Topography of the Photosystem I Core Proteins of the Cyanobacterium Synechocystis sp. PCC 6803*

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PsA and PsB are homologous integral membrane proteins that form the heterodimeric core of photosystem I. Domain-specific antibodies were generated to examine the topography of PsA and PsB. The purified photosystem I complexes from the wild type strain of Synechocystis sp. PCC 6803 were treated with eight proteases to study the accessibility of cleavage sites in PsA and PsB. Proteolytic fragments were identified using the information from N-terminal amino acid sequencing, reactivity to antibodies, apparent mass, and specificity of proteases. The extramembrane loops of PsA and PsB differed in their accessibility to proteases, which indicated the folded structure of the loops or their shielding by the small subunits of photosystem I. NaI-treated and mutant photosystem I complexes were used to identify the extramembrane loops that were exposed in the absence of specific small subunits. The absence of PsAD exposed additional proteolytic sites in PsB, whereas the absence of PsAE exposed sites in PsA. These studies distinguish PsA and PsB in the absence of PsAD exposed additional proteolytic sites in PsB, whereas the absence of PsAE exposed sites in PsA. These studies distinguish PsA and PsB in the absence of PsAD exposed additional proteolytic sites in PsB, whereas the absence of PsAE exposed sites in PsA. These studies distinguish PsA and PsB in the absence of PsAD exposed additional proteolytic sites in PsB, whereas the absence of PsAE exposed sites in PsA. These studies distinguish PsA and PsB in the absence of PsAD exposed additional proteolytic sites in PsB, whereas the absence of PsAE exposed sites in PsA.

Photosystem I (PS I) from cyanobacteria and chloroplasts is a multisubunit membrane-protein complex that catalyzes electron transfer from reduced plastocyanin (or cytochrome c₆) to oxidized ferredoxin (or flavodoxin) (1–4). The PsA and PsB subunits of PS I form the heterodimeric core of the complex which harbors approximately 100 antenna chlorophyll a molecules, 10–12 β-carotenes, the primary electron donor P700, and a chain of electron acceptors (A₃, A₁, and F₅₇). PsA and PsB also interact directly with plastocyanin or cytochrome c₆ (5–7). In addition to the core proteins, the cyanobacterial PS I complex contains 9 small subunits (1–3). PsAC binds the terminal electron acceptors F₅₇ and F₆₅₅, which are two [4Fe-4S] center (8). PsD provides an essential ferredoxin-docking site on the reducing side of PS I (9–11) and is required for the stable assembly of PsAC and PsAE into the PS I complex (12, 13). PsAE is involved in ferredoxin docking (10, 14–16) and in cyclic electron flow around PS I (17, 18). PsAF provides a component of the docking site for plastocyanin in the plant PS I (17, 19, 20) but not in the cyanobacterial PS I (6, 21). PsAL is essential for the formation of PS I trimers in cyanobacteria (22). PsA and PsB are required for the correct organization of PsAL and PsAF, respectively (23, 24). The absence of PsAM in cyanobacterial mutants causes deficiency in growth at high light intensity and affects stable assembly of PsAD.²³ The role of PsAK has not been identified.

During the past few years, major advances in x-ray crystallography (25, 26), electron microscopy (27), molecular genetics (10, 22), and biochemical studies (28, 29) have provided a framework for understanding the overall architecture of PS I. The PS I complex has an elongated shape with a local pseudo-2-fold symmetry. PsAC, PsAD, and PsAE are peripheral subunits, located on the n side (stromal side in chloroplasts and cytoplasmic side in cyanobacteria) of photosynthetic membranes, with PsAC positioned in the center of each monomeric PS I on the axis of symmetry (26, 27). The recent crystal analysis of PS I has indicated the location of [4Fe-4S] clusters F₆₅₅, F₅₇, A₁, and F₅₇, 71 chlorophyll a molecules, 31 transmembrane α-helices, and 9 surface and 3 stromal α-helices (26). A monomer of PS I consists of a “catalytic domain” and a smaller “connecting domain” that links monomers to form a trimer. The connecting domain contains three transmembrane helices which may belong to PsAL and PsAI (22, 23). The remaining helices belong to the PS I core and other subunits in the catalytic domain. Twenty-two transmembrane and eight peripheral helices in the catalytic domain are arranged in an approximate symmetry (25, 26). Therefore, in agreement with the hydrophathy analysis, the PsA and PsB cores are proposed to contain 11 transmembrane helices each (30).

Although the x-ray crystallography studies provided information of the PS I core, PsA and PsB could not be distinguished at the present resolution (26). Similarly, the interaction between PS I core and small subunits, the surface domains, and residues of the PS I core and positioning of extramembrane loops with respect to the photosynthetic membranes have not been elucidated clearly. Topographical studies provide a valuable tool to understand these unresolved structural features of PS I. In this paper, we describe biochemical studies that used subunit-deficient mutants, limited proteolysis,...
sis, and domain-specific antibodies. We studied the accessibility of PsaA and PsaB to different proteases, the shielding of PsaA and PsaB by smaller PS I subunits, and the position of the N terminus of PsaA with respect to the membrane plane.

EXPERIMENTAL PROCEDURES

Cyanobacterial Strains and Culture—Strains of Synechocystis sp. PCC 6803 that were used in this study are listed in Table I. Cultures of wild type and mutant strains were grown in BG-11 with or without 5 mM glucose and antibiotics (30 μg/ml chloramphenicol or 40 μg/ml kanamycin) under a light intensity of 21 μmol m⁻² s⁻¹. Cells were harvested at the late exponential growth phase and resuspended in 0.4 M sucrose, 10 mM NaCl, 1 mM PMSF, 2 mM benzamidine, and 10 mM MOPS, pH 7.0, for isolation of thylakoids.

Isolation of the Photosynthetic Membranes and Purification of PS I Complexes—Photosynthetic membranes were isolated after cell breakage with a bead beater (Biospec Products) (28). To isolate PS I, the membranes were solubilized with Triton X-100 and subjected to DEAE-cellulose chromatography and sucrose gradient centrifugation (31). Chlorophyll concentrations in the thylakoid membranes and PS I complexes were determined in 80% (v/v) acetone (32).

Preparation of Osmotically Shocked Cells of Synechocystis sp. PCC 6803—Cells were harvested at the late exponential growth phase by centrifugation (7000 × g, 10 min). The pellet was resuspended in 0.4 M sucrose, 5 mM EDTA, and 10 mM HEPES, pH 7.0, pelleted again, and resuspended in the same buffer with 0.2% (v/v) lysozyme to 1 g of cells per 10 mL. Cell wall was digested by incubation at 27 °C for 12 h under illumination (21 μmol m⁻² s⁻¹) with constant shaking. Cells were harvested by centrifugation (7000 × g, 10 min), resuspended in osmotic shock solution (50 mM potassium phosphate, pH 6.8, 30 mM sodium citrate, 0.2 mM CaCl₂, 1 mM PMSF, and 2 mM benzamidine), incubated on ice for 30 min, and harvested by centrifugation (7000 × g, 10 min) again. This osmotic shock treatment was repeated two additional times. The cells were pelleted and resuspended twice with 10 mM NaCl, 10 mM HEPES, pH 7.0, and finally resuspended in 0.4 M sucrose, 10 mM NaCl, 10 mM HEPES, pH 7.0, for protease treatment (33).

Treatment of PS I with Proteases and NaI—To study the accessibility of PS I subunits to proteases, purified wild type and mutant PS I complexes, wild type thylakoid membranes, and osmotically shocked cells were incubated with protease at a final chlorophyll concentration of 2 μg/mL. The protease reaction conditions are listed in Table II. To remove peripheral subunits from the PS I core, purified wild type PS I complex was incubated with 3 μM NaI for 30 min on ice (34). The samples were diluted with an excess amount of 10 mM MOPS-HCl, pH 7.0, 0.05% Triton X-100, and desalted by ultrafiltration through a Centricon-100 (Amicon).

Oxygen Uptake Measurements—Purified PS I complexes that had been treated with proteases were used for oxygen uptake measurements. In a total volume of 1 mL, PS I containing 10 μg of chlorophyll was used for the reaction. PS II inhibitor, electron donors, and electron acceptor were added to a final concentration of 50 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, 1 mM ascorbate, 1 mM 3,6-diaminodurene, and 2 mM methyl viologen. Oxygen uptake was measured on the Oxygen Monitoring System (Hansatech, UK) under the light density of 2430 μmol m⁻² s⁻¹.

Preparation of Antibodies—Domain-specific antibodies were generated using overexpressed fusion proteins as antigens. DNA fragments coding the appropriate peptides in PsaA and PsaB of Synechocystis sp. PCC 6803, PsaA2 for residues 2-AI39FPEREAKAVKSDKNVDPVPT-SFEKRWKGPCHFDRTL of PsaA, and PsaB450 for residues B450-QILIEPVAQWQATSKRALYGFVDLLSNDPIASSTTGAAWLPG-WLDAINSGINSLF of PsaB were amplified by polymerase chain reaction and inserted into expression vector pGEX-KG between EcoRI and XhoI sites. The expression of fusion proteins was induced by isopropyl β-D-thiogalactopyranoside. Cells were harvested by centrifugation and lysed by a probe sonicator. The fusion proteins in inclusion bodies were isolated from membranes by centrifugation through 10% sucrose and applied directly for electrophoresis. The gel was stained by Coomassie Blue. The fusion proteins were excised from the gel and used for raising antibodies at Cocalico Biological. Antibody anti-PsaB718 was raised against the C terminus of PsaB (35). Anti sera were evaluated by Western blotting against both thylakoid membranes and purified PS I complex. All three domain-specific antibodies anti-PsaA2, anti-PsaB450, and anti-PsaB718 recognized only the 66-kDa diffuse band corresponding to the comigrating PsaA and PsaB proteins.

Analytical Gel Electrophoresis, Immunodetection, and N-terminal Amino Acid Sequencing—Isolated PS I complexes and photosynthetic membranes 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 for better resolution of the PS I subunits (10). After electrophoresis, gels were electrotransferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Immunodetection was performed using the enhanced chemiluminescence reagents (Amersham Corp.). For sequencing of N termini of proteolytic cleavage fragments, peptides were separated by electrophoresis, transferred to Immobilon-P membranes, stained with Coomassie Blue containing 1% acetic acid for several minutes, destained with 50% methanol, and rinsed with deionized water. The N-terminal sequences were determined on an Applied Biosystems 477A Sequencer.

RESULTS

Activity of the Protease-treated PS I Complexes—In this study, we used limited proteolysis to map surface domains in the PsaA-B core of PS I. To examine activity of the protease-treated complexes, we determined PS I-mediated oxygen consumption rates by Mehler reaction (36, 37). Purified wild type PS I complexes were treated by eight proteases and employed in oxygen uptake measurements (Table III). The PS I activity of untreated sample was 377.5 μmol of O₂/mg of chlorophyll/h. The PS I activities in all eight protease treatments ranged from 99 to 133% control activity. An active reaction center and functional electron transfer chain are required for the PS I activity that is measured by the oxygen uptake. Thus the protease treatments did not damage the electron transfer chain within the PS I complex. This may imply that the limited proteolysis could only access extramembrane loops. In most protease-treated samples, the PS I activity was higher than that in the untreated control. Proteases might have degraded the extramembrane loops and small subunits, thereby facilitating access of electron donor or acceptor to the electron transfer centers in the PS I complex.

Accessibility of PsaA and PsaB to Proteases—Purified PS I complexes from the wild type strain were treated with proteases to study the accessibility of cleavage sites in PsaA and PsaB. The PS I complexes were treated with different concentrations of proteases and the resulting fragments were stained by Coomassie Blue or immunodetected by the three domain-specific antibodies (Fig. 1). The apparent masses of protein
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| Proteases          | Thermolysin (EC 3.4.24.4) | Glu-C (EC 3.4.21.19) | Chymotrypsin (EC 3.4.21.24) | Papain (EC 3.4.22.2) | Lys-C (EC 3.4.21.50) | Trypsin (EC 3.4.21.4) | Clostripain (EC 3.4.22.8) | Pepsin (EC 3.4.23.1) |
|-------------------|-----------------------------|----------------------|----------------------------|----------------------|----------------------|-----------------------|--------------------------|------------------------|
| Specificity       | [VILWMF]                    | [E]\*                | [FWY]                      | [KKILG]\*             | [KR]\*                 | [KR]\*                | [FLYW]\*                 |                        |
| Source            | Bacillus thermoproteolyticus (Sigma) | Staphylococcus aureus (Boehringer Mannheim) | Bovine pancreas (Boehringer Mannheim) | Carica papaya (Promega Biotech) | Pseudomonas aeruginosa (Promega Biotech) | Porcine pancreas (Promega Biotech) | Clostridium histolyticum (Boehringer Mannheim) | Porcine stomach (Boehringer Mannheim) |
| Final protease concentration Chl | 250 µg/mg Chl | 250 µg/mg Chl | 250 µg/mg Chl | 10 µg/mg Chl | 250 µg/mg Chl | 250 µg/mg Chl | 250 µg/mg Chl | 250 µg/mg Chl |
| Proteolysis condition\* | 10 mM MOPS (pH 7.0); 5 mM CaCl\_2 | 50 mM Tris-HCl (pH 7.5); 1 mM EDTA | 10 mM MOPS (pH 7.5); 0.5 mM CaCl\_2 | 10 mM MOPS (pH 7.5); 1 mM EDTA; 1.5 mM CySH | 25 mM Tris-HCl (pH 8.0); 1 mM CaCl\_2 | 50 mM Tris-HCl (pH 8.0); 1 mM CaCl\_2; 2 mM DTT | 20 mM Tris-HCl 20 mM NaH\_2PO\_4 | 20 mM Tris-HCl 20 mM NaH\_2PO\_4 |
| Incubation        | 37 °C, 30 min 15 °C, 30 min 25 °C, 1 h 25 °C, 1 h 37 °C, 1 h | 37 °C, 1 h | 37 °C, 1 h | 37 °C, 1 h | 37 °C, 1 h | 37 °C, 1 h | 37 °C, 1 h | 37 °C, 1 h |
| Termination       | 20 mM EDTA; 20 mM PMSF | 20 mM PMSF | 20 mM PMSF | 20 mM PMSF | 20 mM PMSF | 20 mM PMSF | 20 µM pepstatin A |

\* 0.05% Triton X-100 was added for purified PS I complexes.
\* Chl, chlorophyll.
\* DTT, dithiothreitol.

TABLE III

Oxygen measurements of protease-treated PS I complexes

| Protease treatment | PS I activity (µmol O₂/mg chlorophyll · h) |
|--------------------|--------------------------------------------|
| Control            | 377.5                                      |
| Chymotrypsin       | 416.0                                      |
| Clostripain        | 371.9                                      |
| Glu-C              | 408.8                                      |
| Lys-C              | 472.9                                      |
| Papain             | 390.9                                      |
| Pepsin             | 431.5                                      |
| Thermolysin        | 455.9                                      |
| Trypsin            | 503.4                                      |

fragments were determined from the migration of prestained protein molecular weight standards (Life Technologies, Inc.) upon electrophoresis. The protein fragments that were visible in Coomassie Blue staining were subjected to N-terminal amino acid sequencing. Three criteria, the N-terminal sequences, the apparent mass, and the immunoreactivities of proteolytic fragments, were used to identify the proteolytic fragments (Table IV).

The ThII, GII, and PaIII fragments were deduced accurately from their N-terminal sequence and from their immunodetection by anti-PsaA2. Also, a diffused band that migrated slightly faster than the PsaA-B band was visible in Coomassie Blue staining. This can be explained as a cleavage in the N-terminal sequence of PsaA. The N-terminal sequence of the TrI fragment revealed the cleavage site in the N-terminal extramembranous domain of PsaA. Thus these fragments, grouped as type I fragments, could be identified with accuracy. Similar to many membrane proteins, the electrophoretic behavior of the PS I core proteins was anomalous compared with the migration of soluble proteins. The comigrating PsaA and PsaB formed the 66-kDa diffuse band in PAGE, whereas the deduced mass is more than 80 kDa. The predicted mass was higher than the apparent mass. This was also true for the type I fragments and was considered during the prediction of the other fragments.

Table V lists the results of Western blotting, the apparent mass, and the predicted proteolysis regions for the protein fragments that could be immunodetected with domain-specific antibodies. The identity of these fragments could be predicted by several ways. The prediction of type I fragments was described in the previous paragraph. Type 2 and type 3 fragments contained epitope for the N terminus of PsaA or epitope at the C terminus of PsaB, as shown by the immunodetection results. Based on their apparent mass, there was only one reasonable cleavage site for the fragments of type 2. For example, the GIV fragment, a 44.5-kDa polypeptide recognized by anti-PsaA2, could result from the proteolysis at Glu-512 in PsaA with a predicted mass of 47.5 kDa. The adjacent Glu-C cleavage sites were Glu-342 or Glu-686 in PsaA which made the predicted mass of 37.9 or 76.9 kDa unreasonable for GIV. In type 3 fragments, there were several possible cleavage sites located within one extramembrane loop or in a region containing two extramembrane loops. For example, the GII fragment, a 33.7-kDa peptide immunodetected with anti-PsaA2, could result from four reasonable cleavages between Glu-323 and Glu-342 in PsaA with the predicted mass from 35.8 to 37.9 kDa. These four possible cleavage sites were located in the E loop. The LyI fragment is the only type 4 fragment. It accumulated similarly as the TrI fragment, so it was predicted to have the similar cleavage at the N-terminal loop of PsaA. The type 5 fragments contained the peptide PsaB450 but there was not enough data
to identify them accurately. Overall, the limited proteolysis of the wild type PS I provided extensive information about the residues that are exposed on the surface of the PS I complex.

**Treatment of Mutant PS I Complexes with Proteases**—As the components of the multiprotein PS I complex, the PsaA and PsaB core proteins interact with the small subunits. To study these interactions, subunit-deficient mutant PS I complexes (Table I) and NaI-treated PS I complexes were used for limited proteolytic treatments. Different PS I complexes were incubated with proteases, and resulting fragments were detected by Western blotting (Fig. 2). When specific small subunits were absent, the proteolytic sites in the core proteins that are shielded by the small subunits were expected to be exposed to proteases. Indeed, additional peptide fragments that reacted

| Proteolytic fragments | N-terminal sequence | Position of the first amino acid | Predicted fragment | Apparent mass kDa | Predicted mass kDa |
|----------------------|---------------------|----------------------------------|--------------------|------------------|-------------------|
| ThI                  | ATKFPKF             | PsaB, 2                          | PsaB, 2–497        | 45.0             | 54.7              |
| ThII                 | INSGINS             | PsaB, 198–731                    | 23.8               | 26.4             |
| ThIII                | WGIWGS              | PsaB, 304                        | PsaB, 304–497      | 15.2             | 21.1              |
| GIll                 | KQILIE              | PsaB, 449                        | PsaB, 449–731      | 27.6             | 31.7              |
| ChI                  | SQDXA               | PsaB, 9                          | PsaB, 9–506        | 43.2             | 54.8              |
| PaII                 | QATXGGK             | PsaB, 462                        | PsaB, 462–731      | 26.2             | 30.1              |
| TrI                  | WGPXG               | PsaA, 28                         | PsaA, 28–?         | 53.6             | ?                 |

TABLE IV

*N-terminal amino acid sequence analysis of the proteolytic fragments in Fig. 1*

The position of the first amino acid was identified after comparison with the deduced amino acid sequence of PsaA and PsaB.
with the three domain-specific antibodies were obtained in mutant PS I complexes compared with the protease-treated wild type PS I complexes. The apparent mass and the immunoreactivity were used in prediction of the additional fragments (Table VI).

In general the ADC4, AEK2, and NaI-treated PS I complexes yielded more additional proteolytic fragments than the AFI6 and AIC9 complexes, indicating that the peripheral PS I subunits shield more extramembrane proteolytic sites in the core proteins than the integral membrane proteins. When the ADC4 and NaI-treated PS I complexes were incubated with proteases, PsaA and PsaB were digested much more rapidly than the core proteins than the integral membrane proteins. When the ADC4 and NaI-treated PS I complexes were treated with proteases, small additional proteolytic fragments (AFI6-F, AIC9-F, and CIC) resulted from the cleavages in the N-terminal domain of PsaA. Also, a 44-kDa fragment (CIA) resulted from accessible sites in the C-terminal domain of PsaA. The cleavages in the N-terminal domain of PsaA could be detected only upon protease treatment of the PsaE-less AEK2 complexes, implying that the N-terminal domain of PsaA may interact with PsaE. The NaI treatment of PS I complexes removed PsaD, PsaE, and PsaC from the core proteins. When the NaI-treated PS I complexes were treated with proteases, the anti-PsaA2 antibody immunoreacted with the 42-kDa degradation product that was similar to the one in the protease-treated ADC4 complex and also with the large proteolytic fragments similar to the ones in the protease-treated AEK2 complex.

When detected with the anti-PsaB450 antibody, the protease-treated PS I complexes of the AEK2 strain were treated with proteases, small additional proteolytic fragments (ThC-F, ChC-F, and CIC) resulted from the cleavages in the N-terminal domain of PsaA. Also, a 44-kDa fragment (CIA) resulted from accessible sites in the C-terminal domain of PsaA. The cleavages in the N-terminal domain of PsaA could be detected only upon protease treatment of the PsaE-less AEK2 complexes, implying that the N-terminal domain of PsaA may interact with PsaE. The NaI treatment of PS I complexes removed PsaD, PsaE, and PsaC from the core proteins. When the NaI-treated PS I complexes were treated with proteases, the anti-PsaA2 antibody immunoreacted with the 42-kDa degradation product that was similar to the one in the protease-treated ADC4 complex and also with the large proteolytic fragments similar to the ones in the protease-treated AEK2 complex.

| Proteolytic fragments | Immunodetected | Apparent mass | Predicted fragment | Accessible site locationa | Predicted mass | Prediction type |
|-----------------------|---------------|--------------|-------------------|--------------------------|---------------|----------------|
| Thi Anti-PsaB450      | 45.0          | PsaB, 2–497  | PsaB, H           | 54.7                     | 1             |               |
| ThII Anti-PsaB718     | 26.7          | PsaB, 488–731| PsaB, H           | 26.9                     | 1             |               |
| ThIII Anti-PsaB450    | 16.2          | PsaB, 295–497| PsaB, E and H     | 22.1                     | 1             |               |
| ThIV Anti-PsaB450     | 15.2          | PsaB, 304–497| PsaB, E and H     | 21.1                     | 1             |               |
| GII Anti-PsaB450 and B718 | 41.2          | PsaB, 304–731| PsaB, E           | 47.5                     | 2             |               |
| GIII Anti-PsaA2       | 33.7          | PsaA, 1–(323–342)| PsaA, E       | 35.8–37.9                 | 3             |               |
| GIV Anti-PsaB450 and B718 | 27.6          | PsaB, 448–731| PsaB, H           | 31.7                     | 1             |               |
| GIV Anti-PsaA2        | 21.0          | PsaA, 1–(241–251)| PsaA, D       | 26.7–27.8                 | 3             |               |
| GIV Anti-PsaA2        | 44.4          | PsaA, 1–512  | PsaA, H           | 56.7                     | 2             |               |
| GIVI Anti-PsaA2       | 29.1          | PsaA, 1–(323–342)| PsaA, E       | 35.8–37.9                 | 3             |               |
| GIVII Anti-PsaA2      | 12.0          | PsaA, 1–101  | PsaA, E           | 11.5                     | 2             |               |
| GIVIII Anti-PsaB450 and B718 | 45.9       | PsaB, 202–267–731| PsaB, D  | 51.7–58.5                 | 3             |               |
| GIX Anti-PsaB450      | 36.9          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| GIX Anti-PsaB450      | 23.4          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| ChII Anti-PsaB450     | 43.2          | PsaB, 9–506  | PsaB, A and H     | 54.8                     | 1             |               |
| ChI Anti-PsaB450      | 17.3          | PsaB, 445–731| PsaB, H           | 32.1                     | 4             |               |
| PaI Anti-PsaA2        | 40.6          | PsaA, 1–(414–433)| PsaA, G       | 45.8–48.1                 | 3             |               |
| PaI Anti-PsaA2        | 26.4          | PsaA, 1–(320–344)| PsaA, D or E    | 31.1–38.1                 | 3             |               |
| PaI Anti-PsaA2        | 30.3          | PsaA, 1–462–731| PsaB, H           | 30.1                     | 1             |               |
| PaIV Anti-PsaA2       | 22.7          | PsaA, 1–(223–269)| PsaA, D       | 24.7–29.8                 | 3             |               |
| PaV Anti-PsaA2        | 13.0          | PsaA, 1–(120–151)| PsaA, B       | 13.6–16.7                 | 3             |               |
| PaVI Anti-PsaB718     | 28.6          | PsaB, 477–549–731| PsaB, H or I   | 21.4–28.6                 | 3             |               |
| PaVII Anti-PsaB718    | 13.7          | PsaB, 565–614–731| PsaB, J       | 13.6–15.7                 | 3             |               |
| PaVIII Anti-PsaB450   | 42.6          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| PaIX Anti-PsaB450     | 36.4          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| PII Anti-PsaA2        | 53.4          | PsaA, ?      | PsaA, A           | ?                        | 4             |               |
| PII Anti-PsaA2        | 53.8          | PsaA, 28–?   | PsaA, A           | ?                        | 1             |               |
| PII Anti-PsaA2        | 39.5          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| PIII Anti-PsaB450     | 16.7          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| PeI Anti-PsaB450      | 41.4          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| PeII Anti-PsaB450     | 35.9          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| PeIII Anti-PsaB450    | 14.6          | PsaB, ?      | PsaB, ?           | ?                        | 5             |               |
| CII Anti-PsaB450 and B718 | 43.8       | PsaB, 283–731| PsaB, E           | 48.8                     | 2             |               |

a For nomenclature of loops, see Fig. 4.
the ADC4 and NaI-treated PS I complexes were treated with the Lys-C protease. From its apparent mass and the presence of the C-terminal epitope, this fragment was predicted to have N terminus from one of five possible cleavage sites in the G and H loops to the C terminus of PsaB. Proteolysis at these sites should result in fragments with a predicted mass of 31.0–36.8 kDa. When the PS I complexes of AFK6 were treated with trypsin, the 22.7-kDa TrB fragment was detected by the anti-PsaB718 antibody. This fragment could have resulted from a cleavage at Lys-449 or Lys-467 in the H loop of PsaB. In the ADC4 and NaI-treated PS I complexes, an additional 40.0–45.0-kDa fragment shown as LF in Fig. 2 was detected in most protease treatments. This fragment was absent in the untreated PS I complex. The possible cleavage sites may be lo-

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FIG. 2. Treatment of the subunit-deficient PS I complexes with proteases. The PS I complexes were incubated with different proteases at the final concentration as described in Table II. The samples equivalent to 5 μg of chlorophyll were analyzed by Tricine/urea/SDS-PAGE and Western blotting with three domain-specific antibodies. For treatments: −, control; Ch, chymotrypsin; Cl, clostripain; Gl, Glu-C; Ly, Lys-C; Pa, papain; Pe, pepsin; Th, thermolysin; and Tr, trypsin.
Table VI

| Proteolytic fragments | Mutant PS I complexes | Immunodetection | Apparent mass | Predicted fragment | Predicted mass | Accessible site location |
|-----------------------|-----------------------|-----------------|--------------|-------------------|----------------|-------------------------|
| ChA                   | NaI                   | Anti-PsaA2      | 15.1         | PsaA, 1–(144–190) | 15.9–21.2      | PsaA, B, C             |
| ChB                   | NaI, ADC4             | Anti-PsbB718    | 15.9         | PsaB (551–751)–731 | 18.8–20.8      | PsaB, I                 |
| ChC                   | AEK2                  | Anti-PsbA2      | 30.4         | PsaA, 1–(277–341) | 30.8–37.7      | PsaA, D, E              |
| ChD                   | AEK2                  | Anti-PsbA2      | 19.8         | PsaA, 1–(189–239) | 21.1–32.0      | PsaA, C, D              |
| ChE                   | AEK2                  | Anti-PsbA2      | 17.6         | PsaA, 1–(189–239) | 21.1–32.0      | PsaA, C, D              |
| ChF                   | AEK2                  | Anti-PsbA2      | 11.4         | PsaA, 1–(100–156) | 11.4–17.3      | PsaA, B                 |
| Cla                   | NaI, AEK2             | Anti-PsbA2      | 49.1         | PsaA, 1–(462–751) | 51.5–63.1      | PsaA, H, I              |
| CIB                   | NaI                   | Anti-PsbB718    | 15.9         | PsaB, (540–562)–731 | 19.7–22.1      | PsaB, I                 |
| CIC                   | AEK2                  | Anti-PsbA2      | 24.0         | PsaA, 1–312       | 34.6           | PsaA, E                 |
| LyA                   | NaI, ADC4, AKF6, AIC9 | Anti-PsbB718    | 15.5         | PsaB (543–549)–731 | 21.1–21.8      | PsaB, I                 |
| LyB                   | NaI, AEK2, AKF6, AIC9 | Anti-PsbB718    | 16.8         | PsaB, 534–731     | 22.7           | PsaB, I                 |
| LyC                   | NaI, ADC4             | Anti-PsbB718    | 28.2         | PsaB, (403–450)–731 | 31.0–36.8      | PsaB, G, H              |
| ThA                   | NaI, ADC4, AEK2       | Anti-PsbB718    | 15.5         | PsaB (532–570)–731 | 19.0–22.9      | PsaB, I                 |
| ThB                   | AEK2                  | Anti-PsbB718    | 26.0         | PsaB, (440–498)–731 | 26.4–32.6      | PsaB, H                 |
| ThC                   | AEK2                  | Anti-PsbA2      | 37.4         | PsaA, 1–(350–429) | 42.0–47.5      | PsaA, F, G              |
| ThD                   | AEK2                  | Anti-PsbA2      | 29.0         | PsaA, 1–(314–343) | 34.8–38.0      | PsaA, E                 |
| ThE                   | AEK2                  | Anti-PsbA2      | 22.8         | PsaA, 1–(222–289) | 24.6–32.0      | PsaA, D                 |
| ThF                   | AEK2                  | Anti-PsbA2      | 14.3         | PsaA, 1–(129–192) | 14.4–21.5      | PsaA, B, C              |
| TrA                   | AKF6                  | Anti-PsbA2      | 40.0         | PsaA, 1–(462–519) | 51.5–57.4      | PsaA, H                 |
| TrB                   | AKF6                  | Anti-PsbB718    | 22.7         | PsaB (450–460)–731 | 29.5–31.6      | PsaB, H                 |
| LF                    | NaI, ADC4             | Anti-PsbB718    | 40.4–45.0    | PsaB (158–327)–731 | 45.0–63.4      | PsaB, C, D, E           |

*For nomenclature of loops, see Fig. 4.

DISCUSSION

PS I is a multisubunit protein complex that contains at least 11 polypeptides in cyanobacteria (1). In this study, we used domain-specific antibodies, limited proteolysis, and subunit-deficient PS I complexes to understand the topography of the PsaA and PsaB proteins that form the catalytic hydrophobic core of PS I. Domain-specific antibodies were generated against specific epitopes in PsaA and PsaB. Western blotting with thylakoid membranes and purified PS I complexes showed that the antibodies are specific to PsaA or PsaB. N-terminal amino acid sequences of the fragments recognized by antibodies (Table IV) demonstrated the domain specificity of the antibodies to PsaA and PsaB proteins. The GIII fragment with the N terminus at Lys-449 of PsaB was recognized by the anti-PsaB450 antibodies, whereas the ThII fragment with the N terminus at Ile-498 of PsaB was not recognized by anti-PsaB450. These

cated in the C, D, and E loops of PsaB with a predicted mass of 45.0–63.4 kDa. However, no such fragment was observed in the AEK2, AKF6, and AIC9 PS I complexes. Therefore, the N-terminal domain of PsaB is protected by PsaD but may not interact with the other subunits.

Previous work has indicated that a 16-kDa proteolytic fragment detected by anti-PsbB718 in thermolysin-treated ADC4 PS I complex resulted from a cleavage at Leu-531 in the I loop of PsaB (28). Similar proteolytic fragments, such as ChB, CIB, LyA, LyB, ThA in different mutant PS I complexes, were detected. These proteolytic fragments could result from cleavages in the I loop of PsaB indicating that the I loop in the C-terminal domain of PsaB may interact with several subunits. In the Lys-C treatments, two close fragments from the cleavages in the I loop of PsaB were obtained in the AKF6, AIC9, and NaI-treated PS I complexes with apparent mass about 15.5 and 16.8 kDa. Only one such fragment was detected in ADC4 and AEK2 each, with a slight difference in their apparent mass. One of the four lysyl residues in the I loop may be involved in the cleavage by the Lys-C endoprotease. The cleavage at Lys-533 may yield a C-terminal fragment with a predicted mass of 22.7 kDa, and the cleavage at Lys-542, Lys-547, or Lys-548 would result in the fragment containing C-terminal epitope with a predicted mass of 21.1–21.8 kDa. According to these results, LyB was predicted as the cleavage at Lys-C and trypsin treatments expected to be exposed to proteases during the treatment. Small fragments in Lys-C and trypsin treatments were recognized by anti-PsaD indicating the digestion of PsaD in membranes from both control and osmotically shocked cells. Digestion of PsaD in the osmotically shocked cells was not as extensive as in isolated membranes. Due to a large amount of cytoplasmic proteins, protease to protein ratio in the cell treatments was expected to be lower than in the thylakoid treatments. If the thylakoid membranes were intact during the protease treatments, PsbO which is located on the p side of photosynthetic membrane was expected to remain intact. In membranes, PsbO was not detected after protease treatment. In contrast, most PsbO remained intact in the osmotically shocked cells. These results showed that the osmotically shocked thylakoid membranes remain largely intact during the protease treatments. In Lys-C and trypsin treatments, significant degradation of PsaA was observed when anti-PsaA2 was used for detection. As described previously, the degradation of PsaA in Lys-C and trypsin treatments resulted from the cleavage in the N-terminal loop of PsaA. Therefore, the N terminus of PsaA was exposed to proteases in the osmotically shocked cells showing that it is on the n side of photosynthetic membranes.

The domain-specific antibody anti-PsaA2 was used to determine the orientation of PsaA (Fig. 3). PsaD, which is located on the n side of photosynthetic membrane, was expected to be exposed to proteases during the treatment. Small fragments in Lys-C and trypsin treatments were recognized by anti-PsaD indicating the digestion of PsaD in membranes from both control and osmotically shocked cells. Digestion of PsaD in the osmotically shocked cells was not as extensive as in isolated membranes. Due to a large amount of cytoplasmic proteins, protease to protein ratio in the cell treatments was expected to be lower than in the thylakoid treatments. If the thylakoid membranes were intact during the protease treatments, PsbO which is located on the p side of photosynthetic membrane was expected to remain intact. In membranes, PsbO was not detected after protease treatment. In contrast, most PsbO remained intact in the osmotically shocked cells. These results showed that the osmotically shocked thylakoid membranes remain largely intact during the protease treatments. In Lys-C and trypsin treatments, significant degradation of PsaA was observed when anti-PsaA2 was used for detection. As described previously, the degradation of PsaA in Lys-C and trypsin treatments resulted from the cleavage in the N-terminal loop of PsaA. Therefore, the N terminus of PsaA was exposed to proteases in the osmotically shocked cells showing that it is on the n side of photosynthetic membranes.
results indicated that the specific binding domain of anti-PsaB450 should be located between Lys-449 and Ile-498 of PsaB protein, the corresponding peptide of anti-PsaB450. N terminus of the TrI fragment which was not recognized by anti-PsaA2 started at Trp-28 of PsaA. Therefore anti-PsaA2 specifically recognizes the N terminus of PsaA. Limited proteolysis has been successfully used to probe transmembrane topology of membrane proteins. In these analyses, it is necessary to establish conditions that allow cleavage of the extramembrane loops but not of the transmembrane regions. We ensured integrity of the hydrophobic core of PS I after proteolysis from measuring PS I activity using artificial electron donor and acceptor (Table III).

The structure of cyanobacterial PS I complex at 4-Å resolution has indicated that PsaA and PsaB have 11 transmembrane helices each. These helices span the lipid bilayer completely (26). Based on the available information and the results in this paper, we proposed a model for transmembrane folding of the PsaA and PsaB core proteins (Fig. 4). The homology between PsaA and PsaB proteins implied that they would fold similarly. Therefore, the model in Fig. 4 should be applicable to both PsaA and PsaB proteins. In this model, 11 transmembrane helices span the thylakoid membrane completely with the N termini on the n side of the thylakoid membrane. Consequently, the C termini are on the p side of the thylakoid membrane (26). Such orientation is supported by the following observations. The 4-Å crystal structure indicated clearly the complete spanning of the 11 transmembrane helices (26). The Lys-C and trypsin treatments of the osmotically shocked cells in this paper suggested that the N terminus of PsaA is located on the n side of the thylakoid membrane. Xu and Chitnis (28) showed that the K loop interacts with PsaD which is located on the n side of the thylakoid membrane. Vallon and Bogorad (29) located the G loop on the n side and the F and H loops on the p side by using immunogold labeling. The I loop with the FX-binding domain should be located on the n side of the thylakoid membrane. Also, the six n side extramembrane loops contain the majority of the positive residues, which agrees with the “positive inside” rule (39, 40). Therefore, there is sufficient evidence from different sources that collectively demonstrate the transmembrane orientation of the PsaA and PsaB proteins (Fig. 4).

Because of the folded structure of the substrate proteins, proteases cannot cleave at every recognition site in the primary sequence. The accessibility of extramembrane loops in PsaA and PsaB to proteases is determined by folding of the loops. Some loops may form loose conformation that will be exposed readily to proteases. In contrast, some loops may have compact conformation, like the helices that are parallel to the membrane plane. Many protease recognition sites in the compact loops may not be accessible to proteases. Also, the protection from other proteins will reduce the surface exposure of the loops. For these reasons, the accessibility of extramembrane loops in PsaA and PsaB differed for each loop (Fig. 4). The protease recognition sites in the C, F, and K loops of PsaA and PsaB could not be cleaved, whereas the H and E loops of PsaB were readily accessible to proteases. The H loop is on the p side of the thylakoid membrane in the model. It is only one transmembrane helix from the I loop which contains the FX-binding motif and interacts with PsaC on the n side (41, 42). From the arrangement of helices in the 4-Å model of PS I, both I and H loops should be located in the center of the PS I core. The extensive accessibility of the H loop to proteases and its p side location imply that the H loop of PsaB could be accessible to interaction with plastocyanin or cytochrome c₅₅₃, the electron carriers that transfer electrons from cytochrome b₅₆₃ complex to the P700 reaction center of PS I complex in the p side of the thylakoid membrane. PsaF is involved in docking of plastocyanin in the plant PS I (7, 19, 20) but not in the cyanobacterial PS I (6, 21). This difference is ascribed to a lysine-rich sequence that is present in the N-terminal region of the plant PsaF but not in the cyanobacterial PsaF (43).

Besides PsaF, the H and J loops are the only two domains exposed to p side and located in the center of the PS I complex. When the H and J loop sequences of PsaA and PsaB from high plants and cyanobacteria are aligned, there is a major difference in a 12-residue sequence in the H loop of PsaB but not in others (4). This information suggests that the H loop of PsaB in cyanobacterial PS I complex may help the docking of plastocyanin or cytochrome c₅₃₃ from the p side of the thylakoid membrane and even contribute to the electron transfer from these two electron donors to the P700 reaction center.

In the 4-Å crystal structure of PS I, the C-terminal domains from both PsaA and PsaB core proteins form a cage where the electron transfer chain is located. This important cage is the catalytic center of the PS I complex and is protected by PsaD, PsaC, and PsaE subunits from the n side and by the surface helices I and I’ in the J loop of PsaA and PsaB from the p side of the thylakoid membrane (26). The protection of the C-terminal domains in PsaA and PsaB is also observed in the protease treatments of the wild type PS I complex. The only cleavages that may occur in the I, J, K, and L loops were the PaVI and PaVII fragments from the papain treatment accompanying the complete degradation of the small subunits PsaD and PsaF. With intact small subunits, the C-terminal loops I, J, K, and L are well protected from the limited proteolysis. Protection by the small subunits is also shown by the numerous proteolytic fragments from the cleavages in the I loop of PsaB in the mutant PS I complexes (Table VI).

The additional fragments in protease treatments of the subunit-deficient PS I complexes provide information about the interactions between the small subunits and the loops of PsaA and PsaB. PsaE is the only missing subunit in the PS I complex from the AEK2 strain. The additional fragments from the protease treatments of AEK2 complexes indicated that the absence of PsaE resulted in possible cleavages in the B–I loops of PsaA and the I loop of PsaB (Table VI). As an n side subunit, PsaE may interact with the C, E, F, and I loops in the n side of the thylakoid membrane. The cleavages in the p side loops may result from further digestion of the degradation products. Therefore, PsaE may interact with the n side loops in the N-terminal domain of PsaA and the I loops of both PsaA and
PsaB (Fig. 4). In the protease treatments of the ADC4 PS I complexes, additional cleavages were located in the C–E and G–I loops of PsaB (Table VI). Thus, PsaD may interact with the n side loops in the N-terminal domain of PsaB and the I loop of PsaB (Fig. 4). Additionally, Xu and Chitnis (28) identified that PsaD may shield the I and K loops of PsaB. When the PS I complexes from the AKF6 and AIC9 strains were treated by proteases, no additional fragments from the N-terminal domains of PsaA and PsaB were observed. The AKF6 and AIC9 mutant PS I complexes do not contain PsaF–PsaJ and PsaI–PsaL, respectively. Therefore, the extramembrane loops in the N-terminal domains of PsaA and PsaB may not interact with these subunits. The trypsin treatment of the AKF6 PS I complex yielded the TrA and TrB fragments. These two fragments resulted from the cleavages in the H loops of PsaA and PsaB (Table VI). Between the two subunits absent in the AKF6 complex, PsaF is mainly a transmembrane helix, whereas PsaF has been proposed to contain a peripheral domain on the p side of membrane (44). Therefore, the p side domain of PsaF may interact with the H loops of PsaA and PsaB on the p side of the thylakoid membrane (Fig. 4). In the Ysa-C treatments of the AKF6 and AIC9 PS I complexes, additional cleavages were observed in the I loop of PsaB. The missing subunits in these mutant PS I complexes may not directly interact with the I loop of PsaB because the predicted positions of these subunits in the 4-Å crystal structure were not close enough to the I loop of PsaB (26). However, chemical cross-linking studies have yielded the following cross-linked products: PsaC–PsaD, PsaC–PsaE, PsaD–PsaL, and PsaE–PsaF (44, 45). The interactions between the cross-linked subunits may cause some conformational changes of PsaD, PsaE, or even PsaC in the mutant PS I complexes. Therefore, the I loop of PsaB that was protected by PsaD and PsaE subunits could be exposed to protease in the AKF6 and AIC9 PS I complexes. Correspondingly, the absence of PsaE may cause the conformational change in PsaF and result in the exposure of the H loops of PsaA and PsaB that are shielded by PsaF. In the thermolysin treatment of the AEK2 PS I complex, the ThB fragment resulted from the cleavage in the H loop of PsaB (Table VI). As described above, PsaF may interact with the H loops of PsaA and PsaB, whereas PsaI, PsaL, and PsaJ may not shield the extramembrane loops of the core proteins (Fig. 4).

In the 4-Å crystal structure of PS I, core proteins PsaA and PsaB contribute their C-terminal domain to form the central cage of PS I core, and the N-terminal domain of PsaA and PsaB may form the peripheral helices. PsaA and PsaB may crossover in the central cage so both the C-terminal domains of PsaA and PsaB may be protected by a single subunit. For this reason, PsaF can interact with both H loops of the core proteins, and PsaE can shield both I loops of PsaA and PsaB (Fig. 4). However, the peripheral helices in one of the two symmetry regions should be donated from one of the core proteins. The absence of PsaE results in further cleavages in the N-terminal domain of PsaA, and the absence of PsaD results in further cleavages in the N-terminal domain of PsaB in the protease treatments of the mutant PS I complexes (Fig. 4). This asymmetrical interaction indicated the arrangement of the N termini of the PsaA and PsaB core proteins related to the PsaD and PsaE subunits. However, the electron microscopy study has revealed that PsaD and PsaE are located in different sides of the central axis, and they do shield different parts of the core proteins (46). Combining this information, we propose a model to distinguish PsaA and PsaB in the 4-Å crystal structure of PS I (Fig. 5). In this model, the region partly covered by PsaD and not by PsaE...
belongs to PsaB, and the region only covered by PsaE belongs to PsaA. Therefore, our data indicate that the primed helices in the 4-Å map belong to PsaA, and the unprimed helices are assigned to PsaB if the primed and unprimed helices are contributed from each of the core proteins (Fig. 5).

To conclude, the topographical analyses of the PsaA and PsaB proteins have allowed us to examine the accessibility of their extramembrane loops to proteases, the shielding and protection of these loops by small subunits, the location of the N terminus of PsaA, and the assignment of the two core proteins relative to small subunits in the 4-Å map. The biochemical techniques are indeed valuable for deciphering the structural details of membrane proteins and to complement the biophysical techniques that cannot be applied readily to the structural analysis of membrane proteins.

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REFERENCES
1. Chitnis, P. R., Xu, Q., Chitnis, V. P., and Nechushtai, R. (1995) Photosynth. Res. 44, 25–40
2. Fromme, P. (1996) Curr. Opin. Struct. Biol. 6, 473–484
3. Chitnis, P. R. (1996) Plant Physiol. (Bethesda) 111, 661–669
4. Golbeck, J. H. (1994) in The Molecular Biology of Cyanobacteria (Bryant, D. A., ed) pp. 179–220, Kluwer Academic Publishers Group, Dordrecht, The Netherlands