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Site-directed Mutagenesis of the Photosystem I Reaction Center in Chloroplasts

THE PROLINE-CYSTEINE MOTIF*

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Site-directed mutagenesis has been used to introduce specific amino acid changes into the photosystem I reaction center in the green alga *Chlamydomonas reinhardtii*. Plasmids containing mutated copies of the chloroplast *psaB* gene, encoding a polypeptide of the photosystem I reaction center heterodimer, were introduced into the chloroplast genome by particle bombardment. Successful transformants were selected by two procedures. The first involved complementation of a nonphotosynthetic mutant of *Chlamydomonas*, CC-2341 (*ac-u-g*-2.3), which has a frameshift mutation in the *psaB* gene, and selection of photosynthetic transformants on minimal medium. The second procedure utilized a co-transformation procedure with a plasmid containing a rRNA gene that confers spectinomycin resistance. Homologous replacement of the *psaB* gene was confirmed by screening for a unique restriction enzyme site within the transforming *psaB* sequences. These procedures have been used to specifically mutate a highly conserved proline-cysteine motif suggested to be important in coordinating the [4Fe-4S] iron-sulfur center F_X. Our results show that the cysteine is essential for assembly of the photosystem I reaction center although the adjacent proline fulfills no identifiable function. The approach described in this paper will be of value to future studies of the structure, function, and assembly of photosystem I.

The photosystem I reaction center of plants, algae, and cyanobacteria mediates the light-induced transfer of electrons from plastocyanin to ferredoxin. In chloroplasts, the photosystem I reaction center consists of at least 13 individual polypeptides encoded by genes located in both the chloroplast and the nucleus. The primary electron donor P700 and early electron acceptors A_0, A_1, and F_X are coordinated by two related polypeptides of approximately 83 kDa encoded by the chloroplast *psaA* and *psaB* genes. Two additional electron acceptors, F_A and F_B, are coordinated by an 8-kDa polypeptide encoded by the chloroplast *psaC* gene (reviewed in Scheller and Møller (1990), Golbeck and Bryant (1991), and Golbeck (1992)).

The electron acceptors F_X, F_A, and F_B are all [4Fe-4S] iron-sulfur centers. The amino acid sequence of the PsaC polypeptide contains conserved cysteine motifs similar to those found in bacterial ferredoxins (Dunn and Gray, 1988). Studies using *in vitro* reconstitution of depleted photosystem I preparations with mutant PsaC proteins have demonstrated that the cysteines are involved in coordinating centers F_A and F_B (Zhao *et al.*, 1992). It is well established that F_X is a [4Fe-4S] iron-sulfur center generally expected to be coordinated by 4 cysteine residues (Golbeck, 1992). Inspection of the deduced amino acid sequences of the *psaA* and *psaB* genes from a wide range of prokaryotic and eukaryotic organisms reveals 3 conserved cysteines in the PsaA protein and 2 conserved cysteines in PsaB (reviewed in Golbeck and Bryant (1991) and Golbeck (1992)). Therefore, F_X is probably an inter-polypeptide iron-sulfur center coordinated by cysteine ligands from both PsaA and PsaB. The cysteines predicted to coordinate F_X are located in identical highly conserved regions of the PsaA and PsaB polypeptides with the following sequence of amino acids, Phe-Pro-Cys-Asp-Gly-Pro-Gly-Arg-Gly-Gly-Thr-Cys, see Fig. 1. This cysteine-rich region is located in a putative stromal-exposed extramembrane loop (Fish *et al.*, 1985), a location suited to F_X since it interacts closely with the F_A/F_B centers. Recently, amino acid sequencing of the reaction center protein of *Heliobacillus mobilis* has shown that a similar cysteine region is present (Trost *et al.*, 1992), strengthening arguments that this region plays an important structural role in photosystem I. A cysteine-proline motif conserved in nearly all [4Fe-4S] ferredoxins (Golbeck, 1992) is also found in this region and also is present in the *Heliobacillus* reaction center protein. However, it cannot be concluded for certain that any of the conserved cysteines coordinate F_X since it has been reported that aspartic acid can serve as a ligand to at least one of the iron atoms in some 4Fe-4S ferredoxins (Armstrong *et al.*, 1989a, 1989b; Canover *et al.*, 1990).

Specific mutagenesis would provide a powerful tool for our further understanding of assembly and function of the photosystem I reaction center. Unlike the purple bacterial photosynthetic reaction center and photosystem II, site-directed mutagenesis of the photosystem I reaction center has not been reported. The aim of the research reported here is to develop strategies for site-directed mutagenesis of the *psaB* gene in *Chlamydomonas reinhardtii* so that this technique can be used to address some of the questions raised above. *C. reinhardtii* is able to grow in the absence of photosystem I function using acetate as a carbon source (Harris, 1989). Together with the ability to transform the chloroplast genome using the biolistics technique, it should be possible to intro-

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duce site-directed mutations into the photosystem I reaction center and recover mutants with both photosynthetic and nonphotosynthetic phenotypes. We have previously demonstrated that a chloroplast photosystem I mutant of *Chlamydomonas* can be transformed with wild-type *psaB* sequences to complement an existing chloroplast frameshift mutation in this region (Bingham *et al.*, 1991). In this paper we report the utility of this procedure for introducing site-directed amino acid changes into the photosystem I reaction center and as an example have targeted the proline-cysteine motif to examine its role in complex assembly.

EXPERIMENTAL PROCEDURES

Strains and Culture—*C. reinhardtii* strains were obtained from the *Chlamydomonas* Culture Collection at Duke University. CC-125 is a wild-type mt^+ strain (Harris, 1989). CC-2341 (*ac-u-g-2.3*) is a nonphotosynthetic strain that carries a frameshift mutation in the chloroplast *psaB* gene (Shepherd *et al.*, 1979; Bingham *et al.*, 1991). The CC-2341 (*ac-u-g-2.3*) strain has been transformed with wild-type copies of the chloroplast *psaB* gene to give a photosynthetic phenotype and is referred to as CC-2341T. The cells were maintained on HS (Sueoka, 1960) media supplemented with acetate (HSA).

Plasmids and in Vitro Mutagenesis—The chloroplast DNA *Eco*RI restriction fragment 14 (nomenclature in Harris (1989)), containing the *psaB* and *rbcL* genes, was subcloned into pUC19. A 1.5 kbp[1] *Bam*HI-*Pst*I fragment was then isolated and cloned into M13mp19. Oligonucleotide-mediated site-directed mutagenesis was then used to introduce a silent mutation into codon 541 to produce a unique *Xba*I restriction enzyme site using a kit from Amersham following manufacturer's protocols. The *Bam*HI-*Pst*I fragment containing the *Xba*I site was subcloned into pUC19 to give plasmid pL541L-3 (Fig. 1). Plasmid pL541L-3 was then used as a template for PCR-mediated site-directed mutagenesis following procedures essentially as described by Sakar and Sommer (1990). This procedure utilized two successive rounds of PCR to produce a DNA fragment that contains the desired mutation. The first PCR reaction used the mutagenic primer (Fig. 1) and reverse universal primer to amplify a 1.39-kbp fragment from pL541L-3. The amplified fragment was then purified from a low melting point agarose gel and used as a "megaprimer" (Sakar and Sommer, 1990) with the forward universal primer to amplify a 1.5-kbp fragment from pL541L-3. The 1.5-kbp fragment was then digested with *Xba*I and *Ban*II and a 423-bp fragment, containing the site-directed mutation, was subcloned into pL541L-3. The resulting plasmids were directly sequenced to confirm that they contained the mutation. After the mutation was confirmed, the *Bam*HI-*Pst*I fragment was subcloned into the *Eco*RI-*Pst*I fragment of the chloroplast DNA restriction fragment Eco14 for transformation into *Chlamydomonas*. The plasmid p228 contains the chloroplast 16 S rRNA gene with a mutation for spectinomycin resistance (Harris *et al.*, 1989) and was a gift from Dr. John Boynton.

Chloroplast Transformation and Analysis of Transformant Cells—Chloroplast transformation was performed using the biolistics technique following procedures essentially described in Boynton *et al.* (1988) and Newman *et al.* (1990). CC-2341 cells grown to approximately 3×10^6 cells per ml were concentrated by centrifugation and resuspended in HS media to a concentration of 5×10^7 cells per ml. One-half milliliter of cells were then dispersed on the surface of plates containing 1.2% agar in HS medium. Gold particles were deglomerated and washed, and 5 μg of the donor plasmid DNA precipitated onto the particles as previously described (Newman *et al.*, 1990). The DNA-coated particles were then bombarded into the cells using the Biolistics PDS-1000 Particle Delivery System (Du Pont-New England Nuclear). The bombarded plates were placed under low irradiance (100 μmol of photons m^{-2} s^{-1}) until photosynthetic colonies appeared. Single colonies were then streaked onto HS plates and grown under dim light.

For co-transformation, 5-fluorodeoxyuridine (final concentration 0.5 mM) was added to CC-125 cultures at a density of 5×10^4 cells ml^{-1}, and the cultures were grown to a density of 5×10^6 cells per ml. 2.5×10^7 cells were bombarded with an equal molar mixture of the plasmids pC560H and p-228 as described. Following bombardment, cells were transferred to HSA plates containing 100 μg ml^{-1} spectinomycin (HSA-S) and grown under dim light until colonies were obtained. Single transformant colonies appearing after 2 weeks were streaked onto HS or HSA-S plates.

Total cellular DNA was isolated from confluent regions (approximately 0.25 cm^{-2}) of the transformed cells using a rapid DNA minipreparation procedure essentially as reported (Newman *et al.*, 1990). Cells were scraped up using a sterile toothpick and resuspended in a 1.5-ml Microfuge tube in 0.4 ml of TEN buffer (150 mM NaCl, 10 mM Na_2EDTA, 10 mM Tris-HCl, pH 8.0). The cells were briefly vortexed, and then 40 μl of 20% SDS and 40 μl of 50 mg ml^{-1} heat-treated pronase were added. Samples were incubated at 50 °C for 30 min and then extracted with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1, v/v) as described. Nucleic acids were precipitated from the aqueous phase with 95% ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 100 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). One microliter of the cellular DNA was then used as a template to amplify an 800-bp fragment of chloroplast DNA using primers that flank the *Xba*I site and the introduced mutation. Following digestion of the PCR-amplified DNA with *Xba*I, the DNA was size-fractionated on a 2% agarose gel. Both the photosynthetic or spectinomycin-resistant cells were screened for the presence of *Xba*I site in the PCR-amplified DNA. Sequential rounds of single colony isolations were performed until homoplasmic cell lines were obtained from which the amplified DNA cut completely with *Xba*I. The amplified DNA from the homoplasmic strains was then sequenced using a Cycle Sequence kit (Bethesda Research Laboratories) 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 were purified by centrifugation through a sucrose step gradient following previously published procedures (Chua and Bennoun, 1975). Thylakoid membranes were then solubilized in gel loading buffer (5% lithium dodecyl sulfate, 100 mM dithiothreitol, 10% glycerol, and 50 mM Tris, pH 8.8), and polypeptides were 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 nitrocellulose membrane. The immobilized polypeptides were incubated with antisera against the PsaA/B (a gift from Dr. N. Nelson), PsaL (from Dr. N.-H. Chua), and PsaF (from Dr. R. Malkin) polypeptides. For immunodecoration a goat-anti-rabbit IgG-horseradish peroxidase was used followed by color development according to procedures supplied by Bio-Rad Laboratories.

Fluorescence Emission Spectra and Kinetics—Chlorophyll fluorescence emission kinetics were measured from whole cells using a pulse-amplitude modulated fluorimeter (Walz). Low temperature fluorescence emission spectra were obtained from whole cells frozen in 60% glycerol using a SPEX Fluorolog 2 scanning spectrometer.

Electron Transport and P700 Determinations—Photosystem I activity was measured polarographically as light-induced oxygen uptake in the presence of methyl viologen (50 μM), 2,6-dichlorophenolindolphenol (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). P700 content was determined from ferricyanide-oxidized minus ascorbate-reduced difference spectra using published procedures and extinction coefficient (Hiyama and Ke, 1972).

RESULTS

Site-directed Mutagenesis of the Photosystem I Reaction Center—A *Bam*HI-*Pst*I subclone of chloroplast DNA restriction fragment Eco14 was used as a template for site-directed mutagenesis to introduce a silent base change into the *psaB* gene at codon position 541 in order to produce a unique *Xba*I site. The final plasmid construct was termed pL541L-3., Fig. 1. This plasmid was then used as a template to introduce base changes at codon positions 559 and 560 encoding the proline-cysteine motif. The primers were designed to change proline 559 to either a leucine or an alanine and cysteine 560 to a histidine. Following mutagenesis, an *Xba*I-*Ban*II fragment, containing the desired mutation, was subcloned into the *Eco*RI-*Pst*I fragment of Eco14 containing the entire *psaB* gene (Fig. 1). The resulting plasmids were termed pP559A, pP559L, and pC560H.

[1] The abbreviations used are: kbp, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction.

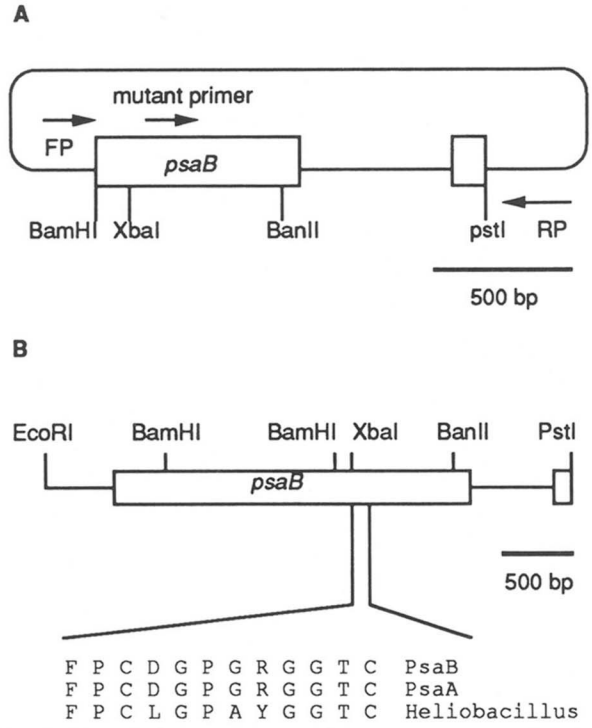

FIG. 1. **Restriction enzyme map of plasmids pL541L-3 and pL541L.** *A*, the plasmid pL541L is a 1.5-kbp *Bam*HI-*Pst*I fragment from the chloroplast DNA *Eco*RI fragment E14 containing the *psaB* gene cloned into pUC19. The *Xba*I site was introduced into the *psaB* gene using site-directed mutagenesis (see "Experimental Procedures"). *FP* and *RP* are forward and reverse universal primers complementary to sequences in pUC19. The mutagenic primers were designed to introduce specific base changes into *psaB*. *B*, following mutagenesis and sequencing of pL541L-3, the *Bam*HI-*Ban*II fragment is subcloned into the *Eco*RI-*Pst*I fragment of *Chlamydomonas* chloroplast DNA, termed pL541L, that contains the entire *psaB* gene. The amino acid sequence shows the putative iron-sulfur binding region of the *Chlamydomonas* PsaB protein, the analogous regions in PsaA, and the reaction center of *H. mobilis*.

The CC-2341 strain of *C. reinhardtii* contains the chloroplast *ac-u-g 2.3* mutation (Shepherd *et al.*, 1979) which maps to the *psaB* gene region (Girard-Bascou, 1987). The *ac-u-g-2.3* mutation has been identified as a single AT base pair deletion causing a reading frameshift and premature termination of the PsaB polypeptide (Bingham *et al.*, 1991). Since the AT base pair deletion is located within 6 codons of the sequences encoding the proline-cysteine motif, complementation of the CC-2341 (*ac-u-g-2.3*) strain could provide a rapid and convenient way to introduce site-directed mutations into this region of the *psaB* gene, providing that the mutation results in a photosynthetic phenotype. The plasmids pP559L, pP559A, and pC560H were precipitated onto gold particles and introduced into the chloroplast genome of CC-2341 (*ac-u-g-2.3*) using the biolistics technique (Boynton *et al.*, 1988). Following particle bombardment, cells were transferred to minimal HS (acetate minus) plates and grown under dim light to select for photosynthetic transformants. Transformation with both pP559L and pP559A resulted in photosynthetic transformants at a frequency of 5×10^{-6} which is well in excess of the reported reversion frequency of the *ac-u-g-2.3* mutation of 1×10^{-8} (Bingham *et al.*, 1991). We also transformed CC-2341 (*ac-u-g-2.3*) with the plasmid pL541L to repair the *ac-u-g-2.3* mutation and produce a strain called CC-2341T, which is a suitable control for photosynthetic measurements. Photosynthetic transformants were not recovered when transformed with pC560H.

Single colonies of the photosynthetic transformants were then streaked onto HS media and subsequently screened for the presence of the *Xba*I site by PCR amplification of a fragment of the *psaB* gene from minipreps of total cellular DNA. The 800-bp fragment amplified from DNA minipreps could be cut completely with *Xba*I indicating that all copies of the chloroplast genome contained the unique restriction site (Fig. 2). Typically, only one round of single colony selection was required to obtain homoplasmic cell lines containing the mutation. To confirm that the P559L and P559A mutations were introduced into the chloroplast genome along with the *Xba*I site, the PCR product was directly sequenced. As shown in Fig. 3, both the P559A and P559L mutants contain the introduced base changes.

For selection of transformants from pC560H, we utilized a co-transformation procedure with the plasmid p-228 (Newman *et al.*, 1990) that contains the 16 S rRNA gene with a mutation that confers resistance to spectinomycin (Harris *et al.*, 1989). Following particle bombardment with a mixture of

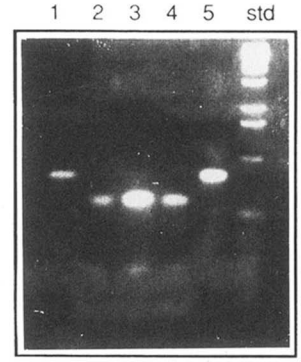

FIG. 2. **Analysis of *Chlamydomonas* transformants by PCR.** Total *Chlamydomonas* cellular DNA was amplified by PCR using primers complementary to *psaB* that flank the *Xba*I site. The PCR products (10-μl aliquots) were digested with *Xba*I and size-fractionated by electrophoresis through 2% agarose gels. The amplified DNA in *lane 1* is from wild-type cells and does not cut with *Xba*I. Amplified DNAs from *Chlamydomonas* mutants P559A (*lane 2*), P559L (*lane 3*), and C560H (*lane 4*) digested with *Xba*I. *Lane 5* shows undigested DNA from P559L. *Lane 6*, 1-kbp size markers (Bethesda Research Laboratories).

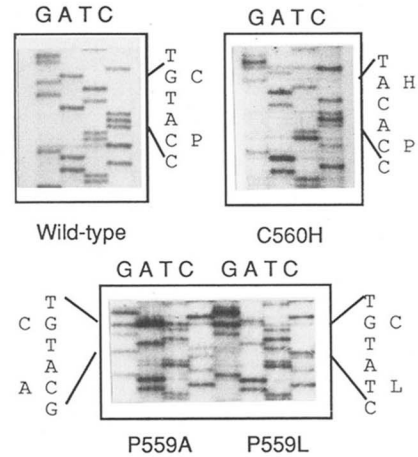

FIG. 3. **DNA sequence analysis of amplified PCR products from mutant *Chlamydomonas* cells.** Total cellular DNA from *Chlamydomonas* wild-type, P559A, P559L, and C560H cells was amplified by PCR and purified through Sephadex G-50 spin columns. Fifty fmol of the purified DNA was directly sequenced by PCR. The wild-type cysteine triplet, TGT, was changed to CAT encoding histidine. The wild-type proline triplet, CCA, was changed to either GCA (alanine) or CTA (leucine).

the plasmids pC560H and p-228, transformed cells were transferred to HSA media containing 100 $\mu g\ ml^{-1}$ spectinomycin (HSA-S). After growth in dim light for approximately 2 weeks, single colonies were transferred to HSA-S plates. Potential co-transformants were screened by PCR for the presence of the *XbaI* site. Approximately 75% of the initial spectinomycin colonies screened by PCR contained a detectable number of copies of *psaB* that contained the *XbaI* site. Co-transformants containing the *XbaI* site were then put through successive rounds of single colony cloning until fully segregated colonies homoplasmic for the *XbaI* site were obtained (Fig. 2). Two rounds of single colony selection were generally required to generate homoplasmic cell lines. The amplified DNA was then sequenced to confirm the presence of the C560H mutation in the chloroplast genome (Fig. 3).

Fluorescence Emission Characteristics—To determine if the nonphotosynthetic C560H mutant has characteristics of a photosystem I minus *Chlamydomonas* mutant, we have examined the kinetics of fluorescence emission from whole cells, which exhibits a characteristic pattern over a 2-s time period, Fig. 4. Upon irradiation, fluorescence emission rises rapidly to a maximum level and subsequently drops to a steady-state level. The quenching of fluorescence emission on this time scale is in part attributed to the reoxidation of the plastoquinone pool driven by photosystem I turnover and has been useful as a qualitative indicator of photosystem I activity (Takahashi *et al.*, 1991). Fig. 4 shows that the fluorescence emission kinetics from the P559A and P559L mutants are indistinguishable from wild-type cells. There is no quenching of fluorescence emission from C560H mutant cells within the same time scale indicative of a blockage in photosystem I electron transport.

The 77 K fluorescence emission spectra of wild-type and photosystem I minus mutants of *Chlamydomonas* have been well characterized and can provide a valuable indication as to the presence or absence of chlorophylls associated with the photosystem I core complex (Lin and Knox, 1991). The steady-state fluorescence emission spectra from whole cells at 77 K is shown in Fig. 5. In wild-type cells, the emission peaks at 685 nm and 695 nm arise from photosystem II, and the emission band at 718 nm is associated with fluorescence from the photosystem I core complex (Lin and Knox, 1991). The 718 nm emission is absent from C560H cells and is replaced by a strong emission at 708 nm. The 708 nm emission band is attributed to fluorescence from LHCI (Lin and Knox, 1991), which is no longer able to transfer excitation energy to the photosystem I core complex. The absence of a 718 nm band indicates that chlorophylls associated with photosystem I core complex are not present in the C560H cells. An identical fluorescence emission spectrum was also obtained from CC-2341 cells which are known not to contain a photosystem I

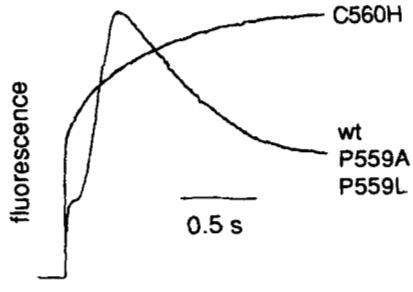

FIG. 4. **Kinetics of fluorescence emission from *Chlamydomonas* cells.** *Chlamydomonas* cells grown in HSA medium were transferred to the Walz PAM fluorimeter chamber and dark-adapted for 10 min before measurement on a time scale of 2 s.

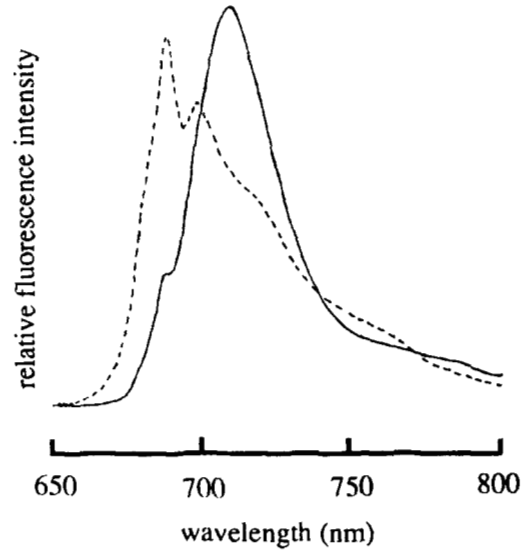

FIG. 5. **Fluorescence emission spectra of *Chlamydomonas* cells at 77K.** *Chlamydomonas* cells were frozen at 77K in the presence of 60% glycerol. Fluorescence emission spectra were generated by excitation at 435 nm. The emission spectra (- - -) from wild-type, P559A, and P559L cells were identical. Spectra from CC-2341 and C560H cells are shown by the *solid line*.

TABLE I

Photosystem I content and activity of thylakoid membranes from mutants

Strain[a]	Oxygen uptake[b]	Chl/P700
	μmol/mg chl/h	
Wild-type	1260	920
C560H	0	0
P559A	1263	924
P559L	1255	912
CC-2341T	1250	915

[a] Chlamydomonas strains are C125, wild-type; C560H, P559A, and P559L, site-directed mutants; CC-2341T, strain in which the frameshift mutation in *psaB* has been repaired by chloroplast transformation with the wild-type *psaB* gene.

[b] Using methyl viologen as electron acceptor.

core complex (Girard-Bascou *et al.*, 1987). This result also shows that, in the C560H mutant, the LHCI complex can assemble in the absence of the photosystem I core complex, also confirmed by Western blotting (not presented).

Photosystem I Activity—To further examine the effect of mutations on photosystem I electron transport, thylakoids were isolated from the mutant and wild-type cells lines, and photosystem I-mediated light-induced electron transfer from dichlorophenolindolphenol to methyl viologen was determined by measuring oxygen uptake. The data in Table I show that thylakoids from both P559L and P559A exhibit rates of photosystem I electron transfer equivalent to thylakoids isolated from the CC-2341T and CC-125 cell lines. As previously reported, very low levels of light-induced oxygen uptake by methyl viologen are obtained even in the absence of photosystem I activity (Fujii *et al.*, 1990). Therefore, we determined light-induced oxygen uptake from thylakoids isolated from CC-2341, that lack photosystem I, and subtracted that from the rates of light-induced oxygen uptake obtained from the mutant thylakoid membranes. After subtraction of the background rates, the C560H mutant thylakoids show no oxygen uptake (Table I). Table I also shows the Chl/P700 ratio in thylakoid membrane preparations determined from the ferricyanide-oxidized minus ascorbate-reduced difference spectra of P700. The P700 content of the 2341T, P559A, and P559L

cells are all similar (Table I). The C560H thylakoids contain no detectable P700, further confirming the absence of active photosystem I complexes in this mutant.

Photosystem I Polypeptides—Our results show that the C560H mutant contains no photochemically active photosystem I. We were also interested to determine the impact of the mutations on assembly of individual polypeptides of the photosystem I complex. Thylakoid membranes were isolated from the mutant cells, and the polypeptides were size-fractionated by polyacrylamide gel electrophoresis. Proteins were either visualized by staining with Coomassie Blue or electroblotted to a nitrocellulose support and probed with antisera. The PsaA and PsaB polypeptides are visible in the Coomassie-stained gels as a single diffuse band, labeled PsaA/B in Fig. 6. The PsaA/B band is visible in both the P559A and P559L mutants, confirmed by probing with antisera against PsaA/B, Fig. 6. The immobilized polypeptides were also probed with antisera raised against PsaF and PsaL. These polypeptides are also present in the P559A and P559L mutants.

A PsaA/B band is absent in the stained polypeptide profiles of thylakoids from the C560H mutant, and no cross-reaction with PsaA/B antisera could be detected, Fig. 6. This indicates that the photosystem I reaction heterodimer does not stably assemble to a detectable level in the C560H mutant. Fig. 6 also shows that there is no cross-reaction of C560H membranes when probed with antisera against PsaF and PsaL. These results demonstrate that the *psaB* gene product is required for assembly of the photosystem I complex and that in its absence the hydrophilic PsaF and the hydrophobic PsaL and PsaA polypeptides do not assemble.

DISCUSSION

We have presented results demonstrating that site-directed mutations can be introduced into the photosystem I reaction center heterodimer, and that stable assembly of a functional mutant photosystem I complex can be obtained. The use of site-directed mutagenesis has previously been restricted to purple bacterial reaction centers and photosystem II. The fact that *Chlamydomonas* can grow heterotrophically and that several photosystem I mutants in the chloroplast genome are known (see Harris, 1989) has always suggested that site-directed mutagenesis of the photosystem I reaction center was feasible. The use of a chloroplast frameshift mutant as a recipient of genes altered by site-directed mutagenesis is a novel method for introducing site-directed mutations into the plastid genome. The *ac-u-g-2.3* mutation is of particular value since the frameshift occurs within the most highly conserved region of the PsaB polypeptide predicted to be involved in the binding of electron transfer co-factors. Photosynthetic growth in the absence of acetate is a powerful selection for successful transformation, and, in the case of the mutations reported here, transformation frequencies are far in excess of the reversion frequency of the frameshift mutation. The segregation rate of the chloroplast mutation was found to be very rapid, and homoplasmic mutants were obtained by the second round of single colony cloning. Introducing a unique restriction enzyme site within the transforming DNA can be used as a convenient marker to screen for homologous recombination. We did not encounter problems with recombination of donor and recipient DNA between the restriction enzyme site and the desired mutation. All transformants were found to contain the desired mutation, which greatly reduced the number of photosynthetic transformants required for screening. The approach described in this paper is particularly appealing since, ultimately, the most useful photosystem I mutants will be those that have modified but not lethal photosynthetic phenotypes.

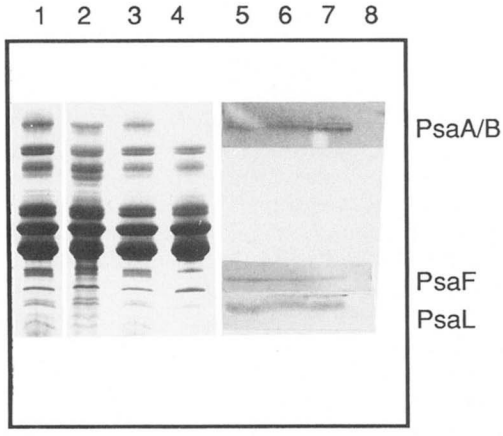

FIG. 6. **Polypeptides profile and immunodecoration of immobilized polypeptides.** Thylakoids from CC-125 (*lanes 1* and *5*), P559A (*lanes 2* and *6*), P559L (*lanes 3* and *7*), and C560H (*lanes 4* and *8*) cells were fractionated by SDS-PAGE and stained with Coomassie Blue (*lanes 1–4*) or electrophoretically transferred to nitrocellulose membrane. Immobilized polypeptides were probed with antisera raised against PsaA/B, PsaF, and PsaL (*lanes 5–8*).

We have used these procedures of site-directed mutagenesis and chloroplast transformation to specifically alter amino acids of a putative F_X binding region of the photosystem I reaction center. Changing cysteine 560 to histidine results in the lack of assembly of the photosystem I reaction center. This demonstrates that C560 is important for assembly of photosystem I. It is possible that cysteine is not specifically required, but that this mutation alters the secondary or tertiary structure of PsaB and prevents assembly. If the secondary structure of this region were critical for PsaB assembly, then changing an adjacent amino acid, especially a proline, would also dramatically alter assembly. However, neither P559A nor P559L has any impact on the accumulation of the photosystem I reaction center. This confirms that a cysteine residue is required at this position for correct assembly. Since *psaB* contains only two cysteine ligands, this supports the hypothesis that C560 is required for coordination of F_X. The fact that a proline residue is not critical at position 559 was a surprising result considering that it is conserved in all organisms containing photosystem I (Golbeck and Bryant, 1991) or "photosystem I-like" (Trost *et al.*, 1992) reaction centers. We are further exploring the properties of photosystem I complexes isolated from the P559A and P559L mutants. Whatever the consequences of the proline mutations on isolated complexes, they do not impact cell growth or overall photosystem I activity.

Our results show that the *psaB* gene product plays a crucial role in the stable assembly of the photosystem I complex, similar to conclusions based on biochemical and genetic analysis of chloroplast frameshift mutants (Girard-Bascou *et al.*, 1987). The PsaA protein does not assemble in the absence of PsaB, indicating that PsaA cannot independently form a stable homodimer. Also, in the absence of the PsaB reaction center polypeptide, at least two additional photosystem I polypeptides, PsaF and PsaL, do not stably assemble, most probably due to rapid proteolytic degradation. Further analysis of these and other site-directed mutants will be of value in investigating the mechanism of assembly of the photosystem I complex.

The use of site-directed mutagenesis will be of great value in further understanding the structure and function of the photosystem I reaction center. We are currently using the

described approach to investigate the role of conserved histidines in binding chlorophyll molecules involved in excitation energy and electron transfer and the role of a putative leucine zipper in reaction center dimerization (Kossel *et al.*, 1990; Webber and Malkin, 1990). The cloning and sequencing of genes encoding reaction center proteins from green sulfur bacteria (Leibel *et al.*, 1992), considered the progenitor of the photosystem I reaction center, will be of further assistance in identifying potentially important amino acid residues. However, as demonstrated here, changing highly conserved and structurally important amino acids such as proline 559 may not necessarily result in any identifiable phenotype.

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