Evidence That the F_X Domain in Photosystem I Interacts with the Subunit PsaC: Site-Directed Changes in PsaB Destabilize the Subunit Interaction in *Chlamydomonas reinhardtii*†

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ABSTRACT: The highly conserved amino acid sequence PCDGPGRGGTC in both photosystem I reaction center core proteins PsaA and PsaB has been predicted to contribute the four cysteine ligands for coordination of the 4Fe−4S iron−sulfur cluster F_X, and we have proposed a working model for the binding of PsaC to this domain of the reaction center core heterodimer [Rodday et al. (1993) *Photosynth. Res. 36,* 1−9]. We have investigated structure−function relationships between this domain and the PsaC subunit by site-directed mutagenesis of the conserved prolines P560 and P564, and the charged residues D562 and R566 in the eucaryotic alga *Chlamydomonas reinhardtii.* The D562N and R566E mutants did not accumulate the PsaA and PsaB reaction center proteins, indicating that these residues are essential for the stable assembly of photosystem I. The P560A, P560L, and P564L mutants accumulated functional reaction centers but showed an impaired interaction between the reaction center core complex and the PsaC subunit. We observed that the reaction centers of the proline mutants dissociated more readily in urea, and reconstitution of the mutant core preparations using PsaC and Fe−S cluster insertion protocols *in vitro* were incomplete. We suggest that P560 and D562 contribute to the stability of the F_X cluster, most likely by providing essential hydrogen bonding to the C561 ligand. The data obtained from the P564 and R566 replacements provide direct evidence that the intercysteinyl region in PsaB is a domain involved in the interaction between PsaC and the reaction center core.

The PS I[1] reaction center of cyanobacteria, algae, and higher plants mediates electron transfer from plastocyanin to ferredoxin. Following charge separation and creation of the primary radical pair, $P_{700}^+-A_0^-$, charge stabilization occurs via rapid electron transfer through the transient intermediates phylloquinone, F_X, and F_AF_B. P_{700}, A_0, F_X, and phylloquinone are localized on the PS I reaction center core complex, which is a heterodimer of the PsaA and PsaB proteins. The iron−sulfur centers F_AF_B are coordinated by the 9-kDa PsaC subunit bound to the stromal surface of the core complex. Additional subunits contribute to the overall structure of PS I and are involved in stabilization of the reaction center and the docking of extrinsic redox components [see Golbeck (1992, 1993) for reviews]. Crystals of PS I have been prepared and a structure has been obtained at a resolution of 6 Å (Krauss et al., 1993). Despite subatomic resolution, the 4Fe−4S centers can be clearly identified because of their high electron density. This confirms that the F_X center is located on the stromal side of the reaction center complex as expected. The nearest iron−sulfur center to F_X is located at a distance of 15 Å (Krauss et al., 1993) and indicates that the PsaC protein docks directly to the reaction center core complex. Ultimate refinement of the crystal structure will require additional X-ray studies, and its confirmation will necessitate insights from biochemical and biophysical investigations.

Comparative analysis of the derived amino acid sequence of PsaA and PsaB has revealed the presence of two highly conserved regions predicted to be involved in coordinating F_X (Fish & Bogorad, 1986). The four cysteine ligands required for coordination of F_X (McDermott et al., 1989; Petroulas et al., 1989) are contributed equally by both subunits and are localized in homologous segments of each polypeptide (Fish et al., 1985). Thus F_X is predicted to be an interpolypeptide iron−sulfur center. The amino acid sequence of the F_X-binding region of PsaB from *Chlamydomonas reinhardtii* is shown in Figure 1C. The amino acids located between the two cysteines of both PsaA and PsaB are comprised of predominantly small hydrophobic amino acids. Conserved prolines are located at position 560, adjacent to a cysteine ligand, and at position 564 in PsaB. Also, within the intercysteine region are the highly conserved charged residues D562 and R564. The small hydrophobic residues predominant in the sequence suggest considerable flexibility in the structure which may be important for allowing the cysteine residues to coordinate F_X.

This region is of extreme interest because the close protein−protein interaction between the PS I reaction center core and PsaC, which is most likely required for efficient

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[1] Abbreviations: Chl, chlorophyll; DTT, dithiothreitol; LDS, lithium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PS I, photosystem I; SDS, sodium dodecyl sulfate; TAP, Tris−acetate−phosphate buffer; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; Tris, tris[hydroxymethyl]aminomethane; Tricine, *N*-tris[hydroxymethyl]methylglycine.

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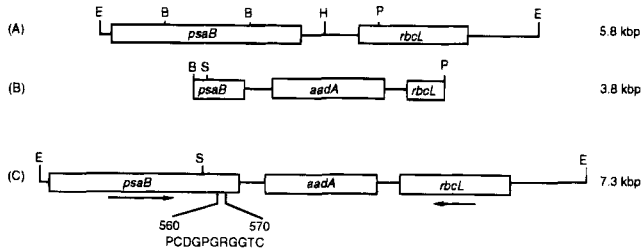

FIGURE 1: Map of chloroplast DNA containing the *psaB* gene. (A) The *psaB* gene is located on chloroplast DNA restriction enzyme fragment *Eco*14. (B) A fragment of *psaB* containing site-directed mutations was subcloned into a *Bam*HI−*Pst*I fragment of *Eco*14 and subsequently the *aadA* gene was introduced into a *Hin*cII site located in the intergenic region between *psaB* and *rbcL* (Cui et al., 1995). This fragment was termed pG528G-S and was used as the donor plasmid for chloroplast transformation. (C) Following chloroplast transformation, the donor plasmid integrates into the chloroplast genome by homologous recombination. The resulting transformant cells contain the *aadA* gene and the site-directed mutation in *psaB*. The amino acid sequence of the F_X domain of PsaB is indicated. B, *Bam*HI; E, *Eco*RI; H, *Hin*cII; P, *Pst*I; and S, *Stu*I.

electron transfer between the redox centers F_X and F_AF_B, may be mediated by this domain (Golbeck, 1993). We have proposed a molecular structure for it based upon the coordinates of the four cysteine ligands predicted from the atomic structure of 4Fe−4S clusters (Adman et al., 1973) and intensive molecular modeling of the intervening sequence (Rodday et al., 1993). We considered the possibility that the intercysteine regions may form two loop structures in the F_X domain and proposed that they may comprise a binding site for the PsaC subunit. The model was tested experimentally and we reported that both the chemical modification and proteolysis of P_{700}−F_X cores prevented the *in vitro* reconstitution of the cores using PsaC. The results were consistent with the model and confirmed that an arginine-containing domain is involved in the interaction between the reaction center heterodimer and the PsaC subunit.

Recently it has become possible to generate site-directed mutants of the PS I reaction center in both cyanobacteria (Smart et al., 1993) and the chloroplast of *Chlamydomonas reinhardtii* (Webber et al., 1993), which has allowed the initiation of investigations to test specific models of the F_X binding domain. Mutants generated thus far have provided direct experimental evidence that the cysteine residues in PsaB are ligands for the F_X iron−sulfur center (Smart et al., 1993; Webber et al., 1993). We have previously reported that mutation of the highly conserved P560 and PsaB in *C. reinhardtii* had no apparent effect on assembly of PS I complexes (Webber et al., 1993). However, a site-directed change of R561 in the interhelical loop of PsaB in *Synechocystis* sp. PCC 6803, corresponding to R566 in *C. reinhardtii*, resulted in a phenotype with greatly reduced photoautotropic growth (Rodday et al., 1994). Investigation of PS I complexes from the cyanobacterial R561E mutant revealed that the reaction center was destabilized, and the interaction between PsaC and the core heterodimer was impaired. We concluded that the observations were in direct support of the model previously proposed (Rodday et al., 1993) and that residue R561 is important for binding of PsaC to the PS I reaction center core.

This investigation was initiated to study further the structure−function relationships in the F_X domain of PS I complexes in the eucaryotic alga *C. reinhardtii*, and chloroplast transformation was used to introduce mutations into the *psaB* gene. We report here on the site-directed mutagenesis of P560 and D562, which are residues adjacent to the F_X ligand C561, and P564 and R566, which are two residues in the F_X interhelical loop (Figure 1C). The data presented indicate that both the charged residues and the conserved prolines are important for reaction center stability and provide additional confirmation that the F_X interhelical loop on the PsaB subunit of the core is involved in a protein−protein interaction with PsaC.

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 was used as a template for oligonucleotide-directed mutagenesis. The oligonucleotides used to engineer the mutants D562N, P564L, and R566E were 5′-TTCCCATGTAACGGTCCTGGT-3′ (D562N), 5′-TGTGACGGTCTTGGTCGTGGC-3′ (P564L), and 5′-GACGGTCCTGGTGAAGGCGGTACTTGT-3′ (R566E). Following mutagenesis, a *Stu*I−*Ban*II fragment was subcloned from M13 into pG528G-S and used in transformation experiments. The procedures used to produce the mutants P560A and P560L are described elsewhere (Webber et al., 1993).

Culture Conditions, Chloroplast Transformation, and Analysis of Transformant Cells. A wild type strain of *C. reinhardtii* CC125 mt^+ was used as the recipient of donor plasmids in the transformation experiments. CC2341 (*ac-u-g*-2.3) has a frameshift mutation in the chloroplast *psaB* gene and accumulates no PS I (Girard-Bascou, 1987; Girard-Bascou et al., 1987; Bingham et al., 1991). The cells were maintained on HS medium (Sueoka, 1960) supplemented with acetate (HSA medium) 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 medium 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 for PCR amplification of a 1-kbp fragment of chloroplast DNA using primers that flank the *Stu*I site and introduced mutations. The PCR-amplified DNA was then digested with *Stu*I and analyzed on a 2% agarose gel. Sequential rounds of single-colony isolations were performed until homoplasmic cell lines (i.e., amplified DNA cut completely with *Stu*I) were obtained. 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.

Thylakoid Membrane Preparations and Protein Analysis. For thylakoid membrane isolation, wild type and mutant cells were broken by passage through a French pressure cell and the thylakoid membranes were 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 the polypeptides were size-fractionated by SDS−PAGE using buffers and acrylamide concentrations as described (Ikeuchi & 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 and PsaB proteins (a gift from Dr. J. Guikema). For immunodecoration, a goat anti-rabbit IgG−horseradish peroxidase was used followed by color development according to procedures supplied by Bio-Rad.

For PS I dissociation and *in vitro* reconstitution experiments, *C. reinhardtii* wild type and mutant cells were grown on TAP medium and thylakoids were isolated from the cells after breakage using a French pressure cell at 4000 psi (Chua & Bennoun, 1975), but the sucrose gradient step was not performed. The final thylakoid pellet was resuspended in a minimum amount of 10 mM Tricine, pH 7.5, and frozen at −80 °C. PS I was prepared by differential centrifugation of thylakoids solubilized using Triton X-100 (Harris, 1989) and stored frozen at −80 °C. The concentration of P_{700} in the thylakoids was determined by chemical difference spectrophotometry using an extinction coefficient of 64 000 M^{-1} cm^{-1} at 700 nm (Hiyama & Ke, 1972).

Reaction Center Dissociation and Reconstitution. The dissociation of PsaC from PS I on the stromal surface of thylakoids was by minor modification of the procedure of Golbeck and co-workers (Golbeck et al., 1988; Parret et al., 1989, 1990; Zhao et al., 1990) as follows. The thylakoids were incubated at room temperature in 6 M urea for 60 min. The preparations were then rapidly desalted by passage through a small (2 × 15 cm) Sephadex G-75 column equilibrated with 50 mM Tris-HCl, pH 8.2. Reconstitution of the desalted urea-treated preparations was performed following the procedure developed by Li et al. (1991). Reconstitutions were performed in dim light anaerobically by purging the sample for 30 min with nitrogen followed by addition of 2-mercaptoethanol to a final concentration of 0.5% (v/v). After 5 min of additional purging, apo-PsaC was added at concentrations as indicated in the figure legends, followed by 150 μM $FeCl_3$. Another 5-min purge was performed before addition of 150 μM Na_2S. The preparations were then incubated at 4 °C overnight or as indicated in the figure legends. The recombinant PsaC apoprotein used in the reconstitutions was overexpressed in *Escherichia coli* (Zhao et al., 1990; Li et al., 1991) and generously provided by Drs. D. A. Bryant and J. H. Golbeck. The concentration of apo-PsaC was calculated using an extinction coefficient of 13 200 M^{-1} cm^{-1} at 280 nm (J. H. Golbeck, personal communication).

Flash Kinetic Spectrophotometry. Recombination reactions between P_{700}^+ and reduced PS I acceptors were measured at room temperature in the microsecond and millisecond time ranges by flash absorption spectroscopy using equipment as described previously by Biggins (1990). The absorption transients due to P_{700}^+ were measured at 820 nm, and an extinction coefficient of 6500 M^{-1} cm^{-1} at 820 nm was used to calculate the concentration of photoactive P_{700}^+ (Mathis & Sétif, 1981). The kinetic data were analyzed using Sigma-plot version 4.0 (Jandel Scientific), and the decay curves were fit using three exponents with $t_{1/2} = 100$ μs, 1.2 ms, and 30 ms. The 100-μs component was chosen because it corresponded to the time response of the instrument but undoubtedly represented much faster kinetic components in the decay. The millisecond components were chosen on the basis of the published $t_{1/2}$ times for the recombination reactions between P_{700}^+ and F_X^- (1−2 ms) and between P_{700}^+ and $F_AF_B^-$ (*ca.* 30 ms).

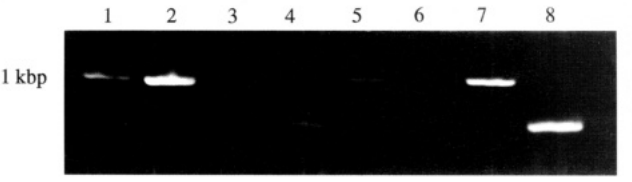

FIGURE 2: Analysis of transformant cells by PCR. Total cellular DNA was used as a template to amplify a 1-kbp fragment of *psaB* using primers that flank the site of *Stu*I in the donor plasmid. The product was either loaded directly onto the 2% agarose gel (odd-numbered lanes) or digested with *Stu*I 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; D562N, lanes 3 and 4; P564L, lanes 5 and 6; and R566E, lanes 7 and 8.

RESULTS

Generation of PS I Site-Directed Mutants by Chloroplast Transformation. Chlamydomonas strains carrying mutations in the F_X binding region of PsaB were generated by chloroplast transformation by particle bombardment. Creation of the P560A and P560L mutants has been described previously (Webber et al., 1993). Mutant strains D562N, P564L, and R566E were generated using chimeric *aadA* gene constructs cloned 3′ to the *psaB* gene (Figure 1). Homologous recombination of the donor plasmid into the chloroplast genome also incorporates the *aadA* gene and renders cells resistant to spectinomycin (Figure 1). Spectinomycin-resistant colonies were 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 1). Following digestion with *Stu*I, the amplified fragment was fractionated on a 2% agarose gel as shown in Figure 2. 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 2). 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). In all cases, transformants containing the *Stu*I site also contained the desired mutation.

Western Blot Analysis of Thylakoid Proteins. To determine the impact of the mutations on PS I reaction center accumulation, we quantified the amount of the PsaA and PsaB 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

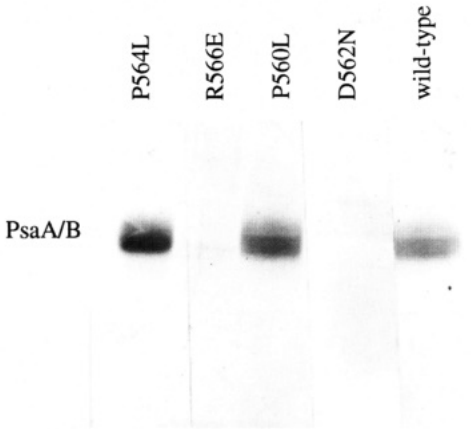

FIGURE 3: Western blot of wild type and mutant proteins. Thylakoid membranes were isolated as described in Materials and Methods. Twenty micrograms of chlorophyll was loaded for each sample and separated by SDS−PAGE followed by transfer to a nitrocellulose membrane. After blocking, the membrane was probed with antisera raised against the PsaA and PsaB proteins.

nitrocellulose membranes and immunodecorated with antisera against the PsaA/PsaB proteins as shown in Figure 3. The Western data shows that the P_{700} binding proteins did not accumulate to a detectable level in the D562N and R566E mutants. However, P564L mutants accumulated the PsaA/PsaB polypeptides to a level equivalent to wild type. Similar results showing normal accumulation of PS I have been reported previously for the P560A and P560L mutants (Webber et al., 1993).

Electron Transfer on the PS I Acceptor Side of Wild Type and the P Mutants. The stability of the PS I reaction centers and interaction of the subunit PsaC with the wild type and P mutant core heterodimers was investigated by measuring the reaction center dissociation using urea and by reconstitution of the urea-treated cores with apo-PsaC and insertion of FeS clusters *in vitro*. The extent of dissociation and reconstitution was followed spectrophotometrically by measuring the electron transfer kinetics between P_{700}^+ and various reduced acceptors in recombination reactions following an activation flash. The complete experimental protocol is illustrated in Figure 4 which shows control experiments using wild type PS I. Figure 4, trace A shows the flash transient obtained at room temperature and the $t_{1/2}$ for the major component of the decay (92% signal) is *ca* 30 ± 3 ms, indicative of recombination between P_{700}^+ and the reduced terminal acceptors F_AF_B on the subunit PsaC. This is generally accepted as evidence for complete forward electron transfer as a result of photoactivation of the reaction center. Treatment of PS I with urea dissociates several small subunits from the reaction center core, including PsaC (Parrett et al., 1989), and Figure 4, trace B shows the flash transient obtained in a preparation after such treatment. The amplitude of the signal was routinely found to be about 60% of the control (Figure 4, trace A) and the decay was polyphasic with components in the microsecond as well as millisecond ranges. The decay curves were satisfactorily fit as illustrated using three kinetic phases of $t_{1/2}$ = 100 μs, 1.2 ms, and 30 ms, corresponding to the recombination of P_{700}^+ with phylloquinone$^-$ (63% signal) or F_X^- (18% signal) and the direct reduction of P_{700}^+ by ascorbate/TMPD in the reaction mixture (19% signal), respectively. The assignment of the 100-μs phase to the P_{700}^+−phylloquinone$^-$ backreaction is

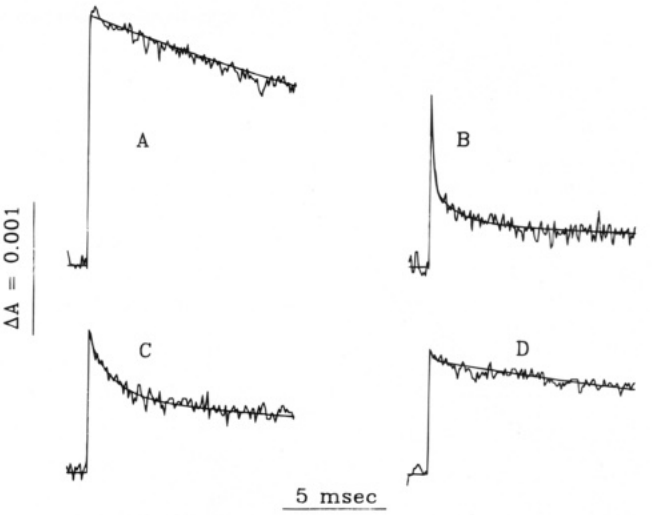

FIGURE 4: Flash-induced absorption transients measured at 820 nm showing recombination reactions between P_{700}^+ and reduced acceptors in wild type *C. reinhardtii* PS I preparations. Trace A, untreated PS I; trace B, after treatment with 6 M urea for 60 min at room temperature; trace C, urea-treated sample after anaerobic incubation with $FeCl_3$, Na_2S, and 2-mercaptoethanol according to the reconstitution protocol in Materials and Methods; trace D, sample from (C) after further incubation with PsaC at a ratio PsaC/P_{700} = 20. The chlorophyll concentration was 150 μg/mL, and the reaction mixture in (A) and (B) contained 5 mM ascorbate and 100 μM TMPD.

based on recent observations on P_{700}−phylloquinone core preparations and PS I mutants that have forward electron transfer terminating at phylloquinone because of a nonfunctional F_X cluster. These samples show a biphasic decay with components at $t_{1/2}$ = 10 μs and 110 μs in a 2:1 ratio (J. H. Golbeck, personal communication). The result shown in Figure 4, trace B, is quite different to that normally observed when thylakoids or PS I preparations from cyanobacteria or higher plants are treated with chaotropic agents. In those cases the majority of the *ca*. 30-ms recombination between P_{700}^+ and the reduced terminal acceptors is replaced by a 1−2-ms decay due to the P_{700}^+-F_X^- backreaction and components in the microsecond range are not usually observed (Parrett et al., 1989). This indicates that the F_X cluster in PS I of *C. reinhardtii* is more susceptible to destruction by chaotropes, and this suggests the possibility of some differences in the protein structure or subunit organization.

The flash transient observed from urea-treated PS I preparations after incubation with Fe^{3+} and S^{2-} in the presence of 2-mercaptoethanol to insert FeS centers is presented in Figure 4, trace C. Analysis of the decay kinetics (100-μs component, 10%; 1.2-ms component, 41%) reveals that the majority of the 100-μs phase observed in Figure 4, trace B, (P_{700}^+−phylloquinone$^-$ backreaction) was replaced by the 1.2-ms component, indicating the reconstruction of a large proportion of the damaged F_X clusters. Finally, inclusion of PsaC in the reaction mixture allowed insertion of the terminal FeS clusters, F_AF_B, in the subunit PsaC and functional interaction of the subunit with the core binding site as shown in Figure 4, trace D. The transient shown is characterized by a predominant (88% signal) 30-ms decay due to the backreaction between P_{700}^+ and the newly assembled F_AF_B iron−sulfur centers (Li et al., 1991; Zhao et al., 1990).

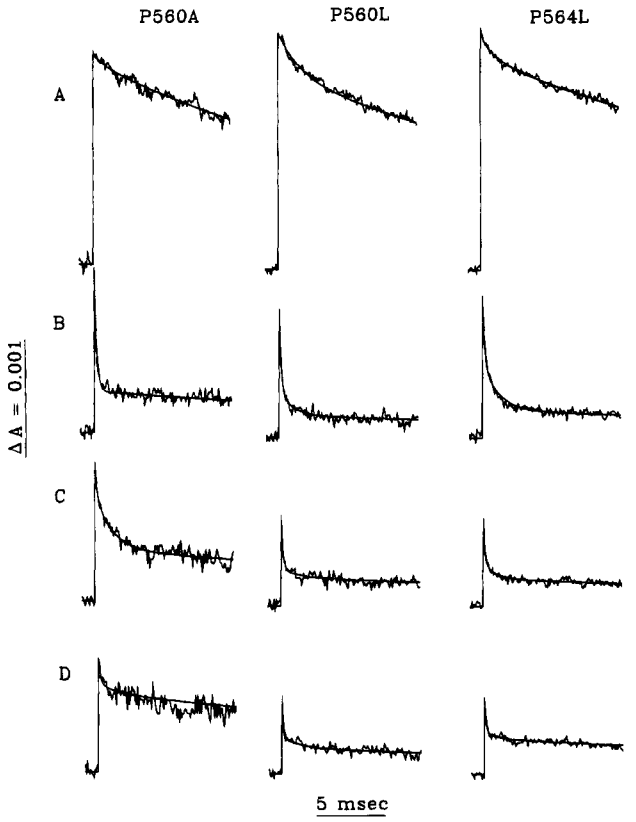

FIGURE 5: Flash-induced absorption transients due to P_{700}^+ in recombination reactions in mutant PS I preparations from *C. reinhardtii*. The reaction conditions for the samples in rows A−D were the same as in Figure 4, panels A−D, respectively.

PS I preparations from the three P mutants of the F_X domain were investigated using the same experimental protocol as used for the wild type above, and Figure 5 shows the kinetic behavior observed during dissociation and reconstitution. All three untreated P mutants display wild-type flash kinetics (Figure 5, row A) and show a $t_{1/2}$ *ca.* 30-ms recombination reaction indicative of complete forward electron transfer to the terminal centers. The leucine mutants, P560L and P564L, however, show a minor contribution of a faster millisecond phase, which we did not observe in the P560A mutant, possibly due to a disruption in electron transfer between F_X and F_AF_B in these two mutants. Figure 5, row B, shows the effect of urea treatment to dissociate the peripheral subunits. The flash transient of the P564L mutant was similar to wild type (Figure 4, trace B) but the F_X cluster was almost totally destroyed in the P560A and P560L mutants, mostly likely due to effects on the adjacent C561 ligand of the F_X cluster.

Attempts were made to reconstitute the urea-treated mutant preparations using FeS cluster insertion protocols and addition of PsaC as shown for wild type in Figure 4, traces C and D. We observed a partial (30−40%) reconstitution of both F_X and F_AF_B centers in the case of the P560A preparation (Figure 5, rows C and D), but the overall signal amplitudes of the P560L and P564L mutants declined and reconstitution of the acceptor side was marginal. These results show that the replacement of P560 by alanine results in destabilization of the PS I acceptor side, but the residue change was not as severe as the replacement by leucine. The observations also show that the replacement of P564 by leucine results in an F_X domain that is severely modified with respect to F_X cluster insertion and the subunit interaction between PsaC and the PS I reaction center core.

Table 1: Dissociation of PS I by Urea

	% residual forward electron transfer[a]			
[urea] (M)	wild type	P560A	P560L	P564L
0	100	100	100	100
3.5	96	89	91	80
4.0	93	84	69	65
4.5	85	71	68	60
5.0	53	45	44	41
5.5	40	32	29	*b*
6.0	26	14	17	16

[a] The percent residual forward electron transfer was estimated from the amplitude of the 30-ms decay component in the flash optical transient due to P_{700}^+ in the backreaction between P_{700}^+ and reduced terminal accptors. [b] Not determined.

Stability of the P Mutant Reaction Centers. We then studied the stability of the proline mutant PS I preparations in more detail by investigating the dissociation of the acceptor side using various concentrations of urea. The dissociations were followed by measuring the loss of the *ca.* 30-ms recombination reaction between P_{700}^+ and F_AF_B spectrophotometrically as shown above for wild type (Figure 4, traces A and B) and the mutants (Figure 5, rows A and B). Table 1 lists the percent residual forward electron transfer for wild type and mutant PS I preparations after incubation using increasing concentrations of urea to disrupt the subunit interactions on the surface-exposed regions of PS I. The P560A mutant is less stable than wild type, showing more dissociation at all concentrations of urea used. However, the replacement of P560 and P564 by leucine residues results in a much greater instability, with the 564 site being affected the most. In all cases there remained a residual slow phase of the signal in the ≥30 ms time range, representing 16−26% of the overall transient. This contrasts with the behavior of PS I reaction center preparations prepared from spinach (Rodday et al., 1993) and thylakoids from *Synechocystis* sp. PCC 6803 (Rodday et al., 1994). Using identical methodology, we usually observed a conversion of all the absorption signal to a transient with a 1−2-ms decay after treatment of such preparations with urea, in agreement with others (Parrett et al, 1989). We tentatively suggest that the residual ≥30-ms signal observed in the *C. reinhardtii* preparations after treatment with 6 M urea most likely reflects direct reduction of P_{700}^+ by ascorbate/TMPD rather than a recombination reaction.

Reconstitution of Electron Transfer in the P Mutant Core Preparations. The reconstitution of the mutant core preparations obtained by exhaustive urea treatment was studied as a means of assessing the interaction of PsaC with its binding site on the PS I reaction center core. Reconstitution was measured using the protocol shown (Figure 4, traces C and D, and Figure 5, rows C and D), where the recovery of the $P_{700}^+-F_AF_B^-$ backreaction was taken as restoration of full forward electron transfer to the terminal acceptors. The extent of the reconstitution of the preparations was investigated as a function of PsaC concentration as shown in Figure 6. The data presented are those acquired for all experiments performed and, therefore, represent observations on several wild type and mutant preparations. The values for percent reconstitution at saturating PsaC ($PsaC/P_{700} = 20$) were wild type (47%), P560A (37%), P560L (16%), and P564L (18%).

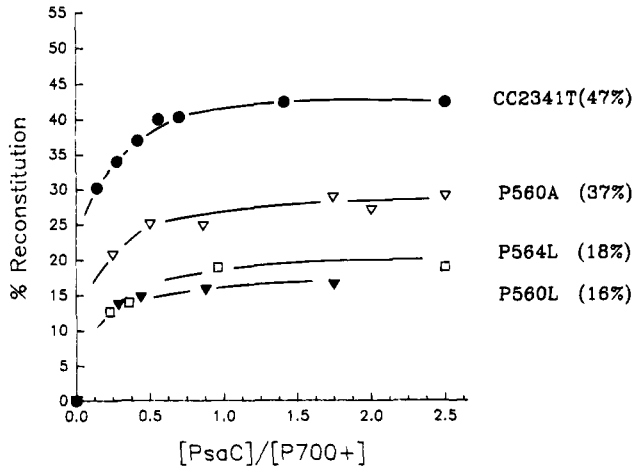

FIGURE 6: Reconstitution of the acceptor side of PS I in wild type and mutant thylakoids from *C. reinhardtii*. The preparations were treated with urea as for the sample shown in Figure 1, trace B, and then reconstituted using Fe^{3+}, S^{2-}, 2-mercaptoethanol, and PsaC as indicated. The reconstitution mixtures were incubated overnight at 4° C. Flash transients were then measured as in Figure 4, trace D. The percent reconstitution was estimated from the amplitude of the 30-ms kinetic phase of the signal decay due to the backreaction between P_{700}^+ and reduced terminal acceptors.

The results presented in Figure 6 reveal that wild type and all mutants almost reach their saturated PsaC values at 0.5 PsaC/reaction center after an overnight incubation at 4 °C. The replacement by alanine at P560 results in a 20% reduction in reconstitution but the leucine substitutions at P560 and P564 were less tolerable with an over 60% reduction compared to wild type. These observations are consistent with the dissociation data presented in Table 1 and indicate that the residues P560 and P564 play a key role in maintaining the structural integrity of the F_X domain. The results also confirm that the F_X interhelical loop of PsaB is a domain involved in the interaction between PsaC and the PS I core (Rodday et al., 1994).

DISCUSSION

Mutations Adjacent to the C561 Ligand of the F_X Cluster. The results presented above confirm that the site-directed mutation of P560 to leucine or alanine results in mutants that are capable of full assembly of PS I and growth under photoautotrophic conditions (Webber et al., 1993). From the flash kinetic studies on the mutant preparations treated with urea to prepare the F_X core, and reconstitution of the F_X core using PsaC and cluster insertion protocols, we conclude that although the residue changes allowed assembly of the normal complement of functional PS I, they led to a decrease in the stability of the reaction center and an impaired interaction of the reaction center core with PsaC. The leucine substitution resulted in a greater destabilization and less efficient reconstitution of the core than the substitution by alanine. This suggests that the isopropyl side chain of leucine may have resulted in an unfavorable interaction through increased hydrophobicity or by modification of the conformation of the subunit binding site. In either case the cluster F_X was unaffected and normal (wild type) PS I recombination kinetics were observed between P_{700}^+ and the reduced acceptors phylloquinone, F_X^-, and $F_AF_B^-$.

These results are very similar to those reported by Quinkal et al. (1994), who investigated the role of conserved proline residues adjacent to the 2[4Fe−4S] ferredoxin of *Clostridium pasteurianum*. They showed by site-directed mutagenesis that either proline could be substituted without changing the protein fold or causing significant changes in reduction potential and enzyme function. However, 2-D NMR studies of the proteins with proline replacements indicated destabilization of the structures most likely associated with changes in hydrogen bonding within the cluster domain. In particular, two hydrogen bonds to the adjacent cysteine ligand were found to be weakened. It is likely that the F_X cluster domain in PS I is destabilized in an analogous fashion. This also raises the interesting question of how PS I is assembled *in vivo* in the P560 mutants, and perhaps additional factors such as prolyl isomerases or molecular chaperones may be involved.

In contrast to the proline changes, the site-directed mutation of D562, which is also adjacent to the C561 ligand of F_X, to the uncharged asparagine resulted in a mutant which was found to be incapable of stable PS I assembly and unable to grow photoautotrophically. This result implies that D562 plays an essential role in the structure of the F_X domain in PsaB and we suggest that the change in the D562N mutant affects the assembly of the F_X cluster. Alternatively, an F_X cluster may form in the mutant but with an incorrect redox potential, rendering it nonfunctional in forward electron transfer. This would then result in rapid degradation of the PS I polypeptides. These alternatives are currently being investigated by rapid pulse-labeling of chloroplast proteins.

Mutations in the Interhelical Loop of the Conserved F_X Region in PsaB. Site 564 is within the interhelical loop in the conserved region between the two cysteine ligands of F_X in PsaB. We proposed previously (Rodday et al., 1993) that this domain, which is localized on the stromal surface of the PS I core, is involved in PsaC binding. Again, although assembly of PS I occurred in the P564L mutant and the cells were shown to grow autotrophically, we observed that the reaction center stability and interaction between the core was modified and was comparable to the mutant P560L. Our previous molecular modeling study of this domain (Rodday et al., 1993) indicated considerable flexibility and plasticity of the interhelical loop regions because of the three glycine residues in the eight-residue loop. Therefore, we suggest that the decreased interaction between the F_X core and PsaC in the P564L mutant is not due to a radical change in conformation of the loop backbone but is more likely a result of either increased local hydrophobicity or steric effects in the PsaC binding site.

The arginine at site 566 occupies a prominent central position in the interhelical loop and made possible our earlier experiments where the specific chemical modification and proteolysis of P_{700}−F_X cores provided strong evidence for the involvement of a surface-exposed domain containing arginine in the interaction between the core and the PsaC subunit (Rodday et al., 1994). More recently, we have acquired direct evidence that the interhelical loop of PsaB in the cyanobacterium *Synechocystis* sp. PCC 6803 is involved in the interaction with PsaC and that the corresponding arginine residue may be implicated in the binding mechanism (Rodday et al., 1994). In that investigation the mutant R561E in *Synechocystis* was engineered and, in contrast to the corresponding R566E mutant of *C. reinhardtii* studied here, assembled 40% PS I and was weakly photoautotrophic. The R566E mutant of *C. reinhardtii* prepared

for this study was found to be totally devoid of PS I reaction center proteins, indicating that the photosynthetic apparatus of the eucaryotic alga is much more susceptible to changes in the primary structure of the PS I subunits. The fact that the neighboring proline can be changed with no effect on PS I assembly suggests that the effect of the R566E mutation is due to a change in side chain charge rather than a large perturbation in secondary structure. The contrasting effect between corresponding mutations in *C. reinhardtii* and cyanobacteria is similar to the effect of insertional inactivation of *psaC* on PS I stability. In *C. reinhardtii*, Takahasi et al. (1991) reported that PS I did not assemble in the absence of PsaC and the subunits rapidly turned over, whereas for *Anabaena variablis*, Mannan et al. (1991) observed assembly of a PS I core with normal Chl/P_{700} ratio and forward electron transfer to F_X. The fact that PsaC is critical for PS I reaction center assembly in *C. reinhardtii* may explain the lack of PS I in the R566E mutant, since an impaired interaction between the PS I reaction center core and PsaC would lead to destabilization of PS I. The reason for a greater sensitivity of *C. reinhardtii* to mutational changes that cause severe functional disruptions is unknown, but it may be a result of the greater structural complexity of the reaction centers and the light-harvesting apparatus associated with PS I in eucaryotes.

Structure of the F_X Domain and PsaC Binding. The characterization of the mutants P560L, P560A, P564L, D662N, and R566E detailed in this paper lend support to the model we have proposed for the interaction between the PS I reaction center core and the subunit PsaC. Specific information could not be obtained on the failure of the D562N and R566E mutants to assemble a PS I reaction center as a result of the severe perturbations induced by the site-directed changes at these two sites. However, we can definitely conclude that the two residues are important in maintaining the stability of the reaction center in the F_X domain, in confirmation of the results we obtained on the R561 mutant of *Synechocystis* 6803 (Rodday et al., 1994).

Data obtained on preparations of PS I from cells with mutations of the proline residues at positions 560 and 564 also corroborate our previous findings and confirm that the introduction of structural changes in the F_X loop region leads to both a decrease in stability of the acceptor side of PS I and a decreased interaction between the reaction center core and PsaC. These observations lend further support for the model we have proposed for the organization of the F_X domain and binding of the PsaC subunit to the core heterodimer (Rodday et al., 1993). This domain is crucial in forward electron transfer between F_X on the core heterodimer and the terminal F_AF_B centers on the PsaC subunit. Work now in progress on the site-directed mutagenesis of surface-exposed residues of PsaC *in vivo* and *in vitro* is expected to provide additional experimental evidence to test the model.

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