Site-Directed Mutagenesis of the Subunit PsaC Establishes a Surface-Exposed Domain Interacting with the Photosystem I Core Binding Site†

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ABSTRACT: We have postulated that the orientation of PsaC on the photosystem I core involves electrostatic interactions between charged residues on the core binding site and the subunit [Rodday, S. M., Jun, S.-S., & Biggins, J. (1993) *Photosynth. Res. 36*, 1−9]. We, therefore, changed eight acidic residues on PsaC to arginine and examined the efficiency of the mutant subunits in the reconstitution of P_{700}−F_X cores *in vitro*. Reconstitution of the cores by the mutant subunits was determined by analysis of the kinetics of recombination reactions between P_{700}^+ and reduced acceptors as measured optically. Restoration of complete forward electron transfer, indicative of efficient subunit binding, was estimated from the ca. 30 ms decay component in the flash transients. Slightly reduced levels of reconstitution were observed for the mutants D24R, E46R/D47R, D61R, and E72R. In contrast, mutants D9R, E27R, and D32R showed significantly lower efficiencies. The presence of the iron−sulfur centers, F_A and F_B, in these three mutant subunits was confirmed by low-temperature EPR spectroscopy indicating that the polypeptides had folded correctly. We conclude that the introduction of positively charged side chains at positions 9, 27, and 32 seriously disrupts PsaC binding. However, when the wild-type acidic residues in these positions were changed to alanine, only mutant D9A showed a reduced level of reconstitution, suggesting that this aspartate is the most important residue participating in the electrostatic interaction with the core. The results are discussed in relation to the photosystem I crystal structure and support an orientation of PsaC on the core such that center F_B is proximal to F_X.

The primary photochemical reactions catalyzed by the PS I[1] reaction center occur on a core heterodimer composed of the subunits PsaA and PsaB and a small subunit, PsaC. The primary donor and acceptor, P_{700} and A_0, respectively, and the transient acceptors phylloquinone and F_X reside on the core, whereas the terminal acceptors F_A/F_B are located on PsaC (Golbeck, 1992, 1993). We have been engaged in a detailed study of the interaction between the PsaC subunit and the core heterodimer in order to identify the binding site for PsaC, the mechanism of the PsaC−core interaction, and, ultimately, the orientation of PsaC in the binding site. We developed a working model for the protein−subunit interaction based upon predictions of the structure of a domain on the core heterodimer in the vicinity of the F_X cluster (Rodday et al., 1993). Briefly, we proposed that the two conserved regions between the cysteine ligands of the F_X cluster on PsaA and PsaB (CDGPRGGTC) are two flexible loops that form a surface-exposed cavity to accommodate the PsaC subunit. Molecular modeling studies confirmed the feasibility of the proposal which was then tested experimentally. We reported that P_{700}−F_X cores, prepared by removal of PsaC and other small subunits by chaotrope treatment, could not be functionally reconstituted using PsaC after chemical modification of arginine residues and tryptic cleavage of the cores (Rodday et al., 1993). These results supported the model that was then tested more directly by the site-directed mutagenesis of residues in the putative binding site on the core (Rodday et al., 1994, 1995).

Results obtained from two species showed that the oligonucleotide-directed mutagenesis of the arginine residue occupying the central position in the F_X loop of PsaB resulted in a 60% reduction of assembly of PS I in *Synechocystis* sp. PCC 6803 (Rodday et al., 1994) and no assembly of PS I in *Chlamydomonas reinhardtii* (Rodday et al., 1995). The 40%-assembled PS I in *Synechocystis* was found to be unstable, and the *in vitro* reconstitution of the mutant P_{700}−F_X cores using wild-type PsaC was incomplete. A similar instability of PS I and impaired interaction between the core and PsaC was demonstrated in *C. reinhardtii* when the conserved prolines P560 and P564 in the F_X domain of PsaB were changed to A560, L560, and L564. Recently, Hallahan et al. (1995) conducted EPR spectroscopy on the PS I mutant D576L of *C. reinhardtii* and obtained evidence for the involvement of the F_X interhelical loop of the PsaA subunit in the binding of PsaC to the core.

Collectively, our results indicate that the F_X loop in PsaB is involved in binding PsaC, and the mechanism of binding probably involves electrostatic interactions between R561 in *Synechocystis* (or the corresponding R566 in *Chlamydomonas*) and a surface-exposed acidic domain on the PsaC

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[1] Abbreviations, DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PS I, photosystem I; SDS, sodium dodecyl sulfate; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Tris, tris(hydroxymethyl)aminomethane.

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Table 1: Design of Oligonucleotides Used for the Site-Directed Mutagenesis of PsaC

mutant	wild-type codon[a]	mutagenic oligonucleotide[a,b]
D9R	GTC	5′GCCGATGCAAGTGCGGTAAATCTTGACCG
D9A	GTC	5′GCCGATGCAAGTGGCGTAAATCTTGACCG
D24R	ATC	5′GGGCACCATTTCCAGTACACGGAGAGGGCACGCCCG
E27R	TTC	5′CCCAAGGCACCATTCTCAGTACATCGAGAGGGC
E27A	TTC	5′CCCAAGGCACCATTGCCAGTACATCGAGAGG
D32R	GTC	5′CCGGCTTTACAGCCGCGCCAGGGCACC
D32A	GTC	5′CCGGCTTTACAGCCGGCCCAGGGCACC
E46R/D47R	ATCTTC	5′CGCTTACAGCCCACACACCGTCGGGTTCGCGGAGAAGCAGCG
D61R	GTC	5′CCCGAATGCTCAAAAAGCGCGTGGGGCAAGCCG
E72R	TTC	5′CCCATGCTGCGCGTCGTTCTGGCACCCAGGTAGACCCG

[a] The nucleotide sequences are of the complementary strand because *psaC* was subcloned into the mutagenic vector in the inverted orientation. [b] The site-directed changes were as underscored.

subunit. We have suggested that ion pairs between residues on the PsaC subunit and the core binding site serve to orient PsaC during assembly of the PS I reaction center. Once the subunits were correctly oriented, hydrophobic interactions between residues on the contact surfaces of the PsaC subunit and the core binding site then stabilize the overall structure (Rodday et al., 1994).

We have now extended our experimental strategy to test these proposals further by changing all the acidic residues on PsaC to arginine or alanine by site-directed mutagenesis with the aim of identifying the interaction domain on PsaC. In a preliminary report, we presented data indicating that the PsaC mutant D9R was only partially effective in the reconstitution of wild-type cores whereas the mutant E72R had a wild-type response (Biggins et al., 1995). We concluded that an N-terminal domain including residue D9 is probably involved in the subunit−core interaction but the C-terminal extension of PsaC containing E72 is not implicated. We now report on the complete set of PsaC arginine mutants, D9R, D24R, E27R, D32R, E46R/D47R, D61R, and E72R, and three PsaC alanine mutants, D9A, E27A, and D32A.

MATERIALS AND METHODS

Biological Preparations. PS I was isolated from spinach according to Anderson and Boardman (1996) using high-grade digitonin from the Gallard-Schlesinger Industries Corp. (Carle Place, NY). The D-144 fractions were stored as pellets at −80 °C. The concentration of P_{700} was determined by difference spectrophotometry using extinction coefficients of 64 000 M^{-1} cm^{-1} at 700 nm (Hiyama & Ke, 1972) and 6500 M^{-1} cm^{-1} at 820 nm (Mathis & Sétif, 1981). P_{700}−F_X cores were prepared fresh when required by dissociation of the low-molecular mass subunits from PS I using urea as described previously (Rodday et al., 1993). After urea treatment, the PS I preparation was passed through a Sephadex G-75 sizing column equilibrated with 50 mM Tris-HCl (pH 8.3) to desalt the P_{700}−F_X cores and to remove PsaC subunits and other small polypeptides. Functional reconstitution of the P_{700}−F_X cores using PsaC *in vitro* was performed at 4 °C by modifications of the procedure developed by Golbeck and co-workers (Parrett et al., 1989, 1990; Li et al., 1991) as described previously (Rodday et al., 1993). The PS I dissociation and the extent of reconstitution of P_{700}−F_X cores were followed by flash kinetic spectrophotometry as described below.

Plasmids and Site-Directed Mutagenesis. *psa*C from *Synechococcus* sp. PCC 6301 (Herman et al., 1994) was kindly provided by Dr. J. H. Golbeck and Dr. M. P. Scott as an insert between the *Afl*III and *Bam*HI restriction sites of a pET 3d vector (Novagen Inc., Madison, WI). For site-directed mutagenesis, a *Sal*I-*Bam*HI fragment containing the *psa*C insert was subcloned in the inverted orientation into the pAlter-1 vector of the Altered Sites II *in vitro* mutagenesis system (Promega Corp., Madison, WI). The manufacturer's protocol using a double-stranded template was used exactly except that electroporation was used for cotransformation of *Escherichia coli* ES 1301 after the mutagenesis reaction. Complementary strand oligonucleotides obtained from Operon Technologies (Alameda, CA) of the sequences shown in Table 1 were used for the mutagenesis of targeted codons of acidic residues on PsaC to codons for arginine or alanine as indicated. The mutagenic oligonucleotides were purified via urea−PAGE before use, and the site-directed changes were confirmed directly by dideoxy-DNA sequencing using Sequenase T7 DNA polymerase (USB Corp., Cleveland, OH).

Production and Purification of Wild-Type and Mutant PsaC Apoproteins. The mutant *psa*C genes were subcloned into pET 3d vectors and established in the expression host *E. coli* BL21(DE3) pLysS for protein production. Large scale growth of the host was conducted in 10 L batch cultures on LB medium supplemented with 30 μg of ferric ammonium sulfate per milliliter, 50 μg of ampicillin per milliliter, and 34.5 μg of chloramphenicol per milliliter in a Microferm 14 fermentor (New Brunswick Scientific Co., Edison, NJ). The expression of *psaC* was induced using 0.4 mM IPTG, and protein overproduction was continued for 4 h (Zhao et al., 1990; Li et al., 1991). The cells were harvested, washed, and suspended in 50 mM Tris-HCl (pH 8.3) containing 10 μg of DNase per milliliter and stored frozen.

Wild-type and mutant PsaC apoproteins were purified as follows. The *E. coli* cells were broken by three passes through a French pressure cell at 20 000 psi. Inclusion bodies containing PsaC were isolated by centrifugation of the homogenate at 7600*g* for 5 min and washed three times using 20 mM Tris-HCl (pH 8.3) and 10 mM NaCl and once with the same buffer containing 1 M urea. The washed inclusion bodies were then solubilized using 7 M urea, 50 mM Tris-HCl (pH 8.3), and 10 mM DTT (<100 mg of protein in 10 mL) for 1 h at room temperature. The protein was then diluted to a concentration of <1 mg/mL with 7 M urea, 50 mM Tris-HCl (pH 8.3), and 2 mM DTT (UTD buffer) and held at 4 °C overnight. The preparation was centrifuged at 27000*g* for 10 min, and PsaC protein was recovered from the dilute supernatant by ion exchange.

Excess DEAE-Sepharose CL-6B was added to the supernatant at a low protein to exchanger ratio to minimize aggregation upon binding (15−30 μmol/mg of protein, typically ca. 10−20 mL packed volume) and the mixture stirred gently at room temperature for 1 h to bind PsaC. The exchanger with bound PsaC was then recovered by low-speed centrifugation and washed once using 5 volumes of UDT buffer. The washed exchanger was then loaded onto the surface of an equivalent volume of DEAE-Sepharose in an FPLC column and washed further with UTD buffer until the eluate was free of protein. PsaC was chromatographed using UTD buffer containing 0.1 M NaCl, dialyzed against 20 mM Tris-HCl (pH 8.3) and 0.2% 2-mercaptoethanol, and then frozen in aliquots (ca. 500 μg/mL) for storage at −80 °C.

We found that the above procedure could not be used for purification of the PsaC double mutant E46R/D47R because it had a much higher tendency to aggregate, particularly during elution on the anion exchange column. Therefore, inclusion bodies isolated from the E46R/D47R mutant were solubilized in 6 M guanidine hydrochloride, 50 mM Tris-HCl (pH 8.3), and 10 mM DTT at a concentration of 10 mg of protein per milliliter. After centrifugation at 27000*g* for 10 min to remove the insoluble material, a sample (30 mg in 3 mL) was loaded onto a Superose 12 sizing column (2.5 × 40 cm) equilibrated with UDT buffer and eluted with the same buffer at a flow rate of 0.5 mL/min. The E46R/D47R mutant PsaC eluted at ca. 1.5 void volumes. The concentration of urea was then lowered gradually by stepwise dialysis versus 6, 4, 2, and 1 M urea and finally against 50 mM Tris-HCl (pH 8.3) and 0.2% 2-mercaptoethanol. We found that it was essential that the protein concentration be less than 5 μg/mL before dialysis to avoid aggregation of the protein during removal of the chaotrope. The purity of the wild-type and mutant PsaC apoproteins was verified by SDS−PAGE.

Large Scale Reconstitution of P_{700}−F_X Cores for EPR Spectroscopy. PS I equivalent to 1 mg of chlorophyll was treated with 7 M urea (3 mL final volume) for 1.5 h at room temperature and then passed down a Sephadex G-100 sizing column (2.5 × 40 cm) to remove excess urea and the low-molecular mass subunits that had dissociated from the P_{700}−F_X cores. The P_{700}−F_X cores were then diluted to 50 mL using 50 mM Tris-HCl (pH 8.3) including 5 mg of wild-type or mutant PsaC apoprotein of interest and placed in a 50 mL Amicon protein concentration apparatus fitted with a YM-100 membrane. The cap of the cell was retrofitted with a port (Swagelock) which could accept either a serum cap to facilitate the introduction of reagents during low-pressure purging of the reactants with inert gas or a steel plug for the high-pressure concentration of the product after protein folding, FeS cluster insertion, and reconstitution of the cores. Prior to protein folding and cluster insertion, the reconstitution mixture was flushed with argon for 1 h and the following reagents were then added at 30 min intervals: 0.3% 2-mercaptoethanol, 150 μM $FeCl_3$, and 150 μM Na_2S. The reaction mixture was stirred anaerobically overnight at room temperature. The mixture was concentrated to 10 mL, adjusted to 0.1 M glycine (pH 10.3) using a 1 M stock solution, and concentrated further to 1 mL. Samples were transferred anaerobically to EPR tubes, reduced using 2 mM sodium hydrosulfite, frozen in liquid nitrogen, and stored at −80 °C.

Spectroscopy. Flash kinetic spectrophotometry was performed at either 700 or 820 nm to monitor the recombination kinetics between P_{700}^+ and reduced acceptors following flash activation using standard procedures (Hiyama & Ke, 1972; Mathis & Sétif, 1991) and apparatus and signal-processing methods as described (Biggins, 1990). EPR spectroscopy was performed using a Bruker ESP300 instrument with an ER 023 signal channel, an ER 032 M field controller, an ER 4102 STO standard rectangular cavity with a 100% light-transmitting front flange, and an ER 041 MR bridge. Operating system version 9 (OS9/680) was used with a 1 MB base memory. An Oxford ITC4 helium temperature controller and an ESR 900 cryostat were used to achieve the low temperatures. The EPR conditions were as follows: field modulation frequency, 100 kHz; field modulation amplitude, 14.2 G; receiver gain, 1×10^5; sweep time, 20.97 s; time constant, 5.12 ms; conversion time, 20.48 ms; and temperature, 10 K. All spectra were the average of 10 scans.

RESULTS

Reconstitution of Wild-Type P_{700}−F_X Cores Using Mutant PsaC Subunits with Arginine Replacements. The interaction of the mutant PsaC subunits with the P_{700}−F_X core was determined by measuring the extent of the recovery of the recombination reaction between P_{700}^+ and the terminal acceptor(s), F_A/F_B, by flash kinetic spectrophotometry at room temperature. The P_{700}−F_X cores typically showed a $t_{1/2} = 1-2$ ms recombination reaction between P_{700}^+ and F_X^- (Parrett et al., 1989), and reconstitution of the cores using wild-type PsaC resulted in complete restoration of forward electron transfer as shown by replacement of the $t_{1/2} = 1-2$ ms decay kinetics with a 30 ms component (Zhao et al., 1990; Li et al., 1991). The optical transients observed (not shown) were similar to those reported previously with regard to signal to noise ratio and decay kinetics (Rodday et al., 1993, 1994, 1995). As shown in Figure 1, complete reconstitution of the P_{700}−F_X cores required an excess of 35 wild-type PsaCs per reaction center for the assay conditions used. The effectiveness of the mutant PsaC subunits in the reconstitution of P_{700}−F_X cores was compared with that of wild-type PsaC, and the data (Figure 1) represent the entire experimental results obtained involving many P_{700}−F_X core samples and several productions of the wild-type and mutant PsaC apoproteins. All seven PsaC arginine mutants promoted lower levels of functional reconstitution of the cores in comparison to the wild-type PsaC, but they fell into two distinct categories shown in the two panels of Figure 1. The mutants D9R, E27R, and D32R (Figure 1, left panel) showed significantly lower levels of reconstitution and a final value of ca. 60% of that of the wild-type PsaC. These mutants also saturated at about 35 subunits per reaction center. The right panel of Figure 1 shows the response of the second category of PsaC mutants, D24R, E46R/D47R, D61R, and E72R, which promoted a much higher level of reconstitution. The final level of reconstitution of these four PsaC mutants was generally between 80 and 90% of that of the wild type; however, we frequently observed wild-type responses for E72R and D24R, whereas D61R was always the least effective. We consider these mutants (Figure 1, right panel) to be basically wild type, and the slightly reduced level of reconstitution of the P_{700}−F_X cores by these mutants might be caused by minor changes in the tertiary structure of the holoproteins affecting binding and reaction center stability.

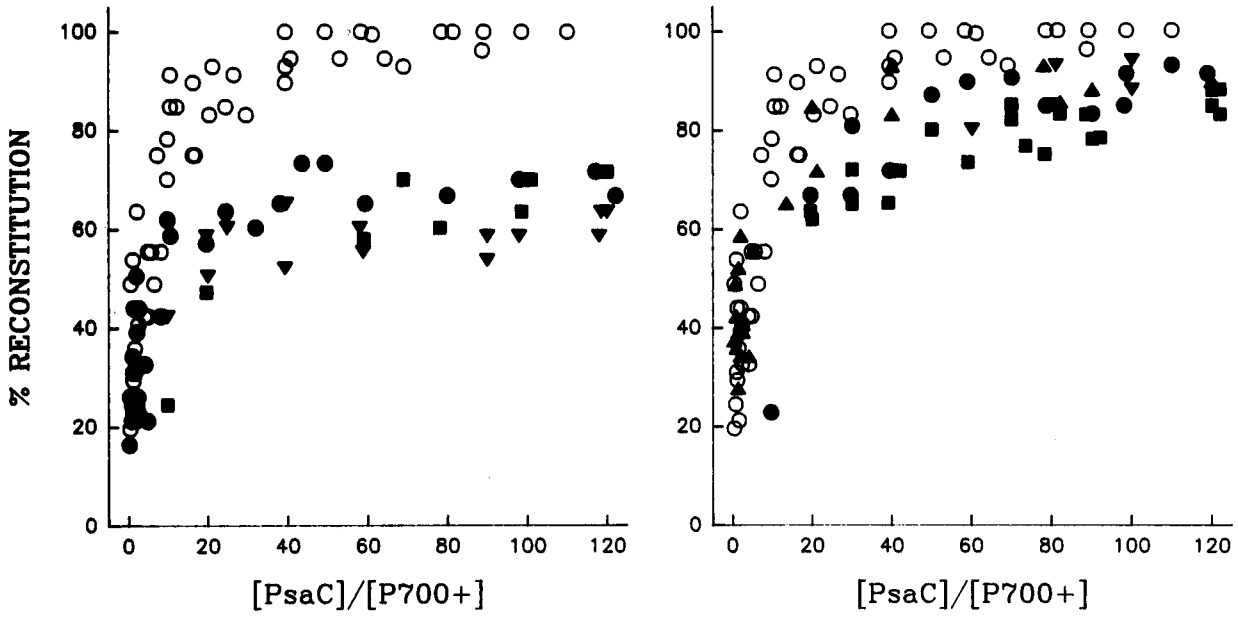

FIGURE 1: Reconstitution of $P_{700}-F_X$ cores by wild-type PsaC and PsaC arginine mutants. Reconstitution was measured by flash kinetic analysis to determine the recombination reactions between P_{700}^+ and the acceptors F_X^- and F_A^-/F_B^- after a flash. The percent reconstitution was estimated from the signal amplitude of the ca. 30 ms component of the absorption transients representing the extent of restoration of the $P_{700}^+-F_A^-/F_B^-$ recombination. The samples contained 10−12 μg of chlorophyll per milliliter, 5 mM ascorbate, 100 μM TMPD, and PsaC protein as indicated. Left panel: ○, wild type; ●, D9R; ▼, E27R; and ■, D32R. Right panel: ○, wild type (data as in left panel); ●, D24R; ▼, E46R/D47R; ■, D61R; and ▲, E72R.

Table 2: Reconstitution of $P_{700}-F_X$ Cores by PsaC Alanine Mutants[a]

PsaC mutant	% reconstitution[b]
D9A	71.7 ± 3.4
E27A	95.1 ± 2.1
D32A	95.8 ± 2.0

[a] Experimental conditions as noted in the legend to Figure 1. [b] The values listed are for saturating concentrations of mutant PsaC protein.

In contrast, the three PsaC mutants shown in the left panel of Figure 1 promoted significantly reduced levels of reconstitution that could be caused by factors such as a major change in PsaC protein conformation, an alteration in the FeS ligand motifs affecting the assembly of the FeS centers, or a change in the binding of PsaC to the $P_{700}-F_X$ core.

Reconstitution of Wild-Type $P_{700}-F_X$ Cores Using Mutant PsaC Subunits with Alanine Replacements. The results above show that the introduction of positively charged residues at positions 9, 27, and 32 resulted in a much reduced ability of the PsaC subunits to reconstitute $P_{700}-F_X$ cores. The experiments were then extended to determine whether the loss of negative charge alone at each position was significant. The acidic residues at positions 9, 27, and 32 were, therefore, changed to alanine, and the resulting PsaC mutants were tested in the core reconstitution assay. The results in Table 2 show that the mutants E27A and D32A were similar to wild-type PsaC whereas D9A reconstituted ca. 70%, a level slightly higher than that for D9R (Figure 1, left panel). This indicates that the reconstitution of $P_{700}-F_X$ cores by PsaC requires the negatively charged side chain at position 9 in the subunit, but not at positions 27 and 32. However, the introduction of positively charged residues at positions 27 and 32 interferes with the reconstitution.

Verification of the Insertion of the F_A/F_B Centers in the Mutant PsaC Holoproteins by EPR Spectroscopy. The question of whether the three PsaC mutants, D9R, E27R, and D32R which show significantly lower levels of restoration of electron transfer function, as determined in the core reconstitution assay, were deficient in the FeS redox centers, F_A and F_B, was tested directly by conducting EPR spectroscopy on the holoproteins after protein folding and cluster insertion *in vitro*. Figure 2 shows low-temperature EPR spectra of $P_{700}-F_X$ cores reconstituted using wild-type and mutant PsaC subunits in the absence of PsaD. These conditions were identical to those used for the preparation of samples for flash kinetic measurements but on a larger scale followed by concentration of the material and chemical reduction using hydrosulfite at pH 10.3. The EPR spectra of the preparations reconstituted with the mutants D9R, E27R, and D32R are very similar to those of wild-type PsaC with clearly defined high-field resonances due to the F_A and F_B clusters at $g = 1.888$ and 1.910, although the g anisotropy

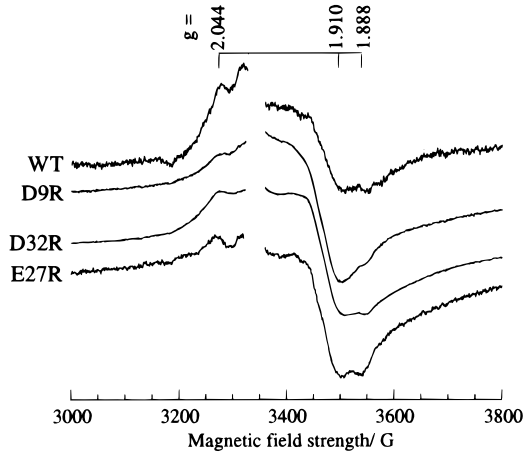

FIGURE 2: Low-temperature EPR spectra of $P_{700}-F_X^-$ core preparations reconstituted with wild-type (WT) PsaC and three PsaC arginine mutants as indicated. The signals in the $g = 2.00$ region were removed from the spectra for clarity. The EPR conditions were as described in Materials and Methods.

is less resolved than that seen in previously published spectra of the same PsaC clone in the absence of PsaD [in Li et al. (1991), see Figure 7B]. These control experiments confirm that the F_A and F_B clusters were successfully introduced into the three mutant PsaC proteins, and the inability of the three mutant subunits to fully reconstitute $P_{700}-F_X$ cores was, therefore, not a result of major changes in the conformation of the proteins. We conclude that the observed decreases in the level of reconstitution by the mutant PsaC subunits reflects a reduced ability of the subunits to bind to the $P_{700}-F_X$ core.

DISCUSSION

The results presented show that, when the PsaC residues D9, E27, and D32 were changed to arginine by site-directed mutagenesis, the *in vitro* reconstitution of $P_{700}-F_X$ cores by the mutant subunits was significantly reduced. This suggests that the acidic side chains at positions 9, 27, and 32 on the subunit may be involved in the mechanism of PsaC binding. However, when the same three residues were changed to alanine, only the mutant D9A showed a reduced level of reconstitution. We interpret these results to indicate that the presence of a negatively charged residue at position 9 is required in the mechanism of binding PsaC to the core. However, the introduction of an arginine residue at position 27 or 32 reduces the *in vitro* reconstitution of the core presumably because the large positively charged side chain in the interaction domain causes sufficient charge repulsion to disrupt the favorable ion pairing between D9 and the core. The mutation of additional residues in this domain to arginine and the study of multiple mutants such as D9R/E27R would be desirable to clarify this point. Nevertheless, the findings are consistent with and complement our previous report on the effect of the structural change R561E on the F_X loop of PsaB in *Synechocystis* sp. PCC 6803 (Rodday et al., 1994). The R561E mutation resulted in a phenotype with an unstable PS I reaction center, and the mutant $P_{700}-F_X$ core failed to reconstitute using wild-type PsaC. The two results provide strong evidence that electrostatic interactions between specific negative charges on PsaC and a specific arginine on the $P_{700}-F_X$ core play an important role in the structure of PS I.

In a recent report (Biggins et al., 1995), we presented the results of computer modeling of the interaction between PsaC and the $P_{700}-F_X$ core using the atomic structure of the ferredoxin of *Peptococcus aerogenes* (Adman et al., 1972) as a simulator for the PsaC subunit. The coordinates and distances between the three FeS centers F_A/F_B and F_X were obtained from the 6 Å crystal structure of PS I (Krauss et al., 1993). Although PsaC contains an eight-residue insert between strands three and four and a C-terminal extension, the overall protein fold is most likely very similar to that of *P. aerogenes* ferredoxin and other soluble ferredoxins (Fukuyama et al., 1988; Kissinger et al., 1991) because the cysteine ligand motifs for the two 4Fe4S clusters are the same and the distance between the clusters is identical. The modeling results confirmed the possibility of several favorable interactions between the arginine residues in the putative binding site and predicted acidic residues on the surface of PsaC, and they were observed for both orientations of the subunit, placing either F_A or F_B adjacent to F_X. Recently, Kamlowski et al. (1995) analyzed single crystals of PS I by low-temperature EPR and experimentally determined the **g** tensor orientations of the FeS centers with respect to the crystalline *c*-axis (membrane normal), and they proposed two models for the absolute orientation of PsaC on the PS I reaction center. Their models depict two cases for when either F_A or F_B is proximal to F_X, but they were unable to distinguish between the two possibilities because 180° rotation around the c_2-axis of the subunit gave the same set of angles.

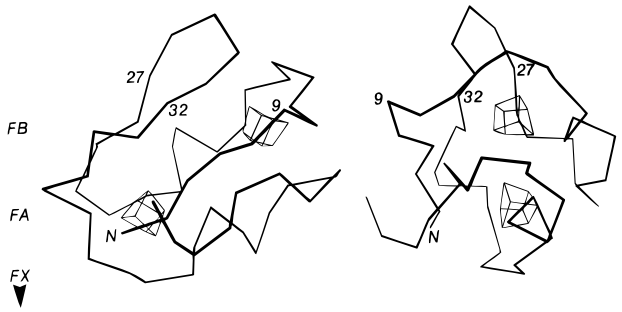

FIGURE 3: Tertiary structure of the ferredoxin of *P. aerogenes* as a simulator for PsaC showing the main chain and the two FeS centers. The left view shows the orientation of the subunit with the cluster analogous to F_A proximal to F_X on the PS I core as proposed by Kamlowski et al. (1995). The representation on the right is a −90° *y*-axis rotation of the left structure. The two views correspond to the irregular triangle (left) and vertically aligned (right) arrangement of the FeS centers shown by Krauss et al. (1993). The residue numbers shown are the predicted PsaC positions based upon sequence alignment.

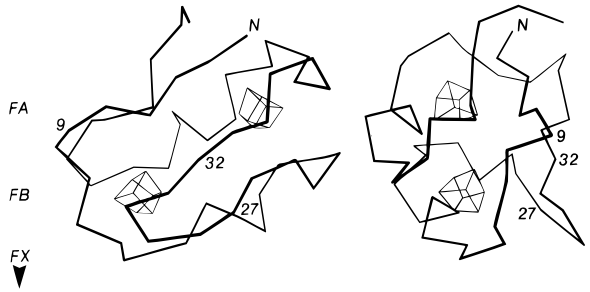

FIGURE 4: Tertiary structure of the ferredoxin of *P. aerogenes* as a simulator for PsaC shown with the cluster analogous to F_B proximal to F_X on the PS I core as proposed by Kamlowski et al. (1993). The representation on the right is a +90° *y*-axis rotation of the structure shown on the left. Residue numbering is as in Figure 3.

We now relate our results of the site-directed mutagenesis of PsaC presented above to the two models of Kamlowski et al. (1995). Figures 3 and 4 show the backbone structure of the *P. aerogenes* ferredoxin as a simulator for PsaC aligned with either F_A (Figure 3) or F_B (Figure 4) proximal to F_X in the two orientations proposed by Kamlowski et al. (1995). For each orientation, we present two views, one corresponding to the irregular triangle formed by the three FeS centers (view direction perpendicular to the F_X-F_A/F_B plane) and the other corresponding to 90° rotation around the y-axes such that the three FeS centers align vertically. These views correspond exactly to the two arrangements of the three FeS centers shown in the 6 Å crystal structure of PS I [in Krauss et al. (1993), see Figure 4]. The eight-residue insert present in PsaC most likely enlarges the loop between strands three and four.

Examination of these two figures shows that positions 9, 27, and 32 are localized on a surface-exposed domain in the vicinity of the N-terminal FeS cluster which has been shown

to be center F_B of PsaC (Zhao et al., 1992). The domain is on the face of the subunit opposite the two one-turn α-helices. We have presented a strong case for the involvement of R561 of the PsaB interhelical loop (*Synechocystis* 6803) in the interaction with PsaC (Rodday et al., 1994), and assuming ion pairing occurs between this residue and one or more acidic residues on the subunit, then the results of the mutagenesis experiments reported here are consistent with orientation of PsaC on the core such that F_B is proximal to F_X as shown in Figure 4. We have demonstrated that conversion of residues 9, 27, and 32 to arginine interferes with the reconstitution of PsaC on the core, and we propose that this is due to the inability of mutant PsaC subunits to form favorable ion pairs with residues in the binding site. Examination of the positions of residues 9, 27, and 32 relative to F_X indicates that such negative interactions would occur readily for the orientation of PsaC shown in Figure 4. However, rotation of the PsaC subunit 180° along the axis of the two FeS clusters so that F_A is proximal to F_X (Figure 3) positions residues 9, and 27 and 32 in particular, beyond any possibility of interaction with either F_X interhelical loop on the $P_{700}-F_X$ core [in Biggins et al. (1995), see Figure 1].

Although all three PsaC arginine mutants showed a reduced level of reconstitution of the $P_{700}-F_X$ core, the conversion of the same residues to alanine revealed that only D9A was impaired in interacting with the core. This suggests that the loss of negative charge at position 9 is critical, and therefore, D9 in wild-type PsaC is probably the most important residue in forming electrostatic interactions with the $P_{700}-F_X$ core. At this stage, we cannot claim that D9 of PsaC pairs specifically with an arginine in either F_X interhelical loop, but this possibility is being tested by compensatory experiments in progress using the PsaC mutants D9A, D9R, E27R, and D32R in reconstitutions with the PsaB core mutant R561E studied previously (Rodday et al., 1994).

We suggested that, following alignment of the PsaC subunit on the $P_{700}-F_X$ core by the electrostatic interactions discussed above, the overall structure be stabilized by hydrophobic interactions between residues on the contact surfaces of the subunit and the core (Rodday et al., 1994). It is noteworthy that the orientation of the PsaC subunit shown in Figure 4, which is supported by the mutagenesis results presented here, also aligns the structure such that 11 hydrophobic residues of PsaC are positioned on the lower surface of the subunit adjacent to the core. These residues are I12, G13, V18, A20, P22, L23, V25, L26, M28, V29, and P30.

We now evaluate our revised model for the binding of PsaC to the $P_{700}-F_X$ core in relation to relevant reports in the literature. Naver et al. (1996) recently reported on the effects of deletion of the C-terminal extension and the eight-residue internal "loop" in PsaC. Both deletion mutants reconstituted $P_{700}-F_X$ cores *in vitro* at levels less than that of wild-type PsaC, but the loop deletion mutant was the least efficient, showing only 55% reconstitution, and required PsaD for binding. Our results are generally consistent with their findings because the PsaC mutant E72R was similar to wild-type PsaC in our experiments and is a residue within the C-terminal extension. We, therefore, concur with Naver et al. (1996) that the C-terminal extension is probably not required for positioning PsaC on the core and is most likely localized on the stromal surface of the PS I reaction center. Residues E27 and D32 are two of the eight residues in the loop that was deleted by Naver et al. (1996), and they suggested that these charged residues may be involved in ion pairing with an arginine residue on the core and face the thylakoid membrane. Our results with the PsaC arginine mutants are in agreement with their observations and point to the domain containing E27 and D32 being in the contact region with the core binding site. However, our observations with the corresponding alanine mutants, E27A and D32A, suggest that the negative charges provided by the side chains at positions 27 and 32 are not required for PsaC binding. It is not known for the mutants E27A and D32A whether the residual negative charge at the other position may be important. Clearly, the construction and examination of the double mutant E27A/D32A could settle this point.

The question of whether F_A or F_B is proximal to F_X and details of forward electron transfer through the centers of the PsaC subunit are presently unresolved. If electron transfer through the PsaC redox centers is serial, then placing center F_B proximal to the core cluster, F_X, is compatible with expectations based upon the redox potentials of the centers (Evans et al., 1974) and the greater extent of photoreduction of F_A over F_B at cryogenic temperatures. On the other hand, reports on the effects of the selective destruction of the center F_B using modifiers such as urea−ferricyanide (Golbeck & Warden, 1982) or mercurials (Kojima et al., 1987; Fujii et al., 1990; Sakurai et al., 1991; He & Malkin, 1994; Jung et al., 1995) indicate that F_A is proximal to F_X. The treatments used to selectively destroy one of the PsaC centers all result in the destruction of F_B, implying that this center may be closer to the reaction center surface facilitating attack by the reagents. The cluster modification experiments also show that F_A can be photoreduced in the absence of F_B, but F_B is required for $NADP^+$ photoreduction (Fujii et al., 1990; He & Malkin, 1994; Jung et al., 1995). In the most recent study, Jung et al. (1995) reported that rebuilding the F_B cluster after destruction resulted in restoration of $NADP^+$ photoreduction and concluded that their data provided circumstantial evidence for placing F_A proximal to F_X. The collective structural information we have presented in this report and elsewhere (Rodday et al., 1994, 1995) is consistent with orientation of PsaC on the reaction center core as shown in Figure 4 with cluster F_B proximal to F_X, but the most definitive evidence may only be forthcoming following increased resolution of the PS I crystal structure itself.

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REFERENCES

Adman, E. T., Sieker, L. C., & Jensen, L. H. (1973) *J. Biol. Chem. 248,* 3987−3996.

Anderson, J. M., & Boardman, N. K. (1966) *Biochim. Biophys. Acta 112,* 403−421.

Biggins, J. (1990) *Biochemistry 29,* 7259−7264.

Biggins, J., Rodday, S. M., & Do, L. T. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp 111−114, Kluwer Academic Publishers, Dordrecht.

Evans, M. C. W., Reeves, S. G., & Cammack, R. (1974) *FEBS Lett. 49,* 111−114.

Fujii, T., Yokayama, E., Inoue, K., & Sakurai, H. (1990) *Biochim. Biophys. Acta 1015,* 41−48.

Fukuyama, K., Nagahara, Y., Tsukihara, T., Katsube, Y., Hase, T., & Matsubara, H. (1988) *J. Mol. Biol. 199*, 183−193.
Golbeck, J. H. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 43*, 293−324.
Golbeck, J. H. (1993) *Curr. Opin. Struct. Biol. 3*, 508−514.
Golbeck, J. H., & Warden, J. T. (1982) *Biochim. Biophys. Acta 681*, 77−84.
Golbeck, J. H., Mehari, T., Parrett, K. G., & Ikegami, I. (1988) *FEBS Lett. 240*, 9−14.
Hallahan, B. J., Purton, S., Ivison, A., Wright, D., & Evans, M. C. W. (1995) *Photosynth. Res. 46*, 257−264.
He, W., & Malkin, R. (1994) *Photosynth. Res. 41*, 381−388.
Herman, P. L., Adiwilaga, K., Golbeck, J. H., & Weeks, D. P. (1994) *Plant Physiol. 104*, 1459−1461.
Hiyama, T., & Ke, B. (1972) *Biochim. Biophys. Acta 267*, 160−171.
Jung, Y.-S., Yu, L., & Golbeck, J. H. (1995) *Photosynth. Res. 46*, 249−255.
Kamlowski, A., van der Est, A., Fromme, P., & Stehlik, D. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp 29−34, Kluwer Academic Publishers, Dordrecht.
Kissinger, C. R., Sieker, L. C., Adman, E. T., & Jensen, L. H. (1991) *J. Mol. Biol. 219*, 693−715.
Kojima, Y., Niinomi, Y., Tsuboi, S., Hiyama, T., & Sakurai, H. (1987) *Bot. Mag., Tokyo 100*, 243−253.
Krauss, N., Hinrichs, W., Witt, I., Fromme, P., Pritzkow, W., Dauter, Z., Betzel, C., Wilson, K. S., Witt, H. T., & Saenger, W. (1993) *Nature 361*, 326−331.
Li, N., Zhao, J., Warren, P. V., Warden, J. T., Bryant, D. A., & Golbeck, J. H. (1991) *Biochemistry 30*, 7863−7872.
Mathis, P., & Sétif, P. (1981) *Isr. J. Chem. 21*, 316−320.
Naver, H., Scott, P. M., Golbeck, J. H., Møller, B. L., & Scheller, H. V. (1996) *J. Biol. Chem. 271*, 8996−9001.
Parrett, K. G., Mehari, T., Warren, P. G., & Golbeck, J. H. (1989) *Biochim. Biophys. Acta 973*, 324−332.
Parrett, K. G., Mehari, T., & Golbeck, J. H. (1990) *Biochim. Biophys. Acta 1015*, 341−352.
Rodday, S. M., Jun, S.-S., & Biggins, J. (1993) *Photosynth. Res. 36*, 1−9.
Rodday, S. M., Schulz, R., McIntosh, L., & Biggins, J. (1994) *Photosynth. Res. 42*, 185−190.
Rodday, S. M., Webber, A. N., Bingham, S. E., & Biggins, J. (1995) *Biochemistry 34*, 6328−6334.
Sakurai, H., Inoue, K., Fujii, T., & Mathis, P. (1991) *Photosynth. Res. 27*, 65−71.
Zhao, J., Warren, P. V., Li, N., Bryant, D. A., & Golbeck, J. H. (1990) *FEBS Lett. 276*, 175−180.
Zhao, J., Li, N., Warren, P. V., Golbeck, J. H., & Bryant, D. A. (1992) *Biochemistry 31*, 5093−5099.

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