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Organization of Photosystem I Polypeptides[1]

Identification of PsaB Domains That May Interact with PsaD

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PsaA and PsaB are homologous integral membrane-proteins that form the heterodimeric core of photosystem I (PSI). We used subunit-deficient PSI complexes from the mutant strains of the cyanobacterium *Synechocystis* sp. PCC 6803 to examine interactions between PsaB and other PSI subunits. Incubation of the wild-type PSI with thermolysin yielded 22-kD C-terminal fragments of PsaB that were resistant to further proteolysis. Modification of the wild-type PSI with *N*-hydroxysuccinimidobiotin and subsequent cleavage by thermolysin showed that the lysyl residues in the 22-kD C-terminal domain were inaccessible to modification by *N*-hydroxysuccinimidobiotin. The absence of PsaE, PsaF, PsaI, PsaJ, or PsaL facilitated accumulation of the 22-kD C-terminal fragments of PsaB but did not alter their resistance to further proteolysis. When the PsaD-less PSI was treated with thermolysin, the 22-kD C-terminal fragments of PsaB were rapidly cleaved, with concomitant accumulation of a 16-kD fragment and then a 3.4-kD one. We mapped the N termini of these fragments by N-terminal amino acid sequencing and the C termini from their positive reaction with an antibody against the C-terminal peptide of PsaB. The cleavage sites were proposed to be in the extramembrane loops on the cytoplasmic side. Western blot analyses showed resistance of PsaC and PsaI to proteolysis prior to cleavage of the 22-kD fragments. Therefore, we propose that PsaD shields two extramembrane loops of PsaB and protects the C-terminal domain of PsaB from in vitro proteolysis.

PSI of cyanobacteria and chloroplasts is a multisubunit membrane-protein complex that catalyzes electron transfer from reduced plastocyanin (or Cyt c_6) to oxidized Fd (or flavodoxin) (Chitnis and Nelson, 1991; Bryant, 1992; Golbeck, 1993; Chitnis et al., 1995). PsaA and PsaB, the 82-kD transmembrane subunits of PSI, form a heterodimeric core that harbors approximately 100 antenna Chl *a* molecules, the primary reaction center P700, and a chain of electron acceptors A_0, A_1, and F_X. In addition, PsaA and PsaB may directly interact with plastocyanin or Cyt c_6 (Kuhn et al., 1994; Xu et al., 1994e). The PsaC subunit binds the terminal electron acceptors F_A and F_B, each a [4Fe-4S] cluster. In addition to PsaA, PsaB, and PsaC, the PSI complex contains at least eight other proteins in cyanobacteria (PsaD, PsaE, PsaF, PsaL, PsaK, PsaJ, PsaI, and PsaM) and three additional ones in chloroplasts (PsaG, PsaH, and PsaN) (Chitnis et al., 1995). These PSI proteins do not bind any redox centers, but some have important functions in docking of soluble electron carriers or in organization of the complex (Chitnis et al., 1995).

X-ray crystallographic analysis of the PSI complex from the thermophilic cyanobacterium *Synechococcus* sp. has been used to propose a model for its three-dimensional structure at 6-Å resolution (Krauss et al., 1993). Each monomer of the trimeric PSI complex consists of a "catalytic" domain and a smaller "connecting" domain that links the monomers. The electron density could be fitted to include the three [4Fe-4S] clusters F_X, F_A, and F_B, 28 α-helices, and 45 Chl *a* molecules. The PsaC, PsaD, and PsaE are peripheral subunits that are located on the *n* side (stromal in chloroplasts and cytoplasmic in cyanobacteria) of the photosynthetic membranes with PsaC positioned in the center of each monomeric PSI on a local pseudo-2-fold axis (Krauss et al., 1993; Kruip et al., 1993). Additional information about the organization of PSI subunits comes from various biochemical studies (Chitnis et al., 1995). Specific domains of the PsaD, PsaE, and PsaL subunits are exposed to proteases (Lagoutte and Vallon, 1992; Zilber and Malkin, 1992; Xu et al., 1994b). Cross-linking and in vitro reconstitution experiments have revealed that PsaD, PsaE, and PsaC are in contact with each other and that a considerable part of PsaC is buried under PsaD and PsaE (Oh-oka et al., 1989; Li et al., 1991; Chitnis and Nelson, 1992). Topological characterization of subunit-deficient cyanobacterial mutants has revealed additional structural interactions among PsaE, PsaF, or PsaJ (Xu et al., 1994d, 1994e), PsaD and PsaL (Xu et al., 1994a), or PsaL and PsaI (Q. Xu and P.R. Chitnis, unpublished data).

Deduced amino acid sequences of PsaA and PsaB from cyanobacteria to higher plants show a high degree of similarity and many common structural features. Among them, the conserved motif FPCDGPGRGGTC serves as the binding site for the [4Fe-4S] cluster F_X (Golbeck, 1993). Based on hydropathy analyses, PsaA and PsaB have been predicted to contain 11 hydrophobic helices each, connected by hydrophilic loops (Chitnis et al., 1995). At least eight of the hydrophobic domains form transmembrane helices and one may be oriented parallel to the membrane (Krauss et al., 1993; Kuhn et al., 1994). The positioning of

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Abbreviation: NHS-biotin, *N*-hydroxysuccinimidobiotin.

extramembrane loops in PsaA-PsaB relative to the photosynthetic membranes has been examined using "on-grid" immunolabeling with domain-specific antibodies (Vallon and Bogorad, 1993) and accessibility to proteolysis (Zilber and Malkin, 1992; Vallon and Bogorad, 1993). The domains of PsaA and PsaB that interact with other PSI subunits have not been identified.

We previously reported that thermolysin treatment of the PsaF-PsaJ-less PSI complexes from a cyanobacterial mutant strain results in the accumulation of a C-terminal domain of PsaB that is resistant to further proteolysis (Xu et al., 1994e). This domain contains the F_X-binding loop and may be important in interactions between PsaA and PsaB (Golbeck, 1993; Smart et al., 1993). The resistance of the C-terminal domain of PsaB to in vitro proteolysis may be due to the folded structure of PsaB or may result from shielding by smaller PSI subunits. To test these possibilities, we investigated the topology of the C-terminal domain of PsaB in PSI complexes from the wild-type and mutant strains of the cyanobacterium *Synechocystis* sp. PCC 6803 that lack PsaD, PsaE, PsaF-PsaJ, or PsaL by mapping the protease cleavage sites. We also examined the accessibility of the C-terminal domain of PsaB to modification by NHS-biotin.

MATERIALS AND METHODS

Materials

Prestained protein molecular weight standards were from GIBCO-BRL. Thermolysin (protease type X from *Bacillus thermoproteolyticus*; EC 3.4.24.4), avidin-peroxidase, and NHS-biotin were from Sigma. The polyvinylidene difluoride (Immobilon-P) membranes were from Millipore. The enhanced chemiluminescence reagents for western blotting were from Amersham. 3,3′,5,5′-Tetramethylbenzidine, a chromogenic substrate for horseradish peroxidase, was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Other chemicals were purchased from Sigma or Fisher Scientific.

Cyanobacterial Strains and Culture

Strains of the cyanobacterium *Synechocystis* sp. PCC 6803 that were used in the present study are listed in Table I. Cyanobacterial cultures of wild-type and mutant strains were grown in BG-11 with or without Glc (5 mM) and selective antibiotics (40 μg/mL kanamycin or 30 μg/mL chloramphenicol) under a light intensity of 21 μmol m^{-1} s^{-1}. Cells at the late exponential phase of growth were harvested and resuspended in 0.4 M Suc, 10 mM NaCl, 5 mM PMSF, 5 mM benzamidine, and 10 mM Mops, pH 7.0, for isolation of thylakoids.

Isolation of the Photosynthetic Membranes and PSI Complexes

Photosynthetic membranes were isolated after cell breakage with a bead beater (Chitnis and Chitnis, 1993). To isolate PSI, the photosynthetic membranes were solubilized with Triton X-100, followed by DEAE-cellulose chromatography and Suc gradient centrifugation (Reilly et al., 1988). The PSI isolated by this procedure is functional as a light-driven Cyt c_6-Fd oxidoreductase (Xu et al., 1994e). Chl concentrations in the photosynthetic membranes and PSI complexes were determined in 80% (v/v) acetone (Arnon, 1949). The isolated photosynthetic membranes or PSI were stored at −20°C until use.

Treatment of PSI with Thermolysin

To study the accessibility of PSI subunits to proteases, purified wild-type and mutant PSI complexes at 150 μg Chl/mL were incubated with thermolysin at a concentration of 20 or 40 μg protease/mg Chl in the presence of 5 mM $CaCl_2$ at 37°C. Thermolysin primarily hydrolyzes peptide bonds on the N-terminal side of hydrophobic amino acid residues (Matsubara, 1970). The proteolytic reactions were terminated with 20 mM EDTA.

Table I. *Strains of Synechocystis sp. PCC 6803 that were used in this study*

Strain	Description	Characteristics of the Purified PSI Complexes
Wild type	Glc-tolerant strain	Eleven subunits resolved upon electrophoresis (Xu et al., 1994c); capable of light-driven Cyt c_6 oxidation and Fd reduction
AEK2	*psaE* replaced by a kanamycin-resistance gene (Chitnis et al., 1989); strain deficient in cyclic electron flow (Chitnis et al., 1995)	Only PsaE missing (Chitnis et al., 1989); drastically decreased Fd-mediated $NADP^+$ photoreduction (Xu et al., 1994c)
ADC4	*psaD* replaced by a chloramphenicol-resistance gene (Cohen et al., 1993)	PsaD and PsaL missing (Xu et al., 1994a); no Fd-mediated $NADP^+$ photoreduction (Xu et al., 1994c)
AFK6	*psaF* replaced by a kanamycin-resistance gene (Chitnis et al., 1991); *psaJ* transcriptionally inactivated (Xu et al., 1994e)	PsaF and PsaJ missing and level of PsaE decreased (Chitnis et al., 1991; Xu et al., 1994e); normal rate of P700 rereduction by Cyt c_6 (Xu et al., 1994e)
ALC7–3	*psaL* replaced by a chloramphenicol-resistance gene (Chitnis et al., 1993)	Only PsaL missing (Chitnis et al., 1993; Chitnis and Chitnis, 1993); no major effect on electron transport activity; no PSI trimers (Chitnis and Chitnis, 1993)

Modification of PSI Subunits with NHS-Biotin

Modification of protein with NHS-biotin has been used to examine exposure of protein surfaces to the aqueous phase (Bayer and Wilchek, 1990; Frankel and Bricker, 1992). The wild-type and PsaE-less PSI complexes at 150 μg Chl/mL were incubated with 58 μM NHS-biotin, 10 mM Mops (pH 7.0), 0.05% Triton X-100, and 0.05% DMSO for 30 min at room temperature (Xu et al., 1994b). The reaction was quenched with 50 mM ammonium bicarbonate (pH 7.8). The biotinylated PSI was proteolytically cleaved by thermolysin. PSI subunits were separated by Tricine-urea-SDS-PAGE and electroblotted to Immobilon-P membranes. The blot was probed with an avidin-peroxidase conjugate and developed with hydrogen peroxide and 4-chloro-1-naphthol (Frankel and Bricker, 1992).

Analytical Gel Electrophoresis and Immunodetection

Proteins in the isolated PSI complexes were solubilized and resolved by a modified Tricine-urea-SDS-PAGE (Xu et al., 1994c) for better resolution of the PSI subunits. After electrophoresis, gels were stained with Coomassie blue or silver. Alternatively, proteins were electroblotted to Immobilon-P membranes. Immunodetection was performed using enhanced chemiluminescence except where otherwise indicated. The antibodies against PsaC and PsaD were from Dr. John H. Golbeck (University of Nebraska, Lincoln). The anti-$PsaB_c$ antibody was raised using a peptide corresponding to the Y^{720}AAFLIASTSGKFG^{733} sequence at the C terminus of pea PsaB (Henry et al., 1992) and was provided by Dr. James A. Guikema (Kansas State University). Antibodies against PsaE were generated against the protein from *Synechocystis* sp. PCC 6803 (Xu et al., 1994a). The antibody against PsaI was generated using GSH-S-transferase-PsaI fusion protein that was expressed in *Escherichia coli* (Q. Xu and P.R. Chitnis, unpublished data).

N-Terminal Amino Acid Sequencing

For N-terminal amino acid sequencing, the peptides were separated by electrophoresis, blotted to Immobilon-P membranes, stained with Coomassie blue containing 1% acetic acid for 3 min, destained with 50% methanol, and rinsed extensively with deionized water. N-terminal sequences for the peptides immunoreactive with the anti-$PsaB_c$ antibody were determined at the Biotechnology Core Facility of Kansas State University or at the Protein Chemistry Core Facility of Baylor College of Medicine.

RESULTS

The C-Terminal 22-kD Domain of PsaB in the Wild-Type PSI Is Resistant to Proteolysis

When the subunits of the wild-type PSI preparation were separated by Tricine-urea-SDS-PAGE, 11 proteins were resolved (Fig. 1A). A clear separation of all PSI subunits allowed us to examine the relative accessibility of PSI proteins to proteases. In the wild-type PSI complex, there was a differential sensitivity among PSI subunits to thermolysin (Fig. 1A). Incubation of wild-type PSI complexes with 20 μg thermolysin/mg Chl resulted in a progressive cleavage of the PsaA-PsaB polypeptides. Among them, BI and BII fragments accumulated prior to a significant proteolysis of other PSI subunits. The BI and BII fragments could not be extracted by 3 M NaI, the concentration at which PsaD, PsaE, and PsaC were completely removed (data not shown). Among the remaining PSI subunits, PsaK was notably susceptible to cleavage by thermolysin. Spinach PsaK is also susceptible to thermolysin (Zilber and Malkin, 1992). When the proteolytically cleaved fragments were probed with the anti-$PsaB_c$ antibody, and the antigen-antibody complexes were visualized by enhanced chemiluminescence, both BI and BII fragments were immunoreactive (Fig. 1B). N-terminal amino acid sequencing of these fragments has revealed that BI and BII correspond to I^{482} . . . ^{731} and I^{498} . . . G^{731} fragments of PsaB, respectively (Xu et al., 1994e). The BI fragment accumulated prior to the BII fragment, whereas the latter resisted further cleavage during prolonged incubation with the protease. The C-terminal fragments of PsaB accumulated prior to significant proteolysis of smaller PSI subunits, including PsaD (Fig. 1). Therefore, shielding by smaller PSI subunits may be responsible for resistance of the C-terminal domain of PsaB to in vitro proteolysis.

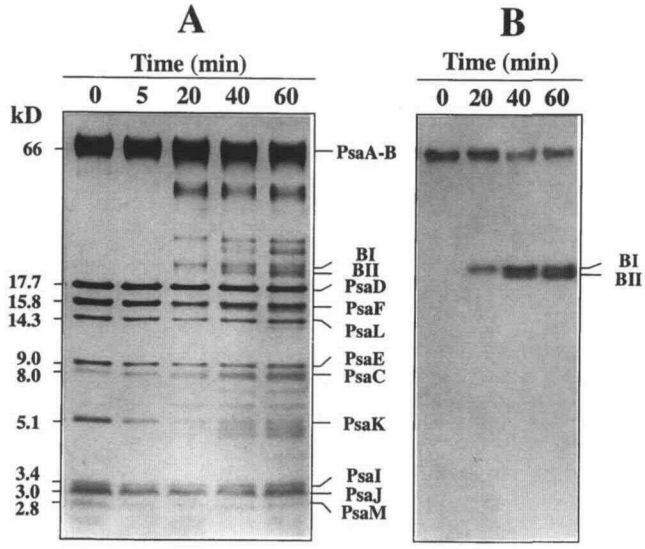

Figure 1. Accessibility of wild-type PSI subunits to digestion by thermolysin and accumulation of the C-terminal domain of PsaB. A, The wild-type PSI was digested with thermolysin at 20 μg protease/mg Chl for 0, 5, 20, 40, and 60 min. The samples equivalent to 5 μg of Chl were analyzed by Tricine-urea-SDS-PAGE. The gel was silver stained. The identification of PSI subunits is based on mutational analyses of PSI subunits, immunoreaction, or N-terminal amino acid sequencing (Xu et al., 1994c). Thermolysin migrated at 32 kD. The molecular masses of PSI subunits were determined from migration of the following protein markers: insulin (2.9 kD), bovine trypsin inhibitor (6.2 kD), lysozyme (14.3 kD), β-lactoglobulin (18.4 kD), carbonic anhydrase (29 kD), ovalbumin (43 kD), and BSA (68 kD). B, The resolved proteins in the above samples were electroblotted to Immobilon-P membranes and probed with the anti-$PsaB_c$ antibody. The immunoreaction was visualized by enhanced chemiluminescence.

The C-Terminal Domain of PsaB in PsaE-, PsaL-, or PsaF-PsaJ-less PSI Complexes Is Also Resistant to Proteolysis

To identify the PSI subunits that may shield the C-terminal domain of PsaB from in vitro proteolysis, PsaE-less, PsaF-PsaJ-less, or PsaL-less PSI complexes were purified from cyanobacterial mutant strains (Table I). These complexes are functional and stable. Incubation of the wild-type and subunit-deficient PSI complexes with thermolysin yielded a series of similar cleavage products from the PsaA-PsaB proteins (Xu et al., 1994c). When we probed these fragments with the anti-$PsaB_c$ antibody and visualized the antigen-antibody complexes by enhanced chemiluminescence, a 22-kD species was immunoreactive in the wild type (Fig. 2). The absence of PsaE, PsaF, PsaJ, or PsaL led to increased accumulation of the 22-kD fragment but it did not impair its resistance from further proteolysis (Fig. 2). When the mutant PSI complexes were treated with a higher concentration of thermolysin, such as 40 μg thermolysin/mg Chl, the 22-kD species accumulated as the predominant PsaB degradation product that contained C-terminal epitope (data not shown). Therefore, PsaE, PsaF, PsaJ, or PsaL does not shield the 22-kD C-terminal domain of PsaB from thermolysin.

The C-Terminal Domain of PsaB Cannot Be Modified with NHS-Biotin

Sequence analysis of the PSI proteins indicates that PsaB has 20 lysyl residues, eight of which are in the protease-resistant, 22-kD C-terminal domain (Smart and McIntosh, 1991). NHS-biotin reacts with the N terminus and the ϵ-amino group of lysyl residues (Bayer and Wilchek, 1990). Modification of a protein with NHS-biotin has been used to probe the protein surfaces exposed to the aqueous phase (Frankel and Bricker, 1992; Xu et al., 1994b). When the wild-type PSI was labeled with NHS-biotin and the blot was subsequently probed with avidin-peroxidase, PsaA-PsaB, PsaD, PsaF, PsaL, PsaE, and PsaC were biotinylated (Fig. 3A). Since thermolysin cleavage of PsaE-less PSI complexes resulted in an extensive accumulation of the C-terminal fragment of PsaB (Fig. 2), the proteolytically cleaved PsaE-less complexes were used to detect biotinylation of the C-terminal domain of PsaB. The absence of PsaE did not significantly alter the biotinylation patterns of PSI subunits (Fig. 3A). To determine whether any lysyl residue(s) in the C-terminal protease-resistant domain of PsaB can be modified by NHS-biotin, the biotinylated PsaE-less PSI was exposed to thermolysin. Protease digestion resulted in a series of PsaA-PsaB cleavage products (Fig. 3B), including the 22-kD fragments that were immunoreactive with the anti-$PsaB_c$ antibody. When the blot was probed with avidin-peroxidase and the reaction was detected using a chromogenic substrate, neither of the C-terminal PsaB fragments could be visualized, indicating that the lysyl residues in these fragments were not biotinylated. In contrast, many other cleavage products of PsaA-PsaB were labeled with NHS-biotin. The inability of NHS-biotin to react with the lysyl residues in the C-terminal domain of the PsaB was consistent with the resistance of this domain to proteolysis (Fig. 2).

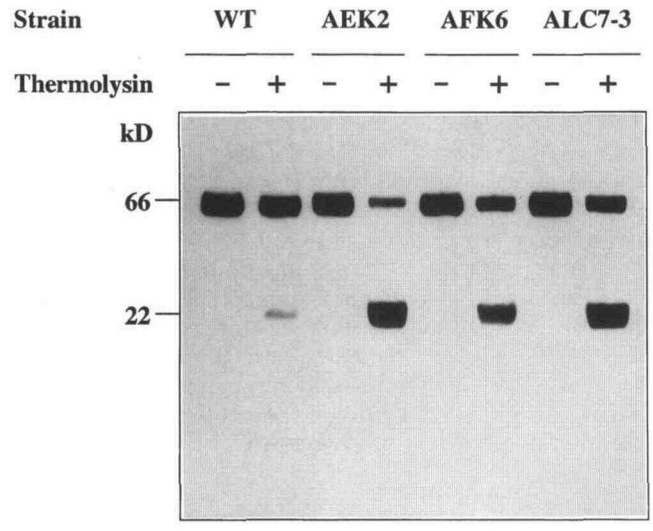

Figure 2. Western blot analysis of PsaB cleavage products upon treatment of the wild-type (WT) and mutant PSI with thermolysin. The wild-type and mutant PSI were digested with thermolysin at 20 μg protease/mg Chl for 30 min. The thermolysin-treated PSI preparations (5 μg of Chl per lane) were analyzed using Tricine-urea-SDS-PAGE. The separated proteins were transferred to Immobilon-P membranes and probed with the anti-$PsaB_c$ antibody. The immunoreaction was visualized by enhanced chemiluminescence.

The C-Terminal 22-kD Domain of PsaB Can Be Degraded Further in the PsaD-less PSI

We examined protease accessibility of the C-terminal domain of PsaB in the PsaD-less PSI, which retains the wild-type levels of PsaE and PsaC (Xu et al., 1994a). Incubation of the wild-type PSI with thermolysin resulted in a progressive cleavage of PsaB with concomitant accumulation of the 22-kD C-terminal domain of PsaB (Fig. 4A). Extended treatment with thermolysin yielded a small amount of a 16-kD fragment that contained the antigenic site at the C terminus of PsaB. In contrast, exposure of PsaD-less PSI to thermolysin caused a rapid proteolysis of the 22-kD C-terminal domain of PsaB with a concomitant accumulation of the 16-kD species (labeled BIII). With a longer exposure to thermolysin, a 3.4-kD fragment (labeled BIV), which was immunoreactive with the anti-$PsaB_c$ antibody, steadily accumulated. An examination of intensity of the BIII and BIV fragments and their sequential accumulation suggested that proteolysis in the N-terminal region of BIII fragment yielded the BIV fragment. The antibody used here would not recognize proteolytic products without the C-terminal epitope. This observation suggested that shielding by PsaD, rather than the intrinsic folding of the PsaA-PsaB core, made the C-terminal domain of PsaB inaccessible to thermolysin or NHS-biotin.

PsaD, PsaE, and PsaC are closely associated with each other on the *n* side of thylakoids (Oh-oka et al., 1989). Based on molecular modeling, PsaC has been proposed to interact with the F_X-binding motif in PsaA-PsaB (Rodday et al., 1993). We assessed the roles of PsaE and PsaC in shielding of the C-terminal domain of PsaB in the PsaD-less PSI by examining the degradation of PsaE and PsaC (Fig.

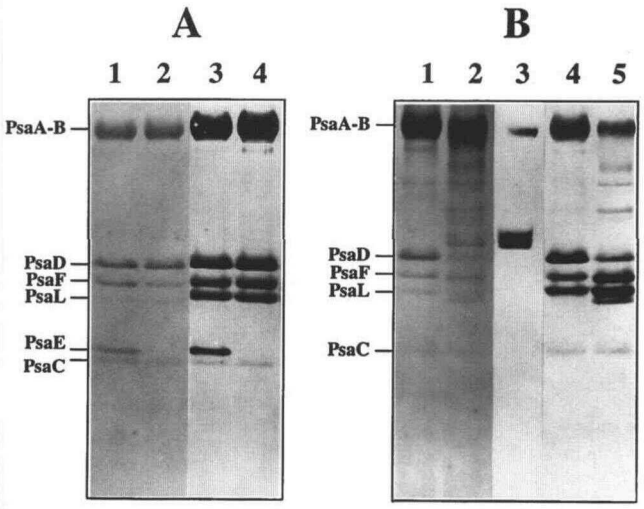

Figure 3. Labeling of the wild-type and PsaE-less PSI by NHS-biotin. A, The biotinylated wild-type (lane 1) and PsaE-less (lane 2) PSI equivalent to 10 μg of Chl were solubilized. The proteins were separated by Tricine-urea-SDS-PAGE, electroblotted on Immobilon-P membranes, and stained with Coomassie blue. A replica of proteins in lanes 1 and 2 (lanes 3 and 4, respectively) were probed with avidin-peroxidase conjugate and the immunoreaction was visualized using 4-chloro-1-naphthol and hydrogen peroxide. B, The biotinylated PsaE-less PSI (lane 1) was treated with thermolysin as in Figure 1 for 30 min (lane 2) and samples containing 10 μg of Chl were solubilized. The proteins were separated by Tricine-urea-SDS-PAGE, electroblotted on Immobilon-P membranes, and stained with Coomassie blue. The proteins in the thermolysin-cleaved, biotinylated PsaE-less PSI containing 1 μg of Chl were separated by electrophoresis and probed with the anti-PsaB$_c$ antibody (lane 3). Replicas of lanes 1 and 2 (in lanes 4 and 5, respectively) were probed with avidin-peroxidase conjugate. The immunoreaction was visualized using 4-chloro-1-naphthol and hydrogen peroxide.

4A). Immunodetection using an antibody against PsaE revealed that PsaE in the wild-type and PsaD-less PSI was not significantly degraded by thermolysin (Fig. 4A). Therefore, the exposure of additional thermolysin cleavage sites in PsaB of PsaD-less PSI was not due to degradation of PsaE. We also immunodetected the level of PsaC in the proteolytically cleaved wild-type and PsaD-less complexes. Although there was an increased degradation of PsaB following protease treatment of the wild-type PSI (Fig. 2), PsaC was not cleaved by thermolysin (Fig. 4A). PsaC in the PsaD-less PSI was also resistant to in vitro proteolysis (Fig. 4A). Similarly, PsaI was also resistant to thermolysin cleavage in the PsaD-less PSI, prior to accumulation of BIII and BIV fragments (Fig. 4A). Therefore, susceptibility of the 22-kD C-terminal domain of PsaB to proteolysis in the PsaD-less PSI was due to the absence of PsaD but not to altered degradation of PsaE, PsaC, or PsaI by thermolysin.

The positive immunoreaction of BIII and BIV fragments to the anti-PsaB$_c$ antibody showed that these fragments have the C-terminal epitope. We estimated the N termini of the BIII and BIV fragments from their apparent mass and from their N-terminal amino acid sequences. The 16- and 3.4-kD fragments were identified by western blotting as illustrated in Figure 4B prior to subjecting them to N-terminal amino acid sequence analysis. A single lane of resolved peptides was bisected and the half-lane was probed with the anti-PsaB$_c$ antibody and the other half was stained with Coomassie blue to position the peptide. N-terminal amino acid sequencing of the 16- and 3.4-kD peptides revealed identifiable sequences (Table II). Since the BIII and BIV fragments were recognized by anti-PsaB$_c$ antibody, the amino acid residue at each sequencing cycle should match the deduced sequence in the C-terminal domain of PsaB (Smart and McIntosh, 1991). N-terminal amino acid analysis of the 16-kD peptide was performed three times at two protein-sequencing facilities. The results suggested that the 16-kD band consisted of a mixture of peptides. N-terminal amino acid sequences of one peptide corresponded to A^{307}HKGPLTGAG of PsaB. The A^{307} . . . G^{731} fragment has a predicted molecular mass of 47.2 kD (Table II) and would migrate with an apparent molecular mass much higher than 16 kD. Similarly, the peptide bond before I^{482} is the thermolysin cleavage site on PsaB that is

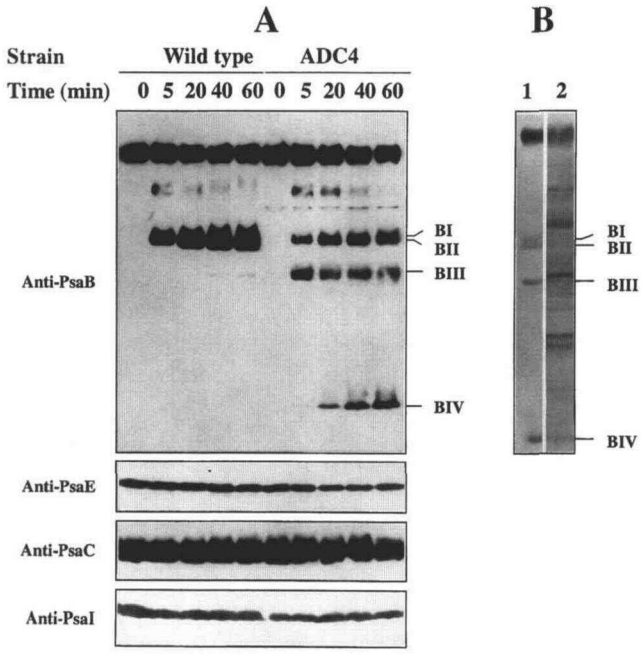

Figure 4. Accumulation and identification of species containing the C terminus of PsaB following proteolysis of PsaD-less PSI. A, The PsaD-less PSI complex at 150 μg Chl/mL was cleaved with 40 μg thermolysin/mg Chl for 0, 5, 20, 40, and 60 min. Proteins in the proteolytically cleaved PSI complex containing 5 μg of Chl were resolved, blotted to Immobilon-P membranes, and probed with antibodies against the C terminus of PsaB, PsaE, PsaC, or PsaI. The immunoreaction was visualized by enhanced chemiluminescence. B, The PsaD-less PSI complex was cleaved as above for 30 min. Proteins in the proteolytically cleaved PSI complex containing 20 μg of Chl were separated by electrophoresis and electroblotted onto Immobilon-P membranes. To precisely determine a peptide of interest, the Immobilon-P membranes were split. A portion was either probed with the anti-PsaB$_c$ antibody (lane 1) or stained with Coomassie blue containing 1% acetic acid (lane 2). The immunoreaction was visualized using a chromogenic substrate of horseradish peroxidase. The BIII and BIV fragments in lane 2 were excised for N-terminal amino acid sequencing.

Table II. *N-terminal amino acid sequence analysis of the thermolysin-generated BIII and BIV fragments as described in Figure 4*

The position of the first amino acid was identified after comparison with the deduced amino acid sequence of PsaB (Smart and McIntosh, 1991).

Thermolysin-Generated Fragments	N-Terminal Amino Acid Sequence[a]	Position of the First Amino Acid	Apparent Mass[d]	Predicted Mass[e]
			kD	*kD*
BI[b]	IASTTG	482	21.9	28.050
BII[b]	INSGINS	498	20.7	26.442
16-kD peptide[c]	AHKGPLTGAG	307	16	47.205
BIII[c]	LXKGXLXSXG	531	16	23.049
BIV	LVRXKDKPVA	687	3.4	4.844

[a] The standard single-letter codes for the amino acid residues are used. X indicates positions where unambiguous amino acid assignments could not be made. [b] See Xu et al. (1994e). [c] These sequences were verified by Dr. Richard Cook at Protein Chemistry Core Facility of Baylor College of Medicine. [d] Average of four independent estimates from the electrophoretic mobility on Tricine-urea-SDS-PAGE. [e] The mass is predicted with an assumption that the proteolytic fragment retains the C-terminal epitope.

exposed early during proteolysis (Table II). Therefore, the 16-kD peptide was co-purified with BIII and might have resulted from cleavage of PsaB at two sites, one at A^{307} and the other at or before I^{482}.

Another 16-kD peptide had an N-terminal amino acid sequence of LXKGALXSXG, with X indicating positions where unequivocal amino acid assignments could not be made. This sequence may correspond to the L^{531}IKGAL sequence, which is predicted to be in the region prior to the F_X-binding domain in PsaB (Fish et al., 1985). We propose that the L^{531} . . . G^{731} peptide represents the BIII fragment. First, the L^{531} . . . G^{731} peptide has a predicted mass of 23 kD. PsaB and its hydrophobic fragments have anomalous electrophoretic migration, leading to underestimation of apparent molecular mass (Fish et al., 1985; Fish and Bogorad, 1986; Zilber and Malkin, 1992; Vallon and Bogorad, 1993). Second, a major portion of the F_X-binding domain that follows Leu^{531} lacks thermolysin cleavage sites. A cleavage after the F_X-binding sequence would result in a smaller peptide. Third, some amino acids in the F_X-binding motif have been predicted to interact with PsaC, which was resistant to cleavage by thermolysin in the PsaD-less PSI (Fig. 4A). Therefore, L^{531} . . . G^{731} is the most plausible identification of the BIII fragment. The low abundance of the L^{531} . . . G^{731} peptide in the 16-kD band may represent less accumulation of BIII because of its subsequent cleavage to yield the BIV fragment. The N-terminal amino acid sequence of BIV fragment corresponded to L^{687}VRWKDKPVA of PsaB. This domain is predicted to be in the extramembrane loop on the *n* side of thylakoids between the putative transmembrane helices X and XI (Fish et al., 1985).

DISCUSSION

PSI is a multisubunit enzyme that contains at least 11 polypeptides in cyanobacteria and 3 additional ones in higher plants (Chitnis et al., 1995). Despite the recently published x-ray diffraction analysis of PSI crystals at 6-Å resolution (Krauss et al., 1993), details of subunit interactions and topography remain largely unknown. Accessibility of PSI subunits to proteases has been used as a criterion to analyze PSI organization (Zilber and Malkin, 1992). Here, we show that a 22-kD C-terminal domain of PsaB is inaccessible to proteases and NHS-biotin. We also show that PsaD shields this PsaB domain from thermolysin.

The structure of cyanobacterial PSI complex at 6-Å resolution indicates that PsaA and PsaB have at least nine α-helices each, eight of which span a lipid bilayer (Krauss et al., 1993). The domains of PsaA-PsaB that interact with other PSI subunits have not been identified. When the wild-type PSI complexes were treated with thermolysin or with NHS-biotin, the 22-kD C-terminal domain of PsaB was inaccessible to both probes. A similar fragment was detected when spinach thylakoids were treated with trypsin (Vallon and Bogorad, 1993). The C-terminal domain is highly conserved among cyanobacteria and higher plants, thus reflecting its importance in function or in structural stability. It contains four putative transmembrane helices and loops joining them (Fig. 5). Among these loops, the loop joining helices VIII and IX contains the motif FPCDGPGRGGTC that binds the [4Fe-4S] cluster F_X (Smart et al., 1993; Warren et al., 1993). From the comparison of reaction centers of PSI, PSII, heliobacteria, green-sulfur bacteria, and purple bacteria, it has been proposed that C-terminal halves of PsaA and PsaB may be crucial in coordinating the electron transfer centers (Vermaas, 1994). The electron transfer centers of PSI are highly electronegative, making them susceptible to oxidative damage. Therefore, the inaccessibility of the C-terminal domain, which binds at least some of the electron transfer centers, may have a vital role in the function and stability of PSI.

The C-terminal domain of PsaB may be shielded from proteases and NHS-biotin by peripheral subunits or the extramembrane loops of integral proteins of PSI. To determine which subunits of PSI interact with the conserved C-terminal domain of PsaB, the subunit-deficient PSI complexes were treated with thermolysin, and the degradation products were probed with the anti-$PsaB_c$ antibody and

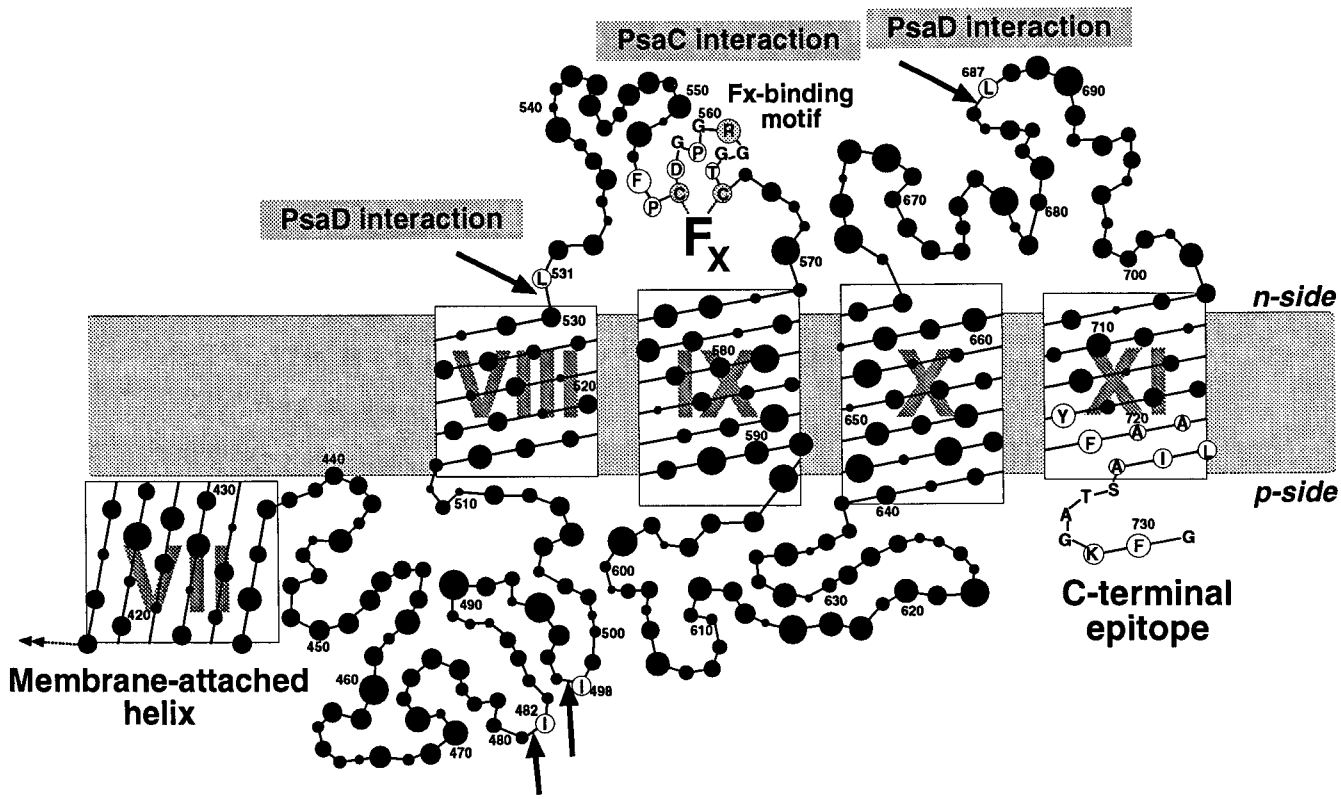

Figure 5. A topological model for the C-terminal domain of PsaB. The putative transmembrane helices are based on the hydropathy analyses of PsaB from *Synechocystis* sp. PCC 6803. Only the amino acids mentioned in "Discussion" are identified. Other amino acids are shown as filled circles, with diameters proportional to their volumes. Helices have been numbered according to the 11-helix model of Fish et al. (1985). The membrane-attached helix has been proposed by Kuhn et al. (1994). The model shows the F_X-binding motif (Smart et al., 1993; Warren et al., 1993) and its interaction with PsaC (Rodday et al., 1993), accessible thermolysin cleavage sites (arrows) (Table II), the epitope of the anti-$PsaB_C$ antibody (Henry et al., 1992), and the proposed PsaD-interacting domains. The amino acid numbers are for the sequence of PsaB from *Synechocystis* sp. PCC 6803 (Smart and McIntosh, 1991).

subjected to mapping by N-terminal amino acid sequencing. The thermolysin-resistant, C-terminal, 22-kD domain of PsaB accumulated in the absence of PsaE, PsaF-PsaJ, or PsaL (Fig. 2). PsaK was easily accessible to cleavage by thermolysin (Fig. 1) and other proteases (Zilber and Malkin, 1992). Therefore, PsaE, PsaF, PsaJ, PsaL, or PsaK may not be crucial in shielding the C-terminal region of PsaB. The increased accessibility of the C-terminal PsaB domain to thermolysin in the absence of PsaD strongly suggested a direct role of PsaD in protecting this domain. Electron paramagnetic resonance studies of thylakoids from the PsaD-less strain has revealed that PsaC is correctly oriented on the PSI core (Y.-S. Jung, V.P. Chitnis, P.R. Chitnis, and J.H. Golbeck, unpublished data). The absence of PsaD causes loss of PsaL during isolation of PSI complex (Xu et al., 1994a). PsaL-less PSI retains a wild-type level of other PSI subunits (Chitnis et al., 1993) and the absence of PsaL has little impact on protease resistance of the 22-kD C-terminal domain of PsaB (Fig. 2). Taken together, the enhanced protease accessibility of the C-terminal domain of PsaB in the PsaD-less PSI can be directly attributed to the absence of PsaD and is not due to gross topological alterations in PsaB or other subunits.

N-terminal amino acid sequencing of the BIII and BIV fragments mapped the thermolysin cleavage sites at Leu^{531} and Leu^{687}, respectively (Table II). These cleavage sites may be located on the *n* side of photosynthetic membranes. Since the absence of PsaE had little impact on proteolysis of the C-terminal domain of PsaB (Fig. 2) and since the absence of PsaD did not alter the resistance of PsaC to proteolysis (Oh-oka et al., 1989; Zilber and Malkin, 1992) prior to significant accumulation of BIII and BIV fragments containing a C-terminal epitope (Fig. 4), it is possible that PsaD is positioned in close proximity to the thermolysin cleavage sites in two extramembrane loops, thus shielding them from proteolytic cleavage (Fig. 5). Alternatively, the presence of PsaD may simply impede the access of proteases to the extramembrane loops. The VIII-IX loop also includes the F_X-binding motif, which is on the stromal surface (Krauss et al., 1993). It has been postulated that PsaC may interact with a surface-exposed domain in the immediate vicinity of the F_X-binding domain (Rodday et al., 1993). The BIV fragment, which contains the last predicted transmembrane helix XI (Fish et al., 1985), exhibited a high degree of resistance from further proteolysis (Fig. 4), although it

contains 13 thermolysin-sensitive sites based on the deduced sequence of PsaB (Smart and McIntosh, 1991).

The results presented here, along with previously published data and hypotheses, can be integrated to propose a topographical model for the C-terminal domain of PsaB (Fig. 5). It has been proposed that the helix VII (nomenclature according to the 11-helix model of Fish et al., 1985) is a membrane-attached helix (Kuhn et al., 1994). From its proposed role in plastocyanin docking, the VII helix is likely to be on the luminal surface. The VII-VIII loop contains cleavage sites at Ile^{482} and Ile^{498} that are accessible to thermolysin (Xu et al., 1994e), indicating that at least parts of this loop are exposed on the surface. The VII-VIII loop is separated from the F_X-binding domain by one putative transmembrane helix VIII (Fish et al., 1985; Kirsch et al., 1986). The VIII-IX loop has several crucial roles. It contains the F_X-binding motif (Smart et al., 1993; Warren et al., 1993). Three [4Fe-4S] clusters, F_X, F_A, and F_B, are within the 35 Å-high "hump" protruding from the 40-Å-thick membranes (Krauss et al., 1993), with the F_A and F_B clusters (indistinguishable at 6 Å) located approximately 14 and 21 Å from F_X (Krauss et al., 1993). Thus, the F_X-binding motif must be located on the *n* side of the membrane as predicted earlier (Fish et al., 1985; Smart et al., 1993). Our results show that the amino-terminal region of the putative VIII-IX loop is exposed to proteases in the absence of PsaD. Therefore, in addition to binding F_X, the VIII-IX loop may interact with PsaC and PsaD. The IX-X loop is proposed to be on the *p* side. An alternative would be that both IX and X helices are parallel to the lipid bilayer on the *n* side. However, this alternative is unlikely because x-ray crystallographic studies have failed to detect four α-helices (two each from PsaA and PsaB) that are parallel to the *n* side of the membranes. The results presented here demonstrate that a site in the putative X-XI loop is exposed to a protease in the absence of PsaD, thus suggesting its presence on the *n* side of the membranes. This model predicts the C terminus of PsaB on the *p* side and is consistent with the studies on the spinach thylakoids (Vallon and Bogorad, 1993).

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