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Biochimica et Biophysica Acta 1410 (1999) 7–18

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Structural features and assembly of the soluble overexpressed PsaD subunit of photosystem I

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Received 2 July 1998; received in revised form 13 October 1998; accepted 17 November 1998

Abstract

PsaD is a peripheral protein on the reducing side of photosystem I (PS I). We expressed the *psa*D gene from the thermophilic cyanobacterium *Mastigocladus laminosus* in *Escherichia coli* and obtained a soluble protein with a polyhistidine tag at the carboxyl terminus. The soluble PsaD protein was purified by Ni-affinity chromatography and had a mass of 16716 Da by MALDI-TOF. The N-terminal amino acid sequence of the overexpressed PsaD matched the N-terminal sequence of the native PsaD from *M. laminosus*. The soluble PsaD could assemble into the PsaD-less PS I. As determined by isothermal titration calorimetry, PsaD bound to PS I with 1.0 binding site per PS I, the binding constant of 7.7×10^{6} M^{-1}, and the enthalpy change of -93.6 kJ mol^{-1}. This is the first time that the binding constant and binding heat have been determined in the assembly of any photosynthetic membrane protein. To identify the surface-exposed domains, purified PS I complexes and overexpressed PsaD were treated with N-hydroxysuccinimidobiotin (NHS-biotin) and biotin-maleimide, and the biotinylated residues were mapped. The Cys^{66}, Lys^{21}, Arg^{118} and Arg^{119} residues were exposed on the surface of soluble PsaD whereas the Lys^{129} and Lys^{131} residues were not exposed on the surface. Consistent with the X-ray crystallographic studies on PS I, circular dichroism spectroscopy revealed that PsaD contains a small proportion of α-helical conformation.

Keywords: PsaD; Photosystem I; Topography; (*Mastigocladus laminosus*)

1. Introduction

Photosystem I (PS I) from cyanobacteria and chloroplasts is a multisubunit membrane–protein complex that catalyzes electron transfer from reduced plastocyanin in the thylakoid lumen to oxidized ferredoxin in the chloroplast stroma or cyanobacterial cytoplasm [1–5]. In cyanobacteria, plastocyanin and ferredoxin can be replaced by cytochrome c_6 and flavodoxin, respectively, depending on the nutritional availability. The heterodimeric core of the PS I complex is formed by the PsaA and PsaB subunits which harbor approximately 100 antenna chlorophyll *a* molecules, 10–12 β-carotenes,

Abbreviations: PS, photosystem; MOPS, 3-[*N*-morpho]linepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl *b*-D-thiogalactopyranoside; Tris, tris[hydroxymethyl]aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NHS-biotin, *N*-hydroxysuccinimidobiotin; DTT, dithiothreitol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ECL, enhanced chemiluminescence

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0005-2728/99/$ – see front matter
PII: S0005-2728(98)00169-8

the primary electron donor P700, and a chain of electron acceptors (A_0, A_1 and F_X). In addition to the core proteins, the cyanobacterial PS I complex contains three peripheral proteins (PsaC, PsaD and PsaE) and six integral membrane proteins (PsaF, PsaI, PsaJ, PsaK, PsaL and PsaM) [1–3]. PsaC binds the terminal electron acceptors F_A and F_B, which are two [4Fe–4S] centers. PsaE facilitates ferredoxin docking [6–9] and may be involved in the cyclic electron flow around PS I [10,11]. Functions of other subunits have been studied using subunit-deficient mutants of cyanobacteria and algae [12–19].

The PsaD subunit of PS I is a conserved peripheral protein on the reducing side of PS I. The cyanobacterial PsaD protein contains 139–144 amino acids whereas the plant PsaD has about 23 additional residues at the N-terminus [20]. This extension of eukaryotic PsaD is accessible to proteases [21,22]. Chemical crosslinking and subunit-deficient mutants have demonstrated physical interactions of PsaD with PsaC, PsaL and ferredoxin [23–28]. In addition, PsaD interacts with several extramembrane loops of PsaB [29]. PsaD provides an essential ferredoxin-docking site [6,23]. The docking may involve electrostatic interactions between the basic PsaD protein and the electronegative surfaces of ferredoxin [23]. Site-directed mutagenesis study revealed that the Lys^{106} of PsaD from *Synechocystis* sp. PCC 6803 PS I is a dispensable component of the docking site [30]. PsaD is also required for the stable assembly of PsaC and PsaE into the PS I complex [31–33].

A model for PS I structure at 4-Å resolution is available from X-ray crystallography [34,35], although the structure of PsaD could not be described due to the lower resolution. Being a peripheral membrane protein, PsaD should be able to be produced as a soluble protein and then may be used for high-resolution structural analysis by X-ray crystallography and NMR studies. The *psaD* genes from *Nostoc* sp. PCC 8009 and *Synechocystis* sp. PCC 6803 have been overexpressed in *Escherichia coli*, but yielded insoluble protein that is deposited in inclusion bodies [30,36]. We describe here overexpression of the *psa*D gene from the thermophilic cyanobacterium *Mastigocladis laminosus*, purification of the functional soluble overexpressed protein, and analysis of its assembly and topography.

2. Materials and methods

2.1. Cyanobacterial strains and culture

Cultures of *M. laminosus* were grown in medium D (pH 8.2) of Castenholz [37] at 55°C under 66 μmol m^{-1} s^{-1} light with constant stirring, and aerated by a mixture of air and CO_2 at a ratio of 10:1. Cultures of *Synechocystis* sp. PCC 6803 were grown in BG-11 medium under a light intensity of 66 μmol m^{-1} s^{-1} at 25°C and were aerated by bubbling with air. Cells were harvested at the late exponential growth phase and resuspended in 0.4 M sucrose, 10 mM NaCl, 10 mM MOPS–HCl (pH 7.0). PS I complexes were purified according to previously described methods [38,39].

2.2. Overexpression of the PsaD protein

The *psaD* gene of *M. laminosus* [40] was amplified by the polymerase chain reaction using primers that added *Nde*I and *Xho*I restriction sites at the two ends of the *psaD* gene of *M. laminosus*. The amplified fragment was inserted into pET-21b(+) vector between *Nde*I and *Xho*I sites. The resulting plasmid was introduced into BL21 (DE3) strain of *E. coli*. To induce expression of the *psaD* gene of *M. laminosus*, the cultures were grown aerobically till the absorbance at 600 nm of the cultures reached 0.6–1.0 and IPTG were added at a final concentration of 1 mM. Cells were harvested after 3 h of induction and were broken by a sonicator. After centrifugation, soluble overexpressed PsaD protein was purified from the supernatant by Ni-affinity chromatography with His·Bind Buffer kit (Novagen). Purified proteins were finally eluted in 1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, desalted twice in 20 mM phosphate buffer (pH 7.0) using Econo-Pac 10DG column (Bio-Rad) to remove the imidazole completely, and stored at −20°C for further use. The concentration of the purified PsaD protein was determined using the extinction coefficient of 57.9 μM^{-1} cm^{-1} at 280 nm. The molecular mass of the purified PsaD–His protein was measured by matrix-assisted laser desorption/ionization (MALDI) mass analysis on a Finnigan MAT Lasermat 2000 MALDI mass analyzer at the Protein Facility of Iowa State University. The *psa*D gene from *Synechocystis* sp.

PCC 6803 was also overexpressed in *E. coli* and the resultant PsaD protein of *Synechocystis* sp. PCC 6803 was further purified [30].

2.3. Analytical gel electrophoresis and N-terminal amino acid sequencing

The overexpressed PsaD proteins were denatured at 100°C for 5 min in the presence of 1% SDS and 0.1% 2-mercaptoethanol, and the PS I complexes were solubilized at 37°C for 2 h with 1% SDS and 0.1% 2-mercaptoethanol. Proteins were resolved by a modified Tricine–urea SDS–PAGE [6]. After electrophoresis, gels were stained with Coomassie blue. Alternatively, the proteins were electrotransferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). The blot was probed with peroxidase-conjugated avidin (Cooper Biomedical) and then developed with chromogenic reagents of hydrogen peroxide and 4-chloro-1-naphthol [41] or with ECL reagents (Amersham). For protein sequencing, the proteolytic fragments on Immobilon-P membranes were stained with Coomassie blue containing 1% acetic acid for several minutes, destained with 50% methanol, and rinsed with deionized water. The membrane pieces were used for protein sequencing son a 477A Protein Sequencer/120A Analyzer (Applied Biosystems) at the Protein Facility of Iowa State University.

2.4. Assembly of PsaD protein into PS I complex

A typical assembly reaction included ADC4 thylakoid membranes (PsaD-less) and PsaD proteins in MM buffer (10 mM MOPS (pH 7.0), 1 mM $MgCl_2$). The incubation was carried out at 25°C for 60 min. After incubation, the samples were divided into two parts. Half volume of the assembly mixture was denatured for Western analysis. This fraction contains all (incorporated and free) PsaD proteins in the assembly reaction. The other half of the assembly reaction was transferred on ice and diluted with 10-fold volume ice-cold MM buffer. The thylakoid membranes were then pelleted at $50\,000 \times g$ for 60 min, resuspended in MM buffer, and treated with the chaotropic reagent 2M NaBr for 15 min on ice [32]. The treated thylakoid membranes were pelleted, washed, and finally resuspended. This fraction contains only the PsaD proteins incorporated into the PS I complex. Samples were subjected to analytical gel electrophoresis and the presence of PsaD protein in the thylakoid membranes was detected by antibody against PsaD protein [16]. The ferredoxin-mediated $NADP^+$ photoreduction by the assembly mixture was performed as described [39].

2.5. Isothermal titration calorimetry (ITC)

The calorimetric experiments were performed with a CSC4200 Isothermal titration calorimeter (Calorimetry Sciences Corp., Provo, UT). The titration was carried out at 25°C. The preparations of the PsaD-less PS I complex and overexpressed PsaD protein were dialyzed against the working buffer (10 mM MOPS (pH 7.0), 0.05% Triton X-100). The titration was conducted by injecting 10 μl of 50.0 μM PsaD protein into 1.3 ml of 4.0 μM PsaD-less PS I 16 times at an interval of 5 min. Data analysis was carried out using the method described in [44] with the BindWorks software provided with the instrument.

2.6. Biotin modification and limited endoproteinase treatment

A typical biotin labeling reaction included purified PS I complexes (500 μg chlorophyll ml^{-1}) or purified overexpressed PsaD protein (500 μg ml^{-1}) and 60 μM NHS-biotin or 60 μM biotin-maleimide. The reactions were carried out at room temperature for 60 min in the dark, and terminated by addition of 100 mM glycine for NHS-biotin modification or 100 mM DTT for biotin-maleimide modification. For limited proteolysis treatment, the NHS-biotin-labeled PsaD (250 μg ml^{-1}) were treated with endoproteinase clostripain (10 μg ml^{-1}) in the presence of 20 mM Tris (pH 7.5), 1 mM $CaCl_2$, 2 mM DTT for 60 min at 37°C. The reactions were quenched by addition of 20 mM EDTA.

2.7. Circular dichroism analysis

Circular dichroism spectra of the recombinant proteins were determined under nitrogen in a cuvette with a 0.1 cm pathlength using a Jasco-700 circular dichroism spectropolarimeter. Four spectra were tak-

en for each sample at wavelength from 260 nm to 190 nm. The data were smoothed linearly and analyzed with the K2D program [42,43].

3. Results

3.1. Overexpression and purification of the PsaD protein

The *psa*D gene of *M. laminosus* was cloned into pET-21b(+) vector and introduced into the *E. coli* BL21 strain. The constructed plasmid would produce the PsaD protein followed by two additional residues Leu–Glu, from the *Xho*I site, and ending with a hexahistidine tag at the C-terminus. The resulting PsaD protein is referred to as PsaD–His in the following description. IPTG was used to induce overexpression of the *psa*D gene. To examine the production of PsaD–His in *E. coli*, uninduced and induced cells were heated at 100°C for 5 min in the presence of 1% SDS and 0.1% 2-mercaptoethanol, and the proteins were resolved by SDS–PAGE (Fig. 1A). The induced cells contained a dominant protein with an apparent mass of 18.5 kDa. The predicted molecular mass of the PsaD–His protein was 16 752 Da. The native PsaD protein has a predicted mass of 15.6 kDa and migrates as a 17.7 kDa band (data not shown). These observations suggested that the 18.5 kDa protein was the overexpressed product of the *psaD* gene from *M. laminosus*. We found the optimal conditions for *psa*D overexpression to be

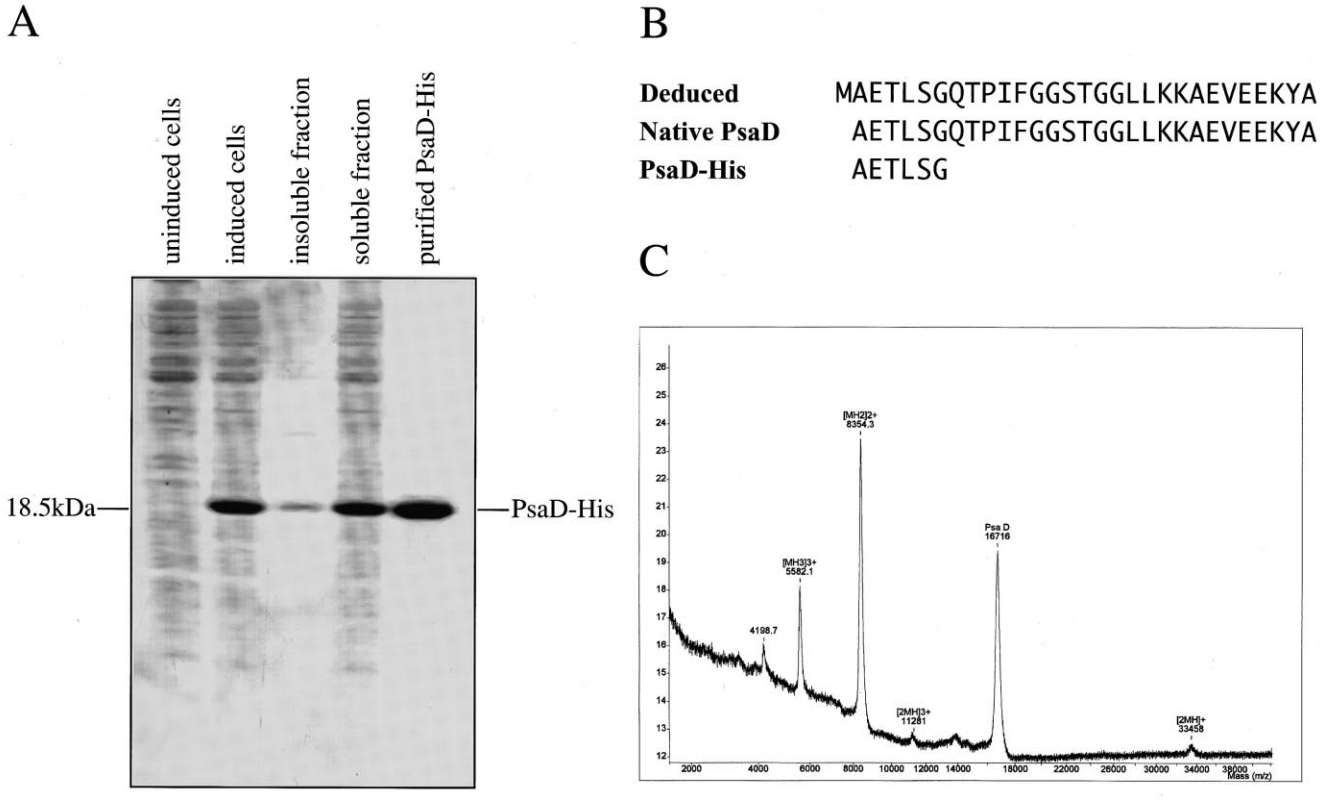

Fig. 1. Overexpression and purification of the PsaD protein of *M. laminosus*. (A) The PsaD proteins were resolved by Tricine–urea SDS–PAGE and detected by Coomassie blue staining. IPTG was used to induce the overexpression of the *psaD* gene. Soluble PsaD protein was purified by Ni-affinity chromatography. The molecular masses of protein fragments were determined from migration of the following protein markers: insulin (2.9 kDa), bovine trypsin inhibitor (5.8 kDa), lysozyme (14.7 kDa), β-lactoglobulin (18.5 kDa), carbonic anhydrase (28.9 kDa) and ovalbumin (44.0 kDa). (B) The N-terminal amino acid sequences of the native PsaD and the purified PsaD–His are compared to the deduced amino acid sequence of PsaD from *M. laminosus*. The deduced N-terminal amino acid sequence of PsaD is shown the first 30 amino acid residues. (C) The purified PsaD–His protein was analyzed by MALDI with a molecular mass of 16 716 Da. Other peaks are mass standards.

incubation for 3 h at a final IPTG concentration of 1 mM. To examine if PsaD is overexpressed as a soluble protein, we centrifuged cell lysate and examined proteins in the soluble fraction and the insoluble pellet by SDS–PAGE (Fig. 1A). Most of the overexpressed PsaD protein was in the soluble fraction. A small amount of PsaD was found in the insoluble fraction that may contain some unbroken cells. The overexpressed PsaD–His protein was purified by Ni-affinity chromatography (Fig. 1A). The purification requires the binding of the C-terminal polyhistidine tag to the Ni-affinity matrix. Therefore, we can conclude that the purified PsaD protein has an intact C-terminus. To identify the N-terminus of the purified PsaD, we determined the N-terminal amino acid sequence as AETLSG, which matched the N-terminal amino acid sequence of the native PsaD and the amino acid sequence that was deduced from the gene sequence (Fig. 1B). Thus the N-terminal methionine was removed posttranslationally in both cyanobacteria and *E. coli*. Therefore, the overexpressed PsaD protein has an intact N-terminus. MALDI analysis of the purified PsaD protein determined the molecular mass of the purified PsaD–His protein to be 16716 Da (Fig. 1C). The variation between the MALDI molecular mass of PsaD–His and the predicted molecular mass of PsaD–His (16752 Da) was 0.2%, which is within the error of MALDI analysis. To conclude, the overexpressed PsaD protein of *M. laminosus* was soluble and could be purified in an intact form.

3.2. Assembly of the PsaD protein into the PS I complex

We examined assembly of the purified PsaD protein into the PS I complexes that had been purified from the PsaD-less ADC4 mutant strain [6,26]. Different amounts of the purified PsaD proteins and the ADC4 PS I complexes were used for the assembly, and the total and incorporated PsaD proteins in the assembly reaction were detected by a PsaD-specific antibody (Fig. 2). When the total PsaD proteins that were used for assembly into ADC4 thylakoid membranes containing 5 μg of chlorophyll increased from 0.5 μg to 7 μg, the amount of incorporated PsaD proteins increased correspondingly. The increase from 7 μg to 25 μg of total PsaD had small

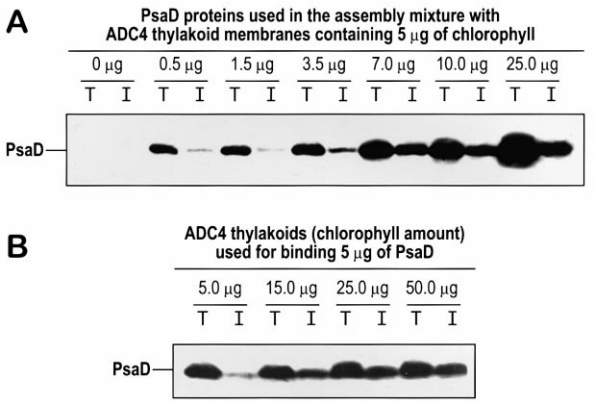

Fig. 2. The assembly of PsaD into ADC4 PS I complex. (A) Different amounts of PsaD proteins were incubated with ADC4 thylakoid membranes containing 5 μg of chlorophyll. T, total PsaD proteins in the assembly reactions; I, incorporated PsaD proteins in the PS I complex which is resistant to the chaotropic reagent treatment. (B) Different amount of ADC4 thylakoid membranes was incubated with 5 μg of PsaD proteins. T, I, same as in A.

effect on the amount of incorporated PsaD proteins. When 5 μg PsaD protein was used for the assembly, the amount of incorporated PsaD increased with the increase of ADC4 thylakoids from 5 μg chlorophyll to 50 μg chlorophyll. Therefore, the purified PsaD protein was capable of assembly into PS I and the amount of assembled PsaD could be saturated.

PsaD provides an essential ferredoxin-docking site on the PS I complex. Consequently, the PsaD-less thylakoid membranes are completely deficient in ferredoxin-mediated $NADP^+$ photoreduction [6]. To test the functional incorporation of PsaD protein into the ADC4 PS I complex, ferredoxin-mediated $NADP^+$ photoreduction activity was measured after assembly of PsaD. The ferredoxin-mediated $NADP^+$ photoreduction activity was normalized on the equal P700 basis and was increased from 0% in the ADC4 membrane to 60% of the wild-type activity in the ADC4 membrane with PsaD. Therefore, the purified PsaD proteins are functional. The partial recovery may be due to a competition between the free PsaD proteins with the incorporated PsaD proteins for binding to ferredoxin. Alternatively, the C-terminal His-tag of the PsaD–His protein might alter ferredoxin docking and reduce the activity. The C-terminal region of PsaD has been shown to be involved in ferredoxin docking [11].

The binding of PsaD to PS I complex was examined by isothermal titration calorimetry. This technique provides thermodynamic information about binding between two molecules [44,45]. The PsaD proteins were injected into the ADC4 PS I complexes 16 times, and the cell feedback (CFB) signals were recorded and integrated over time to measure the total heat change during the titration. Dilution heats were subtracted out from the total heat to provide reaction heat. The thermodynamic parameters of binding were obtained through nonlinear least-squares fit of the reaction heat [44]. The reaction heat and the theoretical fit of the data are shown in Fig. 3. The binding stoichiometry was determined as 1.0 PsaD binding site per ADC4 PS I complex, which perfectly matched the result from biochemical and structural studies that PS I complex contains only one molecule of PsaD [34,35]. The estimate of one PsaD molecule per complex also showed that the soluble overexpressed PsaD is homogeneous in its ability to assemble into PS I complex. The binding constant for the incorporation was determined as 7.7×10^{6} M^{-1}. With the binding constant, the free energy change can be calculated as -39.3 kJ mol^{-1}. The binding of PsaD to the PS I complex was an exothermic reaction and the enthalpy change for the binding was -93.6 kJ mol^{-1}. Therefore, the entropy change of the binding reaction can be calculated as -182.2 J mol^{-1} K^{-1}.

3.3. Modification of surface-exposed residues

Analysis of the deduced amino acid sequences revealed that the PsaD protein of *M. laminosus* contains 12 lysyl residues and 1 cysteinyl residue. NHS-biotin reacts specifically with the N-terminus and the ε-amino group of lysyl residues [46], while biotin-maleimide modifies the sulfhydryl groups of cysteinyl residues. Modification of a protein with NHS-biotin has been used to probe the protein surface exposed to the aqueous phase [30,47]. To identify the surface-exposed residues of the soluble overexpressed PsaD protein and of the PsaD subunit that is in the PS I complex, both the PsaD–His and the PS I complexes of *M. laminosus* were treated with biotinylation reagents. The biotinylated proteins were resolved by SDS–PAGE and electroblotted. Proteins on the membranes were detected by Coomassie blue staining. The peroxidase-conjugated avidin and the chromogenic substrates for peroxidase were used to detect the protein modification (Fig. 4). During electrophoresis, the PsaD subunit in PS I migrated faster than the PsaD–His protein, reflecting the difference in their molecular mass. Both the PsaD–His protein and the PsaD subunit in PS I showed significant amounts of biotin incorporation when treated with NHS-biotin, showing the presence of surface-exposed lysyl residues. The PsaF and PsaL subunits of the PS I complex from *M. laminosus* also con-

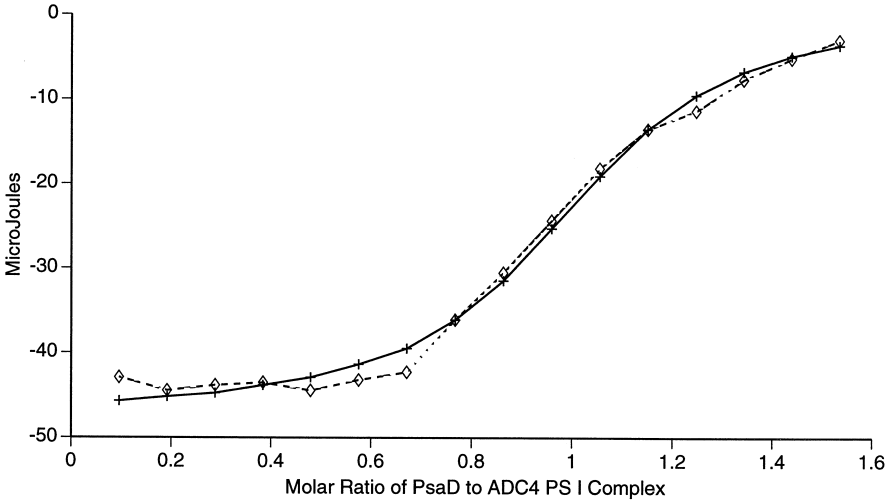

Fig. 3. The binding of the soluble PsaD to the ADC4 PS I complex. ITC was used to study the binding of PsaD to ADC4 PS I complex. The dashed line with '◇' shows the actual ITC data. The solid line with '+' shows the theoretical fit of the data for $n=1$. The thermodynamic parameters of binding were obtained through nonlinear least-squares fit of the reaction heat [44].

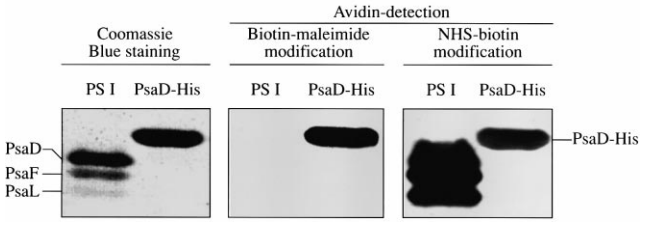

Fig. 4. Modification of surface-exposed residues. The PS I complex from *M. laminosus* and the PsaD–His protein were modified with biotin-maleimide and NHS-biotin. The proteins were resolved by Tricine–urea SDS–PAGE and detected by Coomassie blue staining or avidin peroxidase with chromogenic reagents.

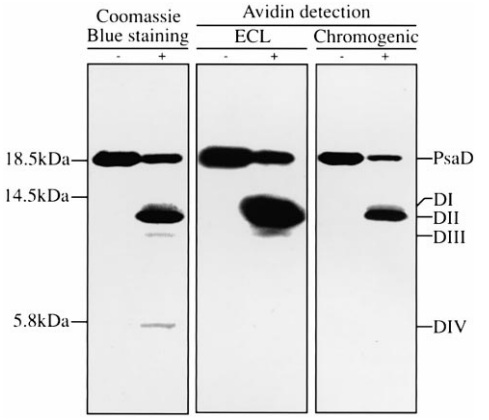

Fig. 5. Accessibility of the Soluble PsaD Protein to clostripain. The PsaD–His protein was labeled with NHS-biotin and the labeled proteins was treated with endoproteinase clostripain. Proteolytic fragments were resolved by Tricine–urea SDS–PAGE and detected by Coomassie blue staining or avidin peroxidase with ECL reagents and with chromogenic reagents. −, labeled PsaD–His without protease treatment. +, labeled PsaD–His with protease treatment.

tained surface-exposed lysyl residues. When the PsaD–His protein and the PS I complexes were treated with the biotin-maleimide, only the PsaD–His protein was labeled by biotin indicating that the only cysteinyl residue in the PsaD–His is exposed on the surface. However, the same residue in the PsaD subunit that was assembled in the PS I complex was not modified by biotin-maleimide. These results indicated that the Cys^{66} residue may be located in the region that interacts with other subunits and is buried inside the complex. The PsaF and PsaL subunits of the PS I complex were not modified by biotin-maleimide. The *M. laminosus* PsaL does not contain cysteine whereas mature PsaF contains two cysteinyl residues [48,49]. Therefore, the two cysteinyl residues of PsaF are not exposed on the surface of the PS I complex from *M. laminosus*.

3.4. Accessibility of the soluble PsaD protein to clostripain

To study topography of the soluble PsaD protein, the PsaD–His protein that had been modified with NHS-biotin was subjected to limited proteolysis. After clostripain treatment, the proteins were denatured, separated by SDS–PAGE, and electrotransferred to Immobilon-P membranes. The proteins on the membranes were stained with Coomassie blue, detected by avidin peroxidase with ECL reagents or with chromogenic reagents (Fig. 5). The fragments that were visible in the Coomassie blue staining were subjected to N-terminal amino acid sequencing. The N-terminal sequences and the apparent mass of the proteolytic fragments were used to predict the sites of protease cleavage (Table 1). When the biotinylated PsaD was treated with clostripain which cleaves specifically at the carboxylic side of argininyl residues, four small detectable fragments were generated. The DI and DII fragments which contain 10 lysyl residues were detected by Coomassie blue staining, avidin peroxidase with ECL reagents and with chromogenic reagents. These two fragments contained the N-terminus of the intact PsaD protein

Table 1
N-Terminal amino acid sequence analysis of the proteolysis fragments in Fig. 5

Peptide	N-Terminal sequence	Avidin detection	Predicted fragment[a]	Apparent mass (kDa)	Predicted mass (kDa)
PsaD–His	AETLSG	+	A2-H148	18.5	16.8
DI	AETLSG	+	A2-R119	13.0	13.3
DII	AETLSG	+	A2-R118	12.8	13.2
DIII	KAEVEE	+	K22-R118 (or K22-R114)	10.8	11.2 (or 10.7)
DIV	RIGQNP	−	R119-H148	5.6	3.6

[a]The position of the first amino acid was identified after comparison with the deduced amino acid sequence of PsaD.

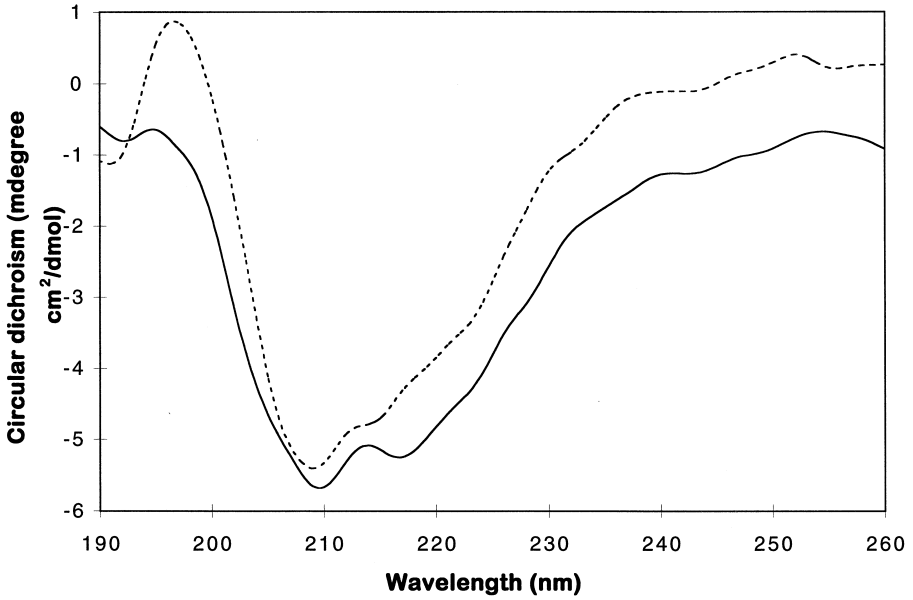

Fig. 6. Circular dichroism spectra of the PsaD proteins from *Synechocystis* sp. PCC 6803 and *M. laminosus*. Circular dichroism data were collected every 0.2 nm from 190 nm to 260 nm with a protein concentration of 0.1 mg ml^{-1} at 20°C. The data were smoothed linearly. The spectra are shown as solid line for PsaD of *M. laminosus* and dashed line for PsaD of *Synechocystis* sp. PCC 6803.

and showed only a minor difference in their apparent mass, indicating closely located proteolysis sites. The Arg^{118} and Arg^{119} residues were the most possible cleavage sites for the DI and DII fragments. The DIII fragment that was visible in Coomassie blue staining was detected only with ECL reagents, but not with chromogenic reagents. This may be because of different sensitivity of the two detection methods. The N-terminal sequence of the DIII fragment was resulted from the cleavage at Lys^{21}, which may be due to nonspecific proteolysis. The most possible cleavage site for the C-terminus of the DIII fragment was Arg^{114} or Arg^{118}. A 3.4 kDa domain at the C-terminus of the PsaD–His protein which contain two lysyl residues (Lys^{129}, Lys^{131}) was cleaved by clostripain at Arg^{119} to generate the DIV fragment. The DIV fragment did not react with avidin peroxidase, indicating that both Lys^{129} and Lys^{131} could not be modified by NHS-biotin and are not exposed to the surface. In contrast, the clostripain cleavage sites Lys^{21}, Arg^{118} and Arg^{119} are exposed outside and accessible to the protease. In addition, Arg^{114} may also be accessible to clostripain.

3.5. Secondary structural features of the PsaD protein

Both PsaD proteins of *M. laminosus* and *Synechocystis* sp. PCC 6803 were used for circular dichroism analysis (Fig. 6). The circular dichroism spectra of the PsaD proteins from two sources varied considerably. This difference could arise from the difference in the primary sequences of the two PsaD proteins, or due to the difference in the preparation of these two proteins. The PsaD protein of *Synechocystis* sp. PCC 6803 was produced in *E. coli* as inclusion bodies that were denatured with guanidine–HCl and refolded by removing the chaotrope. This method could lead to heterogeneity in the solubilized PsaD protein. In contrast, the PsaD protein of *M. laminosus* was produced in *E. coli* as a soluble protein and is likely to be homogenous in its conformation. The circular dichroism data were used to determine the secondary structure proportion. Singular ellipticity values at 222 nm of the PsaD proteins were compared with the standard values obtained from myoglobin and lysozyme to estimate the proportion of α-helix [50]. Also, the ellipticity values from 200 nm to 240 nm were used to calculate the secondary structure fractions by the K2D program [42,43]. The amino acid sequences of both PsaD proteins were analyzed by GeneWorks software with the Garnier protein structure prediction [51] and the Chou–Fasman indices [52]. Table 2 lists the secondary structure fractions determined by different

methods. The predictions by different methods varied considerably. For PsaD of *M. laminosus*, the percentages of α-helix were 21–25% with the singular value, the Garnier and the Chou–Fasman methods, while the percentage of α-helix determined by K2D program was only 11%. The percentage of β-sheet content was predicted to be 30–40% by different methods. There was a large variation in predicted values for PsaD of *Synechocystis* sp. PCC 6803. The percentage of α-helix was estimated to be 20% by single value method, 7% by K2D program, and about 28% from primary sequence. Despite the variation in the predicted numerical values, all methods predict that small fraction of PsaD is α-helical, but a relatively larger fraction forms β sheets. In the 4-Å structural model for PS I, PsaD contains a single short α-helix [34,35]. Therefore, the 11% (about 16 amino acid residues) of α-helix in *M. laminosus* PsaD and the 7% (about 10 amino acid residues) of α-helix in *Synechocystis* PsaD, which were estimated by K2D program with the circular dichroism data, are likely the more accurate estimates.

4. Discussion

The peripheral PsaD protein has been suggested to serve distinct functions in the PS I complex of cyanobacteria, algae and higher plants. It provides an essential ferredoxin-docking site on the reducing side of PS I [6,23] and is required for the stable assembly of PsaC and PsaE into the PS I complex [31,32]. PsaD interacts with PsaB, PsaC, PsaL and ferredoxin [3]. These interactions are specific and crucial for the structural organization and function of the PS I complex. Despite its pivotal role, little is known about the structure of PsaD and its assembly into the PS I complex.

We expressed the *psaD* gene of *M. laminosus* in *E. coli* and obtained a soluble protein. Both bacteria and cyanobacteria have similar prokaryotic translational and posttranslational processes. Therefore, the overexpressed PsaD protein from *E. coli* should contain structural features similar to the cyanobacterial PsaD before its assembly into the PS I complex. The one-step purification of the overexpressed PsaD protein provides a convenient method to obtain large amounts of pure and functional PsaD. Therefore, the overexpressed PsaD is suitable for detailed structural studies such as X-ray crystallography, NMR, CD spectroscopy and topographical biochemistry. The overexpressed protein is also useful in studying assembly of PsaD into PS I.

The overexpressed PsaD protein was able to assemble into the PsaD-less PS I complex and the incorporation of PsaD into the PsaD-less PS I complex led partial recovery of the photoreduction activity of the chimerical PS I complex. Due to the availability of purified functional soluble PsaD proteins, we could perform ITC experiments to determine thermodynamic and binding parameters of the assembly of PsaD into the complex. This is the first time that the binding constant and binding heat were determined for the assembly of any photosynthetic membrane protein.

The soluble overexpressed PsaD protein provided

Table 2
Secondary structure analysis of the PsaD proteins from *Mastigocladus laminosus* and *Synechocystis* sp. PCC 6803

Protein	Secondary	Prediction from			
		Circular dichroism		Garnier protein structure prediction	Chou–Fasman indices
		singular values method	K2D program		
Mastigocladus laminosus	α-Helix	25%	11%	21%	25%
	β-Sheet		40%	30%	35%
Synechocystis sp. PCC 6803	α-Helix	20%	7%	28%	29%
	β-Sheet		45%	17%	32%

The secondary structure fractions were obtained from the circular dichroism spectra using singular value methods [50], and the K2D program [42,43]. The Garnier protein structure prediction [51] and the Chou–Fasman indices [52] were obtained using GeneWorks software.

an unprecedented opportunity to study structural features of a protein subunit before its assembly into an integral membrane complex and compare the details with the topography of the assembled protein. The modification and limited proteolysis studies on PsaD in the PS I complex and the soluble PsaD protein revealed their different topographical features. For example, the only cysteinyl residue Cys^{66} in PsaD could be modified with biotin-maleimide in the soluble protein while it was not labeled in the PS I complex (Fig. 4). When PsaD assembles into the PS I complex, it interacts with the PsaB, PsaC and PsaL subunits. The residues of PsaD in the interaction region would be exposed in soluble protein, and they would be buried after assembly into the complex which were inaccessible for modification. Therefore, the Cys^{66} residue of PsaD is located in the domain of PsaD that interacts with other subunits. In addition, this study identified several surface-exposed residues of the soluble PsaD and determined two residues may be buried inside the polypeptide.

The topography of the PsaD protein in the PS I complex has been studied by chemical cross-linking [23–26], protease accessibility [21,22,47] and mutagenesis [30,53]. However, the three-dimensional structure or a topographical model of PsaD is still not available. Based on our results and previous studies, here we propose structural features for PsaD that incorporate topographical and functional information.

The N-terminal region of cyanobacterial PsaD contains about 60 residues. The limited proteolysis of the modified PS I complex indicated that the lysyl residues before Glu^{92} can not be labeled by NHS-biotin [24]. In our studies, cleavage at Lys^{21} of PsaD of *M. laminosus* indicated that this lysyl residue is exposed on the surface of soluble protein. Similarly, Cys^{66} is surface-exposed in soluble PsaD protein, but not in PsaD of PS I complex. Therefore, these two residues may be located in the region that interacts with other subunits of the PS I complex. These results suggest that the N-terminal region may interact with other subunits in the assembly of PS I complex. Crystallographic studies have shown that PsaD is positioned in the stromal side of photosynthetic membrane. It contains a single short α-helix located near the common interface with PsaC and the integral membrane core subunits which may be important for the stable association of both PsaC and PsaD with the PS I core [34]. The analysis of the PsaD sequences indicated that a putative α-helix presents in the N-terminal region. We suggest that the Leu^{20}–Thr^{32} residues of PsaD may form the single short α-helix of PsaD which is located near the common interface with PsaC and the membrane-integral core subunits. PsaD shields extramembrane loops of PsaB [29]. Thus, the N-terminal region of PsaD may interact with the PsaB and PsaC subunits.

The central region, from Arg^{72} to Arg^{86} of PsaD in *Synechocystis* sp. PCC 6803, forms the basic domain of PsaD [20]. The mutations in the basic domain disturbed the interaction between PsaD and PsaL [54]. Therefore, the central region, forming a basic domain, may interact with PsaL in the assembly of the PS I complex. This region is crucial for the in vivo accumulation of PsaD [54]. Biochemical analysis has shown that several residues in the C-terminal half of PsaD are exposed on the surface of PsaD [47]. The surface region contains residues that form the ferredoxin binding domain [23,30,53]. In our study, Arg^{114}, Arg^{118} and Arg^{119} of soluble PsaD were accessible to protease. It is likely that they are exposed in the surface of PS I complex as well. These residues may be involved in the electrostatic interactions with the acidic residues of ferredoxin. The deletions of the C-terminus of PsaD impaired the reductase activity of PS I in the ferredoxin-mediated $NADP^+$ photoreduction [11]. The Lys^{129} and Lys^{131} residues in the C-terminus of PsaD were not exposed on the surface. Therefore, the C-terminal region of PsaD may position the ferredoxin-binding domain properly and its deletion could cause the observed defect in ferredoxin-mediated electron transfer. Alternatively, the C-terminus could be involved directly in ferredoxin docking.

In summary, we expressed the *psaD* gene from *M. laminosus* to obtain the soluble PsaD protein, and identified the topographical features of the functional PsaD protein. The topographical and circular dichroism spectrometric studies presented here and the available information were used to propose a topographical model for the interaction of PsaD with other proteins which will greatly benefit the structural studies of PsaD protein.

Acknowledgements

We thank Calorimetry Sciences Corporation for the ITC measurements. We also acknowledge Vaishali P. Chitnis for determining the N-terminal amino acid sequence of native PsaD from the purified PS I complexes from *M. laminosus*. We thank Dr. Louisa B. Tabatabai for help with circular dichroism spectrometry and Dr. Rachel Nechushtai for insightful discussions. This work is supported in part by a grant from the National Institutes of Health (GM53104), Journal Paper No. J-17501 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3416, and supported by Hatch Act and State of Iowa funds.

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