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Interaction of Plastocyanin with Photosystem I: A Chemical Cross-Linking Study of the Polypeptide That Binds Plastocyanin†

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ABSTRACT: Plastocyanin has been covalently cross-linked to photosystem I (PSI) by using a water-soluble cross-linker, *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The cross-linking reaction is light stimulated and results in the disappearance of a single 19-kDa subunit of PSI with the formation of a new protein-staining component of 31 kDa. The new product at 31 kDa reacts with both plastocyanin and 19-kDa subunit antibodies. Carboxyl group modified plastocyanin does not form a cross-linked product with PSI, implying that the negatively charged surface-exposed groups on plastocyanin are necessary to stabilize binding. These results demonstrate a specific interaction of plastocyanin with PSI and further implicate a specific protein to which plastocyanin binds to facilitate electron transfer to the P700 reaction center.

Electron transport in oxygen-evolving membranes of cyanobacteria, algae, and higher plants occurs through the cooperative interaction of two photochemical systems which results in the reduction of NADP with water as the electron donor. Electrons are shuttled between these photosystems by a third membrane complex, the cytochrome b_6–f complex. The cytochrome complex mediates electron flow from photosystem II (PSII) to photosystem I (PSI)[1] through two soluble electron

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carriers: plastoquinone and the copper-containing protein plastocyanin. Photosystem I transfers electrons from reduced plastocyanin through six intrinsic redox centers [P700, A_0, A_1, Fe–S_X, Fe–S_A, and Fe–S_B; for recent reviews, see Haehnel (1984) and Malkin (1987a,b)] to ferredoxin. Thus, PSI acts as a light-dependent reduced plastocyanin–ferredoxin oxidoreductase.

A great deal of spectroscopic information concerning PSI is now available (Rutherford & Heathcote, 1985; Ke & Shuvalov, 1987). However, structural and functional aspects of PSI have received less attention, perhaps due to the lack of reaction center preparations similar to those from photosynthetic bacteria (Okamura et al., 1982; Glazer & Melis, 1987). Recently, the purification and characterization of a "native" PSI complex which retains functional, structural, and spectroscopic properties of the in vivo PSI but is free from other contaminating complexes (Mullet et al., 1980; Ortiz et al., 1984) have renewed interest in studying the functional role of PSI subunits.

The native PSI complex, isolated from higher plant chloroplast membranes after detergent solubilization, contains a Chl/P700 ratio of approximately 200 and at least 10 polypeptide subunits (Mullet et al., 1980; Ortiz et al., 1984; Lam et al., 1984a,b). This isolated PSI complex can be further fractionated into two chlorophyll-containing complexes: a reaction center core complex and an antenna complex LHCP I (Haworth et al., 1983; Lam et al., 1984a,b). The PSI core complex (~100 Chl/P700) contains two higher molecular weight polypeptides (58K and 62K from SDS–PAGE analysis) (Fish & Bogorad, 1986; Vierling & Alberte, 1983) and several low molecular weight subunits as well as the entire complement of bound electron acceptors. The LHCP I fraction contains several subunits in the 24–27-kDa range that bind chlorophylls *a* and *b* and function as an antenna for the reaction center (Lam et al., 1984a,b; Nechushtai et al., 1987). It is also possible to isolate a chlorophyll–protein complex known as CP1 which contains P700, A_0, A_1, and Fe–S_X but only has the two high molecular weight subunits (Vierling & Alberte, 1983; Golbeck et al., 1987).

The two electron-transfer partners for PSI, plastocyanin and ferredoxin, are well-characterized proteins for which X-ray crystallographic structures are available (Fukuyama et al., 1980; Freeman, 1981). Only a few studies have been done on the nature of the interaction between ferredoxin and PSI. Recent results from cross-linking studies show that a 20-kDa subunit from PSI (22 kDa in our gel system) may be involved in binding ferredoxin (Zanetti & Merati, 1987). These recent findings suggest a specific interaction of the soluble electron carrier, ferredoxin, with a membrane-bound polypeptide associated with the PSI complex.

In the case of plastocyanin, several groups have attempted to address the mechanism of interaction with PSI. Bengis and Nelson (1977) first studied the question of the relationship of individual PSI subunits to electron donation by reduced plastocyanin. They found that Triton X-100 concentrations of approximately 1% were sufficient to remove one PSI subunit (denoted subunit III in their terminology) and that the loss of this subunit led to a loss in the plastocyanin-dependent activity of PSI. The explanations proposed for the activity loss were either that this polypeptide contained an essential electron-carrying intermediate between plastocyanin and P700 or that this subunit aided in maintaining the proper conformation for efficient electron transfer between plastocyanin and P700. Haehnel et al. (1980) proposed on the basis of kinetic studies that this subunit was essential for activity but that it was not an electron carrier. These results suggest that subunit III helped to mediate electron transfer between plastocyanin and the functional PSI complex. Recently, several studies (Burkey & Gross, 1981, 1982; Anderson et al., 1987) have focused on the interaction of plastocyanin with PSI, mainly through the use of chemically modified plastocyanin, but the involvement of a specific PSI subunit in binding has not been considered.

In an attempt to provide greater insight into the interaction of plastocyanin with PSI, we have covalently linked these two reaction partners by utilizing the zero-length chemical cross-linker *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). Our results indicate that a PSI subunit of 19 kDa (by SDS–PAGE) cross-links to plastocyanin, resulting in the appearance of a new molecular weight component of 31 kDa (by SDS–PAGE). This cross-linked product showed positive cross-reactivity to antibodies prepared against both polypeptides. The properties of PSI which has been covalently modified with plastocyanin are also described. These results are considered in terms of current structural and functional models for PSI.

Materials and Methods

Materials. EDC, glycine ethyl ester (GEE), NADP, digitonin, and Triton X-100 were obtained from Sigma. Zwittergent 16 and dodecyl β-D-maltoside were obtained from Calbiochem. Horseradish peroxidase conjugated IgG was purchased from Bio-Rad. All other chemicals were of reagent grade. Plastocyanin, ferredoxin, and ferredoxin–NADP reductase were prepared in our laboratory and were generous gifts of Richard Chain. Antibodies to PSI subunit III (19-kDa subunit) were provided by J. P. Thornber, to CP1 by R. Alberte, and to the 17.8- and the 17.3-kDa subunits by N.-H. Chua, while antibodies to spinach plastocyanin were prepared in our laboratory.

Triton X-100 and Digitonin-Solubilized PSI Preparations. Mature greenhouse-grown spinach leaves were homogenized at 4 °C in a Waring blender for 30 s in ice-cold STNM (0.3 M sucrose, 0.05 M Tris-HCl, pH 7.8, 0.01 M NaCl, and 0.005 M $MgCl_2$). The homogenate was filtered through four layers of nylon mesh, and the filtrate was centrifuged at 10000*g* for 10 min to pellet the chloroplasts. The pellet was resuspended in 100 mM NaCl and allowed to incubate for 10 min followed by centrifugation at 10000*g* for 10 min. This procedure resulted in lysis of the chloroplasts and separation of photosynthetic membranes from the soluble extract. The chloroplast membranes were then resuspended in STNM which contained 2 M NaBr and allowed to stand for 30 min at 4 °C, followed by dilution with an equal volume of STNM and centrifugation at 15000*g* for 10 min. The pelleted membranes were resuspended in STNM and centrifuged at 10000*g* for 10 min. The chlorophyll-containing pellet was resuspended in distilled water to a chlorophyll concentration of 0.8–1.1 mg/mL, and Triton X-100, from a 20% (w/v) stock solution, was added to give a final concentration of 1.0%. This suspension was incubated at 25 °C for 45 min with gentle stirring. Following incubation, unsolubilized material was removed by centrifugation at 48000*g* for 20 min. The supernatant, containing solubilized PSI and LHCP complexes, was loaded onto a 0.4–1 M sucrose gradient that contained 0.02% Triton X-100 with a 2 M sucrose cushion and centrifuged at 360000*g* for 4–5 h in a Ti 60 rotor at 2 °C. The narrow dark green band at the top of the 2 M cushion contained the native PSI complex (PSI-200).

[1] Abbreviations: PSI, photosystem I; DCPIP, 2,6-dichlorophenolindophenol; EDC, *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; LHCP, light-harvesting chlorophyll protein.

For digitonin PSI preparations, the chlorophyll-containing pellet, following the NaBr treatment, was resuspended in distilled water to a chlorophyll concentration of 0.3–0.5 mg/mL, and digitonin was added from a freshly prepared 4% (w/v) stock solution to give a final concentration of 0.5% (w/v). The remainder of the preparation of PSI particles was as described by Boardman (1972). Digitonin preparations were used immediately while those made with Triton X-100 were either used fresh or stored at −20 °C prior to use.

Preparation of PSI Core Complex (PSI-100) and PSI Reaction Center Complex (CPI). PSI-200 (as described above) was further fractionated into a core complex (PSI-100) and an antenna complex (LHCP I). PSI-200 was diluted with 5 volumes of 20 mM Tris-HCl buffer (pH 7.6) and centrifuged at 255000*g* for 1 h. The pellet was resuspended in water to a chlorophyll concentration of 0.5 mg/mL. To the suspension were added solid Zwittergent 16 and dodecyl β-D-maltoside to final concentrations of 0.2% and 0.15%, respectively. This suspension was gently stirred on ice for 1 h. The suspension was then loaded onto a sucrose density gradient (0.1–1 M) in 20 mM Tricine–NaOH buffer (pH 7.8) containing 0.1% Triton X-100 and centrifuged at 280000*g* for 4 h in a Beckman SW-41 rotor. The lower green band contained the PSI core complex, and the top band contained LHCP I as well as some LHCP II.

CPI was isolated from PSI-200 essentially as described by Bengis and Nelson (1977) using SDS. PSI-200 was concentrated by using an Amicon concentrating cell with a YM-50 membrane to give a chlorophyll concentration of 0.5 mg of Chl/mL, and solid SDS was added to give a 50/1 SDS/Chl (w/v) mixture. This mixture was then overlaid on a (0.15–0.75 M) sucrose density gradient containing 50 mM Tris-HCl, pH 7.8, and 0.1% Triton X-100. The gradient was centrifuged overnight at 280000*g*. The lower green band, which contained only the two uppermost SDS–PAGE subunits, was the CP1 fraction.

Chemical Modification of Plastocyanin. Oxidized plastocyanin (0.3 mM) was modified by a procedure similar to that described for ferredoxin by Vieria and Davis (1985), in 20 mM MOPS, pH 6.5. GEE was added from a 2.0 M stock solution to give a final concentration of 20 mM. EDC was added from a freshly prepared 100 mM stock solution to give a final concentration of 5 mM. The reaction was allowed to proceed for 3 h at 4 °C followed by quenching of the reaction with ammonium acetate (0.1 M final concentration). The modified protein was stored at −20 °C until needed.

Chemical Cross-Linking of PC to PSI Using EDC. PSI from digitonin solubilization (0.3 mg of Chl/mL) was treated in the presence or absence of 15 μM plastocyanin with 3 mM EDC (freshly prepared) which contained 5 mM $MgCl_2$ and 20 mM MOPS (pH 6.5). This reaction mixture was incubated for 30 min at 25 °C followed by quenching the reaction with the addition of 0.1 M ammonium acetate from a 5 M concentrated stock solution. The samples were diluted with distilled water and concentrated by ultracentrifugation at 200000*g* for 1 h. The pellet was resuspended in TNM buffer (0.05 M Tris-HCl, pH 7.8, 0.01 M NaCl, and 0.005 M $MgCl_2$). This preparation was used for all assays described below as well as for the isolation of the PSI complex using Triton X-100 as described above. PSI-200 (40 μg of Chl/mL) was treated in the presence and absence of 10 μM plastocyanin with 3 mM EDC containing 5 mM $MgCl_2$, 20 mM MOPS (pH 6.5), and 2 mM sodium ascorbate. The reaction mixture was incubated as before and quenched with ammonium acetate. GEE–plastocyanin was used in place of native plastocyanin in some cross-linking experiments.

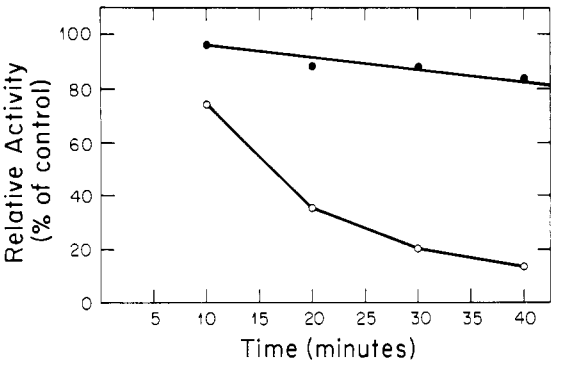

FIGURE 1: Effect of cross-linking on PSI activity. The reaction mixture contained 20 mM MOPS buffer (pH 6.5), 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.2 mM DCPIP, 200 μM methylviologen, 10 μM plastocyanin, 60 μg of digitonin PSI chlorophyll, and, where present, 3 mM EDC. Activity was measured as O_2 uptake in an oxygen electrode with saturating white light. (○) +EDC; (●) no EDC.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Analytical SDS–PAGE of PSI complexes and of EDC-cross-linked PSI complexes was performed at 25 °C with a slight modification of the Laemmli buffer system (Laemmli, 1970) using a 1.5-mm slab gel with a 4% stacking and a 10–20% gradient resolving gel. Prior to electrophoresis, samples were solubilized with 200 mM DTT, 2% SDS, and 20 mM Tris-HCl buffer (pH 8.3) at 50 °C for 20 min. Electrophoresis was done at a constant current of 20 mA for 6–8 h. Following electrophoresis, the gel was either stained for protein with Coomassie Brilliant Blue or electroblotted for antibody probing. For quantitation of stained gels, the gels were scanned with a Hoefer scanning densitometer. The resulting densitometric traces were digitized on a digitization pad connected to a Hewlett-Packard 86B computer.

Western blotting was performed essentially as described by Towbin et al. (1978) with the following modifications: 0.1 μM nitrocellulose paper was used, 0.5% SDS was included in the transfer buffer, and Carnation notfat dry milk (2% w/v) was used as a blocking reagent. The cross-reactions were visualized as purple bands by using the HRP color development reagents (Bio-Rad).

Assay Methods. P700 estimations were made optically by using an Aminco DW-2 spectrophotometer (Lam et al., 1984a,b) and the extinction coefficient of Hiyama and Ke (1972). Photochemical O_2 uptake experiments were conducted in the presence and absence of EDC as described by Malkin (1984). NADP photoreduction was measured in a Gilford spectrophotometer modified for actinic illumination (McSwain & Arnon, 1968). Chlorophyll determinations were made by using acetone/H_2O (80/20) with the extinction coefficients of Arnon (1949). EPR spectra were recorded at liquid helium temperatures using an X-band Bruker spectrometer as previously described (Malkin, 1987a).

RESULTS

Treatment of PSI with plastocyanin in the presence of the water-soluble cross-linker EDC produced a plastocyanin–PSI-cross-linked complex. Figure 1 shows the effect of EDC cross-linking of plastocyanin to PSI on PSI activity as measured by O_2 uptake. The results indicate a greater than 80% inhibition of activity when EDC plus plastocyanin is present with PSI as compared with a ~10–15% loss of activity under similar conditions in the absence of EDC. In addition, Table I provides data obtained by monitoring $NADP^+$ photoreduction from a number of preparations. As can be seen in Table I, EDC treatment alone only slightly inhibits electron transfer

Table I: $NADP^+$ Photoreduction of PSI Preparations Cross-Linked with EDC in the Presence and Absence of Plastocyanin (PC) and Modified Plastocyanin[a]

	activity			
preparation	control	control + EDC	control + EDC + PC	control + EDC + modified PC
digitonin PSI	216 ± 25	162 ± 10	4 ± 2	150 ± 10
PSI-200	153 ± 15	110 ± 15	0	95 ± 12
PSI-100	56 ± 10	31 ± 8	0	28 ± 6
CP1	0	0	0	0

[a] PSI preparations (25–50 μg of Chl/mL of ~250 pg of P700) were incubated with 20 μg of ferredoxin, 30 μg of plastocyanin, and 20 μg of ferredoxin–NADP reductase in 50 mM Tris–HCl, pH 7.5. When present, the EDC concentration was 3 mM. Initial rates were measured as described under Materials and Methods, and activity is expressed in terms of micromoles of $NADP^+$ reduced for milligram of chlorophyll per hour.

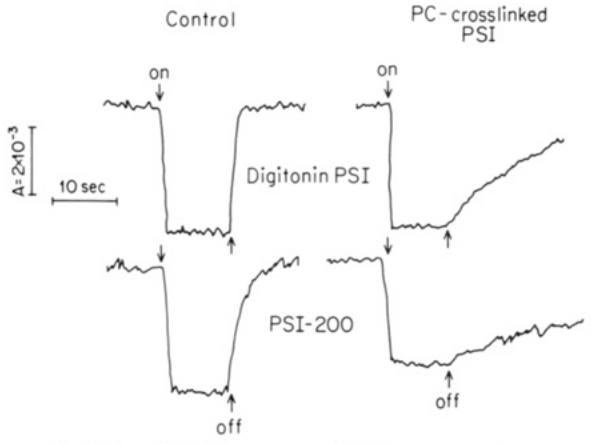

FIGURE 2: Effect of EDC treatment of PSI on P700 kinetics. The reaction mixture contained 20 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.2 mM DCPIP, 2 μM plastocyanin, and 10 μg of chlorophyll for PSI-200 and 15 μg of chlorophyll for digitonin PSI. Samples were cross-linked with plastocyanin and EDC as described under Materials and Methods. P700 photooxidation and reduction were measured at 435–444 nm in an Aminco DW-2A spectrophotometer with saturating red actinic light.

to $NADP^+$. Treatment with EDC plus GEE-modified plastocyanin results in only a slight additional loss of activity compared with EDC treatment alone, suggesting that the modified plastocyanin can no longer interact with PSI. However, a PSI sample treated with EDC plus plastocyanin is totally inactive. These studies demonstrate that inactivation requires both EDC and plastocyanin and that internal cross-linking in the PSI complex is not the cause of the inactivation.

Figure 2 shows kinetics of P700 photooxidation in steady-state light with two PSI complexes and the reduction of $P700^+$ by reduced plastocyanin. While it is difficult to measure exact rates of $P700^+$ reduction by plastocyanin because of instrument-limited time responses, one can see qualitative differences between the different preparations. The rate of $P700^+$ reduction is much higher for the digitonin PSI preparation when compared with PSI-200. Occasionally, the rates were so fast that no photooxidation of P700 could be measured. For both preparations, reduction rates were substantially inhibited by the treatment with EDC in the presence of plastocyanin. This suggests that the electron-transfer block occurs at the site of interaction between plastocyanin and PSI, not at the reaction center which is involved directly in P700 photooxidation. This fact is further supported by EPR measurements of plastocyanin photooxidation. A sample of plastocyanin cross-linked to PSI was only 15% photooxidized compared to the photooxidation

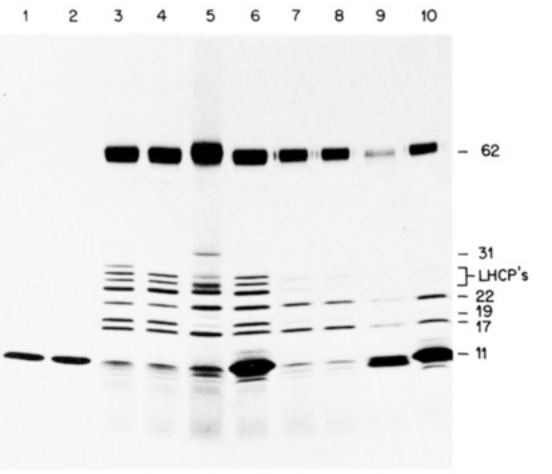

FIGURE 3: SDS–PAGE analysis of EDC-cross-linked PSI preparations. PSI-200 isolated from the digitonin PSI preparation contained 10 μg of chlorophyll per lane while PSI-100 contained 5 μg of chlorophyll per lane. Conditions for cross-linking were as described under Materials and Methods. Lane 1, plastocyanin (PC); lane 2, PC + EDC; lane 3, PSI-200; lane 4, PSI-200 + EDC; lane 5, PSI-200 + EDC + PC; lane 6, PSI-200 + EDC + GEE–PC; lane 7, PSI-100; lane 8, PSI-100 + EDC; lane 9, PSI-100 + EDC + PC; lane 10, PSI-100 + EDC + GEE–PC. Molecular weight standards were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

of the control sample containing plastocyanin and PSI (data not shown).

Figure 3 shows the SDS–PAGE analysis of various EDC treatments on samples of PSI. It can be seen (lane 5) that incubation of plastocyanin with PSI in the presence of EDC produced two major effects: the appearance of a new molecular weight band at 31K and the disappearance of the 19-kDa subunit relative to the other subunits in the complex. The PSI core complex, prior to EDC treatment, showed decreased amounts of the 19-kDa subunit, and treatment with EDC plus plastocyanin (lane 9) also resulted in the complete loss of the 19-kDa subunit. Since the 19-kDa subunit is depleted in the core complex, this preparation shows less of the new 31-kDa protein component. Furthermore, no cross-linked products were observed in samples in which carboxyl-modified plastocyanin was used for the cross-linking experiments (lanes 6 and 10). The control experiments with EDC (no plastocyanin) only showed minor changes from the experiments without EDC (see lanes 3, 4, 7, and 8). These results suggest a specific interaction of plastocyanin with one subunit of PSI. Densitometric scans of the lanes containing the cross-linked product indicate that >80% of the 19-kDa subunit is cross-linked. One of the contaminating LHC II proteins (28-kDa subunit) also seems to be affected by the cross-linking treatment, but in preparations where this protein is depleted, such as the PSI core complex, this pattern is not observed. It is tempting to suggest that there is a 1/1 product formed between plastocyanin and the 19-kDa subunit and that this results in the new 31-kDa protein band. In order to test this idea, Western blots were done and probed with the corresponding antibodies. Figure 4 shows the Western blot which was probed with an antibody to plastocyanin. As can be seen from the EDC-treated samples of both PSI-200 (lane 5) and the PSI core complex (lane 9), the new 31-kDa band in SDS–PAGE shows positive cross-reactivity with a plastocyanin antibody. Plastocyanin was also run for a control (lane 1) and clearly cross-reacts with the antibody. Plastocyanin treated with EDC alone (lane 2) migrates at essentially the same position as native plastocyanin. No cross-linked component of 31 kDa was present in the experiments in which PSI was

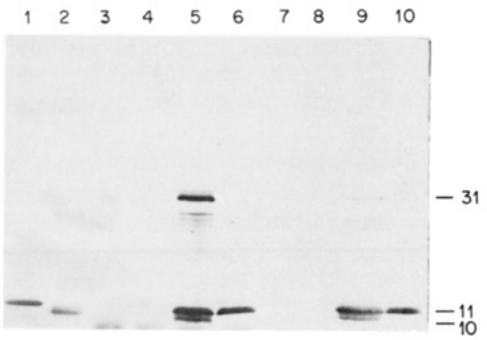

FIGURE 4: Immunoblot analysis of EDC-cross-linked preparations of PSI probed with a plastocyanin antibody. All lanes and cross-linking conditions are as in Figure 3.

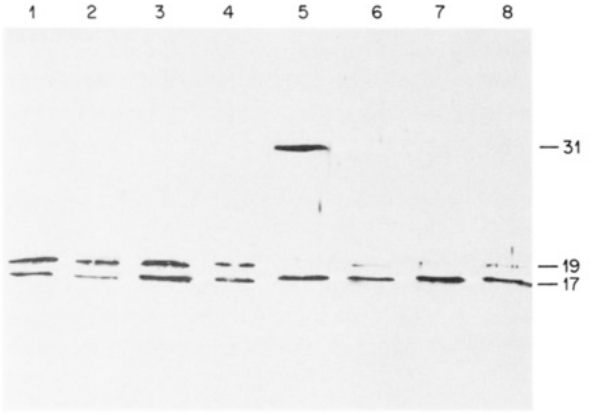

FIGURE 5: Immunoblot analysis of EDC-cross-linked PSI preparations probed with an antibody to the 19-kDa subunit of PSI. Chlorophyll concentrations were the same as those used in Figure 3. Lane 1, PSI-200; lane 2, PSI-200 + PC; lane 3, PSI-200 + EDC; lane 4, PSI-200 + EDC + GEE–PC; lane 5, PSI-200 + EDC + PC; lane 6, PSI-100 + EDC + PC; lane 7, PSI-100 + EDC; lane 8, PSI-100.

reacted with modified plastocyanin (lanes 6 and 10) in the presence of EDC. These results, as well as those already discussed, support the view that ionic interactions are important in stabilizing the complex prior to covalent cross-linking with EDC. Figure 5 shows a similar Western blot as in Figure 4, except that antibodies to the 19-kDa protein were used for probing. Several positive cross-reacting bands are visible. The two lowest bands correspond to the positions of the 17.8- and 17.3-kDa subunits of PSI and are presumed to originate from contamination in the 19-kDa antibody. Lane 5 shows a decreased amount of the 19-kDa subunit and the appearance of the 31-kDa subunit. Since this sample was treated with EDC and plastocyanin, the tentative conclusion was that this positive cross-reaction at 31 kDa is from the 19-kDa + PC cross-linked product. However, since the 17.8- and 17.3-kDa subunits showed positive cross-reactivity in this experiment, an additional analysis was done using antibodies to the 17.8- and 17.3-kDa subunits. As can be seen in Figure 6, neither the 17.8- nor the 17.3-kDa subunit shows any positive cross-reactivity, other than to their respective subunits. We would therefore propose that the 19-kDa subunit is responsible for the positive cross-reactivity at 31 kDa.

Cross-linking studies were also conducted with CP1 which only showed cross-linking between 62- and 58-kDa peptides but no cross-linking with plastocyanin. These results support the earlier $NADP^+$ photoreduction and plastocyanin-mediated P700 reduction experiments. Also, antibodies to CP1 never yielded any higher molecular weight bands that showed positive cross-reactivity. These results suggest that plastocyanin does not interact in a way that facilitates cross-linking to either one of the CP1 subunits.

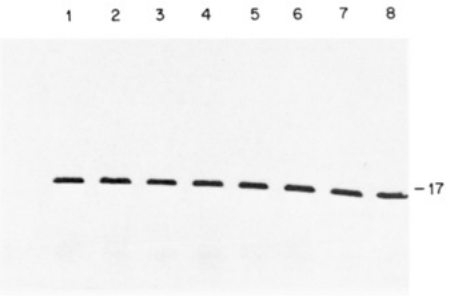

FIGURE 6: Immunoblot analysis of EDC-cross-linked PSI preparations probed with an antibody to the 17.3–17.8-kDa subunits of PSI. Samples in lanes were identical with those of Figure 3 (lanes 3–10).

One interesting feature of the above-mentioned cross-linking experiments was the finding that this reaction, which couples plastocyanin to PSI, is light-stimulated. In cross-linking experiments which were conducted in an oxygen electrode in the light (which allowed equilibration of the reaction mixture with O_2), a 50% increase in cross-linked product was found (data not shown) compared to similar experiments conducted in the dark. As a result of these studies, all cross-linking reactions used for this study were conducted in 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light.

Discussion

The present results show that plastocyanin can be cross-linked to PSI via a single specific PSI subunit. These results also indicate that plastocyanin is bound close to or at the in vivo binding site and that cross-linking blocks further binding of non-cross-linked plastocyanin to PSI. This observation is most dramatically displayed in the photooxidation measurements of plastocyanin cross-linked to PSI, in which only ~15% of the plastocyanin is photooxidizable compared to the control complex plus plastocyanin which shows complete photooxidation. The blockage may be in the form of steric constraints that no longer allow for favorable interaction of plastocyanin with P700. It has been shown that divalent cations are also required for the reaction of plastocyanin with P700 (Lockau, 1979; Haehnel et al., 1980; Takabe et al., 1983). These groups have suggested that divalent cations provide a "shielding" environment which allows the two reaction partners to approach each other for favorable interaction. A more precise picture is beginning to emerge for the interaction of plastocyanin with its redox partners cytochrome *f* and PSI from plastocyanin modification studies by Anderson et al. (1987). It is clear from these experiments that the localized negative changes on plastocyanin form electrostatic interactions which stabilize binding and promote favorable electron transfer to occur between redox partners.

Recent results of Bottin and Mathis (1987) have argued for two binding sites for plastocyanin on the PSI complex, one "distant" and one "close". The present results do not address this model directly but argue that the covalent binding of plastocyanin at a binding site in PSI inhibits all plastocyanin-dependent activity. A nonbound plastocyanin is unable to function with a complex which has been covalently modified with plastocyanin, a result which would suggest the two binding sites envisioned by Bottin and Mathis (1987) must be in close proximity or overlap to some extent.

Particularly striking is the observation that GEE-modified plastocyanin showed no cross-linking in the presence of EDC and PSI preparations. This result is consistent with the fact that modified plastocyanin is a very poor electron donor to PSI-200 as measured by the rate of $P700^+$ reduction. Furthermore, addition of $MgCl_2$ ($\geq$50 mM) inhibited the cross-linking reaction. This further indicates the importance of the

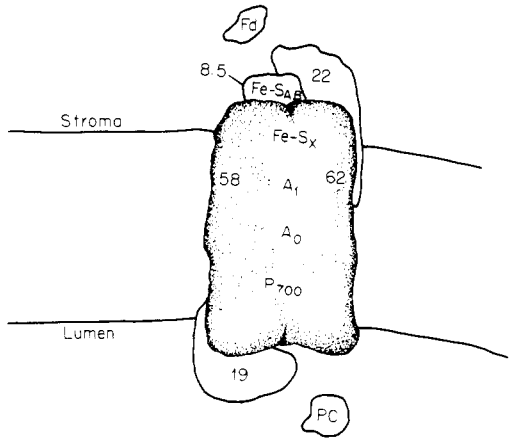

FIGURE 7: Model for the structural and functional organization of subunits in the PSI complex. This model is based on studies of Ortiz et al. (1985), Zanetti and Merati (1987), Golbeck et al. (1987), Wynn and Malkin (1988), and the present work.

electrostatic interaction between the 19-kDa protein and plastocyanin.

While it is difficult to decide if the 19-kDa subunit in our gel system corresponds to the same peptide (subunit III) in the Bengis and Nelson (1977) study, the two studies are strongly suggestive of a similar peptide that directs the binding of plastocyanin to PSI. As mentioned previously, this 19-kDa subunit has been shown not to contain any prosthetic groups (Haehnel et al., 1980), and, instead, it may serve to neutralize the negative charge repulsion between plastocyanin and PSI by providing a "docking site" on PSI. The fact that depletion of this protein subunit in the PSI core complex results in a lowered $NADP^+$ photoreduction rate and a lower rate of reduction by $P700^+$ is suggestive that this protein provides an important recognition site for plastocyanin. The role of the 19-kDa subunit has been questioned by Bhardwaj et al. (1982), but recent results of Nechushtai et al. (1986) implicate its involvement in interacting with plastocyanin. From our results, the later conclusion fits quite well with a model in which the function of the 19-kDa subunit is as a docking protein for plastocyanin binding. One problem which arises from this work is that the $NADP^+$ photoreduction rates (Table I) for the PSI preparation made with Triton X-100 were lower than rates of the digitonin preparations even though both complexes appear to be very similar in their polypeptide compositions. Since Triton X-100 has been shown (Nechushtai et al., 1986) to affect fluorescence emission spectra in PSI, detergent binding to the isolated complexes may play a more critical role than has previously been indicated. Obviously, different detergents affect the fractionation of the native PSI complex, and the mechanism of this effect is not understood.

From all available data to date, Figure 7 seems to present a reasonable model for the functional organization of the higher plant PSI complex. Since it is known that ferredoxin interacts specifically with the 22-kDa subunit (Zanetti & Merati, 1987) and that plastocyanin interacts with the 19-kDa subunit (this study), it stands to reason that these two proteins are surface-exposed on opposite sides of the thylakoid membranes. This conclusion is supported by earlier topography data using membrane-impermeant probes (Ortiz et al., 1985). Recent results on Fe–S centers A and B indicate these are bound to a single subunit of ~9 kDa (Høj et al., 1987; Oh-Oka et al., 1987; Wynn & Malkin, 1988) and presumably electrons from these centers are donated to ferredoxin. This would also suggest a critical orientation of the 9-kDa subunit with respect to the other subunits in the complex. This model relates to the overall function of the complex in terms of electron transfer from plastocyanin to ferredoxin. Further details will become available as more detailed information on specific subunits, such as amino acid sequences, is obtained.

One final result of interest relates to the observed increase in the covalent binding of plastocyanin in the light as compared to dark samples. This would argue for a light-dependent conformational change in PSI. A similar effect has been noted by Selman et al. (1974) and Malkin (1984) in studies of diazoniumbenzenesulfonate (DABS) modification of PSI where inhibition in the light was substantially greater than in the dark. Since DABS reacts at the stromal surface while plastocyanin reacts at the lumenal surface, these conformational changes appear to occur across the membrane, affecting both electron donor and electron acceptor reactions. Such conformational changes may offer a mechanism for subtle controls of electron-transfer processes involving PSI, but further detailed studies are required to examine these effects in more detail.

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Cytochrome b_5, Cytochrome *c*, and Cytochrome P-450 Interactions with NADPH–Cytochrome P-450 Reductase in Phospholipid Vesicles

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ABSTRACT: Upon incubation of detergent-solubilized NADPH–cytochrome P-450 reductase and either cytochrome b_5 or cytochrome *c* in the presence of a water-soluble carbodiimide, a 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), covalently cross-linked complex was formed. The cross-linked derivative was a heterodimer consisting of one molecule each of flavoprotein and cytochrome, and it was purified to 90% or more homogeneity. The binary covalent complex between the flavoprotein and cytochrome b_5 was exclusively observed following incubation of all three proteins including NADPH–cytochrome P-450 reductase, cytochrome b_5, and cytochrome *c* in L-α-dimyristoylphosphatidylcholine vesicles, and no heterotrimer could be identified. The isolated reductase–cytochrome b_5 complex was incapable of covalent binding with cytochrome *c* in the presence of EDC. No clear band for covalent complex formation between PB-1 and reductase was seen with the present EDC cross-linking technique. More than 90% of the cross-linked cytochrome *c* in the purified derivative was rapidly reduced upon addition of an NADPH-generating system, whereas approximately 80% of the cross-linked cytochrome b_5 was rapidly reduced. These results showed that in the greater part of the complexes, the flavin-mediated pathway for reduction of cytochrome *c* or cytochrome b_5 by pyridine nucleotide was intact. When reconstituted into phospholipid vesicles, the purified amphipathic derivative could hardly reduce exogenously added cytochrome *c*, cytochrome b_5, or PB-1, indicating that the cross-linked cytochrome shields the single-electron-transferring interface of the flavoprotein. These results suggest that the covalent cross-linked derivative is a valid model of the noncovalent functional electron-transfer complex.

Hepatic microsomal NADPH–cytochrome P-450 reductase is a membrane-bound flavoprotein which is responsible for electron transfer from NADPH to cytochrome P-450 in the oxidative metabolism of numerous endogenous and foreign compounds (Conney, 1976; Gillette et al., 1972). This reductase is also involved in electron transfer to other heme proteins, such as ferric heme oxidase and hemoglobin (Guengerich, 1978). In addition, the reductase can transfer electrons to cytochrome b_5, and it was recently demonstrated that it may replace NADH–cytochrome b_5 reductase to support NADPH-dependent desaturation of fatty acids (Enoch & Strittmatter, 1979; Daily & Strittmatter, 1980). It is anchored to the microsomal membrane by its hydrophobic amino-terminal region (Black et al., 1979; Gum & Strobel, 1981). This hydrophobic tail, which is readily cleaved from the intact protein by various proteases including trypsin, is essential for the proper interaction of reductase with either cytochrome P-450 (Black et al., 1979; Gum & Strobel, 1981) or cytochrome b_5 (Enoch & Strittmatter, 1979). However, a flavoprotein which loses its tail by protease digestion retains the ability to reduce cytochrome *c* (Masters et al., 1975; Lu et al., 1969). In fact, proteolysis of the cross-linked covalent

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