Ferredoxin Cross-Links to a 22 kD Subunit of Photosystem I

APRIL L. ZILBER and RICHARD MALKIN*
Division of Molecular Plant Biology, University of California, Berkeley, California 94720

ABSTRACT

We have used a cross-linking approach to study the interaction of ferredoxin (Fd) with photosystem I (PSI). The cross-linking reagent N-ethyl-3-(3-dimethylaminopropyl) carbodiimide was found to cross-link spinach Fd to a 22 kilodalton subunit of PSI in both isolated spinach (Spinacia oleracea) PSI complexes and spinach thylakoid membranes.

The product had an apparent molecular weight of 38 kilodaltons on sodium dodeyl sulfate-polyacrylamide gel electrophoresis and was identified as a cross-linked product using specific antibodies to Fd and the 22 kilodalton subunit. In both a native PSI complex (200 Chl/P700) and a PSI core complex (100 Chl/P700), a second cross-linked product at 36 kilodaltons was seen. The latter cross-reacted with an antibody to Fd but did not cross-react with antibodies directed against the 24.3, 22, 19, 17.3 or 8.5 kilodalton, or psaC subunits of PSI. Its composition remains to be determined. In thylakoids only the 38 kilodalton product was observed along with a cross-linked complex of Fd and Fd:NADP+ reductase.

Chloroplast thylakoid membranes contain three integral membrane protein complexes which cooperate in the transfer of electrons from water to NADP. These are PSI, PSII, and the Cyt b6-f complex. In this noncyclic electron transport chain, PSI catalyzes light-induced electron transport from reduced plastocyanin to Fd (10).

The PSI complex contains P700 as its reaction center Chl and a series of electron acceptors, Ao, A1, Fe-Sx, Fe-Sa, and Fe-Sb, which accept the electron lost from the reaction center Chl upon photooxidation (10, 16). Studies with isolated PSI complexes have shown that approximately 12 polypeptide subunits are present in the complex (8, 12). The functions of some of these subunits have been elucidated in the past few years. By gentle detergent treatment, a light-harvesting antenna complex (LHCP2 I) containing Chl a and b can be removed from PSI (5, 14).

Three polypeptide subunits in the 24 to 27 kD range have been associated with this complex. P700, Ao, A1, and Fe-Sx appear to be bound to two high mol wt subunits of ~60 kD (4, 18) while Fe-Sa and Fe-Sb reside on a 9 kD subunit recently identified as the psaC gene product (6, 13, 19). There are still several subunits in the 6 to 22 kD mol wt range with no known function.

In the present study, we have examined the interaction of Fd with PSI in order to identify a possible Fd-binding subunit. We have used a chemical cross-linking approach. A water soluble carbodiimide, EDC, has been used to cross-link Fd to PSI in unfractionated thylakoid membranes and to purified PSI complexes. This cross-linker has previously been used to cross-link Fd to Fd:NADP+ reductase (20) and to cross-link plastocyanin to Cyt f (3). In this study, cross-linked products have been identified by immunoblotting with specific antibodies. Using this approach, a 22 kD subunit of PSI has been identified as a possible Fd-binding protein in the PSI complex.

MATERIALS AND METHODS

Thylakoid and PSI Preparation. Spinach (Spinacia oleracea) was grown hydroponically in a greenhouse. Washed, deribbed leaves were homogenized in blending buffer (50 mM Tris-HCl [pH 7.8], 0.3 M sucrose, 10 mM NaCl, 5 mM MgCl2). The homogenate was passed through filtering silk and centrifuged for 3 min at 3000 g. The pellets were resuspended in 10 mM MOPS buffer (pH 6.5) for use in cross-linking, or in blending buffer for activity assays. PSI-200 was prepared by the method of Mullet et al. (12). PSI-100 was prepared as described by Ortiz et al. (14).

The preparations used in this work contained 185 Chl/P700 and 120 Chl/P700, respectively.

Cross-Linking. Ferredoxin was cross-linked to thylakoids as described by Merati and Zanetti (11) with the following changes. The cross-linking reaction was carried out in 10 mM MOPS buffer (pH 6.5), 2 mM MgCl2 with 2.5 mM EDC. After quenching the reaction, blending buffer was used to dilute the membranes, which were then pelleted and resuspended in blending buffer.

PSI-200 (0.5 mg Chl/mL) was incubated in the presence or absence of 30 μM Fd and 1 mM EDC in the MOPS-MgCl2 buffer for 30 min at room temperature. This EDC concentration was chosen because higher concentrations led to the formation of high mol wt aggregates on SDS-PAGE. The reaction was stopped by the addition of one-fourth volume of solubilization buffer containing 30% (v/v) glycerol, 8% (w/v) SDS, 200 mM Tris-HCl pH 6.8, 20% (v/v) β-mercaptoethanol and 0.04% (w/v) bromphenol blue. PSI-100 (0.5 mg Chl/mL) was treated as described above with the exception that 45 μM Fd was used. The concentration of Fd used in these experiments was calculated based on a ratio of 12 Fd:1 P700. This ratio was chosen because it provides a slight excess over the amount of Fd required to saturate the NADP+ photoreduction activity of PSI-200 in our assay system.

Electrophoresis and Western Blotting. Samples were denatured by incubation in solubilization buffer (described above) for 2 h at room temperature and were applied to a SDS-PAGE slab gel with a 10 to 15% linear acrylamide gradient in the resolving gel (7). Electrophoretic transfer to nitrocellulose (Schleicher and Schuell) and immunoblotting were done essentially as described by Towbin et al. (17). The blocking solution contained 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% (v/v) Tween 20, and 5% (w/v) nonfat dry milk powder. The blots were visualized with the horseradish peroxidase system (BioRad).

Assays. PSI electron transport activity was assayed by measuring Cyt c and NADP+ photoreduction, using a Gilford spectrophotometer modified for side illumination (9). Chl was determined by the method of Arnon (1). P700 was measured in an...
Aminco DW-2a spectrophotometer as described by Lam et al. (8).

Materials. Ferredoxin was purified from spinach according to an unpublished procedure used in our laboratory and was a gift from R. K. Chain. Ferredoxin with an $A_{420}/A_{276}$ of 0.46 to 0.48 was used in all experiments. Antibodies against Fd:NADP$^+$ reductase and the 24.3 kD LHCP I subunit were prepared in our laboratory. Antibodies against Fd and the 17.3 kD PSI subunit were a gift from Dr. N.-H. Chua, antibodies against the 22, 19, and 8.5 kD PSI subunits were a gift from Drs. R. Nechushtai and J. P. Thornber, and antibody against the psaC protein was a gift from Dr. H. Matsubara. EDC was purchased from Sigma and all other reagents were of analytical grade.

RESULTS

The PSI complexes used in studying Fd binding have been analyzed by SDS-PAGE; a Coomassie-stained gel is shown in Figure 1. The polypeptide subunits which will be referred to in following sections are indicated. The PSI-200 complex (lane 1) contains subunits in the 24 to 29 kD range which arise from LHCP I and a small amount of contaminating LHCP II. In addition, numerous low $M_t$ subunits and the ~60 kD subunits are present. The PSI-100 complex (lane 2) has lost most of the LHCP subunits and is depleted of the 19 kD subunit as well.

The results of a cross-linking experiment with Fd and the PSI-200 complex are shown in Figure 2. Lanes 1 and 2 were probed with an anti-Fd antibody. Lane 1 shows PSI-200 treated with EDC in the absence of Fd, and lane 2 shows PSI-200 treated with EDC in the presence of Fd. Two new bands appear in lane 2 with apparent $M_t$ of 36 and 38 kD. Lanes 3 and 4 show identical samples on the same blot which were probed with antibody directed against the 22 kD PSI subunit. Lane 3 shows that the antibody cross-reacted with the 22 kD subunit and that no internal cross-linking occurred when PSI-200 was treated with EDC. Lane 4 shows that the intensity of cross-reaction at 22 kD has decreased and a new band at 38 kD has appeared. We conclude that Fd and the 22 kD subunit are cross-linked by EDC and yield a 38 kD product.

The experiment shown in Figure 2 was carried out with 12 Fd/P700 so one would expect to detect a cross-reacting band corresponding to excess unreacted Fd in lane 2. The inability to detect this band could possibly arise from poor binding of monomeric Fd to the nitrocellulose membrane (see also Fig. 4, lane 2 and Fig. 5, lane 4).

In an attempt to identify the 36 kD product shown in lane 2 of Figure 2, blots were probed with antibodies raised against a number of different PSI subunits: the 24.3 kD LHCP I subunit, the 19 kD subunit, the 17.3 kD subunit, the psaC subunit (which has an apparent $M_t$ of 7.5 kD in our gel system), and an 8.5 kD subunit (the antibody to the latter also cross-reacts with the psaC protein). The results of this analysis are presented in Figure 3. Lanes 1, 3, 5, 7, and 9 show samples of PSI-200 treated with EDC alone, and lanes 2, 4, 6, 8, and 10 show samples of PSI-200 plus Fd, treated with EDC. No new bands arising from cross-
linked products appear on the blots probed with the antibodies described above. It is also apparent that under the conditions used, EDC did not lead to internal cross-linking of subunits within the PSI complex. Some of the antibodies produce a cross-reaction at 60 to 66 kD, but these bands are not dependent on the presence of Fd during the cross-linking incubation. A similar band is also seen with PSI samples not treated with EDC (not shown) and is, therefore, not considered to be significant in the present context.

To confirm the cross-linking results obtained with the PSI-200 complex, an analogous study (Fig. 4) was done with the PSI-100 complex, which lacks LHCP I and the 19 kD subunit. Lanes 1 and 2 were probed with anti-Fd antibody and lanes 3 and 4 were probed with antibody to the 22 kD subunit. As shown in lane 2, when PSI-100 plus Fd were treated with EDC, the 36 and 38 kD bands appeared. Lane 4 shows that the 38 kD band is a cross-linked product of Fd and the 22 kD subunit.

In order to determine whether the cross-linked products seen in the isolated PSI complexes represented artifacts resulting from detergent-solubilized material, similar experiments were carried out with unfractionated spinach thylakoid membranes. After quenching the cross-linking reaction, the membranes were washed to remove excess unreacted Fd. The results are shown in Figure 5. Lanes 1 and 2 were probed with antibody raised against Fd: NADP⁺ reductase (which appears as a dark band at 36 kD), lanes 3 and 4 were probed with antibody against Fd, and lanes 5 and 6 were probed with antibody against the 22 kD subunit. Lane 4 shows that a 38 kD product, a 48 kD product, and a faint 55 kD product can be detected with antibody against Fd. Lane 6 shows that the 38 kD product contains the 22 kD subunit, and lane 2 shows that the 48 and 55 kD products cross-react with antibody to Fd: NADP⁺ reductase. This result indicates that Fd is cross-linked to the reductase by EDC treatment, as expected based on previous results of Merati and Zanetti (11).

cross-reacting bands observed in the samples treated with EDC alone probably arise from small residual amounts of Fd associated with the thylakoids at the time of cross-linking. The cross-linked thylakoids were able to catalyze Cyt c photoreduction with H₂O as electron donor in the absence of added Fd. Thylakoids treated with EDC alone had a low activity which may have been due to the residual Fd mentioned above. The cross-linked thylakoids were unable to catalyze NADP⁺ photoreduction (data not shown). These findings are similar to those reported by Merati and Zanetti (11).

**DISCUSSION**

The results of cross-linking Fd to thylakoids, PSI-200, and PSI-100 show that the 22 kD subunit of PSI interacts with Fd. Possibly this represents a nonphysiological interaction of Fd with the 22 kD subunit. However, the observation that Fd becomes cross-linked both to Fd: NADP⁺ reductase and to PSI in thylakoids under the same conditions suggests that Fd is binding to a physiological binding site on PSI. The Cyt c photoreduction activity of the cross-linked complex provides further support for this interpretation.

In this study we have used EDC, a zero-length cross-linker, which forms a covalent bond between carboxyl and amino groups on proteins (2). As Fd has many acidic amino acids and only four lysines, it seems likely that carboxyl groups on Fd are being cross-linked to lysine amino groups on the 22 kD subunit. Labeling studies with TNBS (15) have shown that the 22 kD subunit (referred to as a 19 kD subunit in that work) is stroma-exposed. This orientation in the thylakoid membrane is consistent with the observed interaction with Fd described here.

Since Fd is thought to be reduced by Fe-Sₐ and/or Fe-S₈, it was expected that Fd would bind to and be cross-linked to the pscC protein (19). However, this protein may not interact with Fd via charged amino acid side chains which would be reactive.
with EDC. Other types of cross-linking reagents could be used to investigate this possibility.

The 36 kD cross-linked product seen in PSI-200 and PSI-100 cross-linking experiments may result from the exposure of a PSI subunit which is not exposed in the thylakoid membrane. Possibly this unidentified subunit is not a part of the Fd binding site but may occupy a nearest neighbor position. The 36 kD product does not seem to be a multimer of cross-linked Fd since Fd treated with EDC does not produce a new, higher mol wt band on SDS-PAGE (data not shown).

As this work was nearing completion, Zanetti and Merati (21) reported that Fd was cross-linked to a 20 kD subunit of PSI, which is the same as the 22 kD subunit in our gel system. In this paper we provide immunological evidence for the interaction of the 22 kD subunit with Fd. Future work will attempt to clarify the nature of this interaction.

Acknowledgment—We thank Drs. N.-H. Chua, H. Matsubara, R. Nechushtai, and J. P. Thornber for their kind gifts of antibodies used in this work. Thanks to Dr. R. M. Wynn and Dr. M. Droux for useful discussions.

LITERATURE CITED

5. HAWORTH P, JL WATSON, CJ ARNTZEN 1983 The detection, isolation and characterization of a light-harvesting complex which is specifically associated with photosystem I. Biochim Biophys Acta 724: 151–158
13. OH-OKA H, Y TAKAHASHI, K WADA, H MATSUBARA, K OHYAMA, H OZEKI 1987 The 8 kDa polypeptide in photosystem I is a probable candidate of an

FIG. 4. Immunoblot analysis of a cross-linking experiment with Fd and PSI-100. Lanes 1 and 3 show samples of PSI-100 treated with EDC alone; lanes 2 and 4 show samples of PSI-100 treated with EDC in the presence of Fd. Lanes 1 and 2 were probed with antibody to Fd and lanes 3 and 4 were probed with antibody to the 22 kD PSI subunit. Samples containing 8 µg Chl were applied to each lane. The band at ~17 kD does not arise from Fd but from a PSI subunit which cross-reacts weakly with the anti-Fd antibody used in this work.

FIG. 5. Immunoblot analysis of a cross-linking experiment with Fd and spinach thylakoid membranes. Lanes 1, 3, and 5 show samples of thylakoids treated with EDC alone; lanes 2, 4, and 6 show samples of thylakoids treated with EDC in the presence of Fd. Lanes 1 and 2 were probed with antibody to Fd:NADP+ reductase (FNR), lanes 3 and 4 were probed with antibody to Fd, and lanes 5 and 6 were probed with antibody to the 22 kD PSI subunit. Samples containing 15 µg Chl were applied to each lane.
iron-sulfur center protein coded by the chloroplast gene frxA. FEBS Lett 218: 52–54
17. Towbin H, T Staehelin, J Gordon 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76: 4350–4354