP:        35      S I     I  W         A   R   66

C. fusiformis 10 kD PS I:    2   LNLQTPFPXFGG      13
[19]O. sinensis PsaD:        3              T      14
[20]C. reinhardtii PsaD:    58        PD   S I      69
[21]Barley PsaD:            69       DPS   S I      80
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[1]pirA23613;[2]pirA60731;[3]spP12853;[4]spP14226;[5]pirA38889;[6]spP27665;[7]gpZ11999;
[8]spP11472;[9]spP46483;[10]pirS42130;[11]pirS47487;[12]pirS42129;[13]pirS42132;
[14]pirS42133;[15]pirS42134;[16]gpU10069;[17]gpU10068;[18]pirS12030;[19]gpZ67753;
[20]pirS47088;[21]pirJQ2247.

Figure 4.8. Alignment of N-terminal amino acid sequences from selected thylakoid membrane polypeptides of *Cylindrotheca* with sequences obtained from GenBank.

Chapter 5

Isolation of a 65kD Chlorophyll-Protein Complex from a Marine Diatom That Contains Only PsaA: Implications for the Stability of PS I[1]

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[1]Prepared for publication in Biochimica et Biophysica Acta

5.1 Abstract

Photosystem I (PS I) activity (ascorbate/DCPIP to methylviologen) is easily lost upon purification of thylakoid membranes from the marine diatom *Cylindrotheca fusiformis*. We have developed protocols for the purification of diatom thylakoids that retain PS I activity (Martinson et al., in prep.; Chapter 4) and compared them to inactive thylakoids. Based on several assays, we determined that, relative to higher plants, green algae, cyanobacteria, and red algae, the stability of PS I in diatoms is exceedingly low. PS I particles prepared from active and inactive thylakoids using dodecylmaltoside had similar properties except that particles from inactive thylakoids were depleted in vitamin K_1. This suggested an alteration in the stability of PS I components in inactive thylakoids, which was investigated further using mild LDS-PAGE. CPI was resolved from both active and inactive thylakoids, however, its abundance was reduced in inactive thylakoids. Coincident with the reduction in CPI was the appearance of a novel green band at 65 kD in inactive thylakoids. This complex was shown to be a dissociation product derived from CPI, and was named CPI_{65kD}. CPI_{65kD} did not fluoresce at room temperature, but had a 77K fluorescence emission peak at 703 nm. CPI_{65kD} contained Chl *a* and ß-carotene bound to a single apoprotein; immunological and N-terminal amino acid sequence data strongly suggest that this apoprotein is PsaA. From these results we conclude that at least some of the pigments responsible for the low fluorescence yield of PS I reside on the PsaA subunit. Moreover, we conclude that the lability of the diatom PS I is related to the inherent instability of the PsaB-pigment complex. Immunoassays using an antibody specific to the C-terminus of PsaB in pea indicate that this region of PsaB may be posttranslationally removed in diatoms. We suggest that the proteolytic modification of PsaB first affects the stability of the PsaA/PsaB heterodimer *in vitro* and subsequently the stability of the PsaB-pigment complex. The physiological implications of these putative modifications to PsaB are discussed.

5.2 Introduction

The process of photosynthesis occurs via special membrane-bound pigment-protein complexes. One of these pigment-protein complexes, Photosystem I (PS I), is a supramolecular complex containing at least 12 polypeptides (Ikeuchi, 1992). Of the many different preparations of PS I that have been described (for a review, see Golbeck, 1988), the simplest is CPI, which contains the two core polypeptides, PsaA and PsaB. The genes encoding these two polypeptides, *psa*A and *psa*B (Fish et al, 1985; Fish and Bogorad, 1986), are highly conserved across species (Bryant, 1992). The two proteins are approximately 44% homologous at the amino acid level, but the level of homology approaches 55% when conservative substitutions are taken into account (Fish et al., 1985). These hydrophobic proteins are 83.2 and 82.5 kD, respectively, and consist of eleven transmembrane helices each. The CPI complex is highly resistant to denaturation, perhaps due in part to the extreme hydrophobic character of the PsaA/PsaB heterodimer. Located within CPI are the components necessary for efficient charge separation and electron transfer: the P700 reaction center chlorophyll(s), the primary acceptor A_0, two vitamin K_1 (phylloquinone) molecules, and a 4Fe-4S center, X.

Much of our knowledge of PS I comes from studies of higher plants, green algae, and cyanobacteria. Several different PS I preparations have been isolated, with chlorophyll to P700 ratios ranging from 200 to as low as 7 (Ikegami and Katoh, 1975; Golbeck, 1992). CPI generally has a chlorophyll *a*/P700 ratio between 40 and 100 (Golbeck, 1992), and contains 8-10 carotenoid molecules per chlorophyll *a* (Sonoike and Katoh, 1986). PS I particles/complexes have also been isolated from chromophyte algae (Barrett and Anderson, 1980; Berkaloff et al., 1990) and found to be highly similar to those of green plants and cyanobacteria in terms of pigment and polypeptide composition. In addition, the electron transfer components within the core seem to be largely conserved. Some variations do occur,

however, in that the higher plant PS I core has a 77K fluorescence emission maximum at 720-725 nm, while in algae the emission maximum is generally around 717 nm (Goedheer, 1981). Nevertheless, room temperature fluorescence yields of all PS I complexes are extremely low, a property which is presumably due to the presence of Chl species with short fluorescence lifetimes (Searle et al., 1977).

In this communication, we report that the PS I complex of diatoms is atypical in that it is easily dissociated. We have taken advantage of this instability to isolate and characterize two non-fluorescent pigment-binding complexes from a marine diatom. One complex, migrating at 110 kD during LDS-PAGE, is CPI, while the other (M_r of 65 kD) contains only a single PS I apoprotein. Immunological and N-terminal protein sequence data strongly suggest that the apoprotein present in the 65 kD complex is PsaA. The formation of this complex was correlated with a loss of PS I activity in purified diatom thylakoids. The immunological data also indicate that the C-terminus of PsaB may be posttranslationally modified by proteolytic cleavage. We propose that these modifications are responsible for the instability of PS I in diatoms.

5.3 Materials and Methods

5.3.1 Cultures and Growth Conditions

Cultures of the chlorophyte *Chlamydomonas reinhardtii* (WT137), and the diatom *Cylindrotheca fusiformis* (Watson's strain 13) were grown and harvested as described previously (Plumley et al., 1993).

5.3.2 Purification of Thylakoid Membranes

Thylakoid membranes were purified from *Chlamydomonas* by flotation through sucrose density gradients as described by Chua and Bennoun (1975), with slight modifications

(Plumley et al., 1993). Thylakoid membranes were purified from *Cylindrotheca* as described in Plumley et al. (1993), with the following modification: after harvesting by centrifugation, the cells were frozen in buffer at -60°C prior to breaking by two passages through a French Press at 10,000 psi. This procedure yields thylakoids with inactive PS I reaction centers (ascorbate/DCPIP (2,6-dichlorophenol-indophenol) to methylviologen; Martinson et al., in prep.; Chapter 4). Purified thylakoid membranes were stored in a minimal volume of gel sample buffer (100 mM Tris (Tris[hydroxymethyl]aminomethane)-HCl pH 8.5, 100 mM dithiothreitol (DTT), 5 mM ε-aminocaproic acid (ACA), and 1 mM benzamidine (BAM)). Chlorophyll concentrations were determined in 90% (w/v) acetone using the equations of Jeffrey and Humphrey (1975), and membranes were stored at -60°C. Thylakoids that retain PS I activity were purified and stored as described (Martinson et al., in prep.; Chapter 4).

5.3.3 Purification of PS I particles and detection of Vitamin K_1

Thylakoids with and without PS I activity were solubilized at 1 mg Chl/ml in 50 mM Tris-HCl pH 8.5, 5 mM ACA, 1 mM BAM, 1% (w/v) ß-dodecylmaltoside (DM) for 15 min at 0°C. PS I particles were isolated by centrifugation at 284,000 g, 4°C, for 16 h on gradients containing 0-30% sucrose, 5 mM Tris-HCl pH 8.5, 5 mM ACA, 1 mM BAM. Fractions enriched in PS I were collected and Vitamin K_1 extracted using *sec*-butanol (Martinson and Plumley, 1995). Vitamin K_1 was resolved on a Rainin Microsorb RP-18 (25 cm x 4.6 mm) column using a methanol/isopropanol (80:20) mobile phase, and detected at 270 nm (Malkin, 1986; Martinson and Plumley, 1995).

5.3.4 Sample Preparation for Mild LDS-PAGE

All sample preparation for mild LDS-PAGE was done at 0-4°C. Aliquots of thylakoid membranes corresponding to 60 μg chlorophyll were solubilized at a final concentration of 1

mg Chl/ml in 134 mM Tris-HCl pH 8.5, 134 mM DTT, 10 mM ACA, 2 mM BAM, 5% (w/v) sucrose, 1% (w/v) LDS (LDS:Chl = 10:1). Samples were vortexed briefly and then centrifuged at 16,000 g for 10 min immediately prior to mild LDS-PAGE. Samples solubilized with DM were handled identically except that a detergent to chlorophyll ratio of 20:1 was used and samples were incubated for 20 min at 0°C prior to the centrifugation step.

5.3.5 Electrophoresis

Mild ("green gel") LDS-PAGE was carried out on 20 x 30 cm x 1.5 mm slab gels consisting of 8% (w/v) acrylamide, 0.21% (w/v) bis-acrylamide, 375 mM Tris-HCl, pH 8.8, and 10% (v/v) glycerol. Gels for re-electrophoresis of green bands were of similar composition, but contained 6% (w/v) acrylamide, 0.16% (w/v) bis-acrylamide. All gels had a 3.5 cm stacking gel of 5% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, and 125 mM Tris-HCl pH 6.8. Reservoir buffers contained 50 mM Tris Base, 383 mM glycine, with 0.1% (w/v) SDS in the upper reservoir (Laemmli, 1970). Gels and buffers were cooled at 4°C for 5-6 h prior to use and electrophoresis was done in darkness at 4°C for 10-12 h at 16 mA.

Denaturing electrophoresis of chlorophyll-protein complexes isolated from green gels was carried out on slab gels containing a 6-12% (w/v) gradient of polyacrylamide, a 5-12.5% (w/v) gradient of sucrose; 375 mM Tris-HCl pH 8.8, with a stacking gel of 5% (w/v) polyacrylamide, 125 mM Tris-HCl pH 6.8. Running conditions were the same as used for mild SDS-PAGE except that the upper reservoir buffer contained 0.2% (w/v) SDS, and gels were run at 20°C for 16-18 h.

5.3.6 Sample Preparation for Denaturing SDS-PAGE

Immediately after mild LDS-PAGE, gel slices containing chlorophyll-protein complexes were excised and denatured by incubation in 60 mM Tris-HCl pH 8.5, 10 mM DTT, 2% (w/v) SDS at room temperature for 1 h before being placed into gel sample wells and sealed with 0.1% (w/v) agarose, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS.

Aliquots of thylakoid membranes corresponding to 0.5 mg Chl/ml were solubilized in 60 mM Tris-HCl pH 8.5, 60 mM DTT, 2% LDS, 12% sucrose and heated at 100°C for 1 min prior to electrophoresis.

5.3.7 Western blotting

Following SDS-PAGE, proteins were transferred to nitrocellulose using a wet transfer apparatus (Hoefer Scientific). Transfer was performed at 250 mA for 4-5 h at RT. Proteins were visualized with Ponceau S (Mishkind et al., 1987). For immunodetection, blots were developed with antibodies against either the P700 complex of cyanobacteria, the N-terminus of PsaA, or the C-terminus of PsaB (Henry et al., 1992), and detected using alkaline phosphatase conjugated goat-anti-rabbit IgG in conjunction with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Mishkind et al., 1987).

5.3.8 N-terminal sequencing

Proteins were resolved using denaturing SDS-PAGE and transferred to PVDF (Millipore) using a wet transfer apparatus (Hoefer Scientific). Blotting was done at 4°C for 48 h. After transfer, proteins were detected using Coomassie Brilliant Blue G-250 as recommended by Millipore. Sequencing was performed at the University of Georgia Molecular Genetics Facility (Athens, GA) using a Milligen Prosequencer 6000 (Millipore).

5.3.9 Spectroscopic Analyses

For absorbance measurements, green bands were excised from the polyacrylamide gel, ground in ddH_2O, and eluted overnight at 4°C in darkness. Spectra were obtained with an HP8452A diode array spectrophotometer. For 77K fluorescence, green bands were excised, placed in 6 x 50 mm disposable borosilicate culture tubes (Kimble), covered with 50% glycerol, and frozen in liquid N_2. Spectra were obtained with a Perkin Elmer MPF-66 spectrofluorometer, using 4 nm bandpass for both emission and excitation. To correct for the decrease in efficiency in the detector at red wavelengths, sample spectra were divided by a "blank" spectrum obtained by scanning a piece of white paper. The sample spectra were divided by the blank. Circular dichroism (CD) spectra were obtained by sandwiching gel slices between microscope slides. Spectra were recorded with a Jasco 720 series spectropolarimeter using 60 μm slits.

5.3.10 Pigment Analyses

Pigments were extracted for HPLC analysis using *sec*-butanol (Martinson and Plumley, 1995). Pigments were resolved on a Rainin Microsorb MV C-18 column (4.6 mm x 25 cm) as described (Martinson and Plumley, 1995).

5.4 Results

Photosystem II (PS II) and Photosystem I (PS I) activity are easily lost during the purification of thylakoid membranes from diatoms (Martinson et al., in prep.; Chapter 4). Thylakoids purified using methods previously described by us (Plumley et al., 1993), are completely inactive in both oxygen evolution (H_2O to ferricyanide) and oxygen uptake (ascorbate/DCPIP to methylviologen); we refer to these thylakoids as "inactive". In contrast, thylakoids prepared using milder breaking conditions and buffers of higher osmotic strength

retain PS I activity (Martinson et al., in prep.; Chapter 4); these thylakoids were designated "active".

To investigate the nature of the inactivation of PS I, active and inactive thylakoid membranes of *Cylindrotheca* were solubilized with ß-dodecylmaltoside (DM) and fractionated by sucrose density gradient ultracentrifugation. Dark green-colored fractions were obtained from each of the two types of thylakoids, and found to be enriched in PS I proteins (Fig. 5.1A). There were no apparent differences in the polypeptide composition of the two fractions, except that some PS II polypeptides were present in the fraction obtained from active thylakoids. Fluorescence emission spectra (77K) for the two fractions revealed a major peak at 717-718 nm, and except for a slight blue-shift in the preparation from inactive thylakoids, were essentially identical (Fig. 5.1B). The two fractions differed markedly in their vitamin K_1 content, however. The PS I-enriched fraction from inactive thylakoids contained significantly less vitamin K_1 relative to the PS I-enriched fraction from active thylakoids (Fig. 5.1C). This suggests that loss of PS I activity seen in some thylakoid preparations may be due to the loss of vitamin K_1, and moreover, implies that the stability of PS I components may be profoundly altered in inactive thylakoids.

The fluorescence emission properties of PS I particles purified from active and inactive thylakoids using DM were essentially identical (Fig. 5.1B), suggesting that there were no major structural differences between the two types of PS I. However, since a stabilizing effect on PS I has been documented for DM (Nechushtai et al., 1986), this detergent may not be suitable for analyzing potential differences in the stability of PS I. Therefore, to compare the stability of the PS I reaction centers in active versus inactive thylakoids, solubilization was done using another detergent, LDS, and pigment-protein complexes fractionated by mild LDS-PAGE (Martinson and Plumley, in press; Chapter 2).

From active thylakoids (Martinson et al., in prep.; Chapter 4), two green bands and several brown bands were resolved, ranging in size from ≈110 to 50 kD (not shown). The brown bands were identified as Chl *a*/*c*-fucoxanthin light-harvesting complexes based on their pigment and protein compositions. These complexes will be described in a subsequent communication (Martinson and Plumley, in prep.). The fastest migrating green band contained polypeptides that cross-reacted with antibodies against the PsbB and PsbC polypeptides (not shown), and will not be discussed further here.

For inactive thylakoids, a pattern similar to that observed for active thylakoids was obtained, except that the abundance of the 110 kD green band was reduced and an additional green band was observed at 65 kD (Fig. 5.2 lane 3). The two green bands with M_rs of ≈110 and 65 kD did not fluoresce at room temperature when excited with UV light (not shown), a property specifically associated with PS I (Searle et al., 1977). The lack of fluorescence from the 110 kD green band (Fig. 5.2, lanes 3,4) was not surprising because its mobility during LDS-PAGE suggested that it was homologous to CPI of *Chlamydomonas* (Fig. 5.2, lanes 1,2), cyanobacteria, and higher plants (not shown). The resolution of a non-fluorescent green band at 65 kD, on the other hand, was striking in that no counterpart to it was visible in *Chlamydomonas* (Fig. 5.2, lane 1), higher plants, red algae, or cyanobacteria (not shown). Moreover, a 65 kD green band was not resolved from active thylakoids of *Cylindrotheca* solubilized under the same conditions (not shown).

We hypothesized the 65 kD green band was related to PS I since its appearance was correlated with the reduction in CPI abundance and because it did not fluoresce at room temperature. To test this hypothesis, we investigated the relationship between the 110 kD band (which we conclusively showed to be CPI; see below) and the 65 kD band. Re-electrophoresis of the 110 kD green band obtained from inactive thylakoids on a second non-denaturing gel resulted in two green bands with M_rs of 110 kD and 65 kD (Fig. 5.2, lane 4).

This was in sharp contrast to results with both the 110 kD green band obtained from active thylakoids of *Cylindrotheca* (not shown) and *Chlamydomonas* (Fig. 5.2, lane 2) where re-electrophoresis of CPI yielded only CPI. Re-electrophoresis of the 65 kD band from *Cylindrotheca* resulted in only a single band of 65 kD (Fig. 5.2, lane 5). These results unequivocally demonstrated that the 65 kD band was derived from the 110 kD band of inactive *Cylindrotheca* thylakoids.

The re-electrophoresis experiments described above were based on the assumption that the 110 kD green complex was analogous to CPI. To confirm the identity of the 110 kD band as CPI and to determine the polypeptide composition of the 65 kD band, denaturing SDS-PAGE and immunoblotting were performed. When the 110 and 65 kD green bands from *Cylindrotheca* were excised and subjected to denaturing SDS-PAGE, several major polypeptides were visible. The polypeptide profile of the 110 kD complex showed three apoproteins of about 83 kD, two apoproteins of approximately 65 and 60 kD, and a diffuse band between 50 and 55 kD (Fig. 5.3A, lane 3). This pattern was similar to that for the CPI complex isolated from *Chlamydomonas* (Fig. 5.3A, lane 2), which showed bands at 84, 65, and 60 kD, as well as a diffuse band at 50-55 kD. The 65 kD complex from the diatom contained primarily a 60 kD apoprotein, but a diffuse band around 55 kD was also visible (Fig. 5.3A, lane 4). Since the PS I subunits, PsaA and PsaB, typically show anomalous migration during SDS-PAGE resulting in bands with M_rs of 60-65 kD, but frequently ranging from ≈50 to 85 kD (Henry et al., 1992), these results suggest that the apoproteins present in the 110 and 65 kD green complexes were PsaA and PsaB.

Conclusive identification of the apoproteins present in the 110 and 65 kD green bands was obtained via immunological methods. Western blots using antibodies to the P700 apoproteins from cyanobacteria confirmed that the antibody recognized the corresponding polypeptides in thylakoids from both *Cylindrotheca* and *Chlamydomonas* (Fig. 5.3B, lanes

1,5). The antibody also cross-reacted with the polypetides of 65 and 60 kD present in the *Chlamydomonas* CPI and the 110 kD green band from *Cylindrotheca* (Fig. 5.3B, lanes 2,3). These results demonstrated that the 110 kD green band of the diatom is structurally homologous to PS I, and by inference, is CPI. Importantly, the 60 kD polypeptide present in the 65 kD green band was also recognized by the cyanobacterial PS I antibody (Fig. 5.3B, lane 4). This result confirmed the identity of the 65 kD band as a component of PS I. Based on its relationship to CPI, we will refer to the 65 kD band from *Cylindrotheca* as CPI_{65kD}. Since only a single protein was present in CPI_{65kD}, we conclude that it contains only one of the two reaction center subunits, either PsaA or PsaB.

A high degree of variability in the relative migration of the PsaA and PsaB polypeptides during denaturing SDS-PAGE has been documented (Henry et al., 1992). Thus, one cannot conclusively distinguish between these two proteins based solely on their M_rs. To identify the apoprotein of CPI_{65kD}, immunological analyses were performed using antibodies specific to either PsaB or PsaA. These antibodies were raised against synthetic polypeptides corresponding to either the N-terminus of PsaA (residues 2-15) or the C-terminus of PsaB (residues 721-734) from pea (Henry et al., 1992). The PsaA antibody did not cross-react with any proteins in *Cylindrotheca* thylakoids, but recognized polypeptides in the 60-65 kD range in thylakoids from romaine lettuce (not shown). The PsaB antibody failed to recognize any of the polypeptides present in either CPI or CPI_{65kD} (Fig. 5.3C, lanes 3,4), but did recognize an 83 kD polypeptide in thylakoids from *Cylindrotheca* (Fig. 5.3C, lane 5). Since the predicted molecular weights of both PsaA and PsaB are approximately 82 kD, we investigated the possibility that the 83 kD protein in thylakoids was a PS I component. The protein was purified, and its N-terminal amino acid sequence determined. The sequence (KLSDLEKKLFEEQXQ) has no homology with any proteins present in the data bank. We conclude that the cross-reactivity of the PsaB antibody with the 83 kD protein was

nonspecific. Reactivity of the PsaB antibody to proteins in *Chlamydomonas* thylakoids was low (Fig. 5.3C, lane 1), however, the antibody recognized both the 65 kD and the 60 kD polypeptides present in CPI from *Chlamydomonas* (Fig. 5.3C, lane 2).

A logical explanation for the lack of a cross-reaction with the PsaA- and PsaB-specific antibodies is that the N-terminal amino acid sequence of PsaA and the C-terminal amino acid sequence of PsaB in *Cylindrotheca* are significantly different from those of green plants. The deduced N-terminal amino acid sequence of PsaA from the diatom *Odontella sinensis* (Kowallik, 1996) is only 33% identical to the sequence used to generate the antibody. Thus, the PsaA antibody may not cross-react with the diatom PsaA protein because of sequence variability. However, the deduced C-terminal amino acid sequence of PsaB in *Odontella* (Kowallik, 1996) is 86% identical to the C-terminal sequence of PsaB in pea, which was used to make the antibody. This suggests that the C-terminus of PsaB in *Cylindrotheca* may be posttranslationally modified by proteolysis although we cannot presently exclude the possibility that the two different amino acids (L for F, and V for L) in the diatom are responsible for the lack of cross-reactivity. The possible posttranslational cleavage of the C-terminus of PsaB is currently being investigated in our lab.

Since we could not distinguish between PsaA and PsaB using immunological methods, the best way to specifically identify these subunits was through partial amino acid sequencing. The N-terminal sequence of the 65 kD apoprotein present in CPI (Fig. 5.3A, lane 3) was found to be 94% identical to the deduced amino acid sequence for PsaB from another diatom, *Odontella*, and 88% homologous to PsaB from *Anabaena variabilis*, *Euglena gracilis*, *Synechocystis* sp. (PC 6803), *Synechococcus*, and liverwort (Fig. 5.4). The N-termini of each of the 60 kD apoproteins from CPI and CPI_{65kD} (Fig. 5.3A, lanes 3,4) were blocked; a blocked N-terminus has been observed previously for PsaA (M. Ikeuchi, pers. comm.). Together, these data support the identification of the 60 kD apoprotein present in CPI_{65kD} as PsaA.

The isolation of a Chl-binding complex containing only PsaA provides a unique opportunity to compare the biochemical composition of the PsaA subunit to that of the holocomplex (e.g. CPI). The pigment composition of CPI_{65kD} was compared to that of the CPI complexes isolated from *Cylindrotheca* and *Chlamydomonas* using absorption spectroscopy (Fig. 5.5) and HPLC (Fig. 5.6). The CPI complex obtained from *Cylindrotheca* in this study had an absorption spectrum essentially identical to that of CPI obtained from *Chlamydomonas*, with major peaks at 422, 436, and 676 nm due to Chl *a*. These spectra agree with those observed for purified PS I complexes from a cyanobacterium (Tsiotis et al., 1993). In addition, the spectra from both samples exhibited small peaks at 500 and 584 nm, indicating the presence of carotenoids, and a minor peak at 630 nm, due to Chl *a*. The CPI_{65kD} complex had an absorption spectrum that was nearly identical to the intact CPI complex (Fig. 5.5). Importantly, there was no shift in the absorption maxima for Chl *a*, suggesting that no major alterations in the binding of Chl *a* to the PsaA apoprotein had occurred relative to CPI. There were, however, quantitative differences in absorbance at 676 nm and in the 500-600 nm range between CPI_{65} and CPI, but the differences amounted to less than 10% of the total absorbance, as determined from difference spectra (not shown).

HPLC pigment profiles (Fig. 5.6) were similar for the different CPI complexes, revealing the presence of only Chl *a* and ß-carotene as expected for CPI (Thornber et al., 1991). Since the Chl concentrations of the pigment extracts were not known, the *precise* ratio of Chl *a* to ß-carotene could not be calculated. However, the *relative* ratio of Chl *a* to ß-carotene in each sample could be calculated by determining the areas under the Chl *a* and ß-carotene peaks within a sample. These calculations gave relative Chl *a* to ß-carotene ratios of 2.4, 2.4, and 1.6 for the *Chlamydomonas* CPI, the diatom CPI, and CPI_{65kD}, respectively, suggesting an enrichment in ß-carotene relative to Chl *a* for CPI_{65kD}. Interestingly, there was no corresponding increase in absorption at wavelengths expected for ß-carotene (500 and 584

nm) in CPI_{65kD} relative to CPI (Fig. 5.5); in fact, there was a slight decrease in absorption at these wavelengths in CPI_{65kD}. The reason(s) for the discrepancy between the absorption data and the HPLC data are discussed below. From these data we conclude that the PsaA apoprotein binds both ß-carotene and Chl *a* as expected for PS I, although at this time we are unable to say precisely what proportion of the total Chl *a* and ß-carotene present in the CPI holocomplex is bound by PsaA.

As noted previously, the fluorescence yield of PS I is low at physiological temperatures. Therefore, low temperature (77K) fluorescence spectroscopy was used to study the fluorescence properties of CPI and CPI_{65kD} in more detail (Fig. 5.7). The CPI complex isolated from *Chlamydomonas* showed an emission peak at 717 nm, typical of CPI complexes isolated from green algae (Goedheer, 1981). The 77K fluorescence emission spectrum of CPI isolated from *Cylindrotheca* had a broad maximum between 708-712 nm, significantly blue-shifted from the 717 nm maxima seen for both the *Chlamydomonas* CPI (Fig. 5.7) and PS I particles purified from DM-solubilized thylakoids of *Cylindrotheca* (Fig. 5.1B). The 77K fluorescence emission maximum of CPI_{65kD} was at 703 nm, slightly blue-shifted relative to the emission peak of the CPI complex (Fig. 5.7). These data suggest that either the Chls responsible for the 717 nm emission in PS I have been lost during isolation of CPI and CPI_{65kD} from *Cylindrotheca*, or that their organization within the complex has been altered in such a way as to change their fluorescence emission properties.

The organization of pigments within CPI_{65kD} and the two CPI complexes was studied using circular dichroism (CD) spectroscopy (Fig. 5.8). The CD spectrum for *Chlamydomonas* exhibits a negative peak at 685 nm and a positive peak at 670 nm, and is similar to previously published results for PS I particles from pea (Nabedryk et al., 1984) and *Synechocystis* (van der Lee et al., 1993). The spectrum obtained for *Cylindrotheca* showed a negative peak at 682 nm and a positive peak at 665 nm; the 682(-) peak was somewhat broadened toward the

red. Although this spectrum is similar to that for CPI from *Chlamydomonas*, the shift in the positive peak from 670 to 665 nm and the broadening of the major negative peak (682 nm) indicates that some organizational changes have occurred in the diatom CPI. The CD spectrum of CPI_{65kD} is unlike any previously published spectra for PS I complexes, with a major positive peak at 664 nm, and two negative peaks at 678 and 690 nm, separated by a small positive peak at 683 nm (Fig. 5.8). These data indicate that the organization of Chls in CPI_{65kD} differs from that in CPI.

Further characterization of the biophysical and biochemical properties of CPI_{65kD} is limited by the use of PAGE to resolve the complex and by low yields. Moreover, in order to acquire material of sufficient purity for biophysical and biochemical analyses, CPI_{65kD} had to be obtained not by re-electrophoresis of CPI_{65kD}, but by re-electrophoresis of CPI (not shown). Another method of resolving this complex is necessary to provide sufficient material for biophysical analyses such as EPR spectroscopy. Attempts to purify CPI_{65kD} using sucrose density gradient fractionation, however, were unsuccessful (not shown). This suggested to us that the sieving of CPI through the polyacrylamide matrix during electrophoresis may be required to dissociate PsaA (e.g. CPI_{65kD}) from PsaB.

5.5 Discussion

In the course of purifying thylakoid membranes from the marine diatom *Cylindrotheca fusiformis* (Martinson and Plumley, in prep.; Chapter 4), we observed a remarkable susceptibility of PS I to inactivation. PS I activity, measured as oxygen uptake upon illumination in the presence of ascorbate/DCPIP and methylviologen, was completely lost in thylakoids that had been purified using buffers of low osmotic strength and/or harsh breaking conditions. A comparison of PS I-enriched particles purified by DM-solubilization of thylakoids which retain PS I activity (active; Martinson and Plumley, in prep.; Chapter 4) and thylakoids

that have lost PS I activity (inactive) revealed nearly identical polypeptide compositions (Fig. 5.1A) and virtually indistinguishable 77K fluorescence emission properties. However, a substantial reduction in the vitamin K_1 content of the PS I particles purified from inactive thylakoids was observed (Fig. 5.1C). Since vitamin K_1 functions as the secondary electron acceptor A_1, this could explain the lack of electron transport capacity in these thylakoids; further studies employing rapid (pico- or femtosecond) kinetics are needed to accurately define the role of vitamin K_1 in PS I electron flow. A recently established collaboration with Drs. M. Mimuro and S. Itoh (Iwaki et al., 1992; National Institute of Basic Biology, Okazaki, Japan) should help resolve this question.

Given the integral role of vitamin K_1 in PS I, it seemed logical to investigate the stability of the PS I reaction centers of inactive thylakoids. As noted above, the DM PS I particles from inactive thylakoids appeared to be structurally similar to those isolated from active thylakoids (Fig. 5.1A,B). In addition, we noted that when inactive thylakoids are solubilized with DM and subjected to nondenaturing LDS-PAGE, only the 110 kD green band, CPI, is obtained (Fig. 5.9, lane 1). Moreover, re-electrophoresis of this CPI yields little to no CPI_{65kD} (Fig. 5.9, lane 2) indicating that DM can stabilize CPI (Fig. 5.9) as well as PS I particles (Fig. 5.1) even in the absence of vitamin K_1. These data are consistent with the conclusion of Nechushtai and coworkers (1986) that DM has a stabilizing effect on PS I. Finally, it should be noted that vitamin K_1 has been shown to be somewhat labile, and is easily displaced by other quinones (Rustandi et al., 1992). We conclude that although vitamin K_1 loss is correlated with the loss of electron transport activity, it is unlikely that this loss is the cause of PS I instability in inactive thylakoids.

Since the detergent LDS does not exert a stabilizing effect on PS I (Nechushtai et al., 1986), we chose this detergent to study the stability of PS I complexes from inactive and active thylakoids. Solubilization of thylakoids with low concentrations of LDS (LDS:Chl = 10),

followed by mild LDS-PAGE resolved a novel green complex at 65 kD from inactive, but not from active, thylakoids. Subsequent analysis showed this complex to be derived from the PS I core complex, CPI (Fig. 5.2), and to contain only the PsaA subunit (Figs. 5.3,5.4). Based on its relationship to CPI, we refer to this band as CPI_{65kD}. From these results, we conclude that PsaA in *Cylindrotheca* is more stable than PsaB, and surmise that the susceptibility of CPI to dissociation is due to the instability of PsaB. In addition, these results demonstrated a clear relationship between the loss of PS I activity in thylakoids and the dissociation of the PS I reaction center.

The results obtained in this study differ from those previously reported by us for LDS-PAGE of inactive thylakoids in that CPI_{65kD} was not resolved (Plumley et al., 1993). We suggest that this may be due to differences in the amount of Chl loaded on the gel (8.5 μg in the earlier study vs. 60 μg for the experiments presented here). It is probable that CPI_{65kD} was present in the earlier gels but was not detected.

The isolation of a pigment-binding complex containing only PsaA provides a unique opportunity to compare the biochemical composition of the PsaA subunit to that of the PS I holocomplex (e.g. CPI). Since most of this work was completed before techniques were perfected for obtaining active thylakoids (Chapter 4) we could not rule out alterations in the CPI complex from *Cylindrotheca*. Therefore, we used CPI isolated from the green alga *Chlamydomonas* as the "standard" by which to compare the biochemical and biophysical properties of the diatom CPI and CPI_{65kD}. Work is currently planned to repeat key experiments with both active and inactive thylakoids.

As a first step in characterizing this unique complex, the pigment composition of both CPI complexes and CPI_{65kD} were compared. The absorption spectra for all complexes were nearly identical (Fig. 5.5) suggesting that there were no differences in the relative amounts of Chl *a* and carotenoid present in each of the complexes. In contrast, HPLC pigment profiles

revealed an apparent enrichment in ß-carotene relative to Chl *a* in CPI_{65kD} (Fig. 5.6). At first glance, these results seem contradictory to the absorption data in that there is no enhancement in absorption in the 500-600 nm range for CPI_{65kD} (Fig. 5.5). The discrepancy in the absorption data and HPLC could be due to an absorption artifact (e.g., particle flattening, light scattering) or to changes in the absorption properties of either ß-carotene or Chl *a* caused by conformational changes in the protein. Alternatively, ß-carotene may be more readily extracted from CPI_{65kD} by *sec*-butanol. The differences in extractability could be a function of the extent to which pigments are exposed to *sec*-butanol in CPI vs CPI_{65kD} or because the number of ß-carotene molecules that are covalently bound (Makewicz et al., 1994) to PsaB is greater than the number bound to PsaA. More information (e.g. concentrations of Chl and protein for each sample) is needed before conclusions can be drawn regarding the precise ratios of Chl, ß-carotene and protein in each of the complexes.

The low fluorescence emission yields of PS I complexes at physiological temperatures is well-known, and has been attributed to Chl *a* molecules with short fluorescence lifetimes (Searle et al., 1977). Presumably, excitation energy transferred to these Chls is rapidly dissipated via vibrational processes, and therefore fluorescence is negligible. A decrease in the dissipation of excitation energy by vibrational processes would be expected upon cooling to 77K, resulting in an increase in fluorescence emission. The presence of one or two long-wavelength (e.g. 705 nm) absorbing Chl *a* species has been invoked to explain the temperature-dependence of fluorescence in PS I particles from *Chlamydomonas* (Werst et al., 1992). These "red" Chls were predicted to be located in the immediate vicinity of P700 (Jia et al., 1992), but it is not known if they are bound to PsaA, PsaB, or both subunits. The exceedingly low room temperature fluorescence emission from CPI_{65kD} suggests that at least one "red" Chl is bound to the PsaA polypeptide. At this time, it is not clear whether there are one or two "red" Chls in PS I (Jia et al., 1992; Werst, et al., 1992). The isolation of CPI_{65kD}

may prove useful in addressing this issue and our collaboration with Drs. M. Mimuro and S. Itoh (Iwaki et al., 1992; National Institute of Basic Biology, Okazaki, Japan) should help resolve this question.

The organization of Chls in the three complexes was studied further using low temperature (77K) fluorescence emission spectroscopy (Fig. 5.7). For CPI complexes isolated from algae, the fluorescence emission maximum typically occurs at ~717-720 nm (Goedheer, 1981). The CPI complex isolated from *Chlamydomonas* using mild LDS-PAGE had the expected 717 nm peak. In contrast, CPI isolated from *Cylindrotheca* exhibited a broad peak from 708-712 nm, substantially blue-shifted from the peak for *Chlamydomonas* CPI. The emission maximum for CPI_{65kD} was at 703 nm, slightly more blue-shifted than that for CPI. A blue-shift in the fluorescence emission maximum of PS I particles purified using LDS has been observed, and attributed to conformational changes in the proteins (Nechushtai et al., 1986). In addition, the blue-shift in the fluorescence emission could be reversed by exchanging LDS for DM. We attempted to restore the fluorescence emission maximum of the diatom CPI to 717 nm by incubating the gel slice in DM, but were unsuccessful (not shown). Similarly, incubation of the CPI_{65kD} complex in DM did not significantly alter its emission peak (not shown). These results indicate that the alterations that have occurred with the diatom CPI and CPI_{65kD} are irreversible, at least with respect to DM treatment, and indicate that the diatom PS I is more susceptible to LDS-induced conformational changes than green plant PS I. In addition, we hypothesize that the blue-shift and general broadening of fluorescence seen in CPI isolated from diatoms using LDS is not merely an artifact caused by the binding of LDS, but is instead due to conformational changes in CPI as it is being dissociated into CPI_{65kD}.

The possibility that the diatom CPI had undergone conformational changes was investigated further using CD spectroscopy. The CD spectrum of CPI from *Cylindrotheca* was characterized by a negative peak at 682 nm and a positive peak at 665 nm. The major

positive peak (665 nm) was substantially blue-shifted in the diatom CPI relative to CPI from *Chlamydomonas* (670 nm; Fig. 5.8), romaine lettuce, or *Porphyridium* (not shown), and is more similar to the major positive peak in CPI_{65kD} (664 nm). In addition, the major negative peak in the diatom CPI was shifted to 682 nm (from 685 nm in *Chlamydomonas* CPI), and appeared somewhat broader than the peak in the *Chlamydomonas* CPI. Occasionally, a distinct negative shoulder at 690 nm was observed in the spectrum for the diatom CPI (not shown). In the CD spectrum for CPI_{65kD}, the broad 682(-) peak seen in CPI has been resolved into two negative peaks at 678 and 690 nm, along with the generation of a new positive peak at 683 nm (Fig. 5.8). These data suggest that the CPI isolated in the gel slices is in the process of being dissociated, and hence its spectroscopic signals are a combination of the individual signals from "native" CPI and CPI_{65kD}. This would explain the broad fluorescence emission spectrum of CPI (which overlaps the emission spectrum of CPI_{65kD}) as well as the overall blue-shifting of the CD peaks relative to those of *Chlamydomonas* CPI (Figs. 5.7, 5.8).

The CD spectrum of CPI_{65kD} is unique compared to CD spectra published for PS I particles (Nabedryk et al., 1984; Ikegami and Itoh, 1986; Shubin et al., 1986). There are, however, striking similarities between the CPI_{65kD} spectrum and that of the reduced-minus-oxidized CD spectrum of ether-extracted PS I particles containing 7-10 Chls (Ikegami and Itoh, 1986). The spectrum for CPI_{65kD} contains signals at 664(+)/678(-) and 683(+)/690(-) (Fig. 5.8), while the difference spectrum presented by Ikegami and Itoh (1986) contains signals at 663(+)/673(-) and 683(+)/694(-). The negative signal observed in CPI_{65kD} is at 690 nm (as opposed to 694 nm), but it is possible that a negative signal at 694 nm is masked by the presence of additional dichroic Chl pairs. It should be noted that the signals observed by Ikegami and Itoh (1986) were observed only in the difference spectrum; the oxidized and reduced spectra were dominated by a large CD signal at 678(+)/688(-) that was shown to originate from a redox-insensitive Chl species absorbing at 684 nm. A possible explanation

for the appearance of the 683(+)/690(-) signals in the CD spectrum of CPI_{65kD}, then, is that some portion of the Chls responsible for the 678(+)/688(-) signal are either absent from the complex or are no longer dichroic.

The similarities between the CD signals from CPI_{65kD} and the 663(+)/673(-) and 683(+)/694(-) signals observed in the reduced-minus-oxidized spectrum described above are interesting in that the latter signals were attributed to dimeric Chls in the vicinity of P700, and to P700, respectively (Ikegami and Itoh, 1986). In addition, the reduced-minus-oxidized fluorescence emission spectrum of ether-extracted PS I particles revealed a major component at 701 nm that was attributed to P700 (Iwaki et al., 1992). One interpretation of these data is that CPI_{65kD} contains P700. Although the possibility that P700 is present in CPI_{65kD} is an intriguing one, it must be pointed out that this is contrary to all current interpretations of the structure of P700 (Golbeck, 1988; Moënne-Loccoz et al., 1990). However, it is also important to realize that the organization of electron transfer components within the PS I reaction center has yet to be determined (Krauss et al., 1993). Without additional evidence, we must conclude that P700 is most likely not present in CPI_{65kD}, and that the assignment of certain spectroscopic signals to P700 should perhaps be reassessed.

A complex similar to CPI_{65kD} was isolated from the PS I reaction center core of a thermophilic cyanobacterium, *Synechococcus* sp. (Sonoike and Katoh, 1986). This complex (designated CP60) was enriched in the 60 kD PS I polypeptide, although the precise identity of this apoprotein was not addressed. Although at first glance it would seem that CPI_{65kD} and CP60 represent identical complexes, there are significant differences in the spectroscopic properties of these two complexes. For example, the absorption maxima of CP60 was at 672 nm, while that of the PS I complex used to purify CP60 (e.g. CPI-e) occurred at 675 nm. In contrast, the absorption maxima for both CPI_{65kD} and CPI was 676 nm (Fig. 5.5). More dramatic differences are evident in the 77K fluorescence emission spectra of the two

preparations. CP60 exhibited a maximum between 717 and 725 nm, depending on the preparation, whereas the emission maximum of CPI_{65kD} was found to be 703 nm (Fig. 5.7). Since their preparation did not have P700 activity, Sonoike and Katoh (1986) concluded that CP60 functioned as the internal antenna of PSI. Based on the differences in the spectroscopic properties of CP60 and CPI_{65kD}, we hypothesize that the apoprotein in the CP60 complex isolated by Sonoike and Katoh (1986) is PsaB. Efforts are being made to confirm this hypothesis.

The isolation of CPI_{65kD} is correlated with a loss of PS I activity in purified diatom thylakoids. Previous studies suggested that the loss of activity was caused by damage to the Fe-S centers, F_B and/or F_A, in purified thylakoids (Martinson and Plumley, in prep.; Chapter 4). In addition, work presented here suggests that vitamin K_1 is readily lost from damaged PS I reaction centers (Fig. 5.1C), which would disrupt electron flow within PS I. The reasons for the extreme lability of PS I in diatoms are unclear at this time. The results obtained in this study, however suggest a differential stability of PsaA and PsaB, with PsaA being more stable. We suggest that structural modification of the PsaB polypeptide in diatoms may be the cause of this instability.

The presence of structural modification in the diatom PsaB polypeptide is suggested by the lack of cross-reactivity of this polypeptide with an antibody specific for the C-terminus of PsaB (Fig. 5.3). As discussed above, the lack of cross-reactivity is not likely due to differences in the C-terminal amino acid sequence of the diatom PsaB. Another possibility is that the C-terminus of PsaB has been cleaved, perhaps during the purification of thylakoids (e.g. by a protease insensitive to the serine-protease inhibitors, ACA and BAM). In this regard, it should be noted that no cross-reactivity is observed between this antibody and the PsaB polypeptide of active thylakoids. This does not entirely rule out the possibility that the cleavage of PsaB is an artifact of purification, however, and studies are in progress to answer

this question (Chapter 6). A more intriguing possibility is that the C-terminus (e.g. residues 721-734) of PsaB in *Cylindrotheca* may be missing as a result of *in vivo* processing events. This is particularly interesting in that the sequence to which the antibody was generated constitutes most of a predicted transmembrane helix (Golbeck, 1988). Since 60% of PS I has been shown to be located in the inner membranes of the thylakoid stacks in *Phaeodactylum* (Pyszniak and Gibbs, 1992), it is likely that slight structural modifications of PS I are necessary. We propose that C-terminal processing of PsaB may be the means by which this occurs, and are currently investigating this possibility (Chapter 6).

5.5.1 Conclusions

In summary, a unique pigment-protein complex (CPI_{65kD}) was isolated from thylakoid membranes of a marine diatom using mild LDS-PAGE. The complex is obtained only from thylakoid membranes inactive in PS I-dependent O_2 uptake (ascorbate/DCPIP to MV). We have shown that CPI_{65kD} is derived from CPI, and immunochemical and N-terminal sequencing data suggest that the complex contains only the PsaA polypeptide. In addition, CPI_{65kD} has many of the biochemical and biophysical properties associated with PS I, including the presence of one or more "red" Chls responsible for the low fluorescence yield of PS I. This complex will provide a unique opportunity to study the organization of electron transport components within the PS I core complex.

The isolation of CPI_{65kD} is of interest from a structure-function standpoint. Several authors have documented the mutual requirement of PsaA and PsaB in the stable assembly of the PS I reaction center (Toelge et al., 1991; Webber et al., 1993). This may explain why we were unable to obtain a pigment-binding complex consisting of only PsaB or PsaA from *Chlamydomonas*, romaine lettuce, or *Porphyridium* (not shown). When CPI begins to dissociate, PsaA and PsaB also dissociate because each is unstable in the absence of the

other. In contrast, the diatom PsaA is stable, perhaps even more so than PsaA from other plants and algae. This raises the intriguing hypothesis that PsaA in the diatom could accumulate in the absence of PsaB (i.e., in deletion mutants with a deleted *psa*B gene) or that the PsaA-chlorophyll complex could be reconsituted in vitro. Either of these avenues could provide a creative means of answering some of the many outstanding questions concerning electron flow through PS I and the components required for assembly and function of this complex.

5.6 References

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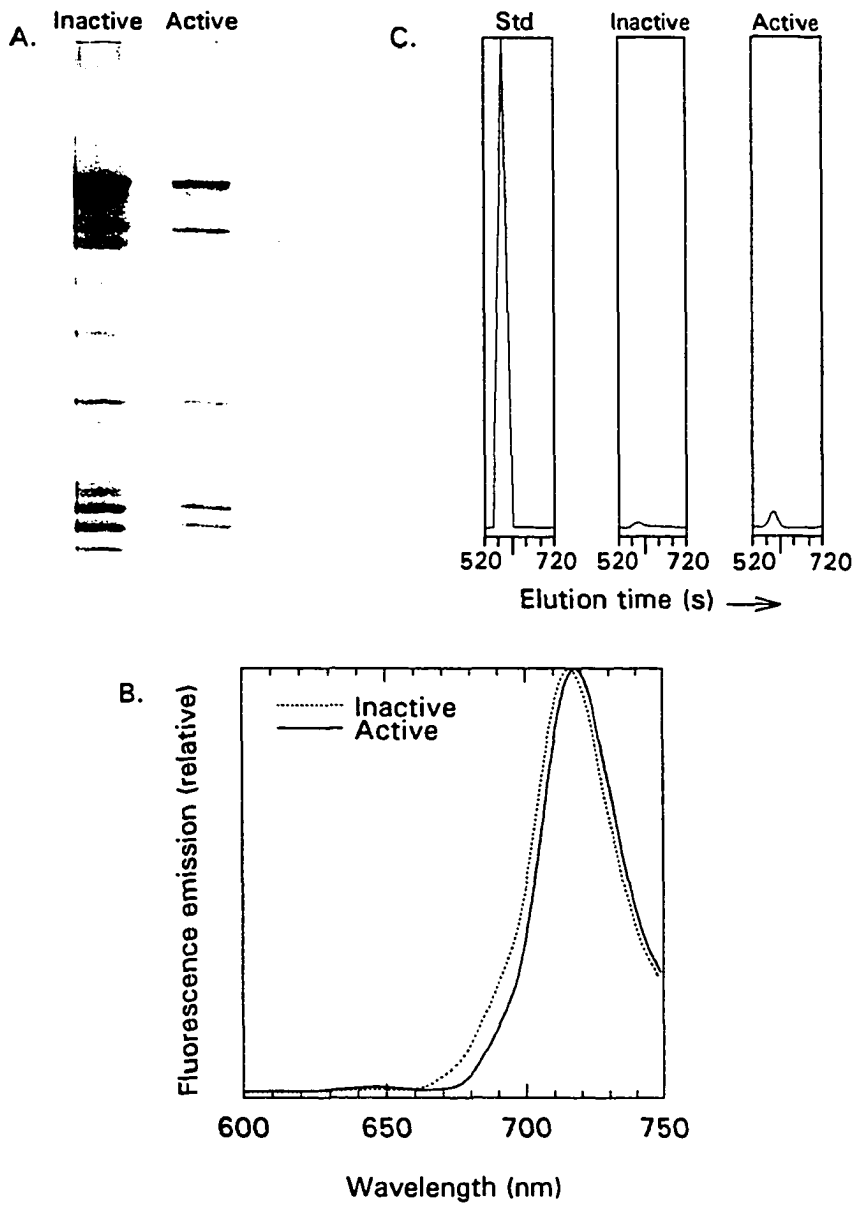


Figure 5.1. Comparison of PS I-enriched particles purified from thylakoids with (active) or without (inactive) PS I activity. A. Coomassie-stained SDS-PAGE gel showing polypeptide composition of PS I particles purified from inactive and active thylakoids. B. Fluorescence emission spectra (77K) of same particles excited with 440 nm light. C. Vitamin K_1 content of same particles. For the standard, 20 μl of a 215 μM vitamin K_1 standard was injected.

Figure 5.2. Mild LDS-PAGE of LDS-solubilized thylakoids. Lane 1: *Chlamydomonas* thylakoids. Lane 2: Re-electrophoresis of *Chlamydomonas* CPI. Lane 3: *Cylindrotheca* thylakoids (inactive). Lane 4: Re-electrophoresis of *Cylindrotheca* CPI. Lane 5: Re-electrophoresis of *Cylindrotheca* CPI_{65kD}. Gel was not stained. CPI^{*}: oligomeric form of CPI. CP47: 47 kD antenna complex of PS II.

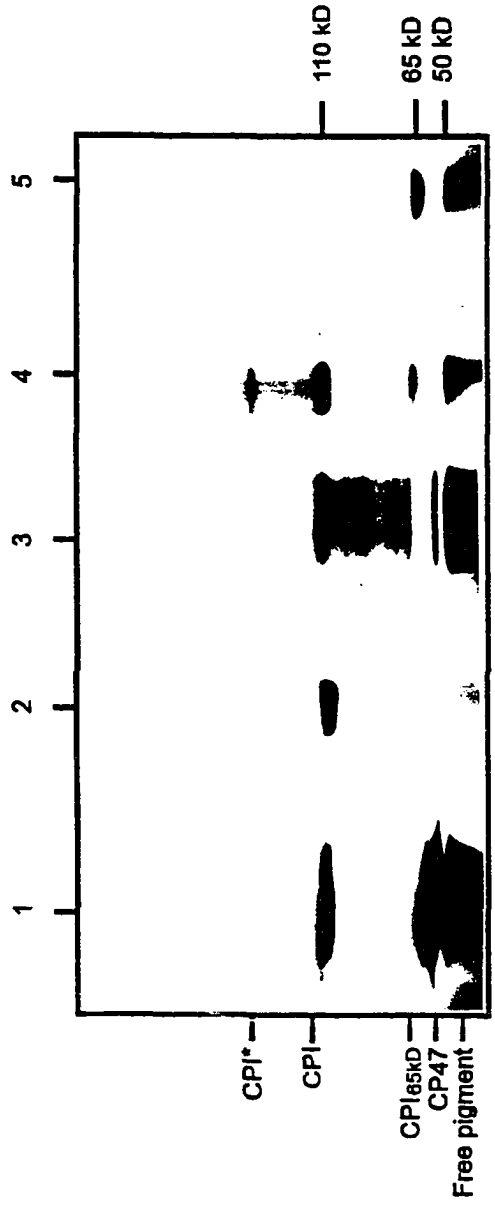
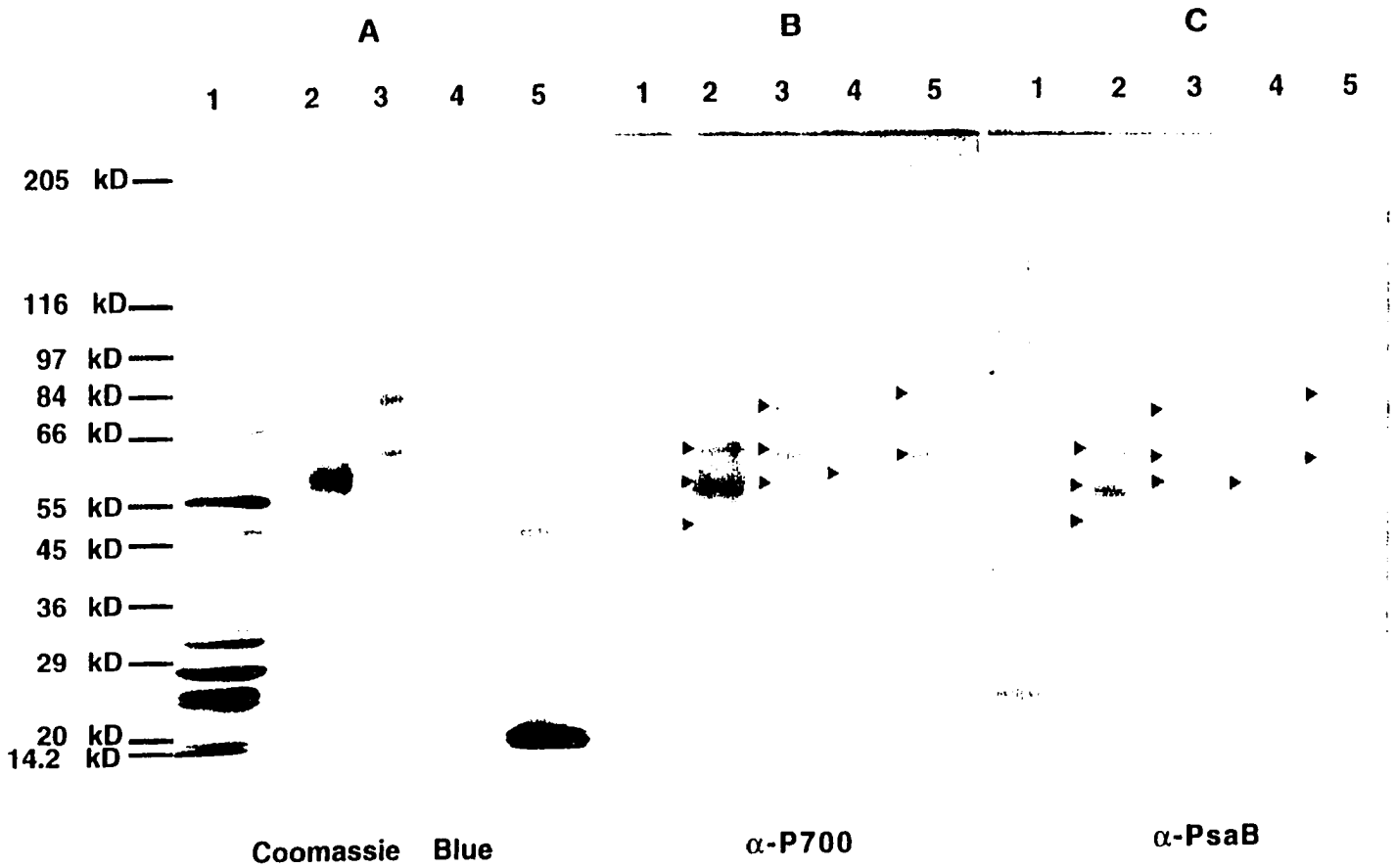


Figure 5.3. Polypeptide composition of CPI and CPI_{65kD} complexes. Apoproteins were resolved from CPI complexes obtained after two rounds of mild LDS-PAGE to remove comigrating polypeptide contaminants, or from CPI_{65kD} complexes obtained by re-electrophoresis of the diatom CPI complex. A. Coomassie Blue-stained gel. B. Western blot of gel in A, reacted with an antibody against the PsaA/B heterodimer of cyanobacteria. C. Western blot of gel in A, reacted with an antibody against a synthetic peptide corresponding to the C-terminus of the PsaB polypeptide from pea. Lane 1: *Chlamydomonas* thylakoids. Lane 2: *Chlamydomonas* CPI. Lane 3: *Cylindrotheca* CPI. Lane 4: *Cylindrotheca* CPI_{65kD}. Lane 5: *Cylindrotheca* thylakoids.



CPI upper band:	1	STKFPKFSQALAQDPAT	17
[1]Odontella sinensis PsaB:	1	A	17
[2]Anabaena variabilis PsaB:	1	A D T	17
[3]Eugena gracilis PsaB:	2	A G T	18
[4]Synechocystis PCC 6803 PsaB:	2	A D T	18
[5]Marchantia polymorpha PsaB:	2	ASR G S T	18
[6]Antirrhinum majus PsaB:	4	R R G T	18
[7]Chlamydomonas moewusii PsaB:	6	G T	19
[8]Chlamydomonas reinhardtii PsaB:	6	G R	19
[9]Oryza sativa PsaB:	4	R R G T	18
[10]Spinacia oleracea PsaB:	4	R R G T	18
[11]Nicotiana tabacum PsaB:	4	R R G T	18
[12]Zea mays PsaB:	4	R R G T	18
[13]Pisum sativum PsaB:	4	RI R GI T	18

Figure 5.4. Partial amino acid sequence of the 65 kD apoprotein of CPI from *Cylindrotheca* aligned with PsaB proteins. Alignments were done using BLAST (Altschul et al., 1990) with sequences obtained from GenBank. Accession numbers: [1]gnZ67753;[2]spP31088;[3]spP19431; [4]pirS18243;[5]spP06408;[6]gpX84152;[7]spP36492;[8]spP09144;[9]spP12156;[10]spP06512;[11]spP 06407;[12]spP04967;[13]spP05311.

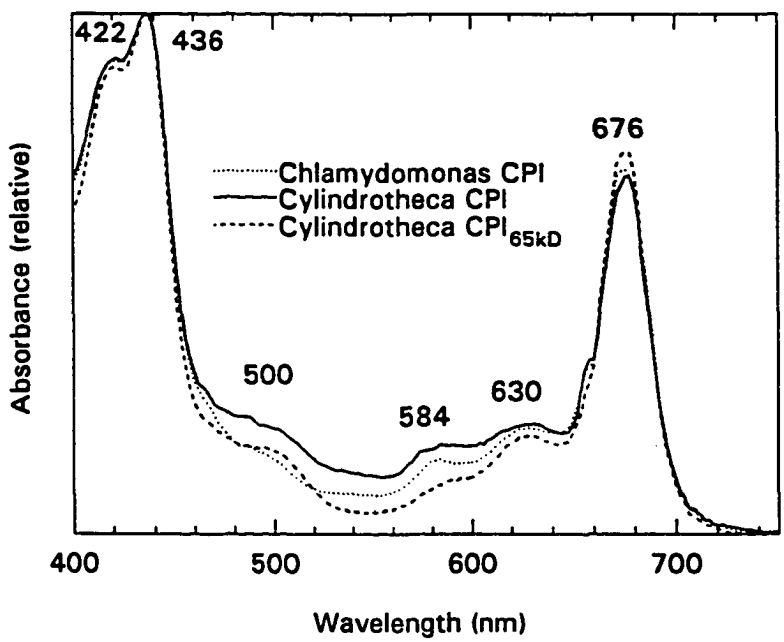


Figure 5.5. Absorption spectra of CPI_{65kD} and CPI complexes.

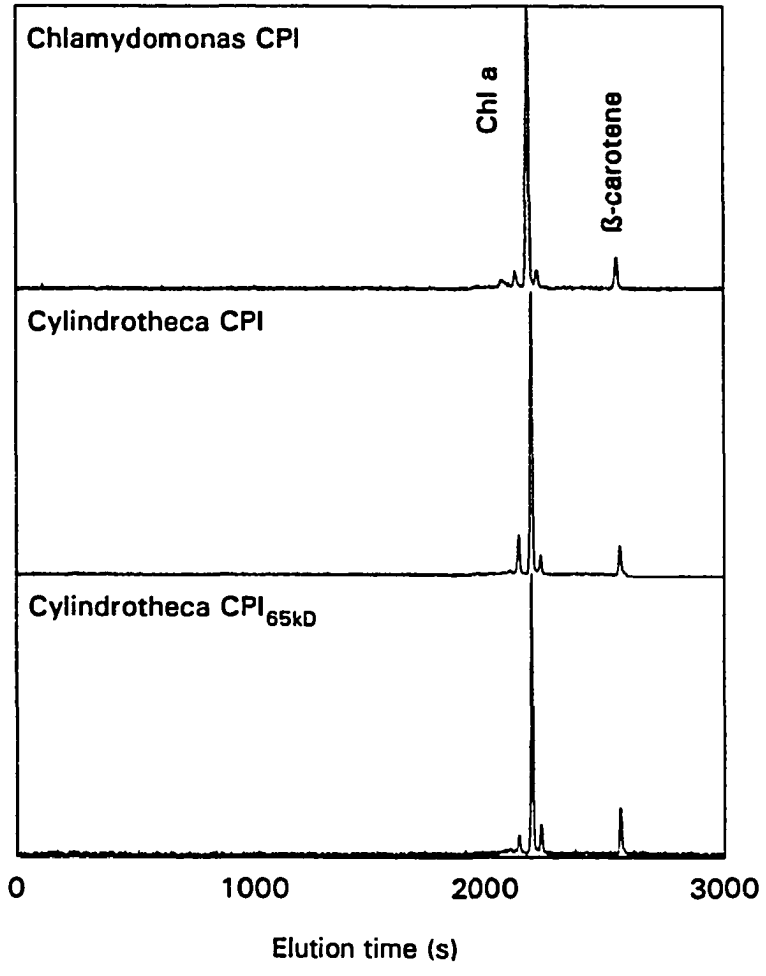


Figure 5.6. Pigment compositions of CPI_{65kD} and CPI complexes.

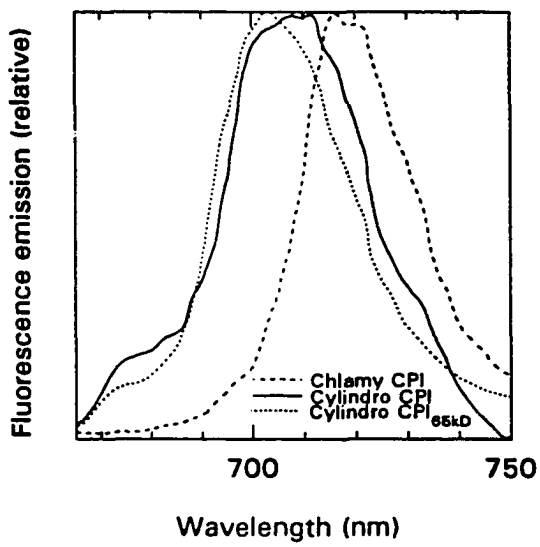


Figure 5.7. Fluorescence emission spectra (77K) of CPI_{65kD} and CPI complexes.

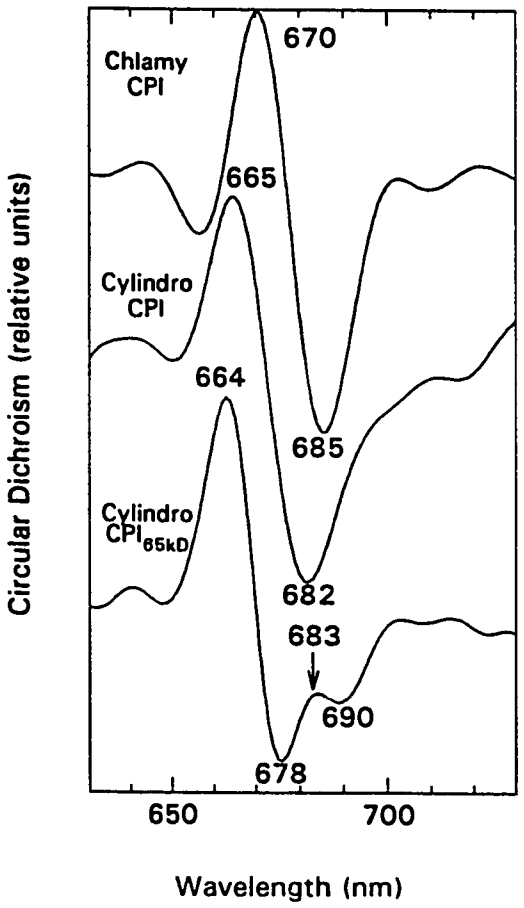


Figure 5.8. Circular dichroism spectra (630-730 nm) of CPI_{65kD} and CPI complexes.

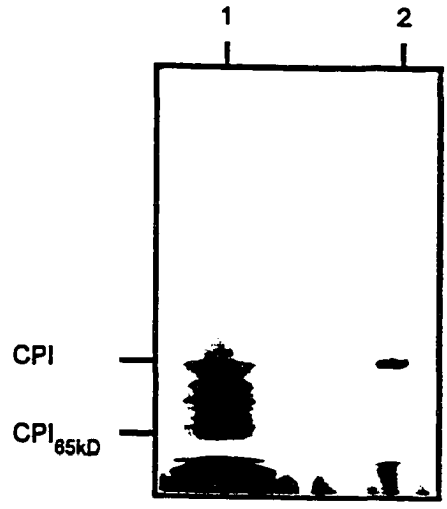


Figure 5.9. Mild LDS-PAGE of dodecylmaltoside-solubilized thylakoids from *Cylindrotheca*. Lane 1: Thylakoids (inactive). Lane 2: Re-electrophoresis of CPI resolved from dodecylmaltoside-solubilized inactive thylakoids.

Chapter 6

Summary and Prospects for Further Research

6.1 Summary of contributions

The work presented in this dissertation represents a major contribution to the study of the photosynthetic apparatus of diatoms. One of the major achievements was the development of a method for purifying high quality thylakoid membranes from diatoms that are capable of high rates of O_2 evolution (Chapter 4). The importance of a high quality (i.e., photosynthetically active) thylakoid preparation for the isolation of photosynthetic pigment-protein complexes (PPCs) from diatoms cannot be underestimated. As I have shown, the activities of PS II and PS I are remarkably sensitive (compared with other plants and algae) to the methods used to purify thylakoids. Moreover, the loss of electron transport activity seems to be followed closely by dissociation of the reaction center complexes. This was demonstrated for PS I in Chapter 5; evidence for the dissociation of PS II will be presented below (see 6.2.1). The dissociation of the reaction center complexes makes their isolation and subsequent characterization difficult, if not impossible, and leads to additional difficulties in identifying the LHCs specifically associated with each reaction center (see 6.2.1).

A second major contribution was the biophysical and biochemical characterization of the purified thylakoids (Chapter 4). Much of the work regarding the photosynthetic PPCs of diatoms and other chromophyte algae has been done with thylakoid membranes that have not been characterized with respect to their biophysical and biochemical properties. Conclusive statements regarding the properties of individual PPCs can be made only with a solid understanding of the biochemical and biophysical characteristics of the thylakoid membrane preparation used to isolate the PPCs. Moreover, the precise identification of photosynthetic polypeptides is difficult because, as I have demonstrated (Chapter 4), there are a number of proteins present in purified thylakoids with the same M_rs. Hence, without the extensive immunological and protein sequence data provided by this work, it would be impossible to unequivocally identify a particular polypeptide present in an isolated PPC.

In addition to the contributions described above, a method for the rapid extraction of pigments from PPCs using *sec*-butanol was developed (Chapter 3) which should prove useful in the study of PPCs from all photosynthetic organisms. The method may also be of benefit to the oceanographic community in that it provides for the simultaneous extraction and concentration of pigments and acyl lipids from dilute aqueous samples without the use of DMSO. Moreover, the stability of extracted pigments in *sec*-butanol is much greater than it is in acetone, thus eliminating many of the difficulties associated with sample storage.

Finally, a unique PS I-derived complex was isolated from diatom thylakoids lacking PS I activity, and shown to contain only the PsaA polypeptide (Chapter 5). This complex contained at least one of the small number (1 or 2) of "red" Chls believed to be responsible for the low fluorescence yields observed for PS I (Werst et al., 1992), and moreover, exhibited spectroscopic characteristics similar to those previously attributed to P700. Although it is likely that PsaA and PsaB each bind one of the Chls constituting the P700 reaction center dimer (Maeda et al., 1993; Krauss et al., 1993), this has not been unequivocally demonstrated. Hence, the isolation of this complex represents a potentially significant contribution to the understanding of photosynthesis in all oxygen-evolving plants in that it may provide insight into the organization of the PS I reaction center.

6.2 Prospects for further research

6.2.1 Isolation and characterization of PPCs from diatoms

As mentioned above, the importance of a highly purified, functionally active thylakoid preparation for isolating PPCs from diatoms cannot be underestimated. Loss of electron transport capabilities leads to dissociation of PS I, as shown in Chapter 5, as well as to PS II, as will be described now. The dissociation of PS II was demonstrated clearly by the following experiment. Thylakoids that did not exhibit electron transport ("inactive") were solubilized

with dodecylmaltoside and PPCs fractionated using sucrose density gradient ultracentrifugation (Chapter 2); PPCs were also resolved from O_2-evolving ("active") thylakoids solubilized using the same conditions. The sucrose density gradient pattern of PPCs was nearly identical for both types of samples except that an additional green fraction was resolved from active thylakoids and a pellet was obtained from inactive thylakoids (Fig. 6.1). Denaturing SDS-PAGE of these fractions revealed striking differences in the polypeptide compositions of similar fractions obtained from the two types of thylakoids (Fig. 6.2). For example, the dark brown fractions isolated from active thylakoids contained primarily the 18, 17.5, and 17 kD LHCPs (Fig. 6.2, lanes 11,12), with only traces of PS II-related polypeptides. In contrast, the dark brown fractions resolved from inactive thylakoids contained substantially more PS II polypeptides (Fig. 6.2, lanes 4,5). At first glance it appeared that the dark brown fractions from inactive thylakoids consisted of PS II reaction centers with their associated LHCs. Three lines of evidence, however, suggest that the presence of PS II polypeptides in the dark brown LHC fractions from inactive thylakoids was an artifact caused by the dissociation of PS II in these thylakoids.

1. The 77K fluorescence emission maxima for dark brown fractions from both inactive and active thylakoids were at ≈682 nm (Fig. 6.3), a wavelength normally ascribed to LHCs. If PS II was functionally associated with the LHC in the dark brown fractions from inactive thylakoids, the fluorescence emission maximum should be at, or near, 685-686 nm, the fluorescence emission for PS II. These results rule out a functional association of PS II and LHC in the dark brown fractions from inactive thylakoids.

2. The dark brown fractions isolated from each of the two types of thylakoids sedimented at the same position on the gradients (Fig. 6.1). This suggested that LHC and PS II in the dark brown fractions from inactive thylakoids were not associated in a complex because the LHC-PS II holocomplex would sediment farther down on the gradient than either the LHCs or PS II. These sedimentation results also suggested that the PS II polypeptides present in these fractions were not assembled in a complex (e.g. the PS II reaction center was dissociated) because the density of PS II reaction center particles is greater than that of LHC particles (see 3). These results are consistent with a dissociated PS II complex in the dark brown fractions of inactive thylakoids.

3. A green complex was resolved from active thylakoids (Fig. 6.1) that was enriched in PS II polypeptides, but contained some PS I components as well (Fig. 6.2, lane 13). This fraction exhibited 77K fluorescence emission peaks at 686, 689, 692, and 715 nm (Fig. 6.3). The 715 nm peak was due to PS I (Chapter 5); the 686 nm peak is similar to the 685 nm peak observed *in vivo* (Chapter 4) and is presumably due to the PS II core. A peak at 688 nm has been assigned to the CP47 and CP43 proximal antennae of PS II (Martinson and Plumley, 1994). The origin of the 692 nm peak is not clear at this time, but it may arise from a unique LHC (e.g. see Chapter 4). None of the particles isolated from inactive thylakoids had fluorescence emission properties that could be attributed to PS II (not shown).

These data, together with the loss of PS II-dependent electron transport activity, provide convincing evidence that the PS II reaction centers are dissociated in inactive thylakoids.

These results illustrate how the use of inactive thylakoids to purify PPCs from diatoms can lead to erroneous conclusions about the association of LHCPs with the PS II reaction center. Therefore, the claims made by others that the major LHCPs of diatoms (e.g. the 17-18 kD polypeptides) are specifically associated with the PS II reaction center (Bhaya and Grossman, 1993) must be reassessed.

Now that a method for purifying high quality thylakoid membranes from diatoms is available, the isolation and characterization of PPCs from these algae can proceed. This work is important for several reasons. First, the ability to purify intact reaction center complexes will be useful in studies of trace element (e.g. iron) limitation in diatoms. For example, the PS I reaction center contains a substantial amount of iron (8-12 moles per mole of P700; Golbeck, 1988), all of which is absolutely required for electron transport. Both purified thylakoids and isolated reaction center preparations should prove useful in assessing the effects of Fe-limitation and/or Fe-starvation on the abundance, activity, and stability of PS I in diatoms. in addition, the development of molecular and/or biophysical markers for the detection of trace element limitation in natural phytoplankton populations will be aided by these types of studies.

Second, while there is a vast amount of knowledge available regarding the LHCs of Chl *a*/*b*-containing plants, almost nothing is known about the fucoxanthin-Chl *a*/*c*-protein complexes (FCPCs) of diatoms. The isolation and characterization of FCPCs has been hindered primarily by the lack of a high quality thylakoid preparation. Many "purified" FCPCs described in the literature are contaminated with PS II and PS I polypeptides (Owens and Wold, 1986; Caron and Brown, 1987), making it impossible to specifically identify them as being associated with PS II or PS I. At present, we do not know the number of FCPCs present in diatoms, their biochemical and biophysical properties, nor the nature of their associations with the reaction centers. Preliminary fractionation experiments have identified two LHCPs that appear to be associated with PS I (Chapter 4). Collaborative relationships have been established with

several scientists regarding this work, and it is expected that rapid advances will be made concerning diatom FCPCs.

6.2.2 Identification of light-harvesting genes in *Cylindrotheca*

While very little is known about FCPCs in diatoms, there is some data available on the genes encoding the LHCP genes. Six genes have been sequenced from the diatom *Phaeodactylum tricornutum*, and were designated *fcp*A-F (Bhaya and Grossman, 1993). As with the Chl *a*/*b*-binding proteins (Cabs; Green et al., 1991), the diatom *fcp* genes are located in the nucleus, and are part of a multigene family (Grossman et al., 1990). We were able to confirm that the three major LHCPs in *Cylindrotheca* thylakoids are homologous to the *fcp*E and *fcp*F gene products (e.g. FcpE and FcpF) of *Phaeodactylum* (Chapter 4).

An interesting outcome of the work reported here was the identification of two PS I-associated LHCPs in *Cylindrotheca* (Chapter 4). The N-terminal sequence of one of these proteins did not show homology to any of the deduced amino acid sequences of known Fcps or Cabs. The two proteins were identified as LHCPs by their cross-reactivity with an antibody to the 29 kD LHCP of *Chlamydomonas*. The cross-reactivity of the novel LHCPs with the *Chlamydomonas* antibody is not surprising, as we have previously suggested that this antibody preparation contains epitopes to regions that appear to be conserved in LHCPs from a number of divergent plants and algae (Plumley et al., 1993). These results suggest that there are additional light-harvesting genes present in *Cylindrotheca*; work is currently in progress to clone and sequence these genes.

The isolation of these genes is important for a number of reasons. First, knowledge of the gene sequences will aid in the identification of LHCPs in diatoms, as demonstrated in this work (Chapter 4). Second, it is important to know precisely how many *fcp* genes are present in diatoms in order to understand the expression of these genes *in vivo*. For example,

although LHCPs homologous to FcpE and FcpF were conclusively identified in *Cylindrotheca* (Chapter 4), we have yet to find a homolog to FcpA or FcpB. There are at least three possible explanations for the apparent absence of FcpA and FcpB in thylakoids of *Cylindrotheca*.

1. It is possible that homologous proteins are present in *Cylindrotheca* and we just haven't found them yet. However, since we sequenced the three major LHCPs, this would imply that FcpA and FcpB are minor components of the light-harvesting apparatus in *Cylindrotheca* grown under the conditions described in Chapter 4.
2. It is possible that *fcp*A and *fcp*B are not expressed in *Cylindrotheca* under the growth conditions used in this work. The expression of the *fcp* genes in *Phaeodactylum* has not been investigated, however, some evidence suggests that expression of the *fcp* genes in the brown alga, *Macrocystis pyrifera*, is regulated by light quality (K. Apt and A. Grossman, unpublished results).
3. It is possible that these genes are not present in *Cylindrotheca*. The cloning and sequencing of *fcp* genes in *Cylindrotheca* is currently in progress.

Finally, diatom *fcp* genes may be a better tool for studying the function and evolution of LHCs in general because these genes appear to be present in only one copy (Bhaya and Grossman, 1993). In contrast, there can be as many as 16 copies of some *cab* genes of higher plants (Jansson, 1994), each of which could be slightly different. This makes site-directed mutagenesis/gene knockout studies difficult with green plants such as *Arabidopsis* and *Chlamydomonas*. The presence of only a single copy of each of the *fcp* genes, coupled with the recent development of transformation protocols for diatoms (Dunahay et al., 1995), should facilitate these types of studies.

We have previously shown by immunological methods that LHCPs from a diverse group of plants and algae are structurally similar (Plumley et al., 1993). In addition, the deduced

amino acid sequences of LHCPs of Chl *a/b*-containing (e.g. CAB polypeptides) share two regions of homology with the LHCPs of Chl *a/c*-containing algae (e.g. FCPs) (Grossman et al., 1990). We are currently trying to map these conserved domains more precisely in *Chlamydomonas* and *Cylindrotheca* in order to determine their functional roles. To date, we have cloned and partially sequenced one LHCP gene of *Chlamydomonas* (Fig. 6.4). Using degenerate primers derived from the regions of the CAB polypeptides that are similar to the FCP polypeptides (Fig. 6.4), we have amplified DNA from *Cylindrotheca* in order to clone the diatom LHCP genes. The goal of this work is to develop protocols for *in vitro* reconstitution of FCPs with pigments, and then to reconstitute these FCPs with reaction center complexes from either *Chlamydomonas* or *Cylindrotheca*. These assays should help determine if the conserved domains in LHCPs are important for pigment-binding and/or functional association of LHCs with reaction centers.

6.2.3 Stability of PS I in diatoms

The stability of PS I in diatoms appears to be considerably less than that observed in higher plants, green algae, red algae, and cyanobacteria (Chapter 5). At this time, the precise reason for this is unclear, however, we can conclude that the source of instability is PsaB, not PsaA (Chapter 5). We hypothesized that slight structural modifications of the PsaB polypeptide in diatoms may be the cause of this variation in stability. Structural modifications of diatom PS I may be necessary in order for this complex to be located in the inner membranes of the thylakoid stacks (Pyszniak and Gibbs, 1992).

As discussed in Chapter 5, the presence of structural modifications in the diatom PsaB polypeptide is suggested by the lack of cross-reactivity of these polypeptides with antibodies specific for the C-terminus of PsaB from pea (Fig. 5.3). There are two possible explanations for these results, the first of which is that the C-terminal sequence of PsaB in *Cylindrotheca*

is significantly different from that of PsaB in the majority of other plants and algae, including another diatom. We have already shown that the N-terminus of the PsaB polypeptide in *Cylindrotheca* is 94% identical to that of PsaB in *Odontella* (Chapter 5). Although it seems highly unlikely that the sequence of *psa*B in *Cylindrotheca* is that different from those in other plants and algae, this possibility cannot be entirely ruled out. To address this issue, DNA sequence data is needed. In *Odontella*, the *psa*B and *psa*A genes are contiguous, separated by only 180 bp (Kowallik, 1996). If the same holds true for *Cylindrotheca*, it should be possible to use the *Odontella* sequence to synthesize degenerate primers to conserved regions that will permit amplification over the *psa*B/*psa*A boundary in *Cylindrotheca* (e.g. from the last 90 bp of *psa*B through the first 90 bp of *psa*A). Assuming a 180-220 bp noncoding region between the two genes in *Cylindrotheca*, the amplified DNA would be short enough (e.g. ~400 bp) that amplifying, cloning, and sequencing it would not pose difficulties.

The second possibility is that the C-terminus of PsaB has been cleaved, either by *in vivo* processing or during purification of the thylakoid membranes. Proteolytic cleavage during purification of thylakoids, if it is occurring, must be due to a non-serine-type protease, as these would be inhibited by the protease inhibitors, ACA and BAM, used in all purification steps. It should be possible to rule out this possibility by adding boiling SDS or concentrated NaOH to a rapidly-harvested cell pellet to denature any endogenous proteases. Total membrane proteins would then be resolved using denaturing SDS-PAGE, blotted, and the membrane reacted with the PsaB antibody. A positive reaction would provide strong evidence in favor of proteolytic cleavage of PsaB during thylakoid membrane purification. However, a negative reaction would lend support to the hypothesis that processing of this protein occurs *in vivo*. Another possibility would be to determine if the PsaB polypeptide in *Odontella* cross-reacts with the PsaB antibody (using the same method described above). A negative reaction would provide strong evidence in support of *in vivo* processing, while a positive reaction would

suggest that the C-terminal sequence of PsaB is significantly different in *Cylindrotheca*.

It is hoped that the successful completion of one or more of the studies outlined above will permit the development of a model of PS I in diatoms. To my knowledge, post-translational processing of PsaB has not been demonstrated in any plant or alga. The occurrence of such an event in diatoms could perhaps explain both the instability of PsaB in purified thylakoids (Chapter 5) and the ease with which PS I activity is lost in purified thylakoids (Chapter 4).

A physiological role for the processing of the C-terminus of PsaB is difficult to imagine, particularly because the sequence that the antibody was generated against constitutes most of a putative membrane-spanning helix (see Golbeck, 1988). At present, the role of many of the helices in PsaA and PsaB is unclear; only 18 of the predicted 22 transmembrane helices could be tentatively identified from the crystal structure of PS I (Krauss et al., 1993). Nevertheless, it is not difficult to imagine how the cleavage of this helix could lead to some rather significant structural changes in the PsaB polypeptide of diatoms. The absence of this helix could explain the exceptionally low stability of PsaB in diatoms. More significantly, the putative removal of the C-terminus might be physiologically necessary in diatoms and other algae that have their thylakoid membranes arranged in groups of three. Approximately 60% of the PS I reaction centers are located in one of the four appressed membranes of diatoms (Pyszniak and Gibbs, 1992), thus the PS I reaction centers in these algae are likely to be in an environment that is quite different from that of green plant PS I. It would not be surprising if a posttranslational processing event evolved to accommodate the assembly and/or subsequent functioning of PS I in diatoms.

6.3 References

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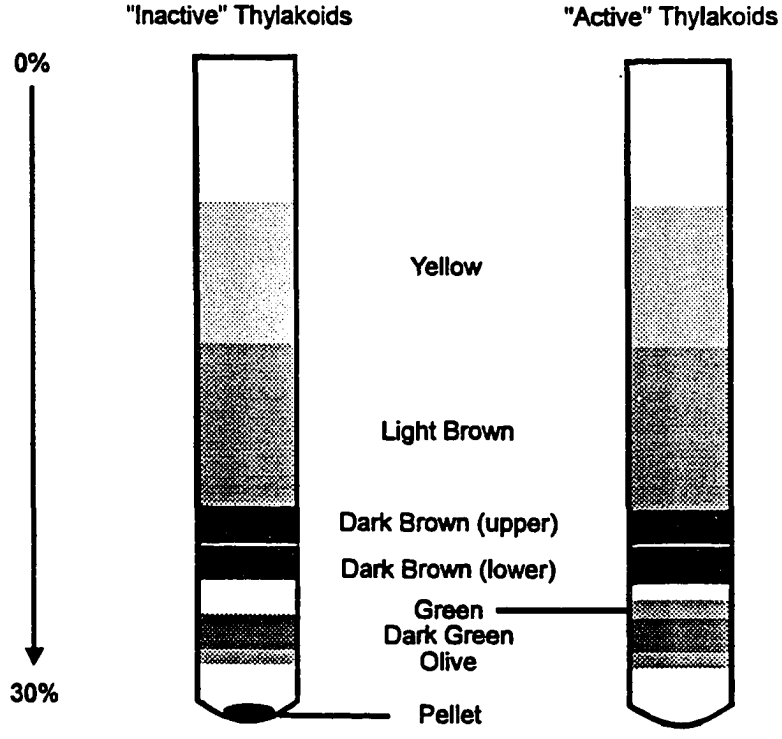
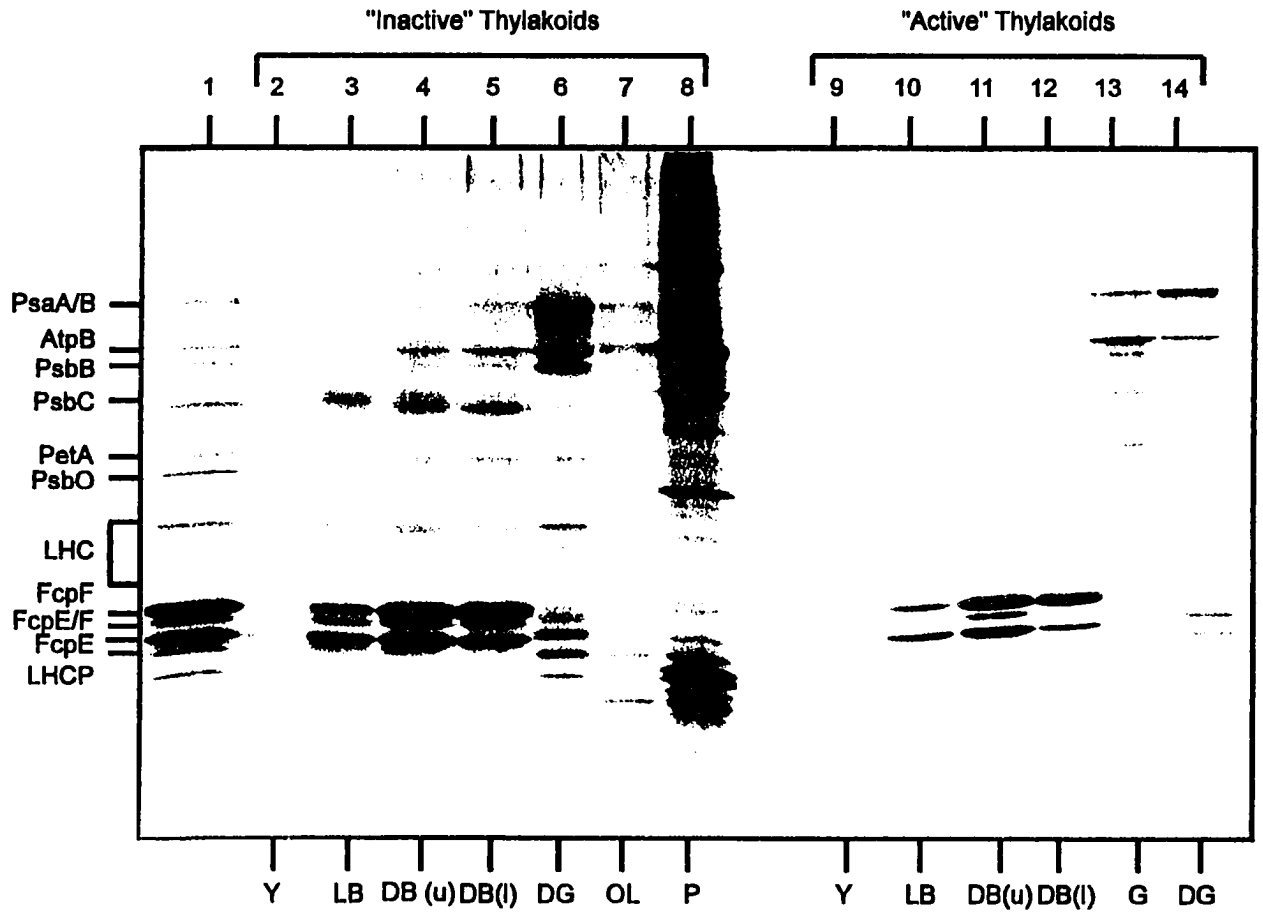


Figure 6.1. Sucrose density gradient fractionation of inactive and active thylakoids. Thylakoids were solubilized with 1% dodecylmaltoside (DM:Chl = 10) and fractionated on 0-30% (continuous) sucrose gradients as described in Chapter 2. The colors of the pigmented fractions are indicated in the center of the diagram.

Figure 6.2. Denaturing SDS-PAGE of sucrose density gradient fractions obtained from inactive and active thylakoids. Lane 1: active thylakoids (10 μg Chl). Lanes 2-8: fractions resolved from inactive thylakoids. Lanes 9-14: fractions resolved from active thylakoids. Y: Yellow; LB: light brown; DB: dark brown; DG: dark green; OL: olive; P: pellet; G: green. The dark brown fraction on each gradient was collected in two aliquots, an upper (u) and a lower (l). Labels on the left indicate the positions of the major polypeptides present in thylakoids. PsaA/B: PS I reaction center subunits; AtpB: β-subunit of ATP Synthase; PsbB and PsbC: antenna polypeptides of the PS II reaction center; PetA: cytochrome f; PsbO: 33kD component of the water-oxidation complex; LHCP, FcpE, and FcpF: light-harvesting polypeptides (see Chapter 4 for more detail).



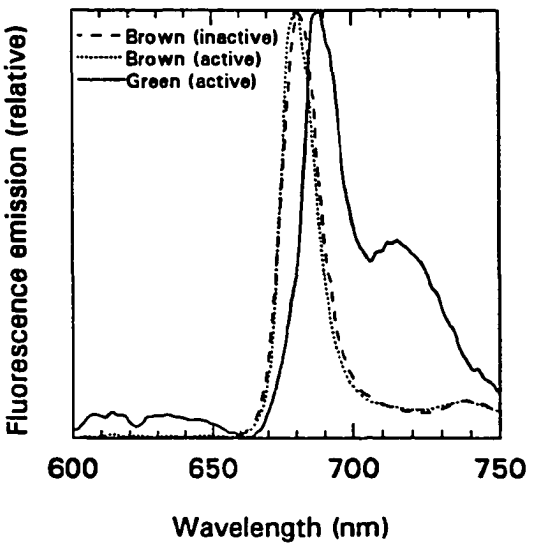
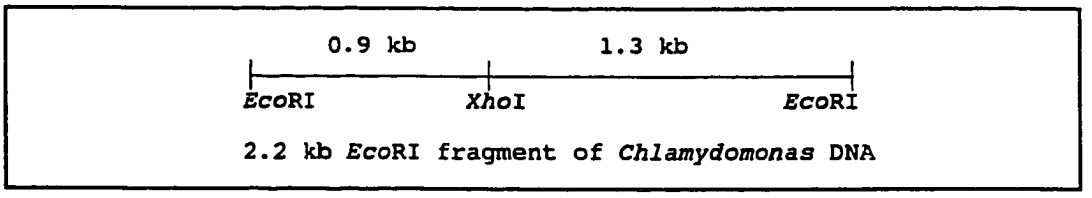


Figure 6.3. Fluorescence emission spectra (77K) of dark brown fractions from inactive and active thylakoids, and of the green fraction from active thylakoids. Excitation was at 440 nm, with a slit width of 4 nm for both emission and excitation. Spectra were not corrected for the decrease in detector sensitivity at long wavelengths.

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       CGAAGAAGCCGAACATGGAGAACATGGCCAGGCGGCCGTTCTTGATCTCCTTCACCTTCNNNGCTCA
       GCGAAGGTGTCG

0.9U   GAATTCGCCCCTCTCCCAGGGCCCCTACTCTGAGAACTCGACCCCCGCCTACCTGACTGGCGAGTTC
       CCCGGCGACTACGGCTGGGACACTGCTGGTCTGTCCGCTGACCCGGAGACCTTCAAGCGCTACCGCG
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JS0075   YGWDTAGLSADPETFKRYRELELIHARWAMLGALGCLTPELLAKSGTKFGEAVWFKAGAQIFSE
0.9U     YGWDTAGLSADPETFKRYRELELIHARWAMLGALGCLTPELLAKNGTKFGEAVWFKA
Shared     WD  GL AD E F R R +E+ H R  ML   G LT E
FcpA       WDPLGLVAD EKFDRLRYVEIKHGRICMLAVAGYLTQE

JS0075   GGLDYLGNPSLVHAQNIVATSAVQVILMGLIEGYRVNGGPAGEGLDPLYPGESFDPLGLADDPD

JS0075   TFAELKVKEIKNGRLASFSMFGFFVQAIVTGKGPVQNLDDHLANPTVNNAFAFATKFTPSA
0.9R          KVKEIKNGRLAMFSMFGFFVQAIVTGKGPVQNLDDHLANPTVNNAFAFATKFTPSA
Shared           EIK+GR++M ++ G+ VQ
FcpC/D           EIKHGRISMLAVVGYLVQ
```

Figure 6.4. Comparison of deduced amino acid sequence of *Chlamydomonas* LHCP gene with the deduced sequence of a diatom LHCP gene. Top: Gene map of KG11a, a cloned *Chlamydomonas* DNA fragment. Middle: Partial DNA sequence of a subcloned 0.9 kB fragment using the universal (U) and reverse (R) primers. Bottom: Deduced amino acid sequences of a *Chlamydomonas* LHCP (JS0075), the subcloned 0.9 kB fragment (0.9R and 0.9U), and diatom LHCP genes (FcpA/C/D). The regions of similarity between the *Chlamydomonas* and diatom proteins are indicated (Shared).