Plant Physiol. (1997) 115: 1699–1705

The PsaD Subunit of Photosystem I[1]

Mutations in the Basic Domain Reduce the Level of PsaD in the Membranes

Vaishali P. Chitnis, An Ke[2], and Parag R. Chitnis*

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

The PsaD subunit of photosystem I (PSI) is a peripheral protein that provides a docking site for ferredoxin and interacts with the PsaB, PsaC, and PsaL subunits of PSI. We used site-directed mutagenesis to determine the function of a basic region in PsaD of the cyanobacterium *Synechocystis* sp. PCC 6803. We generated five mutant strains in which one or more charged residues were altered. Western blotting showed that replacement of lysine (Lys)-74 with glutamine or glutamic acid led to a substantial decrease in the level of PsaD in the membranes. The mutant PSI complexes showed reduced $NADP^+$ photoreduction activity mediated by ferredoxin; the decrease in activity correlated with the reduced level of PsaD. Using protein synthesis inhibitors we showed that the degradation rates of the mutant and wild-type PsaD were similar, indicating a defect in the assembly of the mutant protein. Treatment of the mutant PSI complexes with a different concentration of NaI showed that the mutations decreased affinity between PsaD and the transmembrane components of PSI. With glutaraldehyde, the mutant and wild-type PsaD proteins could be cross-linked with PsaC, but the PsaD-PsaL cross-linked product was reduced drastically when arginine-72, Lys-74, and Lys-76 were mutated simultaneously. These studies demonstrate that the basic residues in the central region of PsaD, especially Lys-74, are crucial in the assembly of PsaD into the PSI complex.

PSI is a multiheteromeric pigment-protein complex in the thylakoid membranes of cyanobacteria and chloroplasts (Golbeck, 1994; Chitnis, 1996). It catalyzes electron transfer from reduced plastocyanin (or Cyt c_6) to oxidized Fd (or flavodoxin). The PsaA and PsaB subunits form a heterodimeric core that harbors approximately 100 antenna chlorophyll *a* molecules, the primary electron donor, P700, and a chain of electron acceptors, A_0, A_1, and F_X. The PsaC, PsaD, and PsaE subunits of PSI constitute its reducing side, on which Fd can dock and accept electrons. PsaC contains the (4Fe-4S) clusters F_A and F_B, which donate electrons to Fd. PsaD and PsaE facilitate Fd docking, and PsaE may be involved in cyclic electron flow around PSI (Chitnis, 1996).

PsaD is a crucial component on the reducing side of PSI. The insertional inactivation of the *psaD* gene in the PSI complexes of the cyanobacterium *Synechocystis* sp. PCC 6803 indicated that PsaD is essential for efficient function of the cyanobacterial PSI (Chitnis et al., 1989). PsaD has several roles in the function and organization of PSI. First, it interacts with at least three proteins of PSI and stabilizes their organization within the complex. Cross-linking experiments have demonstrated a close association of PsaD with PsaC and PsaL (Xu et al., 1994a; Armbrust et al., 1996; Jansson et al., 1996). Limited proteolysis experiments showed that PsaD shields extramembrane loops of PsaB (Sun et al., 1997b). Second, PsaD influences assembly of PsaC into PSI (Li et al., 1991) and electron paramagnetic resonance properties of the terminal electron donors F_A and F_B (Chitnis et al., 1996; Hanley et al., 1996). Third, PsaD is an essential component of the docking site for Fd. It can be cross-linked to Fd using a hydrophilic, zero-length cross-linker, *N*-ethyl-3(3-dimethylaminopropyl)-carbodiimide (Zanetti and Merati, 1987; Zilber and Malkin, 1988); the cross-linked product is redox-active (Lelong et al., 1996). The Lys-106 residue of PsaD in *Synechocystis* sp. PCC 6803 can be cross-linked with the Glu-93 residue of Fd (Lelong et al., 1994). The PsaD-less mutants of *Synechocystis* sp. PCC 6803 were used to demonstrate the functional significance of PsaD in electron transfer to Fd (Xu et al., 1994c; Hanley et al., 1996). The Lys-106 residue is a dispensable component of the docking site, and an ionic interaction between Lys-106 of PsaD and Glu-93 of Fd is not essential for electron transfer to Fd (Chitnis et al., 1996; Hanley et al., 1996). Analysis of site-directed mutations in PsaD indicated that the roles of PsaD in stabilization of PsaC on the reaction center and in facilitating Fd-mediated $NADP^+$ photoreduction on the reducing side of PSI are independent of each other (Chitnis et al., 1996).

The three-dimensional structure of PsaD is not known. The x-ray crystallographic analysis of PSI at 4Å resolution indicates that PsaD may contain one short α-helix (Krauss et al., 1996). Biochemical and molecular genetic studies were used to identify the domains in PsaD that are exposed on the surface of PSI. Modification of surface residues and limited proteolysis with endoproteinase Glu-C and thermolysin have been used to understand the topography of PsaD (Xu et al., 1994b). These studies indicated that the surface-exposed proteolytic sites and lysyl residues are

[1] This work was supported in part by grants from the National Science Foundation (nos. MCB9696170 and MCB9723001). The Kansas Agricultural Experiment Station supported initial stages of this work. This is journal paper no. J-17,457 of the Iowa Agriculture and Home Economics Experiment Station, Ames, project no. 3416, and was supported by Hatch Act and State of Iowa funds.

[2] Present address: Division of Biology, Kansas State University, Manhattan, KS 66506.

* Corresponding author; e-mail chitnis@iastate.edu; fax 1–515–294–045.

located in the C-terminal domain of PsaD. Examination of the primary sequence of PsaD reveals a basic domain between Arg-72 and Lys-86 in the central region of the protein (Manna and Chitnis, 1997). Although the length and exact sequence of the basic domain vary in different species, it contains four conserved basic residues. One or two additional lysyl or arginyl residues are found at various positions in the basic region. To investigate the role of the basic domain in assembly and function of PsaD, we generated site-directed mutations in two conserved and one variable basic residue in this region and introduced the mutant genes in a PsaD-less strain of *Synechocystis* sp. PCC 6803. Here we present biochemical characterization of the mutant strains.

MATERIALS AND METHODS

PsaD Mutagenesis

All PsaD mutants used in this experiment were generated by a PCR-mediated mutation technique (Higuchi, 1989). The detailed strategy for PsaD mutagenesis was described previously (Chitnis et al., 1996). A degenerate oligonucleotide was used for replacing the residues in the basic domain. The sequence of the mutagenic primer was T(TGC) GGG TT(TGC) AAA TT(TGC) TGT (CG)(CT)(CG) TAG CTG G. The *psaD* gene was amplified by PCR using the mutagenic primer and cloned into an appropriate vector (Chitnis et al., 1996). The amplified regions in 20 DNA clones were sequenced completely to identify sequence alterations. Although the primer was designed to make one or more replacements in Arg-72, Lys-74, Lys-76, and Lys-78, none of the sequenced DNAs contained mutations that altered Lys-78. In one case, an additional residue, Glu-62, was mutated, probably because of an error by *Taq* DNA polymerase. Five DNAs with representative mutations were selected for further studies (Table I). The natural transformation was used to introduce the mutated DNAs into the PsaD-less ADC4 strain of *Synechocystis* sp. PCC 6803 (Xu et al., 1994c). After several generations of segregation on BG-11 plates with 30 μg/mL kanamycin, stable mutant strains were generated. We extracted the genomic DNA from the mutant strains, amplified the mutant *psaD* genes by PCR, and confirmed the appropriate nucleotide changes in the genome by DNA sequencing. The KD1 strain contains the unaltered *psaD* gene introduced back into the ADC4 strain and was used in these studies as the wild type.

Characterization of Mutants

The strains were grown aerobically in BG-11 medium under 21 μmol m^{-2} s^{-1} light for isolation of membranes and PSI complexes. Previously published methods were used for isolation of thylakoid membranes (Chitnis and Chitnis, 1993), purification of PSI complexes using Triton X-100 (Chitnis et al., 1993), isolation of monomeric PSI complexes with *n*-dodecyl β-maltoside (Chitnis and Chitnis, 1993), treatment of membranes with NaI (Xu et al., 1994e), cross-linking of PSI proteins with glutaraldehyde (Xu et al., 1994a), and analytical PAGE and immunodetection (Xu et al., 1994c). The reductase activity of the mutants was determined using an Fd-mediated $NADP^+$ photoreduction assay (Jung et al., 1995). The reaction mixture contained thylakoid membranes (5 μg chlorophyll/mL), 5 μM Fd, 50 mM Tricine, pH 8.0, 10 mM $MgCl_2$ 15 μM Cyt c_6, 5 mM sodium ascorbate, 0.05% *n*-dodecyl β-maltoside, 0.5 mM $NADP^+$, and 0.8 μM Fd:$NADP^+$ oxidoreductase. The changes in the absorption of NADPH at 340 nm were monitored using a 160A spectrophotometer (Shimadzu, Tokyo, Japan) fitted with red-light-emitting diodes (LS1, Hansatech, Norfolk, UK) for sample illumination. To examine protein turnover, we grew cells for 6, 11, and 24 h at 30°C in BG11 medium that was supplemented with two protein-synthesis inhibitors, streptomycin and tetracycline, each at 80 μg/mL. Membranes were prepared and relative levels of PSI proteins was determined by western blotting. The anti-PsaD and anti-PsaC antibodies were kindly provided by Dr. John H. Golbeck (Pennsylvania State University, State College, PA). Other antibodies were raised against respective proteins from *Synechocystis* sp. PCC 6803 (Sun et al., 1997a). Cyt c_6 was purified from *Microcystis aeruginosa* according to published procedures (Gomez Lojero and Krogmann, 1996). Fd was overproduced in *Escherichia coli* from a gene of *Synechocystis* sp. PCC 6803 and was purified by previously published methods (Xu et al., 1995b).

RESULTS

Function of PSI in the Mutant Strains

To study the physiological impact of mutations, we estimated the doubling time of the mutant strains under photoautotrophic growth conditions. The growth of cultures in BG11 medium was monitored from the changes at A_{730}. All strains contained similar amounts of chlorophyll (approximately 4 μg) per unit at A_{730}. Consistent with a previous report (Xu et al., 1994c), the doubling time for the PsaD-less mutant was longer than that for the KD1 strain (Table I). The strains with site-directed mutations grew at approximately the same rate as the wild type. PsaD functions as an essential docking site for Fd. Consequently, Fd-mediated electron transfer is reduced drastically in the PsaD-less PSI complexes (Chitnis et al., 1996). When we determined Fd-mediated $NADP^+$ photoreduction as a measure of the reductase activity of PSI, the rate in the wild-type membranes was 285 μmol mg^{-1} chlorophyll h^{-1}, whereas the activity in the PsaD-less membranes was barely detected (Table I). The mutations reduced the reductase activity by 26 to 81% when compared with the wild-type membranes. The thylakoids of strain 7 showed 19% of the wild-type activity, whereas the membranes of strain 3 had 74% of the normal activity. Other mutations had intermediate activities. Despite the decreased Fd-mediated $NADP^+$ reduction activity, the growth rates were not significantly affected by the mutations. This could be due to the presence of flavodoxin in cells during growth conditions. Traces of flavodoxin can be recovered from the cells grown under normal conditions and the amount of fla-

Table I. *Effects of mutations on properties and function of PsaD*

Strain	Mutation(s)	Predicted Protein Features		Doubling Time	$NADP^+$ Photoreduction Rate
		Mass	pI		
		D		*h*	*μmol mg^{-1} chlorophyll h^{-1}*
KD1	Wild type	15,626	9.38	16	285
3	K74Q	15,744	9.06	15	210
5	E62V, K74E, K76E	15,716	7.17	18	60
7	R72W, K74E	15,775	7.17	18	55
13	R72W	15,774	9.06	16	215
19	R72W, K74Q, K76E	15,775	5.82	15	195
ADC4	PsaD-less strain			28	15

vodoxin dramatically increases under iron-deficient growth conditions (Bottin and Lagoutte, 1992). Alternatively, the normal growth rates in the mutants indicated that PSI activity is not a limiting factor for autotrophic growth.

Accumulation of PSI Proteins in the Mutant Strains

The effects of mutations on PSI function could result from a defect in Fd docking or from decreased accumulation of mutant proteins in the PSI complexes. To examine these possibilities, relative levels of PsaD and other PSI proteins in thylakoids and in purified PSI complexes were estimated by western analyses (Fig. 1). The epitopes for the anti-PsaD polyclonal antibody are located in the region between Glu-93 and the carboxyl terminus (Xu et al., 1994b). Therefore, mutations in the basic domain between Arg-72 and Lys-86 are not expected to alter the affinity of this antibody for PsaD. In addition, when the protein subunits of the purified PSI complexes were resolved by electrophoresis and the gel was stained with Coomassie blue, the band intensity of PsaD correlated with the corresponding signal in western blotting (data not shown). Thus, immunodetection was expected to provide reliable estimates of the relative levels for PsaD.

Western analysis of thylakoid proteins was performed on an equal chlorophyll basis. The level of PsaD in mutant strains 5 and 7 was considerably lower than in the wild-type strain (Fig. 1). From densitometry, we estimated that these strains contained approximately 25% of the wild-type level of PsaD. In contrast, thylakoids of the mutant strain 19 had approximately 65% of the wild-type level of PsaD. Other mutants had intermediate levels of PsaD in their thylakoid membranes. Mutations in the surface-exposed residues, such as Lys-106, do not affect the steady-state level of PsaD in membranes (Chitnis et al., 1996). Mutant strain 19 contained a reduced level of PsaK. Since the PsaD-PsaK interaction has not been demonstrated or anticipated, we do not know the significance of this observation. Mutations in PsaD did not have a major influence on the accumulation of other PSI subunits in thylakoid membranes (Fig. 1). When the monomeric PSI complexes that had been purified in the presence of Triton X-100 were

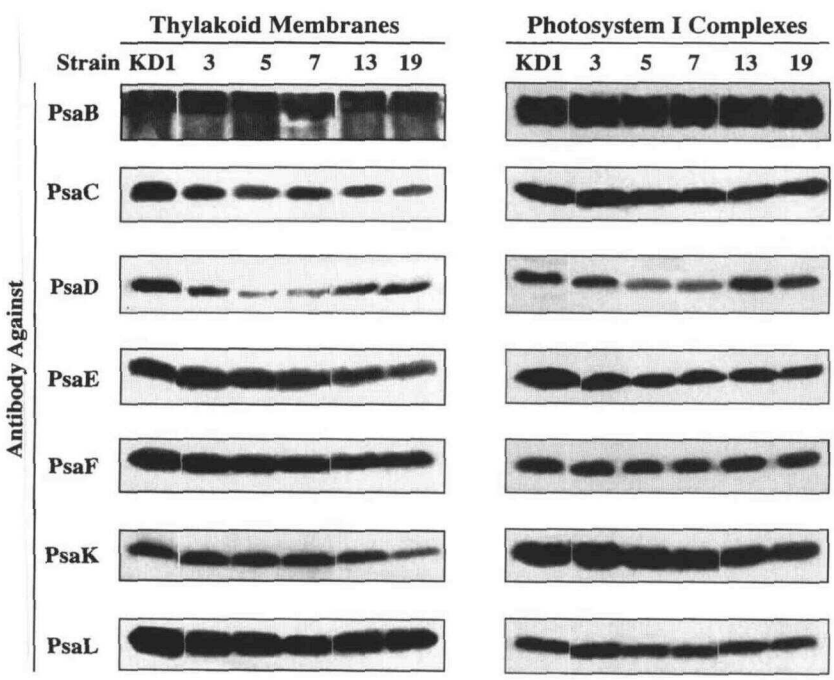

Figure 1. Western analyses of PSI subunits in the thylakoid membranes and purified PSI complexes. The thylakoids and PSI complexes were purified from the PsaD-less (ADC4), the wild-type (KD1), and the PsaD mutant strains. The membranes and PSI complexes (containing 10 μg of chlorophyll) were solubilized and resolved by Tricine/urea/SDS-PAGE. The separated proteins were transferred to an Immobilon-P membrane (Millipore) and probed with several antibodies. The immunoreaction was visualized by enhanced chemiluminescence.

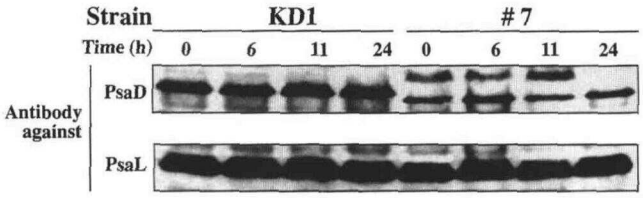

Figure 2. Levels of PsaD and PsaL after treatment with protein synthesis inhibitors. The cells of the wild type and mutant 7 strains (#7) in the wild-type and DE strains were grown at 38°C in the presence of the protein-synthesis inhibitors streptomycin and tetracycline at a final concentration of 80 μg/ml each. The cells were harvested at 6, 11, and 24 h after initiation of the experiment. Subsequently, the photosynthetic membranes were isolated. The proteins in the preparations containing 10 μg of chlorophyll were separated on Tricine-SDS-urea-PAGE, transferred to Immobilon-P membranes, and probed with anti-PsaD and anti-PsaL antibodies. The immunoreaction was visualized by enhanced chemiluminescence.

used for western blotting, we observed similar trends in accumulation of PsaD (Fig. 2). The PSI complexes of mutant strains 5 and 7 contained the least amount of PsaD among the PSI complexes used in this experiment. In addition, the level of PsaL in these complexes was reduced; a similar decrease in accumulation of PsaL has been reported for the PsaD-less strain of *Synechocystis* sp. PCC 6803 (Xu et al., 1994a). It is interesting that PsaD of mutant strains 3, 5, and 7 migrated slightly faster than the wild-type PsaD protein (Fig. 1). The changes in charges on the protein might affect SDS binding and thus alter migration during electrophoresis. Thus, the site-directed mutations in the basic region reduced the abundance of PsaD in the PSI complexes and in the thylakoid membranes. The decreased levels of PsaD might have caused a decrease in the Fd-mediated electron transfer activity of the mutant PSI complexes.

Stability of PsaD in Mutant Strain 7

The reduced level of PsaD in the mutant strains could be due to the decreased expression of the mutant genes, impaired assembly of mutant proteins into membranes, or increased turnover of mutant proteins. Although we cannot rule out effects of mutations on transcription, translation, or RNA stability, these possibilities are less likely than the effects on assembly or degradation. The mutations caused relatively minor changes in the nucleotide sequence and are more than 200 nucleotides downstream from the regions involved in transcription of the *psaD* gene or translation of its mRNA. To test the effects of mutations on PsaD turnover, we grew the KD1 and strain-7 cells in the presence of antibiotics that inhibited protein synthesis. We monitored levels of PsaD and PsaL in thylakoid membranes by western blotting (Fig. 2). The wild-type PSI complexes and its protein constituents are turned over very slowly (Chitnis and Nelson, 1992b). Cellular chlorophyll content, PSI activity, or level of the PSI proteins is not altered for up to 24 h of antibiotic treatment (data not shown). In contrast, the level of the D1 protein of PSII rapidly decreased under these conditions. The relative amounts of PsaD and PsaL did not decrease significantly after up to 24 h of treatment with antibiotics in the mutant and KD1 strains. Variation in the immunodetection intensity was within the error of these analyses. Therefore, the mutation in strain 7 does not cause increased degradation of PsaD that has been assembled in the complexes. When we used cytoplasmic fractions in western analysis, we failed to detect soluble PsaD protein. It is likely that the unassembled PsaD is rapidly degraded by the cytoplasmic proteases. Overall, our results imply that the mutation in strain 7 affects assembly of PsaD into PSI.

Treatment of Membranes with NaI

To study the defect in assembly of the mutant PsaD proteins, we examined interaction of PsaD with other PSI proteins. Resistance of PSI subunits to chaotropic treatments has been used to assess the relative strength of their interactions within the PSI complexes (Xu et al., 1994d, 1994e; Chitnis et al., 1996). When thylakoid membranes of the wild-type and PsaD mutants were incubated with 0, 1, 2, 3, and 4 M NaI, their subunits differed in their susceptibility to removal by NaI (Fig. 3). PsaC and PsaE in the PsaD-less ADC4 strain were more susceptible to removal by NaI treatment than in the wild type (KD1) or in mutant strain 7. One mole of NaI could remove PsaC and PsaE subunits from the thylakoid membranes from ADC4. To remove PsaC and PsaE from the thylakoids of the KD1 strain, 3 M NaI or more was needed. Treatment with 2 M NaI could remove PsaD from the thylakoid membranes of mutant strain 7, but 3 M NaI was needed to remove PsaC, showing different effects of the PsaD mutations on these subunits. The results of NaI treatment demonstrated that

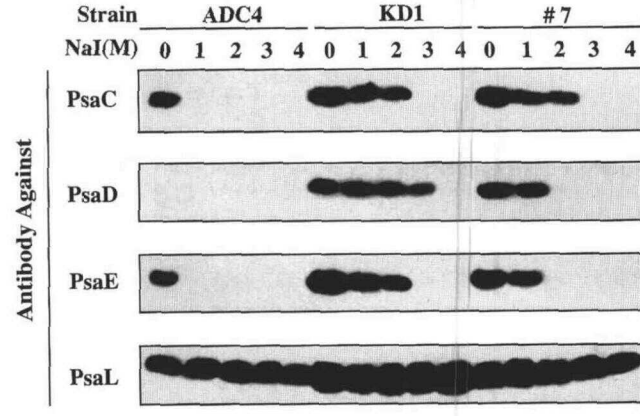

Figure 3. Removal of peripheral proteins of PSI by NaI treatment. Relative contents of the PsaC, PsaD, PsaE, and PsaL subunits in the thylakoid membranes purified from the PsaD-less (ADC4), the wild-type (KD1), and a PsaD mutant (#7) strain after treatment with NaI. The membranes were incubated with 0, 1, 2, 3, or 4 M NaI for 15 min on ice. Samples containing 10 μg of chlorophyll were solubilized and resolved by Tricine/urea/SDS-PAGE. Separated proteins were transferred to an Immobilon-P membrane and probed with different antibodies. The immunoreaction was visualized by enhanced chemiluminescence. For immunodetection of PsaD in membranes of mutant strain 7, samples with 25 μg of chlorophyll were used in each lane and the blots were exposed longer to the x-ray film.

the PsaE subunit in mutant strain 7 could be removed from PSI by a lower concentration (2 M) of NaI, compared with that of the wild type (Fig. 3). The easy removal of PsaE from the mutant membranes could result from the decreased amount of PsaD in them. The requirement of PsaD for stable assembly of PsaE has been demonstrated by in vitro reconstitution (Chitnis and Nelson, 1992a). In contrast to the peripheral proteins, PsaL, an integral membrane protein, could not be dissociated from the wild type or mutant PSI complexes by up to 4 M NaI (Fig. 3). Therefore, one or more mutations in mutant strain 7 decreased the strength of interactions between PsaD and the remaining PSI.

Chemical Cross-Linking

Chemical cross-linking was used to investigate subunit interactions in the mutant PSI complexes. Glutaraldehyde is a bifunctional cross-linking reagent that reacts primarily with amino groups of lysyl residues (McIntosh, 1992). Previous experiments have shown that PsaD can be cross-linked with PsaC and PsaL when PSI of *Synechocystis* sp. PCC 6803 is treated with glutaraldehyde (Xu et al., 1994a; Armbrust et al., 1996). We purified the PSI complexes from the KD1 and mutant membranes with Triton X-100, treated them with glutaraldehyde, and identified cross-linked products by western analysis with anti-PsaD antibody (Fig. 4). Upon cross-linking, PsaD migrated slower than the non-cross-linked PsaD, possibly due to the chemical linkage of gluteraldehyde molecules to the protein. In addition, the wild-type PSI complexes contained 25- and 29-kD cross-linked proteins that were recognized by the PsaD antibody. The 25- and 29-kD products have previously been identified as the PsaD-PsaC and PsaD-PsaL cross-linked proteins, respectively (Xu et al., 1994a; Armbrust et al., 1996). Both cross-linked pairs could be detected when the complexes from mutant strains 3, 7, and 12 were used. However, the 29-kD product was detected at a drastically reduced level in the PSI complexes of mutant strain 19 that had been treated with glutaraldehyde. It is possible that Triton X-100 disturbs PsaD-PsaL interactions more easily in the mutant than in the wild type. To test this possibility, we purified monomeric PSI complexes using *n*-dodecyl β-maltoside from the KD1 mutant and mutant strain 19 and used them for cross-linking experiments. The 29-kD cross-linked product was barely detected in mutant strain 19 but could be observed in the wild-type complexes upon treatment with glutaraldehyde. In contrast, the 25-kD product was detected at comparable levels in the wild type and the mutant strain (Fig. 4). Therefore, the mutations in strain 19 affect the PsaD-PsaL interaction but not the PsaD-PsaC interaction.

DISCUSSION

The PsaD subunit is a 16-kD peripheral protein on the reducing side of PSI. It interacts with PsaB, PsaC, PsaL, and Fd (Chitnis, 1996). These interactions are specific and crucial for structural organization and function of the complex. Consequently, the primary sequence of PsaD contains information for the molecular recognitions between PsaD and its interacting partners. Analyses of the primary structure of PsaD subunits from different sources have indicated the presence of α-helices and a central domain that contains four to six basic amino acids among 15 residues (Fig. 5). Limited proteolysis experiments, chemical cross-linking, modification of surface-exposed ε-amino groups, and deletion mutations in PsaD have shown that several residues in the C-terminal half of PsaD are on the surface of PSI (Lelong et al., 1994; Xu et al., 1994b; Chitnis et al., 1995). The present study is focused on the elucidation of the function of the basic domain in PsaD.

Electrostatic interactions are important in the assembly and function of PsaD. The precursor of PsaD assembles into the PSI complexes without the aid of any molecular chaperones (Chitnis and Nelson, 1992a), and ionic strength affects the PsaD assembly into thylakoid membranes (Minai et al., 1996). Similarly, electrostatic interactions are crucial in Fd-PSI electron transfer (Hervas et al., 1992; Setif and Bottin, 1994). The wild-type PsaD is a basic protein with a deduced pI of 9.38, whereas the mutant strains 5 and 7 have a pI of 7.17 (Table I). These mutants also had less

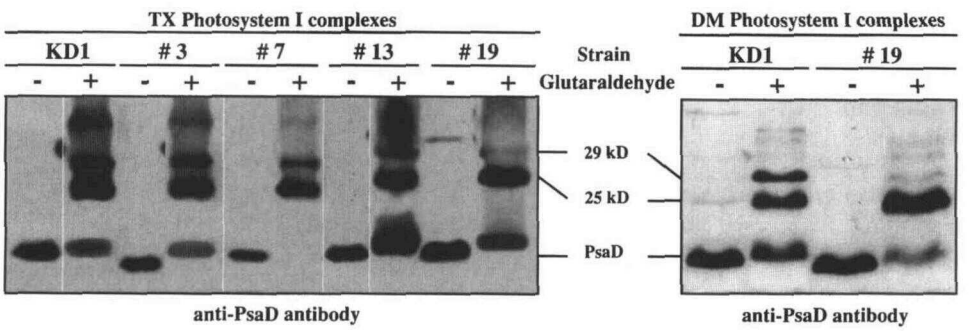

Figure 4. Chemical cross-linking of protein subunits of the purified PSI complexes. The PSI complexes of the wild-type (KD1) and the mutant strains were prepared in Triton X-100 (TX PSI complexes). The monomeric PSI complexes of the wild type (KD1) and the mutant 19 strains (#19) were isolated with *n*-dodecyl β-maltoside (DM PSI complexes). The PSI complexes were incubated with (+) or without (-) 10 mM glutaraldehyde for 30 min on ice, and the reactions were terminated by adding 100 mM Gly. The control (-) and cross-linked (+) samples (10 μg of chlorophyll/lane) were analyzed by Tricine/urea/SDS-PAGE and western blotting with anti-PsaD antibody.

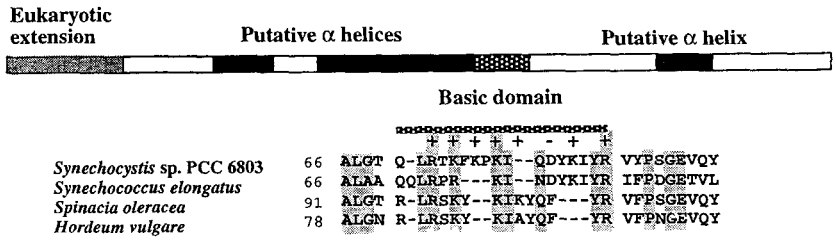

Figure 5. The basic domain in PsaD. Predicted structural features and a comparison of selected sequences of the basic domain are shown. Although two to three regions of PsaD are predicted to form α-helices, only one α-helix has been assigned to PsaD in the 4 Å structure of PSI (Krauss et al., 1996). The conserved residues in the basic domain are shaded. The numbers indicate positions of the first amino acid in the sequence shown in the figure.

PsaD in the thylakoid membranes than the wild type (Fig. 1). However, assembly of mutant strain 19, which showed the greatest change in pI value of PsaD, was not affected as much as in mutant strains 5 and 7. Therefore, the regional charge distribution may be more important than the overall electrostatic properties in the assembly and accumulation of PsaD into PSI.

To identify which of the four mutated residues have a crucial role(s) in PsaD assembly, we compared the mutations and their phenotypes. Examination of mutations in the mutant strains 5 and 7 reveals a common replacement; the Lys-74 residue changed to Glu. We propose that the replacement of Lys-74 by an oppositely charged residue caused decreased accumulation of the mutated PsaD in membranes. The single mutant of Lys-74 to an uncharged polar amino acid (Gln) in mutant strain 3 impaired PsaD assembly into PSI to a lesser extent. The substitution of Arg-72 by Trp in mutant strain 13 (the same as another residue change in strain 7) did not show a drastic effect on the amount of PsaD in the membranes. Mutant strain 19 had three residues altered; its membranes had significantly more PsaD than mutant strain 5. This mutant had an identical replacement (K76E) as one of the mutations in mutant strain 5, and its other residue substitution (R72W) was also present in mutant strain 7. The third residue change in strain 19 was Lys-74 replaced by Gln (the same as that in mutant strain 3) instead of by Glu, as that in strains 5 and 7. Therefore, we can eliminate the effect of the E62V mutation on the phenotype in strain 5. It can be concluded that the Lys-74 residue is important for PsaD to assemble into PSI complexes, considering the phenotypes of these mutant strains.

Replacement of Lys-74 by an acidic residue may alter folding of PsaD and, thus, impair assembly. Alternatively, the mutation may lessen the affinity of PsaD to its interacting subunits in the PSI complex. NaI extraction removed the mutant PsaD in strain 7 more easily than in the wild type (Fig. 3). To remove the PsaD subunit from wild-type membranes, 4 M NaI was needed, but only 2 M NaI could remove the mutated PsaD from thylakoid membranes. This result indicates that the mutant PsaD subunit has a lower affinity to the PSI complexes. PsaD interacts with PsaC, PsaL, and the extramembrane loops of PsaB (Xu et al., 1994a, 1995a; Armbrust et al., 1996; Jansson et al., 1996; Sun et al., 1997b). Among these interactions, we propose that PsaD-PsaC interactions are not altered by the mutations based on the following results. First, complete removal of PsaC from the membranes of both KD1 and the mutant required 3 M NaI, (Fig. 3). Second, when we used the wild-type and mutant PSI complexes for cross-linking experiments, the 25-kD PsaD-PsaC cross-linked product was observed at substantial levels (Fig. 4). Third, the PsaC proteins from various sources show extremely high conservation, with approximately 95% sequence identities. The protein regions that interact with PsaC are expected to be highly conserved. Although the basic region of PsaD is conserved in the charged character, the primary sequence shows considerable variation (Fig. 5). Therefore, the results from chaotropic extraction and chemical cross-linking, and consideration of primary sequence conservation indicate that the interaction between PsaC and the mutant PsaD proteins are not altered.

Our results indicate that the interaction between PsaD and PsaL are disturbed by mutations in the basic region. When two types of PSI complexes of mutant strain 19 were used for cross-linking, the 29-kD PsaD-PsaL product was reduced substantially in comparison with the wild type (Fig. 4). The ϵ-amino residue of Lys-41 and the N terminus of PsaL are the most likely candidates for cross-linking reaction with PsaD (Xu et al., 1994a). Since the PsaD-PsaL interaction was not completely abolished, we believe that the mutated residues in PsaD may not be involved directly in cross-linking, but may provide electrostatic interactions to position PsaD and PsaL in close proximity. Mutant strain 19 contains a replacement of the Lys-76 residue that is not altered in mutant strains 3, 7, and 12. Thus, Lys-76 may be crucial in PsaD-PsaL interactions, although this residue is less important than Lys-74 in maintaining normal steady-state levels of PsaD. The amino-terminal extramembrane region of PsaL and the basic domain of PsaD contain two acidic and four basic residues, respectively, that are found in all available deduced primary sequences. Yet, the remaining primary sequences of these regions are not highly conserved (Manna and Chitnis, 1997). We could not examine the interaction of mutant PsaD with PsaB; the stromal extramembrane loops are highly conserved and may contain acidic residues that interact with the residues of the basic region in PsaD. Thus, our results indicate that substitution of Lys-74 and/or Lys-76 of PsaD by negatively charged residues significantly affects its interactions with the transmembrane subunits of PSI.

In summary, the present study shows that the basic domain of PsaD, especially its Lys-74 residue, is crucial for in vivo accumulation of PsaD. This region may interact with the acidic residues in the amino-terminal region of PsaL or the stromal extramembrane loops of PsaB.

ACKNOWLEDGMENTS

We thank Dr. David Krogmann (Purdue University, West Lafayette, IN) for the gift of Cyt c_6 and for critically reading the manuscript. We also acknowledge Dr. John Golbeck for anti-PsaD and anti-PsaC antibodies.

Received June 23, 1997; accepted September 4, 1997.

LITERATURE CITED

Armbrust TS, Chitnis PR, Guikema JA (1996) Organization of photosystem I polypeptides examined by chemical cross-linking. Plant Physiol **111:** 1307–1312

Bottin H, Lagoutte B (1992) Ferredoxin and flavodoxin from the cyanobacterium Synechocystis sp PCC 6803. Biochim Biophys Acta **1101:** 48–56

Chitnis PR (1996) Photosystem I. Plant Physiol **111:** 661–669

Chitnis PR, Chitnis VP, Xu Q, Jung Y-S, Yu L, Golbeck JH (1995) Mutational analysis of photosystem I polypeptides. *In* P Mathis, ed, Photosynthesis: from Light to Biosphere. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 17–22

Chitnis PR, Nelson N (1992a) Assembly of two subunits of the cyanobacterial photosystem I on the *n*-side of thylakoid membranes. Plant Physiol **99:** 239–246

Chitnis PR, Nelson N (1992b) Biogenesis of photosystem I: the subunit PsaE is important for the stability of PS I complex. *In* J Argyroudi-Akoyunoglou, ed, Chloroplast Biogenesis. Plenum Press, New York, pp 285–290

Chitnis PR, Reilly PA, Nelson N (1989) Insertional inactivation of the gene encoding subunit II of photosystem I from the cyanobacterium *Synechocystis* sp. PCC 6803. J Biol Chem **264:** 18381–18385

Chitnis VP, Chitnis PR (1993) PsaL subunit is required for the formation of photosystem I trimers in the cyanobacterium *Synechocystis* sp. PCC 6803. FEBS Lett **336:** 330–334

Chitnis VP, Jung Y-S, Albee L, Golbeck JH, Chitnis PR (1996) Mutational analysis of photosystem I polypeptides: role of PsaD and the lysyl 106 residue in the reductase activity of photosystem I. J Biol Chem **271:** 11772–11780

Chitnis VP, Xu Q, Yu L, Golbeck JH, Nakamoto H, Xie DL, Chitnis PR (1993) Targeted inactivation of the gene *psa*L encoding a subunit of photosystem I of the cyanobacterium *Synechocystis* sp. PCC 6803. J Biol Chem **268:** 11678–11684

Golbeck JH (1994) Photosystem I in cyanobacteria. *In* DA Bryant, ed, The Molecular Biology of Cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 179–220

Gomez Lojero C, Krogmann DW (1996) Large scale preparations of photosynthetic catalysts from cyanobacteria. Photosynth Res **47:** 293–299

Hanley J, Setif P, Bottin H, Lagoutte B (1996) Mutagenesis of photosystem I in the region of the ferredoxin cross-linking site: modifications of positively charged amino acids. Biochemistry **35:** 8563–8571

Hervas M, Navarro J, Tollin G (1992) A laser-flash spectroscopy study of the kinetics of electron transfer from spinach photosystem I to spinach and algal ferredoxins. Photochem Photobiol **56:** 319–324

Higuchi R (1989) Using PCR to engineer DNA. *In* HA Erlich, ed, PCR Technology. Stockton Press, New York, pp 61–70

Jansson S, Anderson B, Scheller HV (1996) Nearest-neighbor analysis of higher plant photosystem I holocomplex. Plant Physiol **112:** 409–420

Jung Y-S, Yu L, Golbeck JH (1995) Reconstitution of iron-sulfur center F_B results in complete restoration of $NADP^+$ photoreduction in Hg-treated photosystem I complexes from *Synechococcus* sp. PCC 6301. Photosynth Res **46:** 249–255

Krauss N, Schubert W-D, Klukas O, Fromme P, Witt HT, Saenger W (1996) Photosystem I at 4 Å resolution represents the first structural model of a joint photosynthetic reaction centre and core antenna system. Nature Struct Biol **3:** 965–973

Lelong C, Boekema E, Kruip J, Bottin H, Rogner M, Setif P (1996) Characterization of a redox-active cross-linked complex between cyanobacterial photosystem I and soluble ferredoxin. EMBO J **15:** 2160–2168

Lelong C, Setif P, Lagoutte B, Bottin H (1994) Identification of the amino acids involved in the functional interaction between photosystem I and ferredoxin from *Synechocystis* sp. PCC 6803 by chemical cross-linking. J Biol Chem **269:** 10034–10039

Li N, Zhao J, Warren PV, Warden JT, Bryant DA, Golbeck JH (1991) PsaD is required for the stable binding of PsaC to the photosystem I core protein of *Synechococcus* sp. PCC 6301. Biochemistry **30:** 7863–7872

Manna P, Chitnis PR (1997) Function and molecular genetics of photosystem I. *In* GS Singhal, G Renger, SK Sapory, eds, Concepts in Photobiology: Photosynthesis and Photomorphogenesis. Kluwer Academic Publishers, Dordrecht, The Netherlands (in press)

McIntosh DB (1992) Glutaraldehyde cross-links Lys-492 and Arg-678 at the active site of sarcoplasmic reticulum Ca^{2+}-ATPase. J Biol Chem **31:** 22328–22335

Minai L, Cohen Y, Chitnis PR, Nechushtai R (1996) The precursor of PsaD assembles into the photosystem I complex in two steps. Proc Natl Acad Sci USA **93:** 6338–6342

Setif PQ, Bottin H (1994) Laser flash absorption spectroscopy study of ferredoxin reduction by photosystem I in *Synechocystis* sp. PCC 6803: evidence for submicrosecond and microsecond kinetics. Biochemistry **33:** 8495–8504

Sun J, Ke A, Jin P, Chitnis VP, Chitnis PR (1997a) Isolation and functional study of photosystem I subunits in the cyanobacterium *Synechocystis* sp. PCC 6803. Methods Enzymol (in press)

Sun J, Xu Q, Chitnis VP, Jin P, Chitnis PR (1997b) Topography of the photosystem I core proteins of the cyanobacterium *Synechocystis* sp. PCC 6803. J Biol Chem **272:** 21793–21802

Xu Q, Armbrust TS, Guikema JA, Chitnis PR (1994a) Organization of photosystem I polypeptides. A structural interaction between PsaD and PsaL subunits. Plant Physiol **106:** 1057–1063

Xu Q, Chitnis VP, Ke A, Chitnis PR (1995a) Structural Organization of Photosystem I. *In* P Mathis, ed, Photosynthesis: From Light to Biosphere. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 87–90

Xu Q, Guikema JA, Chitnis PR (1994b) Identification of surface-exposed domains on the reducing side of photosystem I. Plant Physiol **106:** 617–624

Xu Q, Hoppe D, Chitnis VP, Odom WR, Guikema JA, Chitnis PR (1995b) Mutational analysis of photosystem I polypeptides in the cyanobacterium *Synechocystis* sp. PCC 6803. Targeted inactivation of *psa*I reveals the function of PsaI in the structural organization of PsaL. J Biol Chem **270:** 16243–16250

Xu Q, Jung YS, Chitnis VP, Guikema JA, Golbeck JH, Chitnis PR (1994c) Mutational analysis of photosystem I polypeptides in *Synechocystis* sp. PCC 6803. Subunit requirements for reduction of $NADP^+$ mediated by ferredoxin and flavodoxin. J Biol Chem **2693:** 21512–21518

Xu Q, Odom WR, Guikema JA, Chitnis VP, Chitnis PR (1994d) Targeted deletion of psaJ from the cyanobacterium *Synechocystis* sp. PCC 6803 indicates structural interactions between the PsaJ and PsaF subunits of photosystem I. Plant Mol Biol **2624:** 291–302

Xu Q, Yu L, Chitnis VP, Chitnis PR (1994e) Function and organization of photosystem I in a cyanobacterial mutant strain that lacks PsaF and PsaJ subunits. J Biol Chem **269:** 3205–3211

Zanetti G, Merati G (1987) Interaction between photosystem I and ferredoxin: identification by chemical cross-linking of the polypeptide which binds ferredoxin. Eur J Biochem **169:** 143–146

Zilber A, Malkin R (1988) Ferredoxin cross-links to a 22-kD subunit of photosystem I. Plant Physiol **88:** 810–814