Site-Directed Mutagenesis of Conserved Histidines in the Helix VIII Domain of PsaB Impairs Assembly of the Photosystem I Reaction Center without Altering Spectroscopic Characteristics of P_{700}[†]

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ABSTRACT: The chloroplast *psaB* gene encodes one of the polypeptides of the photosystem I reaction center heterodimer that coordinates the electron transfer components P_{700}, A_0, and A_1. Histidine residues in the most highly conserved region of the PsaB protein are predicted to coordinate the P_{700} reaction center chlorophyll(s) and the initial electron acceptor, A_0. Oligonucleotide-mediated site-directed mutagenesis and chloroplast transformation of *Chlamydomonas reinhardtii* have been used to determine the importance of these conserved histidines in photosystem I reaction center biogenesis and function. It is demonstrated that these histidine residues are essential for stable accumulation of the photosystem I reaction center. Protein pulse-labeling shows that changing the histidine residues impairs a post-translational step in reaction center assembly. Photosystem I complexes from the mutants have been characterized by Electron Nuclear Double Resonance and Electron Spin Echo Envelope Modulation spectroscopy to determine the impact of any mutations on P_{700}^+. In all cases we determine that spectroscopic characteristics of P_{700}^+ remain unchanged. The implications of these results to current models of the photosystem I reaction center and related bacterial reaction centers are discussed.

In plants and algae, the electron transfer events of photosynthesis are initiated in the photosystem II (PSII)[1] and photosystem I (PSI) reaction center complexes located in the thylakoid membrane. PSI is a multisubunit membrane protein complex that uses light energy to transfer electrons from plastocyanin to ferredoxin. The PSI complex in chloroplast thylakoid membranes consists of at least 13 individual polypeptides and an associated chlorophyll *a/b* (Chl *a/b*) binding light-harvesting complex (Golbeck & Bryant, 1991; Golbeck, 1992). The reaction center core of PSI is a heterodimeric complex of two related polypeptides of 83 kDa called PsaA and PsaB, encoded by the chloroplast *psaA* and *psaB* genes. PsaA and PsaB coordinate the primary electron donor P_{700} and early electron acceptors A_0, A_1, and the 4Fe-4S iron-sulfur center F_X (Golbeck, 1992). Two additional electron acceptors, F_A and F_B, are coordinated by a chloroplast-encoded 9 kDa polypeptide, PsaC, located on the stromal side of the thylakoid membrane (Golbeck & Bryant, 1991; Golbeck, 1992). The cofactor P_{700} is probably a Chl *a* dimer, A_0 a Chl *a* monomer, and A_1 a vitamin K1 (phylloquinone) (Setif, 1992).

The *psaA* and *psaB* genes encoding the PSI reaction center heterodimer have been isolated and sequenced from a number of prokaryotic and eukaryotic organisms. Both the PsaA and PsaB proteins are highly hydrophobic and are predicted to contain 11 membrane-spanning α-helices based on analysis of hydrophobicity profiles (Fish *et al.*, 1985). A 6 Å resolution model of the PSI complex has been derived by X-ray diffraction from PSI crystals (Krauss *et al.*, 1993). This model predicts nine α-helical regions, eight of which are membrane-spanning, for both the PsaA and PsaB proteins. As yet, the resolution is insufficient to derive further information on which specific amino acids, or which regions of the PsaA and PsaB proteins, coordinate P_{700}, A_0, and A_1. In the absence of suitable X-ray diffraction data, comparative analysis of derived amino acid sequences from a number of species has been used to develop models that predict ligands to several of the cofactors, most notably F_X and P_{700}. The most highly conserved region of both PsaA and PsaB lies within a stretch of approximately 100 amino acids that includes a cysteine-rich region and a preceding stretch of predominantly hydrophobic amino acids (Webber & Malkin, 1990; Golbeck & Bryant, 1991). On the basis of the folding model of PSI developed by Fish *et al.* (1985), this conserved hydrophobic region is predicted to form membrane span VIII (Figure 1). From these amino acid comparisons, it has been predicted that the conserved cysteines provide ligands to F_X to form an interpolypeptide [4Fe-4S] iron-sulfur cluster (Figure 1) (Golbeck, 1992). The conserved histidines, 523 and 530 (from the derived amino acid sequence of *Chlamydomonas reinhardtii* PsaB), and

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[1] Abbreviations: bp, base pair(s); Bchl *a*, bacteriochlorophyll *a*; Bph *a*, bacteriopheophytin *a*; Chl *a*, chlorophyll *a*; CP47, antenna complex of photosystem II core; ENDOR, Electron Nuclear Double Resonance; ESEEM, Electron Spin Echo Envelope Modulation; FT, Fourier transform; hfc, hyperfine coupling constant; hfi, hyperfine interaction; LHC, light-harvesting complex; PSI, photosystem I; PSII, Photosystem II; P_{700}, primary electron donor in PSI; $\nu 1_H$, Nuclear Larmor Frequency of ^1H; $\nu 14_N$, nuclear Larmor frequency of ^{14}N.

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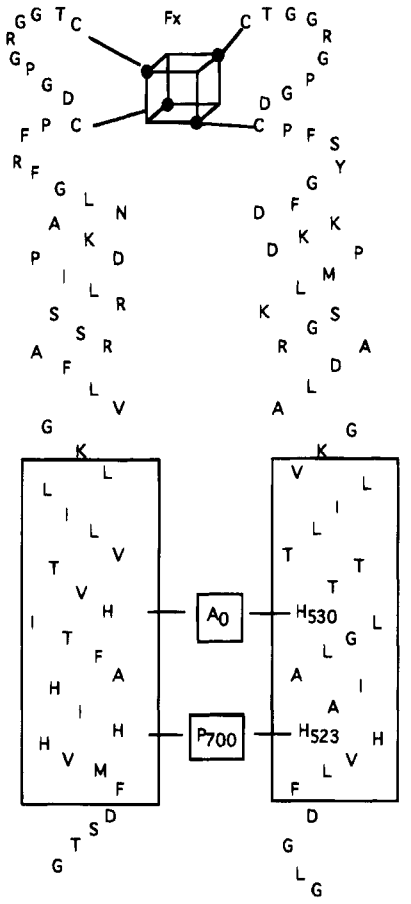


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          518                 538
           |                   |
PsaB  DFLVHHAIALGLHTTTLILVK
PsaA  DFMVHHIHAFTIHVTVLILLK
H. mobilis  DFVAAHAIAGGLHFTMVPLWR
C. limicola DWVMAHVITAGSLFSLIALVR
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FIGURE 1: (Top) Model for coordination of PSI reaction center cofactors P_{700}, A_0, and F_X. The [4Fe-4S] iron–sulfur center F_X is coordinated by two cysteines from each of the PsaA and PsaB polypeptides. Conserved histidines in the membrane span preceding the F_X binding region are predicted to coordinate the P_{700} Chl(s) and A_0. (Bottom) Comparison of the derived amino acid sequence that forms membrane span VIII of PsaA and PsaB (sequence from *C. reinhardtii*) and the equivalent region derived from *H. mobilis* and *C. limicola* reaction center genes. Histidines 522, 523, and 530 of PsaB are also conserved in PsaA and in all the plant and algal sequences so far determined. Histidine 523 (boldface) is further conserved in both *H. mobilis* and *C. limicola* reaction center proteins.

complementary histidines in PsaA have been suggested to function as ligands to the primary donor, P_{700}, and A_0, respectively (Robert & Moenne-Loccoz, 1990; Golbeck, 1992).

Recently, genes encoding the reaction center polypeptide of *Chlorobium limicola* (Büttner *et al.*, 1992) and *Heliobacillus mobilis* (Liebl *et al.*, 1993) have been isolated and sequenced. *Chlorobium* and *Heliobacillus* both have iron-sulfur center containing reaction centers that are ancestrally related to PSI. Comparison of the bacterial amino acid sequences with those from PsaA and PsaB has provided further information on amino acids conserved over a wide evolutionary time scale (Figure 1). A histidine, equivalent to H523 of PsaB, is conserved in all the reaction center

Table 1: Oligonucliotides Used for Generating Mutations

mutation	oligonucleotide
H522Y	5′-CCTTGTT**TA**CCACGCTATTGC-3′
H522Q	5′-CCTTGTT**CAA**CACGCTATTGC-3′
H523Y	5′-CTTGTTCAC**TA**CGCTATTGCT-3′
H523Q	5′-CTTGTTCAC**CAA**GCTATTGCT-3′
H523L	5′-CTTGTTCAC**CTA**GCTATTGCT-3′
H530L[a]	5′-TTAGGCCTTC**TA**ACTACAACA-3′

[a] Contains an additional silent mutation, underlined, that generates a unique *Stu*I site.

sequences, and it has been proposed to function as a ligand to the primary donor, P_{840}, of the *Chlorobium* reaction center and P_{798} of the *Heliobacillus* reaction center (Büttner *et al.*, 1992; Liebl *et al.*, 1993), as well as P_{700} of PSI.

The ability to generate site-directed mutants in the PSI reaction center proteins of cyanobacteria (Smart *et al.*, 1993) and the chloroplast of *Chlamydomonas reinhardtii* (Webber *et al.*, 1993) now allows us to test models that predict the function of conserved amino acids in [Fe-S]-type reaction centers. Only a limited number of site-directed mutations have been reported in the PsaB protein of PSI. These mutations have provided strong supporting evidence that the conserved cysteine residues, depicted in Figure 1, provide ligands for the iron-sulfur center F_X (Smart *et al.*, 1993; Webber *et al.*, 1993). To determine the function of the conserved histidine residues in membrane span VIII, we have introduced site-directed mutations into the chloroplast *psaB* gene of *Chlamydomonas reinhardtii*. We report that the mutations have an impact on PSI reaction center accumulation in thylakoid membranes, but no changes in P_{700} characteristics have been found.

MATERIALS AND METHODS

Plasmids and in Vitro Mutagenesis. Plasmid pG528G is an *Eco*RI–*Pst*I fragment of chloroplast DNA that encodes the *psaB* gene and a portion of *rbcL* (Bingham *et al.*, 1991). In addition, this plasmid contains a single silent site-directed mutation at codon position 528 that creates a unique *Stu*I site. The chimeric *aadA* (Goldschmidt-Clermont, 1991; Bingham & Webber, 1994) gene construct was cloned into a *Hin*cII site within the intergenic region between *psaB* and *rbcL* in pG528G to give plasmid pG528G-S. A *Bam*HI–*Pst*I fragment of pG528G was subcloned into M13mp19 and single-stranded DNA used as a template for oligonucleotide-directed mutagenesis. Oligonucleotides used are listed in Table 1. Following mutagenesis, a *Stu*I–*Ban*II fragment was subcloned from M13 into pG528G-S and used in transformation experiments.

Culture Conditions, Chloroplast Transformation, and Analysis of Transformant Cells. A wild-type strain of *Chlamydomonas reinhardtii* CC125 *mt*+ was used as the recipient of donor plasmids in the transformation experiments. CC2341 (*ac-u-g*-2.3) has a frame-shift mutation in the chloroplast *psaB* gene and accumulates no PSI (Girard-Bascou, 1987; Girard-Bascou *et al.*, 1987; Bingham *et al.*, 1991). The cells were maintained on HS (Sueoka, 1960) medium supplemented with acetate (HSA) when required. Chloroplast transformation was performed by the biolistics technique as previously described (Boynton *et al.*, 1988; Webber *et al.*, 1993). Bombarded cells were transferred to plates containing HS media supplemented with acetate, 100

μg mL^{-1} spectinomycin, and 1.2% agar and placed under dim light for 7−10 days until colonies appeared. Single colonies were restreaked onto solid medium. Total DNA was isolated from cells taken from confluent regions of the plates as previously described (Webber *et al.*, 1993) and resuspended at a final volume of 100 μL. One microliter of this DNA was then used as a template to PCR-amplify a 1 kbp fragment of chloroplast DNA using primers that flank the *Stu*I site and introduced mutations. Following digestion of the PCR-amplified DNA with *Stu*I, the DNA was size-fractionated on a 2% agarose gel. Sequential rounds of single-colony isolations were performed until homoplasmic cell lines were obtained from which the amplified DNA cut completely with *Stu*I. To confirm the presence of the desired mutations, the amplified DNA from the homoplasmic strains was sequenced using a Cycle Sequence kit (BRL) following the manufacturer's procedures.

Protein Analysis. For thylakoid membrane isolation, wild-type and mutant cells were broken by passage through a French press and thylakoid membranes purified by centrifugation through a sucrose step gradient following previously published procedures (Chua & Bennoun, 1975). Thylakoid membranes were then solubilized in gel loading buffer (5% LDS, 100 mM DTT, 10% glycerol, and 50 mM Tris, pH 8.8) and polypeptides size-fractionated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis using buffers and acrylamide concentrations described in Ikeuchi and Inoue (1988). Following electrophoresis, polypeptides were either visualized by staining with Coomassie blue or electroblotted onto a nitrocellulose membrane. The immobilized polypeptides were incubated with antisera against PsaA/B (a gift from Dr. A. Melis). For immunodecoration, a goat- anti-rabbit IgG−horseradish peroxidase was used followed by color development according to procedures supplied by Bio-Rad. Nondenaturing (green) gel electrophoresis was performed following published procedures (Delepelaire & Chua, 1979; Herrin *et al.*, 1992). Thylakoid membranes were solubilized in LDS sample buffer at 4 °C and size-fractionated through 10% acrylamide gels in the absence of SDS at 4 °C. PSI complexes were purified by solubilization of thylakoid membranes with dodecyl maltoside following published protocols (Takahashi, 1991).

Labeling with [^{14}C]Acetate. Cells were labeled with [^{14}C]acetate following published procedures (Kuras & Wollman, 1994). Cells grown to a density of 1 × 10^6 cells/mL in acetate-supplemented medium were washed and resuspended at the same volume in medium lacking acetate. After incubation for 1 h, cells were again washed with HS medium. Cycloheximide (10 μg mL^{-1}) was then added to the media followed by 25 μCi/mL [^{14}C]acetate (specific activity 60 mCi/mM). After 10 min, cells were pelleted by centrifugation and thylakoid membranes rapidly isolated and stored at −20 °C.

RNA Isolation and Northern Blotting. Total cellular RNA was isolated from *Chlamydomonas* cells, grown to a density of 1 × 10^6 cells/mL in acetate-supplemented medium as described previously (Xu *et al.*, 1993). Glyoxal-denatured RNA (10 μg) was size-fractionated by agarose gel electrophoresis and transferred to a Hybond-N membrane (Amersham) by capillary blotting. A *Bam*HI DNA fragment from pG528G, specific for *psaB*, was labeled using the random primer method (Feinberg & Vogelstein, 1983). DNA−RNA hybridizations were carried out in 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4), 50% formamide, 5 × Denhardt's, 1% SDS, and 100 μg mL^{-1} carrier DNA at 42 °C. Following hybridization, blots were washed twice in 5 × SSPE at 42 °C for 15 min each followed by washing in 0.1% SSPE/0.1% SDS at 50 °C for 1 h and a final 15 min rinse at room temperature with 0.1% SSPE.

Electron Transport and Optical Spectroscopy. PSI activity was measured polarographically as light-induced oxygen uptake in the presence of methyl viologen (50 μM), 2,6-dichlorophenolindophenol (0.1 mM), ascorbate (5 mM), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (20 μM), and sodium azide (5 mM) in 50 mM Tricine buffer (pH 8.0). Transient absorption difference measurements on the millisecond time scale were performed using a laboratory-built single-beam spectrophotometer described elsewhere (Kleinherenbrink *et al.*, 1994). The sample was held in a 1 cm optical cuvette and protected from the measuring light by an electronic shutter until approximately 100 ms before the saturating excitation flash at 532 nm generated by an Nd-YAG laser (Surelite, continuum). P_{700}^+ concentrations were calculated using published extinction coefficients (Hiyama & Ke, 1972).

ENDOR and ESEEM Spectroscopy. Electron Nuclear Double Resonance (ENDOR) spectroscopy and Electron Spin Echo Envelope Modulation (ESEEM) spectroscopy were performed on the primary donor cation radical P_{700}^+ of PSI samples in frozen solution. P_{700} was oxidized optically by irradiating a concentrated PSI preparation in EPR quartz capillaries of 4 mm o.d. Chl concentration for wild-type spectra was 4.0 mg mL^{-1} and for mutant spectra was 2 mg mL^{-1}. PSI from *Synechococcus* (9.8 mg of Chl mL^{-1}) was prepared as in Krauss *et al.* (1993). Illumination was performed at ambient temperature using a 1000 W tungsten lamp equipped with a water filter (10 cm path length) and a red filter (Schott RG 665). After 1 min, the samples were frozen under continued irradiation in liquid nitrogen and transferred to the spectrometer.

The ENDOR (Electron Nuclear Double Resonance) measurements were performed on a Bruker ESP 300E X-band EPR spectrometer with home-built ENDOR accessories (Käß *et al.*, 1994b). The sample temperature was controlled using a nitrogen flow Bruker ER4111 VT system (minimum temperature ≈100 K). The ESEEM (Electron Spin Echo Envelope Modulation) experiments were performed in a Bruker ESP 380E FT-EPR spectrometer equipped with a dielectric ring cavity (ESP 380−1052 DLQ-H) and an Oxford cryostat CF935 at approximately 10 K (Höfer, 1989). A four step phase-cycle was employed in all experiments to avoid artifacts in the spectra. Subtraction of the relaxation decay, corrections for eventually occurring base-line offsets, and the subsequent Fourier transformations were all done using the ESP380 software.

RESULTS

Generation of PSI Site-Directed Mutants by Chloroplast Transformation. Chlamydomonas strains carrying mutations in the histidine residues of membrane span VIII of PsaB were generated by chloroplast transformation by particle bombardment. The strains carrying the H522Y, H523Y, and H523Q mutations were created by cotransformation of the wild-type strain CC125 with plasmids carrying the mutant *psaB* gene

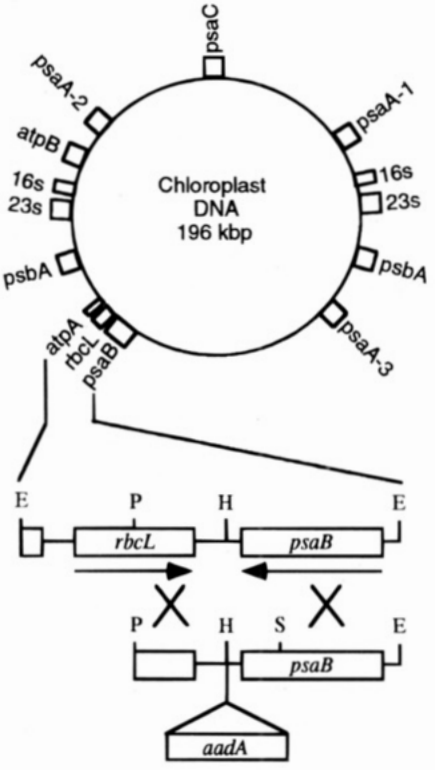

FIGURE 2: *Chlamydomonas* chloroplast DNA map showing the location of *psaB* and the strategy employed to introduce mutations. The *psaB* gene lies within the *Eco*RI (E) restriction enzyme fragment E14 (Harris, 1989; Bingham *et al.*, 1991). The donor *Eco*RI−*Pst*I (P) fragment contains a silent *Stu*I site (S), the site-directed mutation, and the chimeric *aadA* gene cloned into a *Hin*cII site (H) within the intergenic region between *rbcL* and *psaB*.

(Webber *et al.*, 1993) and plasmid p-228 (Newman *et al.*, 1990) that contains a 16S rDNA with a mutation that confers resistance to spectinomycin. Mutant strains H522Q, H523L, and H530L were generated using chimeric *aadA* gene constructs cloned 3′ to the *psaB* gene (Figure 2). Homologous recombination of the donor plasmid into the chloroplast genome also incorporates the *aadA* gene and renders cells resistant to spectinomycin (Figure 2).

Spectinomycin-resistant colonies appearing 7−10 days following transformation were picked and restreaked onto plates containing 100 μg mL^{-1} spectinomycin. Total cellular DNA was isolated from cells collected from confluent regions on the plates and amplified by PCR using primers that flank the site of the introduced mutation and a unique *Stu*I restriction enzyme site that is not present in the wild-type chloroplast *psaB* gene (Figure 2). The 1 kbp amplified fragment was digested with *Stu*I and size-fractionated on 2% agarose gels (Figure 3). Transformed cells that were heteroplasmic for the introduced mutation, i.e., amplified DNA could not be completely digested with *Stu*I, were subject to further rounds of single-colony isolation. Transformant strains selected for further characterization were homoplasmic, and the 1 kbp PCR fragment amplified from each mutant DNA preparation could be completely digested by *Stu*I (Figure 3). To ensure that the desired mutation was introduced into the chloroplast genome along with the *Stu*I site, the PCR-amplified DNA was directly sequenced (not shown). Typically, the frequency of cointegration of the *Stu*I site with the *aadA* cartridge ranges between 5% and 50% depending on the mutation. In all cases, transformants containing the *Stu*I site also contained the desired mutation.

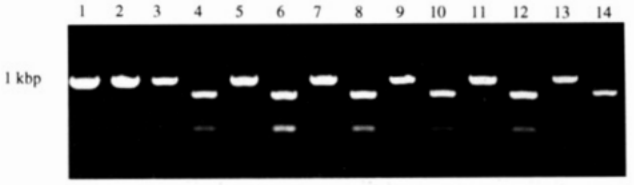

FIGURE 3: Analysis of PCR products from mutant cells. A 1 kbp fragment of *psaB* was amplified from total cellular DNA using primers that flank the site of the silent mutation in the donor plasmid that generates a *Stu*I site. The product was either loaded directly onto the 2% agarose gel (odd-numbered lanes) or digested with *Stu*I before electrophoresis (even-numbered lanes). Complete digestion with *Stu*I indicates that all copies of the wild-type *psaB* gene have been replaced with the mutant copy. Wild-type, lanes 1 and 2; H522Q, lanes 3 and 4; H522Y, lanes 5 and 6; H523Q, lanes 7 and 8; H523L, lanes 9 and 10; H523Y, lane 11 and 12; H530L, lanes 13 and 14. Note that the PCR product from wild-type cells is not digested with *Stu*I.

Table 2: Photosystem I Activity and Content of Thylakoid Membranes from Wild-Type and Mutants

mutant name[a]	oxygen uptake[b] [μmol (mg of Chl^{-1} h^{-1}]	Chl/P700 ratio
wild-type	1145	910
H522Y	0	0
H522Q	345	3040
H523Y	0	0
H523Q	824	1200
H523L	137	7500
H530L	570	1730

[a] Wild-type and mutants are described under Materials and Methods. [b] Using methyl viologen as electron acceptor.

Growth Characteristics and Electron Transport Rates and P_{700} Content. The H522Y and H523Y mutants were unable to grow autotrophically on media lacking acetate. All other histidine mutants, except H523L, were able to grow autotrophically in liquid culture at rates comparable to wild-type. The initial growth rate of the H523L mutant was significantly slower than wild-type and other histidine mutants. The slow growth may be due to impaired function of PSI or to reduced accumulation of PSI in the thylakoid membrane.

To determine effects of the mutations on PSI activities, thylakoids were isolated from the mutant and wild-type strains, and PSI-mediated electron transport from DCPIP to MV was determined by oxygen uptake. As shown by the results presented in Table 2, all the histidine mutations resulted in reduced rates of PSI-mediated electron transport on a total Chl basis. The H522Y and H523Y mutants showed no PSI activity. When histidine 522 was replaced with glutamine, the PSI activity was approximately 30% of the wild-type rate. The H523L mutant showed a rate of electron transport that was approximately 10−15% of the wild-type, and when H523 was replaced with glutamine, the rate of PSI electron transport was restored to 75% relative to wild-type. The H530L mutation showed electron transfer rates approximately 50% of wild-type. The Chl/P_{700} ratio in thylakoids isolated from the mutants closely paralleled the observed change in PSI electron transfer rates suggesting that the PSI reaction center concentration was decreased (Table 2). Light saturation curves of PSI electron transfer indicated an increased light-harvesting capacity for all mutants, probably due to an increased LHCI/PSI ratio (not shown). We, and others, have previously shown that LHCI accumulates independently of the PSI reaction center core (Lin & Knox, 1990; Webber *et al.*, 1993), so an increased

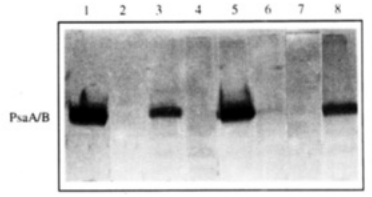

FIGURE 4: Western blot of thylakoid proteins probed with antisera raised against the PsaA/B reaction center proteins. Twenty micrograms of chlorophyll was loaded for each sample. Lane 1, wild-type; lane 2, CC2341 (PSI minus mutant); lane 3, H522Q; lane 4, H522Y; lane 5, H523Q; lane 6, H523L; lane 7, H523Y; lane 8, H530L.

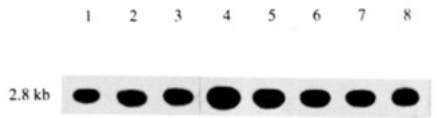

FIGURE 5: Northern blot of total cellular RNA probed with a *Bam*HI fragment of the chloroplast *psaB* gene. An equal amount (10 μg) of glyoxal-denatured RNA was loaded for each sample. The radiolabeled RNA band migrates at approximately 2.8 kb, corresponding to the size of the mature *psaB* transcript. Lane 1, CC125; lane 2, CC125-*aadA*; lane 3, H522Q; lane 4, H522Y; lane 5, H523Q; lane 6, H523L; lane 7, H523Y; lane 8, H530L.

light-harvesting capacity would be expected with a decrease in PSI concentration. We have no evidence from these data of impairment in excitation energy transfer to the PSI reaction center in any of these mutants, as has been observed for histidine mutations in the CP47 Chl *a*-binding protein of PSII (Shen *et al.*, 1993). This may be due to the large number of Chls associated with the PsaA and PsaB polypeptides.

Synthesis, Accumulation, and Assembly of PSI Reaction Center Proteins. To further determine the impact of mutations on PSI reaction center accumulation, we have quantified the amount of the PsaA/B reaction center proteins by Western blotting. Thylakoid proteins were solubilized in SDS-containing denaturation buffer and size-fractionated on 10−18% polyacrylamide gels. Following electrophoresis, proteins were transferred to a nitrocellulose membrane and immunodecorated with antisera against the PsaA/PsaB proteins (Figure 4). The Western data show that the P_{700} binding proteins accumulated to varying extents and confirm that the reduced Chl/P_{700} content of many of the mutants is due to reduced levels of the PsaA/B proteins. The sensitivity of Western blots is low, and so only very weak cross-reaction was observed with thylakoid proteins from the H523L mutant.

The mutations could lead to lower levels of PSI reaction centers for several reasons including reduced mRNA levels, impaired translation, unstable apoproteins, or lack of assembly of translated proteins into a functional PSI reaction center. To determine if the mutations impair mRNA accumulation, total RNA was isolated from the mutant cells, size-fractionated through agarose gels, and transferred to a nylon support. The immobilized mRNA was then hybridized with a ^{32}P-labeled *Bam*HI fragment of the *psaB* gene. As shown in Figure 5, the probe DNA hybridizes with a 2.8 kb RNA species that is the expected size of the mature *psaB* transcript (Xu *et al.*, 1993). Messenger RNA from *psaB* accumulates to approximately equivalent levels in each of the different mutant strains (Figure 5). Thus, any effect of individual mutations on protein accumulation is at the translational or posttranslational stage.

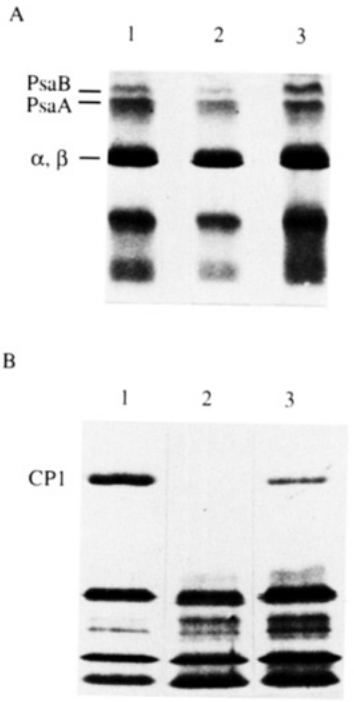

FIGURE 6: Autoradiographs of ^{14}C-labeled thylakoid proteins following fractionation by polyacrylamide gel electrophoresis. (A) Wild-type and mutants cells were labeled with [^{14}C]acetate in the presence of cycloheximide (see Materials and Methods). Thylakoid membranes were isolated from the cells and solubilized with SDS-containing sample buffer, and proteins were size-fractionated through a 10−18% acrylamide gel in the presence of 8 M urea. Lane 1, wild-type; lane 2, H522Y; lane 3, H523L. (B) Thylakoid membranes were solubilized in LDS-containing sample buffer at 4 °C and size-fractionated through a 10% nondenaturing acrylamide gel. The nondenaturing gel was dried and exposed to X-ray film for 2 days. Lane 1, wild-type; lane 2, H522Y; lane 3, H523L.

We have selected two mutants impaired in PSI accumulation, H522Y and H523L, to examine apoprotein synthesis and holoprotein assembly. Chloroplast proteins were labeled *in vivo* using [^{14}C]acetate in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis (see Materials and Methods). Following a 10 min labeling period, thylakoid membranes were isolated from the cells. Thylakoid proteins were then analyzed by denaturing and nondenaturing gel electrophoresis. Incorporation of radioactivity into both the PsaA and PsaB polypeptides was detected when labeled proteins were solubilized with SDS and size-fractionated on 12−18% acrylamide gels in the presence of 8 M urea. Following electrophoresis, the gels were dried and exposed to X-ray film. Assignment of the PsaA and PsaB bands was according to Takahashi *et al.* (1991). The autoradiograph presented in Figure 6A shows that similar amounts of ^{14}C were incorporated into PsaB and PsaA in H523L and wild-type cells, although incorporation was slightly reduced, relative to the α and β subunits of CF1, in H522Y (Figure 6A). Densitometric scanning of the autoradiographs confirmed these conclusions (not shown). These results indicate that absence or reduced levels of PSI in mutant thylakoids are not due to a block in translation of *psaB* mRNA. The slightly reduced incorporation of label into PsaA and PsaB in the H522Y mutant may be due to the spectinomycin resistance mutation present in the 16S rDNA, which could impair rates of chloroplast protein synthesis, particularly of large proteins such as these.

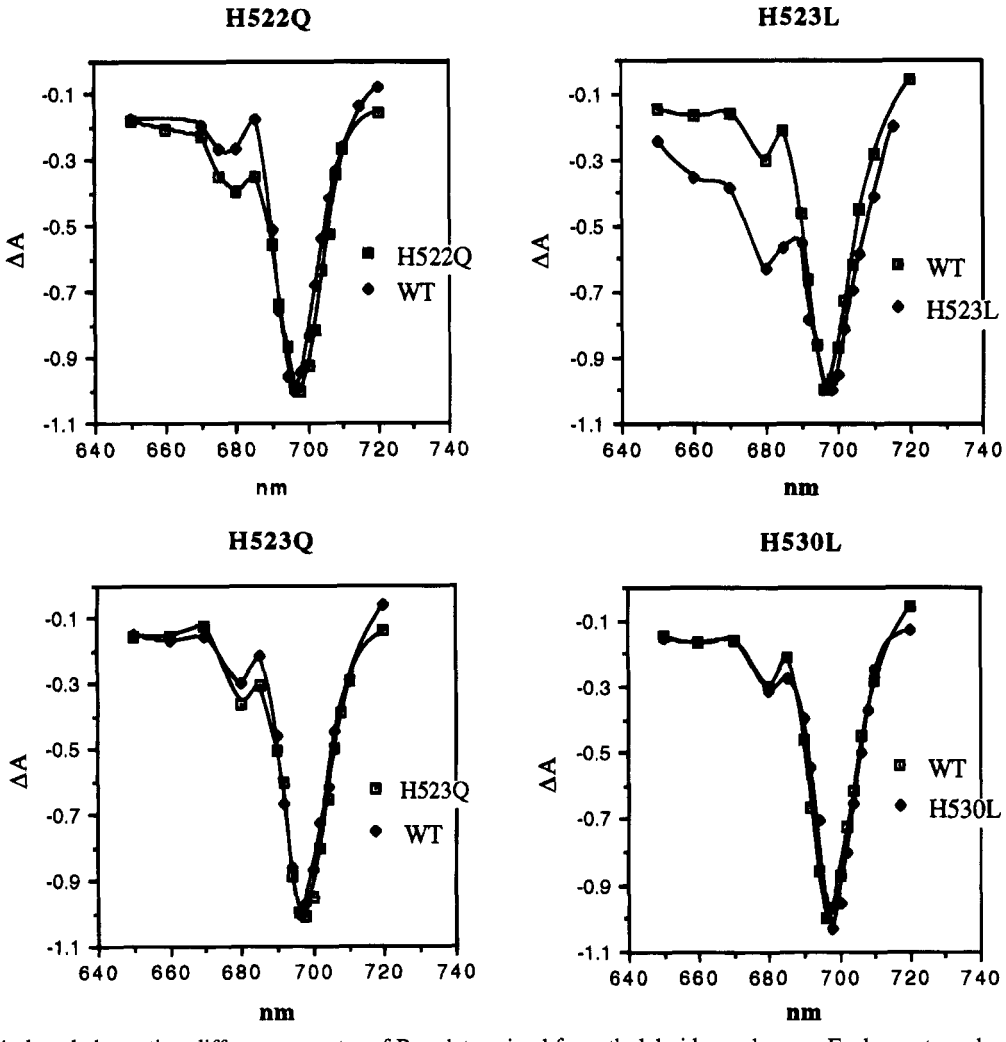

FIGURE 7: Flash-induced absorption difference spectra of P_{700} determined from thylakoid membranes. Each spectrum has been normalized to the wild-type spectra for better comparison. Each point is the average of 100 flashes.

We wanted to determine if the newly synthesized PsaA and PsaB polypeptides are incorporated into a reaction center complex in the mutant thylakoids. Therefore, the same ^{14}C-labeled thylakoids used for the SDS denaturing gels were solubilized with LDS and fractionated through a 10% acrylamide gel in the absence of urea and SDS at 4 °C (see Materials and Methods). Under these conditions, the PsaA and PsaB proteins are resolved as a single Chl-containing band termed CPI. Following electrophoresis, the green gel was dried and exposed to X-ray film. As shown in Figure 6B, incorporation of radioactivity into CPI can be clearly visualized. In the H523L mutants, the level of incorporation of radioactivity into CPI, determined by densitometric scanning of the autoradiograph, was reduced by approximately 85% in H523L, and was not detected in H522Y. This correlated with the steady-state level of CPI determined by nondenaturing gel electrophoresis and the results of Western blotting. If the newly synthesized PsaA and PsaB proteins, shown in Figure 6A, were incorporated into the CPI complex before degradation, then similar incorporation of radioactivity into CPI would be expected in both mutant and wild-type thylakoids. However, since incorporation into CPI is severely reduced, we conclude that the H523L and H522Y mutations impair the posttranslational assembly of the full-length PsaA and PsaB proteins into a Chl-containing CPI complex. Further work on these and other PSI mutants is being undertaken to further examine the effect of site-directed mutations on CPI assembly.

Optical, ENDOR, and ESEEM Spectroscopy of P_{700}. Histidine 523 of membrane span VIII is highly conserved in PSI reaction center proteins, and a histidine is also conserved in an equivalent position in reaction center proteins of *Chlorobium* and *Heliobacillus*. Thus, as has been discussed above, histidine 523 has frequently been proposed to serve as a ligand to P_{700}. Replacement of a histidine residue with a leucine would be expected to result in replacement of Chl *a* with Phe *a*, as found in bacterial reaction center mutants (Bylina & Youvan, 1988) and mutants of Chl *a*-binding proteins of PSII (Shen & Vermaas, 1994). Production of such a primary donor in PSI would be expected to result in significantly altered spectroscopic characteristics of P_{700}^+. Figure 7 shows the spectra of the flash-induced absorbance difference change associated with PSI measured in thylakoid membranes from the histidine mutant strains. Each mutant preparation showed a 30 ms decay of the flash-induced absorption difference change characteristic of charge recombination between F_A/F_B^- and P_{700}^+ (not shown). No P_{700}^+ signal could be detected in the H522Y and H523Y mutants, confirming the conclusion drawn from the electron transport assays and Western blots that PSI is absent. The absorption difference spectrum peaks at 696 nm, characteristic of the *Chlamydomonas* PSI

complex. As shown in Figure 7, there were no significant differences in the optical absorption difference spectra in any of the histidine mutants of membrane span VIII.

The characterization of the primary donor by optical spectroscopy suffers from some drawbacks. With increasing Chl/P_{700} ratio, the primary donor band becomes weak as compared to those of antenna Chl's. EPR spectroscopy of the related radical cation P_{700}^+ opens a way to solve this problem because the nonparamagnetic antenna species do not yield EPR signals. Furthermore, the use of more sophisticated EPR techniques allows determination of the electron−nuclear hyperfine coupling constants between the delocalized unpaired electron spin and the magnetic nuclei in the radical cation P_{700}^+ created in the charge separation process. The two techniques used here to resolve the hfc's, ENDOR and ESEEM, are complementary with respect to the detection of proton and nitrogen nuclei (Dikanov & Tsvetkov, 1992). The former show high sensitivity for proton hyperfine transitions, the latter for the nitrogens.

For the above-mentioned mutant in bacterial reaction centers of *Rhodobacter sphaeroides* containing a BChl *a*−BPhe *a* heterodimer as primary donor (McDowell *et al.*, 1991), ENDOR spectroscopy clearly revealed drastic changes in hyperfine couplings (hfc's) of several megahertz as compared with wild-type (Huber *et al.*, 1990). Therefore, it can be expected that the proton hfc's of P_{700}^+ in PSI also serve as a sensitive probe for the detection of structural changes of this species and its immediate surrounding.

Figure 8 shows cw ^{1}H-ENDOR spectra of P_{700}^+ of PSI preparations isolated from wild-type and the H523Q and H523L *Chlamydomonas* mutants. For comparison, the spectrum on P_{700}^+ of the cyanobacterium *Synechococcus elongatus* is also depicted (Käß *et al.*, 1994a). The analysis of the latter has recently become possible by studies of P_{700}^+ in PSI single crystals of this species (Käß *et al.*, 1994a; Käß *et al.*, unpublished results).

The analysis showed that the three observed methyl proton hfc's have to be assigned to one Chl *a* molecule. Although some of the smaller hfc's in the spectra could belong to a second Chl *a* forming the dimer ("special pair") P_{700}^+, this has not been fully established in our analysis so far. P_{700}^+ could, therefore, be a strongly asymmetric dimer as suggested by the EPR line width and reduction of some of the hfc's (Käß, 1994a), or it could be a monomer disturbed by its environment (O'Malley & Babcock, 1984). In *Synechococcus*, the isotropic hfc's of the three methyl group protons in the spin carrying Chl *a* were calculated from the hf tensors determined in PSI single crystals to be 5.1, 3.4, and 2.8 MHz for positions[2] 12, 7, and 2, respectively.

The overall appearance of the four ENDOR spectra shown in Figure 8 is quite similar. Thus, no significant structural differences exist between P_{700}^+ in *Chlamydomonas* (wild-type and mutant) and *Synechococcus*. The three line pairs (1,1′), (2,2′), and (3,3′) are assigned, in analogy to P_{700}^+ in *Synechococcus*, to the ^{1}H hfc's of the three CH_3 groups at molecular positions 2, 7, and 12 in the spin carrying Chl *a* of P_{700}^+. The isotropic methyl proton hfc's, A_{iso}, are estimated to be 5.1, 3.5, and 2.8 MHz for molecular positions 12, 7, and 2, respectively. These prominent hfc's remain the same within experimental error (±100 kHz) in the three

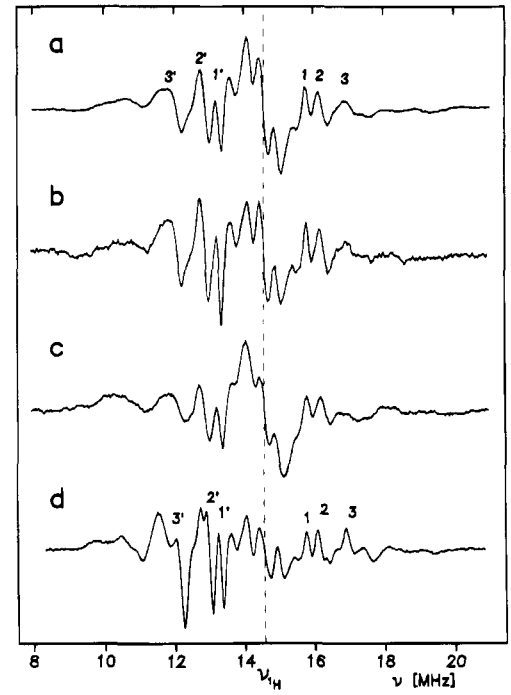

FIGURE 8: ^{1}H ENDOR spectra of P_{700}^+ in PSI from *Chlamydomonas reinhardtii* in frozen solution. (a) Wild-type at 160 K; (b) mutant H523Q at 130 K; (c) mutant H523L at 130 K. For comparison the ENDOR spectrum of P_{700}^+ in PSI from *Synechococcus elongatus* at 120 K is shown in (d). Experimental conditions: microwave power 5.1 mW, RF power 200 W, time constant 640 ms, total averaging time 60 min, frequency modulation amplitude (FM) was 100 kHz in all experiments.. The line pairs (1,1′), (2,2′), and (3,3′) indicated in spectra a and d are assigned to the methyl proton hfc's of the CH_3 groups at molecular positions 2, 7, and 12 (Käß *et al.*, 1994a; Käß *et al.*, unpublished results). The isotropic hfc's extracted for *Chlamydomonas* from the powder ENDOR lines are 2.8 (position 2), 3.5 (position 7), and 5.1 MHz (position 12). The respective hfc's for *Synechococcus* are 2.8, 3.4, and 5.1 MHz (extracted from the powder ENDOR spectrum (Käß *et al.*, unpublished results).

ENDOR spectra of *Chlamydomonas* PSI preparations. In addition, even the outermost patterns of the spectra remain unchanged (hfc's 7−9 MHz). In analogy to the results obtained for monomeric Chl a^+ (Scheer *et al.*, 1977; Käß *et al.*, 1994b) and for P_{700}^+ in PSI single crystals (Käß *et al.*, unpublished results), these have been assigned to β-proton hfc's in positions 17 and 18 at ring D of the Chl *a* bearing the unpaired electron spin in P_{700}^+. This is indicative for a conservation of the local geometry of ring D in all three *Chlamydomonas* preparations. The ENDOR results lead to the conclusion that the electronic structure of P_{700}^+ is the same in wild-type, H523Q, and H523L, and no evidence exists for the occurrence of a heterodimer in mutant H523L. Similar conclusions have been drawn for the H522Q and H530L mutants, that show unaltered EPR spectra.

Since ENDOR only yields proton hfc's and, therefore, essentially allows only an investigation of geometrical changes near the periphery of the porphyrin macrocycle, stimulated echo ESEEM experiments were performed with the aim to determine nitrogen (^{14}N) hfc's near the center of the Chl *a* molecules. The evaluation of these spectra is complicated by the quadrupole moment of ^{14}N (nuclear spin $I = 1$). Furthermore, for an exact analysis, spectral simulations are necessary since tensor principle values do not directly correlate with peaks in the experimental ESEEM spectra (Dikanov & Tsvetkov, 1992; Lin *et al.*, 1986; Britt *et al.*, 1991). Nevertheless, such spectra can serve as

[2] Positions numbered after IUPAC−IUB Joint Commission Biochemical Nomenclature (1979).

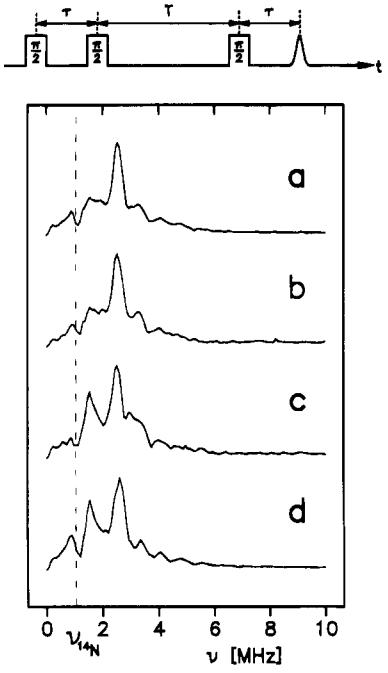

FIGURE 9: Stimulated echo (three-pulse) ESEEM of P_{700}^+ in PSI from *Chlamydomonas reinhardtii*. (a) Wild-type; (b) H523Q; and (c) H523L. For comparison, the result of a similar experiment on P_{700}^+ in *Synechococcus elongatus* is shown in (d). Experimental conditions: temperature 10 K; $T \times \tau = 350 \times 100$ points. Start values and time increments: $T_0 = 200$ ns, $\tau_0 = 120$ ns, $\Delta T = 24$ ns, $\Delta\tau = 8$ ns; $\pi/2$ pulse = 16 ns; $B_0 = 3498$ G; a four-step cycle was employed; the total accumulation time for the whole 2D arrays was approximately 5 h. For definition of time intervals see pulse sequence above. Data manipulation using ESP 380 software: baseline correction for relaxation decay (third-order polynomial), zero-filling to 512 × 128 points, application of a hamming window [full width at half-maximum (FWHM) 170 points] in T direction prior to 2D FT. All spectra shown are skyline projections on the ν_T axis after Fourier transform and calculation of magnitudes of the full 2D data sets.

sensitive fingerprints for the different samples which should indicate changes in structure.

Figure 9 shows skyline projections of stimulated echo experiments of P_{700}^+ in the *Chlamydomonas* wild-type and mutants H523Q and H523L and, for comparison, of P_{700}^+ in *Synechococcus elongatus*. The pronounced peaks in the spectra indicate that the so-called cancellation condition, $A = 2\nu_0$ (ν_0, free nuclear Larmor frequency; A, effective hfc), is fulfilled for at least one of the nitrogen nuclei (Mims & Peisach, 1978). Even a slight deviation from the cancellation condition by a variation of A drastically changes the intensities of the observed peaks (Dikanov *et al.*, 1983) whereas their positions remain constant. From the peak positions, the ^{14}N quadrupole coupling constant and asymmetry parameter can be obtained (Dikanov *et al.*, 1983). They depend on the electric field gradient at the respective nucleus that is a sensitive measure of its immediate electronic surroundings.

The *Chlamydomonas* wild-type and H523Q mutant essentially yield identical ESEEM spectra. The main differences between the H523L mutant and wild-type are the increased intensity of the peak at 1.5 MHz, and the shoulder emerging around 3.14 MHz in the former spectrum. However, the changes in the effective hfc A cannot be very large since the peaks are still distinguishable (Britt *et al.*, 1991). The positions of the main features are the same in all ESEEM spectra. The main differences are intensity changes. As already mentioned above, this is indicative of only minor changes in the effective hfc's. Even the small peak at 2.95 MHz and the shoulder at 3.45 MHz in the spectrum of H523L have their counterparts in the ESEEM spectrum of *Synechococcus*. From these ESEEM experiments, we conclude that the surroundings of the nitrogen atoms in the central part of the spin carrying Chl *a* molecule(s) in P_{700}^+ differ, if at all, only very slightly in the four PSI preparations investigated.

DISCUSSION

Using site-directed mutagenesis of the *psaB* gene in the chloroplast genome of *Chlamydomonas reinhardtii*, we have shown that the conserved histidines in membrane span VIII are important for accumulation of the PSI reaction center in thylakoid membranes. The high conservation of one of these histidines in PSI reaction center proteins, H523 of PsaB, as well as in reaction center proteins in iron−sulfur center containing bacterial reaction centers, has led to the proposal that H523 of PsaB coordinates the P_{700} Chl *a* of PSI. Replacement of H523 of PsaB with an alternate nitrogen ligand, glutamine, allows almost normal accumulation of the PSI reaction center complex, whereas replacement with a leucine or tyrosine destabilizes the complex. This is consistent with the fact that a nitrogen atom from either H523 or Q523 provides a fifth ligand to the central magnesium of a Chl. Similar mutations of histidine residues in CP47, a Chl *a*-binding protein of the PSII core antenna, also demonstrate that glutamine and asparagine will substitute for histidine (Shen *et al.*, 1993). Therefore, this is consistent with the hypothesis that H523, or its equivalent in bacterial reaction centers, is a ligand to a Chl or BChl.

Substitution of a histidine with leucine has previously been shown to result in the loss of the central magnesium from Chl and production of Phe (Bylina & Youvan, 1988; Shen & Vermaas, 1994). In purple bacteria, mutation of the histidine ligand to the special pair resulted in pheophytinization of one of the BChl *a* molecules, yielding a BChl *a*−BPhe *a* heterodimer (Bylina & Youvan, 1988). This resulted in a reduced quantum efficiency of charge separation in the reaction center (Bylina & Youvan, 1988) and produced significant changes in the absorption and EPR spectra (Kirmaier *et al.*, 1988; Bylina *et al.*, 1990; Huber, 1990). If H523 were a ligand to a Chl of P_{700}^+, we would expect that substitution of histidine with a leucine would result in replacement of this Chl *a* with Phe *a* and alter the property of P_{700} sufficiently to detect altered optical absorption and/or ENDOR/ESEEM spectra. Such differences should be detectable if P_{700} is a dimer with an asymmetric spin distribution over the two halves as recently observed in mutants of the bacterial reaction center (Rautter *et al.*, unpublished results). PSI preparations from *Chlamydomonas* wild-type and mutant thylakoid membranes produced ENDOR and ESEEM spectra that are essentially identical to those obtained from cyanobacteria. This indicates that the environment surrounding the P_{700}^+ Chl *a* is highly conserved in PSI from both prokaryotic and eukaryotic organisms. Even mutants that accumulate only a fraction of the normal PSI content yield preparations that produce well-resolved and reproducible spectra. Thus, these powerful spectroscopic techniques will be of considerable value in analysis of PSI mutants, including those that are significantly impaired in assembly

of the complex. The fact that we can detect no changes in ENDOR and ESEEM spectra from any of the mutants is evidence that histidine 523 does not coordinate P_{700}^+.

Comparison of the reaction center amino acid sequence from *Heliobacillus*, *Chlorobium*, and PSI reveals other conserved residues that may be a potential ligand to one of the reaction center Chls. These include two conserved histidines in membrane span VI and a conserved histidine in membrane span X (H656 of *Chlamydomonas* PsaB). The site-directed mutant H656L accumulates no PSI reaction center, indicating that this residue indeed fulfills an important function (Webber, Su, and Bingham, unpublished results). It is not totally certain that the ligand to P_{700} will be a histidine since Chl ligands may be provided by a range of different amino acids (Kühlbrandt, 1994). However, it is likely that the reaction center binding site has been conserved throughout evolution, and thus is likely to be provided by amino acids conserved in bacterial and PSI proteins. We are currently generating further mutations in conserved residues in PSI to determine their role in reaction center assembly and function.

The availability of Chl is an important factor for the stable accumulation of Chl-binding proteins in the thylakoid membrane of algae and plants (Mullet, 1988). Incorporation of Chl into the protein complex is thought to occur cotranslationally. Therefore, in the absence of Chl, apoproteins may not accumulate due to a block in translation or rapid degradation of the newly synthesized protein (Klein & Mullet, 1986; Herrin *et al.*, 1992). Recent analysis of Chl *a*-binding protein synthesis in etioplasts has produced results that favor destabilization and rapid degradation of the apoprotein rather than any impairment in translational initiation and elongation (Kim *et al.*, 1994). Mutants impaired in Chl *a* binding also provide some insight into how Chl association with the apoprotein controls Chl-binding protein synthesis. Our evidence indicates that histidine 523 of PsaB is a Chl *a* ligand, and that the H523L mutant is significantly impaired in PSI accumulation. However, we find no evidence for impaired translation of the PsaB protein in this mutant. Similar results obtained from mutants in the PSII complex in cyanobacteria also support the conclusion that altered Chl *a* binding does not impair translation initiation and elongation (Yu & Vermaas, 1993). Rather, the newly synthesized proteins are rapidly degraded. Our results further indicate that impaired Chl binding prevents assembly of the newly synthesized apoproteins into a reaction center heterodimer. The stages of assembly of the PSII and PSI proteins into a reaction center complex are mostly unknown. It is thought that Chl addition and membrane insertion of reaction center proteins occur co-translationally during protein synthesis on membrane-bound ribosomes. However, it is not known whether assembly of the apoproteins into a reaction center complex also occurs co-translationally, or is a post-translational event. Analysis of newly synthesized proteins by nondenaturing gel electrophoresis suggests that the mutations affect a post-translational step in assembly of the PSI reaction center. This supports a model for PSI biogenesis in which apoprotein synthesis is followed by assembly of the individual polypeptides into a functional reaction center complex. Further analysis of these and other site-directed mutants will provide valuable information on the assembly pathway of the PSI reaction center.

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