Mutation of Histidine Residues in CP47 Leads to Destabilization of the Photosystem II Complex and to Impairment of Light Energy Transfer†

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ABSTRACT: Site-directed mutagenesis has been used to change conserved histidine residues in hydrophobic regions of the photosystem II chlorophyll-binding protein CP47 in the cyanobacterium *Synechocystis* sp. PCC 6803. Nine mutants with one, four mutants with two, and four mutants with three His mutations in CP47 have been generated and characterized. Mutation of any one of seven different His residues to Tyr leads to slower photoautotrophic growth and apparent destabilization of the PS II complex. Mutations introduced into multiple His residues in one mutant exhibited a cumulative effect. Replacing His by Asn leads to a much smaller effect than observed upon mutation to Tyr. This is consistent with the hypothesis that the mutated His residues are chlorophyll ligands: Asn can substitute as chlorophyll ligand, whereas Tyr cannot. Further evidence supporting a role of the mutated His residues in chlorophyll binding comes from measurements of the light intensity needed to half-saturate oxygen evolution. All His mutants with impaired PS II function needed higher light intensities for half-saturation than wild type. A possible explanation for this decrease in antenna efficiency in the mutants is a loss of the Mg in the chlorophyll due to a loss of the fifth ligand, and thus the formation of a pheophytin molecule in the antenna. We conclude that conserved His residues in hydrophobic regions of CP47 indeed are chlorophyll ligands and that these ligands are important for PS II stability as well as efficient antenna function.

The light-harvesting system of photosystem II (PS II) consists of a peripheral and a core antenna complex. In cyanobacteria, the peripheral antenna is the phycobilisome, which is a pigment–protein complex on the cytoplasmic side of the thylakoid membrane and which acts as the major light-harvesting system for PS II (Bryant, 1991). The core antenna is formed mostly by pigments associated with the chlorophyll-binding proteins CP47 and CP43 (encoded by the *psb*B and *psb*C genes, respectively) (Bricker, 1990; Vermaas & Ikeuchi, 1991). The CP47 protein appears to be more closely associated with the reaction center complex than CP43: CP43 can be removed from the PS II core complex by treatment with either chaotropic agents such as potassium thiocyanate (Yamaguchi et al., 1988) or by additional detergent treatment (Yamagishi & Katoh, 1985; Akabori et al., 1988), while CP47 remains associated with the D1–D2–cyt *b*559 complex under these conditions.

CP47 not only serves as an antenna protein for PS II, but also performs other functions in the PS II complex. CP47 appears to play an important role in stable assembly of PS II (Vermaas et al., 1986, 1988), while hydrophilic regions of CP47 may be closely associated with the extrinsic 33-kDa manganese-stabilizing protein [reviewed by Bricker (1990)].

The focus of this study regarding the CP47 protein is on the requirements for pigment binding. CP47 binds chlorophyll *a* molecules and some β-carotenes (de Vitry et al., 1984), but the number of chlorophyll *a* molecules bound to CP47 is not well established. The estimates range from 10–12 (Tang & Satoh, 1984; Barbato et al., 1991) to 20–25 (de Vitry et al., 1984; Yamaguchi et al., 1988) chlorophylls per CP47 protein. Also, the sites where the pigments are bound in CP47 are still unknown.

Histidine (His) residues coordinate many of the bacteriochlorophyll molecules to their apoproteins in bacterial pigment proteins (Deisenhofer et al., 1985; Zuber, 1986; Tronrud et al., 1986, Zuber & Brunisholz, 1991). By analogy, His residues could also play a major role in binding chlorophyll molecules to protein in the core antenna components of PS II in higher plants and cyanobacteria: these proteins are relatively rich in His residues, and CP47 contains 12 highly conserved His residues in hydrophobic regions of the protein (Figure 1). The distribution pattern of His residues over the hydrophobic regions of CP47 is quite similar to that of CP43, even though these two proteins, while obviously related, exhibit only limited overall primary sequence homology [reviewed by Bricker (1990)]. The putative membrane-spanning helices I, II, III, IV, and VI of CP47 contain at least 2 His residues each; in many cases, 2 His residues in 1 helix are separated by 13–14 residues, placing them toward opposite sides of the membrane; the spacing is such that in an α-helical arrangement the His residues of 1 pair are expected to point in a similar direction. A similar positioning of His residue pairs in membrane-spanning domains is also found in other chlorophyll-binding proteins, including in the PS I reaction center core proteins. If these His residues indeed would serve as chlorophyll ligands, one would expect that chlorophyll molecules in CP47 and CP43 are arranged somewhat symmetrically in two layers, one near each side of the membrane. Such a notion is compatible with what is observed experimentally by electron microscopic analysis of two-dimensional crystals of the light-harvesting complex II (LHC II) Kühlbrandt & Wang, 1991). In LHC II, 14–15 chlorophylls are arranged rather symmetrically on 2 levels, corresponding roughly to the upper and lower half of the lipid bilayer.

The aim of our research was to approach the question of whether these His residues in hydrophobic regions of CP47

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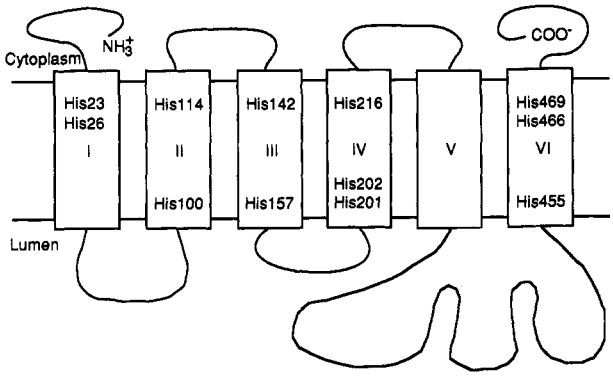

FIGURE 1: Putative membrane folding pattern and distribution of conserved His residues in hydrophobic regions of CP47 from *Synechocystis* 6803. This model is based on the prediction of the CP47 protein structure through its hydropathy profile (Vermaas et al., 1987) and comparison of the derived amino acid sequences of CP47 from four species (Bricker, 1990).

bind chlorophyll, and what significance these residues may have in energy transfer and PS II assembly. Utilizing site-directed mutagenesis of His residues in CP47, we have introduced mutations in His residues in hydrophobic regions of CP47 in the cyanobacterium *Synechocystis* 6803, and show that particularly mutations to residues that are not likely to serve as a ligand to chlorophyll lead to a modification of PS II stability and light energy transfer efficiency, in strong support of the hypothesis that these His residues are chlorophyll ligands.

MATERIALS AND METHODS

Growth conditions and transformation methods for *Synechocystis* 6803 have been described (Vermaas et al., 1987). To measure growth rates, strains were grown in liquid in BG-11 (Rippka et al., 1979) under photoautotrophic conditions (in the absence of glucose) and photoheterotrophic conditions (in the presence of 5 mM glucose). The optical density (cell scattering) at 730 nm was measured with a UV-160 spectrophotometer about every 12 h.

Mutagenesis. The method used for site-directed mutagenesis has been described (Eaton-Rye & Vermaas, 1991). To introduce site-specific mutations in *psb*B, oligonucleotide-directed mutagenesis was applied by using an M13mp19 template containing a part of the *psb*B gene from *Synechocystis* 6803 (from a *Bam*HI site at nucleotide 259 to a *Nco*I site 499 nucleotides downstream of the *psb*B stop codon). A 1.2-kb kanamycin-resistance cartridge was inserted at a *Nco*I site 369 nucleotides downstream of the end of *psb*B. After mutagenesis in M13, the mutated *psb*B gene fragments together with part of the kanamycin-resistance gene was cut out and ligated into a construct in pUC18 that contained a complementary piece of *psb*B and the kanamycin-resistance cartridge [see Eaton-Rye and Vermaas (1991) for details]. The resulting kanamycin-resistant plasmids were used for transformation of a *psb*B-deletion mutant of *Synechocystis* 6803. The introduction of a kanamycin-resistance marker downstream of *psb*B does not significantly affect the phenotype of the mutant (Eaton-Rye & Vermaas, 1991).

To verify the existence of the appropriate mutation(s) in the transformants, the region containing the mutation(s) was amplified from genomic DNA by a polymerase chain reaction (PCR) and sequenced to confirm the presence of the desired mutation(s).

Electron Transport Measurements. Oxygen evolution measurements on mutants and wild-type cells were performed using a Gilson oxygraph, Model KM. In order to excite either phycobilisome pigments or mostly chlorophyll, the actinic light from a xenon arc lamp was filtered through a Corion S40-600-S band-pass filter (bandwidth 50 nm; transmission maximum at 600 nm) or a Schott LG-665 filter (transmits ≥665-nm light), respectively. Neutral density filters were used to adjust the intensity of actinic light. The electron acceptor was 0.5 mM $K_3Fe(CN)_6$, while 0.1 mM 2,5-dimethyl-*p*-benzoquinone was added as redox mediator between thylakoids and the nonpenetrating ferricyanide.

Photosystem II/Chlorophyll Stoichiometry. Herbicide-binding experiments were used to quantify the stoichiometry of functional PS II reaction centers on the basis of chlorophyll concentration. Various concentrations of radiolabeled diuron were added to 1-mL samples containing cells at 30 μg of chlorophyll/mL in 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)/NaOH, pH 7. To correct for the amount of diuron bound to cells at sites other than PS II, control experiments were performed in which diuron binding was measured in the presence of 20 μM unlabeled atrazine, which displaces diuron from its binding site in PS II.

Western Blotting. The procedure for the preparation of the thylakoids from the wild type and mutants was described in Vermaas et al. (1990). Methods used for SDS–polyacrylamide gel electrophoresis and Western blotting were identical to those described in Vermaas et al. (1988).

RESULTS

Mutations. Site-directed mutagenesis was performed using mixed oligonucleotides which were designed to change selected His residues of CP47 to Tyr, Asn, or Asp. Nine strains were isolated carrying a single site-directed mutation in a His residue; these mutants (H100Y, H114Y, H114N, H142N, H157Y, H201Y, H202Y, H216Y, and H216N) are named by the CP47 residue number of the mutated His, followed by the one-letter code for the residue that has been introduced. Four mutant strains containing two mutated His residues (H201Y/H202Y, H142N/H157N, H142N/H100Y, and H216Y/H142Y) and four strains containing three His mutations (H201Y/H202Y/H157N, H201Y/H202Y/H157D, H201Y/H202Y/H216Y, and H201Y/H202Y/H216N) have also been obtained; nomenclature for these double and triple mutants is analogous to that of mutant strains carrying single mutations. In the double mutant H201Y/H202Y, His201 and His202 were changed to tyrosine at the same time by using a single oligonucleotide. In the double mutants H142N/H157N, H142N/H100Y, and H216Y/H142Y, the second mutation was introduced into the template which already carried the first single mutation. To generate the four triple mutants, the third mutation was introduced into the template carrying the double mutation H201Y/H202Y.

Photoautotrophic Competence and Electron Transport Rates. Growth curves of wild type and mutant strains under photoautotrophic and photoheterotrophic conditions were measured. Figure 2 shows the photoautotrophic growth curves of wild type and several mutants with mutated His residues in helix IV. The doubling times of wild type and all mutants under photoautotrophic conditions are listed in Table I. Under photoautotrophic conditions, cells with a single His-to-Tyr mutation exhibit photoautotrophic growth rates which are decreased as compared to wild type. However, mutational effects at some His residues are more pronounced than at other residues. For example, H100Y and H114Y were very hard to grow photoautotrophically (doubling time 5–6 days),

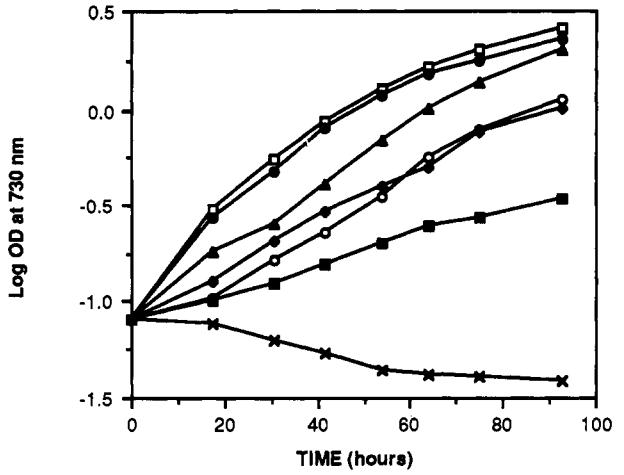

FIGURE 2: Photoautotrophic growth curves of wild type (□) and several mutants with mutation(s) in helix IV, H201Y (▲), H202Y (○), H216Y (♦), H216N (●), H201Y/H202Y (■), and H201Y/H202Y/H216Y (×), measured as the optical density (cell scattering) of the liquid culture at 730 nm.

Table I: Photoautotrophic Growth Rates and Oxygen Evolution Rates in Wild Type and Mutants

name	photoautotrophic doubling time (h)[a]	oxygen evolution rate (%)[a]
wild type	11	100[b]
H100Y	140	6[c]
H114Y	130	8[c]
H114N	45	25
H142N	18	86
H157Y	16	93
H201Y	16	92
H202Y	23	47
H216Y	28	27
H216N	11	98
H201Y/H202Y	62	11[c]
H142N/H157N	19	89
H142N/H100Y	no growth	0
H216Y/H142Y	no growth	0
H201Y/H202Y/H157N	no growth	0
H201Y/H202Y/H157D	no growth	0
H201Y/H202Y/H216Y	no growth	0
H201Y/H202Y/H216N	110	9[c]

[a] Data shown are the average of three experiments; in each case, data are reproducible within 20% of each value reported. [b] Excitation at >570-nm wavelength. The excitation light intensity was 7600 $\mu E \cdot m^{-2} \cdot s^{-1}$. The O_2 evolution of wild type was 420–450 μmol of O_2·(mg of chl)$^{-1}$·h^{-1}. [c] Unstable O_2 evolution rate with half-times of 2 min or less.

while the doubling time of H157Y was 16 h, and thus only slightly different from that of wild type. Photoautotrophic growth of the double His-to-Tyr mutant (H201Y/H202Y) was impaired more than that of the single mutants H201Y and H202Y. The triple mutants H201Y/H202Y/H216Y, H201Y/H202Y/H157D, and H201Y/H202Y/H157N were unable to grow photoautotrophically, while H201Y/H202Y/H216N grew very slowly.

The effect of His-to-Asn mutations on photoautotrophic growth is smaller than that of the comparable His-to-Tyr mutations. H216N grew at a rate close to that of wild type, while H114N also has a better photoautotrophic growth rate than H114Y. Even the double mutant H142N/H157N could grow well photoautotrophically (with a doubling time of 19 h). The growth rate of all mutants under photoheterotrophic conditions is similar to the growth rate of wild type (doubling time 11–13 h; data not shown), indicating that in these mutants the decrease in photoautotrophic growth rate is due exclusively to a change in the PS II complex.

Table II: Chlorophyll/PS II Ratio and [^{14}C]Diuron Dissociation Constant in Wild Type and Mutants

strain	Chl/PS II ratio[a]	K_D (nM)[a]	strain	Chl/PS II ratio	K_D (nM)
wild type	800	12	H216N	900	12
H100Y	10510	37	H201Y/H202Y	4130	18
H114Y	7160	32	H142N/H157N	1010	17
H114N	3630	23	H142N/H100Y	ND[b]	ND
H142N	1020	11	H216Y/H142Y	ND	ND
H157Y	920	12	H201Y/H202Y/H157N	ND	ND
H201Y	1060	12	H201Y/H202Y/H157D	ND	ND
H202Y	1110	14	H201Y/H202Y/H216Y	ND	ND
H216Y	1230	12	H201Y/H202Y/H216N	5700	27

[a] Data reported reflect an average of three experiments. The reproducibility of the results was within 15% of the averages given for all strains except H100Y, H114Y, H114N, H201Y/H202Y, and H201Y/H202Y/H216N; for those strains, the reproducibility was within 25% of the data reported in this table. [b] No measurable [^{14}C]diuron binding could be detected.

In Table I, the oxygen evolution rates of all mutants are presented as the percentage relative to that of wild type. Lower electron transport rates were observed in most of the single mutants, especially for the poorly photoautotrophic mutants H100Y and H114Y. For H216Y/H142Y, H142N/H100Y, and three triple mutants, H201Y/H202Y/H216Y, H201Y/H202Y/H157D, and H201Y/H202Y/H157N, no oxygen evolution could be measured, which is compatible with the lack of photoautotrophic growth of these mutants.

PS II Quantitation. Binding assays with different concentrations of radiolabeled diuron (DCMU, a PS II-directed herbicide) to whole cells are a rapid and accurate method to quantify the number of PS II centers on a chlorophyll basis in wild type and mutants *in vivo*. Each assembled PS II complex contains a single high-affinity diuron-binding site. The diuron-binding affinity and the number of herbicide-binding sites on a chlorophyll basis can be calculated from double-reciprocal plots of the amount of specific binding of diuron to PS II at different concentrations of the herbicide. The results are summarized in Table II. Wild type contains 1 diuron-binding site per 800 chlorophyll molecules. Most of the mutants appear to have lost herbicide-binding sites on a chlorophyll basis, indicating that the PS II/(PS II + PS I) ratio has decreased in these mutants. H100Y and H114Y exhibited an approximately 10-fold increase in their chlorophyll/PS II ratios over that found in wild type. From Table II, it can also be noted that some mutants, such as H100Y, H114Y, and H201Y/H202Y/H216N, appeared to show a decrease of herbicide-binding affinity. However, this decrease in affinity may be partially artifactual because of the low concentration of PS II reaction centers in the thylakoids. The high chlorophyll/PS II ratio in H100Y and H114Y indicates that the number of PS II reaction centers on a chlorophyll basis has decreased in these mutants. The triple mutants H201Y/H202Y/H216Y, H201Y/H202Y/H157D, and H201Y/H202Y/H157N did not bind diuron, and the double mutants H216Y/H142Y and H142N/H100Y also lost herbicide-binding ability. This implies that they do not contain a significant amount of functionally intact PS II reaction centers in their thylakoids, and suggests a complete destabilization of PS II after mutation of several His residues. The relative PS II concentration as measured by diuron binding to some extent parallels the observed photoautotrophic growth rates. Thus, the decreased growth rates observed in several mutants appears to be due in part to a decrease in the amount of intact PS II in the thylakoid. However, other factors may

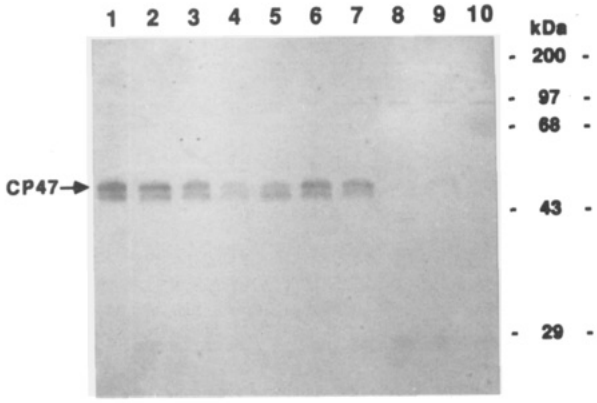

FIGURE 3: Western blot of thylakoid proteins from wild type and mutants probed with antisera against the CP47 protein. Two micrograms of chlorophyll was loaded per lane. The location of the molecular mass markers is indicated. Lane 1, wild type; lane 2, H201Y; lane 3, H202Y; lane 4, H201Y/H202Y; lane 5, H216Y; lane 6, H216N; lane 7, H142N/H157N; lane 8, H201Y/H202Y/H157D; lane 9, H201Y/H202Y/H216N; lane 10, H201Y/H202Y/H216Y.

also be involved in that some mutants with reasonably similar PS II/chlorophyll ratios (for example, H201Y, H202Y, and H216Y) differ in growth rates and rates of oxygen evolution.

Protein Composition. To study the effects of His mutations on PS II reaction center protein assembly and/or stability, blots of thylakoid proteins, size-separated by SDS–PAGE, were exposed to antisera against spinach CP47, D1, and D2. The immunodetection of CP47 is shown in Figure 3. All single and double mutants contain CP47, even though mutants appear to have less CP47 on a chlorophyll basis than wild type. The amount of immunoreaction in the different mutants roughly parallels the respective photoautotrophic growth rates. The triple mutant H201Y/H202Y/H216N (lane 9) has a decreased but detectable amount of CP47. Two other mutants, H201Y/H202Y/H157D (lane 8) and H201Y/H202Y/H216Y (lane 10), lose their normal CP47 bands. Interestingly, in the triple mutants, two new bands that cross-react with the CP47 antiserum appear: a lower band at approximately 28 kDa, along with a high molecular mass band. The presence of protein bands binding CP47 antisera suggests that (at least part of) CP47 can be synthesized in the triple mutants but that the protein is not stable in the thylakoids.

In Figure 4, the results of immunoreaction of thylakoids from mutants and wild type with antisera against the D1 and D2 proteins are shown. In contrast to the situation for CP47, D1 and D2 are measurable in thylakoids from all mutants. However, the apparent amount of D1 and D2 is decreased in a number of mutants, particularly in the triple mutants. It may be interesting to note that the amount of D1 in the various mutants may be decreased more than that of D2.

Energy Transfer. The observation that His-to-Tyr mutations have a much larger effect on PS II stability and PS II-dependent growth than the corresponding His-to-Asn mutations would favor the interpretation that these His residues would interact with chlorophyll: in size, His is closer to Tyr than to Asn, but Asn (and not Tyr) could replace His as chlorophyll ligand. To further examine the possibility of chlorophyll binding to the His residues targeted, we probed whether the mutations affected light-harvesting capabilities of the PS II core antenna. In order to monitor the efficiency of energy transfer from both the core and peripheral antenna system to the PS II reaction center, Hill reaction rates were measured in intact cells at various light intensities. Interestingly, for many mutants the light intensity saturation curve differed significantly from that seen in wild type. As the most sensitive and representative parameter, we chose to present the light intensity at which the initial rate of oxygen evolution is half of that observed at saturating light intensity (7000 $\mu E \cdot m^{-2} \cdot s^{-1}$). The results are presented in Table III. Upon excitation with red light (exciting chlorophyll and phycobilisome pigments absorbing above 665 nm), most mutants (except H216N) were found to require higher light intensities for half-saturation of oxygen evolution than wild type, implying that the mutations in CP47 have decreased the apparent light-harvesting efficiency of PS II. This result was most notable for H201Y/H202Y, which required 1350 $\mu E \cdot m^{-2} \cdot s^{-1}$ to reach half-saturation of its oxygen evolution rate, while this value was 885 $\mu E \cdot m^{-2} \cdot s^{-1}$ for wild type. When excitation at 600 nm (exciting phycobilisome pigments) was employed, mutants showed the same pattern of a higher light requirement for half-saturation of electron transfer. This indicates that the decreased efficiency of light harvesting also extends to light absorbed by phycobilisomes.

To check whether the increased light requirement for half-saturation of PS II electron transfer in the CP47 mutants is caused by a decreased PS II/chlorophyll ratio rather than by a decreased light-harvesting efficiency, the light saturation curve for the D2 mutant G215W [in which Gly215, located next to the His residue that is proposed to bind Q_A and Fe^{2+}, was mutated to Trp (Vermaas et al., 1990)] was measured. In this mutant, the PS II/chlorophyll ratio has decreased by about a factor of 2, with a concomitant decrease in the initial rate of oxygen evolution (Vermaas et al., 1990). In G215W, the rate of oxygen evolution was half of the saturated rate at a lower light intensity (480 $\mu E \cdot m^{-2} \cdot s^{-1}$) than in wild type upon excitation at $\geq$665 nm (not shown); this decrease is expected because the phycobilisome/PS II ratio is increased in G215W (the phycobilisome content is independent of the level of PS II in *Synechocystis* mutants), and thus the effective antenna size per PS II is increased as compared to wild type. In any case, these results imply that the increased light requirement to saturate PS II in CP47 mutants is not a trivial consequence of a decreased PS II/chlorophyll ratio and that the light-harvesting of PS II in these mutants indeed has decreased. These results indeed are compatible with a role of selected CP47 His residues in binding of chlorophyll serving in light absorption and energy transfer.

DISCUSSION

We demonstrate here that in most cases substitution of conserved His residues in hydrophobic regions of CP47 results in a decreased photoautotrophic viability of the mutants and in an apparently decreased stability of PS II. The extent of the decrease depends both on the nature of the mutation and on the number of mutations introduced. In mutants carrying a single mutation, replacement of His by Tyr generally led to slower photoautotrophic growth, lower electron transport rates in oxygen evolution measurements, and a decreased number of PS II reaction centers on a chlorophyll basis. The effect of the double mutations is approximately cumulative as compared to the single mutations. The amount of PS II is impaired further in triple mutants. This is indicative of a progressive destabilization of the PS II reaction center upon successive mutations of His residues.

Do the Conserved His Residues Serve as Chlorophyll Ligands? A central question that triggered this research is whether the conserved His residues in hydrophobic domains of CP47 serve as fifth ligands to the Mg in the chlorophyll ring, thus keeping the pigment in place and providing an anchor for the Mg. Histidine has been shown to serve as ligand to

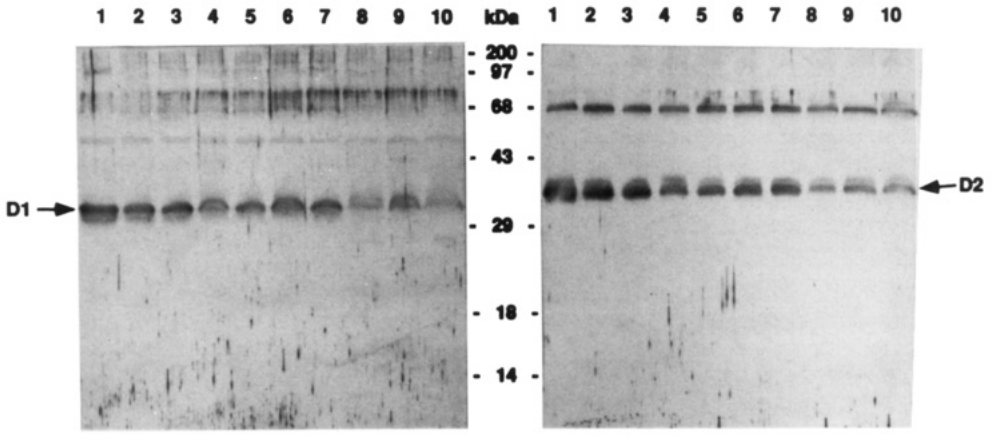

FIGURE 4: Immunoreaction of antisera against D1 and D2 with their respective antigens in thylakoids from wild type and mutants. Two micrograms of chlorophyll was loaded per lane. Lane 1, wild type; lane 2, H201Y; lane 3, H202Y; lane 4, H201Y/H202Y; lane 5, H216Y; lane 6, H216N; lane 7, H142N/H157N; lane 8, H201Y/H202Y/H157D; lane 9, H201Y/H202Y/H216N; lane 10, H201Y/H202Y/H216Y.

Table III: Half-Saturation Light Intensities of Oxygen Evolution Rates in Wild Type and Mutants ($\mu E \cdot m^{-2} \cdot s^{-1}$)

excitation wavelength (nm)	WT	H142N	H157Y	H201Y	H202Y	H201Y/H202Y	H216Y	H216N
>665	890 ± 49[a]	1090 ± 57	1130 ± 38	950 ± 19	1150 ± 62	1350 ± 35	1180 ± 19	910 ± 58
600	370 ± 26	430 ± 21	420 ± 35	410 ± 31	620 ± 49	740 ± 25	460 ± 30	350 ± 28

[a] Standard error from three independent experiments.

bacteriochlorophyll (Deisenhofer et al., 1985; Tronrud et al., 1986; Zuber & Brunisholz, 1991). Substitution of His by Asn appears to have much less effect on PS II stability and function than a similar substitution by Tyr. For example, when His216 was replaced by Tyr, the doubling time of the mutant strain under photoautotrophic growth conditions increased almost 3-fold. This was accompanied by an increase in the ratio of chlorophyll to PS II, a 70% reduction in oxygen evolution activity, and an increase in the half-saturation light intensity for oxygen evolution. In contrast, H216N was quite similar to wild type in all these respects. The capacity for Asn to substitute for His was also evident in the double mutant H142N/H157N (which has a rather wild-type phenotype) and the triple mutant H201Y/H202Y/H216N (the only one we analyzed that is still photoautotrophic). Thus, Asn essentially can functionally replace His in these cases, while Tyr (which in terms of size is more similar to His than Asn is) cannot. These observations are in line with what would be expected if the mutated His residues serve as chlorophyll ligands. While Tyr cannot provide a ligand for the Mg in the chlorophyll, the N of the amide group of Asn can probably serve as an effective ligand to the Mg in the chlorophyll as suggested for the case of bacteriochlorophyll (Zuber, 1986; Wagner-Huber et al., 1988; Bylina & Youvan, 1988). However, the effectiveness with which Asn is able to functionally replace His may be different for different His residues of CP47. In the case of His114 in what is thought to be the second membrane-spanning domain of CP47, a His-to-Asn mutation leads to a phenotype of decreased photoautotrophic growth rates and PS II/chlorophyll ratios. Nonetheless, the disruptive effects of the mutation are milder than the corresponding His-to-Tyr substitution. These results are correlative evidence that indeed the mutated His residues are chlorophyll ligands, and that Asn can replace His to provide a ligand to chlorophyll, but may not lead to as stable an association as is the case with His.

Antenna Function in His Mutants. It may be rather surprising that the various single-site His mutations all lead to a similar and moderate decrease in PS II antenna efficiency and that both the peripheral and core antennas have been affected in their efficiency of transfer to the reaction center. The reasonable assumption that this change in antenna efficiency is due to a modification of pigment binding also implies an important role of the His residues in pigment–protein association. At least two possible hypotheses can be put forward on what the major functional consequence of the modification of His is on a nearby chlorophyll: (1) the chlorophyll is lost *in toto*, and a decrease in antenna function is then explained by a loss of the one chromophore and a loss of energy transfer to the reaction center from selected other chromophores; or (2) His serves as a chlorophyll ligand, and the Mg is lost from the center of the pigment upon removal of the fifth ligand, resulting in pheophytinization; the decrease in antenna efficiency may then be due to less optimal energy transfer parameters (such as the overlap integral and lifetime of the excited state of the pheophytin) between pheophytin and chlorophyll. At this moment, we favor the second explanation (pheophytinization). Precedence for this occurrence is provided by site-directed mutants of purple bacteria, in which the His serving as a ligand to one of the bacteriochlorophylls in the reaction center complex was removed. A bacteriopheophytin resulted (Bylina & Youvan, 1988), accompanied by a moderate decrease in the quantum yield of charge separation. A concept of pheophytinization at a certain location in the various mutants provides a plausible explanation for the generally similar decrease in antenna efficiency in the various single His-to-Tyr mutants: it is likely that an excitation travels through the entire core antenna before it is trapped (Holzwarth, 1987; Schatz et al., 1988) and therefore an excitation is likely to visit any pigment in the core antenna, no matter with which part of the CP47 or CP43 protein it is associated. Under the reasonable assumption that the efficiency of pheophytin-to-chlorophyll energy transfer does not depend on where in the chlorophyll-binding protein the pigments are, one indeed does expect a similar alteration of antenna efficiency in the various mutants. Unfortunately, physical detection of a single extra pheophytin molecule in the PS II antenna needs to await further progress in tailoring the photosynthetic apparatus of *Synechocystis* to contain a more favorable PS II/pigment ratio.

One of the reasons why we do not favor the hypothesis that the chlorophyll is lost (ring and all) when a His-to-Tyr mutation is introduced is that it is not obvious why this would lead to a noticeable decrease in antenna efficiency. The loss of a single chlorophyll out of some 50 antenna chlorophylls associated with PS II is not expected to have a significant impact on antenna function, and would even less affect phycobilisome-to-PS II energy transfer: it is assumed that there are many parallel energy transfer pathways in antenna systems and the loss of one chlorophyll at various locations would not uniformly decrease the transfer efficiency by a little less than a factor of 2.

Effects of His Mutations on PS II Abundance in the Thylakoid. It is clear that the steady-state concentration of CP47 and, to a lesser extent, of D1 and D2 is decreased upon mutation of His residues in CP47. This decrease in the steady-state concentration can result from either an increase in the turnover rate of the protein or a decrease in the rate of synthesis. In general, previous experimentation with *Synechocystis* PS II mutants has not provided any clear evidence for decreased translation rates of PS II genes. Instead, the lack of accumulation of PS II proteins in thylakoids from mutants in which genes for other PS II proteins have been inactivated has been shown to be due to rapid turnover of these proteins (Yu & Vermaas, 1990). Available evidence for the CP47 His mutants supports this notion: from the CP47 immunoblots (Figure 3), it appears that in the triple mutants H201Y/H202Y/H157D and H201Y/H202Y/H216Y the normal CP47 bands were not detected, and also no intact PS II reaction centers could be observed. However, a lower molecular mass band cross-reacting with CP47 appears in these mutants. Judging from its decreased size, this 28-kDa band cross-reacting with CP47 antisera probably originates from a degradation product.

Also, in other systems there is little evidence for translational regulation by chlorophyll binding: studies on LHC II show that absence of chlorophyll leads to a rapid turnover of LHC II (Bennett, 1981) and in general chlorophyll binding is required for the stability of chlorophyll-binding proteins in higher plants (Plumley & Schmidt, 1987; Mullet, 1988; Mullet et al., 1990). In addition, it has been observed that replacement of histidines in LHC II decreases the stability of the complex (Kohorn, 1990). However, evidence also exists for the availability of chlorophyll to affect translation of chlorophyll-binding proteins in certain systems (Eichacker et al., 1992). Future studies on our mutants may clarify the role chlorophyll binding plays in regulation of PS II abundance in cyanobacteria.

Importance of CP47 for the Stability of Other PS II Proteins. CP47 has an important role in the functional assembly of PS II (Vermaas et al., 1988; de Vitry et al., 1989). Interruption of the *psb*B gene with a kanamycin-resistance cartridge led to a PS II$^-$ phenotype with no detectable amounts of CP47, D1, and D2 in the membrane. However, a mutant carrying a 15-residue deletion in CP47 (Eaton-Rye & Vermaas, 1991) as well as 2 chimeric mutants containing portions of the *psb*B gene from spinach and *Synechocystis* exhibited a PS II$^-$ phenotype, but accumulated measurable levels of D1 and D2 in the membrane (Vermaas et al., 1988), suggesting that transiently available CP47 can help stabilizing D1 and D2. This notion is confirmed in the current study, where lower levels of CP47 in His mutants led to a decreased level of D1 and D2, and where mutants in which CP47 was no longer detectable by Western blotting (H201Y/H202Y/H157D and H201Y/H202Y/H216Y) exhibited diminished but measurable levels of D1 and D2. It is interesting to note that the level of D1 in the various mutants may parallel the level of CP47 more than D2 does. This phenomenon has also been observed in *psb*B deletion and *psb*B interruption mutants (Yu & Vermaas, 1990).

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