Mutational Analysis of Photosystem I Polypeptides

ROLE OF PsAD AND THE LYSYL 106 RESIDUE IN THE REDUCTASE ACTIVITY OF PHOTOSYSTEM I*

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The ADC4 mutant of the cyanobacterium Synechocystis sp. PCC 6803 was studied to determine the structural and functional consequences of the absence of PsAD in photosystem I. Isolated ADC4 membranes were shown to be deficient in ferredoxin-mediated NADP \(^+\) reduction, even though charge separation between P700 and F\(_{A}\)/F\(_{B}\) occurred with high efficiency. Unlike the wild type, F\(_{B}\) became preferentially photoreduced when ADC4 membranes were illuminated at 15 K, and the EPR line shapes were relatively broad. Membrane fragments oriented in two dimensions on thin mylar films showed that the g tensor axes of F\(_{A}\) and F\(_{B}\) were identical in the ADC4 and wild type strains, implying that PsAC is oriented similarly on the reaction center. PsAC and the F\(_{A}\)/F\(_{B}\) iron-sulfur clusters are lost more readily from the ADC4 membranes after treatment with Triton X-100 or chaotropic agents, implying a stabilizing role for PsAD. The specific role of Lys\(_{106}\) of PsAD, which can be cross-linked to Glu\(_{93}\) of ferredoxin (LeLong et al. (1994) J. Biol. Chem. 269, 10034-10039), was probed by site-directed mutagenesis. Chemical cross-linking and protease treatment experiments did not reveal any drastic alterations in the conformation of the mutant PsAD proteins. The EPR spectra of F\(_{A}\) and F\(_{B}\) in membranes of the Lys\(_{106}\) mutants were similar to those of the wild type. Membranes of all Lys\(_{106}\) mutants showed wild type rates of flavodoxin reduction and flavodoxin-mediated NADP \(^+\) reduction, but had 10-54% decrease in the ferredoxin-mediated NADP \(^+\) reduction rates. This implies that Lys\(_{106}\) is a dispensable component of the docking site on the reducing side of photosystem I and an ionic interaction between Lys\(_{106}\) of PsAD and Glu\(_{93}\) of ferredoxin is not essential for electron transfer to ferredoxin. These results demonstrate that PsAD serves distinct roles in modulating the EPR spectral characteristics of F\(_{A}\) and F\(_{B}\), in stabilizing PsAC on the reaction center, and in facilitating ferredoxin-mediated NADP \(^+\) photoreduction on the reducing side of photosystem I.

Photosystem I (PS I)\(^2\) in cyanobacteria and chloroplasts is a multisubunit membrane-protein complex that catalyzes electron transfer from reduced plastocyanin (or cytochrome c\(_{553}\)) to oxidized ferredoxin (or flavodoxin) (see Refs. 1 and 2 for recent reviews). The PsA and PsB subunits form a heterodimeric core that harbors -100 antenna Chl a molecules, the primary electron donor, P700, and a chain of electron acceptors A\(_0\), A\(_1\), and F\(_{A}\)/F\(_{B}\). PsAc, PsD, and PsE are peripheral subunits that are located on the n-side (stromal in chloroplasts and cytoplasmic in cyanobacteria) of the photosynthetic membrane. PsAC binds the terminal electron acceptors, F\(_{A}\) and F\(_{B}\), each a [4Fe-4S] cluster. PsAE may be involved in ferredoxin reduction (3-6) and cyclic electron flow around PS I (7). The nature of interactions between PsAE and ferredoxin are not well understood. The remaining proteins of cyanobacterial PS I are integral membrane proteins. Among them, PsAL is required for the formation of PS I trimers (8, 9). PsAF can be chemically cross-linked in vitro to plastocyanin (10, 11). PsAJ and PsAL may be involved in proper assembly of PsAF and PsAL, respectively (12, 13). PsAM has been implicated in cyclic electron flow around PS I.\(^2\) Role of PsAK has not been identified.

The extrinsic protein, PsD, has two reported functions in the PS I complex of cyanobacteria, algae and higher plants. The first function, deduced from in vitro reconstitution experiments, is to stabilize PsAC on the PS I reaction center (14). When PsAC is rebound to a P700-F\(_{X}\) core in the absence of PsAD, F\(_{B}\) rather than F\(_{A}\) becomes photoreduced at 15 K, and the EPR line widths of F\(_{A}\) and F\(_{B}\) are significantly broader than the wild type. These experiments did not investigate the ability of PsAE to bind independently of PsAD. The second function, inferred from cross-linking studies, is to serve as a “docking” protein to facilitate interaction of soluble ferredoxin with the PS I complex (15-17). Recent cross-linking experiments have shown that Lys\(_{106}\) of PsAD from Synechocystis sp. PCC 6803 can be cross-linked to Glu\(_{93}\) in ferredoxin (18). Therefore these two residues come in physical proximity with each other during at least one stage of electron transfer from PS I to ferredoxin. These results indicate a ferredoxin-docking function of PsAD, but do not illustrate a functional requirement of PsAD for NADP \(^+\) photoreduction. The latter issue was addressed with membranes of a PsAD-less cyanobacterial mutant, where it was shown that ferredoxin-mediated NADP \(^+\) photoreduction was severely inhibited (3). This defect may be due to either the lack of ferredoxin-docking site in the PsAD-less membranes or the absence of PsAD may cause alterations in PsAC and its F\(_{A}/F_{B}\) redox centers. In this paper, we further charac-

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\(^1\) The abbreviations used are: PS I, photosystem I; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Chl, chlorophyll; PCR, polymerase chain reaction; Mops, 4-morpholinepropanesulfonic acid; DCPIP, 2,6-dichlorophenolindophenol; NHS, N-hydroxysuccinimide.

\(^2\) D. A. Bryant, personal communication.
was transformed with the recombinant DNAs containing mutations, and mutant strains were obtained according to the procedure described earlier for the KD1 strain. The presence of mutant genes in the resulting strains was confirmed by amplification and sequencing of psaD.

Isolation of Photosynthetic Membranes and PS I Complexes—Previously published methods were used for culture of Synechoystis sp. PCC 6803 (3), for isolation of thylakoid membrane (8), for isolation of PS I trimers using dodecyl-β-D-maltoside (8), and for purification of photochemically active PS I complexes using Triton X-100 (21).

Oriented Thylakoid Membranes—Membrane fragments were oriented in two dimensions on mylar film using the method described in Ref. 36. The membranes were washed five times with 10 mM Tris, pH 8.3, and suspended with 5 mM Tris, pH 8.3, at a chl concentration of 25 mg/ml. This suspension was spread on mylar strips which had been prewashed with detergent and rinsed with deionized, distilled water. The membranes were slowly dried for 3–4 days in darkness at 4 °C in an inert atmosphere maintained at 90% relative humidity.

Removal of PsaC from Synechoystis sp. PCC 6803 Thylakoid Membranes—Thylakoid membranes (25 μg of Chl in a total of 200 μl volume) of wild type and ADC4 strains of Synechoystis sp. PCC 6803 were incubated with 0.05% Triton X-100, 2 mM NaCl, 2 mM NaBr, and different concentrations of NaI for 15 min at ice temperature. The thylakoids were diluted to 1.2 ml with STN (0.4 M sucrose, 100 mM Tris, pH 8.0, 10 mM NaCl) buffer and centrifuged at 20,000 × g for 30 min. Each pellet was suspended in 500 μl of STN buffer, centrifuged again, and used for Western blotting.

Treatment of PS I Complexes with Thermolysin, Glutaraldehyde, or NHS-biotin—Purified wild type PS I complexes at a concentration of 100 μg of Chl/ml were treated with 10 mM glutaraldehyde (Sigma) in the presence of 10 mM Mops-NaOH, pH 7.0, 0.05% Triton X-100 for 30 min on ice. The cross-linking reactions were quenched with 100 mM glycine. To probe topography of the wild type or mutant psaD, PS I complexes (100 μg Chl/ml) were incubated with 50 μg of thermolysin (Sigma)/mg of Chl and with 5 mM CaCl2 at 37 °C for 30 min. The reactions were terminated with 20 mM EDTA. For NHS-biotinylation, PS I complexes at 100 μg of Chl/ml were incubated with 50 μM NHS-biotin (Sigma), 10 mM Mops, pH 7.0, 0.05% Triton X-100, 0.05% Me2SO for 30 min at 25 °C. The reaction was quenched with 50 mM ammonium bicarbonate, pH 7.8.

Analytical Gel Electrophoresis and Immunodetection—Isolated PS I complexes and thylakoids were solubilized with 1% SDS and 0.1% mercaptoethanol at 25 °C for 1 h. Proteins were fractionated by Tricine-urea-SDS-PAGE (3), and proteins were electrotransferred to Immobilon-P membranes. Immunodetection was performed using enhanced chemiluminescence (Amersham Corp.). In some experiments, the relative amounts of these subunits in the membranes were estimated by laser densitometry. The PsaB antibodies were obtained from J. James Guikema, Kansas State University. The PsaD and PsaC antibodies were generated at the University of Nebraska Polyonal Antibody Core Facility using recombinant overexpressed proteins of Synechoystis sp. PCC 7002. The PsaL, PsaF, and PsaE antibodies were raised against respective proteins from Synechoystis sp. PCC 6803 (12, 22). In biotinylation experiments, Immobilon-P membranes were incubated with an avidin-peroxidase conjugate, and labeled proteins were detected with hydrogen peroxide and 4-chloro-1-naphthol (23).

Rebinding of Recombinant Peripheral PS I Proteins to Thylakoid Membranes or to the P700-Fx Core—Nostoc sp. PCC 8009 PsaD and Synechoystis sp. PCC 7002 PsaE were prepared as described previously (14, 24). For overexpressing PsaD of Synechoystis sp. PCC 6803, the gene was amplified using primers that added Ndel site at the 5′ end of the coding region and EcoRI site at the end of the coding region. The amplified fragment was digested simultaneously with Ndel and EcoRI and cloned into pET22a that had been digested with the same enzymes. The plasmid containing psaD was sequenced to ensure fidelity of Taq polymerase and introduced into BL21(DE3) strain of Escherichia coli. Protein overexpression, isolation of inclusion bodies, and PsaD purification were performed by the same methods that have been used for PsaD from Nostoc sp. PCC 8009 (14). PsaD was rebound to the ADC4 thylakoids membranes by adding concentrated PsaD at a molar ratio of 20:1 with P700 and incubating 60 min at 4 °C. The PsaD-less thylakoids and the PsaD-reconstituted thylakoids were separately incubated with 0.05% Triton X-100 for 5 min at 20 °C and pelleted. The supernatant was concentrated by ultrafiltration and used to resuspend the pellet prior to EPR analysis.

The P700-Fx complex and the P700-Fx core were prepared as described previously (26, 27). Reconstitution of the Fx/Fx iron sulfur clusters and re-binding of PsaC, PsaD, and PsaE to the PS I core were performed at ratios of 15 PsaC:20 PsaE:1 P700-Fx core and 7 PsaC:5

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**FIG. 1. Mutagenesis of the PsD Subunit of Photosystem I.** A, the restriction map of DNA region around psaD in the wild type (WT) strain is shown on the top line. The restriction map of insert in the pCD4 plasmid that was used to generate the ADC4 strain (middle line) and of insert in the pKD1 (bottom line) is also shown. The downward and upward arrowheads represent positions of dinucleotides complimentary to the top and upper strands, respectively. Arrow show location, size, and direction of open reading frames for psaD, KmR (gene for kanamycin resistance), and Cmr (gene for chloramphenical resistance). B, Western blot of the membrane proteins from the wild type (WT), ADC4, and K106K (K106K) strains using anti-PsaD antibody. Thylakoid membranes containing 10 μg of Chl were used for electrophoresis and electrotransfer.
Mutations in the PsaD Subunit of Photosystem I

**TABLE I**

| Number       | Sequence                        | Purpose                          |
|--------------|---------------------------------|----------------------------------|
| PC286        | CGTTAAC(CG/A)(TC/T)(TC/T)TCGTTAAGAAACGCCCATCG | Lys<sup>106</sup> replacement by different amino acids |
| PC313        | CGTTAACCTGTTACGAAAAAGCGCCATCG    | Lys<sup>106</sup> to Arg mutation |

*The mutated nucleotides are in bold.

**TABLE II**

Reductase activity of PS I

| Membranes                        | Flavodoxin reduction | NADP<sup>+</sup> reduction mediated by flavodoxin | Ferredoxin | Flavodoxin | Ferredoxin |
|----------------------------------|----------------------|-----------------------------------------------|------------|------------|------------|
| K106K                            | 1100                 | 588                                           | 658        |            |            |
| ADC4                             | 420                  | 180                                           | 60         |            |            |
| ADC4 reconstituted with overexpressed PsaD of Synechocystis sp. PCC 6803 | 800                  | 423                                           | 470        |            |            |

Psae10 PsaE1 P700-F<sub>2</sub> core according to Ref. 14. Dissolved oxygen in the cuvette, as measured in an oxygen electrode, deceased to near-zero levels shortly after addition of β-mercaptoethanol.

Biochemical and Spectroscopic Assays—Chl a was determined in 80% acetone (25). Protein concentrations were determined using a dye binding method (26) after applying correction factors (0.70 for PsaC, 0.54 for PsaD, and 0.50 for PsaE) determined by quantitative amino acid analysis (14). EPR studies were performed with a Bruker ECS-106 X-band spectrometer. All samples contained 1 mM ascorbate and 0.03 mM DCPIP.

Rates of flavodoxin photoreduction by photosynthetic membranes were measured by monitoring the change in the absorption of flavodoxin at 467 nm under saturating actinic light (3, 27). Rates of flavodoxin- or ferredoxin-mediated NADP<sup>+</sup> photoreduction were measured using ferredoxin:NADPH oxidoreductase (Sigma) and by monitoring the change in the absorption of NADPH at 340 nm (3, 27). Spinach ferredoxin was purchased from Sigma. A strain of E. coli with a plasmid containing the flavodoxin gene of Synechococcus sp. PCC 7002 was provided by Dr. D. Bryant (Pennsylvania State University). The expressed flavodoxin was purified by DEAE-Sepharose and gel filtration chromatography.

**RESULTS**

Targeted mutagenesis was used to generate the ADC4 mutant strain of Synechocystis sp. PCC 6803. This strain lacks PsaD in its membranes, but contains all other PS I subunits, including PsaE and PsaC (3). The wild type thylakoids reduce NADP<sup>+</sup> at the rate of 659 μmol·h<sup>-1</sup>·mg Chl<sup>-1</sup> when ferredoxin is used as the electron acceptor (Table II). The ADC4 thylakoids are severely deficient in reducing NADP<sup>+</sup> using ferredoxin as electron acceptor, showing less than 10% of wild type rates. When overexpressed Synechocystis sp. PCC 6803 PsaD was reconstituted with the PsaD-less thylakoids, 72% of the ferredoxin-mediated NADP<sup>+</sup> reductase activity was restored. These results confirm our previous observation that PsaD is essential for electron transfer using ferredoxin as mediator (3). In vitro reconstitution experiments have shown that PsaD influences the binding of PsaC to the reaction center and the EPR spectral characteristics of the F<sub>A</sub>/F<sub>B</sub> clusters (14). The effect of electron transfer to ferredoxin may result from the changes in PsaC and its redox centers that are caused by the absence of PsaD. We therefore examined PsaC in the PsaD-less mutant strain using biochemical and spectroscopic techniques.

Removal of PsaC from the ADC4 Membranes—Immunoblot analyses indicated that the relative amounts of PsaC in untreated membranes from the wild type and ADC4 strains were equivalent (Fig. 2). PsaC was not removed from either the wild type or ADC4 membranes by 2 M NaCl, but 2 M NaBr removed a small amount of PsaC from the ADC4 membranes. Depending on the presence or absence of PsaD, the stronger chaotrope NaI showed a pronounced differential effect on the removal of PsaC. Hence, PsaC in the ADC4 membranes was more susceptible to removal by chaotropic agents than in the wild type membranes.

When wild type membranes were incubated with 0.05% Triton X-100, PsaC was not significantly extracted, but when the ADC4 membranes were incubated similarly for 10 min, about 50% of the PsaC protein was removed (Fig. 2). EPR studies of a similarly treated sample illuminated during freezing showed no removal of F<sub>A</sub> or F<sub>B</sub> from wild type, but the loss of more than 50% of the F<sub>A</sub> and F<sub>B</sub> acceptors from the ADC4 membranes (data not shown). The concurrent loss of the F<sub>A</sub> and F<sub>B</sub> clusters is consistent with the detergent-mediated removal of the PsaC protein rather than the in situ destruction of the iron-sulfur clusters. When the ADC4 membranes were incubated with Nostoc sp. PCC 8009 PsaD and subsequently treated with 0.05% Triton X-100, there was no loss of F<sub>A</sub> and F<sub>B</sub>. There was no change in the appearance of the EPR spectrum when the concentration of Triton X-100 was increased to 1% (data not shown).

EPR Studies of the F<sub>A</sub> and F<sub>B</sub> Clusters in the PsaD-less Membranes—F<sub>A</sub>, characterized by resonances at g = 2.046, 1.943, and 1.855, is the iron-sulfur center primarily photoreduced when wild type thylakoids are frozen in darkness and illuminated at 15 K (28). Under these conditions, usually less than 20% of F<sub>B</sub>, characterized by resonances at g = 2.066, 1.929, and 1.878, is also photoreduced. As shown in Fig. 3A, however, F<sub>B</sub> is the iron-sulfur center primarily photoreduced when membranes from the ADC4 mutant are frozen in darkness and illuminated at 15 K. There is also a small amount of F<sub>A</sub> photoreduced, as shown by the low-field resonance at g = 2.044 and the mid-field resonance at g = 1.949 (the high-field resonance of F<sub>A</sub> is the shoulder on the broad, asymmetrical resonance centered at g = 1.878). When the ADC4 thylakoids are frozen during illumination, both F<sub>A</sub> and F<sub>B</sub> are photoreduced, but the line widths of the resonances are broader than those in wild type thylakoids (Fig. 3C). The low-field and high-field resonances of F<sub>A</sub> and F<sub>B</sub> are merged in the wild type thylakoids at g = 2.046 and 1.885, but in the ADC4 mutant there is a distinct low-field shoulder at g = 2.062 due to F<sub>B</sub> and a high-field shoulder at g = 1.872 due to F<sub>A</sub>. The mid-field resonances are similar to those of the wild type, but the line widths are not as narrow or as clearly defined.

When recombinant Nostoc sp. PCC 8009 PsaD is added to the ADC4 thylakoids, resonances characteristic of F<sub>B</sub> at g = 2.045, 1.941, and 1.857 predominate when the sample is frozen in darkness and illuminated at 15 K (Fig. 3B). There is also a small amount of F<sub>B</sub> photoreduced, as shown by the resonances at g = 2.066, 1.930, and 1.882. The line widths and g values of the F<sub>A</sub> and F<sub>B</sub> resonances, and the pattern of photoreduction at...
the two noninteracting resonances derived from FA and FB. When the reconstituted PS I complex is frozen in darkness and illuminated at 15 K, both FA and FB are reduced, but the broad line resonance at g = 2.073, 1.931, and 1.884. In the presence of PSaD, the ratio of FA to FB reduced by illumination at 15 K is about 4:1. The line widths and g values of the FA and FB resonances, as well as the pattern of photoreduction at 15 K, are very similar to those observed in wild type Synechocystis sp. Synechococcus sp. PCC 6803 membranes. When the reconstituted sample is illuminated during freezing, the low-field and high-field resonances of FA and FB merge at g = 2.047 and 1.886. When the reconstituted PS I complex is frozen in darkness and illuminated at 15 K, FA is still preferentially reduced, as shown by the characteristic resonances at g = 2.073, 1.930, and 1.875 (Fig. 4A). There is also a small amount of FA photoreduced, as shown by the low-field resonance at g = 2.045 and the mid-field resonance at g = 1.952 (the high-field resonance of FA is most likely the high-field shoulder on the broad, asymmetrical resonance centered at g = 1.875). When the reconstituted PS I complex is frozen during illumination, both FA and FB are reduced, but the broad line widths of the FA and FB resonances are even more apparent (Fig. 4C). Unlike wild type PS I, the complex reconstituted without PSaD shows two noninteracting low-field resonances at g = 2.067 (FA) and g = 2.046 (FB). The slope of the mid-field "derivative" resonance at g = 1.935 is less sharp than for either FA or FB in the wild type membranes and probably results from a merging of the two individual resonances. The high-field resonance at g = 1.883 is broad and is probably an envelope of the two noninteracting resonances derived from FA and FB. These results are similar to those of reconstitution with PSaC alone (not shown; see Ref. 14), and confirm that PSaE cannot substitute for PSaD in stabilizing PSaC on the PS I reaction center core.

When Nostoc sp. PCC 8009 PsAD is added to the PSaC and PSaE-reconstituted PS I complex, resonances characteristic of FsA appear at g = 2.046, 1.943, and 1.857 after illumination at 15 K (Fig. 4B). A small amount of FB is reduced, as shown by the resonances at g = 2.070, 1.931, and 1.884. In the presence of PSaD, the ratio of FA to FB reduced by illumination at 15 K is about 4:1. The line widths and g values of the FA and FB resonances, as well as the pattern of photoreduction at 15 K, are very similar to those observed in wild type Synechocystis sp. Synechococcus sp. PCC 6803 complexes (Fig. 4B). When the reconstituted sample is illuminated during freezing, the FA and FB clusters undergo magnetic interaction, resulting in a set of resonances at g = 2.047, 1.939, 1.918, and 1.884 and thus appear similar to wild type complexes isolated from Synechocystis sp. PCC 6803 thylakoids (Fig. 4D). Oriented Thylakoid Membranes—The three-dimensional structure of PSaC is modeled on Peptococcus aerogenes ferre-
Symmetry. The results are summarized in Table III. The clusters are reoriented precisely 180 degrees about the axis of symmetry which runs through the two iron-sulfur clusters. One explanation for the preferential photoreduction of FB is that PsaC may be bound differently in the absence of PsaD, where the crystal structure shows an approximate 2-fold symmetry. The intensity of FA and FB will not match those of the wild type unless the two isomers of PsaC are fixed in a conformation, orienting the FA and FB clusters in a manner that makes the photoreduction of FA more likely than that of FB.

The absence of PsaD in the ADC4 thylakoids impairs their ability to reduce NADP+ using ferredoxin. It also affects association of PsaC with the PS I core and the EPR spectra of FA and FB clusters. These findings raise the issue that the role of PsaD in ferredoxin-mediated electron transfer is an indirect consequence of its function in stabilizing the association of PsaC with the reaction center. To probe this possibility, we generated site-directed mutants in a residue that has been proposed to interact with ferredoxin: the lysyl 106 residue of PsaD. A PCR-based method was used to replace AAA nucleotide sequence for Lys106 in psaD with various nucleotide combinations (Table IV). The resulting mutations included a conservative replacement (K106R), two potentially disruptive replacements (K106E, K106D), two replacements to polar amino acids (K106S, K106N), and a replacement to glycine, the amino acid without a side chain (K106G).

All psaD mutant strains could grow under photoautotrophic conditions, with doubling times comparable with K106K strain. In contrast, the ADC4 strain grew significantly slower than the wild type and K106K strains. We examined the accumulation of mutant PsAD proteins in the membranes using Western analysis (Fig. 5). On an equal Chl basis, all strains contained similar levels of PsAD in the membranes. Within the PS I complex, PsAD interacts with PsaA-PsaB, PsaE, PsaC, and PsaL. When the accumulation of these PS I subunits was examined by Western analysis, membranes of the mutant and K106K strains contained similar levels of PsaA, PsaB, PsaC, PsaE, and PsaL. Therefore, Lys106 mutations in PsAD do not affect accumulation of PsAD and PsAD-interacting subunits in the membrane.

Table III

| EPR signal | Wild type | ADC4 |
|------------|-----------|------|
| A−         | gₓ 2.05   | 68 ± 5 | 64 ± 5 |
|            | gᵧ 1.94   | 50 ± 5 | 50 ± 5 |
|            | gₒ 1.85   | 52 ± 5 | 51   |
| B−         | gₓ 2.07   | 32 ± 5 | 29 ± 5 |
|            | gᵧ 1.92   | 63a   | 69 ± 10 |
|            | gₒ 1.88   | 77b   | 71b  |
| A−B−       | 2.05      | 60 ± 5 | Not determined |
|            | 1.94      | 48 ± 5 | Not determined |
|            | 1.92      | 10 ± 5 | Not determined |
|            | 1.88      | 90 ± 5 | Not determined |

a Deduced from the equation: \( \cos^2 \alpha + \cos^2 \beta + \cos^2 \gamma = 1 \).

b Corrected value; D. Stehlik, personal communication.

maximum intensity at 50° and then falls away in both directions. It is difficult to determine the angle of \( g_{\gamma} \) experimentally; that angle was calculated to be 51° from the equation, \( \cos^2 \alpha + \cos^2 \beta + \cos^2 \gamma = 1 \), where \( \alpha \) is the angle at which a particular tensor has the maximum intensity in the EPR signal. As for FB, the angles that have maximum intensity in \( g_{\alpha} \) and \( g_{\gamma} \) are 29° and 69°, respectively. Within experimental error, the orientation of principal g tensor axes of \( g_{\alpha} \) and \( g_{\gamma} \) in ADC4 is therefore identical with wild type. It is unlikely that the polypeptide can be reoriented precisely 180 degrees about the symmetry axis simply because the binding site for the PS I core is likely to be located on a unique region of the polypeptide backbone. Hence, this result would not be consistent with a function of PsAD in reorienting PsaC on the reaction center core.

Generation of Lys106 Mutations in PsAD—The absence of PsAD in the ADC4 thylakoids impairs their ability to reduce NADP+ using ferredoxin. It also affects association of PsaC with the PS I core and the EPR spectra of FA and FB clusters.

These findings raise the issue that the role of PsaD in ferredoxin-mediated electron transfer is an indirect consequence of its function in stabilizing the association of PsaC with the reaction center. To probe this possibility, we generated site-directed mutants in a residue that has been proposed to interact with ferredoxin: the lysyl 106 residue of PsaD. A PCR-based method was used to replace AAA nucleotide sequence for Lys106 in psaD with various nucleotide combinations (Table IV). The resulting mutations included a conservative replacement (K106R), two potentially disruptive replacements (K106E, K106D), two replacements to polar amino acids (K106S, K106N), and a replacement to glycine, the amino acid without a side chain (K106G).

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Topography and Subunit Interactions of the Lys106 Mutant PsAD Proteins—Effects of mutations on the topography and
subunit interactions of PsAD were investigated using biochemical techniques. When the wild type PS I complexes were treated with glutaraldehyde, the PsAD antibody recognized products. Therefore, mutations in Lys106 do not affect interaction of PsAD with Psal and Psac/Psae. The PsAD-Psal interaction may be crucial in the formation of PS I trimers; the absence of PsAD affects the ability of PS I to form trimers (8). Upon dodecyl-β-D-maltoside treatment and sucrose gradient ultracentrifugation, approximately 7% Chl in ADC4 thylakoids, but 70% Chl in wild type thylakoids, could be obtained in PS I trimers. None of the Lys106 mutations affected the ability of PS I to form trimers (Table IV), again indicating that PsAD-Psal interactions remain unaffected in these mutants.

To investigate the surface-exposed domains in PsAD, purified PS I complexes from different strains were treated with thermolysin (Fig. 6). Previously, we have reported that incubation of wild type PS I complexes with thermolysin resulted in accumulation of distinct fragments from proteolysis at sites in the C-terminal ~3 kDa of PsAD. Proteolytic patterns of Lys106 mutant proteins could be classified into three types. First, K106R and K106D proteins showed fragments that were similar in size and abundance as the wild type. Second, level of K106E, K106N, K106S, and K106K proteins showed fragments that were similar in size and abundance as the wild type. However, the number and sizes of the fragments that were immunoreactive with anti-PsAD antibody were remarkably similar to those in wild type, suggesting that "interaction of Mutant PsAD Proteins—The consequence of mutations at Lys106 on the photoreduction of the Fs/Fp iron-sulfur clusters were investigated by EPR spectroscopy. The spectra of Fs and Fp in membranes isolated from the K106D and K106E strains are shown in Fig. 8. When thylakoids of two mutants are frozen in darkness and illum..."
nated (Fig. 8, A and C), only one electron is transferred from P700 to the terminal iron-sulfur cluster. As indicated before, about 80% of FA becomes photoreduced with \( g \) values of 2.046, 1.943, and 1.855, and 20% of FB becomes photoreduced with \( g \) values of 2.068, 1.928, and 1.880 in wild type PS I complexes. When the thylakoids of the K106D and K106E strains are illuminated during freezing (Fig. 8, B and D), more than one electron is transferred, resulting in a quantitative reduction of both FA and FB. The \( g \) values of 2.049, 1.940, 1.922, and 1.885 are characteristic of an interaction spectrum of FA and FB and appear identical to the wild type. A similar analysis of the remaining mutants showed identical behavior (data not shown). In summary, the preference for FA reduction over FB and the normal magnetic interaction between the two clusters indicate that mutations at Lys106 do not affect the magnetic properties of the iron-sulfur clusters in PsaC.

Thylakoids from the Lys106 mutant strains were used to estimate the reductase activity of PSI in three different assays: direct measurement of the rates of flavodoxin reduction, NADP\(^+\) reduction mediated by flavodoxin, and NADP\(^+\) reduction mediated by ferredoxin (Table IV). The K106K membranes reduced flavodoxin or NADP\(^+\) mediated by flavodoxin at rates of 940 and 525 \( \mu \)mol/mg of Chl/h, respectively. PsaD-less membranes were able to reduce flavodoxin and NADP\(^+\) via flavodoxin at rates 40–50% that of wild type membranes. All Lys\(^{106}\) mutations showed normal electron transfer rates to flavodoxin. This suggests that although PsaD may enhance the interaction of flavodoxin with PS I, it is not an essential component when flavodoxin functions as an electron acceptor of PS I. These results also show that Lys\(^{106}\) is not involved in flavodoxin-PS I interactions. The K106K membranes reduced NADP\(^+\) at the rate of 490 \( \mu \)mol/mg Chl/h when ferredoxin was used as a mediator of electron transfer to ferredoxin-NADP\(^+\) reductase. The PsaD-less membranes showed a drastic decrease in their ability to reduce NADP\(^+\) via ferredoxin. The replacement of Lys\(^{106}\) in K106R, K106G, K106S, and K106N mutants reduced the rates of electron transfer via ferredoxin by 10–34%. Disruptive replacement of Lys\(^{106}\) by aspartate or glutamate led to 43 and 54% reduction in the ferredoxin-mediated NADP\(^+\) photoreduction rates, respectively, compared with the K106K membranes. These results imply that Lys\(^{106}\) is important, but not essential, for the interaction between PS I and ferredoxin.

**DISCUSSION**

The function of PsaD has been studied previously using chemical cross-linking, biochemical resolution, and reconstitution and targeted mutagenesis of the cyanobacterium Synechocystis sp. PCC 6803. These studies have indicated that PsaD has multiple roles: it can be cross-linked to ferredoxin, its absence in the ADC4 cyanobacterial mutant results in loss of NADP\(^+\) photoreduction, and its absence affects the EPR spectral properties of the FA/FB clusters of PsaC. In the present study, we investigated further the different roles of PsaD and examined whether they were correlated using a mutant of Synechocystis sp. PCC 6803. The PsaD-less mutant organism can synthesize and assemble a PS I reaction center containing PsaC and PsaE (3). This result agrees with in vitro reconstitution experiments, which showed that PsaC was able to bind to a P700-F\(_X\) core in the absence of added PsaD (14). We found that low concentrations of Triton X-100 led to release of PsaC...
from membranes isolated from the PsAD-less strain of Synechocystis sp. PCC 6803. This result is also consistent with those from in vitro reconstitution experiments, where it was shown that Triton X-100 removed a large fraction of the added recombinant PsAC from a P700-Fx core (14). Addition of PsAD to the ADC4-thylakoids (this work) and to the PsAC-reconstituted P700-Fx core (14) led equally to an enhanced resistance of PsAC to Triton X-100 extraction.

Low temperature EPR studies of ADC4 membranes showed that Fx was predominantly reduced at 15 K, and the line widths of the reduced Fx and Fb clusters were broader than to the control. The addition of PsAD to the ADC4 membranes resulted in the photoreduction of Fx when the sample was illuminated 15 K and in a sharpening of the EPR line widths. This is also in close agreement with the in vitro reconstitution experiments (14) and shows that Triton X-100 is not responsible for the altered orientation of added PsAC. The presence of PsAE on the ADC4 membranes indicates that this protein does not require PsAD to assemble on the PsI core. PsAE also does not influence the stability of PsAC or the pattern of photoreduction of FxA or Fx, a result which agrees with in vitro reconstitution experiments performed on a P700-Fx core in the presence of added recombinant PsAE (this study).

The similarity of PsAC to P. aerogenes ferredoxin (30) has led to the prediction that the former contains a 2-fold symmetry axis related to the two iron-sulfur binding sites (31, 32). The altered pattern of photoreduction of FxA and Fb in the ADC4 membranes could be explained if one function of PsAD were to orient PsAC on the reaction center core. In the PsAD-less membranes, PsAC would be misoriented; however, EPR studies on the membrane fragments oriented on the thin mylar films have shown that the orientation of principal g tensors of FxA and Fb in ADC4 membranes are very similar with those of wild type. This makes it unlikely that PsAC is oriented differently in ADC4; the explanation of the altered pattern of photoreduction of FxA and Fb must hence be sought elsewhere.

The interprotein electron transfer on the reducing side of PsI has been studied using spectroscopy, chemical cross-linking, and subunit-deficient cyanobacterial mutants. At least three proteins (PsAC, PsAD, and PsAE) in PsI must interact with ferredoxin for efficient electron transfer to occur (3, 4). Recently, kinetics of reduction of soluble cyanobacterial ferredoxin by cyanoabacterial PsI were investigated by flash absorption spectroscopy (33). This study revealed the existence of three different first order components with 1/2 of 500 ns, 20 μs, and 100 μs. The 500-ns phase corresponds to electron transfer from FxA/Fb to ferredoxin. These analyses also pointed to the presence of at least two types of PsI-ferredoxin complexes, all competent in electron transfer. Ferredoxin accepts electrons from FxA/Fb of PsAC, implying that these proteins should be in intimate contact with each other, yet phylogenetic association between PsAC and ferredoxin has not been demonstrated. The major obstacles in the association of PsAC and ferredoxin are their unfavorable electrostatic interactions; both PsAC and ferredoxin have strongly electronegative surfaces at the physiological pH. Docking proteins may be required to facilitate the interaction by providing clusters of amino acids with opposite charges. PsAD and PsAE may fulfill this role, since they are required for the interaction and reduction of ferredoxin (3, 4).

Current evidence suggests that the interactions between ferredoxin and PsI are electrostatic in nature. Spectroscopic study of ferredoxin reduction suggests that complex formation precedes electron transfer and the rate constants for complex formation depend on ion, especially magnesium, concentrations (33, 36). Lys106 of PsAD and Glu93 of ferredoxin can be cross-linked and thus provide one possible pair for ionic interaction. Changing Lys106 to uncharged amino acids, however, does not hinder ferredoxin-mediated electron transfer. Clearly, an ionic interaction between Lys106 of PsAD and Glu93 of ferredoxin is not essential for docking of ferredoxin on the reaction center. Our results show that one and more of the three lysyl residues (at positions 117, 131, and/or 135) may also be exposed on the reducing side of PsI. These residues along with three arginyl residues in the C-terminal domain of PsAD could also provide ionic interactions during docking of ferredoxin on PsAD. Alternatively, Lys106 may participate in ionic interactions between the wild type PsI and ferredoxin, but its function can be replaced by other surface-exposed lysines. Since Lys106 of PsAD can be cross-linked to Glu93 of ferredoxin (18), these residues come in vicinity of each other. Close proximity between these residues may facilitate docking, as the side-directed replacement of Lys106 with negatively charged amino acids drastically reduces rates of electron transfer to ferredoxin. Interestingly, a nonconservative mutation in Anaebaena ferredoxin at Glu94, equivalent of Glu93 in ferredoxin from Synechocystis sp. PCC 6803, does not affect its interaction with spinach PsI (37).

The absence of PsAD has a profound effect on PsAC and its redox centers, which may be partly responsible for the inability of PsAD-less membranes to carry out ferredoxin-mediated NADP+ reduction. However, phenotypes of Lys106 mutants show that PsAD has a distinct docking function that may be its primary role in ferredoxin-mediated photoreduction. K106D and K106E mutants contain normal amounts of PsAC and their FxA/Fb clusters show EPR spectral characteristics similar to the wild type. These membranes also exhibit wild type rates of flavodoxin reduction, again indicating normal electron transfer within the PsI complex. Yet, membranes of these strains are unable to support wild type rates of NADP+ photoreduction using ferredoxin. Therefore, wild type PsAC and its redox centers are not sufficient for obtaining wild type rates of NADP+ photoreduction. These results demonstrate the requirement of PsAD in ferredoxin-mediated electron transfer and provide a strong functional evidence for a ferredoxin-docking role of PsAD.

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