

The PSI-E subunit of photosystem I binds ferredoxin:NADP⁺ oxidoreductase

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A photosystem I complex containing the polypeptides PSI-A to PSI-L, light-harvesting complex I and ferredoxin:NADP⁺ oxidoreductase has been isolated from barley using the non-ionic detergent *n*-decyl- β -D-maltopyranoside. The ratio between bound ferredoxin:NADP⁺ oxidoreductase and P700 is 0.4 ± 0.2 . The complex is highly active in catalyzing light-induced transfer of electrons from plastocyanin to NADP⁺ at rates of 280 ± 150 and $1800 \pm 800 \mu\text{mol NADPH}/(\text{mg chl-h})$, without and in the presence of saturating amounts of exogenously added ferredoxin:NADP⁺ oxidoreductase, respectively. Endogenously bound ferredoxin:NADP⁺ oxidoreductase interacts with the PSI-E subunit as demonstrated by cross-linking experiments using two different types of cross-linkers and identification of the products by Western blotting and the use of monospecific antibodies.

Photosystem I; Ferredoxin:NADP⁺ oxidoreductase; Cross-linking; PSI-E; FNR-binding protein

1. INTRODUCTION

Photosystem I (PS I) is a membrane-bound pigment-protein complex which catalyzes light-dependent electron transfer from plastocyanin to ferredoxin. The subsequent oxidation of ferredoxin and reduction of NADP⁺ is catalyzed by ferredoxin:NADP⁺ oxidoreductase (EC 1.18.1.2) (FNR) [1]. The PS I complex of higher plants contains 12 different polypeptide subunits denoted PSI-A to PSI-L. The composition of PS I and the structure of the subunits is described in several recent reviews [2–4]. FNR is a water-soluble protein, and about 40% of the FNR is easily removed from the thylakoid membrane by washing with EDTA or low salt buffers. The remaining part of the FNR is membrane associated [1,5] and only released after detergent [6] or trypsin [7] treatment and exhibits a heterogeneous binding pattern. It is generally accepted that the binding is mediated by a specific FNR-binding protein but the

identity of this protein has remained controversial [6,8–10].

In the present paper we report the isolation of a highly active PS I complex from barley containing the PS I core polypeptides, light-harvesting complex I (LHC I) as well as bound FNR. Cross-linking experiments demonstrate specific interaction between the PSI-E subunit and FNR suggesting that the PSI-E subunit has an important role in the binding of FNR to the PS I complex.

2. MATERIALS AND METHODS

Isolation of thylakoids from barley (*Hordeum vulgare* L. cv. Svalöfs Bonus) and isolation of PS I after detergent solubilization using Triton X-100 (preparation denoted TX-PS I) were carried out according to Høj et al. [11]. Isolation of PS I using *n*-decyl- β -D-maltopyranoside (DM) (preparation denoted DM-PS I) was carried out as follows: thylakoids suspended to 2 mg chlorophyll (chl) per ml in 20 mM MES (pH 6.3), 5 mM MgCl₂, 15 mM NaCl were incubated with DM (15 mg/ml) for 30 min at 4°C in the dark after which the unsolubilized grana fraction was removed by centrifugation ($48,000 \times g$, 30 min). The supernatant was diluted five-fold with 20 mM Tricine (pH 7.5), 0.3% DM and applied to a column (2.6 $\times$ 12 cm) of DEAE-Sepharose Fast Flow (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in the same buffer. The column was washed with buffer supplemented with 60 mM NaCl and eluted with a 60–500 mM linear NaCl gradient (2 $\times$ 250 ml). The fractions enriched in PS I as monitored by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis [12] were diluted and rechromatographed on the DEAE column. The fractions containing PS I were concentrated in an Amicon ultrafiltration cell fitted with an YM 10 membrane and loaded onto a column (1.6 $\times$ 100 cm) of Ultrogel AcA 34 (IBF Biotechnics, Villeneuve-la-Garenne, France) equilibrated in 25 mM MES (pH 6.5), 250 mM NaCl, 0.25% DM. A homogeneous PS I complex was obtained after rechromatography on the same column. All purification steps were carried out at 2–4°C using a green safe light.

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Abbreviations: chl, chlorophyll; DM, *n*-decyl- β -D-maltopyranoside; DM-PS I, PS I purified with *n*-decyl- β -D-maltopyranoside; DTSSP, 3,3'-dithiobis(sulfosuccinimidylpropionate); EDC, *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; FNR, ferredoxin:NADP⁺ oxidoreductase; LHC I, light-harvesting complex I; MES, 4-morpholine-ethanesulphonic acid; PS I, photosystem I; SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; TX-PS I, PS I purified with Triton X-100.

Cross-linking with *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was carried out as in [13]. Cross-linking with 10 mM 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce Chemical Co., Rockford, IL) was performed at a chlorophyll concentration of 0.5 mg/ml. After incubation for 30 min at room temperature, the reaction was quenched by addition of 1/5 volume of 20 mM *N*-ethylmaleimide, 50 mM ethanolamine, 50 mM Tris-HCl (pH 7.5). The cross-linked products were analyzed by SDS-electrophoresis and Western blot analysis [13]. When DTSSP was used, reductant was omitted in the electrophoresis sample buffer. The S-S bond introduced into the cross-linked products was cleaved by incubation of the excised gel bands in 50 mM dithiothreitol for 30 min at room temperature and the constituent polypeptides were identified by reelectrophoresis on SDS-polyacrylamide gels and Western blot analysis.

NADP⁺-photoreduction was determined as described previously [13], except that FNR was omitted from the reaction mixture when indicated. Ferredoxin, plastocyanin and FNR from barley were purified as described in [14-16]. P700 was determined by chemically induced difference spectroscopy using an extinction coefficient of 64 mM⁻¹ cm⁻¹ [17,18]. Chlorophyll was determined according to Arnon [19]. The amount of FNR bound to the PS I complex was determined according to the previously reported method [20]. Isolated FNR was quantitated by amino acid analysis and the amount of Coomassie brilliant blue R-250 bound to FNR was determined after electrophoresis of known amounts of FNR and DM-PS I.

Monospecific polyclonal antibodies against the individual PS I polypeptides and FNR from barley were prepared as described previously [13].

3. RESULTS

A PS I complex containing the known PS I polypeptides PSI-A to PSI-L and LHC I (*M_r*'s 20,000-25,000) as well as bound FNR (*M_r* 35,000) has been prepared from barley using the detergent DM. The identity of the 35-kDa polypeptide of the DM-PS I preparation was verified by Western blotting and probing with a monospecific antibody raised against isolated FNR. PS I complexes previously prepared using Triton X-100 have been devoid of FNR as evident by SDS-electrophoresis (Fig. 1) and their inability to catalyze light-dependent electron transfer from plastocyanin to NADP⁺ without addition of exogenous FNR (Table I). In contrast, the DM-PS I complex is highly active catalyzing light-mediated electron transfer from plastocyanin to NADP⁺ at a rate of 280 ± 150 μ mol NADPH/(mg chl·h) without any addition of exogenous FNR. The amount of bound FNR varies from one preparation to the other. Ratios of 0.4 ± 0.2 FNR per P700 were determined from the measured specific Coomassie brilliant blue staining intensity of the isolated protein. The addition of a saturating amount of exogenous FNR raises the activity of the DM-PS I complex to 1800 ± 800 μ mol NADPH/(mg chl·h). The increased activity may reflect that less than stoichiometric amounts of FNR is bound to the isolated DM-PS I complex. An initial wash of the thylakoids with 300 mM NaCl or dialysis against 10 mM EDTA before solubilization with DM did not lower the amount of FNR recovered in the isolated complex (data not shown). The ratio of FNR to P700 did not change during the gel filtration chromatography.

Cross-linking of DM-PS I with the cleavable cross-

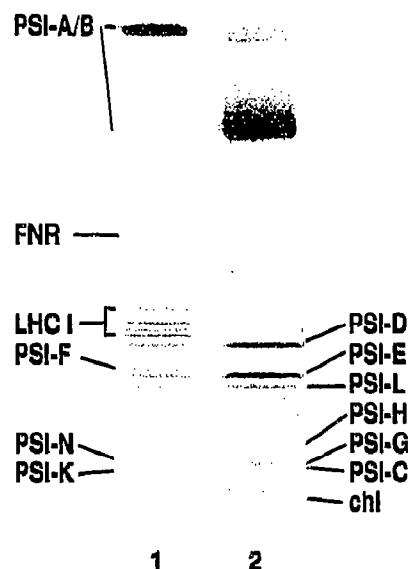


Fig. 1. SDS-polyacrylamide gel of PS I purified with DM (lane 1) and Triton X-100 (lane 2). The gel was stained with Coomassie brilliant blue. The PSI-N subunit has recently been sequenced in barley (J. Knoetzel, unpublished).

linker DTSSP resulted in formation of products with apparent molecular masses of 34, 36 and 55 kDa as detected with monospecific antibodies raised against FNR (Fig. 2). Antibodies raised against the TX-PS I complex from barley recognized the two products with apparent molecular masses of 34 and 36 kDa (Fig. 2). Cross-linked products with the same electrophoretic mobility were formed when EDC was used as cross-linking reagent, although their staining intensity were altered. EDC cross-links neighboring amino and carboxyl groups and the cross-linking reagent is eliminated as a result of the cross-linking reaction [21]. DTSSP cross-links nearby amino groups and introduces a

Table I
Characteristics of photosystem 1 complexes prepared using DM and Triton X-100
The data (mean $\pm$ SD) represent the results obtained with a number of different preparations.

	PS I purified with	
	Triton X-100	DM
chl/P700	78 $\pm$ 18	159 $\pm$ 29
NADP ⁺ reduction, + FNR (μ mol NADPH/mg chl·h)	160 $\pm$ 87	1800 $\pm$ 800
NADP ⁺ reduction, - FNR (μ mol NADPH/mg chl·h)	4 $\pm$ 4	280 $\pm$ 150
FNR (copies per P700)	0	0.4 $\pm$ 0.2
NADP ⁺ reduction, +FNR, after cross-link with EDC (% of control)	89% ^a	28% ^a

^aAverage of two experiments.

spacer arm [22]. The two cross-linking reagents are therefore expected to destroy or alter the accessibility to different epitopes.

The three cross-linked products formed by treatment with DTSSP were detectable as faint bands on SDS-polyacrylamide gels after staining with Coomassie brilliant blue. The stained bands corresponding to the cross-linked products were excised and treated with dithiothreitol to cleave S-S bonds introduced by the cross-linker. The component polypeptides formed were subjected to reelectrophoresis and their identity determined by probing with monospecific antibodies after Western blotting (Fig. 3). The 34-kDa product was only recognized by antibodies against the PSI-E subunit and FNR. The formation of this product indicates a specific interaction between the PSI-E subunit and FNR. The 36-kDa product showed positive reaction with antibodies against PSI-D, PSI-E and FNR. Cross-linking between PSI-D and PSI-E has previously been reported [13,23,24]. Thus the 36-kDa product most likely results from cross-linking of PSI-E to PSI-D as well as to FNR. The 55-kDa product showed positive reaction with antibodies against FNR and the PSI-E subunit (Fig. 3) and could reflect cross-linking between a dimer of FNR and PSI-E. However, silver staining of the cleaved and reelectrophoresed product indicates the presence of low amounts of LHC I polypeptides in the 55-kDa product (data not shown). The 55-kDa product could therefore represent a product between FNR, PSI-E and some LHC I polypeptide. The apparent molecular mass of the cross-linked product between PSI-E and FNR is 34 kDa, which is lower than the molecular mass of FNR alone. Peculiar electrophoretic migration patterns in SDS gels are often observed with cross-linked polypeptides [25,26]. Presumably, chemical cross-linking in some cases prevents formation of the usual extended conformation upon addition of SDS and may lead to abnormal SDS binding [25]. Monospecific antibodies against other subunits of PS I (PSI-A/B, -C, -F, -H, -I, -L) did not recognize any of the products (data not shown).

Treatment of DM-PS I with EDC resulted in an inhibition of the NADP⁺-photoreduction activity (Table I). Previous experiments [13,23] have shown that the NADP⁺-photoreduction activity of TX-PS I was not affected significantly by treatment with EDC. The inhibitory effect exerted by EDC on DM-PS I thus most likely reflects the immobilization of FNR at the reactive site.

4. DISCUSSION

The ability of the DM-PS I preparation to catalyze high rates of light-dependent reduction of NADP⁺ without the addition of exogenous FNR demonstrates that the FNR bound to the PS I complex is physiologically functional. The activity is increased to very high levels

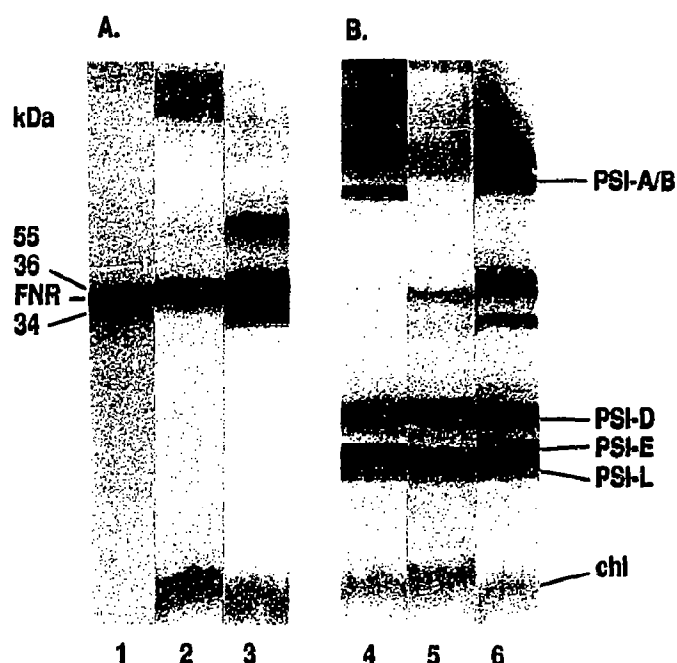


Fig. 2. Western blot analysis of cross-linking of DM-PS I with DTSSP and EDC. (Lanes 1 and 4) DM-PS I; (lanes 2 and 5) DM-PS I cross-linked with EDC; (lanes 3 and 6) DM-PS I cross-linked with DTSSP. Lanes 1-3 were incubated with antibodies against FNR. Lanes 4-6 were incubated with antibodies raised against TX-PS I. The electrophoresis was performed without reductant in the sample buffer resulting in diffuse bands on the Western blot.

by the addition of exogenous FNR as would be expected due to the less than stoichiometric amounts of FNR bound to DM-PS I. To our knowledge no other isolated PS I complex has been reported with such a high activity. Orlich and Hauska [27] report an activity of 1800 $\mu\text{mol NADPH}/(\text{mg chl}\cdot\text{h})$ in PS I isolated from spinach with a low concentration of Triton X-100. However, they used a plastocyanin concentration of 14 μM in the assay for NADP⁺ reduction whereas 2 μM was used in the present investigation. The rate of reduction of P700⁺ is proportional to plastocyanin concentrations below 20 μM [28]. Apparently the detergents previously used for the isolation of PS I complexes of defined polypeptide composition remove or distort components necessary for efficient electron transport. Triton X-100 dissociates the PSI-F subunit from the PS I complex [29-31] thereby lowering the efficiency of plastocyanin as an electron donor to P700. However, other low molecular mass polypeptides like PSI-K are also depleted (Fig. 1) and at present it is not clear whether the detergent effects are primarily manifested at the donor or the acceptor side of PS I, although preliminary kinetic analyses favor the latter (B. Andersen, H.V. Scheller and Ö. Hansson, unpublished data).

The tight binding of FNR to the thylakoid membrane has previously been indicated to involve a specific FNR-binding protein [8-10]. The present demonstration of

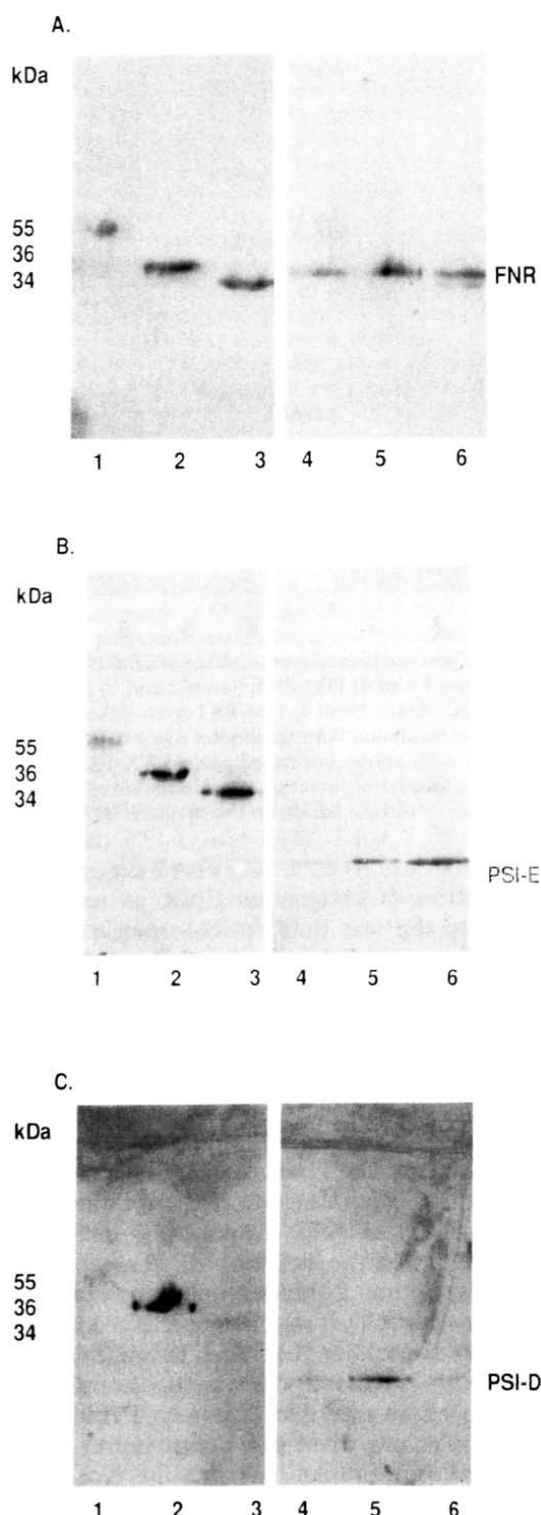


Fig. 3. Western blot analysis of the cross-linked products formed upon treatment with DTSSP. (Lanes 1-3) Cross-linked products with apparent molecular masses of 55, 36 and 34 kDa, respectively, excised from the gel and reelectrophoresed. (Lanes 4-6) Component polypeptides generated from the 55-, 36- and 34-kDa products by treatment with dithiothreitol. The blot in panel A was incubated with antibodies against FNR, the blot in panel B with antibodies against PSI-E, the blot in panel C with antibodies against PSI-D.

the possibility to isolate a PS I complex of defined simple polypeptide composition which still binds FNR restricts the number of possible candidates for such a binding protein. The fact that neither high salt wash nor EDTA treatment of thylakoids affected the recovery of FNR bound to the isolated DM-PS I complex indicates a tight binding. FNR is susceptible to proteolytic degradation resulting in loss of an N-terminal segment of about 11-17 residues [32]. The same type of proteolytic cleavage was also observed during isolation of FNR from barley as evidenced by generation of a product with faster electrophoretic mobility (data not shown). The proteolytic degradation product does not bind to the isolated DM-PS I complex. A partial loss due to proteolytic degradation may explain why the isolated DM-PS I complex binds less than stoichiometric amounts of FNR. Any proteolytic degradation must take place early in the isolation procedure as no degradation or dissociation of FNR occurred during the final gel filtration steps.

Nearest neighbor analysis as monitored using two functionally different types of cross-linkers results in the formation of three products of 34, 36, and 55 kDa. All three cross-linked products obtained comprises PSI-E as well as FNR indicating a close association of the PSI-E subunit and FNR and thus suggesting that the FNR-binding protein could be identical to PSI-E. The PSI-E subunit is stroma exposed [13,23,24,33] as would be expected of an FNR-binding protein. Soluble FNR is able to bind ferredoxin as shown by cross-linking experiments [25, 26]. The interaction between PS I and the soluble electron carrier ferredoxin has been investigated by several groups [13,23,34,35] and the PSI-D subunit has been identified as docking site for ferredoxin. Cross-linking studies have demonstrated that the PSI-D and PSI-E subunits are located very close to one another [13,23,24]. Accordingly, binding of FNR to PSI-E would locate FNR near to the docking site for ferredoxin. It is conceivable that binding of FNR to the PSI-E subunit ensures the proper orientation of FNR for the most efficient electron transfer from ferredoxin to NADP^+ .

Several reports have appeared on the binding of FNR to the thylakoid membrane. Vallejos and co-workers [8] purified a complex of FNR and a 17.5-kDa polypeptide which exhibits the allotropic properties of the membrane-bound FNR. Several suggestions have since been made on the identity of the 17.5-kDa polypeptide. Clark et al. [36] propose that the polypeptide is identical to the 17.5-kDa polypeptide of the cytochrome b_6/f complex and Soncini and Vallejos [37] suggest that the polypeptide is the 16.5-kDa polypeptide of the oxygen-evolving complex of photosystem II. Neither the cytochrome b_6/f complex nor components of the oxygen-evolving complex are present in DM-PS I. If the assignment of the 17.5-kDa polypeptide to components of these complexes is correct, it may be concluded that the 17.5-kDa

polypeptide is not essential for FNR binding. Recently, Berzborn et al. [38] have presented an N-terminal sequence of the claimed FNR-binding protein. This sequence shows no sequence similarity to PSI-E or to other previously reported chloroplast polypeptides. Other experiments [39,40] have indicated a different organization of FNR in the chloroplasts and suggest that the functional form of FNR in NADP⁺-photoreduction is a dimer connected by a 10-kDa polypeptide (denoted connectin). The soluble 10 kDa connectin is thought to mediate the binding of FNR to the membrane. The exact molecular mass of the PSI-E polypeptide of barley is 10,821 Da [41] although the polypeptide migrates with an apparent molecular mass of 16 kDa on SDS-polyacrylamide gels. The molecular masses of the previously reported FNR-binding proteins are thus not significantly different from that of the PSI-E subunit of PS I. Since sequence information is lacking for the 10- and 17.5-kDa polypeptides, conclusions with respect to their possible identity to PSI-E must await further experiments.

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