CROSSLINKING OF PHOTOSYSTEM I CORE POLYPEPTIDES IN *ANACYSTIS NIDULANS*

by

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INTRODUCTION

Photoautotrophic organisms, through an elaborate photosynthetic apparatus, capture and convert solar energy into chemical forms as ATP and NADPH + H. The organisms use these compounds to transform carbon dioxide into carbohydrates that is a cellular source of fixed carbon. The primary photochemistry occurs on specialized membranes, within lipid-protein complexes known as photosystems (PS). This thesis examines the structure of one photosystem - PS I of the cyanobacterium, Anacystis nidulans R2.

I. BIOCHEMICAL STRUCTURE AND FUNCTION OF THYLAKOID MEMBRANE IN ENERGY TRANSDUCTION

Most of the thylakoid proteins are organized into four intrinsic membrane-spanning complexes. Water is oxidized by the PS II complex (38). The cytochrome b/f complex accepts electrons from PS II complex via the plastoquinone pool, acts as a proton pump, and catalyzes the electrogenic Q cycle (39). Plastocyanin transfers electrons from the cytochrome b/f complex to the PS I complex (80). Coupling factor complex uses the proton motive force (PMF) built up by electron transport to produce ATP (64).

A. PS II Complex

The reaction center complex of PS II has been isolated from higher plants (87, 112) and cyanobacteria (9, 88, 98). The core of the PS II complex in higher plants is a set of five hydrophobic polypeptides of 51, 45, 34, 32, and 10 kDa (87, 112). The 51 kDa polypeptide is one of the two prominent proteins of PS II core complex. The 45 KDa protein subunit binds chl a and serves an antenna role in the reaction center

of PS II. The polypeptide composition of PS II from cyanobacteria is very similar to that of PS II from high plants (18, 19,98). The PS II complex of cyanobacteria contains two subunits (45-50 kDa), cytochrome b-599 (10 kDa), and one or more polypeptides in the 30-35 kDa region (98). Topological studies of PS II were done by Guikema and Sherman (34, 35). The results indicated that the 31.5 KDa was atrazine binding protein, the 45 KDa polypeptide and the 42 KDa polypeptide had surface exposed domain.

B. Cytochrome b/f complex

The cytochrome b/f complex functions as a plastoquinol / plastocyanin oxidoreductase which has been isolated from several sources (10, 49, 62). When isolated from higher plants, it consists of at least five polypeptides including cytochrome f (31 kDa), cytochrome b (22.5 kDa), the Rieske Fe-S center (22 kDa), and one or more other proteins of unknown function (48, 49). The role of cytochrome f and the Rieske Fe-S polypeptides has been investigated (114), and was suggested as a linear rapid donor pool to PS I: Fe-S - cytochrome f - plastocyanin - P-700.

C. PS I complex

A detail discussion of the function and structure of PS I will be in part II and III.

D. Coupling Factor Complex

The proton gradient created by electron transfer through PS II and PS I is used by coupling factor complex to generated ATP (64). ATPase complex consists of two parts: coupling factor I (CF1), which contains the active sites for ATP synthesis, and CFo, which functions to bind CF1 to the membrane and as a proton channel (64, 74). CF1 complex of

higher plant is composed of 5 protein subunits of 59, 56, 37, 17.5, and 13 kDa. The proton conducting sector, CF_o, contains 3 subunits of about 8-15 kDa. The CF_1 has been isolated from cyanobacteria (90). This complex also contains five protein subunits of 55.5, 54, 36, 21, and 15 kDa.

II. THE FUNCTION OF PS I REACTION CENTER

A. Electron Donors to PS I

Studies have shown that plastocyanin was the immediate donor to P-700 in higher plants and in most alga (80). Haehnel et. al. (39) observed that the rapid reduction of P-700 could be inhibited by either KCN or $HgCl$, a result implicating plastocyanin as the donor to P-700. Kinetic properties of plastocyanin binding to the PS I complex indicated that two plastocyanin molecules may bind to the reaction center (61).

In contrast with higher plants, cyanobacterial and eukaroytic algae may utilize a cytochrome as the donor to P-700 (90). In several species, the cell is competent to synthesize both proteins, with the choice between them determined by mineral nutrition. If the organisms lack copper, cytochrome will replace plastocyanin (43).

B. Electron Acceptors of PS I

Many studies have shown that the iron-sulfur centers were electron acceptors of PS I. Bearden and Malkin (2) found that P-700 photooxidation was concomitant with photoreduction of a membrane-bound iron-sulfur protein, and that this iron-sulfur protein functioned as an electron acceptor of PS I. Evans et al. (25) also suggested that the electron acceptors of PS I were iron-sulfur centers. As we discuss in detail below, three iron-sulfur centers, Fa, Fb, and Fx, and two

primary electron acceptors, Ao and A1 have been identified in PS I.

1. Iron-sulfur center: Fa, Fb, and Fx

The studies of chemical reduction and illumination at room temperature showed more than one iron-sulfur centers in PS I (53, 54). In early work, Evans et al. (25) proposed that there were two iron-sulfur centers, Fa and Fb, which could be distinguished by EPR. Fb is more electronegative Em than Fa. The direct measurement of the midpoint potential of the iron-sulfur centers in PS I enriched subchloroplast fragments showed that Fa has an Em = -530 mV and Fb has an Em = -590 mV (24). When these fragments were frozen in the dark with ascorbate and illuminated, one electron was moved from P-700 to either Fa or Fb (11).

Another iron-sulfur center, Fx, also has been found in the PS I (5, 65, 66). Evans et al. (23) discovered that the reversibility of P-700 increased after reduction of both Fa and Fb. They proposed an additional electron acceptor functioned prior Fa and Fb (23). Golbeck et al. (29, 110) recently showed the presence of 2Fe-2S cluster and 4Fe-4S clusters among Fa, Fb, and Fx. They suggested that Fx is depicted as a 2Fe-2S cluster shared between the two large molecular weight polypeptides of PS I complex (30, 110).

2. Primary Electron Acceptors: Ao and A1

Two intermediary electron acceptors Ao and A1 were discovered in PS I. In an attempt to determined the redox potential of Fx, Ke et al. (53) found that a more primary acceptor, with a midpoint potential near -735 mV, may exist . The spectroscopic and kinetic data further demonstrated two earlier electron acceptors Ao and A1 in PS I (99). Spectral properties of A1 were found to correspond to that of a chlorophyll a dimer (94). Two electron acceptor species, Ao and A1

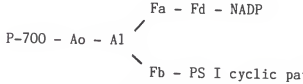
operate in series between P-700 and Fx, as P-700 - Ao - A1 - Fx.

3. Sequence of Electron transport in PS I

It is apparent, therefore, that five electron acceptors can be distinguished using biophysical instrumentation. However, the sequence of electron passage among these acceptors is not entirely clear. In earlier studies, Heathcote et al. (40) have shown that Fb can be photoreduced when Fa has already been reduced. Electron transfer between Fa and Fb was essentially irreversible at low temperature. Yet, when Fa and Fb were reduced the electron transfer from P-700 to Fx was rapidly reversible (66). Evans and Heathcote (25, 40) also confirmed that Fb is involved in electron transfer in PS I between centers Fx and Fa. A linear sequence of electron transfers in PS I was proposed as (77, 96):

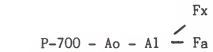
P-700 - Ao - A1 - Fx - Fb - Fa - Fd - NADP

Later studies showed that the Em of Fa can be more negative than that of Fb (45). This result challenged the first model. Golbeck et al. (30, 31) demonstrated a differential decrease in Fe-S centers Fx, Fa and Fb which indicated that Fx serves as a branch point for parallel electron flow through Fa and Fb. Bolton (5) suggested that one bound Fe-S center functions in noncyclic electron transfer pathway to NADP while the second functions in a cyclic pathway around PS I. A alternative model was demonstrated as (61):



Hiyaman and Fork (42) suggested that iron-sulfur centers, Fa, Fb, and Fx were the terminal acceptors in PS I particles at physiological

temperature. Another model for electron transfer in photosystem has been suggested by Setif and Mathis (83).



III. STRUCTURE OF PS I COMPLEX

In higher plants, the PS I complex consists of two component parts: an antenna light harvesting complex (LHCI) and a reaction center core. LHCI serves to harvest light energy and to pass that energy to the reaction center core (61). The composition of LHCI varies widely between species (117) and LHCI is absent in most cyanobacteria (90). The PS I core contained several small polypeptide subunits as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The PS I core has been isolated from spinach (1, 72, 85), swiss chard (3, 4), barley (56, 67), pea (68), maize (22, 63), Chlamydomonas (52) and cyanobacteria (47, 90). The size and complexity of PS I preparations depend on the treatment and electrophoresis conditions (buffers, detergent concentrations, and temperatures) (14, 15, 92).

Studies have shown that PS I proteins were encoded by nuclear and chloroplast DNA (68, 113). Three polypeptides of PS I were synthesized within the chloroplast and eight PS I proteins were coded in nuclear DNA (37, 61). Most of chloropyll antenna proteins of PS I were synthesized on cytoplasmic ribosome (61). Many reports showed that the P-700-chl a-protein (subunit I) were synthesized in the chloroplast (37, 68).

A. PS I Reaction Center Complex

The characteristics of PS I complex have been investigated. Mullet

et al. (67) isolated a native PSI complex from chloroplasts by using low concentrations of Triton X-100. This native PSI contained 110 chlorophyll a molecules per P-700. The chl a / chl b ratio was great than 18. The reaction center of PS I, isolated from PS I particles, contained 40 chlorophyll per P-700 (67). The molecular weight of PS I reaction center was about 130 kDa chl-protein band in SDS-PAGE. The PS I chl-protein complexes of cyanobacteria, which were chromatographically isolated from SDS extracts, have chl a / P-700 ratio of 40, and have molecular weight of 110 kDa on SDS-PAGE (8, 9, 90). Guikema and Sherman (34) have isolated six prominent chl-containing bands with molecular weights 45 to 360 kDa from thylakoid membranes of Anacystics nidulans R2. Four of these chl-protein bands (I, II, III, and V) contained the proteins of PS I.

1. Polypeptides of PS I Reaction Center

PS I reaction center contains one large subunit (60-70 kDa) and other several small polypeptides. Bengis and Nelson (3, 4) showed that the reaction center complex from Swiss chard contained five polypeptides of 70, 25, 20, 18, 16 and 8 kD. The PS I reaction center purified from spinach chloroplast contains six polypeptides of 60-65, 23, 20, 17, 14, and 12 kDa (1, 72).

The polypeptide composition of PS I reaction center from various cyanobcteria are very similar with those from higher plants (90). By digitonin treatment, Newman and Sherman (76) have isolated a P-700 activity PS I particle from Synechococcus cedrorum. This PS I particle contained five polypeptides of 62, 18, 17, 15, and 14 kDa. Lundell et al. (60) found that the PS I particles isolated from Synechococcus 6301 had the full complement of antenna chlorophyll, with 130 chlorophyll

per P-700, and had a protein/chl ratio of 2.9. These PS I particles showed the activity of iron-sulfur centers, Fa, Fb, and Fx. After uniform ^{14}C labeling, this PS I reaction center showed five polypeptides of 70, 18, 17.7, 16, and 10 kDa, with a molar ratios of 4.0 : 0.7 : 1.0 : 0.5 : 1.6. Lundell et al. (60) suggested that the stoichiometry of these five polypeptides was 4 (70 kDa) : 1 (18 kDa) : 1 (17.7 kDa) : 1 (16 kDa) : 2 (10 kDa).

2. Largest polypeptide subunit of PS I reaction center

The largest polypeptide of PS I is P-700 reaction protein. The specific function of this polypeptide from swiss chard has been identified by Bengis and Nelson (3). The results indicated that the primary oxidation and the primary reduction in PS I were associated with the 70 kDa polypeptide. After SDS treatment the low molecular weight subunits are missing and the P-700 signal is retained in 70 kDa polypeptide subunit (4).

Most of chlorophyll in PS I reaction center is associated with the large subunit. Removal of the solubilized chlorophyll from PS I complex, which contained 40 chlorophyll a per P-700, by DEAE-cellulose chromatography resulted in only 10 to 20 chlorophyll a molecules per P-700 (4). It was suggested that the largest subunit, P-700 reaction protein, was specifically associated with about 20 chlorophyll a molecules.

The molecular weight of large subunit range from 50 to 70 kDa. This value changes depending on the species and SDS-PAGE running condition. The molecular mass of this subunit from swiss chard (3), Synechococcus 6301 (116), and Prochloron (89), is 70 kDa, from spinach 60-65 kDa (1, 72), and which is about 62 kDa for Synechococcus.

cedrorum (76) and Synechococcus sp. (102, 103). A broad protein band or two distinct protein bands from 60 kDa to 70 kDa have been observed upon denaturation of chlorophyll-protein complex (108). The explanation of these differences in stoichiometric ratios comes from either partial degradation of a single component or the existence one more copies of subunit I.

Three different models have been suggested to explain stoichiometry for the subunit I of PS I. First, there is only one large polypeptide in PS I reaction center. Ikegami and Ke (50, 51) showed that PS I particle of 160 kDa band contained one P-700. They suggested only one 70 kDa subunit per particle.

Second, two copies of subunit I are present in PS I reaction center (3) Sonoike and Katoh (97) provided direct evidence for the heterogeneity of these two polypeptides (60 and 62 kDa) in the PS I reaction center of cyanobacteria.

Finally, four copies of subunit I present in PS I reaction center. Measurement of the chlorophyll to protein ratio by quantitative amino acid analysis and consderation of the yield of P-700 in the protein isolation suggested that P-700 is associated with a minimum of four subunits of approximately 60 kDa (109). Lundell et al. (60), using ^{14}C labelled study, also suggested a tetrameric structure of protein subunit I for the PS I reaction center.

The genes which encode the P-700 chlorophyll a apoprotein have been sequenced. Fish et al. (27) reported that two genes, designated A1 and A2, coding for P-700-chl a-proteins from maize chloroplasts, have been identified. Both proteins encoded by these two genes were highly hydrophobic and contained eleven putative membrane-spanning domains

(58). Bryant and co-workers (12) used the maize A1 and A2 genes as heterologous hybridization probes to clone cyanobacterium, Synechococcus PCC 7002 A1 and A2 genes. The sequence of gene A1 consisted of 740 amino acids and is 78 % homologous to the sequence deduced for the maize gene.

Subunits I of PS I reaction center corresponding to the bound iron-sulfur protein has been examined. Golbeck et al. (30, 111) found that the unfolding of 64 kDa polypeptide and denaturation of bound iron-sulfur cluster corresponding to Fx. They suggested that Fx was associated with the P-700 polypeptide in the PS I reaction center complex (30, 111). Based on the faces that four cysteinyl ligands were assumed to hold an iron-sulfur cluster, the Fx could be attacked to two 64 KDa proteins abridged by a single 2Fe-2S cluster (29, 30). This structure is further confirmed by sequence of PS I-A1 (contained 5 cysteines) and PS I-A2 (contianed 2 cysteines) genes (27).

3. Smaller polypeptide subunits of PS I reaction center

The molecular mass and the numbers of the small subunits in PS I reaction center vary in species. Spinach PS I reaction center contains five small subunits with molecular mass from 12 to 23 kDa (1, 72), Chlamydomonas has only three small subunits (19, 10, and 8 kDa) (61). In general, cyanobacterial PS I complex consists of three or four low molecular mass polypeptides between 20 to 8 kDa. For example, Mastigocladus laminosus PS I contains three proteins of 16, 11, and 10 kDa (70-73), whereas, the one from Synechococcus 6301 contains four polypeptides of 18, 17, 16 and 10 kDa (60).

Bengis and Nelson (4) suggested that the subunit III (20 kDa) of PS I mediates reduction of P-700 by plastocyanin. Their results

indicated that a reaction center lacking subunit III lost the function of cytochrome f photooxidation or of NADP photoreduction in the presence of plastocyanin (3, 4).

The smallest peptide (8 kDa) of PS I may associate with the iron-sulfur centers. Lagoutte et al. (57) found that most of the cysteine residues were located in the 8 kDa protein of PS I isolated from spinach by in vivo ^{32}S labeling and carboxymethylation with ^{14}C iodoactate. Guikema and Sherman (36), using labeling studies of ^{59}Fe in Anacystis nidulans R2 cell, showed most of ^{59}Fe bond to 8 KDa polypeptide of PS I. They suggested that 8 kDa protein was associated with iron-sulfur centers.

Malkin et al. (61), using chaotropic agents and ESR spectroscopy, suggested that either the 19 kDa (subunit II) polypeptide functions to stabilize the Fa and Fb on the 62 kDa polypeptide or one Fe-S center may located at 19 kDa polypeptide.

Takahashi et al. (102, 103) found 10-14 iron atoms containing in PS I complex of Synechococcus. Lacking two small protein subunits of 10 KDa and 14 KDa in the PS I was total devoid of iron. They indicated that all iron atoms and larger fraction of vitamin K molecules present in the PS I reaction center were associated with the two small subunits of 10 KDa and 14 KDa (102).

B. Membrane Topography of the PS I Reaction

The organization of PS I peptides in thylakoid membrane have been examined (100, 106, 115). Ortiz et al. (81) used the chemical modifier, 2,4,6-trinitrobenzenesulfonate (TNBS) and the proteolytic enzyme, Pronase E, to study the transverse heterogeneity of the protein subunits associated with the PS I complex. The results indicated that

most of the polypeptides of the PS I complex were exposed to the stroma. By employing right side-out and the inverted thylakoid vesicles, six of the nine polypeptides in PS I complex have shown to traverse the membrane (81). The large subunits of PS I encoded by genes A1 and A2 contain eleven putative membrane-spanning domains (58). Enami et al. (20) showed that four protein subunits of the PS I reaction complex from Synechococcus sp. were exposed to the stromal surface of thylakoid membranes by proteolytic digestion with trypsin and pronase E.

Many chemical crosslinking reagents has been used to determine the subunits relationships of protein complexes (13, 59, 118). Topology and neighbor analysis of polypeptide subunits of photosynthesis have been examined from photosynthetic bacteria (44, 104), cyanobacteria (21), and higher plants (16) by several differential crosslinking reagents.

Recently, Enami et al. (21) investigated the neighbor relationships of PS I polypeptide subunits of PS I reaction center in Synethococcus sp. by using three crosslinking reagents, dimethyladipipimidate (hydrophilic), glutaraldehyde (GLU) (hydrophilic), and hexamethylenediisocyanate (hydrophobic) (HMDC). They found that four polypeptides were affected by hydrophilic crosslinker. They suggested that four of five protein subunits in PS I complex were exposed to the stromal surface of thylakoid membranes. Two smaller subunits (13 and 14 kDa) always showed a parallel decrease. Enami et al. (21) suggested that these two subunits are neighbor polypeptides in PS I reaction center complex. They attempted (without success) to identify the crosslinked products by using cleavable chemical reagents.

The goal of this work was to examined the neighbor relationships of PS I polypeptides by using antibodies against five PS I subunits to identify the GLU or HMDC-treated crosslinked bands.

MATERIALS AND METHODS

CELL CULTIVATION

Anacystis nidulans R2 was grown in shaking culture (31 C, 90 rpm, 85 W/m^2) using Allens BG-11 (34) growth media. Cells were harvested by centrifugation (5,000 g for 5 min) and were resuspended in buffer A (50 mM K_2HPO_4, 50 mM KH_2PO_4, 1 mM EDTA, and 1 mM NaN_3, pH 7). Cells were either used immediately or were stored as pellets at -70 C.

ISOLATION OF PS I PREPARATION BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

A PS I preparation was isolated by the method of Lundell et al. (60). Cells were suspended in a buffered salt solution (SPCM), containing 0.6 M sucrose, 80 mM KH_2PO_4, 0.55 mM K_2HPO_4, 0.3 M Na citrate and 10 mM $MgCl_2$, pH 7.0, and were mixed with an equal volume of glass beads. Mechanical disruption was accomplished by a Bead Beater (Biospecs products Inc.) for 10 cycles of 1 min on, 1 min off. Unresolved cells were removed by centrifugation (5,000 g for 5 min). A membrane preparation was obtained by centrifugation (35,000 rpm for 30 min in a T-865 fixed-angle rotor). The green pellet was resuspended in buffer containing 50mM K_2HPO_4, 50 nM KH_2PO_4, 1mM EDTA, 0.1 % Triton X-100, and 1mM NaN_3, pH 7.0 with a glass homogenizer. Menbranes were repelleted in a T-865 fixed-angle rotor at 35,000 rpm for 30 min, resuspended in buffer A, and were used immediately or stored at -70 C.

Membranes (2 mg chl/ml) were solubilized in Triton X-100 (2 %) and stirred for 30 min at room temperature. The insoluable material was

pelleted by centrifugation (35,000 rpm for 30 min in a T-865 rotor). The green suspension was loaded onto a 17 ml step sucrose gradient, consisting of 0.2, 0.4, 0.6, 0.8, and 1.0 M sucrose in buffer A (without Triton X-100), and then centrifuged in an AH-627 Swinging Bucket rotor at 22,000 rpm for 14 hours. The main dark green band located at 0.2 M sucrose gradient was collected and was diluted with buffer A. The green suspension was pelleted in a T-865 fixed-angle rotor at 35,000 rpm for 5 hours. The pellet was resuspended in buffer A (0.5 mg chl/ml) containing 0.5 % Triton X-100, and employed to 5-20 % sucrose linear density gradient (0.1 % Triton X-100). The centrifugation was at 22,000 rpm in a AH-627 Swinging Bucket rotor for 14 hours. A PS I fraction was collected as a dark green band at 0.2 M sucrose gradient.

ISOLATION OF PS I BY DODECYL MALTOSIDE

POLYACRYLAMIDE GEL ELECTROPHORESIS

Cells were broken in a Bead Beater and unbroken cells were removed as described above. The supernatant was centrifuged at 35,000 g for 30 minutes. The green pellets were resuspended in a small volume of SPCM, and slowly diluted by addition of 9 volumes of DW + PI (0.5 mM phenylmethylsulfonyl fluoride, 1.1 mM benzamidine, and 1.2 mM norleucine). This osmotic readjustment detached the phycobilisomes from the photosynthetic membranes. The preparation was centrifuged at 17,000 rpm (35,000 g) for 30 minutes. The green membrane pellet was resuspended in 10 mM Tricine buffer, pH 7.5.

The chlorophyll containing protein complexes (PS I or PS II) were isolated from the thylakoid membranes by polyacrylamide gel electrophoresis in the presence of dodecyl maltoside (32, 33). The gels

were formed with 7.5 % polyacrylamide running gel and 5 % polyacrylamide stacking gel. The running gel and the stacking gel contained 0.1 % dodecyl maltoside. The gel and the running buffer, consisted of 200 mM glycine and 25 mM Tris-base, were chilled before initiation of the electrophoretic run. Samples were prepared by adding dodecyl maltoside to a final concentration of 2.0 %, sucrose to 6 %, Tricine to 20 mM (pH 7.5), resulting in a final chlorophyll concentration of 150 ug/ml. The sample was incubated at 4 C (20 min) and centrifuged for 10 seconds in a microcentrifuge to remove unsolubilized debris. The thylakoid membranes containing 3 ug of chlorophyll were loaded in each well. Electrophoresis was performed at 3 W constant power for two hours at 4 C. The gels were photographed to record the chlorophyll protein patterns immediately after termination of electrophoresis.

The chlorophyll-protein containing bands were separated from the gels by electroelution. Absorption profiles of the eluted material was obtained with an SLM/ AMINCO DW-2C spectrophotometer.

ELECTROPHORESIS

The protein composition of PS I preparation was examined by polyacrylamide gel electrophoresis with lithium dodecyl sulfate (LiDS) in place of SDS (36). Samples were treated with LiDS (2 %), 2-mercaptoethanol (2 %), or 2 M urea, and then kept at 4 C, at room temperature or were heated as described in Fig. legend. Gels were electrophoresed at constant power (3 W) at approximately 4 C. After electrophoresis, gels were fixed in 12 % (w/v) Trichloroacetic acid (TCA) for at least one hour and then replace by 2 % (w/v) TCA. Coomassie brilliant blue G-250 was added to the final concentration of

0.05 % (w/v) (75). The c staining step was repeated at least 1 time after 12 hours shaking. The staining gels were photographed or scanned at 440 nm with a Kontes Fiber Optic scanner.

CROSSLINKING OF PS I PREPARATION

Two crosslinking reagents, glutaraldehyde (GLU) and hexamethylenediisocyanate (HMDC), were used (21). Isolated PS I preparation or thylakoid membranes were incubated with various concentrations of GLU or HMDC at room temperature in 50 mM triethanolamine buffer (PH 8.5). After 20 minutes, the reaction was stopped by addition of glycine to a final concentration of 2 % (w/v).

ANTIBODY PREPARATION AND PURIFICATION

Polyclonal antibodies were raised against the individual subunits of PS I (82). The polypeptides separated by the polyacrylamide gel eletrophoresis were injected into rabbits in the following manner: The polyacrylamide gels were stained and washed. The polypeptide bands were excised and mixed with Freund's complete adjuvant and sonicated briefly to emulsify. The antigens were injected intradermally at about 4-6 sites on the back of rabbits. Subsequent injections were given every 7-10 days with antigens emulsified in Freund's incomplete adjuvant. After about three months, serum was collected from the animals.

The purification of PS I polypeptides was carried out by using a Beckman high-performance liquid chromatograph (HPLC) in an SDS system (41). The PS I preparation collected by sucrose density gradient centrifugation was treated with 5 % (w/v) SDS to denature the proteins (83). The samples were loaded onto a C18 column with mobile phase solvents (acetonitrile and trifluoroacetic acid with 0.1 % SDS). Flow rate was maintained at 1.0 ml/min. A linear gradient from 0 to 80 %

acetonitrile was applied over a 50 min period. Protein-containing peaks were collected, analyzed by LiDS-PAGE, and purified by a second round of HPLC.

HPLC purified proteins were blotted to Zeta-probe membrane in 25 mM Tris, 192 mM glycine, pH 8, (30 min), and blocked in 20 mM Tris, 500 mM NaCl, pH 7.5, 10 % Bovine Albumin (50 C, 12 h), and transfered to PBS buffer (10 mM Pi, 0.9 % NaCl, pH 7.4) (28). These blots were used to affinity-purify antibodies from antisera. The blots were incubated in serum for two hours, washed, and transfered to 200 mM glycine (pH 2.8) for 5 minutes. The eluted material was immediately neutralized with NaOH (78, 79). A single Zeta-probe membrane could be used for at least three cycles of affinity purification.

WESTERN BLOTTING

Protein transfer to nitrocellulose was at 100 V for 2 hours (room temperature) in 25 mM Tris, 192 mM glycine, and 20 % methanol, pH 8.3 (105). The blots were blocked with 2 % milk in PBS, for 2-3 hours at room temperature, and were washed in PBS-T (0.05 % tween-20 in PBS) for 3 cycles of 10 min (55). Blots were incubated with diluted antisera or affinity-purified antibody (1:200 dilution) for 12 hours, and washed for 3 cycles (40 min each) in PBS-T at room temperature. Blots were incubated in PBS containing Goat anti-Rabbit IgG (peroxidase-linked) for 12 hours. After three 40 min washing in PBS-T, blots were transfered to color developing solution (20 % methanol, 0.1 % 4-chloro-naphthal, 0.05 % H202) to resolve Ab binding (107).

RESULTS

ISOLATION OF PS I CORE COMPLEX

Two procedures were employed to isolated a membrane fraction enriched in PS I. First, we used a modification of a protocal by Lundell et al. (60), which involved treatment of membranes with Triton X-100, followed by two sucrose density gradient centrifugation steps. The polypeptide profiles of thylakoid membranes and isolated PS I complexes are shown in Fig. 1. The PS I profile contained five polypeptide bands of 62-64, 16, 14.5, 12.5 and 8 kDa.

Several noteworthy features were observed. First, the migration of the large subunit depended upon the temperature of solubilization with LiDS or urea (Fig. 1-4). We found this band was separated into a 62 and 64 kDa band in LiDS-PAGE containing 2 M urea (Fig. 3 and 4). Second, the dissociation of PS I core was sensitive to detergent concentrations (Fig. 3) and temperature (Fig. 4). When samples were not heated or solubilized with low concentration of LiDS, the 62-64 kDa band decreased correlating with an increase of a dark green chlorophyll-containing band above the 62-64 kDa region before staining. When samples were heated above 70 C for 20 minutes or at 5 % LiDS , the dark green band was converted to the 62-64 kDa bands and free chlorophyll was observed at the dye front. Third, the four small subunits were not as sensitive to detergent concentration and temperature. The intensities of these four bands (Fig. 1, 3 and 4) were almost identical despite differences in LiDS or temperature treatment prior to LiDS-PAGE. Finally, the banding profile was quantified by densiometric scanning of the gel, as showing in Fig. 2. The ratio of the relative

peak area of these bands was 2.1 (64 kDa) : 2.4 (62 kDa) : 1 (16 kDa) : 1.8 (14.5 kDa) : 0.6 (12.5 kDa) : 1 (8 kDa).

Another method to resolve PS I core utilized the dodecyl maltoside PAGE system devised by Guikema (32, 33). Thylakoids were treated with dodecyl maltoside and were resolved on a polyacrylamide gel which also contained maltoside. Figure 5 shows chlorophyll-protein complexes from thylakoid membranes and from isolated PS I on maltoside PAGE . Three chlorophyll containing protein bands, denoted band 1a, 1b, and 2 (32), were observed in thylakoid membrane samples (Fig. 5 Lane 2). Two chlorophyll containing protein bands (1a and 1b) were observed in the PS I sample (Fig 5. Lane 1).

The polypeptide composition of these three chlorophyll-protein bands were examined by LiDS-PAGE folloing electroelution of each green band (Fig. 6). Bands 1a and 1b were identical, and contained 62-64 kDa polypeptide subunits and four small subunits (Fig. 6 Lane 1 and 2). However, bands 1a and 1b differed in migration after mild solubilization. When unheated there were four and two dark green bands in band 1a (Fig. 6 Lane 1) and 1b (Fig. 6 Lane 2), respectively. A broad band in 62-64 region was seen (Fig. 6 Lane 4 and 5) after heat treatment (70 C for 20 min). The polypeptides profile of band 1a and 1b compared with that of the PS I obtained by sucrose density gradient centrifugation showed a similar composition. The proteins of maltoside gels band 2 (Fig. 6 Lane 3) appeared in the region of 30-55 kDa mostly, showing a similar polypeptide composition as that observed in PS II fractions (8, 90).

Fig. 7 shows the light absorption spectral characteristics of the chlorophyll-protein complexes following elution from maltoside gels.

The spectra derived from bands 1a and 1b showed an absorption maximum at 680 nm. However, the spectrum for band 2 showed an absorption maximum at 675 nm. The PS I preparation isolated by sucrose density gradient centrifugation showed a similar absorption peak to band 1a and 1b. We conclude that both bands 1a and 1b represent aggregation states of the PS I core. Both bands 1a and 1b are distinguished from band 2 based on the protein composition and spectral characteristic.

CROSSLINKING OF PS I COMPLEX

Crosslinking reagents provide a powerful tool to understand the organization of photosystem I protein subunits. In preliminary experiments, we screened a number of crosslinking reagents for their effects on PS I peptides. Only two were effective, glutaraldehyde (GLU), a hydrophilic crosslinking reagent and hexamethylenediiaocyanate (HMDC), a hydrophobic crosslinking reagent. Our first goal was to characterize the effects of these reagents on PS I preparations isolated by sucrose density gradient centrifugation.

The polypeptide profile of PS I treated with glutaraldehyde is shown in Fig. 8. Three new polypeptide bands were observed after staining with Coomassie blue. The top band has molecular weight of 95 kDa. Two other bands (25 and 32 kDa) were shown between the large and the small subunits of PS I. One dark green chlorophyll protein band was observed between subunit I and the top crosslinked band.

Fig. 9 shows the banding profile of GLU-treated PS I quantified by densiometric scanning of the gels. The results indicated that the dark green band and four of five subunits decreased with an increased GLU concentration. However, subunit V (8 kDa) was relatively unaltered by GLU. Three crosslinking bands (95, 32, and 25 kDa) decreased sharply

when GLU concentrations were increased to above 0.08 % (w/v) and disappeared completely at 0.12 % (w/v). Perhaps a high GLU induced PS I aggregation which prevented protein migration into the gel.

As shown in Fig. 10, HMDC was also an effective crosslinking reagent with isolated PS I core preparation. HMDC treated PS I samples showed two crosslinked products. The molecular weights of these two bands were similar to the two small crosslinked products of GLU treated samples. Fig. 11 shows the quantification of the banding pattern after crosslinking with HMDC. Notice that the crosslinking of PS I proteins resulted in the reduction of the polypeptide subunits, I, II, III, and IV. Subunit V (8 kDa) was the most resistant to crosslinking with itself or with other subunits. The 62 and 64 kDa polypeptide subunits were readily effected by HMDC treatment.

ANTIBODY PREPARATION AND PURIFICATION

Our preliminary crosslinking experiments convinced us of the need for specific probes to recognize PS I components within crosslinked bands. Therefore, the antibodies were raised in rabbits against the five polypeptide subunits of PS I complex. The specificity of the five antibodies are shown in Fig. 12. The anti-subunit I antibody identified the 62-64 kDa proteins and the chlorophyll-containing band. The anti-subunit V antibody bound only to the 8 kDa subunit. However, the remaining antisera were not as specific as we would have liked. Antibodies raised against subunits II and III crossreacted with both protein subunits II and III. The anti-subunit IV antibodies bound not only to the subunit IV, but also to subunit III slightly .

To purify these antibodies, we attempted an HPLC purification of the antigens. PS I fractions were denatured using SDS (5 %), and

injected into an acetonitrile solvent system containing 0.1 % SDS. Unfortunately, only two proteins were purified by HPLC. Fig. 13 shows the polypeptide subunits III and V separated by HPLC, wich were used to affinity purify antibodies. Fig. 14 shows the purified antibodies blotted with PS I subunit III. Before purification, anti-subunit III antibody identified both subunits II and III (Fig. 14, Lane 1). The purified antibody only identified subunit III (Fig. 14, Lane 2)

IMMUNOLOGICAL IDENTIFICATION OF CROSSLINKED PRODUCTS

The polyclonal antibodies raised against the five protein subunits of PS I complex were used to probe the crosslinked products. Fig. 15 shows the immunoblots of the crosslinking PS I complex with GLU treatment. Two crosslinked bands were observed under this low concentration of GLU. In the control samples (no crosslinking), the antibodies crossreacted only with antigen proteins (Fig.15, Lanes 1, 3, 5, 7, and 9). The anti-subunit I and III antibodies cross-react strongly with the 95 kDa crosslinked band (Fig. 15, Lane 2 and 6). This crosslinked product also can be identified by anti-subunit II and IV antibodies slightly (Fig. 15, Lane 4 and 8). The 32 kDa crosslinked band was identified by the antibodies for subunits II, III, and IV. No immunoblotting crosslinked bands were observed in control samples. Nor was subunit V observed in any crosslinked bands.

Two crosslinked bands were observed in the immunoblot of PS I complex treated with HMDC (Fig. 17 and 18). The molecular weight of these two bands was similar to those observed by Coomassie blue . These two crosslinked products were identified by antibodies for subunits II, III, and IV. No crosslinked band was seen above the 64 kDa polypeptide. The bands above subunit I were chlorophyll-containing bands, not the

crosslinked bands (Fig. 17, Lane 1) The low molecular mass crosslinked band (25 kDa) was a significant crosslinked product by HMDC treatment. The another crosslinked product (32 kDa) was also observed after HMDC treatment, but strongly induced by GLU. The anti-subunit V antibody did not identify any crosslinked band (Fig. 17, Lane 5).

IMMUNOBLOTTING OF PS I COMPLEX FROM

CROSSLINKING OF THYLAKOID MEMBRANE

We have studied the crosslinking characteristics of PS I preparations following isolation protocols involving detergents. Obviously, this protocol has disadvantages. Detergent treatment can yield protein rearragements within membrane complexes. Isolation of particles can force protein-protein proximity which would not normally occur in intact membranes. Isolation of the antibody probes permitted a shift in experimented strategy. We first treated the thylakoid membranes with these two crosslinking reagents, then we isolated the PS I preparations from treated membranes by sucrose density centrifugation. LiDS-PAGE and antibodies were used to examine these preparations.

Fig. 19 shows the polypeptide profiles of PS I isolated from thylakoid membranes treated with GLU and HMDC. We found that there are several large crosslinked products above subunit I. GLU treated samples showed four crosslinked bands (25, 32, 100, and 112 kDa). HMDC treated samples resulted in five crosslinked bands (25, 88, 100, 112, 190, and 210 kDa). The 190 and 210 kDa crosslinked bands contained at least three subunit I proteins based on the molecular weight.

GLU-treated samples showed a more complicated crosslinked pattern in western blotting (Fig. 20). Four crosslinked bands were identified

by anti-subunit I antibody (Fig. 20, Lane 2). The two top crosslinked bands (over 125 kDa) were crosslinked products of two subunit I polypeptides and some smaller subunit proteins. Subunit III antibody identified eight crosslinked products (Fig. 20, Lane 6). The 32 kDa band is a prominent product for both antibodies. The crosslinked patterns are different with probes directed against subunit III and IV. The antibody against subunit IV identified five crosslinked bands. The most significant crosslinked band has a molecular weight of 76 kDa, which may contain subunits I and IV. No crosslinked product was found with subunit V antibody.

Fig. 21 shows the immunoblot of PS I isolated from HMDC treated thylakoid membranes. The PS I polypeptides were readily aggregated by HMDC due to its hydrophobic character. Many crosslinked products were too large to enter the running gel. Several points are noteworthy: (i) two crosslinked bands with molecular mass over 120 kDa; (ii) several crosslinked bands between 62 and 20 kDa region; (iii) no crosslinked band was identified by anti-subunit V antibody.

A summary of crosslinked products of PS I complex treated with GLU and HMDC is given in Table I. We have identified a total of fourteen croslinked bands: GLU treated samples contained nine and HMDC treated samples contained eight. The protein composition was estimated by according to molecular weight and antibody detection.

DISCUSSION

CHARACTERISTICS OF PS I COMPLEX

The major goal of this study was to examine the structure and organization of the reaction center complex of PS I from the cyanobacteria, Anacystis nidulans R2. Two different methods, maltoside-polyacrylamide gel electrophoresis and sucrose density gradient centrifugation, were used to isolate PS I. Both procedures showed the same results: the PS I complex contained a minimum of 6 polypeptides, subunits I (64 and 62 kDa), II (16 kDa), III (14.5 kDa), IV (12.5 kDa), and V (8 kDa).

The polypeptide composition of PS I reaction center complex has been examined in higher plants, spinach (1, 72), swiss chard (3, 4), barley (56, 57), pea (68), maize (22, 63), and cyanobacteria, Mastigocladus laminosus (70-73), Synechococcus 6301 (60). In all of these species, the PS I complex is comprised of at least one large subunit (60-70 kDa) and several other small subunits, ranging in size from 8-22 kDa. The stoichiometry of these subunits is unclear. Many reports suggest that the large subunit is present as a dimer (3, 4, 97). However, Lundell et al. (60), using ^{14}C labelling, suggested a tetrameric arrangement of subunit I. Two genes encode for this subunit I in maize (27), pea (58), and cyanobacteria (12) have identified. Both gene products are expressed and co-migrate at the position of subunit I (26). Our results indicated (Fig. 3 and 4) that the large subunit contained 62 and 64 kDa polypetides. We suggested that at least two polypeptides are present in subunit I.

Our results support the notion that subunit I is the major PS I

chlorophyll binding protein (3). In contrast with higher plants, cyanobacteria are thought to lack LHCI (9, 90) and are devoid of accessory pigment-binding proteins that serve as antenna for PS I. This places a greater emphasis upon the light harvesting role of the PS I core itself. Our results (Fig. 3) are consistent with several other reports showing chlorophyll binding associated only with subunit I (4). An immunoblot of PS I proteins (Fig. 12) shows that anti-subunit I antibodies identify the subunit I polypeptide and a chlorophyll-protein band. However, four antibodies against subunits II, III, IV, and V did not recognize the chlorophyll-protein band. The results suggested that the chlorophyll-containing band houses only subunit I.

The number of lower molecular weight polypeptides vary among higher plant and cyanobacteria. The PS I complex from swiss chard contains five small protein subunits of 25, 20, 18, 16, and 8 kDa (4). The spinach PS I reaction center contains five small polypeptides (23, 20, 17, 14, and 12 kDa) (1, 72). Lundell et al. (60) showed that the PS I complex from Synechococcus 6301 contains four small polypeptides of 18, 17.7, 16, and 10 kDa, whereas the Synechococcus sp. PS I contains proteins of 14, 13, and 10 kDa (102, 103). Our results are very similar to those from Synechococcus 6301 (60).

CROSSLINKING AND IMMUNOBLOT DETECTION OF PS I POLYPEPTIDES

To understand the molecular organization of five polypeptide subunits of PS I complex, we used two crosslinking reagents to analyze the nearest neighbor relationships of these proteins. The first crosslinker we used was GLU, which is a hydrophilic molecule (84). The principal crosslinking observed with GLU is a result of a Schiff base linkage of a lysine with an aldehyde that is in conjugation with a

double bond (84). GLU has a short chain of about 0.7 nm, which restricts the formation of crosslinking products to only closely situated polypeptides. Three crosslinked products were observed in GLU treated PS I (Fig. 8). The 95 kDa crosslinked band contains one large polypeptide (62 or 64 kDa) and two or three small proteins. The 32 kDa band is a significant crosslinked product by GLU treatment. This band may contain three polypeptides.

The second crosslinker used to examine the relationships of PS I polypeptides is HMDC (21). It is a hydrophobic molecule. The functional groups for chemical crosslinking are the amino groups of lysine. The PS I polypeptides seemed more easy aggregated by HMDC treatment. The PS I preparation treated with HMDC showed two crosslinked products (Fig. 10). The composition of these two crosslinked bands was very similar to that by treatment with GLU. At low concentrations of HMDC, the small crosslinked band (24 KDa) did not appear. However, the intensity of this band became more stronger with an increase of HMDC concentration. This crosslinked product may contain two polypeptides.

All of the PS I subunits except subunit V disappeared with increasing concentrations of GLU or HMDC. However, the intensity of subunit V band remained about the same with the concentration changes of both crosslinking reagents. Thus, subunit V of PS I was very resistant to GLU or HMDC.

Enami et al. (21) have examined the molecular arrangement of the PS I reaction center complex from Synechococcus sp. by GLU and HMDC. They found that four polypeptides (62, 60, 14, and 13 KDa) of PS I complex preferentially decreased by these two crosslinker treatment. The 13 and 14 KDa polypeptides decreased always in parallel to each

other. They attempted to identify the crosslinking bands by using cleavable chemical reagents without success.

To identify the crosslinked products of PS I complex treated with GLU or HMDC, we have raised the polyclonal antibodies against the five individual polypeptide. However, it is difficult to obtain the pure antibodies for all five subunits because of the difficulty in obtaining pure antigen. The protein migration on LiDS-PAGE is based in part on the size of the individual polypeptide. To avoid contamination due to the close size of proteins, we attempted to separate the antigen polypeptides by using HPLC. Unfortunately, only subunit III and V were separated by HPLC (Fig. 13). Subunit I, II, and IV proteins were lost and these proteins may still be bound to the matrix of the column.

The method of affinity purification of antibodies from immunoblots was used to purify the antibodies. We obtained a pure antibody against subunit III by this method (Fig. 14).

Our results showed that subunit I is readily crosslinked with subunit III and IV. In Fig. 15, the 95 KDa crosslinked band was identified strongly by antibodies against subunits I and III. This band may contain these two polypeptides and one or two other proteins. In addition, other six crosslinked products possesed subunit I and III (Table 1). Our observations suggest that subunits I and III are close each other. Antibodies against subunit I and IV bound strongly with a 76 KDa crosslinked product (Fig. 20, Lane 2 and 8). This crosslinked band contained only subunits I and IV based on the molecular weight and immuno-detection. Three crosslinked bands were associated with subunit I and IV (Table 1). These results indicated that subunit I and IV were neighbors.

Most of the crosslinked bands contain subunit III. (Table 1). This subunit has most close relationships with other polypeptides in the PS I omplex. Our observations showed that at least three crosslinked bands were associated with subunit III and IV. Both GLU and HMDC treated PS I produced a 32 KD crosslinked band which was extremely identified by the antibodies against these two subunits. From these evidences, we concluded that subunit III and IV are neighbors. Although subunit II and III blottings had the same crosslinking pattern, we can not decide the neighbor relationship between these two subunits due to the mixed antibodies.

Subunit V consistantly resisted crosslinking with other polypeptides. The intensity of subunit V remained unchanged on coomassie blue stained gels when the PS I samples were treated with either of the two crosslinkers. Yet, the other four subunits decreased. Antibody against subunit V did not identify any crosslinked products from GLU or HMDC treated PS I complex or thylakoid membranes. The crosslinking functional groups for both GLU and HMDC are the amino groups of amino acids (especially lysine). Subunit V may lacks of these functional groups. Alternatively, subunit V maybe associate with other subunit in regions where they are devoid of crosslinking sites.

THE MODEL OF PS I COMPLEX IN THYLAKOID MEMBRANE

The results which we obtained for neighbor relationship of PS I polypeptides led directly to a model of PS I organization in thylakoid membranes. Fig. 22 shows a structural model of PS I complex. We suggest several lines of evidence to support this model.

First, four copies of subunit I are present in the PS I complex. Lundell et al (60), using uniform ^{14}C-labeling, observed a tetrameric

arrangement of subunit I in PS I complex from cyanobacteria. This arrangement would increase the role of the large subunit in light harvesting and is consistent with the observation that most cyanobactria lack light harvesting protein complexes. We noted that two forms of subunit I, 64 and 62 KDa polypeptides, were present in PS I from A. nidulans (Fig. 3 and 4). Sonoike and Katoh (97) also have reported that two polypeptide (60 and 62 KDa) were present in subunit I of PS I from cyanobacteria. Our results also showed that two crosslinked products have molecular weights of 190 and 210 KDa. These two crosslinked bands contained at least three subunit I polypeptides based on molecular weights and immunoblot detections.

Second, we favor a dimer of subunit III. The spectrophotometric polyacrylamide gel scans showed that the intensity of subunit III is twice that of subunits II, IV, and V. PS I isolated from GLU-treated membranes blotted with anti-subunit III antibody showed that the molecular weights of two crosslinked bands (49 and 60 kDa) were larger than the sum of the molecular weights of subunits II, III, and IV (Fig. 20). These bands may contained more than one subunit III. We suggest that a dimer of subunit III is present in the PS I complex.

Lastly, subunit V could be a polypeptide associated with subunit I. Guikema and Sherman (35), using ^{59}Fe labelled, suggested that the smallest protein of PS I complex from A. nidulans may contain the Fe-S center, which function for electron transport in PS I. Takahashi (102, 103) also indicated that the smallest subunit and another protein contained most of the iron atoms in PS I. They suggested that these two subunits serve as Fe-S center proteins. A number of reports also showed that subunit I was associated with iron sulfur centers (30, 110, 101).

These results indicated that a close relationship existed between the subunit I and V. Subunit V failed to crosslink with other polypeptides in GLU or HMDC treated samples. This may be due to a lack of functional groups required for crosslinking reactions. Although the exact relationships between subunit V and other proteins can not be found, we still arrange subunit V next to the subunit I based on the functional relationships between these two proteins.

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Fig. 1. LiDS polyacrylamide gel electrophoresis of the thylakoid membrane and PS I preparations of Anacystis nidulans. PS I preparations were obtained by sucrose density gradient centrifugation. Samples were treated with 2 % LiDS and electrophoresed on a 10-20 % polyacrylamide gradient gel at 4 C. Lane 1, thylakoid membrane, heated at 70 C for 20 min; Lane 2, thylakoid membrane, room temperature; Lane 3, PS I preparation, heated at 70 C for 20 min; Lane 4, PS I preparation, room temperature. The molecular weights of PS I polypeptide subunits are given in kDa relative to marker proteins. The gel was stained with coomassie G-250.

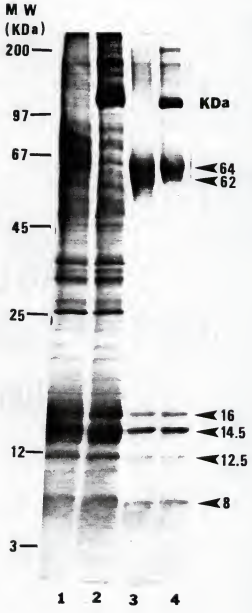

M W
(KDa)
200
97
67
45
25
12
3
KDa
64
62
16
14.5
12.5
8
1
2
3
4

FIG. 2. Polyacrylamide gel scan of PS I isolated by sucrose density gradient centrifugation. Electrophoresis was performed on a 10-20 % polyacrylamide gel. The gel was stained by coomassie blue G-250. The lane was scaned for absorption at 440 nm. The molecular weights of PS I polypeptides were given in kDa.

ABSORBANCE
64
62
M W (KDa)
16
14.5
12.5
8

FIG. 3. Detergent treatment of polypeptide profiles of PS I. The PS I complex was isolated by sucrose density gradient centrifugation. The samples were treated with various concentrations of detergent at room temperature for 30 min, and electrophoresed on 6.5–15 % polyacrylamide gradient gel containing 2 M urea. Lane 1, 1.0 % LiDS; Lane 2, 1.5 % LiDS; Lane 3, 2 % LiDS; Lane 4, 3 % LiDS; Lane 5, 4 % LiDS; Lane 6, 5 % LiDS.

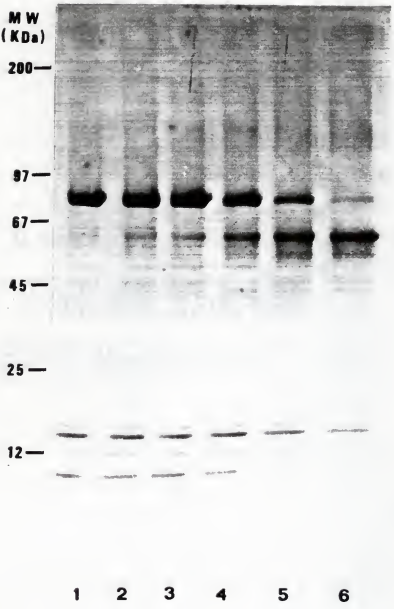

MW
(KDa)
200
97
67
45
25
12
1
2
3
4
5
6

FIG. 4. Temperature treatment of polypeptide profiles of PS I. The PS I complex was isolated by sucrose density gradient centrifugation. The samples were treated with 2 % LiDS at various temperature for 20 min, and electrophoresed on 6.5-15 % polyacrylamide gradient gel containing 2 M urea. Lane 1, 4 C; Land 2, room temperature; Lane 3, 50 C; Lane 4, 70 C; Lane 5, 80 C; Lane 6, 100 C.

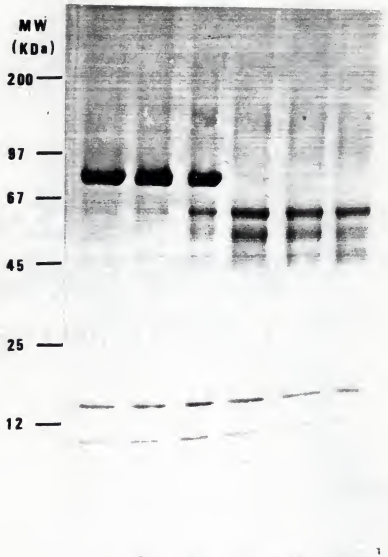

MW
(KDa)
200
97
67
45
25
12
1
2
3
4
5
6

FIG. 5. The unstained chlorophyll-proteins of the PS I preparation and thylakoid membranes in Dodecyl maltoside polyacrylamide gel. Both running gel and stacking gel contained 0.1 % Dodecyl maltoside. Samples were treated with 2 % Dodecyl maltoside at room temperature for 30 min. Thylakoid membranes were electrophoresed on 7.5 % linear gel at 4 C for two hours. Lane 1 showed two chlorophyll-protein bands, 1a and 1b of a PS I preparation. Lane 2 showed three chlorophyll-protein bands, 1a, 1b, and 2 of the thylakoid membranes.

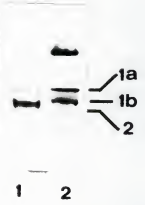

1a
1b
2
1
2

FIG. 6. Polypeptide profiles of chlorophyll-protein complexes. The individual chlorophyll-protein complexes were excised from Dodecyl maltoside polyacrylamide gel and electroeluted. Chlorophyll-protein preparations were treated with 2 % LiDS and was electrophoresed on a 10-20 % polyacrylamide gel at 4 C. A. room temperature: Lane 1, band 1a; Lane 2, band 1b; Lane 3, band 2. B. heated: 70 C, 15 min: Lane 4, band 1a; Lane 5, band 1b; Lane 6, band 2;

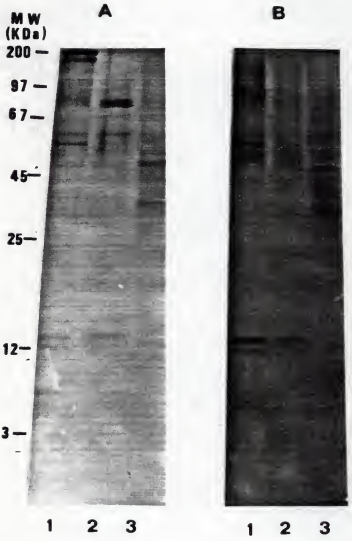

MW
(KDa)
200
97
67
45
25
12
3
A
B
1 2 3
1 2 3

FIG. 7. Light absorption spectra of PS I preparation isolated by sucrose density gradient centrifugation and chlorophyll-protein band 1a, 1b, and 2 isolated by Dodecyl maltoside polyacrylamide gel electrophresis. The PS I preparation has a maximum absorption at 679 nm. The absorption peaks are 680 nm for band 1a and 1b, and 675 nm for band 2.

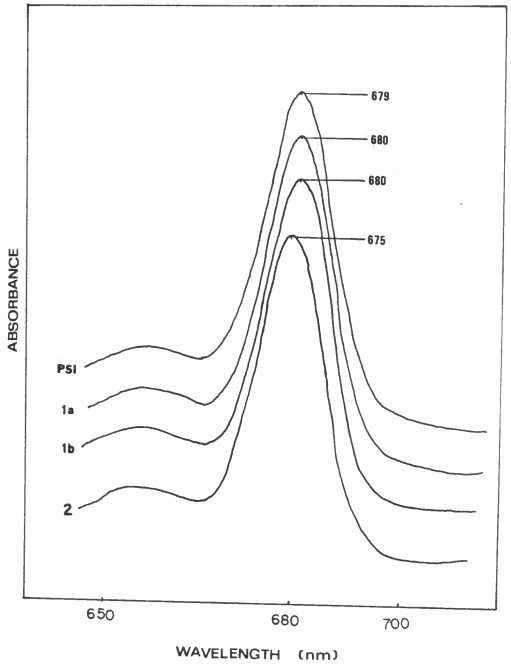

679
680
680
675
PSI
1a
1b
2
ABSORBANCE
650
680
700
WAVELENGTH (nm)

FIG. 8. Polypeptide profiles of PS I preparations crosslinked by glutaraldehyde (GLU) with various concentrations. Samples were treated with GLU for 20 min at room temperature. The chemical reaction was stopped by glycine. The electrophoresis was performed in 6.5-15 % polyacrylamide gels containing 2 M urea. Lane 1, no crosslinking; Lane 2, 0.02 % GLU; Lane 3, 0.04 % GLU; Lane 4, 0.06 % GLU; Lane 5, 0.08 % GLU; Lane 6, 0.1 % GLU.

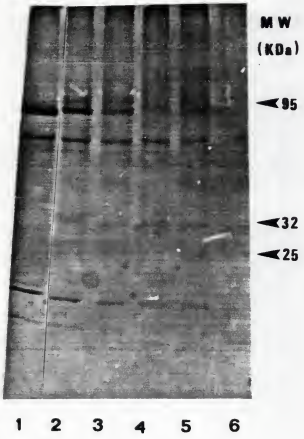
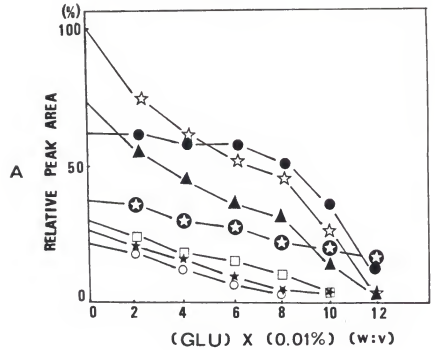

MW
(KDa)
95
32
25
1 2 3 4 5 6

FIG. 9. Polyacrylamide gel scan of PS I preparations crosslinked by GLU. The polypeptides were stained by coomassie blue G-250. The lanes were scanned for absorption at 440 nm. The relative peak areas of protein bands were plotted as a function of GLU concentrations. A:▲ , subunit I (64 kDa); □ , subunit I (62 kDa);★, subunit II; ● , subunit III, ○, subunit IV; ✪ , subunit V; B:☆ , 95 kDa crosslinked band; ○ , 32 kDa crosslinked band; □ , 25 kDa crosslinked band.


A
(%)
100
50
0
RELATIVE PEAK AREA
0 2 4 6 8 10 12
(GLU) X (0.01%) (w:v)

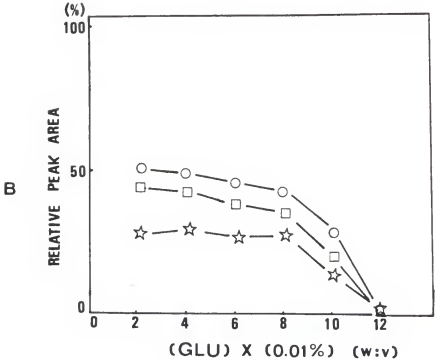

B
(%)
100
50
0
RELATIVE PEAK AREA
0 2 4 6 8 10 12
(GLU) X (0.01%) (w:v)

FIG. 10. Polypeptide profiles of PS I preparation crosslinked by HMDC. Samples were treated with HMDC for 20 min at room temperature. The chemical reaction was stopped by glycine. The electrophoresis was performed in 6.5-15 % polyacrylamide gel containing 2 M urea. Lane 1, no crosslinker; lane 2, 0.002 % HMDC; Lane 3, 0.004 % HMDC; Lane 4, 0.006 % HMDC; Lane 5, 0.008 % HMDC; Lane 6, 0.01 % HMDC; Lane 7, 0.012 % .

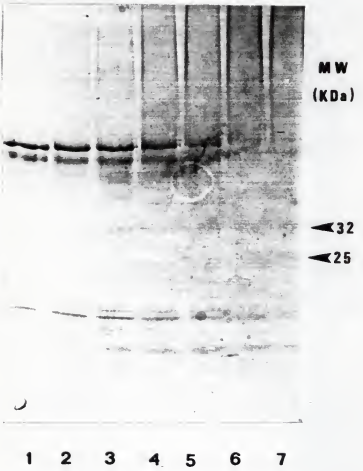
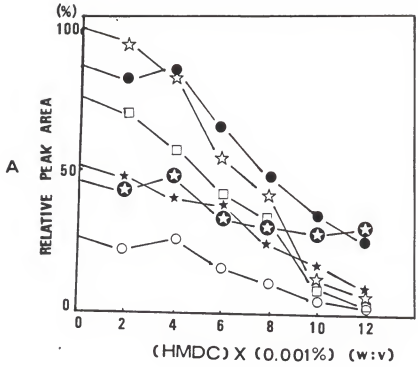

MW
(KDa)
32
25
1 2 3 4 5 6 7

FIG. 11. Polyacrylamide gel scane of PS I preparation crosslinked by HMDC. The polypeptides were stained by coomassie blue G-250. The lanes were scanned for absorption at 440 nm. The relative peak areas of protein bands were plotted as a function of HMDC concentrations. A:☆ , subunit I (64 kDa); □ , subunit I (62 kDa); ★ ,subunit II; ● , subunit III; ○ , subunit IV; ✪ , subunit V; B:●, 32 kDa crosslinked band; ○ , 25 kDa crosslinked band.


A
(%)
100
50
0
RELATIVE PEAK AREA
0
2
4
6
8
10
12
(HMDC) X (0.001%) (w:v)

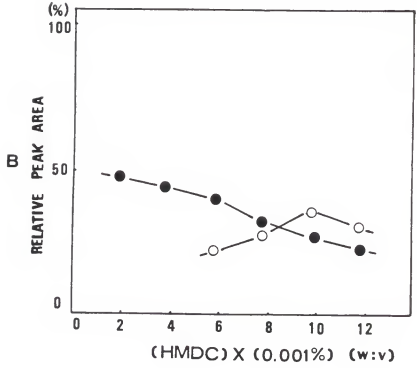

B
(%)
100
50
0
RELATIVE PEAK AREA
0
2
4
6
8
10
12
(HMDC) X (0.001%) (w:v)

FIG. 12. Immunoblot of PS I polypeptides. The antigen proteins were separated from PS I polyacrylamide gels. The five antibodies, raised against the individual polypeptide subunits of PS I, were blotted. Lane 1, anti-subunit I antibody; Lane 2, anti-subunit II antibody; Lane 3, anti-subunit III antibody; Lane 4, anti-subunit IV antibody; Lane 5, anti-subunit V antibody.

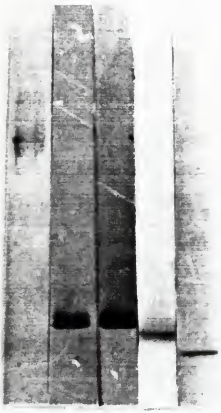
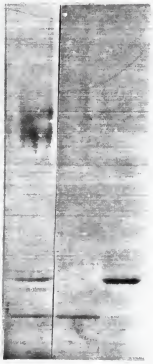

1 2 3 4 5

FIG. 13. Polypeptide profiles of subunit III and V isolated from PS I preparation by HPLC. Prior HPLC, PS I samples were treated with 5 % SDS. HPLC was performed by using a mobile phase solvent of acetonitrile, trifluroacetic acid, and 0.1 % SDS. A linear gradient from 0 to 80 % of acetonitrile was applied at flow rate 1.0 ml/min throughout a 50 min period. Lane 1, PS I preparation; Lane 2, subunit V of PS I; Lane 3, subunit III.



1 2 3

FIG. 14. Immunoblot of the purified anti-subunit III antibody. Subunit III purified by HPLC was blotted in Zeta-probe membrane. After two hours incubation with anti-subunit III serum, the antibody was purified from Zeta-probe membrane under a low pH 2.8 condition. Lane 1, anti-subunit III serum: Lane 2, purified anti-subunit III antibody.


1
2

FIG. 15. Immunological detection of PS I proteins crosslinked by GLU. PS I preparations were treated with 0.06 % of GLU at room temperature for 20 min. The antibodies raised against the PS I proteins were blotted with the crosslinked products. Lane 1, subunit I, no crosslinking; Lane 2, subunit I, crosslinking; Lane 3, subunit II, no crosslinking; Lane 4, subunit II, crosslinking; Lane 5, subunit III, no crosslinking; Lane 6, subunit III, crosslinking; Lane 7, subunit IV, no crosslinking; Lane 8, subunit IV, crosslinking; Lane 9, subunit V, no crosslinking; Lane 10, subunit V, crosslinking. Anti-subunit III antibody was the purified antibody.

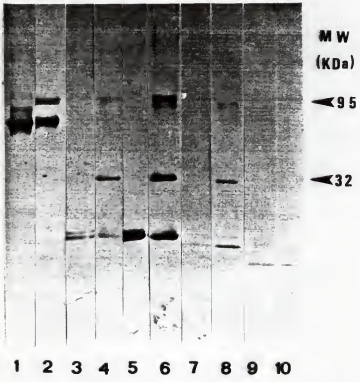

MW
(KDa)
95
32
1 2 3 4 5 6 7 8 9 10

FIG. 16. Immunoblot of anti-subunit III antibody with PS I proteins crosslinked by GLU. PS I samples were treated with various concentration of GLU at room temperature for 20 min. Anti-subunit III antibody was blotted with the crosslinked products. Lane 1, 0.02 % GLU; Lane 2, 0.04 % GLU; Lane 3, 0.06 % GLU; Lane 4, 0.08 % GLU; Lane 5, 0.10 % GLU; Lane 6, 0.12 % GLU;

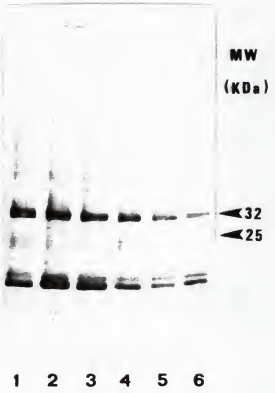

MW
(KDa)
32
25
1 2 3 4 5 6

FIG. 17. Immunological detection of PS I proteins crosslinked by HMDC. PS I preparations were treated with 0.006 % HMDC at room temperature for 20 min. The antibodies raised against the PS I proteins were blotted with the crosslinked products. Lane 1, subunit I, no crosslinking; Lane 2, subunit I crosslinking; Lane 3, subunit II, no crosslinking; Lane 4, subunit II, crosslinking; Lane 5, subunit III, no crosslinking; Lane 6, subunit III, crosslinking; Lane 7, subunit IV, no crosslinking; Lane 8, subunit IV, crosslinking; Lane 9, subunit V, no crosslinking; Lane 10, subunit V, crosslinking.

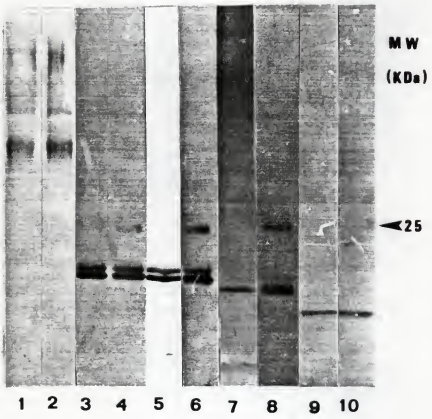

MW
(KDa)
25
1 2 3 4 5 6 7 8 9 10

FIG. 18. Immunoblot of anti-subunit III antibody with PS I proteins crosslinked by HMDC. PS I samples were treated with various concentrations of HMDC at room temperature for 20 min. Anti-subunit III antibody was blotted with the crosslinked PS I. Lane 1, 0.002 % HMDC; Lane 2, 0.004 % HMDC; Lane 3, 0.006 % HMDC; Lane 4, 0.008 % HMDC; Lane 5, 0.010 % HMDC; Lane 6, 0.012 % HMDC.

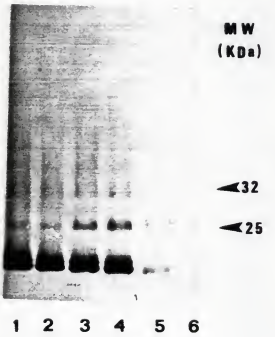

MW
(KDa)
32
25
1 2 3 4 5 6

FIG. 19. Polypeptide profiles of PS I isolated from thylakoid membranes treated with GLU and HMDC. The thylakoid membranes were treated with 0.06 % GLU and 0.006% HMDC at room temperature for 30 min. The electrophoresis was performed in 6.5-15 % polyacrylamide gel containing 2 M urea. Lane 1, PS I from no crosslinking membranes; Lane 2, PS I from GLU-treated membranes; Lane 3, PS I from HMDC-treated membranes.

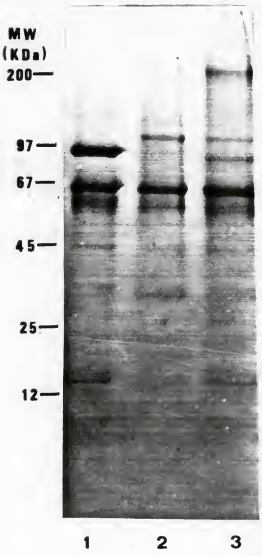

MW
(KDa)
200
97
67
45
25
12
1
2
3

FIG. 20. Immunological detection of PS I isolated from thylakoid membranes crosslinked with GLU. The thylakoid membranes were treated with 0.06 % GLU at room temperature for 20 min. The antibodies were blotted with the crosslinked PS I proteins. Lane 1, subunit I, no crosslinking; Lane 2, subunit I, crosslinking; Lane 3, subunit II, no crosslinking; Lane 4, subunit II, crosslinking; Lane 5, subunit III, no crosslinking; Lane 6, subunit III, crosslinking; Lane 7, subunit IV, no crosslinking; Lane 8, subunit IV, crosslinking; Lane 9, subunit V, no crosslinking; Lane 10, subunit V, crosslinking.

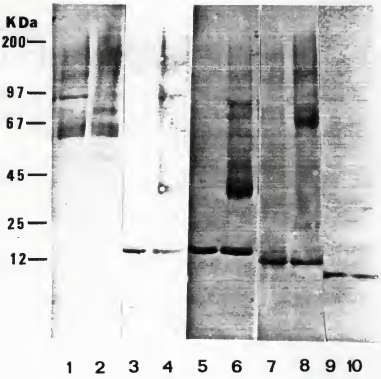

KDa
200
97
67
45
25
12
1 2 3 4 5 6 7 8 9 10

FIG. 21. Immunological detection of PS I isolated from thylakoid membranes crosslinked with HMDC. The thylakoid membranes were treated with 0.006 % HMDC at room temperature for 30 min. The antibodies were blotted with the crosslinked PS I proteins. Lane 1, subunit I, no crosslinking; Lane 2, subunit I crosslinking; Lane 3, subunit II, no crosslinking; Lane 4, subunit II, crosslinking; Lane 5, subunit III, no crosslinking; Lane 6, subunit III, crosslinking; Lane 7, subunit IV, no crosslinking; Lane 8, subunit IV, crosslinking; Lane 9, subunit V, no crosslinking; Lane 10, subunit V, crosslinking.

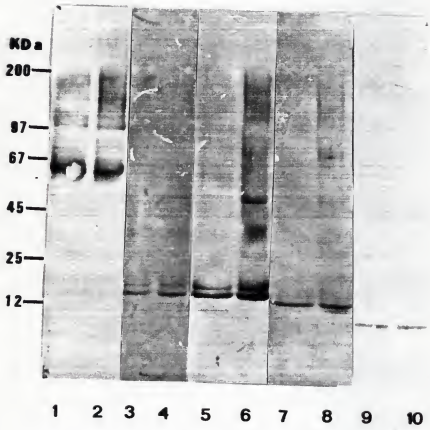

KDa
200
97
67
45
25
12
1 2 3 4 5 6 7 8 9 10

FIG. 22. Tentative model for the spetial arrangement of polypeptide subunits of PS I complex in thylakoid membrane. The model shows the topological neighbor relationship of the PS I polypeptides to the lipid bilayer.

A MODEL OF PS I CORE PEPTIDES

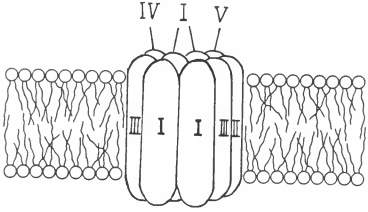


TABLE I

CROSSLINKED BANDS OF PS I POLYPEPTIDES

crosslinked bands (KDa)	GLU	HMDC	protein identity
210		+	3 X I + III + ?
190		+	3 X I + ?
145	+		2 X I + III + ?
130		+	2 X I + III
125	+		2 X I + IV
110		+	I + III + ?
95	+	+	I + III + IV + ?
88	+		I + III + ?
76		+	I + IV
60	+		2 X III + ?
49	+		2 X III + ?
42	+		2 X III + II
32	+	+	II + III + IV
25	+	+	III + IV/II

CROSSLINKING OF PHOTOSYSTEM I CORE POLYPEPTIDES IN *ANACYSTIS NIDULANS*

by

MAOLIN LI

B.S., South China College of Tropical Crop, 1982

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ABSTRACT

A PS I enriched preparation was isolated from thylakoid membranes of Anacystis nidulans by dodecyl maltoside polyacrylamide gel electrophoresis and sucrose density gradient centrifugation. This preparation contained a minimum of 6 polypeptides: I (62 and 64 kDa), II (16 kDa), III (14.5 kDa), IV (12.5 kDa), and V (8 kDa).

The PS I preparation was treated with two crosslinking reagents, glutaraldehyde (GLU), hexamethylenediisocyanate (HMDC). GLU-treated PS I yielded three crosslinking bands (95, 32 and 25 kDa) on polyacrylamide gels. HMDC-treated PS I yielded two crosslinking bands (32 and 25 kDa).

Polyclonal antibodies, raised against these five subunits of PS I, were used to probe the crosslinked bands. Blots of GLU and HMDC-treated PS I indicated that subunit I was readily crosslinked with subunits III and IV; subunit III was readily crosslinked with subunits I, II, and IV; and subunit V did not crosslink with other subunits. The PS I isolated from GLU and HMDC-treated intact membranes showed more crosslinking bands which were identified by antibodies.

Our results suggest that subunit I is associated with subunit III and IV; subunit III is associated with subunit I, II, and IV. We suggested that a tetrameric structure of subunit I and a dimeric structure of subunit III exist in the PS I complex. A structural model of PS I complex was built based on our data.