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**Spectroscopic Studies of *Synechococcus* sp PCC
7002 Phycobilisome Core Mutants**

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Abstract

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by

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The role of the L_{cm} , β^{18} , and α^{AP-B} chromoproteins in the phycobilisome core is investigated in this dissertation. Genetically engineered strains of *Synechococcus* sp PCC 7002 missing different polypeptides and/or chromophores were obtained for this purpose. Intact cells, isolated PBS, and subcore preparations for each mutant were studied to determine the effect of that mutation on energy transfer within the PBS core and from the PBS to the reaction centers. Three mutants lacked the β^{18} and/or α^{AP-B} polypeptides, while the L_{cm} chromophore was altered in the other two mutants. A lower energy absorbing chromophore, $A_{max} = 695$ nm, was substituted for the L_{cm} chromophore.

The deletion of the β^{18} and α^{AP-B} subunits had no discernible effect on energy transfer from the PBS to PSII within the cells. In the cells and the isolated PBS, the altered L_{cm} chromophore acts to both quench the PBS complex and to redirect the energy which would be transferred to PSII.

In the PBS and subcore preparations, the deletion of the α^{AP-B} subunit did not alter energy transfer within the core. The deletion

of the β^{18} subunit from the PBS caused a small decrease in the excited state lifetimes of the final emitters indicating more disorder within the core.

The L_{cm} chromophore was found to absorb at 670 nm and to emit at 683 nm within the intact PBS. The β^{18} chromophore emits at 679 nm while the α^{AP-B} chromophore emits at 682 nm. A strong interaction exists between the L_{cm} chromophore and the β^{18} subunit. Upon deletion of the β^{18} subunit from the PBS core, the L_{cm} chromophore emits at a higher energy. The β^{18} subunit could act to stabilize the L_{cm} chromophore-binding pocket, or exciton coupling could be occurring between the two chromophores.

The role of the α^{AP-B} chromophore is still unclear at this time. The α^{AP-B} chromophore does contribute to the RT emission of the isolated PBS, but it transfers energy to the L_{cm} at 77 K. From this result, one can conclude that the α^{AP-B} subunit is adjacent to the trimer containing the L_{cm} polypeptide.

Table of Contents

Table of Contents	iii
Abbreviations	v
Acknowledgments	vi
Chapter 1 Introduction	
A. Introduction to the phycobilisome	2
B. Rod Composition	4
C. Core Substructure	9
D. Arrangement of Trimers in the Core	11
E. The β^{18} Subunit	17
F. The α^{AP-B} Subunit	18
G. The L_{cm} Polypeptide	19
H. The Project	23
I. References for Chapter 1	25
Chapter 2 Materials and Methods	
A. The Cultures	29
B. Preparation of Intact Phycobilisomes	29
C. Preparation of the 18 S Core Particle	33
D. HPLC of 18 S Preparations	37
E. SDS-PAGE	39
F. Spectroscopy Sample Preparation	45
G. Steady State Spectroscopy	47
I. Time-Resolved Fluorescence Spectroscopy	48
J. Data Analysis of Time-Resolved Fluorescence Decays	51
K. References for Chapter 2	57
Chapter 3 Discussion of the Whole-Cells Results	
A. Absorption Spectroscopy	59
B. Steady State Emission	65

C. Time-Resolved Fluorescence	71
D. Conclusions	82
E. References for Chapter 3	86
Chapter 4 Discussion of PBS Results	
A. Absorption Spectroscopy	88
B. Steady State Fluorescence	94
C. Low Temperature Emission	101
D. Time-Resolved Fluorescence	105
E. Conclusions	116
F. References for Chapter 4	122
Chapter 5 Discussion of 18 S Preparation Results	
A. Absorption Spectroscopy	125
B. Room Temperature Emission	133
C. Low Temperature Emission	138
D. Time-Resolved Fluorescence	142
E. Discussion	151
F. Conclusions	158
G. References for Chapter 5	162
Chapter 6 Conclusions and Future Work	
A. The α^{AP-B} Subunit	163
B. The β^{18} Subunit	165
C. The L_{cm} Chromophore	166
D. Energy Transfer within the Core	168
E. Future Directions	168
F. References for Chapter 6	172

Abbreviations

AP	allophycocyanin
apcD	<i>Synechococcus</i> sp PCC 7002 mutant lacking the α^{AP-B} subunit
apcDF	<i>Synechococcus</i> sp PCC 7002 mutant lacking the α^{AP-B} and β^{18} subunits
apcDE/C186S	<i>Synechococcus</i> sp PCC 7002 mutant lacking the α^{AP-B} subunit along with the substitution of serine for cysteine at position 186 on the L_{cm} polypeptide
apcE/C186S	<i>Synechococcus</i> sp PCC 7002 mutant containing the substitution of serine for cysteine at position 186 on the L_{cm} polypeptide
chl <i>a</i>	chlorophyll <i>a</i>
DAS	decay-associated spectra
DCM	4-dicyanomethylene-2-methylene-6-(<i>p</i> -dimethylamino styrene)-4H-pyrane
HTTC	1,1',3,3',3'-hexamethylindotricarbocyanine
PAGE	polyacrylamide gel electrophoresis
PC	phycocyanin
PE	phycoerythrin
PMSF	phenylmethylsulfonyl fluoride
PSI	photosystem I
PSII	photosystem II
SDS	sodium dodecyl sulfate

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Chapter 1. Introduction to the Phycobilisome

“...the sun is not only the author of visibility in all visible things, but of generation and nourishment and growth...”

Plato, The Republic

Humankind has always had a deep fascination and respect for the process of photosynthesis, and during the last century we are finally solving the mystery of what drives all living things on earth. Many of the processes involved in photosynthesis are starting to be understood in great detail. For example, in the last 10 years crystals of the purple bacterial reaction center and Photosystem I have been grown and structures determined (Deisenhofer, Epp et al., 1984; Witt, Krauss et al., 1992). With the crystal structures known, the research on the reaction centers has accelerated to an overwhelming rate.

As reaction centers become better understood we need to study other aspects of photosynthesis so that in the future we may be able to develop efficient artificial photosynthesis. One major problem that needs to be solved for efficient artificial photosynthesis is how to collect photons and transfer their energy with almost 100% efficiency to the reaction centers. Plants, algae, and photosynthetic bacteria have solved this problem using a variety of methods, none of which is fully understood at this time (Glazer, 1983).

I would like to address the questions of how light energy is able to reach the reaction centers from outlying light-harvesting antennas with greater than 90% efficiency, and the importance of the chromophore placement in the process of the energy transfer. The light harvesting antenna under scrutiny is that found in cyanobacteria and red algae: the phycobilisome.

First, I will discuss the function and the structure of the phycobilisome as it is found in the cyanobacterium, *Synechococcus* sp PCC 7002 (also known as *Agmenellum quadruplicatum*), and I will describe the mutants under study. I will then follow with a methods and materials chapter, three chapters of results, and finally, a discussion of my results and future experiments.

Introduction to the Phycobilisome

The phycobilisome (PBS) is a light-harvesting antenna complex found in cyanobacteria and red algae (Bryant, 1991; Glazer, 1989; Glazer, 1985; Glazer, 1984; Gantt, 1981; Gantt, 1980). Calculations indicate that it transfers light energy with greater than 95% efficiency to reaction centers where the actual charge separation of photosynthesis takes place (Porter, Tredwell et al., 1978). The cyanobacterium gains a tremendous competitive advantage having this antenna because PBS absorb light in the part of the visible spectrum where chlorophyll *a* (chl *a*) and carotenoid absorption is at a minimum. Chl *a* absorbs photons below 450 nm and above 650 nm, while carotenoids usually absorb from 400 to 500 nm. Thus the phycobilisome antenna is able to collect photon energy

from 500 to 650 nm that would normally escape the reaction centers.

The PBS appears to serve other functions also: for example, it can be a source of nitrogen and carbon when the cells are starving (Bryant, 1991; Stevens, Balkwill et al., 1981). I will focus solely on the light harvesting aspects of the PBS, but it is important to be aware of the nutritional role of the PBS in the cyanobacterium.

The PBS transfers most of the energy it collects to Photosystem II (Kura-Hotta, Satoh et al., 1986; Manodori and Melis, 1985; Clement-Metral and Gantt, 1983; Katoh and Gantt, 1979). Recently, independent experiments carried out by Mullineaux and Bryant indicate that energy is transferred to PSI from PBS depending upon the state (State I/State II) of the photosystems (Mullineaux, 1992; Bryant, personal communication). There appears to be a mechanism for energy transfer to PSI; when PSII is getting too many photons some of the energy is 'spilled over' to PSI with some indications of the PSII reaction center becoming uncoupled from the PBS at that point (Mullineaux and Holzwarth, 1990). More work needs to be done to elucidate the actual mechanism, but it seems reasonable that such a mechanism could occur based on the LHCII precedent, although it seems unlikely that the actual PBS antenna would move (Bruce, Brimble et al., 1989).

PBS are found in regular arrays on the stromal or cytoplasmic surface of the thylakoid membrane as shown by electron micrographs (Gantt, 1980). In cyanobacteria, the thylakoid membrane does not contain any grana or stacked regions as seen in higher plants, and the PBS are able to fill the entire membrane surface to a

density of approximately 450 per μm^2 (Dilworth and Gantt, 1981). PBS can be removed from the photosynthetic membrane by rupturing the cell walls and incubating the cell fragments with detergent (Yamanaka, Glazer et al., 1978). The intact, water-soluble PBS can then be isolated from chl *a* and other chromoproteins using a sucrose density gradient.

The structure of the PBS is probably the best known of any light harvesting antenna. It is composed of two macrostructures, (rods and core) as shown in Figure 1-1, based upon electron micrographs of PBS (Glazer, Williams et al., 1979; Yamanaka, Glazer et al., 1978).

The rods are approximately 11.5 nm in diameter and of different lengths, while the core is composed of cylinders approximately 12 nm long and 11 nm in diameter as shown by the electron micrographs. The number of cylinders contained within the core differs with species from two to four. *Synechococcus* sp 7002 has a three cylinder core, the most common PBS type.

Rod Composition

The rods are composed of aggregates of chromoprotein, called phycobiliprotein, held together with colorless linker polypeptides (Tandeau de Marsac and Cohen-Bazire, 1977). For *Synechococcus* sp 7002, the only chromoprotein found in the rods is phycocyanin with an absorption maximum (λ_{abs}) at approximately 620 nm and a fluorescence maximum (λ_{F}) around 650 nm. Other chromoproteins found in rods of PBS from other species include phycoerythrin

Figure 1-1. Schematic of a *Synechococcus* sp PCC 7002 phycobilisome

The PBS is composed of two substructures, rods and core. Rods are made up of hexamers of PC. The different shading in the rods correspond to different linker polypeptides present with the PC hexamer. The rods are 11.5 nm in diameter. The core is made up of three cylinders with each cylinder containing four trimers of AP. The core cylinders are 12 nm long and 11 nm in diameter. The schematic is based upon electron micrographs of PBS (Glazer, Williams et al., 1979, Yamanaka, Glazer et al., 1978). See Figure 1-3 for more information on the composition of both the rods and the core.

($\lambda_{\text{abs}} = 500$ to 565 nm) and phycoerythrocyanin ($\lambda_{\text{abs}} = 568$ to 600 nm).

The phycobiliproteins found in the rods are arranged in hexamers, with the placement and spectral properties of each hexamer strongly influenced by the linker polypeptide associated with it (Yu and Glazer, 1982; Lundell, Williams et al., 1981; de Lorimier, Guglielmi et al., 1990). The colorless linker polypeptides appear to finetune the spectral properties of the chromophores such that the PC hexamers on the rod periphery are bluer-absorbing than the PC hexamers in the middle of the rods which are bluer-absorbing than the PC hexamers next to the cores (Glauser, Sidler et al., 1993). Using the small differences in the absorption spectra of the various components in the rods, the PBS is optimized for unidirectional energy transfer, with photon energy being transferred from the higher energy pigments to the lower energy pigments and finally, to the reaction centers.

The crystal structure for PC hexamers from *Synechococcus* 7002 has been determined to $3\text{\AA}$ resolution (Schirmer, Bode et al., 1987) showing that the hexamers are made up of two trimers added face-to-face, with each trimer made up of three heterodimers, also called monomers. Each heterodimer of PC is composed of an α and a β subunit, with the α subunit carrying one chromophore and the β subunit two chromophores. Free phycobiliproteins in solution commonly form aggregates with two to six heterodimers, depending upon the species of origin.

The structure of the chromophore found in PC, a tetrapyrrole called phycocyanobilin, is shown in Figure 1-2. The chromophore is held in similar geometries in all of the binding pockets of the PC subunits with the exception of the chromophore held at position 155 on the β subunit. Ring D of the chromophore on the β subunit is twisted compared to the other two chromophores (Schirmer, Bode et al., 1987), and the propionic sidechains show large variations between the chromophores. The crystal structure of PC hexamers shows that the tetrapyrrole is held in an extended conformation with ring A covalently bound via a thioether linkage to a cysteine of the protein backbone and the pyrrole nitrogens of rings B and C hydrogen bonded to an aspartate carboxyl. The chromophore displays different spectral properties depending upon the conformation it assumes. For example, without the protein scaffolding to hold the chromophore in a linear configuration, the chromophore apparently assumes a helical, lockwasher conformation (Braslavsky, Holzwarth et al., 1983); in this conformation the visible absorption and the fluorescence yield of the chromophore is greatly reduced. (Scheer, 1986). Braslavsky, Holzwarth, and Schaffner were able to show that biliverdin dimethyl esters (molecules similar to the free chromophore) free in solution could be deactivated radiationlessly by ultrafast intramolecular proton transfer between the nitrogens on the B and C rings in addition to C-C rotational modes. They also found that the biliverdin dimethyl ester fluorescence decay measured using single-photon-counting could be fit to four decay components with the majority of the yield coming from a 20 ps decay component.

Figure 1-2. Structure of phycocyanin chromophore
Based on crystallographic structure from Schirmer, Bode, et al.,
1987.

The chromophore in the denatured protein is acts in a similar manner (Scheer, 1986). This is well illustrated with the denaturation of any of the phycobiliproteins with urea at low pH; the chromoprotein becomes very dull in color with the amount of emission significantly decreasing (Guard-Friar and MacColl, 1984). The decrease in emission is due to increased competition from radiationless deactivation as discussed above, and a concomitant decrease in lifetime would also be expected.

Core Substructure

As with the rod substructure, a hierarchy of structure exists within the cylinders of the core. Each cylinder is composed of four trimers of chromoprotein with each trimer containing three heterodimers, and each heterodimer is composed of an α and a β subunit. Colorless linker polypeptides once again are involved in the ordering of the substructure as illustrated in the model shown in Figure 1-3. The small linker found in the core has also been shown to influence the spectral properties of the chromoproteins (Füglister, Mimuro et al., 1987).

The trimers of chromoproteins found in the core are mostly allophycocyanin (AP) with some unusual phycobiliproteins in lesser quantities. AP is composed of an $\alpha\beta$ heterodimer with each subunit containing only one chromophore. As with PC, the only chromophore found in the core is that shown in Figure 1-3; the protein environment apparently has a great influence on the spectral properties of the chromophore since AP trimers absorb maximally at 650 nm and fluoresce at 660 nm, significantly red-shifted from

A $(\alpha^{AP} \beta^{AP})_3 L_c^8$

B $(\alpha^{AP} \beta^{AP})_2 \beta^{18} L_{cm}$

C $(\alpha^{AP} \beta^{AP})_3$

D $(\alpha^{AP} \beta^{AP})_2 (\alpha^{AP-B} \beta^{AP}) L_c^8$

Figure 1-3. Composition of phycobilisome

The molecular weight of the protein is given as a superscript with the location of the linker polypeptide given as a subscript. r: rod, rc: rod-core, c: core, cm: core-membrane, PC: phycocyanin, AP: allophycocyanin, L: linker polypeptide. The core cylinders next to the membrane are labeled I and II while the apical cylinder is labeled III. The model is from Bryant, 1988.

that of PC hexamers ($\lambda_{\text{abs}} = 620$, $\lambda_{\text{F}} = 650$ nm). At this point, one can begin to see how the PBS has been optimized for efficient energy transfer; the absorption maxima of the AP trimers overlap the fluorescence maxima of the PC hexamers found in the rods. By making small changes in the absorption spectra of the phycobiliproteins found in the antenna, the cyanobacterium ensures that the energy gets funnelled to its reaction centers quickly with little leakage.

The crystal structure of AP has yet to be determined, but due to significant amounts of sequence homology between PC and AP, it is expected to be similar to that of PC.

Arrangement of Trimers in the Core

As noted before, the core is not composed solely of AP trimers; it also contains some trimers with unusual phycobiliproteins similar in amino acid composition to AP subunits but with altered spectral properties. The unusual chromoproteins including $\alpha^{\text{AP-B}}$, L_{cm} , and possibly the β^{18} subunit are thought to mediate energy transfer between the PBS and chl *a* of reaction centers. I will refer to the final energy acceptors in the PBS antenna as terminal emitters. A schematic of the PBS core is given in Figure 1-3, but the arrangement of the trimers relative to each other is still under discussion. To facilitate the discussion of the core organization, I will refer to the large, chromophore-containing protein found in the core as L_{cm} and the β^{AP} -like subunit found with it as the β^{18}

subunit. Of course, the molecular weight of these polypeptides differs somewhat among species.

Extensive work was carried out in A. Glazer's laboratory at the University of California to determine the content and arrangement of core polypeptides using PBS from *Synechococcus* 6301 AN112, a mutant cyanobacterium containing a stripped-down version of PBS with cores consisting of only two cylinders and rods of only one PC hexamer in length (Lundell, Williams et al., 1981; Lundell, Yamanaka et al., 1981; Yamanaka, Lundell et al., 1982; Lundell and Glazer, 1983a; Lundell and Glazer, 1983b; Lundell and Glazer, 1983c). In this very complete study, they dissociated the core cylinders into individual trimers and then separated the trimers using column chromatography and ultracentrifugation. They then carried out SDS-PAGE and isoelectric focusing to identify and quantify the proteins present in the trimers.

In a similar study, using a three cylinder core PBS from *Synechocystis* 6701, Glazer's laboratory was able to show that most of the work on the two cylinder core PBS from *Synechococcus* 6301 seems to correspond well to three cylinder core species of PBS (Gingrich, Lundell et al., 1983). The third (apical) cylinder appears to be composed of two $(\alpha^{AP}\beta^{AP})_3$ trimers and two $(\alpha^{AP}\beta^{AP})_3L^{10}$ trimers. Table 1-1 gives the calculated amounts of each polypeptide found in the PBS for *Synechococcus* sp 7002 based on this work.

A summary of the published spectral properties of each trimer found in the core is given in Table 1-2. The trimers containing L_{cm} , α^{AP-B} , and β^{18} have emission maxima at 678 nm while a simple AP

Table 1-1

Composition of *Synechococcus* sp. PCC 7002 Phycobilisome*

Polypeptide	Copies/PBS
α^{PC}	72
β^{PC}	72
L_r^{33}	6
$\text{L}_r^{8.5}$	6
L_r^{29}	6
α^{AP}	32
β^{AP}	34
$\alpha^{\text{AP-B}}$	2
β^{18}	2
L_c^8	6
$\text{L}_{\text{cm}}^{97}$	2

* From D.A. Bryant, 1988

Table 1-2

Emission Maxima of Isolated Phycobilisome Core Trimers*

Complex	Copies/PBS	F _{max}
<u>In Syn. 6301</u>		
$(\alpha\text{AP}\beta\text{AP})_3$	2	660 nm
$(\alpha\text{AP}\beta\text{AP})_3\text{L}_c^{10}$	2	662 nm
$(\alpha\text{AP}\beta\text{AP})_2(\alpha\text{AP-B}\beta\text{AP})\text{L}_c^{10}$	2	678 nm
$(\alpha\text{AP}\beta\text{AP})_2\beta^{18}\text{L}_{cm}^{75}$	2	678 nm
<u>In Syn. 7002</u>		
$(\alpha\text{AP}\beta\text{AP})_3$	4	660 nm
$(\alpha\text{AP}\beta\text{AP})_3\text{L}_c^8$	4	662 nm
$(\alpha\text{AP}\beta\text{AP})_2(\alpha\text{AP-B}\beta\text{AP})\text{L}_c^8$	2	678 nm
$(\alpha\text{AP}\beta\text{AP})_2\beta^{18}\text{L}_{cm}^{97}$	2	678 nm

* From J.C. Gingrich, D.J. Lundell, and A.N. Glazer, 1983

trimer emits at 660 nm. The β^{18} and L_{cm} polypeptides are found in the same trimer, so it is difficult to separate the contributions of either chromoprotein to the absorption and emission spectrum of the trimer.

The arrangement of the proteins in the core has been studied by several groups. Glazer's laboratory was successful in developing a relatively stable preparation, the 18 S preparation, containing only part of the core (Yamanaka, Lundell et al., 1982; Lundell and Glazer, 1983b). In the 18 S preparation they were able to say that the $(\alpha^{AP}\beta^{AP})_3$ and the $(\alpha^{AP}\beta^{AP})_2 \beta^{18}L_{cm}$ trimers were adjacent in the core. Work carried out by other laboratories indicates that trimers containing the small linker polypeptide should be located on the ends of the core cylinders. This includes dissociation studies by Reuter and Wehrmeyer (Reuter and Wehrmeyer, 1988) and Anderson and Eiserling (Anderson and Eiserling, 1986). In addition, de Lorimier and Bryant at Pennsylvania State University were able to show that the 8 kD polypeptide found in core trimers A and D shown in Figure 1-3 is structurally related to a 8.5 kD protein found capping the rods (Bryant, 1988; de Lorimier, Bryant et al., 1990). Using this information, the only allowed arrangement for the apical core cylinder is shown in Figure 1-3 with the two $(\alpha^{AP}\beta^{AP})_3$ trimers in the interior on the core and the $(\alpha^{AP}\beta^{AP})_3L_c^8$ on the outside.

The cylinders found adjacent to the membrane could be arranged in two different ways. First, the trimers containing the small capping linker (trimers A and D in Figure 1-3) need to be on

the outside. This would make the trimers containing $(\alpha^{AP}\beta^{AP})_3$ (trimer C) and $(\alpha^{AP}\beta^{AP})_2 \beta^{18}L_{cm}$ (trimer B) adjacent as required, but is trimer A adjacent to trimer B or trimer C? The two arrangements are shown in Figure 1-3.

One other thing to consider is the arrangement of the bottom cylinders relative to each other. Are they arranged in a parallel or anti-parallel fashion? The anti-parallel arrangement is preferred, based on the arrangement of the L_{cm} polypeptide, and its efficiency in binding the core together. It has been proposed that the L_{cm} polypeptide acts in binding the core trimers together (Bryant, 1988; Capuano, Braux et al., 1991). If the basal cylinders are arranged in an anti-parallel fashion, one L_{cm} protein will be present in each half of the core, and the L_{cm} should be able to bind that half of the core trimers together in an efficient manner. In the parallel arrangement both L_{cm} polypeptides would be present in one-half of the core. This makes the binding of the core trimers in the other half of the core more difficult to envision. The L_{cm} polypeptide would need to have binding domains the entire length of the core making the parallel arrangement more inefficient. The schematic in Figure 1-3 gives the preferred anti-parallel arrangement.

The functions of the unusual polypeptides (α^{AP-B} , β^{18} , and L_{cm}) found in the core are somewhat ill-defined at this time. I will describe their isolation along with their physical and spectral properties in the following paragraphs.

The β^{18} subunit

The β^{18} was the last core polypeptide to be identified; it was isolated from *Synechococcus* 6301 AN112 using two dimensional mapping (SDS-polyacrylamide electrophoresis followed by isoelectric focusing) (Yamanaka, Lundell et al., 1982). It had never been identified before because the molecular weight of the polypeptide is very similar to that of α^{AP} (18.3 kD versus 18.2 kD), and it ran with the α^{AP} band on SDS-PAGE. The β^{18} polypeptide from *Synechococcus* 6301 was found to carry one phycocyanobilin chromophore, and it was judged to be present in roughly the same concentration as α^{AP-B} .

The β^{18} subunit was shown to be in the same core trimer as the large linker polypeptide in the 18 S core preparation (Lundell and Glazer, 1983b). Using ^{14}C -labeled PBS from *Synechococcus* 6301, Lundell and Glazer found that the trimer contained the ratio of β^{18} : large linker polypeptide: β^{AP} : α^{AP} of 1: 1: 2: 2 giving a core trimer of $(\beta^{AP}\alpha^{AP})_2\beta^{18}$ L⁷⁵. They were successful in isolating the β^{18} subunit from the other polypeptides using preparative isoelectric focusing. The absorption spectrum of the purified subunit closely resembles that of isolated AP subunits with an λ_{abs} of 620 nm. Lundell and Glazer attempted to form AP trimers containing the subunit, but the β^{18} subunit appeared to inhibit the formation of trimers. Based on this evidence they postulated that the β^{18} subunit somehow mediates the interaction between the large linker polypeptide and the AP subunits in the trimer.

The β^{18} subunit has also been isolated from a few other species including *Mastigocladus laminosus* (Füglister, Rümblet et al., 1984; Rümblet, Wirth et al., 1987) and *Synechococcus* sp 7002 (Bryant, de Lorimier et al., 1990). Füglister sequenced the *M. laminosus* subunit and reported that the sequence homology of β^{18} resembles that of the β^{AP} subunit for amino acids 1 - 138 (62% homology), but the final amino acids from 139 - 171 show very little homology with β^{AP} . Bryant reported that the absorption spectrum of $(\alpha^{AP}\beta^{18})_{1-3}$ is slightly red-shifted from that of the AP trimer. It is difficult to predict how the β^{18} spectral properties will be affected upon its incorporation into a trimer with normal AP and L_{cm} .

The α^{AP-B} subunit

The α^{AP-B} subunit was first isolated using column chromatography in 1975 by Glazer and Bryant (Glazer and Bryant, 1975; Ley, Butler et al., 1977). It was immediately identified as a likely terminal emitter in the PBS core, because its spectral properties were red-shifted from those of AP. The polypeptide was originally isolated in a $(\alpha^{AP-B}\beta^{AP})$ trimer, but further work has shown that the substitution of only one α^{AP} subunit with α^{AP-B} is required to give the trimer red-shifted spectral properties (Lundell and Glazer, 1981). AP trimers containing one α^{AP-B} subunit fluoresce at 678 nm, versus 660 nm for a simple AP trimer, with approximately 85% of the emission coming from the α^{AP-B} subunit. Only two copies per

PBS of the subunit were found in *Synechococcus* 6301 AN112 (Lundell and Glazer, 1983c).

The α^{AP-B} subunit was found to be somewhat smaller than α^{AP} (17,800 d versus 18,200 d in *Syn* 6301) with a more basic isoelectric point. Isolated α^{AP-B} also has altered absorption and fluorescence spectra compared to α^{AP} with λ_{abs} of 645 - 650 nm and λ_F of 672 nm, while the α^{AP} subunit maxima are at 617 nm and 642 nm respectively. (Lundell and Glazer, 1981; Lundell and Glazer, 1983c; Yamanaka, Lundell et al., 1982). The quantum yield of fluorescence of the isolated α^{AP-B} subunit was also found to be much smaller than that of the isolated α^{AP} subunit, 0.05 versus 0.2, even though the same chromophore is thought to be in both proteins (Lundell and Glazer, 1981). The α^{AP-B} subunit also has been isolated from other organisms including *Mastigocladus laminosus* (Suter, Füglistaller et al., 1987) and *Rhodella violacea* (Reuter, Nickel et al., 1990). There appears to be a great deal of similarity in the amino acid sequence and spectral characteristics of the subunits from the various organisms.

The L_{cm} Polypeptide

The L_{cm} protein is probably the most interesting polypeptide found in the PBS core, but it also has proved to be the most difficult to study since it is very vulnerable to proteolysis (Yamanaka, Lundell et al., 1982; Gingrich, Lundell et al., 1983). The protein was first isolated with chromophore intact in 1981 by Redlinger and Gantt (Redlinger and Gantt, 1981). It was previously believed

to be colorless since the techniques used in the isolation somehow bleached the chromophore. This protein is also believed to contain the same phycocyanobilin chromophore found in AP and PC (Lundell, Yamanaka et al., 1981).

The L_{cm} polypeptide was successfully isolated from other proteins in the core using a denaturing technique, SDS-PAGE. The protein can be renatured to give an $\lambda_{abs} = 665$ nm and $\lambda_F = 676$ nm (Lundell, Yamanaka et al., 1981). The integrity of the renatured protein was questioned, because the quantum yield was found to be 0.16, lower than that of most phycobiliproteins. Work using core fractionation methods has shown that an isolated trimer of $(\alpha^{AP}\beta^{AP})_2 \beta^{18}L_{cm}$ has a $\lambda_F = 678$ nm, but it is difficult to separate out the individual effects of L_{cm} and β^{18} within the trimer.

The role of the L_{cm} polypeptide must be more than simply that of terminal emitter in the core since it is about 100 kD in size. Originally, the protein was believed to anchor the PBS complex to the photosynthetic membrane; now it appears that the large polypeptide in the core participates in the binding of the rods to the core. I will try to bring together a lot of data on the role of the L_{cm} polypeptide in the next few paragraphs.

A very complete study was carried out on the L_{cm} protein found in *Anabaena variabilis* by Isono and Katoh (Isono and Katoh, 1987). Using a variety of methods they were able to show that very little of the large linker polypeptide is accessible to solvent in intact PBS. First, they tried binding fluorescence probes to intact PBS or intact cores. The probes bound minimally to the linker polypeptide in the intact systems but showed enhanced binding to the L_{cm} in

dissociated PBS. Second, they took advantage of the preferential digestion of L_{cm} with chymotrypsin. In intact PBS the large polypeptide was reduced in molecular weight as judged by SDS-Page from 115 kD to 105 kD, while for intact core it went from 115 kD to 84 kD. Isono and Katoh also digested a subcore preparation called a 14.5 S APC preparation in which L_{cm} was reduced in molecular weight from 115 kD to 42 kD over a thirty minute incubation with chymotrypsin. In none of the digestions did the "far-red fluorescence" spectral properties alter. Isono and Katoh also carried out antibody studies in which antibodies raised against L_{cm} did not react with intact PBS or core but did react with the 14.5 S subcore preparation. They concluded that most of the polypeptide is embedded inside the PBS core with parts becoming exposed upon the loss of rods. They proposed that the large linker polypeptide is involved in the binding of AP trimers within the core.

Based on their chymotrypsin digestion studies of the polypeptide, Isono and Katoh proposed that the polypeptide from *Anabaena variabilis* is composed of four domains of 10, 42, 42, and 21 kD respectively. The 10 kD domain which is removed from intact PBS possibly is the part of the polypeptide that binds the PBS to the photosynthetic membrane. The second domain (42 kD) is the part of the polypeptide that binds the 14.5 S subcore preparation together and contains the chromophore. The third domain (42 kD) is involved in binding the rest of the core while the fourth domain (21 kD) binds the rods to the core.

Better understanding of the role of L_{cm} came with the sequencing of the polypeptide. In recent years much work has been done in determining the amino acid sequences of the L_{cm} polypeptide in different organisms. The sequence has been determined for *Calothrix* sp PCC 7601 (Houmard, Capuano et al., 1990), *Synechococcus* 7002 (Bryant, 1991) and *Synechococcus* 6301 (Capuano, Braux et al., 1991). For all of the proteins studied the sequences can be separated into three types of domains: linker, phycobiliprotein, and lengths of amino acids with no homology connecting the linker and phycobiliprotein domains. For example, in *Calothrix* sp PCC 7601 the L_{cm} polypeptide contains one domain from amino acids 18-237 that is homologous to a phycobiliprotein subunit, especially in terms of the binding pocket. Four other sequence domains are very similar to rod linker proteins, while the segments connecting the different domains have no known homology and are of differing lengths. Similar conclusions were made with the sequences from *Synechococcus* 7002 and *Synechococcus* 6301, although the number of sequences homologous with rod linker polypeptides differs with the type of phycobilisome core found in that species. The L_{cm} can be described as being composed of a phycobiliprotein domain starting at the N terminal end followed by two, three, or four linker domains. The number of linker domains appears to correlate with the number of cylinders contained in the PBS core (Capuano, Braux et al., 1991).

Based on this information it appears that the large polypeptide found in the core plays a dual role in both binding the rod and core cylinders together and accepting energy from chromophores in the

core for transfer to chl *a* pigments. One could also speculate that the large linker polypeptide might also hold the PBS onto the thylakoid membrane, but no transmembrane sequences characteristic of membrane binding have yet been identified in the sequenced proteins.

The Project

To further elucidate the roles played by the unusual polypeptides in the core (α^{AP-B} , β^{18} , and L_{cm}) we studied some mutants that were produced in Bryant's laboratory at Pennsylvania State University (Zhou, 1991). Bryant has been successful in cloning, sequencing, and characterizing all 11 polypeptides found in *Synechococcus* 7002 PBS (Bryant, 1991). In Bryant's laboratory several different core mutations of *Synechococcus* 7002 were created using both interposon mutagenesis and/or point mutations.

The mutants I studied included the *apcF* mutant which is missing β^{18} , the *apcD* mutant missing α^{AP-B} , the double mutant of *apcDF*, the *apcE/C186S* mutant which is missing the chromophore-binding cysteine at position 186, and the double mutant *apcDE/C186S*. The *apcE/C186S* and *apcDE/C186S* mutants proved to be especially interesting because they contain a modified chromophore on L_{cm} instead of losing the chromophore entirely.

Earlier work was done on the *apcD* mutant by Maxson in this laboratory (Maxson, Sauer et al., 1989); I have reproduced some of that work along with expanding the study on the *apcD* mutant. I have a relatively complete set of data on all of the mutants including steady state absorption and emission and time-resolved

emission on whole cells, intact PBS, and isolated 18 S preparations; I am only missing the time-resolved emission data on the apcDE/C186S 18 S preparation.

The deletion mutants of apcD, apcF and apcDF were created by interrupting the gene of interest with an antibiotic resistance marker using recombinant DNA methods (Bryant, 1988). First, the gene had to be cloned, sequenced, and identified. Next, the DNA for that gene was expressed in *E. coli*, and the gene was interrupted or replaced using a sequence of DNA that encodes for antibiotic resistance using homologous recombination DNA techniques. (Golden, Brussian et al., 1987). The altered DNA was then added back into the cyanobacterium, which is transformable by exogenously added homologous DNA; *Synechococcus*. 7002 is a strain of cyanobacteria that can incorporate foreign DNA with homologous sequences into its own DNA. The cells with the altered DNA were selected for by antibiotic resistance. DNA from the selected cells was then sequenced to ensure that the antibiotic resistance marker had been incorporated into the DNA at the correct position.

In the following chapters I will discuss the spectral properties of the whole cells, intact PBS, and core preparations from the mutant and wild-type strains. From these data I will try to assign spectral maxima to the polypeptides, and identify their roles in the antenna complexes.

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Chapter 2. Materials and Methods

The Cultures

The wild-type and mutant strains of *Synechococcus* sp. PCC 7002 were obtained from D.A. Bryant's laboratory at Pennsylvania State University (Zhou, 1991). The cultures were grown using Medium A+ (see Table 2-1) with $200 \mu\text{E m}^{-2}\text{s}^{-1}$ of white fluorescent light at approximately 32°C . The cultures were aerated with an approximate 5% (v/v) CO_2/N_2 gas mixture with constant stirring. Doubling times for the various strains are given in Table 2-2 (Bryant, personal communication).

Continuous stock cultures used to provide starting stock were grown in 125 ml Erlenmeyer flasks under the same temperature and light conditions as above but without $\text{CO}_2(\text{g})$. The stock cultures were grown with the antibiotics listed in Table 2-1 to prevent reversion to wild-type while the larger cultures were generally grown without the antibiotics. The large cultures were harvested during the end of the log phase of growth which generally was 7 to 10 days for a 1.5 l culture.

Preparation of Intact Phycobilisomes

The preparation for intact PBS was taken from Maxson's thesis with slight modifications (Maxson, 1988) and is described in the following paragraphs. Cells near the end of the log phase of growth were concentrated by centrifugation in a Dupont GS-3 Rotor for 5 min at 8500 rpm. The cells were resuspended in growth

Table 2-1

Medium A+ Recipe

Chemical	Amount (g/l) ^a	Concentration of Stock (g/l) ^a	Volume(ml) of Stock per 1 liter of Medium
NaNO ₃	1.0	500	2.0
NH ₄ Cl	0.15	150	1
K ₂ HPO ₄ ·3H ₂ O	0.050	50	1
CaCl ₂ ·2H ₂ O	0.23	230	1
<u>Salt Solution</u>			100
MgSO ₄	2.44	24.4	
NaCl	18	180	
KCl	0.6	6	
Na ₂ EDTA ^b	0.030	30	1
1.0M Tris, pH 8	8.5 mM	1.0 M	8.5
FeCl ₃ ·6H ₂ O	4.0 mg/l	4.0	1
<u>Trace Metals</u>			1
H ₃ BO ₃	34.2 mg/l	34.2	
MnCl ₂ ·4H ₂ O	4.3 mg/l	4.3	
ZnSO ₄ ·7H ₂ O	0.63 mg/l	0.63	
Na ₂ MoO ₄ ·2H ₂ O	3.9 mg/l	3.9	
CuSO ₄ ·5H ₂ O	30 µg/l	0.03	
CoCl ₂ ·6H ₂ O	12.2 µg/l	0.0122	
Vitamin B ¹²	5 µg/l	50 mg/l	0.1
<u>Antibiotics</u>			
Kanamycin ^c	100 mg/l	80	1.25
Ampicillin ^d	5 mg/l	5	1
Erythromycin ^e	20 mg/l	10	2

^a amounts in g/l unless specified otherwise

^b Can substitute with 1 ml of 0.1M EDTA pH 8

^c Add Kanamycin to all mutant cultures

^d Add Ampicillin to apcE/C186S and apcDE/C186S cultures

^e Add Erythromycin to apcDF and apcDE/C186S cultures

Table 2-2

Doubling Times of Mutants

Strain	High Light Intensity ^a	Low Light Intensity ^b
Wild-Type	4.3 h	11.1 h
apcF	5.1 h	14.0 h
apcDF	5.4 h	16.7 h
apcD	4.2 h	11.4 h
apcE/C186S	20.4 h	57.6 h

a 250 $\mu\text{E m}^{-2} \text{s}^{-1}$

b 80 $\mu\text{E m}^{-2} \text{s}^{-1}$

medium and centrifuged at 12000 rpm for 5 min in a Dupont SS-34 Rotor to determine an accurate wet weight of cells. The cells were resuspended to 0.12 g ml⁻¹ with 0.75 M NaH₂PO₄ pH 7.8 (Buffer A) containing 1 mM NaN₃ and approximately 20 µM phenylmethylsulfonyl fluoride (PMSF, Sigma). The PMSF should be added just prior to using, because it degrades rapidly in water-based solvents.

The suspension was homogenized to remove large clumps of cells and then French pressed four times at 1.4 kbar (20,000 psi) at 4 °C. The ruptured cells were immediately incubated with 1.2% (w/w) Triton X-100 for 30 min at room temperature with vigorous stirring; the detergent concentration is critical to the quality of the preparation obtained. Unbroken cells and large cell fragments were removed by centrifuging for 30 min at 16,000 rpm in a Dupont SS-34 rotor at room temperature.

The suspension separated into three phases upon centrifugation; unbroken cells and large cell membranes pelleted to the bottom of the tube, chlorophyll and detergent collected at the top of the tube, and the phycobilisomes were found in the blue supernatant below the green chlorophyll detergent layer. The blue supernatant was carefully removed from the other components in the centrifuge tube and loaded onto a sucrose step gradient composed of 7.0 ml each of 1.0 M, 0.8 M, 0.6 M, 0.4 M, and 0.2 M sucrose in Buffer A in a Beckman SW-28 or SW-27 centrifuge tube. The tubes were spun for 16 h at 21,000 rpm and 15 °C with no brake in a Beckman ultracentrifuge.

A typical sucrose gradient is shown in Figure 2-1 with the intact phycobilisomes found in the 0.6 M sucrose layer. More free phycobiliprotein was found in the 0.4 M sucrose layer for the mutants than for the wild-type preparations. The intact PBS were removed from the gradient after removing all of the upper bands to minimize contamination. All spectroscopy was carried out within 16 h after the protein was removed from the gradients.

Preparation of the 18 S Core Particle

The following procedure for preparation of the 18 S core preparation is a combination of several different procedures taken from Glazer's and Bryant's laboratories (Yamanaka, Lundell et al., 1982; Gingrich, Lundell et al., 1983; Bryant, de Lorimier et al., 1990). Cells were harvested near the end of the log phase of growth and were concentrated using a Dupont GS-3 rotor for 5 min at 8500 rpm. The cells were resuspended using a minimum of growth medium and centrifuged a second time using a Dupont SS-34 rotor for 5 min at 12,000 rpm to obtain an accurate cell wet weight. The cells were resuspended to 0.12 g ml⁻¹ in 50 mM Tris buffer at pH 7.8 with 0.8 M Na₂SO₄, 1 mM NaN₃, and 20 μM PMSF (Buffer B).

The cells were homogenized and French pressed four times at 1.4 kbar (20,000 psi) and 4 °C. The suspension was incubated with 1.2% (w/w) Triton X-100 for 30 min at room temperature with vigorous stirring. The suspension was then centrifuged for 30 min at 16,000 rpm and 15 °C in a Dupont SS-34 rotor. After centrifugation the suspension separated into three different components: a solid pellet composed of unbroken cells and cell membranes, a

Figure 2-1. Sucrose Step Gradient from Phycobilisome Preparation.

Typical gradient profile in SW-27 tube. Intact PBS are located in the 0.6 M sucrose layer. Free phycobiliproteins are found in the 0.4 and 0.2 M sucrose.

green top layer made up of chlorophyll and detergent, and a blue middle layer containing all of the phycobiliproteins. The blue supernatant was carefully removed from the gradient with special care to minimize contamination from the chlorophyll layer and was loaded onto a sucrose step gradient.

The sucrose step gradient was made up of 3 ml of 2.0 M, 5 ml of 1.0 M, and 7 ml each of 0.75 M and 0.5 M sucrose in Buffer B. The gradients were centrifuged for 2 - 4 h in a Beckman 50.2Ti rotor at 45,000 rpm and 15 °C with no brake.

The gradient profile is shown in Figure 2-2a with the intact PBS found in the three dark blue bands found in the 0.75 and 1.0 M sucrose layers. Using steady-state fluorescence and absorption spectroscopy no significant differences were found in the composition of the PBS within the three bands, so all of the bands were combined for the following steps. Significant amounts of chlorophyll were found in the 1.0 M sucrose layer, but all the contaminating chlorophyll appeared to precipitate during the following steps.

After intact PBS were removed from the gradients they were exchanged into 50 mM Tricine buffer at pH 7.8 with 5 mM CaCl_2 , 10% (v/v) glycerol, 1 mM NaN_3 , and 20 μM PMSF (buffer C) in which the intact PBS partially dissociated. The PBS could be exchanged into the Tricine buffer using either dialysis which takes 18 h or Bio-Rad 10-DG desalting columns (Richmond, CA) which takes about 2 h. Next, the dissociated proteins were concentrated using an Amicon PM-30 Ultrafiltration membrane (Amicon

Figure 2-2a. Gradient containing Intact Phycobilisomes from 18 S Preparation.

Step gradient profile of 18 S preparation in SW-27 tube. Intact PBS are found in the 0.75 and 1.0 M sucrose steps. Free phycobiliprotein is found in the 0.5 M sucrose step.

Company, Danvers, MA) at 3.7 bar (55 psi) of pressure until the absorption is 10 - 15 cm⁻¹ at 630 nm.

Approximately 3 ml aliquots of the concentrated protein were loaded onto linear sucrose gradients composed of 0.9 M to 0.3 M sucrose in Buffer C. The gradients were centrifuged for 11 - 12 h at 41,000 rpm in a Beckman 50.2Ti rotor at 15 °C with no brake. The gradient profile is shown in Figure 2-2b with the 18 S core preparation located in the narrow, lower band. The upper broad band is composed mostly of PC hexamers with $\lambda_{\text{abs}} = 628$ nm and $\lambda_{\text{F}} = 650$ nm. The 18 S core preparation was removed from the gradient using a syringe with a small gauge needle with extra care taken to avoid contamination with the upper broad band.

HPLC of 18 S preparations

To determine the composition of the 18 S preparations HPLC was carried out on wild-type and *apcF* samples using the protocol outlined in Swanson's paper (Swanson and Glazer, 1990). First, protein samples were prepared for HPLC by dialyzing the protein overnight against 5 mM phosphate buffer, pH 7.0 containing 1 mM 2-mercapto-ethanol. The protein was then concentrated as much as possible using an Amicon PM-30 ultrafiltration membrane with 3.7 bar (55 psi) of pressure, 4 °C. Just prior to injection, the protein was diluted 1:1 with 9.0 M urea pH 2.0 and spun for 5 min in a microcentrifuge to remove any precipitates.

The HPLC chromatography was carried out using the Waters equipment (Waters Chromatography Division, Milford, MA) in A. Glazer's laboratory as described in Swanson's paper. The protein,

Figure 2-2b. Linear Sucrose Gradient of an 18 S Preparation.

Typical profile of the linear gradient shown in a 50.2Ti tube.
The 18 S preparation is located in the narrow, lower band.

approximately 350 μ l in volume, is injected in a C₄ analytical column (Hipore RP-304, Bio-Rad, Richmond, CA) previously equilibrated with 65% water/ 35% 2:1 acetonitrile: isopropanol everything with 0.1% trifluoroacetic acid (TFA). The proteins were eluted from the column using the following protocol: 0 - 2 min, 65% water/ 35% 2:1 acetonitrile: isopropanol; 2 - 37 min, linear gradient to 30% water/ 70% 2:1 acetonitrile:isopropanol; 37 - 42 min, linear gradient to 2:1 acetonitrile: isopropanol; 42 - 47 min, linear gradient to 65% water/ 35% 2:1 acetonitrile: isopropanol. The flow rate of the column was 1.5 ml min⁻¹, and the absorption of the protein coming off the column was monitored every 3 s using a Waters 991 photodiode array with 2 nm resolution.

The polypeptides came off the analytical column in a well-resolved fashion with the elution data from the wild-type preparation shown in Figure 2-3. The wild-type preparation contained five chromoproteins and 2 colorless polypeptides. The order of protein elution was L_{cm} at 28 min, β^{AP} at 28.5 min, α^{PC} along with a small amount of colorless polypeptide of greater than 30 kD at 29 min, β^{PC} at 30.5 min, α^{AP} at 33 min, and β^{18} at 36 min. The second colorless polypeptide of approximately 30 kD eluted at about 31.5 min.

SDS-PAGE

One dimensional polyacrylamide gel electrophoresis was carried out on both intact PBS, 18 S core preparations, and HPLC fractions. The method was based on that of Laemmli (Laemmli, 1970) and

Figure 2-3. HPLC elution profile of the wild-type 18 S preparation Lcm eluted at 28.5, β AP at 29, β PC and L33 at 30.5, α AP at 33, L29 at 31.5, and β 18 at 36 min. The absorption of the eluted proteins was monitored at 279, 350, and 654 nm.

was carried out as outlined in Maxson's thesis. The equipment used was from Hoeffer Scientific Instruments (San Francisco, CA). The stock solutions used are given in Table 2-3 with the recipe used for a 15% running gel with a 5% stacking gel given in Table 2-4.

The gels were cast using two 16 x 18 mm glass plates separated by spacers of the appropriate width and held upright in a casting stand. The glass plates were carefully cleaned using water and ethanol to eliminate any fingerprints, and everything was handled using latex gloves.

The solutions for the running and stacking gels were made up just prior to pouring the gel, with the initiators added last. When pouring both gels special care was taken to eliminate any air bubbles. Special care needs to be taken with the acrylamide since it is a neurotoxin.

The running gel was allowed to polymerize for at least 90 min before adding the stacking gel. In the interim, a small amount of water was carefully layered on the top of the running gel to ensure an even, bubble-free surface at the interface. The water was removed before pouring the stacking gel. The stacking gel was poured, and a mold for the sample wells was carefully inserted in the gel to create the sample lanes. Once the stacking gel polymerized (after 30 min), the mold was carefully removed and the wells were rinsed with tank buffer.

Protein samples (containing approximately 40 μ g of protein) were prepared by mixing equal volumes of protein solution and 20%(w/v) trichloroacetic acid. The mixture was kept at 4 °C for a minimum of one hour and then centrifuged to recover the precip-

Table 2-3

Solutions Needed For SDS-PAGE

<u>Monomer Solution</u>		
(Stored at 4°C in Dark)	Acrylamide	58.4 g
	Bis-Acrylamide	1.6 g
	H ₂ O	to 200 ml
<u>Running Gel Buffer</u>		
(adjusted to pH 8.8 with HCl)	Tris	36.3 g
	H ₂ O	to 200 ml
<u>Stacking Gel Buffer</u>		
(adjusted to pH 6.8 with HCL)	Tris	3.0 g
	H ₂ O	to 50 ml
<u>10% SDS Solution</u>		
	SDS	50 g
	H ₂ O	to 500 ml
<u>Initiator</u>		
(Made Fresh with each gel)	Ammonium persulfate	0.1 g
	H ₂ O	to 1.0 ml
<u>2X Treatment Buffer</u>		
(Stored at -20°C)	Tris	2.5 ml of Stacking Gel Buffer
	SDS	4.0 ml of 10% SDS Solution
	Glycerol	2.0 ml
	2-mercaptoethanol	1.0 ml
	1% Bromophenol Blue Solution	20 µl
	H ₂ O	to 10 ml

Table 2-3 (cont)

<u>Tank Buffer</u>		
(Made Fresh with each gel)		
Tris	12.0 g	
Glycine	57.6 g	
SDS	40 ml of 10% SDS Solution	
H ₂ O	to 4.0 l	
<u>Stain Stock</u>		
Coomassie Blue R-250	2.0 g	
H ₂ O	to 200 ml	
<u>Stain</u>		
Coomassie Blue R-250	62.5 ml of Stain Stock	
Methanol	250 ml	
Acetic Acid	50 ml	
H ₂ O	to 500 ml	
<u>Destaining Solution I</u>		
Methanol	500 ml	
Acetic Acid	100 ml	
H ₂ O	to 1.0 l	
<u>Destaining Solution II</u>		
Acetic Acid	140 ml	
Methanol	100 ml	
H ₂ O	to 2.0 l	
<u>Molecular Weight Standards</u>		
(Heated for 5 min at 100°C, stored at -20°C)		
Bio-Rad Standard Protein Mixture	10 µl	
Buffer	100 µl of 2X Treatment Buffer	
H ₂ O	90 µl	

Table 2-4

Recipe for 15% Running Gel/ 5% Stacking Gel

Stock Solution ^a	15% Running Gel	5% Stacking Gel
Monomer Solution	7.5 ml	1.0 ml
Running Gel Buffer	4.0 ml	
Stacking Gel Buffer		1.5 ml
H ₂ O	3.3 ml	3.4 ml
10% SDS Solution	0.15 ml	0.06 ml
Initiator	75 μ l	60 μ l
TEMED	5 μ l	3 μ l

^a Composition of stock solutions given in Table 2-3

itate. The precipitate was resuspended in 20 μ l of 2X treatment buffer (recipe given in Table 2-3) and loaded into the sample wells using a 200 μ l pipetman. Empty sample wells were loaded with 20 μ l of 2X treatment buffer. After loading the wells, tank buffer was carefully layered on top to exclude air.

The gel was run at a constant current of 20 mA until the tracking dye ran off the bottom. The gel was then removed from the casting glass plates and stained with the Coomassie Blue Stain overnight. The gel was destained in two steps; first, a rapid destaining with Destain solution I for 1- 2 h, and then, slow destaining with Destain solution II overnight. After destaining the gel was stored in deionized water until it could be dried under vacuum.

Spectroscopy Sample Preparation

For absorption spectroscopy, samples of PBS and 18 S core preparations were taken directly off the sucrose gradients. The preparations were generally diluted to less than 1 cm^{-1} at the absorption maximum using Buffer A containing 1.0 M sucrose for the PBS preparations, and Buffer C containing 0.9 M sucrose for the 18 S preparations. I have found that both preparations are generally more stable if they are diluted using buffer containing a high concentration of sucrose.

Whole cell samples were prepared by centrifuging cells near the end of the log phase of growth for 5 min at 10,000 rpm in a Dupont SS-34 rotor at room temperature. The cells were immediately

resuspended in fresh growth medium or 0.75 M phosphate buffer pH 7.0 such that the absorption of the samples was less than 2 cm^{-1} at 630 nm.

Steady state fluorescence samples of PBS and 18 S core preparations for room temperature work were diluted to an absorption of less than 0.12 cm^{-1} at the excitation wavelength using the appropriate buffer in 1.0 M sucrose. Samples of whole cells were diluted to less than 0.15 cm^{-1} at 590 nm using fresh growth medium or 0.75 M phosphate buffer, pH 7.

Time-resolved fluorescence spectroscopy samples were prepared as for steady state fluorescence. Fresh samples of PBS and 18 S core preparations were prepared approximately every hour to minimize sample degradation with special care taken to keep the same concentration of protein.

Samples for low temperature spectroscopy were prepared by diluting a minimum volume of sample in 2.0 M sucrose (H. Scheer, personal communication). The sample was put under house vacuum for approximately 10 min prior to freezing to remove most of the gases present. Then the sample was frozen in a 1 cm disposable plastic cuvette without any precooling by simply dropping the cuvette into a square optical dewar containing liquid nitrogen. The sucrose concentration is critical; the best glasses are obtained with the highest concentration of sucrose. For some of the 18 S preparations, I actually concentrated the protein before freezing in the sucrose solution. Other cryoprotectants were tried including PEG-4000 (Mimuro, Yamazaki et al., 1989) which gave a

snow and 50% glycerol which caused immediate uncoupling of proteins in intact PBS as judged by fluorescence spectroscopy.

Steady-State Spectroscopy

Absorption spectroscopy was carried out on an AVIV 14 DS spectrophotometer with uv-vis-nir capability (AVIV, Lakewood, NJ) or on a Varian 2300 spectrophotometer (Varian, Houston, TX) interfaced with a Macintosh SE using Labview 2 (National Instruments Corp., Austin, TX). Slight modifications were made on the AVIV instrument to obtain intact cell absorption spectra with minimal scattering problems (Myers, Graham et al., 1980). Both reference and sample PMTs were covered with a single layer of translucent paper, and the cuvette holders were placed as close to the PMT as possible. The spectra obtained were corrected by subtracting the absorption present at 720 nm.

Steady-state fluorescence spectra were taken on a Spex Fluorolog fluorimeter (Spex Industries, Edison, NJ) interfaced with an Epson computer. Excitation spectra and quantum yield measurements were corrected for lamp intensity using 1,1',3,3,3',3'-hexamethylindotricarbocyanine perchlorate (HITC, 8 g l⁻¹ in acetonitrile, Exciton, Dayton, OH) as a quantum reference counter (Nothnagel, 1987) with a Hamamatsu R928 photomultiplier (Hamamatsu Industries, Japan).

Time-Resolved Fluorescence Spectroscopy

Time-resolved fluorescence spectroscopy was carried out using the reverse-timing, single-photon counting system (Figure 2-4) described in Mukerji's thesis (Mukerji, 1990). It is very similar to the standard single-photon-counting instrument found in the literature. An excellent discussion of single-photon counting can also be found in a book by O'Connor and Phillips (O'Connor and Phillips, 1984). I will give a brief description of our instrumentation in the following paragraphs.

A Spectra-Physics (Mountain View, CA) model 2040 Ar ion mode-locked laser pumps a dye laser (Spectra-Physics, model 375) at 82 MHz and 0.5 W with a pulse duration of 100-120 ps. Approximately 100 mW of the Ar ion beam is split off to a photodiode to provide a trigger pulse for the cavity dumper driver before the beam enters the dye laser. The dye laser output is cavity dumped (Spectra-Physics, Model 344) at a frequency of 4 MHz with 5 to 25 mW of power and a pulse duration of 6 ps. Part of the dye beam is split off to provide a stop pulse for the time-to-amplitude converter (tac) while the rest enters the sample box and excites the sample fluorescence.

The sample fluorescence is collected at 90° to the excitation beam using a camera lens (Nikon) to focus the photons on the active area of a monochromator (SA Instruments, New Jersey) and then to a Hamamatsu MCP-PMT, model R2809U-11 with channels 6 mm in diameter. No more than 4000 counts per second are allowed on the MCP-PMT during data collection to prevent multiple photon events and to prolong the lifetime of the detector. The MCP-PMT provides

Figure 2-4. Instrumental Setup for Single Photon Timing Laser System.

a pulse for each photon it 'sees'; the pulse is amplified with a Hewlett Packard amplifier, discriminated (Tennelec), and sent to the tac.

The tac starts a voltage ramp with the input of the MCP-PMT pulse and stops the ramp with the input of the stop pulse from the photodiode. The voltage which contains the information about the length of time between the start and stop photons is then sent to a multichannel analyzer (Northern Tracor, Madison, WI), and a count is added to the bin or channel corresponding to that voltage. This entire process is repeated thousands of times during the course of the experiment until there is a maximum of 10,000 counts in the peak channel. It can take from 5 min to 2 h to collect a decay depending upon the fluorescence yield of the sample, the length of the lifetimes involved, and the width of the channels on the multichannel analyzer.

The histogram that is built up using the 1024 channels of the multichannel analyzer is transferred to the VAX for data analysis. An instrument response function (irf) is collected just prior to or immediately after each decay measurement using dilute non-dairy creamer as a scattering solution. The width of the irf varies from 45 ps to 70 ps depending upon aperture of the camera lens and monochromator slit widths. The narrower irfs show more structure and are harder to deconvolute for a good fit. The time resolution of the instrument is generally considered to be one-fifth the width of the irf.

Fine adjustments are made to the dye laser and cavity dumper using a fast-triggering oscilloscope (Tektronic) to view the pulse.

The time window of the tac can be adjusted from a minimum of 5 ps per channel to μ s per channel. For my experiments it is set at 10 to 15 ps per channel giving a total time window of 10 to 15 ns. The excitation wavelength can range from 580 nm to 640 nm with Rhodamine 590 dye or 610 nm to 715 nm with DCM dye (Exciton, OH).

Data Analysis of Time-Resolved Fluorescence Decays

The kinetics of phycobiliprotein excited states is expected to be fit by a sum of exponentials according to modeling done by Holzwarth (Holzwarth, Wendler et al., 1987). The fluorescence decays were fit to a sum of exponentials using a software program kindly provided by A. Holzwarth. The program uses a semilinear Marquardt algorithm (Marquardt, 1963). The program calculates the sum of exponentials and convolutes that function with an instrument response function, and the results of the convolution are compared to the experimental decay and a reduced χ^2 is calculated. The program repeats the iterative reconvolution procedure until the χ^2 value is at a minimum. The instrument response function, or irf, used in the data analysis is collected just prior to or immediately after the experimental decay using a scattering solution of nondairy creamer.

Figure 2-5 shows an example of a typical decay with its accompanying irf; the arrows indicate the start and end of the data fit. The data were fit to two, three, or four exponentials as shown in Figure 2-6. The goodness of a fit is judged not only by the χ^2 value

Figure 2-5. Typical fluorescence decay

Data from intact apcDE/C186S PBS excited at 634 nm, emission collected at 675 nm. Fluorescence photons collected using the single photon timing method outlined in the text. Time-to-amplitude converter was set to 11.6 ps per channel. The fwhm of the instrument response function is 55 ps. The arrows mark the start and end of the data which was fit in Figure 2-6.

which should be less than 1.2 but also by the appearance of the residuals. The residuals should not have any systematic deviations present as shown in the two component fit and, to a lesser extent, the three component fit in Figure 2-6. The appearance of the early part of the residual is especially critical; if the sum of exponentials used to fit the data is missing a very fast component the early part of the residual plot will show the fit deviating wildly from the experimental data.

Other factors are also important for a good fit. First, the laser system should be very stable before collecting data such that the pulse shape doesn't vary between the irf and the decay. Also, the bias level on the tac has a strong influence on the fit; we have found that the bias level on our tac must be set within a narrow range otherwise the tac displays nonlinear behavior, and the resulting decays give residuals with almost sinewave-like features.

A third factor to consider is the width of the irf; when the irf is greater than 60 ps the program is able to fit the experimental data with lower χ^2 values. When the apertures and slits are closed to give narrower irfs the fits worsen. This effect could be due to two different factors; either the wider irf tends to mask any small laser instabilities, or the fitting program just isn't as efficient at fitting the prominent features present in the narrower irfs. Also, it's possible that the laser is more unstable when the pulse width is made as small as possible. All of these factors need to be considered prior to data collection when designing the experiment.

The data fits shown in Figure 2-6 show some of the problems of interpreting the fit. The two-component fit is obviously not a good

2 Components

$$\chi^2 = 13.9 \quad \tau_1 = 0.09 \text{ ns} \quad \tau_2 = 1.33 \text{ ns}$$

3 Components

$$\chi^2 = 1.19 \quad \tau_1 = 0.15 \text{ ns} \quad \tau_2 = 0.90 \text{ ns} \quad \tau_3 = 2.09 \text{ ns}$$

4 Components

$$\chi^2 = 1.16 \quad \tau_1 = 0.11 \text{ ns} \quad \tau_2 = 0.93 \text{ ns} \quad \tau_3 = 2.13 \text{ ns} \quad \tau_4 = 0.07 \text{ ns}$$

Figure 2-6. Typical exponential fits to data
2, 3, or 4 exponential fits to data displayed in Figure 2-5.

fit to the decay, as shown by both the χ^2 value and the residual. The three-component fit is indistinguishable from the four component fit in terms of the χ^2 value, but the residual isn't quite as good for the three-component fit. At times, it becomes very difficult to differentiate between three and four lifetimes; I always go with the minimum number of components if there is no obvious improvement with the addition of one more lifetime.

For more meaningful data analysis, global analysis is generally carried out in which all of the decays collected for the sample are fit simultaneously. Typically, sets of decays are taken with one excitation wavelength and emission wavelengths spanning the emission spectrum of the sample. Each decay is first fit individually using the iterative deconvolution software to obtain approximate lifetimes. Then the decays are analyzed as a set using a global optimization algorithm provided by A. Holzwarth. The algorithm assumes that the fluorescent lifetimes are independent of emission wavelength, with the amplitude of the lifetime varying with wavelength (Holzwarth, Wendler et al., 1987; Knorr and Harris, 1981; Knutson, Beechem et al., 1983). This reduces the number of parameters allowed to vary during the fit, so the fits should be more meaningful. The global software is also better at finding the very short lifetimes present, especially when the lifetime has a small or zero amplitude at many wavelengths. Global analysis also works better for finding risetimes or negative amplitudes.

Risetimes are present when energy is transferred from one pigment pool to another pool at a lower excited state energy; the

fluorescence of the second pool is delayed by a time consistent with the lifetime of the first pigment pool. I will present all the global analysis work in the form of decay-associated-spectra or DAS. DAS show the amplitude or yield of the individual components at each wavelength examined.

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Chapter 3. Discussion of the Whole-Cell Results

I will start by first examining the steady state and time-resolved spectroscopy of healthy intact cells from the different strains. In this chapter I will compare the efficiency of energy transfer to the reaction centers in the wild-type and mutant cells. I will try to address the following questions in this chapter. Does the amount of phycobiliprotein present in the cells relative to chl *a* change because of the mutation? Do the antennae from the mutants 'leak' more energy than the wild-type PBS? Are the energy transfer kinetics from the PBS to the reaction centers altered with the mutation?

Absorption Spectroscopy

Absorption spectroscopy was carried out to quantify the amount of phycobiliprotein present in the cells relative to chl *a*. I cannot distinguish between free phycobiliprotein and intact PBS absorption in the cells.

The spectrophotometer's measuring beam is easily scattered from the intact cells, and modifications to the sample box were necessary to obtain relatively scatter-free absorption spectra. The first change made was to cover the windows between the sample and reference holders and the PMT with a translucent paper. The translucent paper causes the measuring light to be more diffuse on the PMT; this makes the position of the measuring beam on the detector less critical to the measurement. In a paper by Myers, Graham, and Wang, they use an opaque Plexiglas in this manner,

but I found a single layer of translucent paper (weighing paper in this case) to work well (Myers, Graham et al., 1980). Alternatively, opal glass can be placed between the sample and the detector, but the corrected background turned out to be more linear with the translucent paper. The opal glass probably scattered the light too much.

The second modification to the spectrophotometer's sample box was to move the sample holders as close as possible to the PMT to minimize the loss of light due to scattering. I also corrected for background scatter by subtracting the absorption at 720 nm, a portion of the spectrum where there should be little absorption (Myers, Graham et al., 1980). Without the changes to the sample box mentioned above the absorption spectra had huge backgrounds due to light scatter with an $1/\lambda^2$ function.

Corrected absorption spectra for whole cells are shown in Figure 3-1a,1b. The cultures were one week old, and all appeared to be healthy and in the log phase of growth. The cultures were grown simultaneously under the same light and with the same CO₂ and N₂ mixture. All of the absorption spectra contained two peaks; peak I at 631 - 633 nm and peak II at 677 - 680 nm. The absorption maxima from Figures 3-1a,1b are given in Table 3-1.

As mentioned previously, one of the goals of these experiments was to calculate the relative amount of phycobiliprotein to chl *a*. I was interested in seeing if some of the mutations would cause cells to produce relatively more chl *a* to compensate for inefficient PBS energy transfer. The amount of phycobiliprotein present in the cells is shown by the 630 nm peak while the chl *a* concentration is

Figure 3-1a. Absorption spectra of whole-cells
 The spectra have been corrected for light scatter as outlined in the text. The location of the absorption maxima are given in Table 3-1. The spectra were collected at RT.

Figure 3-1b. Absorption spectra of whole-cells
The spectra have been corrected for light scatter as outlined in the text. The location of the absorption maxima are given in Table 3-1. The spectra were collected at RT.

Table 3-1
Spectral Data for the Intact Cells

	Absorption Maxima	Emission Maxima
wild-type cells	632 nm 679 nm	657 nm
apcD cells	632 nm 678 nm	659 nm
apcF cells	633 nm 677 nm	659 nm
apcDF cells	632 nm 680 nm	651 nm
apcE/C186S cells	632 nm 680 nm	650 nm 705 nm
apcDE/C186S cells	633 nm 679 nm	653 nm

shown by the 680 nm peak in the absorption spectrum. As shown in Figures 3-1a,1b, there isn't any apparent trend in the relative amounts of each type of antenna complex present.

I found that the ratios of the two peaks fluctuated with experiment in all of the strains including that of the wild-type. I attribute this fluctuation to changing culture conditions, with the number and composition of the PBS to depend upon the culture age. For example, if the culture has been growing for several days and the cells are starting to get relatively thick, the amount of light available per cell will decrease due to shading. The *Synechococcus* sp. PCC cyanobacterium possesses a mechanism for adjusting the rod length to compensate for the variable light conditions (de Lorimier, Smith et al., 1992). At low light intensities the rod length increases, which results in a higher 630 to 680 nm absorption ratio. Also, work done by various laboratories show that the phycobiliproteins can be used as carbon and nitrogen sources during periods of cell starvation (Bryant, 1991; Stevens, Balkwill et al., 1981).

It is hard to correct for the differences in phycobiliprotein concentrations due to cell shading and nutrient availability, because some of the mutants have much different doubling times from that of the wild-type strain, see Table 2-2. Therefore, even though the different strains are grown under the same lights and in the same medium, the composition of the antenna can be quite different depending upon the stage of growth of the culture. I will keep this limitation in mind when discussing the spectral data for the intact cells in this chapter.

Steady State Emission

With steady state emission spectroscopy one should be able to judge the efficiency of energy transfer from the PBS antenna to the PSII reaction center in whole-cells. If energy transfer to the reaction center is not occurring, the fluorescence yield of the PBS in the cells should increase; photochemistry is no longer successfully competing with fluorescence. There are some difficulties in interpreting the data. If the amount of uncoupled phycobiliprotein fluctuates during the growth of the culture, the fluorescence quantum yield of the cell also fluctuates. I define uncoupled phycobiliprotein to be either intact PBS uncoupled from reaction centers or dissociated hexamers or trimers of phycobiliprotein. Intact PBS uncoupled from reaction centers fluoresces from 650 to 680 nm, depending upon the strain as shown in Chapter 4. Dissociated PC hexamers fluoresce around 650 nm, while AP trimers emit at 660 nm.

Relative quantum yields and corrected emission spectra were obtained for wild-type and mutant cells. The cells were illuminated at 580 nm to excite the PC in the rods while directly exciting only a small amount of the chl *a* present.

As with the absorption spectra, the appearance of the emission spectrum and the quantum yield of all of the strains varied with culture age. The *apcD*, *apcF*, and *apcDF* fluorescence yields were comparable to that of the wild-type cells while the *apcDE/C186S* and *apcE/C186S* yields were roughly 150% of wild-type. There does appear to be some fluctuation in the amount of uncoupled PBS or dissociated phycobiliprotein present in the cells. The quantum

yields of the mutants relative to that of the wild-type cells differed considerably with experiment. As an aside, the absolute fluorescence yields of the cells are considerably less than those of the isolated PBS in the wild-type, *apcD*, *apcF*, and *apcDF* strains. Most of the energy collected by the PBS in these strains goes to the reaction centers to carry out photochemistry.

The emission spectra are shown in Figures 3-2a,2b, and they are normalized at the emission maximum. The location of the emission maxima are given in Table 3-1. The emission spectra for the different strains were obtained under identical conditions with the same exciting light intensity. I treated the cells in exactly the same way during the experiment, so the relative number of closed reaction centers present in each strain should not differ if the process of energy transfer within the PBS is unchanged. To give the reader some idea of how much the emission spectrum can fluctuate with culture age and growing conditions, I have overlaid normalized wild-type emission spectra from several different experiments in Figure 3-3. The emission spectra are from wild-type cultures between 4 and 8 days. There didn't seem to be any consistent trends with culture age. The intensity of the exciting light did vary somewhat with each experiment, since the spectra were taken at different times during the lifetime of the fluorimeter lamp.

Keeping these limitations in mind, I feel confident that the *apcD*, *apcF*, and *apcDF* spectra shown in Figure 3-2b are indistinguishable from that of the wild-type while the *apcDE/C186S* and *apcE/C186S* spectra are different. The *apcD*, *apcF*, *apcDF*, and wild-type emission spectra display two emission bands at approximately 650

Figure 3-2a. Steady state emission spectra of whole-cells
The cells were excited at 590 nm and emission was detected at 90° to excitation. The excitation monochromator slits were set to a 7 nm bandpass while the emission monochromator was set to a 0.5 nm bandpass. The spectra were collected at RT.

Figure 3-2b. Steady state emission spectra of whole-cells. The cells were excited at 590 nm and emission was detected at 90° to excitation. The excitation monochromator slits were set to a 7 nm bandpass while the emission monochromator was set to a 0.5 nm bandpass. The spectra were collected at RT.

Figure 3-3. Steady state emission spectra of wild-type cells
The spectra are of cells from different wild-type cultures. The cells were excited at 580 or 590 nm at RT. The spectra are normalized at 660 nm.

to 660 nm and 680 nm. The first band at 650 to 660 nm is due mostly to the emission of dissociated phycobiliproteins with some contribution from intact PBS uncoupled from the reaction centers. Nothing else in the cell should fluoresce there. The amounts of dissociated phycobiliproteins present in the cells from the *apcD*, *apcF*, and *apcDF* mutants are roughly that of the wild-type as shown by the quantum yields. The second emission band at 680 nm must be due to a combination of chl *a* fluorescence and emission from terminal emitters in uncoupled PBS.

In Figure 3-2a the emission spectra of the *apcDE/C186S* and *apcE/C186S* strains are displayed with the emission spectrum of the wild-type cells. The 680 nm emission shoulder in the wild-type spectrum is absent in the *apcDE/C186S* and *apcE/C186S* spectra. I have never observed any significant 680 nm emission in any of the whole-cells emission spectra I've taken with the *apcDE/C186S* and *apcE/C186S* mutants. Another difference between the *apcE/C186S* and wild-type emission spectrum is the presence of a 705 nm emitter in the *apcE/C186S* spectrum. This emission is due to the modified chromophore described in Chapter 1. The 705 nm emission band is not very prominent in the *apcE/C186S* spectrum, since it occurs at the same place in the spectrum as emission from some low lying excited states of PC (PC hexamers emit above 690 nm in addition to the main PC emission band at 650 nm) along with chl *a* emission. The presence of the modified chromophore is more prominent in the emission spectrum from the *apcE/C186S* PBS, as shown in the next chapter. Emission from the modified

chromophore seems to be missing in the apcDE/C186S spectrum; it possibly is obscured by the PC and chl *a* emission in the cells.

Time-Resolved Fluorescence

In a simpler world, one might be able to assign fluorescence lifetimes for the pigment pools present in the intact cells using time-resolved fluorescence. The fluorescence from a pigment pool will predominate at a certain place in the emission spectrum: 650 nm for PC, 660 nm for AP, and 680 nm for terminal emitters, PSII reaction centers, and chl *a*. The lifetimes of phycobiliproteins that transfer energy to reaction centers are much shorter than the emission decay lifetimes measured for the uncoupled phycobiliproteins. Energy transfer between coupled chromophores competes strongly with emission. Typical emission lifetimes for free phycobiliproteins are around 1.5 ns; when the chromophores transfer energy to lower excited states their measured emission lifetimes decrease to less than 0.15 ns. Energy transfer to a pigment pool can be seen by the appearance of a risetime or negative amplitude in the decay-associated spectrum, which indicates a delay in the appearance of emission from that pigment pool.

I will present the time-resolved fluorescence decay data as decay-associated spectra (DAS). The DAS are obtained from simultaneously analyzing the fluorescence decays from several different emission wavelengths using global analysis. With global analysis one obtains lifetimes and the relative yield of each lifetime

component at different emission wavelengths. The lifetimes of the chromophores are assumed to be constant across the spectrum.

The risetime at 680 nm due to energy transfer to the reaction centers or terminal emitters is an important indicator of the efficiency of energy transfer. A longer risetime indicates less efficient transfer from outlying pigments to the terminal emitters because it takes longer for the energy to reach the terminal emitters. I am making the assumption that small changes in the amount of PC present are not detected within the time resolution of the instrument.

The cells were excited at 580 nm, and fluorescence decays were collected every 10 nm over the emission spectrum. The emission was collected for almost 12 ns after the exciting pulse. The DAS obtained from global analysis are presented in Figures 3-4a to 3-4f. A minimum of 5 lifetime components are necessary to fit the fluorescence decays for every strain.

The components resolved in the wild-type decays are very similar to those reported by other laboratories for dark-adapted cells of *Synechococcus* 6301 and *Synechocystis* sp. PCC 6803 (Mullineaux and Holzwarth, 1991; Bittersmann and Vermaas, 1991; Mullineaux, Bittersmann, et al., 1990). Based on their work, I assign the 0.050 ns component to AP and PC chromophore pools which are coupled by energy transfer to the terminal emitters. The 0.050 ns component emits in the blue region of the spectrum. The 0.16 ns component must be due to phycobiliproteins which transfer energy to the reaction center based upon this component's resolved

Figure 3-4a. Decay-associated spectra of wild-type cells. The cells were excited at 580 nm at RT. The tac was set to 11.8 ps per channel. The DAS were obtained from global analysis.

Figure 3-4b. Decay-associated spectra of apcD cells
 The cells were excited at 580 nm at RT. The tac was set to 11.8 ps per channel.
 The DAS were obtained from global analysis.

Figure 3-4c Decay-associated spectra of apcF cells. The cells were excited at 580 nm at RT. The tac was set to 11.8 ps per channel. The IAS were obtained from global analysis.

Figure 3-4d. Decay-associated spectra of apcDF cells. The cells were excited at 580 nm at RT. The tac was set to 11.8 ps per channel. The DAS were obtained from global analysis.

Figure 3-4e. Decay-associated spectra of apcE/C186S cells
 The cells were excited at 580 nm at RT. The tac was set to 11.8 ps per channel.
 The DAS were obtained from global analysis.

Figure 3-4f. Decay-associated spectra of apcDE/C186S cells. The cells were excited at 580 nm at RT. The tac was set to 11.8 ps per channel. The DAS were obtained from global analysis.

emission spectrum. The very long-lived component of 10 ns which contributes very little to the total yield is probably from free chl *a*.

The 0.5 ns component was also reported in all of the studies, and Mullineaux and Holzwarth (1991) attribute it to the PSII reaction center based on studies done with closed and open reaction centers. The remaining component in the wild-type DAS of 1.4 ns is assigned to both dissociated phycobiliprotein and to intact PBS uncoupled from the reaction centers. All of these assignments were made using the resolved emission spectra of the individual components along with the DAS spectra from isolated PBS shown in Chapter 4. For example, the 1.4 ns component, which is associated with emission from 640 to 690 nm, is very similar to the 1.5 ns component found in the DAS for intact wild-type PBS.

Both Bittersmann and Mullineaux resolve a very fast component of 0.020 ns with very low yield, which they attribute to the PC pigment pool (Mullineaux and Holzwarth, 1991; Bittersmann and Vermaas, 1991). I was not able to resolve this component, possibly because I was collecting only one-fourth the number of counts per decay. Contributions from the PC pigment pool are included in the 0.05 ns component.

From the assignments I've described above, the most important components in the decays are the 0.16 ns and 0.05 ns components attributed to coupled chromophores within the intact PBS and the 1.4 ns component assigned to uncoupled phycobiliprotein.

As shown in Figure 3-4b,4c, and 4d, the DAS obtained for the *apcD*, *apcF*, and *apcDF* cells are indistinguishable from that of the wild-type within the resolution of the experiment. The *apcD*, *apcF*,

and apcDF cells do not appear to contain any more free phycobiliprotein than the wild-type cells.

The DAS obtained for the apcE/C186S and apcDE/C186S cells are considerably different from that of the wild-type. In the apcE/C186S DAS, two risetimes are present: one at about 680 nm similar to that found in the wild-type DAS, and one at 710 nm. The risetime at 710 nm is due to energy transfer to the modified chromophore from higher energy pigments. As with the steady state emission data, there is no evidence for the presence of the modified chromophore in the apcDE/C186S cells. The risetimes found at 680 nm for the apcDE/C186S and apcE/C186S cells are slightly longer than that found in the wild-type. Energy transfer from the outlying chromophores to the reaction centers or terminal emitters is not happening as efficiently in the apcDE/C186S and apcE/C186S mutants.

The apcE/C186S DAS displays a 0.57 ns component that emits at 680 nm and that can be attributed to the PSII reaction centers; the reaction center appears to function in the apcE/C186S cells. The apcDE/C186S DAS does not show any comparable component that can be assigned to the PSII reaction center. In fact, there is very little emission at 680 nm shown in the apcDE/C186S DAS. The only component in the apcDE/C186S DAS to show any appreciable fluorescence yield at 680 nm is a 0.9 ns component, which I attribute to intact uncoupled PBS.

The apcDE/C186S DAS does contain the 0.18 ns component, which is analogous to the 0.16 ns component that I attribute to chromophores within the PBS coupled to the reaction centers, but it

is a much smaller percentage of the total yield of the cells. Apparently, energy transfer to the PSII reaction center from the apcDE/C186S PBS is considerably impaired.

Mullineaux et al. reported that closed reaction centers in *Synechococcus* 6301 cells had a longer fluorescence lifetime, 0.56 ns compared to 0.48 ns with open reaction centers (Mullineaux, Bittersmann et al., 1990). It seems unlikely that the lifetime of closed reaction centers would increase to 0.90 ns in *Synechococcus* sp. PCC 7002. I would rather attribute the 0.90 ns component in the apcDE/C186S DAS to uncoupled, intact PBS. In Chapter 4, I will show that apcDE/C186S PBS fluorescence decays with a 1.1 ns component. I find no trace of fluorescence from the PSII reaction center in the apcDE/C186S data, but if it is a very small percentage of the total yield of the cell then I would not resolve the reaction center emission.

The other significant difference between the apcDE/C186S and apcE/C186S DAS and the wild-type DAS is the larger relative yields of components which can be assigned to either dissociated phycobiliproteins or uncoupled, intact PBS. In the apcE/C186S DAS, a 1.39 ns component which appears to be due to emission from a combination of both dissociated phycobiliprotein and uncoupled PBS contributes a much larger portion of the total fluorescence yield compared to the wild-type DAS. The apcDE/C186S DAS has a 1.59 ns component which I attribute to dissociated phycobiliprotein and a 0.90 ns component which I assigned to uncoupled PBS. The shorter component is due to the chromophores coupled to the modified chromophore present in the apcDE/C186S PBS, as I will

show in Chapter 4. These two decay components contribute almost all of the emission present in the *apcDE/C186S* cells.

Conclusions

There are no significant differences in the ratio of phycobiliprotein to chl *a* concentrations found in the different strains as shown in the absorption spectra. None of the mutants appear to make larger amounts of chl *a* to compensate for impaired PBS.

The *apcD*, *apcF*, and *apcDF* strains were indistinguishable from the wild-type using steady state and time-resolved fluorescence spectroscopy. Cells from these mutants have similar quantum yields along with similar concentrations of free phycobiliprotein and uncoupled PBS. The PBS from these mutant transfer energy to 680 nm emitters as efficiently as the wild-type PBS. This conclusion is consistent with the doubling times measured for the *apcD*, *apcF*, and *apcDF* mutants, as shown in Table 2-2. The mutants all had doubling times similar to that of the wild-type strain.

Cells from the *apcDE/C186S* and *apcE/C186S* strains display different steady state and time-resolved emission spectra from the wild-type cells. These mutants contain larger amounts of dissociated phycobiliprotein and uncoupled PBS along with PBS that display impaired energy transfer to the 680 nm emitters (both the reaction centers and the terminal emitters from the PBS). All of these factors contribute to the greater fluorescence yield of cells from the *apcE/C186S* and *apcDE/C186S* mutants relative to the wild-type.

Energy transfer from the PBS to the PSII reaction center occurs in the apcE/C186S cells but only on a small scale in the apcDE/C186S cells. None of the resolved components in the apcDE/C186S DAS could be attributed to the reaction center. From the time-resolved fluorescence data, the apcDE/C186S cells appear to be especially impaired. Only small amounts of energy are transferred to the PSII reaction centers from the antenna.

Both the DAS and steady state emission spectra for the apcE/C186S cells show the presence of a modified chromophore that emits at approximately 710 nm. The modified chromophore emission does not appear to be present in the apcDE/C186S spectra. Energy transfer from PC and AP in the antenna to the modified chromophore occurs at the same rate as energy transfer to the 680 nm emitters, as shown by the risetimes at 710 and 680 nm in the apcE/C186S DAS.

The doubling time of the apcE/C186S strain is five times that of the wild-type strain. The impaired growth of the apcE/C186S strain can be attributed to three things. First, the apcE/C186S cells contain more dissociated phycobiliproteins and uncoupled PBS which the cells waste energy making. Second, energy transfer is less efficient in the PBS that are coupled to the reaction centers. Third, energy that would normally carry out photochemistry is being funneled away from the PSII reaction centers to the modified chromophore to be lost as fluorescence.

I don't have a doubling time for the apcDE/C186S strain from Bryant for the conditions given in Table 2-2, but I know that this strain grows at a significantly slower rate. From my emission data I

attribute the slower growth rate to be due to the decrease in energy transfer from the PBS to the reaction centers. The *apcDE/C186S* cells also appear to contain more dissociated phycobiliproteins.

One question which I haven't address yet is what is the composition of the PBS from the mutants. The *apcD* PBS is missing the α^{AP-B} subunit, the *apcF* PBS is missing the β^{18} subunit, and the *apcDF* PBS is missing both α^{AP-B} and β^{18} . What happens in the PBS core when polypeptides are missing? Do water molecules just fill the space, or does a polypeptide similar in amino acid composition substitute for the missing protein? In the next chapters I will present data which indicate that a bluer-absorbing protein substitutes for the β^{18} polypeptide, and probably the α^{AP-B} protein also. I suggest that an α^{AP} subunit replaces the α^{AP-B} subunit and a β^{AP} subunit replaces the β^{18} subunit. This suggestion is based on the similarity between the amino acids sequences for the α^{AP-B} and α^{AP} proteins and the β^{18} and β^{AP} proteins (Glazer and Bryant, 1975; Füglistaller, Rübéli et al., 1984). Since the proteins have similar amino acids sequences, it seems reasonable to assume that their structures will also be similar. Therefore, the proteins are possibly interchangeable to a certain extent within the PBS core. I will discuss this point further in the next chapters.

The *apcDE/C186S* and *apcE/C186S* mutants did not turn out as originally expected. The substitution of a serine for the chromophore-binding cysteine on the L_{cm} was expected to cause the deletion of the chromophore from the polypeptide, but this obviously did not occur. The chromophore is present in a modified form. It absorbs and emits light at lower energies than the

chromophore normally bound to the L_{cm} protein, as I will show in the following chapters.

It seems reasonable to assume that the modified chromophore is being held noncovalently in the binding pocket of the L_{cm} polypeptide. The spectral properties of the modified chromophore is more similar to that of the bound chromophores found in the phycobiliproteins than to the spectral properties of the free chromophore (Braslavsky, Holzwarth, et al., 1983). As I will show in the next chapter, emission from the modified chromophore decays with a time constant of approximately 1 ns. Braslavsky et al. found that the emission from biliverdin dimethyl esters decayed with lifetimes of less than 50 ps.

I have found that the modified chromophore is easily lost from the PBS core when the PBS is partially dissociated, but it seems relatively stable within the intact PBS. The modified chromophore is possibly 3(Z)-phycocyanobilin, the immediate precursor to the bound pigment as reported by Rhie and Beale (Rhie and Beale, 1992). 3(Z)-phycocyanobilin has a double bond on the carbon that, in the bound chromophore, binds to the cysteine group of the protein.

The addition of this extra double bond in the modified chromophore makes it more conjugated than the bound pigment found on the wild-type L_{cm} . The increased conjugation decreases the energy gap between the lowest excited state of the molecule and the ground state, thus shifting the absorption spectrum of the chromophore further to the red. I will discuss spectral properties of the modified chromophore more in the next chapter.

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Chapter 4. Discussion of PBS Results

In the last chapter the spectroscopy of the whole cells was presented to examine how the mutations in the PBS affected energy transfer to the reaction centers. In this chapter, I will explore the spectral properties of the isolated intact PBS. I am making the assumption that the isolated PBS have spectral properties identical to PBS in the cells except that the terminal emitters in the PBS core should now simply fluoresce instead of transferring energy to chl *a*.

The PBS were easily isolated on sucrose density gradients as discussed in Chapter 2. The amount of free phycobiliprotein present on the gradient increased in order of wild-type < *apcF*, *apcD*, *apcDF* < *apcE/C186S* < *apcDE/C186S*. I had some problems isolating intact PBS from the *apcDE/C186S* mutant; most of the phycobiliprotein isolated on the sucrose gradient was dissociated into smaller structures such as rods or hexamers.

I showed in the previous chapter that the amount of dissociated phycobiliprotein present in the intact cells for the *apcD*, *apcF*, and *apcDF* mutants was similar to that of the wild-type while the *apcE/C186S* and *apcDE/C186S* cells contained more dissociated phycobiliproteins and/or uncoupled PBS. It also appears that the PBS from the *apcD*, *apcF*, and *apcDF* mutants are more unstable under the conditions used for their isolation than the wild-type PBS. I diluted concentrated PBS removed from the gradients using 0.75 M phosphate buffer at pH 7.8 with 1.0 M sucrose. The addition of sucrose to the dilution buffer increased the stability of

the preparations, and the change in solvent did not affect the emission spectrum obtained from the PBS.

Absorption

I compared the absorption spectra of PBS from the wild-type and mutant strains to quantify changes in the composition of the PBS with each mutation. I make the assumption initially that the absorbances of the phycobiliproteins are additive. In other words, the mutations have not affected the absorption of other proteins in the PBS core. None of the missing chromophores are involved in excitonic coupling. For example, the absence of the β^{18} subunit does not affect the absorption spectrum of an adjacent AP monomer in the trimer. If this assumption is correct, the differences that I expect are small, on the order of 0.005 a.u. in the normalized spectrum.

The absorption spectra of the PBS are given in Figures 4-1a,1b. All of the spectra have absorption maxima at 630 nm. The *apcDE/C186S*, *apcE/C186S* and *apcDF* PBS absorb slightly less at approximately 600 nm and above 650 nm. The *apcD* and *apcF* PBS are virtually indistinguishable from the wild-type.

Small variations in the 600 nm absorption are possibly due to changes in PC content or to differences in core composition. As I mentioned earlier, the amount of PC in the rods can vary somewhat with light conditions (de Lorimier, Smith et al., 1992), and these changes will appear in the absorption spectrum. The absorption spectrum of AP trimers also contains a shoulder at 600 nm, so the changes in the PBS core composition will influence the AP

Figure 4-1a. Steady state absorption spectra of intact PBS. Samples were taken directly off of sucrose gradients and diluted to less than 1.0 a.u. at 630 nm with 1.0 M sucrose in 0.75 M phosphate buffer, pH 7.8. The spectra were taken at RT.

Figure 4-1b. Steady state absorption spectra of intact PBS. Samples were taken directly off of sucrose gradients and diluted to less than 1.0 a.u. at 630 nm with 1.0 M sucrose in 0.75 M phosphate buffer, pH 7.8. The spectra were taken at RT. The absorption maxima are given in Table 4-1.

absorption shoulder at 600 nm along with the absorption maximum at 650 nm.

I calculated absorption difference spectra for all of the mutants by normalizing the area under the absorption curves and then subtracting the absorption of the PBS from the mutant from that of the wild-type PBS, as shown in Figures 4-2a,2b. With this subtraction procedure I make the assumption that the number of chromophores present is unchanged from wild-type to mutant. The net change in absorption has to be zero with this procedure.

The L_{cm} and α^{AP-B} chromoproteins have been assigned absorption maxima at 675 nm (Mimuro, Yamazaki et al., 1989; Lundell, Yamanaka et al., 1981; Glazer and Bryant, 1975). AP trimers absorb at 650 nm with a 600 nm shoulder, while PC hexamers absorb at about 625 nm. If the absorption spectra of the chromophores are additive, the absence of specific chromophores will leave holes in the absorption spectra of the PBS from the mutants. The holes should appear in the difference spectra.

The difference spectra for the *apcE/C186S*, *apcDE/C186S*, and *apcDF* mutants are very similar in appearance. The PBS from these mutants absorb more than the wild-type at 580 and 630 nm and less than the wild-type at 655 nm with a definite shoulder at approximately 675 nm. The *apcDE/C186S* and *apcE/C186S* PBS also absorb more than the wild-type PBS at 695 nm. The *apcD* and *apcF* difference spectra are almost reversed in appearance from the other spectra; the *apcD* and *apcF* PBS absorb more than the wild-type at approximately 650 nm, and less than the wild-type at

Figure 4-2a. Absorption difference spectra of intact PBS
Normalized spectra in Figure 4-1a to equal area; mutant is subtracted from wild-type.

Figure 4-2b. Absorption difference spectra of intact PBS. Normalized spectra in Figure 4-1b to equal area; mutant is subtracted from wild-type.

630 nm and 670 nm. The *apcDE/C186S* and *apcE/C186S* absorption differences are approximately twice that of the other mutants.

I tried several other ways to execute the absorption subtraction. I normalized the spectra at several different wavelengths, and I tried a subtraction method in which the wild-type absorption is slightly greater than the absorption of the PBS from the mutants. None of the methods gave consistent results for the wavelength region from 600 to 650 nm for any of the strains. The various difference spectra calculated for the *apcDE/C186S* and *apcE/C186S* PBS consistently showed that the *apcDE/C186S* and *apcE/C186S* PBS absorb more than the wild-type at about 695 and less than the wild-type PBS at about 675. The smaller absorption differences in the rest of the mutants were more easily distorted by the subtraction procedures; I don't feel confident about any of the differences in the *apcF*, *apcD*, and *apcDF* absorption spectra.

Steady State Fluorescence

Steady state emission spectra were obtained for all of the strains, as shown in Figures 4-3a,3b. Quantum yields were measured for the PBS from the mutants relative to that of the wild-type, as shown in Table 4-1. The emission spectra of the PBS should enable us to judge the energy transfer efficiency of the isolated antenna. If the chromophores in PBS are not as well coupled, they won't transfer energy as efficiently to the terminal emitter(s). More emission will be seen from PC and AP chromophores. Energy transfer won't compete as well with fluorescence deactivation as in the wild-type antenna.

Figure 4-3a. Steady state emission spectra of intact PBS
 The PBS were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set between 6 and 3 nm resolution while the emission monochromator resolution was between 0.4 and 0.8 nm. The spectra were collected within 2 hours after the PBS were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 1.0 M sucrose in 0.75 M phosphate buffer, pH 7.8. The spectra were taken at RT. The emission maxima are given in Table 4-1.

Figure 4-3b. Steady state emission spectra of intact PBS
 The PBS were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set between 6 and 3 nm resolution while the emission monochromator resolution was between 0.4 and 0.8 nm. The spectra were collected within 2 hours after the PBS were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 1.0 M sucrose in 0.75 M phosphate buffer, pH 7.8. The spectra were taken at RT. The emission maxima are given in Table 4-1.

Table 4-1
Spectral Data on Intact Phybilisomes

	Absorption Maximum	RT Emission Maximum	Quantum Yield [#]	77 K Emission Maximum
Wild-Type	630 nm	676 nm	100	683 nm
apcF	630 nm	665 nm	102 ± 12	681 nm
apcD	630 nm	666 nm	108 ± 8	683 nm
apcDF	630 nm	661 nm	104 ± 11	681 nm
apcE/C186S	630 nm	651 nm 714 nm	54 ± 5	682 nm 715 nm
apcDE/C186S	630 nm	653 nm 712 nm	52 ± 14	678 nm 713 nm

[#]Quantum yields were calculated at room temperature relative to wild-type PBS. The PBS were excited at 590 nm, and the emission from 600 to 800 nm was integrated to give the relative yield. The numbers are given in percentage of wild-type yield. A minimum of three measurements were averaged for each strain to give the standard deviation.

The appearance of more AP and PC emission could also be due to a change in population distribution because fewer 680 nm emitters are present. Assuming a Boltzmann distribution of populations, more emission will originate from the higher energy states if the number of lower energy states decreases. This conclusion especially holds at room temperature where back transfer is important. Therefore, AP and PC emission will increase in the mutants missing 680 nm fluorescers.

The steady state emission spectra are shown in Figures 4-3a,3b. I was interested in seeing the effect of the decrease in the number of terminal emitters on the steady state emission spectrum of the PBS from the mutants. I used the following formula to calculate the relative Boltzmann populations, where n_i is the fraction of i type chromophores, g_i is the number of i type chromophores, and ΔE is the energy difference between the E_0 levels of the chromophores:

$$\frac{n_i}{n_j} = \frac{g_i}{g_j} e^{(-\Delta E/kT)}.$$

The E_0 levels used corresponded to the average of the absorption and emission maxima.

For these calculations I make the assumption that the β^{18} chromophore emits at 682 nm along with the α^{AP-B} and L_{cm} chromoproteins, that the β^{18} and α^{AP-B} subunits are replaced by β^{AP} and α^{AP} subunits (this assumption had very little effect on the calculations), and that the chromophores are coupled. For the degeneracy of the energy states, I assumed that the wild-type PBS are composed of 324 PC chromophores, 46 AP chromophores, and 6

terminal emitters. I made some gross assumptions concerning the energy levels of the pigments, because I did not take into account any differences in the spectral properties of chromophores from the same pigment pool. The E_0 energies used corresponded to 635 nm for PC, 656 nm for AP, 679 nm for the 680 nm emitters, and 705 nm for the modified chromophore present in apcDE/C186S and apcE/C186S PBS. The changes in the emission spectrum from mutant to wild-type PBS are calculated to occur in the absence of any structural perturbations that affect chromophore coupling.

For wild-type PBS, I calculate that 19% of the steady state emission (emission after equilibration) will originate from PC, 32% from AP, and 49% from the 680 nm emitters. For the apcD and apcF mutants, the calculated percentage of emission from each chromophore type should be 23% from PC, 38% from AP, and 39% from the 680 nm emitters while the apcDF emission contributions will be 28%, 49%, and 23%, respectively.

The calculations for the wild-type emission spectrum qualitatively fit the experimental data. With the decrease in the number of 680 nm emitters present, the Boltzmann calculations predict a blue-shift in the steady state spectrum for the mutants. This prediction qualitatively fits the experimental data on the apcD, apcF, and apcDF mutants. The emission from the apcDF mutant is shifted further blue than the other mutants as expected.

The Boltzmann calculations did not work well for the apcDE/C186S or apcE/C186S PBS. About 75% of the emission should be coming from the modified chromophore, but this is obviously not

the case, as shown in Figure 4-3a. I fit the 650 and 715 nm emission bands in the *apcE/C186S* and *apcDE/C186S* spectra to gaussians to calculate the relative yields of each band. The 715 nm band contributes only about one-third of the total yield in the *apcDE/C186S* and *apcE/C186S* PBS. In the *apcDE/C186S* and *apcE/C186S* PBS, most of the emission is originating from PC chromophores.

The emission spectra for the *apcDE/C186S* and *apcE/C186S* PBS show a virtual absence of 680 nm emission along with a strong emission band at 715 nm attributed to the modified chromophore. Boltzmann calculations indicate that less than 10% of the total emission in the steady state emission spectrum should be from the 680 nm emitters in the *apcDE/C186S* and *apcE/C186S* mutants if all the chromophores are coupled. The β^{18} and α^{AP-B} chromophores should transfer energy to the 715 nm emitter.

I examined the relative quantum yields of the PBS to see if more structural disorganization is present in the PBS from the mutants. If the chromophores are not being held in optimal conformations, more nonradiative deactivation could occur, and the quantum yield of the antenna will decrease. The relative quantum yields are given in Table 5-1. The yields obtained for the *apcD*, *apcF*, and *apcDF* PBS are essentially identical to that of the wild-type, while the *apcE/C186S* and *apcDE/C186S* yields are only one-half that of the wild-type.

Low Temperature Emission

At 77 K the transfer of energy from the 680 nm emitters to AP or transfer from AP to PC is minuscule. The emission bands from PC, AP, and terminal emitters can be resolved; the populated excited states at 77 K are smaller in number than at RT, and the emission bands will narrow. If the chromophores are coupled, the only emission that should be present is that from the terminal emitters.

From the 77 K emission spectra, I am able to make some spectral assignments for some of the terminal emitters. In the *apcDF* mutant only one of the three possible terminal emitters is present, the L_{cm} chromophore. In the *apcDE/C186S* mutant only one 680 nm emitter is found, the β^{18} chromophore, although the lower energy modified L_{cm} chromophore is still present.

I make the assumption that the emission spectra of the chromophores in the PBS are not affected by the freezing process. I was successful in obtaining cracked glass samples at 77 K using 2 M sucrose as a cryoprotectant. The emission spectrum of the samples before freezing and after rewarming were very similar. It does not appear that the freezing process caused any irreversible disruption of the antenna complex. I also found that the low-temperature emission spectra were very reproducible from experiment to experiment; the emission maxima for different preparations were within 0.5 nm of the maxima given in Table 4-1.

The PBS were excited at 590 nm, directly exciting mostly PC in the rods. The experimental setup did not allow for the calculation of quantum yields, so only normalized spectra are present in

Figures 4-4a,4b. I have also collected low temperature emission spectra for AP trimers and PC hexamers (not shown). AP trimers have a emission maximum at 659 nm while PC hexamers emit at 651 nm. As I mentioned earlier, the wild-type emission spectrum has only one emission band present, at approximately 683 nm. The rate of back transfer has slowed enough that emission from only the lowest energy chromophores occurs.

The emission spectra obtained for the *apcDE/C186S* and *apcE/C186S* PBS, shown in Figure 4-4a, are vastly different from that of the wild-type PBS. Instead of the one emission band present at 683 nm seen in the wild-type spectrum, the *apcDE/C186S* and *apcE/C186S* spectra contain three emission bands at approximately 645 nm, 680 nm, and 715 nm. The 645 nm band is due to PC, and the 715 nm band is from the modified chromophore. The 680 nm band is from β^{18} emission in the *apcDE/C186S* mutant and β^{18} and/or α^{AP-B} emission in the *apcE/C186S* mutant.

There is a relative increase in the amount of PC emission compared to 680 nm emission in the *apcDE/C186S* and *apcE/C186S* emission spectra. I don't know whether the absolute amount of PC emission increases in the PBS from the mutants because the quantum yields couldn't be calculated.

The *apcD*, *apcF*, and *apcDF* low-temperature emission spectra are similar to that of the wild-type as shown in Figure 4-4b. The *apcD* emission spectrum matches the wild-type spectrum very well except for slightly less emission at about 690 nm. The *apcF* and

Figure 4-4a. Low temperature emission spectra of intact PBS. The PBS were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set to a 6 - 7 nm resolution while the emission monochromator resolution was between 0.1 and 0.4 nm. The spectra were collected within 12 hours after the PBS were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 2.0 M sucrose. The diluted samples were put under vacuum for 10 min and then frozen in N₂(l). The emission maxima are given in Table 4-1.

Figure 4-4b. Low temperature emission spectra of intact PBS
 The PBS were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set to a 6 - 7 nm resolution while the emission monochromator resolution was between 0.1 and 0.4 nm. The spectra were collected within 12 hours after the PBS were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 2.0 M sucrose. The diluted samples were put under vacuum for 10 min and then frozen in N₂(l). The emission maxima are given in Table 4-1.

apcDF spectra are indistinguishable from each other within the variability of the experiment. The apcF and apcDF spectra are blue-shifted from that of the wild-type by 2 nm.

Time-Resolved Fluorescence Emission

The kinetics of energy transfer from one pigment pool to another within the PBS complex can be elucidated using time-resolved fluorescence emission. Any major impairment in the energy transfer characteristics of the PBS antenna should be discernible using single photon timing because the measured decay rates at different wavelengths should change.

I begin this section of the chapter by discussing some of the theory behind the energy transfer kinetics in PBS. Holzwarth has written several reviews on the energy transfer in PBS with more details (Holzwarth, 1991; Holzwarth, 1986). The kinetics of energy transfer in the PBS measured by single photon timing indicates that excitation energy within pools of PC or AP very rapidly equilibrates onto the lowest energy chromophores. The transfer of energy within pools of the same pigment occurs on a timescale too fast for us to detect using single photon timing. Energy transfer between pools of dissimilar pigments, such as PC to AP, occurs on a longer timescale such as 25 to 100 ps. This energy transfer step is the rate-limiting step measured using this method of detection.

A model for the energy transfer processes in PBS is given in Figure 4-5 taken from a Holzwarth review (Holzwarth, 1986). I have modified the scheme by adding in boldface print the rates I measured for the wild-type PBS from *Synechococcus* sp. PCC 7002.

Figure 4-5. Model of Energy Transfer in Intact PBS.
 The transfer times I measured are in boldface print. Model taken from Holzwarth, 1986.

In this model, most of the longer lived emission originates from the terminal emitters. The Boltzmann calculations from earlier in the chapter indicate that as long as the chromophores are coupled for energy transfer, back transfer between the different chromophore pools is significant at RT. Therefore, I think that the two lifetimes attributed to the terminal emitters in Holzwarth's model really apply to all of the coupled chromophores, including PC, AP, and the terminal emitters. Because the energy transfer times are much shorter than the decay of the fluorescence, the energy should be equilibrated throughout the PBS complex before much emission occurs.

A competing model has been presented by Glazer, Yeh et al. based upon data obtained from *Synechocystis* 6701 PBS (Glazer, Yeh et al., 1985; Glazer, Chan et al., 1985). In their model, energy transfer between the chromophores within each subunit is essentially instantaneous with the disk-to-disk transfer step being the rate-limiting transfer process. In this model, they describe the phycobilisome as a lattice of approximately 625 chromophores in which energy transfer can be described as a linear five-point array. The significant differences between the Glazer and Holzwarth models is examined in the Holzwarth reviews.

Time-resolved emission decays were collected for each PBS type, and decay-associated spectra (DAS) were calculated for each set of data, as shown in Figures 4-6a to 6f. As discussed in Chapter 2, each decay was fit to a sum of exponentials using global analysis. At least three components were required for a good fit. The fluorescence decays for different strains were collected using

Figure 4-6a. Decay-associated spectra of wild-type PBS. The PBS were excited at 615 nm at RT. The tac was set to 14.0 ps per channel. The DAS were obtained from global analysis.

Figure 4-6b. Decay-associated spectra of apcD PBS. The PBS were excited at 626 nm at RT. The tac was set to 15.6 ps per channel. The DAS were obtained from global analysis.

Figure 4-6c. Decay-associated spectra of apcF PBS. The PBS were excited at 600 nm at RT. The tac was set to 11.3 ps per channel. The DAS were obtained from global analysis.

Figure 4-6d. Decay-associated spectra of apCDF PBS. The PBS were excited at 614 nm at RT. The tac was set to 14.0 ps per channel. The DAS were obtained from global analysis.

Figure 4-6e. Decay-associated spectra of apcE/C186S PBS. The PBS were excited at 580 nm at RT. The tac was set to 11.8 ps per channel. The DAS were obtained from global analysis.

Figure 4-6f. Decay-associated spectra of apcDE/C186S PBS. The PBS were excited at 616 nm at RT. The tac was set to 11.6 ps per channel. The DAS were obtained from global analysis.

excitation wavelengths ranging from 580 to 625 nm. Within this range of excitation wavelengths, the decay components required to fit the data for each strain did not seem to change, although the relative yield of each component did vary with excitation wavelength. This conclusion is based on examination of wild-type DAS obtained at many different wavelengths.

The time-resolved data that I obtained for the wild-type PBS are similar to those found in the literature for intact PBS from *Mastigocladus laminosus* (Hefferle, Nies et al., 1983) and *Synechococcus* 6301 (Gillbro, Sandström et al., 1985; Suter, Mazzola et al., 1984). The data in the literature were obtained for single fits at different excitation and emission wavelengths; no global analysis was applied. From the Gillbro paper, excitation at 581 or 620 nm with 680 nm emission gave fluorescence decays which required three components of approximately 0.05 ns, 1.4 ns, and 2 ns for a good fit. For the same excitation and emission wavelengths used in the Gillbro paper, the Suter paper reported three components of 0.13 ns, 1.0 ns, and 1.9 ns. The Hefferle study of *M. laminosus* reported lifetimes of around 0.1 and 1.4 ns. This study used streak camera detection which was why they probably couldn't resolve the longest-lived component. Feis, Friedrich *et al.* also studied energy transfer in PBS from *M. laminosus* using hole-burning techniques; they found an energy transfer time of 147 ps from AP to the terminal emitters (Feis, Friedrich et al., 1992).

As I mentioned earlier in the chapter, a certain amount of emission is expected from the PC and AP chromophores at RT due to the large populations of PC and AP chromophores. Because these

chromophores are coupled with the terminal emitters, their emission will decay with the same time constant as the terminal emitters after a certain equilibration time (approximately 0.15 ns is needed for equilibration between the wild-type chromophores). This is why the lifetime components assigned to the terminal emitters span the emission spectrum from 640 to 690 nm, as shown in Figure 4-6a.

The apcD, apcF, apcDF, and wild-type PBS decays all required three components for good fits. The apcD and wild-type DAS, Figures 4-6a,6b, are indistinguishable from each other with each DAS containing three components of approximately 2.9 ns, 1.6 ns, and 0.15 ns. Both DAS contain a 0.15 ns component, which has a positive amplitude at about 650 nm and a negative amplitude at 680 nm. The negative amplitude or "risetime" indicates energy transfer to the 680 nm emitters from AP and PC in the PBS. One can think of the risetime as the time required for equilibration of energy between the different chromophore types.

The apcF and apcDF DAS are indistinguishable from each other and only slightly different from that of the wild-type, Figures 4-6c,6d. The two long-lived decay components found in the apcF and apcDF DAS, assigned as the lifetimes of the terminal emitters, are slightly shorter than those of the wild-type. The shortest component, 0.14 ns, is very similar to that of the wild-type. The mutations didn't cause any differences in the equilibration time between the chromophores.

The DAS spectra for the apcDE/C186S and apcE/C186S mutants are altered from that of the wild-type; a minimum of four

components was necessary to obtain good fits for the decays. The DAS obtained for the apcDE/C186S PBS did vary somewhat with preparation. The apcDE/C186S PBS were a little more susceptible to dissociation than the other mutants.

The two longer-lived decay components found in the apcDE/C186S and apcE/C186S DAS of approximately 2.5 and 1 ns are assigned to the terminal emitters. The shortest decay component, approximately 0.06 ns, has no counterpart in the wild-type DAS, and it is of very low yield. The remaining decay component of 0.17 ns in apcE/C186S DAS and 0.4 ns in apcDE/C186S DAS is similar to the 0.15 ns component found in the wild-type DAS.

The modified chromophore found in the apcDE/C186S and apcE/C186S PBS appears to decay with a lifetime of approximately 1 ns. It still is coupled to other chromophores in the PBS as shown by the emission of the 1 ns component from 640 to 680 nm and the 0.17 ns risetime present at 715 nm.

Conclusions

PBS from the mutants dissociated easier under the conditions for their isolation than the wild-type PBS. The apcE/C186S and apcDE/C186S PBS proved to be the most unstable. This was determined by looking at the amount of free phycobiliprotein present on the sucrose gradient.

The absorption spectra of the apcD and apcF PBS are very similar to that of the wild-type PBS, but the absorption spectra for the apcDF, apcDE/C186S, and apcE/C186S PBS display less

absorption on the red edge of the spectrum than the wild-type PBS. The difference spectra for each of the mutants confirm that conclusion.

The difference spectra also indicate that PBS from the *apcDE/C186S* and *apcE/C186S* mutants absorb less at approximately 675 to 680 nm (due to the lack of normal L_{cm} chromophore) than the wild-type but more at 695 nm (due to the addition of the modified chromophore). The difference spectra for the other mutants are more inconclusive; the magnitude of the differences are smaller. I will examine the absorption spectra of the core preparations in the next chapter to see if the differences are consistent.

The RT emission spectra for the *apcD*, *apcF*, and *apcDF* PBS were slightly red-shifted from that of the wild-type PBS. The differences could be explained qualitatively by calculating the equilibrated excited state populations using a Boltzmann distribution. When the antenna complex loses two or four of the six 680 nm emitters, the equilibrium populations of the excited chromophores change. This results in more steady state emission originating from PC and AP chromophores.

The *apcD*, *apcF*, and *apcDF* emission spectra qualitatively illustrated this result. The *apcD* and *apcF* PBS are missing one of the three 680 nm emitters, and their emission spectra are blue-shifted about 10 nm from that of the wild-type. The *apcDF* PBS is missing two of the three 680 nm emitters, and its emission spectrum is blue-shifted by about 15 nm from that of the wild-type. The changes in emission spectra of the PBS from the *apcD*,

apcF, and apcDF strains appear to be described by the Boltzmann calculations.

The Boltzmann calculations did not work well for the apcDE/C186S and apcE/C186S mutants. The fluorescence yield from the modified chromophore is low; it was found to be only 30% instead of the calculated 75% of the total yield. The Boltzmann predictions could break down for the modified chromophore because the chromophore undergoes more nonradiative deactivation. The modified chromophore acts to quench the emission from the PBS antenna such that the quantum yield of fluorescence is only 50% of the wild-type.

Also, the Boltzmann calculations predict only small amounts of 680 nm emission should be present in the apcE/C186S and apcDE/C186S spectra assuming all the chromophores are coupled. No 680 nm emission band could be resolved in the apcDE/C186S or apcE/C186S spectra. This indicates that the 680 nm emitters present are coupled to the 715 nm emitter; the α^{AP-B} and β^{18} chromophores transfer energy to the modified chromophore. This result is contrary to what Mimuro, Yamazaki et al. found. They reported that at least two of the terminal emitters are independent of each other (Mimuro, Yamazaki et al., 1989).

At 77K, the back transfer of energy from the 680 nm emitters to the other chromophores is much less important. Only the terminal emitters should fluorescence at 77 K if all the chromophores are coupled. This is the case in the wild-type, apcD, apcF, and apcDF emission spectra. The majority of the emission appears to be from one chromophore type.

The low temperature emission spectra from the *apcDE/C186S* and *apcE/C186S* PBS are much more complicated; more than one emission band is present. At 77 K, emission is seen from the 680 nm emitters and from the modified chromophore along with a third emission band from dissociated PC. It is possible that the PBS from these mutants undergo structural rearrangement when frozen, but I favor another explanation. The spectral overlap between the absorption spectrum of the 680 nm emitter and the emission spectrum of the 715 nm emitters decreases at 77 K because both the absorption and emission spectra narrow with lowering temperature. Because the overlap integral decreases, energy transfer between the two chromophore type is less favorable, and more emission is seen from the 680 nm emitter.

From the low temperature data, I assign emission maxima to the different chromophores. The β^{18} chromophore emits at 678 nm in the absence of the L_{cm} chromophore, as shown by the *apcDE/C186S* spectrum. The α^{AP-B} chromophore emits around 682 nm, as shown by the *apcE/C186S* spectrum. Therefore, from the wild-type spectrum, the wild-type L_{cm} chromophore must emit at 683 nm.

The wild-type and *apcD* emission spectra are almost indistinguishable from each other, as are the *apcF* and *apcDF* spectra. I can explain the differences in the *apcD*, *apcF*, and *apcDF* spectra by attributing the emission at low-temperature to mainly the L_{cm} chromophore. As shown in the wild-type emission spectrum, the L_{cm} chromophore emits at 683 nm when the PBS core is intact with all the polypeptides present. As I will show in the next chapter, the emission spectrum of L_{cm} blue-shifts in the

absence of the β^{18} subunit. Therefore, the low-temperature emission spectra of the apcF and apcDF PBS are slightly blue-shifted from that of the wild-type because the β^{18} subunit is missing.

The α^{AP-B} chromophore emits at a higher energy than the L_{cm} chromophore, so the α^{AP-B} chromophore must transfer its energy to the L_{cm} chromophore at 77 K. Bryant has suggested that the α^{AP-B} subunit is active in transferring energy to the PSI reaction center (Bryant, personal communication). If this hypothesis is correct, then the α^{AP-B} chromophore might only play a role in energy transfer in the PBS under high light conditions.

In the time-resolved fluorescence data, the apcD and wild-type PBS were indistinguishable from each other, as were the apcDF and apcF PBS. The results from the apcF and apcDF PBS were only slightly changed from that of the wild-type with the longest lived decay components shortened slightly. This could possibly be due to a small degree of structural disorganization in the PBS core of the apcF and apcDF PBS.

The time-resolved data obtained for the apcDE/C186S and apcE/C186S mutants is altered from that of the wild-type. The longest lived components were somewhat shorter than those seen in the wild-type DAS, while the shortest component resolved in the apcDE/C186S and apcE/C186S DAS has no counterpart in the wild-type DAS. This component possibly indicates impaired energy transfer from the rods to the core. The process is slowed enough that it is now detectable using single photon timing.

From the time-resolved emission work, I assign a lifetime of approximately 1 ns to the modified chromophore and show that the

modified chromophore is coupled to the other chromoproteins present, as shown by the presence of a risetime at 715 nm. There is other evidence of AP and PC coupling to the modified chromophore; AP and PC emission decays with the same 1 ns lifetime.

In the next chapter I will examine the spectral characteristics of a subcore preparation which is missing the $\alpha^{\text{AP-B}}$ chromophore. I will also discuss the evidence of an interaction between the L_{cm} and β^{18} chromophores.

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Chapter 5. Discussion of 18 S Preparation Results

In the last chapter I examined the absorption and emission characteristics of the intact PBS antenna for wild-type and mutant strains. In this chapter I will discuss the spectral properties of a subcore preparation, the 18 S preparation, in which only a fraction of the core is present with a small amount of PC.

According to the paper by Gingrich, Lundell, and Glazer in which they isolate an 18 S preparation from *Synechocystis* 6701, a cyanobacterium containing PBS with tricylindrical cores, the relative ratios of α^{AP} : β^{AP} : α^{PC} : β^{PC} : L_{cm} is approximately 6: 6: 3: 3: 1 (Gingrich, Lundell, et al., 1983). No α^{AP-B} appeared to be present in their preparation. Also, even though they didn't explicitly say that β^{18} is present, based upon work done with *Synechococcus* 6301, I am assuming that the β^{18} subunit is present in the same concentration as the L_{cm} (Yamanaka, Lundell, et al., 1982; Lundell and Glazer, 1983). This assumption should be valid because the L_{cm} and β^{18} polypeptides are in the same trimer.

I decided to use this preparation to study the spectral properties of the β^{18} and L_{cm} chromophores because the emission spectrum for the subcore preparation was found to be very similar to that of intact PBS (Sandström, Gillbro, et al., 1988).

I ran samples of wild-type and *apcF* 18 S preparations on a HPLC column using the procedure developed by Swanson and Glazer (Swanson and Glazer, 1990). I was able to confirm the presence of both AP subunits, both PC subunits, the L_{cm} polypeptide, and two colorless polypeptides of approximately 30 kD. The wild-type

preparation also contains an additional chromopeptide present at approximately the same concentration as the L_{cm} polypeptide. I found this protein to be approximately 18 kD in molecular weight using SDS-PAGE, and I identified it to be the β^{18} subunit. The apcF preparation was missing that polypeptide.

I did not find any polypeptide corresponding to the α^{AP-B} subunit to be present in the 18 S preparations I ran on the HPLC. I was not able to get consistent data on the relative amounts of β^{AP} and α^{AP} present in the preparations. From the HPLC data I couldn't determine if a β^{AP} subunit is substituting for the missing β^{18} subunit in the apcF preparation.

The modified chromophore present in the apcDE/C186S and apcE/C186S cells and intact PBS is missing in the 18 S preparation. Therefore, the 18 S preparation should be composed only of PC, AP, and the β^{18} chromophores for the apcDE/C186S and apcE/C186S preps and only PC, AP, and L_{cm} chromophores for the apcDF and apcF preps. The absence of the modified chromophore is not unexpected since it was thought to be only noncovalently associated with the PBS antenna.

I successfully prepared 18 S preparations for all of the mutants, but the yield of the preparation from the apcDE/C186S cells was consistently low. It also was contaminated with PC. I did not carry out any time-resolved fluorescence studies on the apcDE/C186S mutant because of the lack of sufficient sample, but I do have steady state absorption and emission data for the apcDE/C186S preparation.

I would like to emphasize one more point before discussing the data. Since the α AP-B is absent from the preparation, I expect the compositions of the apcD and wild-type preparations to be identical. The same should hold true for the apcF and apcDF preparations and the apcDE/C186S and apcE/C186S preparations. You will notice that spectral characteristics of the preparations are not always consistent. I concentrated my energies in preparing good samples of wild-type, apcF, and apcE/C186S preparations as judged by the 77 K emission spectra, and the quality of spectral data from the other three mutants is questionable at times.

Absorption

As I did in Chapter 4 with intact PBS, I make the assumption that the absorption of the chromophores in the 18 S preparation is additive. The absence of a chromophore will leave a hole in the absorption spectrum of the preparation. The absorption spectra of the 18 S core preparations are shown in Figure 5-1a,1b. I found some variability in the absorption spectra of my 18 S preparations. The absorption maxima for different preparations of the same strain differed by as much as 2 nm. The spectra displayed in Figures 5-1a,1b are an average of spectra from different preparations weighted equally. I averaged the spectra to get a more meaningful difference spectra as I will discuss later.

All of the preparations have absorption maxima around 653 nm, with the exact position given in Table 5-1. As shown by the location of the absorption maximum, I was successful in isolating a PBS fraction that contains mostly AP. The preparation isolated from

Figure 5-1a. Averaged absorption spectra of 18 S preparations. Averaged 5 apcE/C186S spectra, 2 apcDE/C186S spectra, and 4 wild-type spectra from different preparations. Samples were taken directly off of sucrose gradients without dilution. The spectra were taken at RT. The absorption maxima are given in Table 5-1.

Figure 5-1b. Averaged absorption spectra of 18 S preparations. Averaged 4 apcF spectra, 3 apcD spectra, 3 apcDF, and 4 wild-type spectra from different preparations. Samples were taken directly off of sucrose gradients without dilution. The spectra were taken at RT. The absorption maxima are given in Table 5-1.

Table 5-1

18 S Preparation Spectral Data

	Absorption Maximum	Room Temperature Emission Maximum	Low Temperature Emission Maximum
wild-type	653 nm	668 nm	682.5 nm 653 nm
apcD	653 nm	668 nm	681.5 nm 653 nm
apcF	651 nm	662 nm	676 nm 651.5 nm
apcDF	652 nm	666 nm	672.5 nm 653 nm
apcE/C186S	653 nm	666 nm	671 nm 646 nm
apcDE/C186S	653 nm	665 nm	670.5 nm 647.5 nm

Synechocystis 6701 was reported to contain a 2: 1 ratio of AP to PC (Gingrich, Lundell, et al., 1983). The shoulder at approximately 600 nm is due to PC absorption with a small contribution from AP. The relative height of the shoulder reflects the amount of PC present in the sample. This point is especially well illustrated in Figure 5-1a; the apcDE/C186S spectrum shows a much larger absorption shoulder at 600 nm due to large amounts of accompanying PC.

The important part of the absorption spectrum in this study is the region from about 655 to 700 nm, the red-edge of the spectrum. As shown in Figure 5-1a, the red-edge of the apcE/C186S and apcDE/C186S absorption bands are indistinguishable from each other. Both show an absorption decrease at about 670 nm compared to the wild-type preparation.

The spectra for the apcD, apcF, and apcDF preparations are shown in Figure 5-1b. The apcDF and apcF absorption spectra are very similar to each other, and the apcD and wild-type spectra are indistinguishable from one another. I found the apcF and apcDF absorption maximum to be consistently blue-shifted from that of all the other strains, as shown in Table 5-1. The blue shift could be partially due to increased PC content, but it also could be due to a structural change in the PBS core. Only the apcF and apcDF preparations are missing an entire polypeptide. The apcDE/C186S and apcE/C186S preparations are missing a chromophore, but the polypeptide is still there. Therefore, I expect to see a larger effect on the absorption spectrum in the apcF and apcDF preparations. The assumption about the additivity of the chromophores in the apcF and apcDF preparations may no longer hold.

As with the PBS absorption spectra from Chapter 4, I have calculated difference spectra for the preparations from the mutants. The difference spectra are calculated from an average absorption spectrum obtained for each preparation. I normalized each spectrum to an equal area and then subtracted the spectrum of the mutant from that of the wild-type preparation. Once again, I make the assumption that the preparations contain equal numbers of chromophores. This assumption breaks down when PC contamination is present, as in the *apcDE/C186S* preparation. I also calculated second derivatives of the difference spectra to show the location of the peaks in the spectra.

In Figure 5-2a, I have displayed the difference spectrum for the *apcDE/C186S* and *apcE/C186S* preparations. The difference spectra are hard to interpret because the preparations contain different amounts of PC. I made the assumption that the number of chromophores was constant to calculate the difference spectra, but the *apcDE/C186S* preparation contains more PC relative to AP than the wild-type. Therefore, in the *apcDE/C186S* difference spectrum, PC contamination will show up as negative peaks at 580 and 630 nm (PC absorption) along with a positive peak at 655 nm (AP absorption). Except for the *apcD* mutant, all of the mutants show signs of increased PC content.

Taking the distortion due to differing amounts of PC present into consideration, the *apcE/C186S* and *apcDE/C186S* difference spectra are indistinguishable. This result is also shown by the appearance of the second derivative of the spectrum. Both spectra show that

Figure 5-2a. Absorption difference spectra of 18 S preparations
 Normalized averaged spectra in Figure 5-1a to equal area; mutant subtracted from wild-type. The smoothed second derivative of the absorption difference is displayed in the insert.

Figure 5-2b. Absorption difference spectra of 18 S preparations
 Normalized averaged spectra in Figure 5-1b to equal area; mutant subtracted from wild-type. The smoothed second derivative of the absorption difference is displayed in the insert.

the preparations from the mutants absorb less at 671 nm than the wild-type.

The difference spectra calculated for the *apcD*, *apcF*, and *apcDF* preparations are shown in Figure 5-2b along with the corresponding second derivatives. The *apcD* absorption spectrum is indistinguishable from that of the wild-type, as illustrated by the difference spectrum. The *apcF* and *apcDF* spectra are more similar to those obtained from the *apcDE/C186S* and *apcE/C186S* preparations. As shown earlier in the *apcDE/C186S* spectrum, the *apcF* difference spectrum also shows the presence of an increased PC to AP ratio relative to that of the wild-type, with two negative peaks at 580 and 630 nm. The *apcF* difference spectrum has a large positive peak at approximately 660 nm, which is at least partially due to the increased PC to AP ratio.

The difference spectrum obtained for the *apcDF* preparation looks very similar to those obtained for the *apcDE/C186S* and *apcE/C186S* preparations, except that the shoulder on the 650 nm peak is closer to 680 nm rather than 670 nm as easily seen in the second derivative of the difference. There is some similarity between the second derivatives of the *apcF* and *apcDF* spectra. Both second derivatives show the presence of a shoulder at about 680 nm.

Room Temperature Emission

As discussed in Chapter 4, the room temperature steady state emission spectrum is a reflection of not only the types of chromophores present within the PBS but also of the number of

each type present. If the chromophores are coupled, back transfer is important at room temperature such that significant amounts of emission will originate from PC and AP in the PBS. This is especially true because larger numbers of PC and AP chromophores are present relative to the 680 nm emitters in the PBS. The same should hold true for the 18 S preparations.

I made some Boltzmann calculations as in Chapter 4 using the ratios of AP: PC: 680 nm emitter as 8:4.5:1 for the wild-type preparation along with the assumption that the β^{18} and L_{cm} chromophores emit at 680 nm. I decided on these ratios of chromophores because it seemed likely that the 18 S preparations consist of the 6 inner AP trimers of the PBS core along with about 3 PC trimers from the rods. I did not find any signs of that the capping L^8 is present in the 18 S preparation from the HPLC data. This would indicate that my core preparation is missing the AP trimers on the ends of the core cylinders. I also assumed that all AP and PC chromophores are equal in energy, and I did not allow for the small differences in the spectral properties of chromophores within each pool.

The Boltzmann calculations show that approximately 60% of the emission from the wild-type 18 S preparations should originate from the 680 nm emitters with most of the rest coming from AP. Only 2% of the emission should originate from PC chromophores if all of the chromophores are coupled for energy transfer. There is a larger PC contribution than expected in the wild-type spectrum, probably from dissociated PC hexamers contaminating the

preparation, but the ratio of AP to 680 nm emission is approximately right.

The emission spectra of the preparations from the mutant and the wild-type strains are shown in Figure 5-3a,3b. In Figure 5-3a, the apcDE/C186S and apcE/C186S emission spectra are almost indistinguishable except that the apcDE/C186S spectrum shows the presence of greater amounts of emission free PC. Boltzmann calculations for the apcDE/C186S and apcE/C186S complexes indicate that 42% of the emission should originate from the remaining 680 nm emitter (the β^{18} subunit) with 55% from AP. The amount of emission originating from the 680 nm emitter in the apcDE/C186S and apcE/C186S spectra appears to be less than 25%, which probably indicates that the β^{18} subunit is emitting at a higher energy than 680 nm.

In Figure 5-3b I have displayed the room temperature emission spectra obtained for the apcD, apcF, and apcDF 18 S preparations. The apcD spectrum is very similar to that of the wild-type; differences between the two spectra can be attributed to a lesser quality apcD preparation. The apcDF and apcF emission spectra were quite different, with the apcF spectrum displaying relatively more AP emission than 680 nm emission.

Boltzmann calculations were also carried out for the apcF and apcDF preparations. I made the assumption that an AP chromophore substituted for the missing β^{18} chromophore. The results of the Boltzmann calculations were very similar to those for the apcDE/C186S and apcE/C186S preparations, with 57% of the emission originating from AP, 40% from 680 nm emitters, and 3%

Figure 5-3a. Steady state emission spectra of 18 S preparations
 The samples were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set between 6 and 3 nm resolution while the emission monochromator resolution was between 0.4 and 0.8 nm. The spectra were collected within 2 hours after the preparations were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 1.0 M sucrose in 50 mM tricine with 5 mM CaCl_2 and 10% (v/v) glycerol, pH 7.8. The spectra were taken at RT. The emission maxima are given in Table 5-1.

Figure 5-3b. Steady state emission spectra of 18 S preparations
 The samples were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set between 6 and 3 nm resolution while the emission monochromator resolution was between 0.4 and 0.8 nm. The spectra were collected within 2 hours after the preparations were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 1.0 M sucrose in 50 mM tricine with 5 mM CaCl_2 and 10% (v/v) glycerol, pH 7.8. The spectra were taken at RT. The emission maxima are given in Table 5-1.

from PC. The apcDF spectrum qualitatively fits the calculations while the apcF spectrum displays too much PC emission.

Low Temperature Emission

AT 77 K back transfer between different chromophore pools within the 18 S preparation is very unlikely. All of the emission should originate from the lowest energy chromophores if the chromophores are coupled for energy transfer. I assume that freezing the 18 S preparations does not cause any significant structural changes within the complex such that energy transfer is impaired. As I mentioned before, the only change I expect to see is that the number of populated energy levels will decrease at 77 K relative to room temperature. Both the emission and absorption bands narrow. I froze the 18 S preparations in the same way I froze the intact PBS.

In Figure 5-4a,4b I have displayed the 77 K emission spectra obtained for the 18 S preparations. All of the spectra contain two different emission bands; one from PC at about 650 nm with a second one from the lowest energy chromophore present at 670 to 683 nm. The emission maxima are given in Table 5-1. I found the apcD and wild-type spectra to be indistinguishable as were the apcDE/C186S and apcE/C186S spectra. The apcDF and apcF spectra are not identical.

Starting with Figure 5-4a, the main band of emission in the apcDE/C186S and apcE/C186S spectra is considerably blue-shifted from that of the wild-type, at 671 nm compared to 682 nm. The apcDE/C186S and apcE/C186S preparations are missing the L_{cm}

Figure 5-4a. Low temperature emission spectra of 18 S preparations
 The samples were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set to a 6 - 7 nm resolution while the emission monochromator resolution was between 0.1 and 0.4 nm. The spectra were collected within 3 hours after the preparations were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 2.0 M sucrose. The diluted samples were put under vacuum for 10 min and then frozen in N₂(l). The emission maxima are given in Table 5-1.

Figure 5-4b. Low temperature emission spectra of 18 S preparations. The samples were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set to a 6 - 7 nm resolution while the emission monochromator resolution was between 0.1 and 0.4 nm. The spectra were collected within 3 hours after the preparations were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 2.0 M sucrose. The diluted samples were put under vacuum for 10 min and then frozen in N₂(l). The emission maxima are given in Table 5-1.

chromophore. Any emission coming from the complex should originate from the β^{18} chromophore, the lowest energy chromophore remaining. In the last chapter, I assigned the β^{18} chromophore to have an emission maximum at 678 nm; this was in intact PBS from the apcDE/C186S cells which contained the modified chromophore. One must infer that the β^{18} chromophore is very sensitive to the integrity of the PBS core.

The apcDF and apcF emission spectra shown in Figure 5-4b also display a blue-shifted emission band compared to that of the wild-type; the apcDF preparation emits at 673 nm while the apcF preparation emits at 676 nm. This is the opposite of what was found with the room temperature emission spectra where the apcDF preparation emits further red than that of the apcF. The apcF preparation also does not contain any greater relative amounts of PC as shown by the height of the emission band at 653 nm. Once again, one can only come to the conclusion that the assumption that the apcF and apcDF preparations have the same composition is false.

From the apcF spectrum, the main emission band is shifted about 6 nm further blue than in the wild-type spectrum. In the apcF preparation the β^{18} chromophore is missing and probably substituted with an AP chromophore. Therefore, the lowest energy chromophore present would be the L_{cm} chromophore; in the apcF preparation the L_{cm} chromophore emits at 676 nm. This is also further blue-shifted than expected.

Time-Resolved Fluorescence Emission

As with the PBS preparations, I obtained time-resolved fluorescence data for the mutant and wild-type preparations with the exception of the *apcDE/C186S* strain. The *apcDE/C186S* 18 S preparations never worked out well enough to obtain time-resolved data; either the yield was too low, or the preparation was too contaminated with PC. I would expect the time-resolved data obtained for the *apcE/C186S* 18 S preparation to be similar to that obtained for the *apcDE/C186S* preparation because their steady state spectral properties have been indistinguishable up to this point.

As with the intact PBS, I expect to see emission not only from the terminal emitters in the preparation but also from other chromophores present because energy transfer from terminal emitters to AP will occur at RT. As I discussed in the steady state RT emission section of this chapter, in the wild-type spectrum almost 40% of the emission originates from AP in the preparation. Since the AP chromophores are coupled to the 680 nm emitters, they will decay with the same time constant.

The important time constant obtained from this study is the time needed for the energy to equilibrate over the antenna complex (the risetime for emission to occur at the terminal emitter). In the intact wild-type PBS, I measured it to be 0.15 ns. Assuming that all the chromophores are still coupled, the equilibration time for the 18 S preparations should be shorter than 0.15 ns. I expect this result because fewer chromophores are present in the core complex, and

fewer transfer steps are needed to transfer the energy to the 680 nm emitters.

Decay-associated spectra were obtained for each of the 18 S core preparations and are presented in Figures 5-5a through 5-5e. The samples were excited at 633 or 634 nm, and emission was collected for almost 12 ns after the exciting pulse. The decays were fit to a minimum of three components for the wild-type, *apcD*, *apcF*, and *apcDF* preparations and four components for the *apcE/C186S* preparation.

As shown in Figure 5-5a, the wild-type 18 S preparation displays very similar kinetics to that of wild-type PBS, Figure 4-4a. The 18 S preparation appears to be a good model for studying the energy transfer properties of PBS except that it is somewhat unstable. Time-resolved fluorescence work was carried out on *Synechococcus* 6301 18 S preparations by Sandström and others using single photon timing (Sandström, Gillbro, et al., 1988). Using global analysis for several different excitation wavelengths, they obtained four lifetimes of 22, 60, 1045, and 1810 ps. The yield of the 22 ps component was very small for 640 nm excitation, which probably is why I didn't resolve this component in my fluorescence data. They also reported results that they obtained with 580 nm excitation and 675 nm emission. Without using global analysis, they resolved three components of 2.7, 1.8, and 0.05 ns; lifetimes quite similar to the ones I obtained from global analysis for the wild-type preparation. They concluded that the "18 S preparation is a functional entity of the PBS in terms of energy transfer

Figure 5-5a. Decay-associated spectra of the wild-type 18 S preparation. The samples were excited at 633 nm at RT. The tac was set to 11.6 ps per channel. The DAS were obtained from global analysis.

Figure 5-5b. Decay-associated spectra of the apcD 18 S preparation. The samples were excited at 633 nm at RT. The tac was set to 11.6 ps per channel. The DAS were obtained from global analysis.

Figure 5-5c. Decay-associated spectra of the apcF 18 S preparation. The samples were excited at 634 nm at RT. The tac was set to 11.6 ps per channel. The DAS were obtained from global analysis.

Figure 5-5d. Decay-associated spectra of the apcDF 18 S preparation. The samples were excited at 634 nm at RT. The tac was set to 11.6 ps per channel. The DAS were obtained from global analysis.

Figure 5-5e. Decay-associated spectra of the apcE/C186S 18 S preparation. The samples were excited at 633 nm at RT. The tac was set to 11.6 ps per channel. The DAS were obtained from global analysis.

kinetics". I also found that the fluorescence decay data for the wild-type PBS and 18 S preparations are very similar.

For the wild-type preparation I resolve three components of 0.078 ns, 1.45 ns, and 2.72 ns, shown in Figure 5-5a. The 0.078 ns component corresponds to the time needed for the energy to be equilibrated over the complex. The longer lifetimes correspond to the fluorescence lifetimes of the terminal emitters along with the AP and PC chromophores coupled to the terminal emitters.

I found that the time-resolved emission data is very sensitive to sample integrity. If the sample is degraded such that the β^{18} and L_{cm} chromophores are no longer coupled, there is the appearance of 673 nm emission in the 77 K emission spectra. The samples which display the 673 nm emission also have DAS in which the shortest lifetime component is longer than usual. This is illustrated with the apcD DAS in Figure 5-5b; the equilibration time is more than four times that found in the wild-type DAS. This longer lifetime is characteristic of the degraded samples. This component also has a unique spectrum because it has only negative amplitude. The unique spectrum makes time-resolved fluorescence a good diagnostic tool to determine the integrity of 18 S preparations.

If the chromophores within the complex are in a different structural organization, or if the complex is partially dissociated, it will take longer for energy to travel from PC to the lowest energy chromophores because angles between transition dipoles or distances between chromophores are altered. Therefore, it will take longer for the energy to equilibrate over the complex, and the measured risetimes will be longer. Also, if the complex is not as

intact structurally as the wild-type, the lifetimes of the chromophores could decrease if the chromophores are not being held in optimal conformations. Therefore, by comparing both the risetimes and the decay times of the chromophores from the 18 S preparations of the wild-type and the mutants, one can get some idea of the structural integrity of the complex along with the degree of coupling between the chromophores.

The DAS obtained for the *apcDF* and *apcF* 18 S preparations are indistinguishable from each other. Both sets of decays required three components of approximately 2.4, 1.3, and 0.18 ns. The two longer components are slightly shorter than those obtained for the wild-type preparations, and the shortest component is twice as long as that for the wild-type.

The DAS obtained for the *apcE/C186S* preparation is shown in Figure 5-5e; a fourth decay component is always necessary to fit the decay data. The two longest decay components obtained for the *apcE/C186S* data are shorter than that of the wild-type, with lifetimes of 2.3 and 1.2 ns versus 2.7 and 1.45 ns.

The two shortest lifetime components resolved in the *apcE/C186S* data are 0.05 ns and 0.5 ns; both components have negative amplitudes from 660 to 680 nm. I don't believe that the 0.5 ns component is due to degraded sample because the component does have positive amplitude from 640 to 650 nm, unlike the 0.36 ns component found in the *apcD* DAS.

Discussion

The study of the 18 S preparations has proved to be a worthwhile task. I was successful in obtaining a subcore preparation of the PBS which is missing the α^{AP-B} subunit and most of the PC as shown by HPLC. The apcD and wild-type preparations and the apcE/C186S and apcDE/C186S preparations were indistinguishable as from each as expected. The spectral data on the apcF and apcDF preparations is harder to interpret because the apcF and apcDF preparations have different absorption and emission spectra. This probably means that the apcF and apcDF preparations have different compositions.

The apcD Preparation

As expected, the apcD preparation is virtually indistinguishable from that of the wild-type. The absorption and emission spectra from the apcD preparation are very similar to those of the wild-type. The small differences in the spectra can be attributed to experimental variability. The apcD and wild-type preparations appear to be of the same composition as expected.

The apcF and apcDF Preparations

The apcF and apcDF preparations were also expected to be of the same composition, but I found significant differences between their spectral data. The absorption spectra for the apcDF and apcF preparations are similar; differences between the absorption spectra can be attributed to differing amounts of PC present. The

apcF and apcDF spectra are blue-shifted from that of the wild-type by 1 or 2 nm.

The calculated difference spectra for the mutants are distorted somewhat because of differing amounts of PC are present in each preparation. I made the assumption that the number of chromophores present in each preparation is constant, and the area under the difference spectrum must equal zero. Because the mutants appear to contain more PC chromophores the difference spectrum is distorted with negative peaks appearing at 580 and 630 nm. To compensate for the negative peaks, the difference spectra contain positive peaks at about 650 nm to make the net area difference equal to zero.

The apcDF difference spectrum contains a positive peak at about 680 nm; the preparation from the mutant absorbs less at 680 nm. The 680 nm peak is hidden under a large 660 nm peak in the apcF difference spectrum, but the peak does show up in the second derivative of the apcF difference spectrum. The large 660 nm peak in the apcF difference spectrum indicates that the changes within the apcF PBS core are probably not additive as assumed.

The β^{18} subunit was expected to absorb at approximately 670 nm. If the β^{18} polypeptide was just omitted from the core without any further changes, the peak in the difference is red-shifted and smaller in magnitude than expected. The difference spectra for the apcF and apcDF preparations can be explained if the β^{18} subunit is substituted with a bluer absorbing protein. The higher energy chromophore will partially compensate for the lack of β^{18} chromophore absorption at higher energies. At lower

energies, the bluer-absorbing chromophore can no longer compensate for the lack of the β^{18} absorption, and this leads to a hole in the absorption spectrum. The substitution would also lead to smaller absorption differences.

The replacement of the β^{18} subunit with a β^{AP} is a reasonable hypothesis based on the similarity in the amino acid sequences of the two proteins. In *Mastigocladus laminosus*, Füglistaller, Rübéli et al. found the first 138 amino acids of the β^{18} and β^{AP} subunits to have 62% homology (Füglistaller, Rübéli et al, 1984).

The RT emission spectra for the apcDF and apcF preparations are blue-shifted from that of the wild-type as expected. More emission originates from the AP chromophores in the preparations from the mutants as expected. The apcDF and apcF preparations are missing a 680 nm emitter.

The RT emission spectrum of the apcDF preparation qualitatively fit the results obtained from Boltzmann calculations while the apcF spectrum displays too much PC emission. All of the apcF emission spectra I have obtained display larger amounts of PC emission. The apcF emission is consistently blue-shifted from that of the apcDF preparations. Apparently, the apcF and apcDF preparations have different polypeptide compositions. The PBS core did not dissociate in the same way for the apcF and apcDF mutants.

The low-temperature emission spectra from the apcF and apcDF preparations also display a blue-shifted emission band compared to that of the wild-type preparation. Unlike the RT emission spectrum, the low-temperature emission spectrum of the apcF

preparation is red-shifted from that of the apcDF preparation. This result also provides further evidence that the apcF and apcDF preparations are of different composition, and that the apcF preparation contains larger numbers of PC chromophores. PC emission contributes a large amount of the total fluorescence yield at RT and shifts the emission maximum blue in the apcF spectrum.

From the low-temperature emission data one can assign emission maximum to some of the chromophores within the PBS core. The apcF preparation contains only one 680 nm emitter, the L_{cm} chromophore. In the apcF preparation, the L_{cm} chromophore emits at 676 nm. The 676 nm emission maximum is 7 nm bluer than what is seen with the wild-type preparation. The blue-shift is caused by the lack of the β^{18} subunit.

The time-resolved data for the apcDF and apcF preparations were indistinguishable and slightly modified from that of the wild-type preparation. The two longest decay components resolved are shorter than those found in the wild-type DAS. The decrease in the lifetimes of the emitting chromophores indicates a larger degree of structural disorder within the apcF and apcDF preparations. The chromophores are no longer being held in optimal conformations, and more nonradiative deactivation occurs.

The shortest decay component which corresponds to the equilibration time necessary for the energy to travel throughout the complex is longer in the mutants than in the wild-type. A longer equilibration time indicates impaired energy transfer within the complex.

In summary, the apcF and apcDF preparations have different polypeptide compositions. The apcF preparation contains larger amounts of PC. Both the apcF and apcDF preparations appear to have a higher energy chromophore substituting for the missing β^{18} chromophore as shown in the absorption difference spectrum. From the low-temperature emission spectra, one can assign the L_{cm} chromophore to have an emission of 676 nm in the absence of the β^{18} subunit. The time-resolved emission data indicate that the preparations from the mutants are more disordered, and that energy transfer within the preparation is impaired.

The apcE/C186S and apcDE/C186S Mutants

As I mentioned earlier, the modified chromophore present in the apcE/C186S and apcDE/C186S PBS is missing from the 18 S preparations. The apcE/C186S and apcDE/C186S preparations were virtually indistinguishable spectrally, except the apcDE/C186S preparation had larger amounts of PC present. Absorption spectra from the mutants overlaid exactly above 650 nm; both spectra displayed a large absorption decrease compared the wild-type preparation at about 670 nm. From the absorption data, I assign the L_{cm} chromophore to absorb maximally at 670 nm.

The RT emission spectra for the apcE/C186S and apcDE/C186S preparations are not well described by the Boltzmann calculations. The apcE/C186S and apcDE/C186S emission spectra contain too much AP and PC emission. In the Boltzmann calculations, I assumed that the β^{18} chromophore emitted at 680 nm. If the β^{18} chromophore emits at a higher energy than expected, more

emission would originate from PC and AP chromophores. I redid the Boltzmann calculations for the RT emission using an energy level for the β^{18} chromophore that corresponds to 670 nm instead of 679 nm. I used the low-temperature emission data to choose the new energy level for the β^{18} chromophore. With the new energy level, I calculate that only 22% of the emission should originate from the β^{18} chromophore while AP will contribute 75%. The revised Boltzmann calculations fit the experimental data from the apcE/C186S preparation. The apcDE/C186S spectrum still contains too much PC emission.

The low-temperature emission spectra for the apcDE/C186S and apcE/C186S preparations are indistinguishable with both spectra considerably blue-shifted from that of the wild-type. The 11 nm blue-shift in the apcDE/C186S and apcE/C186S spectra can be attributed to the absence of the L_{cm} chromophore. In the apcDE/C186S and apcE/C186S preparations, the lowest energy chromophore remaining is the β^{18} chromophore which emits at 671 nm in the absence of the L_{cm} chromophore.

The time-resolved emission from the apcE/C186S preparations is quite different from that of the wild-type preparation. The apcE/C186S emission decay is more complicated and requires the addition of a fourth lifetime component in the fit. The two longer lifetimes which I assign to coupled PC, AP, and the terminal emitters are shorter than those seen in the wild-type DAS. As with the apcDF and apcF preparations, I ascribe the shortening of the coupled chromophore decay times to greater disorder within the preparation.

The additional fast decay component in the apcE/C186S DAS is possibly due to the slowing down of a energy transfer step within the core preparation. Unlike DAS obtained for partially dissociated preparations, both of the fast decay components have positive amplitude in the blue part of the emission spectrum. I don't think either component is due to dissociated sample.

There are two possible explanations for the additional decay component resolved in the apcE/C186S DAS. One possibility is that an energy transfer step, such as transfer from PC to AP, apparently is slowed in the apcE/C186S mutant. The transfer step can now be detected with single photon timing. In this case, the additional lifetime would be the 0.050 ns component and the 0.54 ns lifetime would correspond to the 0.08 ns lifetime from the wild-type DAS. The time necessary for the energy to equilibrate over the antenna is lengthened by a factor of 5.

There is a second possible explanation for the additional component in the apcE/C186S DAS. In the wild-type DAS two decay components very similar in size are not being resolved from each other. In the apcE/C186S DAS, one of the components is sufficiently different such that it now can be resolved from the second component. In this case, the 0.05 ns lifetime is probably unchanged while the 0.54 ns lifetime component is actually lengthened by a factor of 5.

The take home message from the apcE/C186S time-resolved data is that the chromophores within the preparation are more disordered and that energy transfer within the core is impaired in some manner.

The apcDE/C186S and apcE/C186S preparations are indistinguishable spectrally except that the apcDE/C186S preparations typically contain larger amounts of PC contamination. As shown by the absorption spectra, the L_{cm} chromophore absorbs at 670 nm. Also, the β^{18} chromophore emits at an energy greater than 680 nm as shown both by the Boltzmann calculations for the RT emission and by the 77 K emission spectrum. The time-resolved emission data indicates that the chromophores within the apcE/C186S 18 S preparations are more disordered, and energy transfer within the complex is impaired.

Conclusions

Using the low temperature emission spectra of apcDE/C186S PBS, I assigned the emission maximum of the β^{18} chromophore to 679 nm. When the L_{cm} chromophore is missing from the PBS core, the β^{18} chromophore emits at 671 nm, as shown by the low-temperature emission data from apcE/C186S 18 S preparations. If the β^{18} subunit is missing from the intact PBS, the L_{cm} chromophore emits at 682 nm (from the 77 K emission spectra of apcF and apcDF PBS). In the 18 S preparations, the L_{cm} chromophore emits at 676 nm (from the 77 K emission spectrum of the apcF preparation).

I have plotted several low-temperature emission spectra of wild-type preparations in Figure 5-6. Some of the preparations are partially dissociated as shown by the present of an emission band at about 673 nm along with a corresponding decrease in the 682 nm emission. The appearance of 673 nm emission could be due to several factors. The L_{cm} polypeptide could be partially degraded

Figure 5-6. Low temperature emission spectra of wild-type 18 S preparations
 The samples were excited at 590 nm, and emission was collected 90° to excitation. The excitation monochromator was set to a 6 - 7 nm resolution while the emission monochromator resolutions were between 0.1 and 0.4 nm. The spectra were collected within 3 hours after the preparations were removed from the gradient. The samples were diluted to less than 0.1 a.u. at the excitation wavelength using 2.0 M sucrose. The diluted samples were put under vacuum for 10 min and then frozen in N₂(l). Examples of spectra obtained from different wild-type 18 S preparations.

from proteases present; I have noticed that the 18 S preparations degrade much faster if protease inhibitor is not added to the buffers. Isono and Katoh also reported that a core preparation they studied was much more susceptible to chymotrypsin than intact PBS (Isono and Katoh, 1987). The 673 nm emission could be due to partial dissociation of the preparation; the β^{18} and L_{cm} chromophore could be coming uncoupled. The 673 nm emission could also be from the β^{18} chromophore if energy transfer is impaired between the β^{18} and the L_{cm} chromophores.

The point I am trying to make with all this data is that there is an interaction between the β^{18} subunit and the L_{cm} chromophore. Both the L_{cm} chromophore and the β^{18} subunit need to be present to obtain the 683 nm emission seen with both the wild-type PBS and 18 S preparation. The interaction is possibly structural in nature. The β^{AP} subunit, which might substitute for the β^{18} subunit, has a very different C-terminal end; the last 15 amino acids of the β^{18} subunit are more negatively charged than the amino acids from the β^{AP} subunit (Bryant, 1988). The C-terminal end of the β^{18} subunit could interact with the L_{cm} polypeptide to stabilize the chromophore. This interaction becomes more important when the PBS core is partially dissociated as in the 18 S preparations.

The β^{18} and L_{cm} chromophores are probably close in space. The β^{18} chromophore appears to be very sensitive to the absence of the L_{cm} chromophore, and its emission maximum shifts more than 8 nm if the L_{cm} chromophore is missing. I feel that this large of a

blue-shift in the emission spectrum has to mean that the β^{18} chromophore is right next to the L_{cm} chromophore binding pocket.

I will sum up all my conclusions in the next chapter, and I will propose some future projects.

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Chapter 6. Conclusions and Future Work

In this chapter I will try to draw together all my results from the previous chapter and suggest some directions for future work on PBS. I have summarized all the spectral results in Table 6-1.

The α^{AP-B} subunit

The deletion of the α^{AP-B} subunit from the PBS core didn't create any dramatic changes in the workings of the antenna complex. The doubling time for the *apcD* cells is very similar to that of the wild-type under our growth conditions. Energy transfer to the PSII reaction center appears to be unimpaired as shown by time-resolved and steady state fluorescence studies on whole cells.

The deletion of the α^{AP-B} subunit from the PBS antenna had very little effect on the spectral properties of the isolated antenna with the exception of the RT emission spectrum. This result was seen both with the *apcD* and *apcDF* strains. The α^{AP-B} subunit does contribute to the RT emission but not to the low temperature emission spectrum. The α^{AP-B} chromophore is slightly higher in energy than the L_{cm} chromophore; at 77 K, energy transfer to the L_{cm} occurs rather than emission. At RT, back transfer from the L_{cm} occurs readily, and the α^{AP-B} chromophore fluoresces.

The deletion of the α^{AP-B} subunit did not seem to perturb the emitting chromophores within the core as shown by the time-resolved emission measurements on the *apcD* and wild-type PBS. The decays lifetimes did not shorten. Maxson studied the *apcD*

Table 6-1

Emission Maxima Assignments

Chromophore Type	Low Temperature Emission Maximum
α AP-B in Intact PBS	682 nm
β^{18} in Intact PBS	678 nm
β^{18} in Core Prep	671 nm
L_{cm} in Intact PBS with β^{18} present	683 nm
L_{cm} in Intact PBS with β^{18} missing	682 nm
L_{cm} in Core Prep with β^{18} present	683 nm
L_{cm} in Core Prep with β^{18} missing	676 nm

strain earlier, and she reached similar conclusions (Maxson, Sauer, et al., 1989).

The β^{18} subunit

The deletion of the β^{18} subunit from the PBS antenna was found to have a larger effect than the deletion of the α^{AP-B} subunit. The doubling times of the *apcF* and *apcDF* cells were slightly longer than that of the wild-type cells. From the absorption spectrum of the 18 S core preparation, it appears that the β^{18} subunit is replaced by a β^{AP} subunit in both the *apcF* and *apcDF* mutants.

As with the α^{AP-B} chromophore, the β^{18} chromophore contributes to the RT emission of the PBS but not to the low temperature emission. The β^{18} chromophore is slightly higher in energy than both the α^{AP-B} and L_{cm} chromophores. The β^{18} chromophore transfers energy to the L_{cm} at 77 K.

The β^{18} subunit interacts with the L_{cm} chromophore as shown by the low-temperature emission spectra of the 18 S preparations. Both the β^{18} subunit and the L_{cm} chromophore are required for the PBS core to emit at 683 nm. The type of interaction between the two chromophores is unclear at this time. It could be a structural interaction in which the β^{18} subunit acts to stabilize the L_{cm} chromophore. In this case, the chromophores would probably be close so that the removal of the chromophore from the L_{cm} binding pocket perturbs the chromophore binding pocket of the β^{18} subunit.

The β^{18} and L_{cm} chromophores could also be excitonically coupled. In this case, the removal of one of the chromophores

would destroy the exciton states; this could explain the blue-shift of the low temperature emission from the apcF and apcDF PBS. I will discuss some possible experiments which could address this question later in the chapter.

The deletion of the β^{18} from the PBS core caused some disorder within the PBS. The lifetimes of the coupled chromophores decreased significantly in both PBS and core preparations. Some of the chromophores within the core are not being held in their ideal conformations.

The L_{cm} Chromophore

The substitution of a serine for cysteine 186 in the L_{cm} proved to be a mutation resulting in qualitatively different behavior, as shown by the emission spectra from the apcE/C186S and apcDE/C186S mutants. The substitution of the cysteine with serine to alter the binding site of the L_{cm} chromophore did not result in the absence of the chromophore from the PBS. Instead, the chromophore appears to be associated with the PBS complex in some noncovalent fashion. Since the chromophore isn't covalently bound to the protein, it probably is still a 3(Z)-phycocyanobilin, the immediate precursor to the bound chromophore (Rhie and Beale, 1992). The 3(Z)-phycocyanobilin contains an additional double bond leading to increased π -orbital conjugation and altered spectroscopic properties. The modified chromophore was found to absorb at 695 nm and emit at 715 nm.

The presence of the modified chromophore really impairs the growth of the *apcDE/C186S* and *apcE/C186S* cells. I found that the modified chromophore is at a lower energy than the reaction centers, so it diverts energy away from the reaction centers. It also acts to quench the PBS complex as shown by its unusually short decay lifetime. Large amounts of energy normally collected by the PBS for transfer to PSII is lost as emission or in nonradiative ways in the *apcE/C186S* and *apcDE/C186S* cells.

The modified chromophore is missing from the 18 S preparations for the *apcDE/C186S* and *apcE/C186S* cells. The absence of the L_{cm} chromophore leaves a hole in the absorption spectrum of the preparation at approximately 670 nm.

As with the deletion of the β^{18} subunit, the modification to the L_{cm} polypeptide perturbed the emitting chromophores within the PBS core. This was shown by the decrease in the decay lifetimes of both the intact PBS and the core preparations. The PBS core is somewhat disordered with some of the chromophores no longer in ideal conformations.

Energy Transfer within the Core

Using the conclusions I made above, I propose that energy transfer within the PBS core occurs as outlined below.

Future Directions

I still have quite a few unanswered questions that I would like to examine. First, what is the nature of the interaction between the L_{cm} and β^{18} chromophores. Are the chromophores excitonically coupled or just structurally dependent? One way to answer this question would be to create another mutant similar to the *apcE/C186S* mutant, change the cysteine on the β^{18} subunit to serine to create a *apcF/C84S* mutant. In analogy to the *apcE/C186S* mutant, the chromophore associated with the β^{18} polypeptide should be present in modified form in the intact PBS and missing in the 18 S preparation.

If the interaction between the β^{18} subunit and the L_{cm} chromophore is structural in nature, then the low temperature

emission of PBS from the *apcF/C84S* mutant should be identical to that of the wild-type. Using the absorption spectrum from the 18 S preparation from this mutant, one could also gain information about the absorption of the β^{18} chromophore.

Another interesting aspect to this study would be the creation of a *apcDFE/C186S* mutant with the PBS missing all of the unusual polypeptides. I have the feeling that the organism would grow very slowly, but it would probably have some very interesting spectral characteristics. Would energy transfer to chl *a* /PSII happen in this mutant?

There are quite a few questions that need to be answered about the PBS. First, what is the function of the α^{AP-B} subunit? It doesn't seem to have a major role within the PBS. Bryant believes that it acts as the terminal emitter to PSI (Bryant, personal communication). I have already done some preliminary work with epr to investigate that possibility using the *apcD* mutant. If one blocks the electron transfer pathway using DCMU, one can quantify the amount of PSI excited by measuring the ferredoxin signal in the epr spectrum. I excited whole cells at 77 K with 580 nm light to directly excite PC of the PBS without exciting much chl *a*. I monitored the ferredoxin signal and illuminated the sample a second time with blue light to excite all of the chl *a*. I could quantify the changes in the ferredoxin signal to see if different amounts of excitation energy reach the PSI reaction center in the wild-type cells versus the *apcD* cells. I still need to optimize the

illumination times, but the preliminary work looked promising with the *apcD* mutant showing impaired energy transfer to PSI.

I had a lot of problems in quantifying the amounts of phycobiliprotein present in whole cells. A technique for the specific bleaching of phycobiliproteins *in vivo* has been developed for *Synechococcus* 6301 (Zhao and Brand, 1989). The method uses heating at 60 °C to bleach the phycobiliproteins without altering the chlorophyll or carotenoid absorption. One should be able to calculate fluorescence quantum yields for the whole cells more precisely using this method.

Another direction that could be explored is the preparation of PSII-PBS complexes for all of the mutants. The spectral data should be easier to interpret than for whole cells. Also, one could monitor the amount of oxygen evolved by the preparations as a gauge of the efficiency of the antenna. Preparations have already been worked out for *Synechococcus elongatus* (Kashino, Enami, et al., 1992), thermophilic *Synechococcus* sp (Kura-Hotta, Satoh, et al., 1986), *Porphyridium cruentum* (Clement-Metral and Gantt, 1983; Chereskin and Gantt, 1985; Clement-Metral, Gantt, et al., 1985; Chereskin and Gantt, 1986), and *Anacystis nidulans* (Pakrasi and Sherman, 1984). The preparations appear to be difficult to work with, but the results could be very interesting since one could measure of the efficiency energy transfer from the PBS to the reaction centers.

Also, I think we need to address the question of how the PBS complex binds to the membrane. One approach to solving this problem would be to use crosslinkers to bind adjacent proteins in a

PBS-PSII preparation, isolate the crosslinked proteins using a denaturing gel, cleave the crosslinker using light, and identify the pieces. The composition of the PBS is relatively well identified, so it would be only a matter of identifying the proteins from the membrane.

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7 / 8 / 93