Mutation of Chlorophyll Ligands in the Chlorophyll-Binding CP47 Protein As Studied in a *Synechocystis* sp. PCC 6803 Photosystem I-less Background†

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ABSTRACT: Site-directed mutations have been introduced to replace conserved histidine residues in the chlorophyll-binding protein CP47 of photosystem II (PS II) in a PS I-less/*apc*E⁻ background strain of the cyanobacterium *Synechocystis* sp. PCC 6803. In thylakoids isolated from such a system, the degree of loss of the 695-nm fluorescence emission maximum at 77 K compared to that at 685 nm generally was consistent with the decrease in oxygen evolution rates measured at saturating light intensity. Taking into account that in the absence of CP47 and PS I some chlorophyll remains detectable in cells, the relative 695-nm fluorescence emission and the rate of oxygen evolution also correlate with the relative amount of chlorophyll per cell and with the number of PS II reaction centers on a chlorophyll basis. Interestingly, the 77 K fluorescence excitation spectra monitoring 695-nm emission of thylakoids from the CP47 His-to-Tyr mutants in a photosystem I-less/*apc*E⁻ background showed increases in the 413- and 531-nm absorption regions, compared to spectra of thylakoids from the background strain. These wavelengths coincide with absorption maxima of pheophytin. No increase in the 531-nm excitation band was observed in thylakoids from mutants lacking PS II or with a His-to-Asn mutation. These results are interpreted to indicate that replacement of conserved histidine residues by tyrosine in CP47 leads to the loss of Mg^{2+} from chlorophyll, resulting in the formation of pheophytin, or to the binding of pheophytin (rather than chlorophyll) at a particular pigment-binding site of CP47 during biogenesis and assembly of the protein. It was observed that the light-harvesting efficiency of CP47 His mutants was lower judging from the light intensity dependence of electron transport and analysis of fluorescence decay kinetics. This suggests that the presence of pheophytin in the antenna decreases antenna efficiency.

The chlorophyll *a*-binding protein CP47 (encoded by the *psb*B gene) is closely associated with the photosystem II (PS II) reaction center. CP47 together with CP43 (encoded by *psb*C) is viewed to function as the core antenna of the PS II reaction center (Vermaas & Ikeuchi, 1991). The excitation energy absorbed by chlorophyll in the core antenna is transferred to P680, the primary donor of the PS II reaction center. CP47 contains 12–13 highly conserved histidine (His) residues in 5 out of 6 hydrophobic regions that are long enough to span the thylakoid membrane (Vermaas et al., 1987; Bricker, 1990; Vermaas, 1993).

In a previous study, site-directed mutagenesis has been used to replace conserved His residues in hydrophobic regions of CP47 in the cyanobacterium *Synechocystis* sp. PCC 6803. It was demonstrated that replacement of His residues in hydrophobic regions of CP47 generally leads to a decrease in the PS II assembly and function, and to impairment of light energy transfer in the PS II reaction center (Eaton-Rye & Vermaas, 1992; Shen et al., 1993a). We hypothesized that replacement of His by Tyr leads to a loss of the fifth ligand to Mg in chlorophyll, resulting in a loss of this particular Mg and thus in formation of pheophytin at this location. However, at that time, no experimental evidence could be presented to support this hypothesis (Shen et al., 1993a).

Precedence for the absence of the central Mg^{2+} in the absence of a suitable ligand is found in bacteria. In the case of *Rhodobacter capsulatus*, when His200 of the M subunit of the photosynthetic reaction center is replaced by either leucine or phenylalanine, the corresponding bacteriochlorophyll (BChl) of the reaction center special pair is replaced by bacteriopheophytin (BPhe), resulting in formation of a BChl–BPhe heterodimer (Bylina & Youvan, 1988, 1991). In the His^{M200}-to-Leu and His^{M200}-to-Phe reaction centers, the spectral properties of the primary donor are significantly affected, and the quantum yield of the initial electron transfer is drastically reduced. Moreover, in the green bacterium *Chloroflexus aurantiacus*, the residue usually binding the accessory BChl associated with the M subunit is a Leu (Ovchinnikov et al., 1988; Shiozawa et al., 1989), and not a His as in all photosynthetic purple bacteria (Komiya et al., 1988). In *Chloroflexus*, per reaction center three BChl and three BPhe are present (Pierson & Thornber, 1983; Blankenship et al., 1983), suggesting that the lack of a His leads to the formation of a BPhe in this system.

Synechocystis sp. PCC 6803 is highly suitable for genetic modification of various components of PS II. Genetic deletion of the PS I reaction center complex and of the anchor protein linking the phycobilisome to the PS II complex (Shen et al., 1993b) has added to the value of this organism for PS II research. This PS I-less/*apc*E⁻ strain essentially represents an *in vivo* PS II particle, and is highly suitable as a background strain for detailed PS II studies, especially those involving fluorescence analysis.

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Chlorophyll fluorescence spectroscopy and analysis of fluorescence decay kinetics are widely used in the study of the organization of chlorophyll–protein complexes and energy transfer processes among pigment–protein complexes. By fluorescence lifetime determinations, excitation energy transfer can be followed. In principle, the lifetime of fluorescence is analyzed by measuring the fluorescence decline after brief flashes of light, thus following the kinetics of exciton density in spectrally distinguishable components of the pigment bed. Studies have been performed in isolated chlorophyll antenna pigments, in isolated PS I and PS II particles, and in intact cells [reviewed by Holzwarth (1986, 1987, 1991)]. In cyanobacteria, fluorescence decay studies have been carried out in intact cells, such as in *Synechocystis* 6803 (Bittersmann & Vermaas, 1991) and *Synechococcus* 6301 (Mullineaux & Holzwarth, 1990, 1991), and in isolated phycobilisomes and PS II and PS I particles [reviewed by Holzwarth (1991) and Holzwarth and Roelofs (1992)]. Fluorescence lifetime studies on PS I-less *Synechocystis* 6803 strains are shown to be highly informative regarding the energy transfer properties in the PS II complex.

MATERIALS AND METHODS

Growth and Transformation. *Synechocystis* 6803 cell growth and transformation procedures have been described by Vermaas et al. (1987). The PS I-less/*apc*E$^-$ strain and His mutants in this background were grown in BG11 (Rippka et al., 1979) supplemented with 15 mM glucose, 3 times higher than the normal glucose concentration for photoheterotrophic growth. Agar plates with the PS I-less/*apc*E$^-$ strain and the His mutants in this background were kept at low light intensity (5 $\mu E \cdot m^{-2} \cdot s^{-1}$), and liquid cultures were grown under normal light conditions (50 $\mu E \cdot m^{-2} \cdot s^{-1}$).

DNA Isolation and Southern Blotting. DNA was prepared from *Synechocystis* 6803 as described by Shen et al. (1993b). After restriction digestion of genomic DNA, Southern blotting to GeneScreen Plus (NEN-Du Pont) was performed, and blots were hybridized with a ^{32}P-labeled nick-translated *Synechocystis psb*B probe, and washed according to the manufacturer's recommendations.

Electron Transport and Herbicide-Binding Measurements. Oxygen evolution measurements and herbicide-binding experiments were carried out using intact cells as described by Shen et al. (1993a). For oxygen evolution measurements, 0.5 mM $K_3Fe(CN)_6$ was used as electron acceptor, and 0.1 mM 2,5-dimethyl-*p*-benzoquinone was added as redox mediator between thylakoids and the nonpenetrating ferricyanide. The sample was adjusted to a final chlorophyll *a* concentration of 2 μg/mL. Light was provided by a 150-W xenon arc lamp. The light was filtered through water, and subsequently was passed through a cutoff filter, transmitting light with wavelengths above 570 or 665 nm. All light intensity measurements were performed with a probe that is sensitive to visible light only. For herbicide-binding analysis using [^{14}C]-diuron, cells at a concentration equivalent to 10 μg/mL chlorophyll were used.

Preparation of Thylakoids. The procedure for the preparation of thylakoids from the PS I-less/*apc*E$^-$ background strain and the CP47 His mutants was based on the procedure described by Vermaas et al. (1990). Cells of a 2-L culture with an optical density of about 1.0 at 730 nm were collected, washed, and subsequently disrupted by vigorous shaking of cells mixed with 0.1-mm glass beads using a Braun homogenizer. After removal of glass beads and cell debris through centrifugation, the thylakoid membranes were pelleted and washed twice with thylakoid buffer. Then the thylakoid membranes were washed with 2 mM dodecyl β-D-maltoside in order to remove remaining phycobilin-binding proteins from the thylakoid fraction. After a final wash with thylakoid buffer to remove detergent, thylakoid membranes were resuspended in thylakoid buffer at a chlorophyll concentration of about 0.1 mg/mL.

Fluorescence Emission and Excitation Spectra. Fluorescence emission and excitation spectra were determined at 77 K using a SPEX Fluorolog 2 instrument. Thylakoid membranes were diluted to a concentration of 1 μg of chlorophyll/mL in 25 mM HEPES/NaOH, pH 7, in 60% (v/v) glycerol prior to freezing in liquid nitrogen. For emission spectra, the excitation and emission bandwidths were 12 and 2.4 nm, respectively. For excitation spectra, the excitation and emission bandwidths were 6 and 6 nm, respectively. A cutoff filter (transmitting at $\geq$665 nm) was used at the exit of the emission monochromator to minimize contributions from scattered light. Excitation difference spectra were calculated by subtraction of the excitation spectra of mutants versus the excitation spectrum of the PS I-less/*apc*E$^-$ strain, after normalizing the fluorescence intensities of the PS I-less/*apc*E$^-$ strain and mutants to 1 at 525-nm excitation.

Picosecond Fluorescence Decay-Associated Emission Spectra. Picosecond fluorescence kinetics were measured in a single-photon-timing spectrometer (O'Connor & Philips, 1984; Bittersmann & Vermaas, 1991). Fluorescence was excited by a cavity-dumped dye laser system (Coherent), synchronously pumped by a Nd-YAG laser. The excitation wavelength was tuned to 680 nm using the laser dye DCM. Thylakoids were diluted to a chlorophyll concentration of 2 μg/mL in thylakoid buffer. The volume of thylakoid suspension used for a fluorescence decay measurement was typically 500 mL. The sample was pumped through a flow-cell in which cells or thylakoids were excited by laser pulses. The rate of pumping was 15 mL/s to prevent accumulation of a significant amount of closed PS II reaction centers in the cuvette exposed to laser pulses. To obtain fluorescence decay spectra of open PS II reaction centers, the sample reservoir was kept in darkness, and 0.5 mM $K_3Fe(CN)_6$ was added. If closed PS II reaction centers were desired, 20 μM atrazine was added to the reservoir, and the sample was illuminated with white light (600 $\mu E \cdot m^{-2} \cdot s^{-1}$).

Fluorescence decay curves were recorded by single-photon timing. The channel resolution of the time-to-amplitude converter was 5.2 ps. Data were accumulated over 10 000 counts in the peak channel. The excitation wavelength was 680 nm, and fluorescence decays were recorded at five emission wavelengths: 670, 690, 700, 710, and 720 nm. Analysis of decay-associated fluorescence emission spectra was conducted by an iterative deconvolution procedure (global lifetime analysis) (Holzwarth et al., 1987). The best fits were assessed by global χ^2 values. The decay-associated spectra for each fluorescence lifetime were plotted as the fluorescence amplitude versus the emission wavelength (Wendler & Holzwarth, 1987).

RESULTS

Mutant Generation. For the creation of a *psb*B deletion mutant in a PS I-less/*apc*E$^-$ background that is suitable for introduction of mutations to the first 87 amino acid residues of CP47, a 1.1-kb *Eco*RI–*Kpn*I *Synechocystis* 6803 DNA fragment, which carries part of the *psb*B gene and 362 bp upstream region, was cloned into pUC18. Subsequently, a 0.6-kb *Nco*I–*Bam*HI fragment was deleted and replaced by

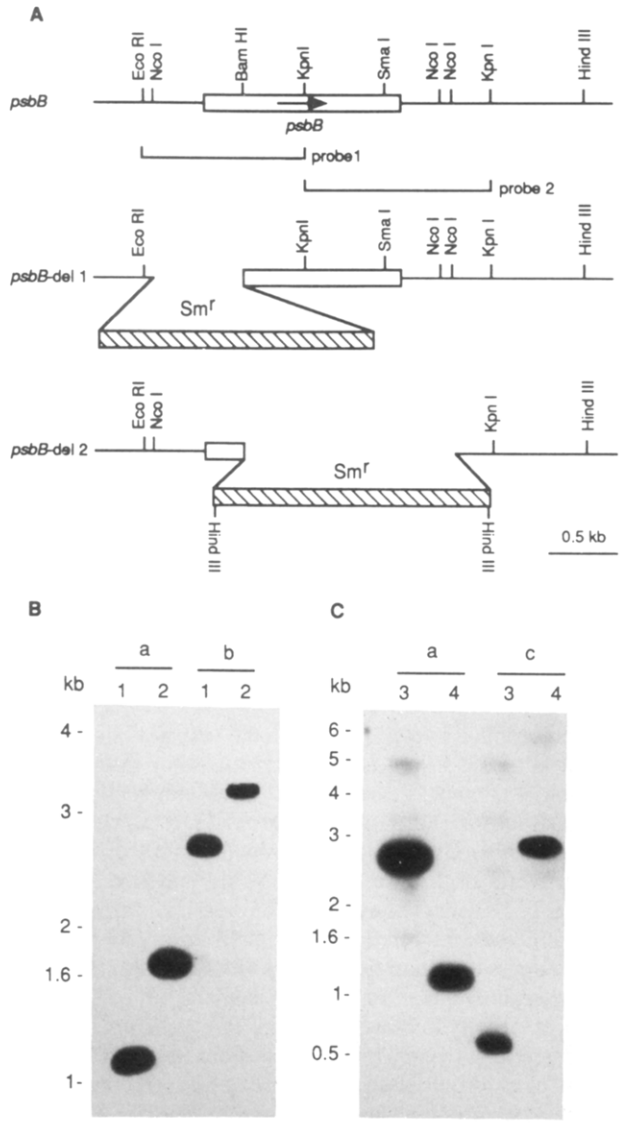

FIGURE 1: Southern blot analysis of the PS I-less/*apc*E$^-$ strain and of the *psb*B deletion mutants in this background. (A) Restriction maps of the *psb*B region in the genome of the *Synechocystis* 6803 PS I-less/*apc*E$^-$ strain (which is considered wild type for this purpose) and of two *psb*B deletion mutants in this background, in which parts of *psb*B have been replaced by a 2-kb spectinomycin-resistance marker (Sm^r). (B) Southern blot of the PS I-less/*apc*E$^-$ strain (a) and of the *psb*B del1/PS I-less/*apc*E$^-$ mutant (b), probed with a ^{32}P-labeled 1.1-kb *Eco*RI–*Kpn*I fragment (probe 1). DNA from the different strains was cut with (lanes 1) *Eco*RI and *Kpn*I and (lanes 2) *Eco*RI and *Sma*I. (C) Southern blot of the PS I-less/*apc*E$^-$ strain (a) and the *psb*B del2/PS I-less/*apc*E$^-$ mutant (c), probed with a ^{32}P-labeled 1.4-kb *Kpn*I–*Kpn*I fragment (probe 2). DNA from the different strains was cut with (lanes 3) *Bam*HI and *Hin*dIII and (lanes 4) *Eco*RI and *Kpn*I.

a 2.0-kb spectinomycin-resistance (Sm^r) cartridge (Prentki & Krisch, 1984), as shown in Figure 1A. This plasmid was used to transform the *Synechocystis* 6803 PS I-less/*apc*E$^-$ strain. The resulting deletion mutant was named *psb*B del1/PS I-less/*apc*E$^-$. As shown in Figure 1B, the deletion mutant is homozygous at the *psb*B locus.

To introduce mutations into the remaining 400 amino acid residue region of CP47, a *psb*B deletion construct was used (Eaton-Rye & Vermaas, 1991), in which a 1.6-kb *psb*B gene fragment between a *Bam*HI site at position 259 of *psb*B and a *Nco*I site 499 nucleotides downstream of *psb*B was deleted and replaced by a 2.0-kb Sm^r cartridge (Figure 1A). This plasmid was used to transform the *Synechocystis* 6803 PS I-less/*apc*E$^-$ strain. As shown in Figure 1C, results of Southern blotting indicated that the deletion mutant lacked the 1.6-kb *Bam*HI–*Nco*I region and contained the appropriate insertion of the Sm^r cartridge. This deletion mutant was designated as *psb*B del2/PS I-less/*apc*E$^-$.

Site-directed mutations were introduced into *psb*B by oligonucleotide-directed mutagenesis, as described by Shen et al. (1993a). To generate mutations in the His9, His23, and His26 residues of CP47, a 1.1-kb *Eco*RI–*Kpn*I *psb*B fragment from *Synechocystis* 6803, containing 760 bp of *psb*B and part of the upstream region, was cloned into bacteriophage M13mp18. A kanamycin-resistance (Km^r) cartridge was inserted at the *Nco*I site 285 bp upstream of the *psb*B start codon for selection of transformants. Mutants were identified by sequencing the appropriate part of *psb*B using single-stranded M13 DNA from single clones. Double-stranded replicative form DNA of the desired mutants was isolated and used to transform the *psb*B del1/PS I-less/*apc*E$^-$ strain. Generation of site-directed mutations in His residues in helices II, III, and IV of CP47 has been described before (Eaton-Rye & Vermaas, 1991; Shen et al., 1993a), and utilizes transformation of the *psb*B del2/PS I-less/*apc*E$^-$ strain.

In this way, all His residues in the putative transmembrane helices I, II, III, and IV of the CP47 protein have been mutated. In addition, as it is currently unknown where exactly helix I is located, also His9 has been mutated. As has been pointed out by Vermaas (1993), this His residue may be part of helix I, because this residue and the next His (His23) share the characteristic spacing of 14 residues found in helix II and helix IV.

PS II Activity and Light Intensity Dependence. The overall PS II activity of the PS I-less/*apc*E$^-$ and CP47 His mutant strains was determined by measuring oxygen evolution rates at saturating light intensity. Whole cells were illuminated with red light ($\geq$665 nm), exciting mostly chlorophyll. The oxygen evolution rates of the PS I-less/*apc*E$^-$ strain and the His mutants in this background are summarized in Table 1. In the PS I-less/*apc*E$^-$ background, all CP47 His mutants exhibited reduced oxygen evolution, but the level of reduction was dependent on the site and nature of the mutation made. No PS II activity could be measured in H23Y/PS I-less/*apc*E$^-$, H114Y/PS I-less/*apc*E$^-$, and two triple mutants. In several mutants with low rates of oxygen evolution on a chlorophyll basis, the oxygen evolution rate was found to be unstable. The reason for this instability is as yet unclear, but may be related to an increased rate of photoinactivation, which has been found also in other PS II mutants [for example, see Vermaas et al. (1990)]. It is possible that structural rearrangements drive this functional inactivation in the light.

The functional effects of mutating His9 to Tyr resemble those observed upon His-to-Tyr mutation of selected residues in hydrophobic regions of the protein. Therefore, the function of His9 may be similar to that of other His residues considered in this study and may involve providing a chlorophyll ligand. However, this functional analogy does not provide firm evidence regarding His9 being in a transmembrane region, as His in domains that are not membrane-spanning also may serve as ligand to chlorophyll in the membrane (Deisenhofer et al., 1985; Kühlbrandt et al., 1994). A high-resolution crystal structure will be needed for the final answer to this question.

In PS I-containing systems, mutation of His residues was found to result in a somewhat higher light requirement for PS II activity (Shen et al., 1993). To test whether this occurred also in the PS I-less/*apc*E$^-$ background, light saturation curves of oxygen evolution were measured in whole cells of the PS I-less/*apc*E$^-$ strain and two CP47 His-to-Tyr mutants upon

Table 1: Chlorophyll Content, Oxygen Evolution, and Chlorophyll/PSII Ratio in Intact Cells of the PS I-less/*apc*E⁻ Strain and of CP47 His Mutants in This Background

strain	μg of Chl·OD_{730}^{-1}·mL^{-1} [a]	μmol of O_2·(mg of Chl)$^{-1}$·h^{-1} [b]	Chl/PS II ratio[c]
PS I-less/*apc*E⁻	0.87	1830	110
H9Y/PS I-less/*apc*E⁻	0.67	1110	160
H23Y/PS I-less/*apc*E⁻	0.28	0	ND[e]
H23N/PS I-less/*apc*E⁻	0.39	420	440
H26Y/PS I-less/*apc*E⁻	0.65	1220	180
H100Y/PS I-less/*apc*E⁻	0.47	530	260
H114Q/PS I-less/*apc*E⁻	0.41	440	370
H114N/PS I-less/*apc*E⁻	0.33	210[d]	1610
H114Y/PS I-less/*apc*E⁻	0.27	0	ND
H142N/PS I-less/*apc*E⁻	0.69	1130	180
H157Y/PS I-less/*apc*E⁻	0.73	1380	160
H201Y/PS I-less/*apc*E⁻	0.71	1330	170
H202Y/PS I-less/*apc*E⁻	0.54	710	280
H216Y/PS I-less/*apc*E⁻	0.52	650	360
H216N/PS I-less/*apc*E⁻	0.79	1510	140
H142N/H157N/PS I-less/-*apc*E⁻	0.65	980	240
H201Y/H202Y/PS I-less/-*apc*E⁻	0.31	120[d]	1830
H201Y/H202Y/H157Q/-PS I-less/*apc*E⁻	0.23	0	ND
H201Y/H202Y/H216Y/-PS I-less/*apc*E⁻	0.26	0	ND
*psb*B del2/PS I-less/*apc*E⁻	0.24	0	ND

[a] Values are the average of three independent experiments; the standard error is ≤10%. [b] Values are the average of four independent experiments; the standard error is <20%. A red cutoff filter (transmitting at ≥665 nm) was used. The light intensity of visible light was 2100 μE·m^{-2}·s^{-1}, which in this wavelength range is close to the intensity needed for saturation. [c] Values are the average of three independent experiments. The standard error is ≤25%. [d] Oxygen evolution inactivated rapidly.[e] ND, no measurable [^{14}C]diuron binding could be detected.

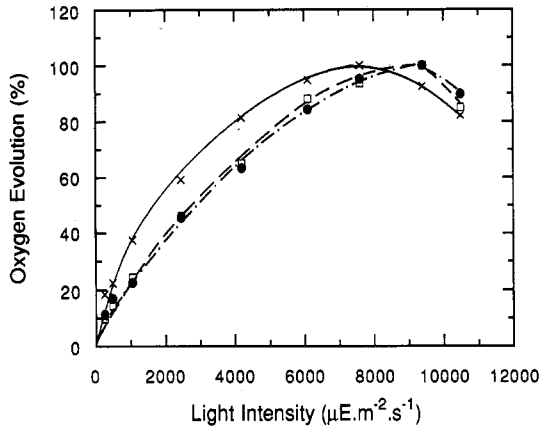

FIGURE 2: Light saturation curves of the PS I-less/*apc*E⁻ strain and two His mutants in this background. Oxygen evolution was measured at different light intensities in intact cells from the PS I-less/*apc*E⁻ strain (×), the H100Y/PS I-less/*apc*E⁻ mutant (□), and the H202Y/PS I-less/*apc*E⁻ mutant (●). For the PS I-less/*apc*E⁻ strain, 100% oxygen evolution represented 2460 μmol of O_2·(mg of chlorophyll)$^{-1}$·h^{-1}. For the H100Y/PS I-less/*apc*E⁻ and H202Y/PS I-less/*apc*E⁻ mutants, this value was 920 and 880 μmol of O_2·(mg of chlorophyll)$^{-1}$·h^{-1}, respectively. An orange cutoff filter (transmitting at ≥570 nm) was used. The data presented here are the average of four independent experiments, and the variability between the results of the different experiments was less than 15% of each of the data points presented.

excitation at >570 nm. Results are presented in Figure 2. For the His mutants, a higher light intensity was required to half-saturate oxygen evolution as compared to the PS I-less/*apc*E⁻ strain, confirming that mutations of these His residues decrease the light-harvesting efficiency of the PS II complex. Note the different wavelength range used for excitation in Figure 2 as compared to the results presented in Table 1. As ≥665-nm light is more efficiently absorbed by PS I-less/*apc*E⁻ strains than >570-nm light, upon illumination with ≥665-nm light a lower light intensity is sufficient for saturation.

PS II/Chlorophyll Ratios and Chlorophyll Quantitation. The chlorophyll/PS II ratios in whole cells of the PS I-less/*apc*E⁻ background strain and CP47 His mutants derived from this strain were determined by herbicide-binding analysis. Results are presented in Table 1. In the background strain, approximately 1 PS II reaction center is found per 110 chlorophyll molecules. Compared to this background strain, all CP47 His mutants (except H216N/PS I-less/*apc*E⁻) showed a decreased PS II concentration on a chlorophyll basis, with the degree of decrease depending on the site and nature of the mutation made. The relative decrease in the number of PS II reaction centers on a chlorophyll basis in various mutants is rather similar to that seen in mutants introduced into a PS I-containing background (Shen et al., 1993a).

The chlorophyll contents of cells from the PS I-less/*apc*E⁻ strain and the CP47 His mutants were determined utilizing cell scattering at 730 nm as a standard for cell concentration and 663-nm absorption of cell extracts in methanol as a measure of the amount of chlorophyll. As shown in Table 1, lower chlorophyll contents were detected in most His mutants compared to the PS I-less/*apc*E⁻ strain. For example, the chlorophyll content of the H100Y/PS I-less/*apc*E⁻ mutant was about half of that of the background strain, while in the *psb*B deletion mutant the chlorophyll content dropped by another factor of 2. The remaining chlorophyll in the *psb*B del2/PS I-less/*apc*E⁻ mutant is from the CP43 protein and polypeptide(s) that bind(s) chlorophyll with 678-nm fluorescence emission (Shen & Vermaas, 1994). Mutants without CP47 essentially lack PS II reaction centers, but retain about 25% of chlorophyll compared to the PS I-less/*apc*E⁻ background strain. Taking this into consideration, the relative amount of chlorophyll on a per-cell basis corrected for the amount of chlorophyll present in the absence of PS I and CP47 in all mutants reported here correlated reasonably well with results of quantitation of PS II content through herbicide-binding measurements.

An important issue in the study of mutants in a PS I-less/*apc*E⁻ background is whether the functional effects of a PS II mutation are comparable in PS I-less and PS I-containing systems. If so, studies of PS II mutants in PS I-less systems provide an accurate assessment of the effects of mutations on PS II properties. To test this, mutational effects on oxygen evolution and the PS II/chlorophyll ratio were compared between a PS I-containing system (Shen et al., 1993a) and in a PS I-less/*apc*E⁻ background. The results are presented in Table 2. The effects of CP47 mutations on PS II content and PS II activity in most cases were found to be relatively similar in the PS I-less/*apc*E⁻ system as in the PS I-containing background. Except for H100Y, the differences are not more than about 2-fold.

Fluorescence Emission Spectra. Measurements of 77 K fluorescence emission spectra provide a sensitive way to monitor the PS II complex. Figure 3 shows the 77 K fluorescence emission spectra of the PS I-less/*apc*E⁻ background strain and several mutants with mutated His residues in helices II and IV of CP47. Upon excitation at 440 nm (exciting chlorophyll *a*), two peaks at 685 nm (in part from CP43) and 695 nm (from CP47) were observed in the PS I-less/*apc*E⁻ strain. The amplitudes of fluorescence emission at 695 nm relative to that at 685 nm and to that in a mutant

Table 2: Comparison of Relative PS II/Chlorophyll Ratios and Oxygen Evolution Rates of CP47 His Mutants in PS I-Containing and PS I-less/*apc*E⁻ Backgrounds, and Relative Amplitude of the 77 K Fluorescence Emission at 695 nm (F_{695}) Measured in Thylakoids of the PS I-less/*apc*E⁻ Strain and Derived CP47 His Mutants[a]

	in PS I-containing background		in PS I-less/*apc*E⁻ background		
strain	PS II/Chl (%)	O_2 evolution (%)	PS II/Chl (%)	O_2 evolution (%)	F_{695} (%)[b]
wild type	100[c]	100	100	100	100
H9Y	81	88	69	61	69
H23Y	0	0	0	0	0
H23N	34	27	31	24	27
H26Y	78	86	61	67	71
H100Y	8	6	42	29	41
H114Q	65	62	30	24	29
H114N	22	25	7	12	8
H114Y	11	8	0	0	0
H142N	78	86	61	62	66
H157Y	87	93	69	75	68
H201Y	76	92	65	73	70
H202Y	72	47	39	38	43
H216Y	65	27	31	36	46
H216N	89	98	79	82	74
H201Y/H202Y	12	11	5	6	7
H201Y/H202Y/H157Q	0	0	0	0	0
H201Y/H202Y/H216Y	0	0	0	0	1

[a] Values reported in this table are the average of four independent experiments. The standard error is ≤10%. [b] Emission spectra were normalized to 1.0 at 685 nm. A 100% amplitude was calculated by subtraction of the fluorescence emission at 695 nm of the *psb*B del2/PS I-less/*apc*E⁻ strain. The amount of 695-nm emission of the *psb*B del2/PS I-less/*apc*E⁻ mutant was taken to be 0%. [c] All values in systems with wild-type PS II were normalized to 100%.

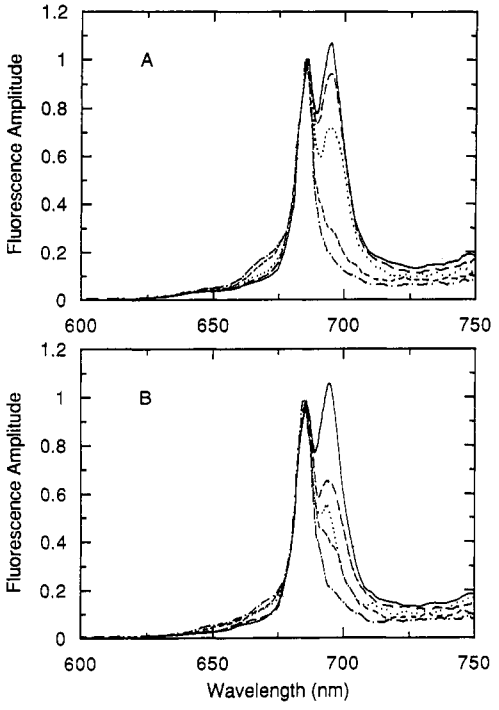

FIGURE 3: 77 K fluorescence emission spectra of the PS I-less/*apc*E⁻ strain and several His mutants in this background. Fluorescence spectra were measured using whole cells upon excitation at 440 nm, at which wavelength mostly chlorophyll is excited. Spectra were normalized to 1.0 at 685 nm. (A) 77 K fluorescence emission spectra were measured using the PS I-less/*apc*E⁻ strain (—) and several mutants with changes in His residues in helix IV of CP47: H201Y/PS I-less/*apc*E⁻ (– –), H202Y/PS I-less/*apc*E⁻ (···), H201Y/H202Y/PS I-less/*apc*E⁻ (- - -), and H201Y/H202Y/H216Y/PS I-less/*apc*E⁻ (– · –). (B) 77 K fluorescence emission spectra were measured using the PS I-less/*apc*E⁻ strain (—) and several mutants with changes in His residues in helix II of CP47: H100Y/PS I-less/*apc*E⁻ (– –), H114Q/PS I-less/*apc*E⁻ (···), H114N/PS I-less/*apc*E⁻ (- - -), and H114Y/PS I-less/*apc*E⁻ (– · –).

lacking CP47 have been presented in Table 2 for thylakoids of the PS I-less/*apc*E⁻ strain and of derived His mutants. A general feature of the results shown in Figure 3 and Table 2 is that replacement of His residues in CP47 led to a decrease of the 695-nm fluorescence emission peak relative to that of the PS I-less/*apc*E⁻ strain.

At His23 and His114, mutation of His to Tyr led to a total loss of the 695-nm fluorescence emission peak. As shown in Figure 3 and Table 2, replacement of His114 by Gln resulted in a 70% decrease in the amplitude of the 695-nm fluorescence emission. Interestingly, in this mutant an approximately 2-nm blue-shift of the 695-nm peak was observed, while in other mutants with decreased emission amplitude at 693–695 nm this shift was not seen. Also, the H114Q emission spectrum in the 695–710-nm region has been shifted to the blue by a few nanometers. In a PS I-containing H114Q mutant, a similar 2–3-nm shift in the 695-nm fluorescence maximum was observed (J. Eaton-Rye and W. Vermaas, unpublished results). The amplitude of the 693-nm emission was decreased further in the H114N/PS I-less/*apc*E⁻ mutant, while in H114Y/PS I-less/*apc*E⁻ no significant 693–695-nm emission could be observed. Also, a His-to-Asn mutation at residue 23 of CP47 had a significant effect on the amplitude of the 693–695-nm emission peak. About a 70% decrease in the 695-nm emission was found in the H23N/PS I-less/*apc*E⁻ mutant. This, together with the fact that the H23Y and H114Y mutants led to a complete loss of PS II, suggests that the His114 and His23 residues in CP47 and the molecules they bind have an important role in stable assembly and function of the PS II complex.

77 K Fluorescence Excitation Difference Spectra. A large advantage of the PS I-less/*apc*E⁻ background strain is that a detailed characterization of the PS II pigment composition is possible. In contrast to PS I-containing strains, where chlorophyll is associated mostly with PS I, in the PS I-less/*apc*E⁻ background small pigmentation changes in PS II may be detected *in vivo* or in thylakoids. For example, if localized pheophytinization would occur as a result of introduced mutations, this might be detectable. To this aim, 77 K fluorescence excitation spectra were recorded for thylakoids of the PS I-less/*apc*E⁻ background strain and derived strains carrying His mutations in CP47. The selected emission wavelength was 695 nm, as this corresponds to fluorescence

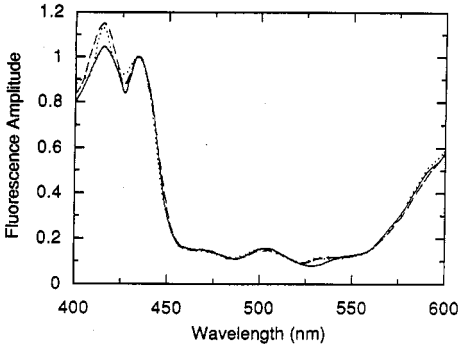

FIGURE 4: 77 K fluorescence excitation spectra of the PS I-less/*apc*E⁻ strain and two derived His mutants. The fluorescence excitation spectra were measured using thylakoids from the PS I-less/*apc*E⁻ strain (—), the H100Y/PS I-less/*apc*E⁻ mutant (···), and the H202Y/PS I-less/*apc*E⁻ (– –) mutant. The emission wavelength was 695 nm, a fluorescence maximum associated with chlorophyll from the CP47 protein. Spectra were normalized to 1 at 435 nm.

Table 3: Ratio of the Fluorescence Amplitude Increase at 413 nm versus That at 531 nm and the Relative Increase at 413 nm As Determined from 77 K Excitation Spectra (695-nm Emission) in Thylakoids

strain	F_{413}/F_{531} ratio	relative increase at 413 nm[a]
H26Y/PS I-less/*apc*E⁻	0.27	0.07
H100Y/PS I-less/*apc*E⁻	0.21	0.13
H201Y/PS I-less/*apc*E⁻	0.22	0.06
H202Y/PS I-less/*apc*E⁻	0.32	0.11
H216Y/PS I-less/*apc*E⁻	0.26	0.12

[a] This value was calculated by comparison of the subtraction of the fluorescence amplitude upon 413-nm excitation in the PS I-less/*apc*E⁻ background from that in the appropriate mutant, after normalization of the fluorescence amplitude upon excitation at 435 nm to 1.

associated with CP47. Figure 4 displays the 77 K fluorescence excitation spectra for the PS I-less/*apc*E⁻ strain and two His-to-Tyr mutants in this background. Differences existed between the PS I-less/*apc*E⁻ background strain and various CP47 His mutants particularly around 413 nm, and a small consistent difference was present in the 531-nm region. The ratio of the fluorescence amplitude at 413 nm versus 531 nm and the relatively increased amplitude at 413 nm in several His-to-Tyr mutants are presented in Table 3. A 6–13% increase at 413 nm was observed in the His-to-Tyr mutants as compared to the amplitude of the 435-nm absorption peak (due to chlorophyll *a*). The absorption increase at 531 nm consistently was about 0.2–0.3 of that at 413 nm in the His-to-Tyr mutants. This ratio of 413- and 531-nm absorption is reminiscent of that found for pheophytin.

To monitor these effects in more detail, a fluorescence excitation difference spectrum in the 525–535-nm region was obtained for various His mutants by subtraction of the PS I-less/*apc*E⁻ background spectrum from the His mutant spectrum, normalizing the spectra to 1 at 525 nm. The results are presented in Figure 5. A number of His-to-Tyr mutants showed a maximum around 531 nm. The largest amplitude of this band was observed in difference spectra with the H100Y/PS I-less/*apc*E⁻, H202Y/PS I-less/*apc*E⁻, and H216Y/PS I-less/*apc*E⁻ mutants. Mutants lacking PS II (such as H114Y/PS I-less/*apc*E⁻ and *psb*B del2/PS I-less/*apc*E⁻) exhibited no 531-nm band, indicating that the presence of the band with a maximum at 531 nm is correlated with the presence of PS II. Also, no 531-nm peak was observed in the His-to-Asn mutant H216N/PS I-less/*apc*E⁻. As Asn may serve as a chlorophyll ligand, the His-to-Asn mutation indeed may not result in a change in pigment composition.

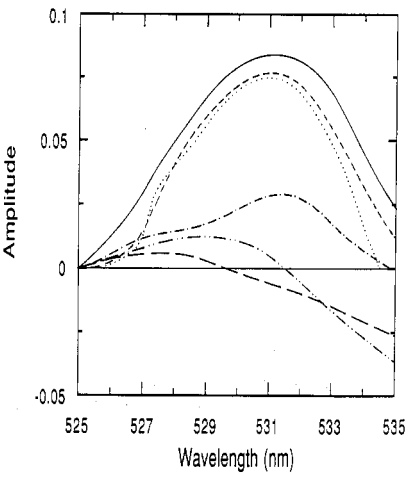

FIGURE 5: 77 K fluorescence excitation difference spectra of the CP47 His mutants compared to the PS I-less/*apc*E⁻ background strain. Difference spectra were calculated by subtraction of the excitation spectrum of the PS I-less/*apc*E⁻ strain from the excitation spectra of the H100Y/PS I-less/*apc*E⁻ (- - -), H114Y/PS I-less/*apc*E⁻ (– –), H201Y/PS I-less/*apc*E⁻ (– · –), H202Y/PS I-less/*apc*E⁻ (···), H216Y/PS I-less/*apc*E⁻ (—), and *psB* del2/PS I-less/*apc*E⁻ (– ·· –) mutants, after normalizing the spectra to 1 at 525 nm. The emission wavelength was 695 nm.

Decay-Associated Fluorescence Emission Spectra. To investigate energy transfer properties in PS II, fluorescence decay kinetics were measured in thylakoids from the PS I-less/*apc*E⁻ background strain and derived His mutants, and from the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant. The latter mutant was created by *psb*C interruption of the *psb*B del2/PS I-less/*apc*E⁻ strain, and lacked all major PS II core polypeptides while retaining some chlorophyll (Shen & Vermaas, 1994). Decay of fluorescence elicited by picosecond pulses at 680 nm was recorded in the wavelength range 670–720 nm. Global analysis of the fluorescence data in the entire wavelength range was performed. The fit quality was evaluated by its global χ^2 value and residual plots (data not shown), and for all strains five lifetime components were needed to fit the data set optimally.

In the decay-associated fluorescence spectra of thylakoids from the PS I-less/*apc*E⁻ strain (Figure 6) and from the H202Y/PS I-less/*apc*E⁻ mutant (Figure 7), the major lifetime component was found to be τ = 42–45 ps. A τ = 20 ps component, which is the major component in the decay-associated fluorescence spectra of PS I-containing *Synechocystis* wild-type cells (Bittersmann & Vermaas, 1991), was not detected in these PS I-less strains. Upon closing PS II by addition of 20 μM atrazine and by irradiation with white light, a 50% decrease in amplitude of the τ = 42–45 ps component was observed in thylakoids of the PS I-less/*apc*E⁻ strain, and a 40% decrease in that from thylakoids of the H202Y/PS I-less/*apc*E⁻ mutant. This τ = 42–45 ps component was not observed in the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant (Figure 8). Thus, the τ = 42–45 ps component is assigned in part to excitation energy transfer and trapping in the open PS II core complex.

The second largest component in systems with PS II in the open state had a lifetime of 180–230 ps. Closing the PS II reaction centers led to a small decrease in its amplitude. This 180–230-ps component was absent in decay-associated spectra from thylakoids of the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant. These results suggest that this component is partially correlated with open PS II reaction centers. This is in line with earlier observations (Bittersmann & Vermaas, 1991).

The relative amplitude of the third lifetime component, τ = 580–690 ps, was observed to increase in the PS I-less/

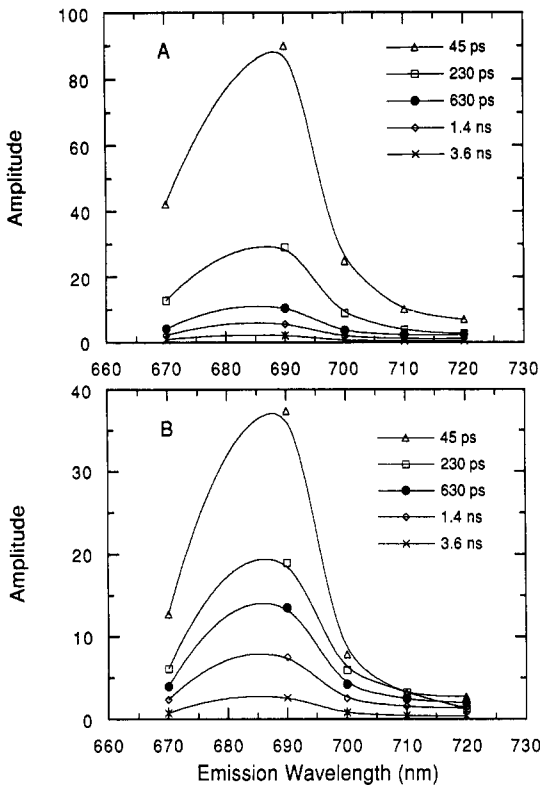

FIGURE 6: Decay-associated emission spectra of thylakoids from the PS I-less/*apc*E⁻ strain. Fluorescence decays were recorded upon 680-nm excitation: (A) using dark-adapted thylakoids in the presence of 0.5 mM $K_3Fe(CN)_6$ (open PS II reaction centers) and (B) after addition of 20 μM atrazine and upon continuous illumination with white light (closed PS II reaction centers).

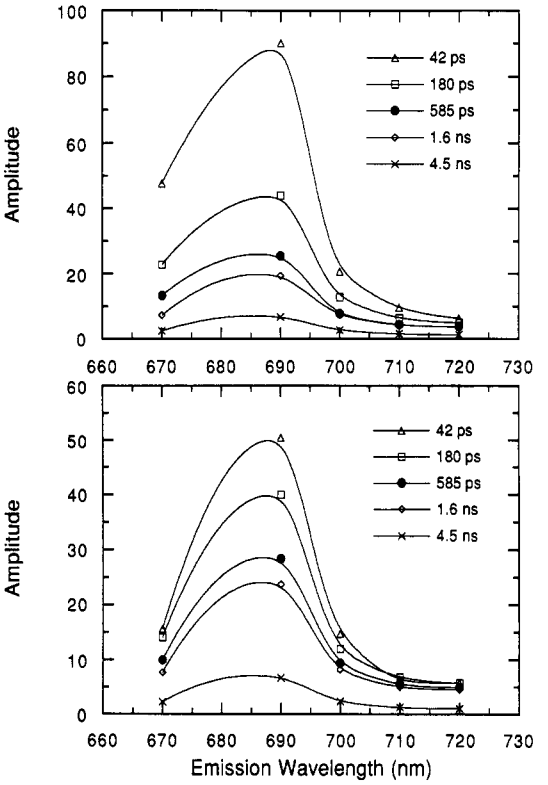

FIGURE 7: Decay-associated emission spectra of thylakoids from the H202Y/PS I-less/*apc*E⁻ mutant. Fluorescence decays were recorded upon 680-nm excitation: (A) using dark-adapted thylakoids in the presence of 0.5 mM $K_3Fe(CN)_6$ (open PS II reaction centers) and (B) after addition of 20 μM atrazine and upon continuous illumination with white light (closed PS II reaction centers).

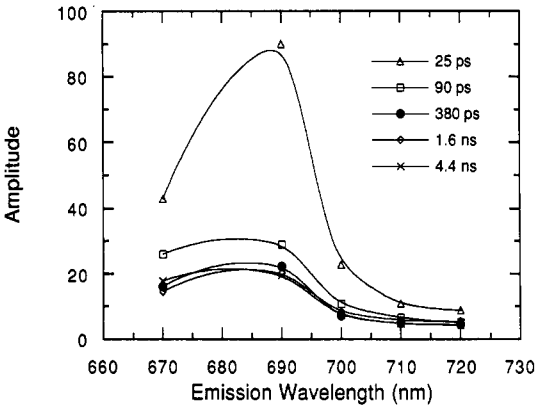

FIGURE 8: Decay-associated emission spectra of thylakoids from the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant. Spectra were recorded upon 680-nm excitation of thylakoids of the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant in the dark-adapted state. Decay-associated spectra determined in the light after addition of 20 μM atrazine (not shown) were identical to the spectra shown here.

*apc*E⁻ strain and the H202Y/PS I-less/*apc*E⁻ mutant. This component again was not detected in thylakoids of the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant. This would suggest that this lifetime component in part is correlated with closed reaction centers. The lifetime of this component in the H202Y/PS I-less/*apc*E⁻ mutant reproducibly was about 10% shorter than in the PS I-less/*apc*E⁻ strain.

The fourth component, with a τ = 1.4–1.6 ns lifetime, was also found to have a small increase in amplitude upon closing PS II centers in the thylakoids of the PS I-less/*apc*E⁻ background strain and the H202Y/PS I-less/*apc*E⁻ mutant. This component may originate, in part, from the PS II reaction center in the closed state.

The amplitude of the slowest component, τ = 3.6–4.5 ns, was larger in thylakoids of the H202Y/PS I-less/*apc*E⁻ mutant than in those of the PS I-less/*apc*E⁻ background strain. A component with a similar lifetime was found in thylakoids from the *psb*B⁻/*psb*C⁻/PS I-less/*apc*E⁻ mutant. Thus, this component most likely is correlated with chlorophyll that is not linked to PS II reaction centers.

DISCUSSION

Similarity of CP47 Mutations in Wild Type versus in the PS I-less/apcE⁻ Background. In a previous study, conserved histidine residues in the hydrophobic regions of CP47 were found to have an important role in PS II assembly and/or stability, and in light harvesting of the PS II core antenna (Shen et al., 1993a). To further investigate the effects of mutation of such His residues in CP47 on PS II assembly and activities and to test the physiological relevance of a PS I-less system for PS II studies, we have introduced His mutations into CP47 of a *Synechocystis* 6803 background strain lacking PS I and phycobilisome function (Shen et al., 1993b). In this system, in which chlorophyll associated with the PS I core is absent, mutation of His to Tyr in CP47 was found to lead to a decrease in the number of functional PS II reaction centers on a chlorophyll basis and in the PS II chlorophyll content. Taking into account that in the absence of CP47 some chlorophyll remains detectable in PS I-less strains, the mutational effects were similar to those found in PS I-containing strains. After correction for remaining chlorophyll in CP47-less PS I-less/*apc*E⁻ mutants, the PS II/chlorophyll ratios and the amount of chlorophyll per cell in the His mutants in the PS I-less/*apc*E⁻ background are consistent with the amount of oxygen evolution observed, suggesting that these

parameters are suitable to estimate the amount of functional PS II in these mutants. These results emphasize the suitability of the PS I-less/*apc*E$^-$ strains to study PS II properties.

77 K Fluorescence Emission at 695 nm. Upon excitation of chlorophyll at 440 nm, two fluorescence emission peaks at 685 and 695 nm are evident, both originating from the PS II complex; the 695-nm component is correlated with CP47 (Nakatani et al., 1984; van Dorssen et al., 1987; Shen et al., 1993b). As shown in Figure 3 and Table 2, the degree of decrease of the 695-nm emission peak correlates with the loss of functional PS II reaction centers on a chlorophyll basis and with the decrease of PS II activity. This also has been observed in PS I-containing systems (Haag et al., 1993). Reduction of the fluorescence emission peak at 695 nm in the CP47 His mutants reflects a decreased content of the number of PS II centers. Thus, 77 K fluorescence emission at 695 nm in the PS I-less/*apc*E$^-$ background system can be used as a quick semiquantitative measurement to probe for the presence of core PS II complexes in CP47 mutants.

Pheophytin Formation in His-to-Tyr Mutants. As outlined in a previous discussion (Shen et al., 1993a) and as observed in homologous systems (Bylina & Youvan, 1988, 1991), upon loss of the fifth ligand of Mg^{2+} in chlorophyll the formation of pheophytin may occur. Pheophytin formation may result either from incorporation of chlorophyll during biosynthesis and loss of the central Mg^{2+} (lacking a fifth ligand) shortly thereafter or from binding of pheophytin at the pigment-binding site during biosynthesis and assembly of the pigment–protein complex. At this moment, no compelling evidence is available to distinguish between these options. The 77 K fluorescence excitation spectra of His-to-Tyr mutants displayed an increase at 413 and 531 nm, compared to the spectrum of the PS I-less/*apc*E$^-$ background strain (Figure 4). Absorption peaks at 409 and 534 nm are found in the absorption spectrum of pheophytin *a in vitro* (Goedheer, 1966). The 413-nm absorption peak is consistent with the absorption peak of pheophytin *a* observed in the isolated PS II reaction center complex (Nanba & Satoh, 1987; Fotinou & Ghanotakis, 1990). However, it is clear that also another thylakoid component absorbing around 413 nm is present, as also in the PS I-less/*apc*E$^-$ strain a pronounced 413-nm peak is visible, while no 531-nm band was observed (Figure 4).

Light-induced absorbance change measurements in isolated PS II particles showed D1-associated pheophytin *a* peaks at 422 and 545 nm, with a 4/1 ratio of absorbance at these wavelengths (Klimov et al., 1977; Nanba & Satoh, 1987). Similar ratios of 413- vs 531-nm absorbance increases were observed in CP47 His-to-Tyr mutants retaining PS II centers. The 7–15-nm wavelength shift between our measurements and the pheophytin difference spectra obtained on PS II reaction center particles may be a concern regarding the interpretation of the 413/531-nm peaks in the CP47 mutants. Pheophytin *a* absorption characteristics depend on the environment of the pigment [compare *in vitro* absorption maxima (Goedheer, 1966) with those in difference spectra of PS II reaction centers], and it is likely that the pheophytin associated with the D2 protein has an absorption maximum that is blue-shifted significantly as compared to the D1-associated pheophytin: in isolated PS II reaction centers, an absorption peak at 540 nm rather than at 545 nm is observed (Nanba & Satoh, 1987). Therefore, it is likely that the absorption maximum of pheophytin *a* is dependent on the environment just like that of chlorophyll *a*, and the interpretation of 412- and 531-nm peaks in His-to-Tyr mutants to originate from pheophytin *a* in CP47 seems reasonable.

This interpretation is strengthened by results obtained on thylakoids from the H216N/PS I-less/*apc*E$^-$ mutant, where no 531-nm peak was observed. Asn can function as a chlorophyll ligand (Zuber, 1986; Wagner-Huber et al., 1988), but Tyr cannot. The fact that a 531-nm maximum is absent from strains with decreased chlorophyll levels excludes the possibility that the observed pheophytin is the consequence of unspecific chlorophyll degradation. These results provide evidence that pheophytin *a* is formed in thylakoid membranes of the CP47 His-to-Tyr mutants. Mutations of His residues to Tyr in the hydrophobic regions of CP47 thus appear to result in the loss of Mg from the chlorophyll molecule.

An important issue is the stoichiometry of pheophytin and chlorophyll in the PS II complex. The increase in the 413-nm peak in various His-to-Tyr mutants is 8–16-fold smaller than the amplitude of the 435-nm peak. Assuming that the extinction coefficient of pheophytin *a* at 413 nm is similar to that of chlorophyll at 435 nm (Goedheer, 1966), this would imply that about 10% of the pigments contributing to the 695-nm emission in the mutants has been converted to pheophytin. As the 413-nm peak was less pronounced in excitation spectra monitoring 685-nm emission than in those monitoring 695-nm emission (data not shown), this suggests that absorption by pigments associated with CP47 is the main origin of 695-nm emission. Therefore, we interpret the 10% increase in the 413-nm band to indicate a ~10% pheophytin content of CP47 rather than of the entire PS II antenna. As the presumed number of chlorophylls associated with CP47 ranges from 10–12 (Tang & Satoh, 1984; Barbato et al., 1991) to 20–25 (de Vitry et al., 1984; Yamaguchi et al., 1988), this implies the formation of 1–2 pheophytins as a result of a single His-to-Tyr mutation in CP47.

These observations provide strong experimental evidence that the His residues that upon mutation to Tyr led to the presence of extra pheophytin in the PS II complex are chlorophyll ligands. However, because of the relative similarity of the effects of all His mutations studied here, also the other His residues investigated in this study most likely serve as chlorophyll ligands. Even the two His residues (His23 and His114) that upon mutation to Tyr led to a virtual disappearance of PS II are implied to be chlorophyll ligands: mutation of these residues to Asn and/or Gln led to less drastic effects on the amount of PS II present, which is in line with the results obtained upon mutation of His216. An interesting question that remains, however, is why mutation of some His residues has a much more drastic effect on the steady-state amount of PS II remaining in the thylakoid membrane. Logical options would be that some pigment molecules are more important contributors to the structural framework of PS II than others, or that pigments and surrounding protein domains have different rigidity, depending on their position. However, resolution of the question why different His residues have different sensitivity to mutations will require more detailed investigations.

Fluorescence Lifetime Components. The fluorescence decay observed is a sum of several lifetime components. Assignment of the fluorescence lifetime components to the various pigment pools in the cyanobacterial photosynthetic apparatus can be based on comparison of fluorescence decay-associated spectra obtained with PS II reaction centers in open and closed states, and with thylakoids from different strains.

(1) Components Associated with Open PS II Reaction Centers. The fastest lifetime component that was found to be associated with open PS II reaction centers is the τ =

42–45 ps component. Upon closing PS II reaction centers, the amplitude of this component was decreased approximately 50% in thylakoids of the PS I-less/*apc*E$^-$ strain and about 40% in thylakoids of the H202Y/PS I-less/*apc*E$^-$ mutant. The smaller reduction of this component upon closing PS II in H202Y/PS I-less/*apc*E$^-$ could be explained by a smaller amount of active PS II reaction centers on a chlorophyll basis (as shown in Table 2). This component did not exist in the PS II-less mutant *psb*B$^-$/*psb*C$^-$/PS I-less/*apc*E$^-$. We attribute this fast component to exciton trapping by P680. A similar assignment of a 40-ps component was made in a study using intact cells of the cyanobacterium *Synechococcus* 6301 (Mullineaux & Holzwarth, 1991).

Closing of PS II reaction centers led to a small (≤10%) decrease in the τ = 180–230 ps component. This component was not observed in the fluorescence decay-associated spectra of the *psb*B$^-$/*psb*C$^-$/PS I-less/*apc*E$^-$ mutant. A small portion of this component thus appears to originate from open PS II reaction centers, which is in agreement with results obtained on intact cells of PS I-containing *Synechocystis* 6803 (Bittersmann & Vermaas, 1991). At this moment, it is unknown what the origin of the remainder of the 180–230-ps component is. However, on the basis of analysis of fluorescence kinetics of the PS II reaction center complex, a 160-ps lifetime has been suggested to be correlated with recombination fluorescence in the PS II reaction center (Roelofs et al., 1991).

(2) Components Associated with Closed PS II Reaction Centers. The τ = 585–690 ps lifetime component was observed prominently in thylakoids of the PS I-less/*apc*E$^-$ strain and the H202Y/PS I-less/*apc*E$^-$ mutant. When the PS II reaction centers were closed, an increase of its amplitude was observed, especially in thylakoids of the PS I-less/*apc*E$^-$ strain. The smaller increase of this amplitude upon closing PS II reaction centers in H202Y/PS I-less/*apc*E$^-$ reflects a decreased number of active PS II reaction centers on a chlorophyll basis. In the *psb*B$^-$/*psb*C$^-$/PS I-less/*apc*E$^-$ mutant, this lifetime component was not found. On the basis of these observations, it is suggested that the τ = 585–690 ps component originates, at least in part, from closed PS II reaction centers. This assignment also was reached in an earlier study of *Synechocystis* 6803 wild-type and PS II mutants (Bittersmann & Vermaas, 1991), and a component with a similar lifetime was found to originate from the PS II core complex in *Synechococcus* 6301 (Mullineaux & Holzwarth, 1991).

Upon closing PS II reaction centers, a small increase in the amplitude of the τ = 1.4–1.6 ns component was observed. This component thus may originate in part from the PS II reaction center in the closed state. In analysis of the picosecond fluorescence kinetics of the cyanobacterial *Synechococcus* 6301 with open (F_0) and closed (F_{max}) PS II centers, a similar lifetime component was also attributed to a small fraction of closed PS II reaction centers (Mullineaux & Holzwarth, 1991). At the same time, the τ = 1.6 ns lifetime component was also observed in the fluorescence decay from thylakoids of the PS II-less *psb*B$^-$/*psb*C$^-$/PS I-less/*apc*E$^-$ mutant. This implies that this component is associated in part with the fluorescence of chlorophyll that is not in the PS II or PS I core.

Antenna Size and Fluorescence Lifetime. The lifetime of a fluorescence decay component associated with an open trap is correlated with the size of the antenna pool (Holzwarth, 1991), as the exciton trapping kinetics appear to be proportional to the antenna size (Schatz et al., 1988). The fluorescence lifetime kinetics of the two components associated with the open state of the PS II reaction center (τ = 42–45 and 180–230 ps) consistently were found to be smaller in four independent experiments in the H202Y/PS I-less/*apc*E$^-$ mutant than in the PS I-less/*apc*E$^-$ background strain, which would suggest that the functional antenna size is smaller in the mutant and/or that an additional fluorescence quencher (or excitation trap) has been introduced. This correlates with the higher light intensity needed for half-saturation of PS II electron transport in the His-to-Tyr mutants. Also, the lifetime of the 585–630-ps component, associated in part with closed PS II reaction centers, consistently was found to be decreased in the H202Y/PS I-less/*apc*E$^-$ mutant. Thus, our data are most consistent with the introduction of a component modestly capable of excitation trapping in the H202Y/PS I-less/*apc*E$^-$ mutant.

Formation of pheophytin in the CP47 protein upon His-to-Tyr mutations (see above) thus not only led to the destabilization of the CP47 protein and to a decrease of PS II assembly but also appeared to decrease antenna function and to lead to a small but significant decrease of the exciton lifetime in the PS II core antenna. This decrease might be caused by impaired energy transfer from pheophytin *a* to chlorophyll *a*, and by radiationless decay from pheophytin *a*.

In conclusion, studies of CP47 mutants in a PS I-less/*apc*E$^-$ background show that His residues in hydrophobic regions of the CP47 protein serve as chlorophyll ligands. Mutation of His residues to Tyr led to a decrease of energy transfer efficiency from the PS II core antenna to the reaction center. Increases in the 413- and 531-nm region, which are coincident with absorption peaks of pheophytin, were observed in the 77 K fluorescence excitation spectra of the CP47 His-to-Tyr mutants, compared to that of the PS I-less/*apc*E$^-$ background strain. The pheophytin in the antenna appears to serve as a shallow excitation trap, leading to decreased antenna function of CP47 carrying His-to-Tyr mutations.

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