Organization of Photosystem I Polypeptides Examined by Chemical Cross-Linking

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Photosystem I from the cyanobacterium Synechocystis sp. PCC 6803 was examined using the chemical cross-linkers glutaraldehyde and N-ethyl-1-3-[3-(dimethylamino)propyl]carbodiimide to investigate the organization of the polypeptide subunits. Thylakoid membranes and photosystem I, which was isolated by Triton X-100 fractionation, were treated with cross-linking reagents and were resolved using a Tricine/urea low-molecular-weight resolution gel system. Subunit-specific antibodies and western blotting analysis were used to identify the components of cross-linked species. These analyses identified glutaraldehyde-dependent cross-linking products composed of small amounts of PsAD and PsAC, PsAC and PsAE, and PsAE and PsAF. The novel cross-link between PsAE and PsAF was also observed following treatment with N-ethyl-1-3-[3-(dimethylamino)propyl]carbodiimide. These cross-linking results suggest a structural interaction between PsAE and PsAF and predict a transmembrane topology for PsAF.

PSI, a multisubunit enzyme complex of the thylakoid membranes in plants and cyanobacteria, catalyzes light-dependent electron flow from the mobile electron carriers plastocyanin or Cyt c₆ in the thylakoid lumen to the stromal mobile electron carrierFd or flavodoxin. A central feature of the PSI reaction center is the heterodimeric core: PsaC, which coordinates two [4Fe-4S] clusters, and PsaF, which is a component of the Fd-docking site (Zilber and Malkin, 1988; Wynn et al., 1989), and PsaI, which is required for PSI trimer formation (Chitnis and Chitnis, 1993); and PsaE, which may be involved in the correct organization of PsaL (Xu et al., 1994c; Zilber and Malkin, 1988; Wynn et al., 1989; Andersen et al., 1990), is required for the stable assembly of PsAC (Li et al., 1991); PsAE, which may be involved in the cyclic electron transport pathway around PSI (Yu et al., 1993), assists in the docking of Fd (Andersen et al., 1992; Sonoke et al., 1993); PsAF, which can be cross-linked to plastocyanin (Wynn and Malkin, 1988; Hippler et al., 1990) and Cyt c₆ (Wynn et al., 1989), suggesting it has a p-side (lumen) domain; PsAL, which is a transmembrane polypeptide that is required for PSI trimer formation (Chitnis and Chitnis, 1993); and PsAD, which is also a transmembrane polypeptide (Chitnis et al., 1995), may be involved in the correct organization of PsAl (Xu et al., 1995). PsAI, PsAK, and PsAM have been identified, but their functions are largely unknown.

The cyanobacterium Synechocystis sp. PCC 6803 provides an attractive system to examine the organization and function of PSI subunits. PSI is easily isolated, and mutant strains with deleted or interrupted genes for PSI subunits provide an elegant system to investigate the organization and topology of these subunits. In this report, we have used the chemical cross-linkers glutaraldehyde and EDC to investigate the organization of PSI polypeptides in Synechocystis sp. PCC 6803. Wild-type and mutant strains were treated with the cross-linkers and examined using antibodies specific to PSI subunits to identify cross-linked products.

MATERIALS AND METHODS

Isolation of Thylakoid Membranes and PSI Particles

Wild-type Synechocystis sp. PCC 6803 and mutant strains AFK6 (PsAE/PsAI-less) (Chitnis et al., 1991; Xu et al., 1994d) and AEK2 (PsAE-less) (Chitnis et al., 1989) were grown in BG-11 with Glc and the appropriate antibiotics. Cells were harvested by centrifugation, resuspended in SMN buffer (10 mM Mops, 400 mM Suc, and 10 mM NaCl, pH 7.0), and broken using a Bead Beater (BioSpec, Bartlesville, OK). PMSF (5 mM) and benzamidine (5 mM) were used to inhibit protease activity during cell breakage. Cell debris was removed by centrifugation for 5 min at 2,000g. Thylakoid membranes were collected by centrifugation at 40,000g and washed three times in SMN buffer. PSI was isolated using Triton X-100 (Sigma) solubilization at a 10:1 (w/w) ratio of Triton X-100 to chlorophyll for 20 min at 4°C. Nonsolubilized material was pelleted by centrifugation at 13,000g for 10 min. The supernatant was loaded on a DEAE column, washed with wash buffer (0.05% [w/v] Triton X-100, 20 mM Hepes, pH 7.0), and eluted with a 50 to 200 mM NaCl gradient. Green fractions were collected, pooled, diluted 4:1 in wash buffer, and loaded on a second DEAE column. Elution was performed with 200 mM NaCl, and eluate was loaded on 6, 12, 18, and 24% (w/v) Suc step gradient and centrifuged for 17 h at 35,000 rpm on a Beckman SW41 rotor. PSI was collected and concentrated on a Centricon 50.

Abbreviations: EDC, N-ethyl-1-3-[3-(dimethylamino)propyl]carbodiimide; EGS, ethylene glycolbis(succinimidylsuccinate); LiDS, lithium dodecyl sulfate.
(Amicon, Beverly, MA). Chlorophyll concentrations were determined in 80% acetone (Arnon, 1949).

Cross-Linking

PSI (0.2 mg chlorophyll/mL) or thylakoid membranes (0.8 mg chlorophyll/mL) were treated with 6 mM glutaraldehyde for 30 min at room temperature, and the reaction was quenched using 50 mM Gly for 15 min at room temperature. EDC treatment was performed on PSI (0.2 mg chlorophyll/mL) for 1 h at room temperature, and the reaction was quenched with 200 mM Tris (pH 7.0) for 15 min at room temperature.

Gel Electrophoresis and Immunodetection

Samples were solubilized in the presence of 2% LiDS and 2-mercaptoethanol. Gel electrophoresis was carried out using a Tricine LiDS-PAGE system containing 6 M urea in the resolving gel according to the method of Xu et al. (1994c), except that LiDS was substituted for SDS. Following electrophoresis, gels were stained with Coomassie blue or the proteins were electrotransferred to PVDF membranes (Immobilon-P, Millipore). The anti-PsaD, anti-PsaE, and anti-PsaF antibodies were prepared in rabbits against purified protein from Synechocystis sp. PCC 6803. The anti-PsaC antibody was a gift from Dr. John H. Golbeck (University of Nebraska, Lincoln). Antigen-antibody interaction was visualized using an alkaline phosphatase-conjugated secondary antibody.

Electron Transport Assays

Oxygen measurements were made on PSI preparations using a Clark-type oxygen electrode (Hansatech, King’s Lynn, UK). Assays were conducted at 25°C with saturating light. PSI electron transport was measured by the addition of 50 μM DCMU, 2 mM methyl viologen, 1 mM 1,4-diaminodurene, and 1 mM ascorbic acid to 20 μg of chlorophyll in a volume of 2 mL. The assay mixture was buffered with 40 mM Hepes, pH 7.0. The rates reported are averages of three to five individual measurements, and the trends shown were repeated at least twice.

RESULTS

Chemical cross-linking, using agents such as glutaraldehyde and EDC, results in the formation of covalent bonds between specific amino acid side chains and has been used to document nearest-neighbor relationships of polypeptides in multisubunit complexes. Formation of such linkages between two polypeptides will render them inseparable during harsh, denaturing conditions such as dodecyl sulfate treatment, causing these polypeptides to migrate as a single species with a higher molecular mass during gel electrophoresis and a reduction in the polypeptide at its native molecular mass. Figure 1 shows such an analysis of isolated PSI preparations from wild-type Synechocystis sp. PCC 6803, with subunits PsaA, PsaB, PsaD, PsaF, and PsaL clearly resolved from nontreated preparations (Fig. 1, lanes 1 and 5). This gel system and the protein amounts loaded on the gel were optimized to visualize cross-linked species in the 20- to 66-kDa range, and subunits below 12 kD, although present in this preparation, were poorly resolved (Xu et al., 1994c). LiDS-PAGE of PSI treated with glutaraldehyde (lanes 2–4) or EDC (lanes 6–8) demonstrated the cross-linking of several PSI subunits, as indicated by the reduction of these subunits at their native molecular mass (Fig. 1). Specifically, glutaraldehyde-dependent cross-linking reduced the levels of PsaA/PsaB, PsaD, PsaF, PsaL, PsaC, and PsaE at their respective molecular masses. EDC-induced cross-linking affected PsaD, PsaF, PsaL, and, to a lesser extent, PsaA/PsaB. Although not visible in Figure 1, neither glutaraldehyde nor EDC had a significant effect on the migration of the other subunits below 12 kD. Cross-linking at 4°C and/or at lower chlorophyll concentrations did not alter the formation of, or the subunit composition of, any identified cross-linked products, suggesting that the cross-linking observed here represents intracomplex and not intercomplex cross-linking (data not shown).

PSI electron transport rates were measured to examine the effects of the chemical cross-linking on PSI function (Table I). Increasing concentrations of glutaraldehyde caused an increase in the rate of PSI electron transport, whereas EDC had an opposite effect, causing a decrease in the electron transport rate. It is likely that the cross-linking reagents influence the surface charge characteristics of specific PSI domains and thus alter the kinetics of electron acceptor and donor interactions with PSI. However, in both cases, cross-linking does not abolish PSI activity; rather, it yields a functional PSI that is capable of catalyzing the flow of electrons to methyl viologen.

Seven glutaraldehyde-dependent cross-linked products from PSI, EDC complexes, were identified by western blot anal-
ysis using antibodies against PsaC, PsaD, PsaE, PsaF, and PsaL; two cross-linked species were observed following EDC treatment. The subunit composition of these species as identified by western blot analyses is summarized in Table II. Two results of these studies were of special interest. First, although we also used antibodies against PsaA and PsaB in these experiments, we observed no cross-linking of the small subunit proteins with the PSI heterodimer core that resulted in species that were identifiable on our PAGE system. This was somewhat surprising, since cross-linking treatment results in a diminished amount of PsaA and PsaB at their native molecular mass, even at the lower concentrations of glutaraldehyde (Fig. 1). Large aggregates (greater than 205 kD) that do not enter the resolving gel but stop at the stacking gel/resolving gel interface (Fig. 1, lane 4) are recognized by anti-PsaA/PsaB antibodies. The possibility of intrasubunit cross-linking within PsaA and PsaB may contribute to aggregate formation by interfering with the ability of detergent to solubilize these hydrophobic molecules. Second, the cross-linked products between PsaF and PsaE (Fig. 2) were of interest because these subunits were thought to be present on the opposite sides of the thylakoid membrane (Wynn and Malkin, 1988; Wynn et al., 1989; Hippler et al., 1990).

It is possible that the cross-linking of PsaE and PsaF may be mediated by a small transmembrane subunit. However, as shown in Table II, the observed migration of the PsaE-PsaF cross-linked species correlated well with the masses deduced from gene sequence information. This correlation suggests that the PsaE-PsaF species is not mediated by a third transmembrane subunit but that PsaF is a transmembrane subunit.

To confirm the subunit composition of these cross-linked products identified by the anti-PsaE and anti-PsaF antibodies, PSI preparations were isolated from mutant strains lacking either PsaE (AEK2) or PsaF (AFK6) and treated with glutaraldehyde (Fig. 3). When PsaF was missing, the cross-linked product of PsaE and PsaF was not observed. The cross-linking between PsaE and PsaC still occurred, whereas no new species were identified involving either PsaE or PsaF. With PsaE missing, no cross-linked products were recognized by the PsaE or PsaF antibodies. New cross-linked products approximately 2 to 4 kD larger than the native molecular masses of PsaE or PsaF would be expected if the PsaE-PsaF cross-link were mediated by a third subunit. Our results (Fig. 3) suggest that PsaE and PsaF are the only subunits involved in the 25- and 26-kD products, and the 1-kD size difference between the two products may have been a result of different internal cross-links, thus altering the electrophoretic migration of the product.

**DISCUSSION**

In this report we have examined nearest-neighbor relationships within the PSI complex by coupling the technique of chemical cross-linking with subunit-specific antibodies. Many attempts have been made to...
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examine the organization of PSI by chemical cross-linking, using hydrophilic and hydrophobic cross-linkers such as dimethyladipimidate, 3,3'-dithiobis(sulfo succinimidyl)propionate), EDC, EGS, glutaraldehyde, and hexamethylenediamine (Enami et al., 1987; Oh-Oka et al., 1989; Andersen et al., 1990; Armbrust et al., 1994). In addition, cross-linking has been used to identify the interaction of PSI with plastocyanin (Wynn and Malkin, 1988; Hippler et al., 1990), Cyt c6 (Wynn et al., 1989), and Fd (Zilber and Malkin, 1988; Wynn et al., 1989; Andersen et al., 1990, 1992; Lelong et al., 1994). The reagent we found most effective for these studies was glutaraldehyde, a hydrophilic molecule that forms cross-links between Lys amino groups (Peters and Richards, 1977). In addition, we used EDC, a zero-length cross-linker that forms a covalent bond between primary amines and carboxyls (Bauminger and Wilchek, 1980). Using both reagents, we observed a cross-linking product containing PsaE and PsaF, in addition to several other cross-linked products.

PsaC and PsaD were identified as components of both glutaraldehyde and EDC cross-linking products. Cross-linking of these subunits has been documented in plants using EGS, a hydrophilic cross-linker (Oh-Oka et al., 1989), and glutaraldehyde (Armbrust et al., 1994). The close physical proximity of PsaC and PsaD on the n-side of thylakoid membranes is also supported by the requirement of PsaD for the correct and stable binding of PsaC to PsaA and PsaB, the heterodimeric core of PSI (Li et al., 1991). PsaD also was observed as a component of a 32-kD cross-linking product minimally comprising PsaD and PsaL (Xu et al., 1994a).

PsaE, like PsaC and PsaD, resides on the n-side (stromal) of the thylakoid membrane, and there is an expected intimacy among these polypeptides on the reducing side of PSI. The proximity of PsaE with PsaC and PsaD, observed by glutaraldehyde-dependent cross-linking (Fig. 2) and EGS-dependent cross-linking in thylakoid membranes of higher plants (Oh-Oka et al., 1989). It is interesting that we did not identify a cross-linked product that contained both PsaE and PsaD, although EGS cross-linking indicates a close proximity of these subunits in higher plants (Oh-Oka et al., 1989).

In this report, it was demonstrated that PsaE and PsaF can be cross-linked to each other. To further support our findings, PsaE-less and PsaF-less cyanobacterial mutant strains were used to demonstrate that the presence of both PsaE and PsaF is required for the formation of the cross-linked product. Two observations argue against the suggestion that PsaE-PsaF products form between different PSI particles that are arranged in opposing orientations. First, the formation of the cross-linked products is not diminished, but rather identical patterns of cross-linked species are produced at lower chlorophyll concentrations, which means that the chances of complex collision are decreased. Second, a PsaE-PsaF product was identified when thylakoid membranes were treated with glutaraldehyde or EDC (data not shown) and the fixed orientation of PSI in thylakoid membranes eliminates the possibility of p-side and n-side subunits coming in contact with each other. Therefore, PsaE and PsaF are in physical proximity in the architecture of the PSI complex, suggesting that PsaF has a stromally exposed domain. The interaction between PsaE and PsaF is further supported through the biochemical characterization of the PsaF-minus mutant where PsaE was easily removed during chaotic extraction as compared with wild type (Cohen et al., 1993; Xu et al., 1994d). Thus, a PsaE-PsaF interaction is likely to be a significant component of PSI stability, with positive and negative charges from each subunit being involved in ionic bonds. Identification of the residues involved in the EDC cross-link may indicate which charged residues are involved in these ionic interactions, since cross-linking has an arm length of zero, indicating that the cross-linked residues are within Van der Waals’ distance of each other.

The cross-linking of PsaF to PsaE suggests a transmembrane topology for PsaF, and several other lines of evidence support this suggestion. The proximity of PsaF with PsaE (Fig. 2), coupled with the n-side localization of the latter subunit, demonstrates an n-side domain to PsaF. The cross-linking of PsaF to plastocyanin (Wynn and Malkin, 1988; Hippler et al., 1990) and Cyt c6 in Synechococcus (Wynn et al., 1989) confirms that PsaF has a domain exposed to the p-side (thylakoid lumen). Furthermore, structural information regarding PsaF and its characteristics within the PSI complex suggests that it is an integral PSI component. Sequence analysis of PsaF from both higher plants and cyanobacteria suggests an a helix, which is highly conserved and sufficiently hydrophobic to be a membrane-spanning domain, near the middle of the sequence (Steppuhn et al., 1988; Chitnis et al., 1989; Franzen et al., 1989; Scott et al., 1994; Golbeck, 1994 [citing unpublished data of D.A. Bryant]). Biochemical analysis of PSI with chaotropic agents, which remove extrinsic polypeptides but do not highly conserve PsaE or PsaF, the PSI complex, indicates a tran-
membrane architecture (Li et al., 1991). When challenged with detergents, PsaF is only extracted from the PSI complex with high Triton X-100 concentrations (Bengis and Nelson, 1975) and partitions with Triton X-114 micelles (Zilber et al., 1990). Both results indicate that PsaF is a transmembrane polypeptide.

Since PsaF has a leader sequence to target the N terminus to the lumen (Chitnis et al., 1991), it is likely that the N terminus is located in the thylakoid lumen. The N-terminal domain of higher plant PsaF also can be cross-linked to plastocyanin (W. Haehnel, personal communication). This information, combined with a predicted transmembrane domain of higher plant PsaF also can be cross-linked to plastocyanin (W. Haehnel, personal communication). Higher resolution crystal models and the identification of the amino acids involved in the PsaE-PsaF cross-links will greatly enhance PSI structural models by identifying the nearest-neighbor relationships among the amino acids of the two polypeptides.

The use of chemical cross-linking to modify isolated PSI allowed us to identify nearest-neighbor relationships among the PSI subunits. In addition, this work coupled with previous cross-linking efforts and biochemical characterizations suggests that PsaF is a transmembrane subunit of the PSI complex. In the current crystal model of PSI by Schubert et al. (1995), helix M could be the transmembrane domain of PsaF, since PsaE is located opposite the PsaF, connecting domain of PSI trimers (E.J. Boekema, personal communication). Higher resolution crystal models and the identification of the amino acids involved in the

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