Interaction of the Soluble Recombinant PsaD Subunit of Spinach Photosystem I with Ferredoxin I[†]

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ABSTRACT: Photosystem I of higher plants functions in photosynthesis as a light-driven oxidoreductase producing reduced ferredoxin. Its peripheral subunit PsaD has been identified as the docking site for ferredoxin I. With the aim of elucidating the structure−function relationship and the role of this subunit, a recombinant form of the spinach protein was produced by heterologous expression in *Escherichia coli*. The PsaD protein was synthesized in soluble form and purified to homogeneity. The interaction of the PsaD subunit with ferredoxin I was investigated using three different approaches: chemical cross-linking between the two purified proteins in solution, affinity chromatography of the PsaD subunit on a ferredoxin-coupled resin, and titration with ferredoxin of the protein fluorescence of the subunit. All these studies indicated that the isolated PsaD in solution has a definite conformation and maintains the ability to bind ferredoxin I with high affinity and specificity. The K_d value of the complex of PsaD and ferredoxin is in the nanomolar range, which is consistent with reported K_m values for ferredoxin I photoreduction by thylakoid membranes. The ionic strength dependence of the K_d suggests that the protein−protein interaction is at least partially electrostatic in nature. Nevertheless, none of the glutamate residues of the acidic cluster of residues 92−94 of ferredoxin I, which have been reported to be involved in the interaction with the subunit, seems to be essential for PsaD binding, as borne out by experiments using ferredoxin I mutants in positions 92−94.

PsaD is a peripheral subunit of photosystem I (PSI[1]), an integral protein complex in the thylakoid membrane of oxygenic photosynthetic organisms (for reviews, see refs *1*−*3*). PSI acts as an oxidoreductase that catalyzes the light-driven transfer of electrons from reduced plastocyanin (or cytochrome c_6) to oxidized ferredoxin (or flavodoxin). The role of several PSI subunits has been elucidated. The two large subunits, PsaA and PsaB, contain the reaction center P_{700} and redox cofactors A_0, A_1, and F_x. At the lumenal oxidizing side of PSI, PsaF plays a role in the docking of plastocyanin at least in higher plants (*4*). At the stromal reducing side of PSI, three extrinsic, small subunits, PsaC (containing the 4Fe4S clusters F_A and F_B), PsaD, and PsaE, are involved in the docking and reduction of ferredoxin. Cross-linking experiments have shown that PsaD serves as a docking site for ferredoxin during photoreduction (*5*, *6*). Deletion of the PsaD gene, indeed, greatly affected the photoautotrophic growth of the mutant (*7*). More recent data confirmed that the PsaD is indispensable for the productive interaction of PSI with ferredoxin (*8*−*10*). The PsaD subunit has also been shown to be required for the assembly of PsaC and PsaE into the PSI complex (*11*). The PsaD is a polypeptide of 139−144 amino acids in cyanobacteria, but has an N-terminal extension of several residues in higher plants, yielding a total length of 158−162 residues (*12*). Topological studies (*13*−*15*) and data from an X-ray structure of PSI at 4 Å (*16*) show that PsaD probably contains an α-helix and is in contact principally with PsaC and PsaE, and also with PsaH and PsaL (*15*). The three-dimensional structure of the higher-plant PSI as determined by electron crystallography has been recently reported (*17*), confirming that the stromal ridge of higher-plant PSI can also be interpreted as being due to the PsaC, -D, and -E subunits. The N-terminal part of the PsaD subunit can be accessed by the proteases, and its C-terminal region is exposed to solvent (*9*, *13*, *14*). Comparison of the amino acid sequences of PsaD from several species shows that the C-terminal part is highly conserved, especially in a region containing many basic residues. Indeed, the polypeptide from spinach leaves is rich in positively charged residues and has a p*I* value of 10.18 (*18*).

Since the amount and quality of the PsaD purified from spinach PSI did not allow further investigation on the structure−function relationship of this subunit and its interaction with ferredoxin, we have tried the heterologous expression of the cloned spinach gene with the aim of obtaining large amounts of the subunit for a detailed characterization. Most recently, the properties of the recombinant PsaD from the cyanobacterium *Nostoc* PCC 8009 and its structure in solution have been published (*19*). Here we

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[1] Abbreviations: Fd, ferredoxin I; PSI, photosystem I; FNR, ferredoxin−$NADP^+$ reductase; GdnHCl, guanidinium chloride; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MBP, maltose binding protein; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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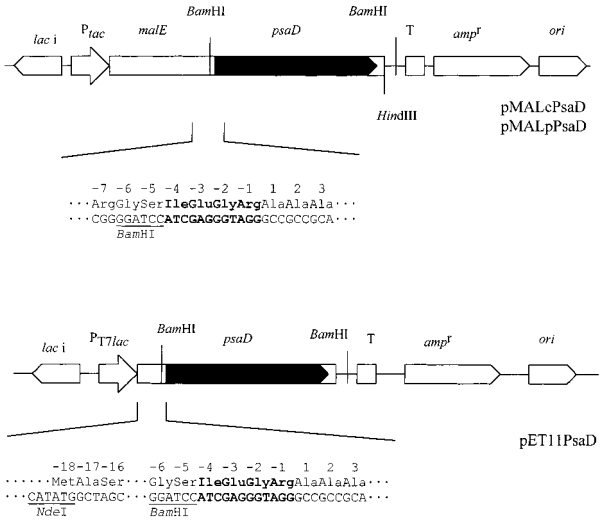

FIGURE 1: Functional map of plasmids pMALcPsaD, pMALpPsaD, and pET11PsaD. Relevant regions are boxed. Sequences at the junction points between PsaD and its N-terminal extensions are shown. The sequences encoding the factor Xa recognition tetrapeptide are bold. P_{tac}, *tac* promoter; P_{T7lac}, fusion between the T7 RNA polymerase promoter and the *lac* operator; *malE*, gene encoding *E. coli* maltose binding protein; T, transcription terminator.

report on the production of a soluble PsaD protein by expression of the spinach gene construct in *Escherichia coli*, its purification, and its structural and functional characterization.

EXPERIMENTAL PROCEDURES

Materials. Recombinant FNR and Fd were purified from *E. coli* as previously described (*20*, *21*). Restriction endonucleases and T4 DNA ligase were purchased from either Gibco BRL or Boehringher Mannheim. All other chemicals were of analytical grade.

Construction of Plasmids. Plasmid pET15PsaD, a construct for PsaD expression based on vector pET15b, was kindly provided by R. G. Herrmann and U. Mühlenhoff. pET15PsaD was obtained by engineering the cDNA encoding the full-length precursor of the *Spinacia oleracea* PsaD subunit (*18*) to remove from its 5′-end the 150-nucleotide portion encoding the transit peptide and to create a *Nco*I site in front of the sequence coding for the mature PsaD peptide. The engineered gene was then inserted into the *Nco*I site of vector pET15b. In this construct, the T7 promoter directs the synthesis of the mature form of PsaD with a methionine residue at its N-terminous. Due to the very low expression levels obtained using pET15PsaD, three additional plasmids were constructed, relying on a fusion-protein strategy (Figure 1). pET15PsaD was digested with *Nco*I, treated with mung bean nuclease to blunt the 5′-end of the insert, and digested with *Hin*dIII. The resulting fragment, harboring the sequence encoding PsaD, was then recloned in both vectors pMAL-c and pMAL-p (for expression in the cytoplasmic or periplasmic space of the host cell, respectively) between the *Stu*I and *Hin*dIII sites, yielding pMALcPsaD and pMALpPsaD, respectively. In these constructs, the sequence encoding PsaD is fused to the 3′-end of the *E. coli* gene *malE*, encoding MBP, whose synthesis is under the control of the *tac* promoter. A sequence encoding the factor Xa recognition tetrapeptide is present at the fusion site, making it possible to yield mature PsaD by proteolytic treatment of the fusion products. Finally, a fragment encoding PsaD, and an N-terminal extension comprising the factor Xa recognition tetrapeptide, was excised from pMALcPsaD by *Bam*HI restriction, and recloned into the *Bam*HI site of vector pET11a, to yield pET11PsaD. In this construct, the T7 promoter directs the synthesis of mature PsaD with an N-terminal extension comprising 14 residues (T7 tag) encoded by the 5′-portion of gene 10 of T7 phage and the four residues of the factor Xa recognition sequence.

Overexpression and Purification of PsaD. *E. coli* strain BL21(DE3) was used as a host for expression using plasmids based on pET vectors, whereas strains RRIΔM15 and Y1090 (*lon*$^-$ strain) were used for expression using pMAL derivatives. Transformed cells were grown in 2×YT medium supplemented with 80 mg/L ampicillin, either in Erlenmeyer flasks or in fermentors (New Brunswick, 1.5 or 12 L). Cultures were grown at 37 °C to an absorbance at 550 nm of about 0.8−1.0. After induction with 0.2 mM IPTG, cells were grown for additional 2 h, and harvested by centrifugation. Cells were resuspended in 2 volumes of lysis buffer [50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 1 mM PMSF] and disrupted by sonication. Cell debris was removed by centrifugation for 1 h at 43000*g*. To purify PsaD from cells transformed with pMALcPsaD, the cell-free extract (9000 mg of total protein) was loaded on a cross-linked amylose column (total volume of 1 L) equilibrated in 20 mM Tris-HCl (pH 7.4) containing 500 mM NaCl and 1 mM EDTA. After extensive washing with the same buffer, the MBP−PsaD fusion protein was eluted by adding 10 mM maltose to the mobile phase. The eluted protein was precipitated with 70% saturation ammonium sulfate. After dialysis, the protein was loaded on a Q-Sepharose fast flow column (70 mL, Pharmacia Biotech) equilibrated in 20 mM Tris-HCl (pH 7.4) containing 5% glycerol. The protein was then eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer. To yield mature PsaD, the fusion protein was treated with factor Xa (mass ratio of 1:1000) for 10 h at 15 °C, in 100 mM Tris-HCl (pH 7.7) containing 100 mM NaCl, 5 mM $CaCl_2$, and 10% glycerol. After desalting, the reaction mixture was loaded on an SP-Sepharose high-performance column (20 mL, Pharmacia Biotech), equilibrated with 10 mM Hepes (pH 7.5) containing 10% glycerol. PsaD was eluted with a linear gradient from 0 to 1 M NaCl, in the same buffer.

To purify PsaD from cells transformed with pET11PsaD, the cell-free extract (2500 mg of total protein) was adjusted to 20% saturation ammonium sulfate and loaded on a phenyl-Sepharose column (50 mL, Pharmacia Biotech) equilibrated in 50 mM Tris-HCl (pH 7.4) containing 20% saturation ammonium sulfate, 10% glycerol, and 1 mM β-mercaptoethanol. The protein was eluted by decreasing the ammonium sulfate concentration with a linear gradient from 20% saturation to 0. The recombinant protein was then concentrated by precipitation with 70% saturation ammonium sulfate and loaded on an SP-Sepharose high-performance column (20 mL, Pharmacia Biotech), equilibrated with 20 mM Hepes (pH 8.0) containing 50 mM NaCl, 10% glycerol, and 1 mM β-mercaptoethanol. The protein was eluted with a linear gradient from 50 to 500 mM NaCl, in the same buffer.

Electrophoresis. PAGE in the presence of SDS was carried out essentially as described in ref *22*.

Mass Spectrometry and N-Terminal Analysis. Matrix-assisted laser desorption/ionization mass spectrometry was carried out in a Vestec apparatus using ferulic acid as a matrix. Sequence determination was performed on a Applied Biosystems 477/A protein sequencer equipped with an on-line HPLC system.

Spectral Analyses. Fluorescence measurements were performed with a Jasco FP-777 spectrofluorometer at 15 °C. Absorption spectra were recorded with a Hewlett-Packard 8453 diode array spectrophotometer. The extinction coefficient of the PsaD protein was determined by measuring the PsaD concentration by three different procedures. The protein subunit was denatured by either 6 M GdnHCl or 0.1 M NaOH and its concentration determined on the basis of PsaD amino acid composition and known extinction coefficients of aromatic residues under these conditions (*23*, *24*). Native or GdnHCl-denatured PsaD was incubated with excess DTNB in 50 mM sodium phosphate (pH 7.0). The amount of released TNB was determined using an extinction coefficient of 13.88 mM^{-1} cm^{-1} at 412 nm (*25*). The PsaD concentration was then calculated from the known content of 1 mol of cysteine per mole of PsaD. These methods yielded a mean value for the PsaD concentration of 23.45 $\pm$ 0.15 μM.

Affinity Chromatography on Fd-Sepharose 4B. Immobilized Fd was obtained by coupling 300 nmol of Fd to 0.6 mL of CNBr-activated Sepharose 4B (Pharmacia Biotech) following the manufacturer's directions. Fd-conjugated resin was packed and used as an affinity column in an FPLC apparatus. PsaD (0.5−1 mg) was loaded on the affinity column, equilibrated in 20 mM Tris-HCl (pH 8.0) containing 5% glycerol. After washing with 5 column volumes of equilibration buffer, a gradient from 0 to 0.5 M NaCl in the same buffer was applied over 10 column volumes.

Cross-Linking Reactions. Mixtures of 40 μM PsaD and 40 μM Fd forms were incubated in 10 mM sodium phosphate (pH 7.0) with 5 mM EDC at 25 °C. When present, FNR was at a concentration of 10 μM. At intervals, aliquots were withdrawn, excess reagent was quenched with ammonium acetate, and samples were analyzed by SDS−PAGE.

RESULTS

PsaD Subunit Overexpression in E. coli. Overexpression of the eukaryotic PsaD gene construct was difficult in the prokaryotic host. We had already experienced such a situation with other spinach cDNA clones (*21*, *26*). Constructs using the pET15b expression vector, in which a cDNA clone encoding the mature form of PsaD was placed under the control of a strong promoter recognized by the T7 RNA polymerase, yielded only a trace amount of the protein. A higher level of expression (10% of that of the *E. coli* total protein) was obtained by using the pMAL-c expression vector that directed the synthesis of PsaD as a fusion protein at the C-terminus of the *E. coli* MBP. The fusion protein was fully recovered in the supernatant of the cell extract and was purified to homogeneity using affinity chromatography on a cross-linked amylose column followed by an ion exchange chromatographic step on a Q-Sepharose column. The fusion protein was then cleaved at the junction by the protease factor Xa. Beside the MBP band, two main protein bands (15−18 kDa) and other minor ones (with lower M_r values) were revealed by SDS−PAGE. N-Terminal sequencing of the 15−18 kDa polypeptides confirmed that they both started with the expected PsaD sequence as deduced from the DNA sequence, i.e., AAAAEGKAATPTE (*18*). Thus, C-terminally truncated forms were produced either in the *E. coli* cells or during purification. An additional chromatographic step on an SP-Sepharose column allowed for elimination of the truncated polypeptides except for one that copurified with the full-length form. By MALDI-TOF measurements, the truncated protein exhibited an M_r of 15 538 versus 17 840 for the full-length PsaD protein. The latter value agrees well with the value of 17 856 for the native mature form (*18*). It can be calculated that the truncated form has lost the 21 C-terminal amino acids, most probably the site of cleavage being between Arg141 and Ser142 of the sequence. To avoid the proteolysis, *E. coli lon*$^-$ strains were used without success. A construct made with the pMAL-p expression vector that is suitable for exporting the expressed fusion protein in the periplasm gave a much lower yield and was not pursued further. Finally, a construct was made using a pET11a expression vector, by which PsaD is synthesized with a N-terminal extension of 14 amino acids encoded by a fragment of the T7 phage gene 10 and the tetrapeptide (IEGR) for factor Xa proteolytic cleavage of the extension. Growth conditions have been selected that allowed the production of the PsaD protein in a soluble form in high yield (ca. 5% of the soluble protein). A purification procedure, different from the previous one, was set up. Two successive chromatographic steps on phenyl-Sepharose and SP-Sepharose columns, respectively, yielded a homogeneous preparation of the PsaD protein (1.2 mg/g of cells) (Figure 2). Removal of the N-terminal extension proved to be difficult on the purified protein and resulted in the appearance of truncated forms if performed at early stages of the purification. This behavior indicates a partial instability in solution of the mature form of PsaD; thus, we did not further attempt to cleave off the N-terminal extension.

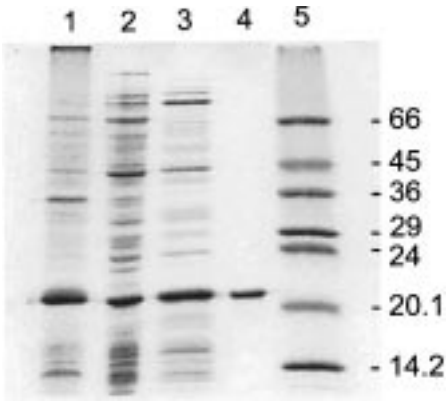

FIGURE 2: Purification profile of the recombinant PsaD. Analysis by SDS−PAGE of the protein fractions at the various purification steps: lane 1, soluble extract from *E. coli* cells harboring pET11PsaD; lane 2, supernatant after 20% ammonium sulfate precipitation; lane 3, fraction off phenyl-Sepharose chromatography; lane 4, fraction off SP-Sepharose chromatography; and lane 5, molecular mass markers (mass values in kilodaltons are listed).

Structural Characterization of PsaD. The purified PsaD protein exhibits a well-defined absorbance spectrum in the near UV which essentially can be ascribed to its content of seven tyrosines and a single tryptophan. We felt it was

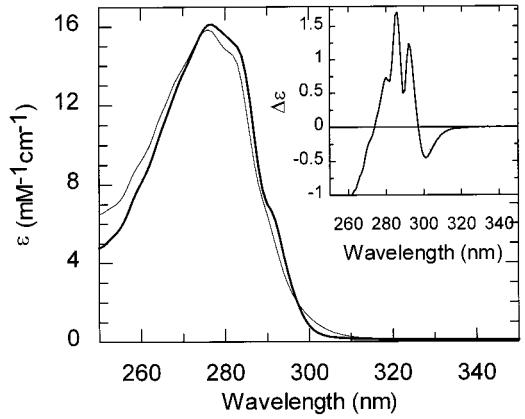

FIGURE 3: Absorbance spectrum of the recombinant PsaD before and after treatment with 6 M GdnHCl: thick line, native PsaD; and thin line, GdnHCl-denatured PsaD. The inset shows the difference spectrum obtained by subtracting the spectrum of denatured PsaD from that of native PsaD.

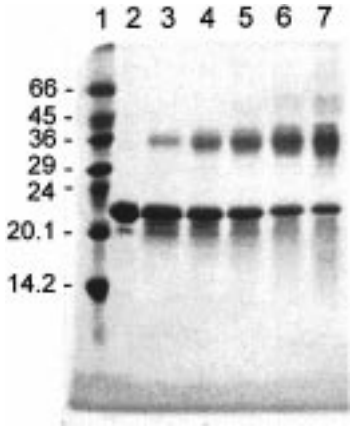

FIGURE 4: EDC-promoted cross-linking of the soluble PsaD with Fd as analyzed by SDS−PAGE. The reaction mixture contained 40 μM PsaD, 40 μM Fd, and 5 mM EDC. Aliquots were withdrawn and loaded on a 14% polyacrylamide gel. Other conditions were as described in Experimental Procedures: lane 1, molecular mass markers (mass values in kilodaltons are listed); lane 2, PsaD protein; and lanes 3−7, aliquots of the mixture analyzed at 5, 10, 15, 25, and 35 min, respectively.

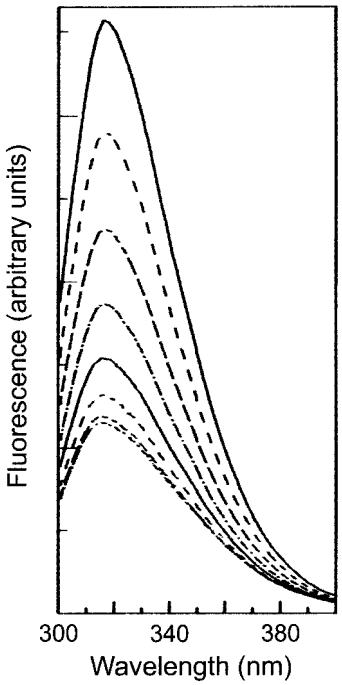

FIGURE 5: Fluorescence emission spectrum of the recombinant PsaD and the effect of Fd addition. The most intense spectrum (continuous line) represents the fluorescence emission spectrum obtained by excitation at 280 nm of a PsaD solution in 25 mM MOPS (pH 7.0) and 10% glycerol. Lower-intensity spectra (broken lines) were recorded after addition of increasing quantities of Fd to the PsaD solution.

important to determine the molar extinction coefficient at the wavelength of maximal absorbance as a mean for an accurate estimation of the PsaD concentration. PsaD is devoid of an easily measurable biological function and did not give reliable figures with the common protein reagents. Three different, established procedures were used for the extinction coefficient determination: analyses of the UV spectra of the PsaD denatured with either 6 M GdnHCl (*23*) or 0.1 M NaOH (*24*) and quantitation of the single cysteine of PsaD in the native and denatured protein through reaction with DTNB (*25*). Whereas the sulfhydryl group in the denatured polypeptide was readily titrated, in the native protein 25 min was required for full reaction, suggesting that the SH group is not exposed to solvent. The values of the PsaD concentration obtained from the four different types of experiments were in excellent agreement. In Figure 3, the UV spectra of native and GdnHCl-denatured PsaD at a concentration of 1 mM are reported. The molar extinction coefficient at 276 nm was determined to be 16.07 $\pm$ 0.1 mM^{-1} cm^{-1}. In the inset, the difference spectrum (native minus denatured) shows two positive peaks at 285 and 292 nm, respectively, indicating that in the native protein the spectrum is slightly red shifted. The PsaD subunit yields an intense emission fluorescence spectrum (see Figure 5) with a maximum at a very short wavelength (315 nm). This indicates that the only tryptophan present, the expected fluorophore, sits in a highly hydrophobic environment. From all these studies, it can be concluded that the PsaD protein can behave as a folded protein in solution.

Functional Characterization of PsaD. To the PsaD subunit of PSI has been assigned the role of topological dock to which Fd must be bound to allow its photoreduction during photosynthesis. Thus, we felt it was essential to show that the isolated subunit is functional by checking its capability to bind Fd. Three different approaches were used to study the interaction between PsaD and Fd. The same procedure used to discover the interaction of Fd with PSI in thylakoid membranes or PSI particles (*5*) was first tried, i.e., chemical cross-linking with the soluble carbodiimide EDC. Analysis by SDS−PAGE of the time course of the cross-linking between the two proteins in solution is shown in Figure 4. A new protein band at about 36 kDa appeared during incubation at low ionic strengths. Such a value for the M_r of the 1:1 covalent complex of the two proteins is due to the anomalous behavior of Fd in SDS−PAGE (*5*, *6*, *21*). The 36 kDa band was not observed if the cross-linking reaction was performed at high ionic strengths. A highly specific procedure was then devised by preparing an affinity column of immobilized Fd by covalent attachment of the protein to a Sepharose 4B resin. At low ionic strengths, the PsaD subunit was fully retained on the column and its elution in a single peak could be achieved by increasing the ionic strength of the eluant (data not shown). Finally, the high protein fluorescence of the PsaD subunit was exploited to

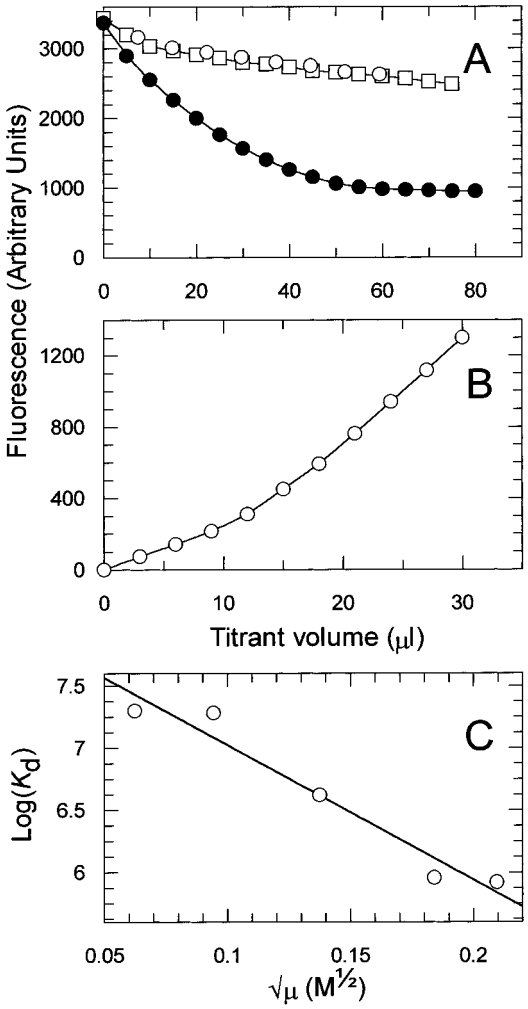

FIGURE 6: Interaction of PsaD with Fd. Determination of the dissociation constant of the complex and evaluation of the ionic strength effect. (A) PsaD (1 μM) in 10 mM MOPS (pH 7.0) and 10% glycerol was titrated with different proteins. The fluorescence emission at 320 nm was recorded upon excitation at 280 nm, after successive additions of buffer (□), of 35 μM Fd (●), and of 36 μM plastocyanin (○). (B) Fluorescence increase after successive additions of 45 μM PsaD to a solution of 0.6 μM Fd. Other conditions were as described for panel A. (C) Ionic strength dependence of the dissociation constant of the complex of PsaD and Fd. Titrations were performed under the conditions described for panel A, but NaCl was added to yield the desired ionic strength.

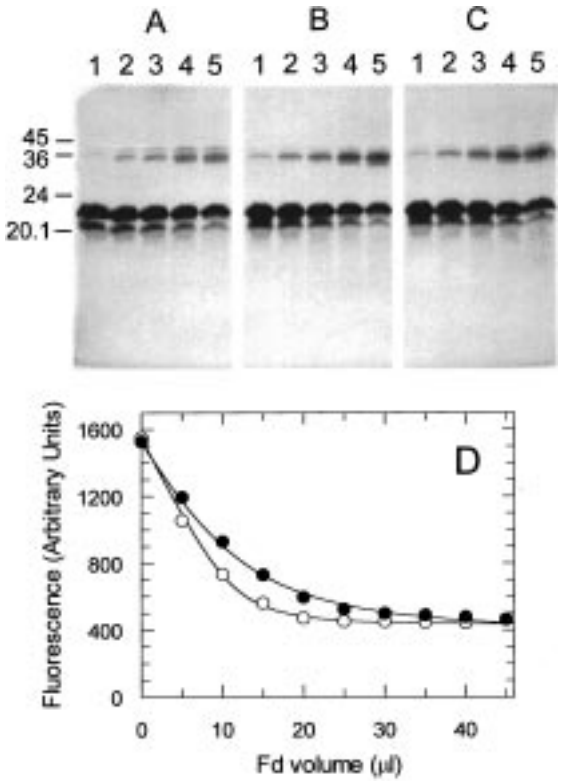

FIGURE 7: Interaction of PsaD with Fd mutants as analyzed by chemical cross-linking and fluorescence titration. (A−C) Cross-linking reaction mixtures were analyzed by SDS−PAGE on 16% polyacrylamide gels at different incubation times (5, 10, 15, 25, and 35 min, in lanes 1−5, respectively). PsaD was reacted with Fd-E93A (A), Fd-E92A/E93A (B), and Fd-E93A/E94A (C), under the conditions described for wild-type Fd in the legend of Figure 4. Mass values in kilodaltons of the protein markers are listed on the left. (D) Fluorescence titrations of PsaD (ca. 0.4 μM) with 30 μM wild-type Fd (○) and 30 μM Fd-E92A/E93A (●). Other conditions were as described in the legend of Figure 6.

quantitatively measure the interaction of the subunit with Fd. Indeed, progressive quenching of PsaD fluorescence was specifically promoted by addition of Fd aliquots (Figure 5). Fd has a very low fluorescence intensity (*20*, *21*). The fluorescence emission spectrum was not modified during the titration, except for the intensity of the peak, which was decreased. At the titration end point, the quenching reached 70% of the initial fluorescence value (Figure 6A). To ascertain that the fluorescence quenching was due to a specific effect induced by Fd binding to PsaD, a titration of the subunit was also performed using plastocyanin, a small, acidic protein, which physiologically interacts with the lumenal side of PSI. The small fluorescence decrease observed by adding plastocyanin aliquots was fully mimicked by buffer additions (Figure 6A). The fluorescence decrease remaining after correction for dilution may be due to protein denaturation at the low protein concentration that was used, following mixing after titrant addition. To avoid as much as possible the loss of PsaD protein due to its low stability, a fixed amount of Fd was titrated with aliquots of PsaD (Figure 6B). The complex with Fd is expected to stabilize the PsaD protein. The mean value for the K_d of the complex calculated from both types of experiments was 51 ± 4 nM at low ionic strengths. In Figure 6C, the ionic strength influence on the K_d of the complex is shown. The increase in the K_d value with increasing ionic strength supports the hypothesis that the interaction is at least partially electrostatic in nature.

The PsaD protein was also tested for its capability to bind Fds mutated in the acidic cluster reported to be involved in the cross-linking with PSI (*8*). The EDC-promoted cross-linking of PsaD with the Fd mutants E93A, E92A/E93A, and E93A/E94A (*27*, *28*), as analyzed by SDS−PAGE (Figure 7A−C), did not differ substantially from the pattern observed with wild-type Fd (Figure 4). Titration of the PsaD fluorescence with the very interesting Fd mutant E92K (*27*), the three-dimensional structure of which we have recently obtained (*29*), yielded practically the same value of K_d obtained with the wild-type Fd. In the case of the Fd-E92A/E93A double mutant, a slightly higher value for the complex K_d was found (Figure 7B).

In the past, it was hypothesized that Fd could form a ternary complex with PSI and the reductase during $NADP^+$ photoreduction. Thus, we investigated whether it was possible to cross-link with EDC the three proteins in solution

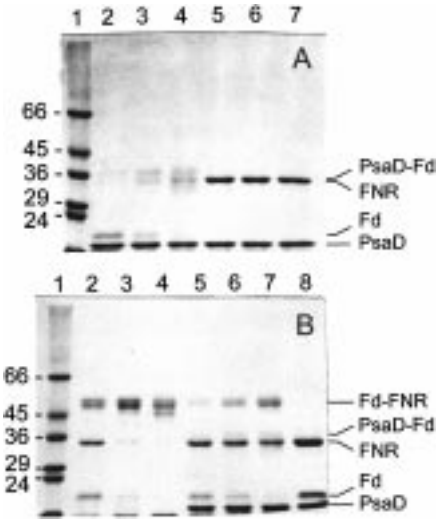

FIGURE 8: Cross-linking between PsaD and Fd in the presence and absence of FNR, as analyzed by SDS−PAGE. Cross-linking conditions were the same as described in the legend of Figure 4, except that the gels were 10% polyacrylamide. (A) Lane 1, molecular mass markers (mass values in kilodaltons are listed); lanes 2−4, cross-linking between PsaD and Fd, as analyzed after reaction for 5, 15, and 45 min, respectively; and lanes 5−7, PsaD reacted as described above, replacing Fd with FNR. (B) Lane 1, molecular mass markers (mass values in kilodaltons are listed); lanes 2−4, cross-linking between FNR and Fd, as analyzed after reaction for 5, 15, and 45 min, respectively; lanes 5−7, cross-linking reactions in the mixture containing FNR, Fd, and PsaD; and lane 8, PsaD, Fd, and FNR in the absence of EDC.

to form a ternary covalent complex. In panels A and B of Figure 8, it is shown that binary complexes of Fd and PsaD, and Fd and FNR, respectively, but not of PsaD and FNR as expected, were formed by incubation of the two specific proteins with EDC. When the three proteins were mixed together with EDC (Figure 8B), no bands in the SDS−PAGE gel corresponding to the M_r of the ternary complex were observed. Both the PsaD−Fd and Fd−FNR covalent complexes were formed, although to a lesser extent. This could be an indication that a competition between PsaD and FNR for the same (or partially overlapping) site of Fd ensued.

DISCUSSION

The peripheral PsaD subunit of spinach PSI could be produced in *E. coli* in a soluble form and could be purified to homogeneity in amounts that are suitable for its structural and functional characterization. We felt it was important to produce the subunit in a soluble form, although the cyanobacterial PsaD protein could be renaturated from inclusion bodies (*19*).

The expression of the spinach PsaD in *E. coli* proved to be difficult compared with that of the cyanobacterial homologue (*11*, *19*), as we have already experienced with other chloroplast proteins, i.e., Fd and FNR. Four different plasmids were assembled; only two of them yielded suitable amounts of the PsaD protein. The subunit obtained as a MBP fusion protein was in part cleaved in the C-terminal region, as revealed by N-terminal sequencing and M_r determination by MALDI-TOF spectrometry. The separation of the truncated forms from the uncleaved native protein was partially unsuccessful. Furthermore, the purified PsaD protein was found to be quite unstable, although addition of glycerol to the protein solution was beneficial. Finally, pET11PsaD allowed us to obtain a homogeneous PsaD protein, with an N-terminal extension of 18 amino acid residues. The planned removal of this N-terminal part was found to be difficult and was not pursued further, the reason being that the N-terminal region of the spinach PsaD subunit, which is 23−25 amino acid residues longer than that of the cyanobacterial subunit, should not be of primary importance for PsaD functionality. Indeed, the 15 N-terminal residues in the subunit of spinach PSI were shown to be cleavable by proteases (and thus exposed to the stroma), and not required for the cross-linking with Fd (*13*, *14*). More recently, the PsaD amino acid residue involved in the cross-link with Fd was identified as Lys106 in the protein C-terminal moiety (*8*), which for most of its length is also exposed to the stroma (*9*, *13*). The achievement of a soluble form of the PsaD subunit of PSI allowed for the possibility of testing the functional role of the subunit in the absence of the other subunits. The isolated PsaD seems to have a folded structure with its only tryptophan residue (Trp56) deeply buried in a hydrophobic environment. Also, the single cysteine residue (Cys89) is not readily titrated, suggesting that it is not exposed to the solvent. Probably, this segment of the polypeptide constitutes the hydrophobic core of the protein. We have experimentally established that the isolated PsaD subunit interacts specifically with Fd with a very high affinity. The value of the K_d of the complex is in the nanomolar range (51 ± 4 nM) and is compatible with the K_m value of 200 nM measured for Fd in the photoreduction by thylakoid membranes (*28*). Instead, no evidence of the interaction of the PsaD subunit with other chloroplast proteins, such as plastocyanin and FNR, which are not expected to bind PsaD, was obtained. Electrostatic interactions are involved in stabilizing the complex of PsaD and Fd, because the increase of the medium ionic strength highly promoted complex dissociation. This is also supported by the fact that it is possible to cross-link the two proteins by using EDC, a carbodiimide that promotes covalent bonds between carboxyl groups of Fd and amino groups of PsaD (*5*, *6*). The partner residues involved in the covalent bonds were identified in the cyanobacterial proteins (*8*) as Lys106 of PsaD and Glu93 of ferredoxin. By site-directed mutagenesis of Lys106, it was shown that this residue is not essential for docking and reduction of Fd (*10*, *30*). Nevertheless, although Lys106 may not be the positive residue directly involved in the interaction with Fd, there is evidence, also from our work, that it is the C-terminal moiety of PsaD that is essential for binding ferredoxin. Indeed, PsaD preparations containing C-terminally truncated forms exhibited a lower titer in Fd titration. In this study, we have probed the interaction of PsaD with Fds mutated in the acidic cluster of residues 92−94. Apparently, none of these residues is essential for complex formation, confirming our previous results on the photoreduction of these Fd mutants (*28*). Furthermore, it was not possible to cross-link both the reductase and the PsaD protein to Fd to form a ternary covalent complex. This would suggest that most probably the same side of Fd is required for interaction with the protein partners.

The high affinity and specificity of the interaction between the soluble form of the PsaD subunit and Fd point to a major role played by this subunit of PSI in the docking of Fd to

the thylakoid membrane. However, several reports (*9*, *31*−*34*) have indicated that other subunits participate in the formation of the binding site of PSI for ferredoxin, namely, the PsaE subunit and, most recently, the PsaC protein in *Chlamydomonas reinhardtii*. The latter subunit, which harbors the clusters F_A and F_B, has the role of reducing Fd, implying that the two proteins should come in contact with each other. Indeed, site-directed mutagenesis of Lys35 of PsaC (*32*) affected both electron transfer from PSI to Fd and cross-linking of Fd to PSI subunits. In the case of the PsaE subunit, its physiological role is still being debated. Several roles have been proposed over the years: docking site for Fd (*9*, *31*, *33*, *34*) and for flavodoxin (*9*, *35*), site for binding FNR (*36*), and involvement in cyclic electron transport (*37*). With the aim of evaluating the contribution of PsaE to the docking platform for ferredoxin, we are pursuing the heterologous expression of PsaE in a soluble and stable form, which for the moment has been only partially successful. By performing studies similar to those described above with the PsaE subunit in the presence and absence of PsaD, we should be able to elucidate the respective roles of the peripheral subunits of PSI in the interaction with ferredoxin.

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