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On the function of subunit PsaE in chloroplast Photosystem I

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Treatment of thylakoids from spinach with NaSCN removes extrinsic stroma-exposed subunits of the Photosystem I complex in addition to CF_1 and some other surface proteins. By increasing the NaSCN concentration, PsaE is released first, followed by PsaD and PsaC. At 0.5 M NaSCN, about 80% of PsaE is resolved without significant loss of other PS I polypeptides. Time-resolved fluorescence spectroscopy showed no significant alteration of PS I isolated from membranes thus treated with regard to energy transfer within the antennas as well as primary charge separation. Washing of thylakoids with NaSCN results in inhibition of electron transport from an artificial electron donor (ascorbate/DAD) to either methylviologen or NADP. Although higher NaSCN concentrations are required for inhibition than for resolution of PsaE, electron transport is restored by reconstitution with isolated PsaE from *Synechococcus*. We conclude that inhibition is due to dislocation of PsaC as a consequence of PsaE resolution, impeding efficient electron transfer from F_x to F_A/F_B. An antibody raised against PsaC inhibits methylviologen reduction only when PsaE has been removed previously. An antibody raised against PsaE inhibits electron transport to NADP, but not to methylviologen. We conclude that binding of this antibody sterically hinders the access of ferredoxin either to the F_A/F_B center or the catalytic site of ferredoxin:NADP oxidoreductase (FNR). Our results suggest an essential role of PsaE in stabilization of the acceptor side of PS I, in particular in maintenance of the functional integrity between the F_A/F_B protein and the membrane-integral sector of the PS I core.

Introduction

Photosystem I is composed of at least 12 different polypeptides which are named according to the respective genes PsaA to PsaL [1]. The two membrane-integral homologous polypeptides PsaA and PsaB form the heterodimer core of PS I and carry the redox centers P700, A_0, A_1 and F_x as well as about 100 antenna chlorophylls [2,3]. The iron-sulfur clusters F_A and F_B are bound within the structure of PsaC, a hydrophilic polypeptide located on the stromal side of the thylakoid membrane [4]. Two other subunits, PsaD and PsaE are stroma-exposed subunits, too [5,6]. PsaD is important for stabilization of PsaC on the heterodimer core [7] and for docking of ferredoxin during transfer of electrons from F_A/F_B [8]. Its functional counterpart on the lumenal side of the membrane is PsaF, a subunit which is supposed to facilitate plastocyanine binding during donation of electrons to $P700^+$ [9,10]. Little is known about the function of the other PS I subunits including PsaE. There is some evidence that PsaE may be involved in ferredoxin reduction by PS I [11,12]. PsaE deletion mutants of *Synechocystis* [13] and *Synechococcus* [14] initially suggested no obvious phenotypic alterations. Subsequently, however, it was found that the deletion mutant of *Synechococcus*-7002 could not grow photoheterotrophically and that growth at low light intensity was considerably reduced [15]. Recently, Yu et al. [16] presented evidence demonstrating that inhibition of growth may be due to inhibition of cyclic electron transport in this mutant.

The extrinsic stroma-exposed polypeptides PsaC, PsaD and PsaE can be removed from isolated PS I and from thylakoid membranes by treatment with 3.9 M NaSCN [4]. By varying the concentration of NaSCN we observed that the three extrinsic PS I subunits were differentially released from spinach thylakoid mem-

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Abbreviations: Chl, chlorophyll; PS I, Photosystem I; DAD, 3,6-diaminodurol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; FNR ferredoxin:NADP oxidoreductase (EC 1.18.1.2); LHCI, light-harvesting protein chlorophyll complex I; Mes, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); SDS, sodiumdodecyl sulfate; SOD, superoxide:superoxide oxidoreductase (EC 1.15.1.1); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

branes, PsaE being most sensitive to the treatment with the chaotropic salt. The technique of differential subunit removal thus gives a means to study the function of PsaE in biochemical experiments. The results indicate an important structural role of PsaE on the acceptor side of higher plant Photosystem I. PsaE seems to cover at least a part of PsaC and to effect tight interaction of PsaC with the PS I core, thus facilitating efficient transport of electrons from F_x to the F_A/F_B center.

Experimental

Preparative methods

Broken chloroplasts were isolated from spinach leaves as in Ref. 17 and adjusted to a chlorophyll concentration of 2 mg/ml. For preparation of PS I we essentially followed the protocol of Høj et al. [4] for PS I preparation from barley. Thylakoid membranes were solubilized by addition of 25 mg Triton X-100/mg Chl and stirred for 30 min at room temperature. The solubilized material was centrifuged at $48\,000 \times g$ for 30 min. The supernatant was 5-times diluted with 20 mM Tricine buffer (pH 7.5), containing 0.2% Triton and then put on a DEAE-cellulose (DE52, Whatman) column (30 cm length, 5 cm diameter), equilibrated with 20 mM Tricine (pH 7.5) containing 0.2% Triton X-100. After washing with one column volume of 60 mM NaCl (containing 20 mM Tricine (pH 7.5) and 0.2% Triton) proteins were eluted with a linear NaCl gradient (60 to 500 mM) containing 20 mM Tricine (pH 7.5) and 0.2% Triton, and fractionated in 10 ml samples. In a NaCl concentration range from 120 to 150 mM a crude PS I preparation with associated LHCI and further proteins was obtained. These fractions characterized by a Chl a/b ratio > 5, were pooled, adjusted to a chlorophyll concentration of 0.5 mg/ml and were further treated with 1% Triton X-100 for 4 h at 4°C. Subsequent gel filtration was carried out on an acrylamide agarose column (ACA34, Pharmacia, 100 cm length, 5 cm diameter) by elution with buffer containing 25 mM Mes buffer (pH 7.3), 0.1% Triton X-100 and 0.25 mM NaCl. In fractions 84 to 105 (8 ml each) a highly enriched PS I preparation (see Fig. 1) was gained. The Chl a/b ratio of this preparation was about 21.

For preparation of antisera, isolated PS I was subjected to preparative SDS-PAGE on 1.5 mm 15% gels (acrylamides : bisacrylamide 30 : 0.8). After staining with Coomassie brillant blue R-250 [18], single bands were excised and the protein was gained by electroelution in a solution containing 0.1% SDS, 2.5% β-mercaptoethanol and 20 mM NH_4HCO_3. After addition of incomplete Freund adjuvant (Difco) the solutions were injected subcutaneously into male rabbits. In order to obtain the isolated IgG fraction, blood serum taken in

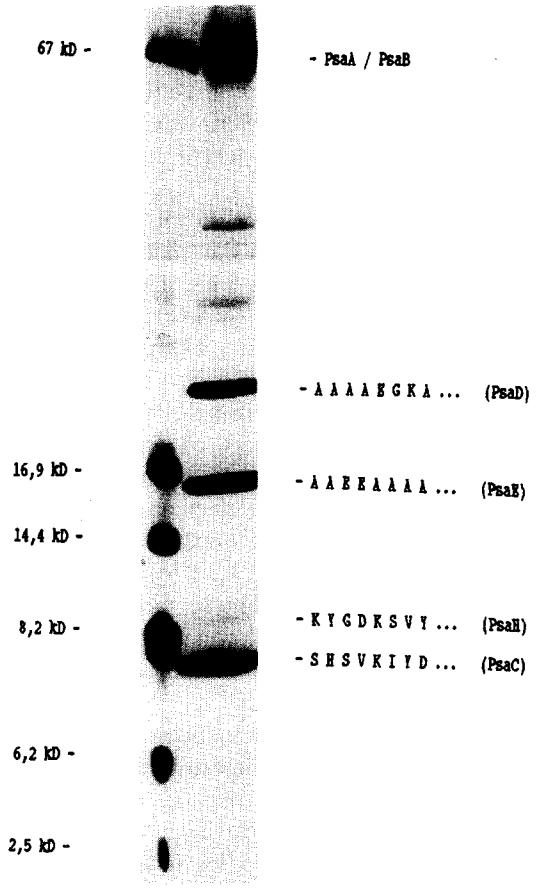

Fig. 1. Identity of the polypeptides of a PS I preparation in an SDS-gel. On the right hand side the obtained N-terminal sequences are shown and assigned to the corresponding PS I subunits. PsaA/PsaB was identified by an antiserum prepared by Prof. N. Nelson. Left lane: protein standard, right lane: PS I preparation.

time intervals of two weeks was worked up according to Ref. 19.

Analytical techniques

For qualitative protein analysis PS I or membrane samples were taken up in 2% SDS, 5% glycerol, 5% β-mercaptoethanol and 50 mM Tris buffer (pH 8.0) containing 0.005% bromophenol blue, and subjected to SDS-PAGE on 1 mm 15% gels. After development, the gels were either silver-stained [20] or transferred to nitrocellulose membranes (Schleicher and Schüll) by electroblotting [21]. For Western blot analysis the nitrocellulose membranes were washed twice with a solution containing 1% casein, 10 mM Tris buffer (pH 8.0) and 150 mM NaCl and then incubated with the antisera for 12 h at 4°C. Afterwards the membranes were washed three times for 10 min with a solution of 10 mM Tris buffer (pH 8.0), 1% Triton and 150 mM NaCl. Then the washing procedure was repeated with the same, but Triton-free medium. Afterwards the membranes were incubated for 1 h at room temperature with anti-rabbit-IgG peroxidase conjugate (Sigma).

After washing with Tris/NaCl medium (see above), the antibody reaction was assayed by the luminol method described in Ref. 22. For semiquantitative determination of relative protein contents the developed X-ray films were scanned with the instrument UltroScan XL from Pharmacia.

For amino acid sequencing the proteins were transferred from the gels to PVDF membranes by electroblotting and stained with amidoblack [23]. The excised bands were analyzed by microsequencing in a pulsed liquid sequencer, model 477A (Applied Biosystems).

Electron transport activity of isolated PS I or thylakoids was measured by oxygen consumption using an oxygen electrode DW1 (Bachofer). The electron donor was ascorbate/DAD and the electron acceptor methylviologen. The medium contained 40 mM Tricine, pH 8.0, 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM DAD, 1 mM ascorbate, 1 mM methylviologen, 1 mM NaN_3 and 2 U/ml SOD. The chlorophyll concentration was 10–30 μg/ml, the samples were illuminated with red light > 630 nm of 1700 $\mu E\ m^{-2}\ s^{-1}$.

PS-I-dependent NADP reduction of thylakoids was assayed with ascorbate/dichlorophenol-indophenol (DCPIP) as donor in a spectrophotometer at 340 nm. The instrument was equipped with a cross-illumination device for excitation of PS I by red light > 630 nm (light intensity 1800 $\mu E\ m^{-2}\ s^{-1}$). The medium contained 25 mM Tricine, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DCPIP, 1 mM ascorbate, 10 μM DCMU, 0.5 mM $NADP^+$, 1.5 μM nigericin and 8 μM ferredoxin. The chlorophyll concentration was 20–50 μg/ml.

Time-resolved fluorescence spectra were measured by the single photon-timing technique and analyzed as described in Ref. 24.

Results

Identification of PS I subunits

Thylakoid membranes were solubilized by Triton X-100 and subjected to ion exchange chromatography. A fraction containing PS I associated with LHCI, FNR, the proteolipid subunit of CF_0 and proteins of the cytochrome b_6/f complex was further treated with 1% Triton X-100. Subsequent gel filtration resulted in an almost pure PS I preparation. The bands obtained in SDS-PAGE (Fig. 1) were identified immunologically (PsaA/B) or by N-terminal amino acid sequencing (PsaC, D, E, G and H). In the final PS I preparation PsaF was missing. As this subunit was still present after the first isolation step, we conclude that it was resolved during the subsequent Triton treatment [25]. The contents of PsaG and PsaH were low and the small subunits PsaI, J and K could be detected only in traces, suggesting that these polypeptides are partly extracted by Triton, too [26].

By aging of isolated PS I the 16 kDa band identified as PsaE disappeared at the expense of an emerging 14 kDa band. Since the N-terminal sequence of the latter was identical to that of PsaE, we conclude a C-terminal retrenchment of PsaE to PsaE'.

Polyclonal antibodies were raised against PsaC, PsaE' and PsaH. The anti-PsaE crossreacted with a protein of 17.8 kDa apparent molecular mass which was present in unwashed thylakoid preparations as well as in stroma extracts of chloroplasts. The protein was isolated and identified as the ribosomal protein L12 by N-terminal amino acid sequencing [27].

Resolution of PS I subunits from thylakoid membranes by NaSCN

Thylakoids were three times washed with hypotonic isolation medium and subsequently treated with NaSCN solutions of different concentrations ranging from 0 to 2 M. The pellets obtained after centrifugation as well as the supernatants were analyzed by SDS-PAGE followed by immunological identification. The Western blots were quantified by scanning. It is known that the extrinsic sector of the thylakoid-ATPase, CF_1, is removed from thylakoid membranes by NaBr and that photophosphorylation can be restored by reconstitution with isolated CF_1 [28]. These results suggest that the integrity of the thylakoid membrane is not affected by treatment with the chaotropic salt. Furthermore some extrinsic stroma exposed subunits of PS I were shown to be resolved by chaotropic salts [4].

The concentration of NaSCN needed for resolution of 50% of the α and β subunits of CF_1 is about 0.5 M (not shown). 50% of the FNR is released at about 1.3 M. The PS I subunit most sensitive towards treatment with NaSCN is PsaE. 50% of this protein is released at a concentration of about 0.35 M and at 0.75 M almost all of PsaE is found in the supernatant. PsaD begins to detach at 0.5 M, 50% being removed at about 1.5 M NaSCN. PsaH and PsaC are found to be fully attached up to NaSCN concentrations of about 1 M (Fig. 2). As expected, the membrane-integral core proteins PsaA/B are resistant against NaSCN treatment (not shown). The differential sensitivity against NaSCN permits the study of a PsaE-deficient PS I. At a concentration of 0.5 M NaSCN about 80% of PsaE is detached without a significant loss of any other of the PS I subunits.

Effect of NaSCN treatment on PS I function

In order to check possible effects of NaSCN treatment on energy transfer reactions and primary charge separation, a ps-fluorescence analysis was carried out at 12°C. To this end, LHCI-containing PS I was isolated from control thylakoids and from membranes

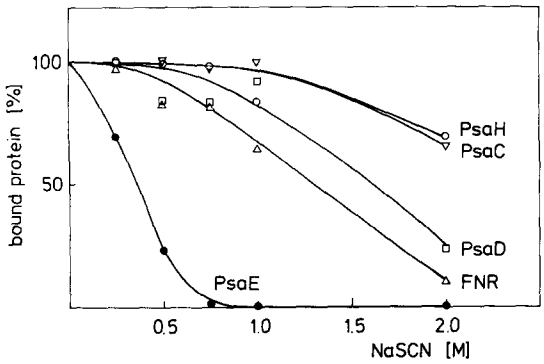

Fig. 2. Resolution of PS I subunits by treatment of thylakoids with NaSCN. Isolated thylakoids (0.5 mg Chl/ml) were stirred with the indicated concentrations of NaSCN for 30 min at 4°C. The suspensions were then diluted 1:1 with hypotonic isolation medium (2 mM Tricine (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$) and centrifuged at 12000× g for 10 min. After two washes with isolation medium, samples corresponding to 30 μg Chl were subjected to SDS-PAGE. The proteins were analyzed by immunodetection and quantified as described in the experimental section.

treated with 0.5 M NaSCN. The time-resolved fluorescence spectrum of untreated controls shows four components with different time constants: $\tau_1 = 12$ ps, $\tau_2 =$

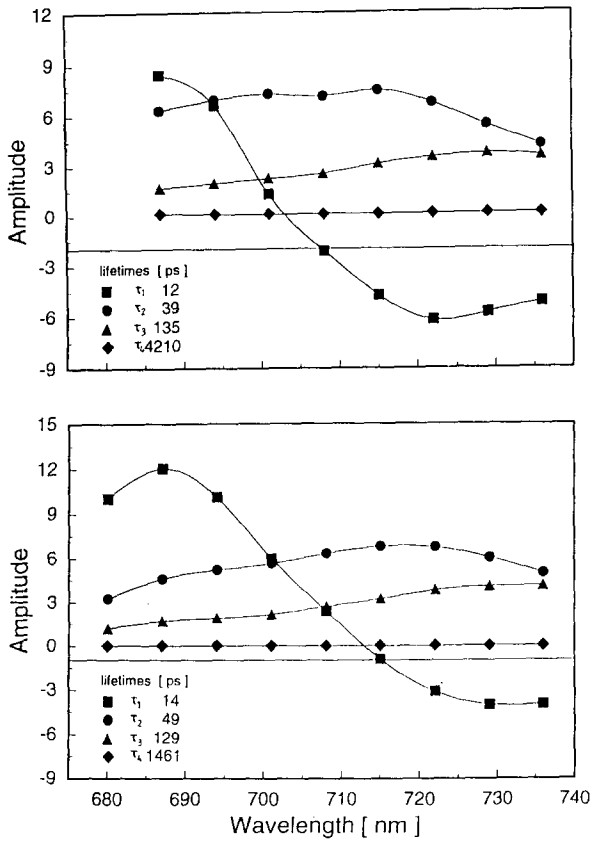

Fig. 3. Time-resolved fluorescence spectra of PS I + LHCI prepared from control thylakoids (upper part) and thylakoids pretreated with 0.5 M NaSCN (lower part). The preparations were taken up in a medium containing 20 mM Tricine buffer (pH 7.5) and 0.05% SB12 to yield a chlorophyll concentration of 8 μg/ml. For experimental details see Ref. 29.

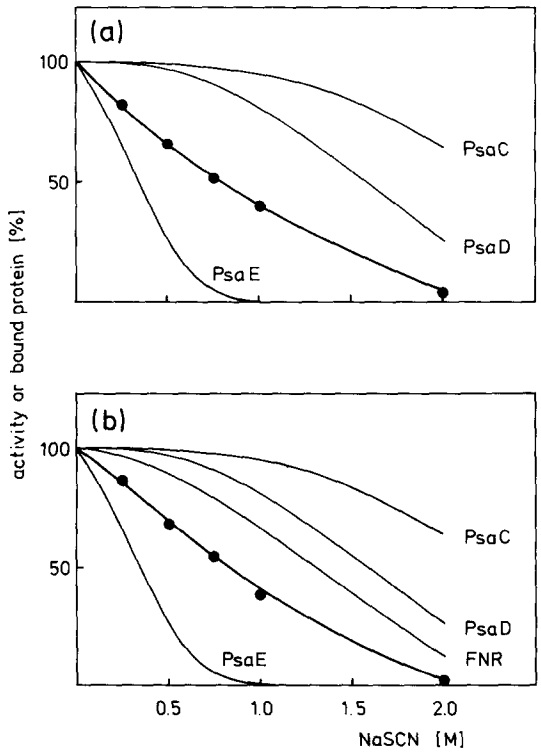

Fig. 4. Effect of NaSCN treatment of thylakoids on PS I activities. NaSCN treatment was carried out as in Fig. 2. (a) Methylviologen reduction (control rate: 420 μmol O_2/(mg Chl h)), (b) $NADP^+$ reduction (control rate: 95 μmol NADPH/(mg Chl h)). The faint curves indicate the relative amounts of the signified PS I polypeptides associated with the membranes. They were taken from Fig. 2.

39 ps, $\tau_3 = 135$ ps and $\tau_4 > 1.5$ ns (Fig. 3). Component 1 which exhibits a positive fluorescence change with a maximum emission at 685 nm and a negative emission maximum at 730 nm, is ascribed to an energy transfer reaction within the antenna of PS I [29]. Components 2 and 3 signify the charge separation step in the heterogenous population consisting of isolated PS I and PS I + LHCI, whereas component 4, having a very low amplitude in our preparation, is most probably due to fluorescence emission of decoupled LHCI-chlorophylls [29]. The fluorescence spectrum of the complex isolated from NaSCN-treated membranes shows the same components with only minor changes in time constants and amplitudes (Fig. 3). We may therefore conclude that treatment with NaSCN, at a concentration which releases most of PsaE, does not significantly affect the primary photoreactions including charge separation in PS I. Moreover, it has been shown by Parrett et al. [30] that treatment of isolated PS I with NaSCN concentrations up to 1.5 M has no effect on the function of F_x.

The steady-state rate of electron transport was measured from an artificial PS I electron donor to methylviologen/O_2 or ferredoxin/$NADP^+$. Thylakoids treated with increasing concentrations of NaSCN show a continuous decrease of both electron transport

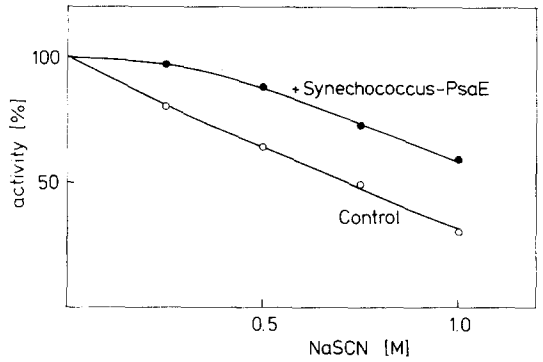

Fig. 5. Rates of methylviologen reduction of thylakoids after treatment with increasing concentrations of NaSCN and reconstitution with isolated PsaE from *Synechococcus*. NaSCN pretreatment was carried out as in Fig. 2. For reconstitution pretreated membranes corresponding to 1 mg Chl/ml were incubated under gentle stirring with PsaE (0.06 mg protein/ml thylakoid suspension) for 5 min at 4°C. Subsequently, the membranes were collected by centrifugation (5 min at 10000× *g*), washed twice with hypotonic isolation medium and after resuspension in the same medium assayed for activity. The control activities which were identical in the samples with and without external addition of PsaE, were 560 μmol O_2 /(mg Chl h).

activities, 50% inhibition being observed at about 0.75 M NaSCN (Fig. 4). As the primary charge separation including reduction of F_x appears not to be affected and an effect of NaSCN to the electron donor side of PS I (located at the inner face of the membrane and therefore inaccessible to NaSCN) is unlikely, we may refer inhibition of electron transport to a block at the membrane-stroma interphase, i.e., between F_x and F_A/F_B. There is no obvious correlation between inhibition and release of PsaE which is effected by lower NaSCN concentrations, or release of other PS I subunits and FNR which are effected by higher NaSCN concentrations. Reconstitution experiments, however, show that NaSCN-induced release of PsaE is indirectly responsible for inhibition of electron transport. In an NaSCN concentration range where PsaE is the only PS

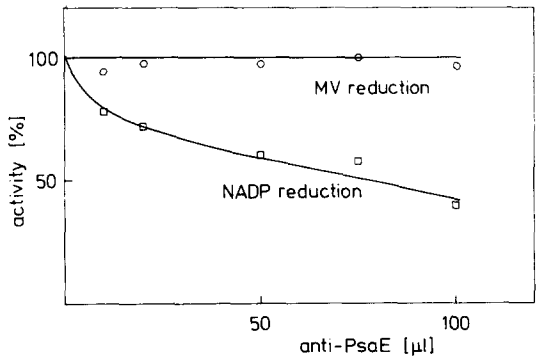

Fig. 6. Effect of anti-PsaE on methylviologen reduction and $NADP^+$ reduction of isolated thylakoids. Thylakoids (1 mg Chl/ml) were incubated with the indicated volumes of the anti-PsaE containing IgG preparation (protein concentration 10 mg/ml) for 5 min at 4°C. The control rates were 425 μmol O_2 /(mg Chl h) and 96 μmol NADPH/(mg Chl h), respectively. All rates were related to the corresponding rates obtained in the presence of control serum of the same protein concentration.

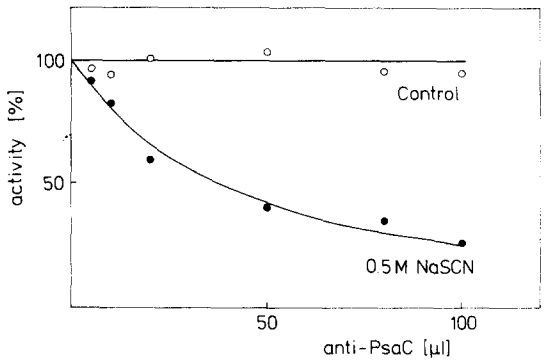

Fig. 7. Effect of anti-PsaC on methylviologen reduction of untreated and 0.5 M NaSCN-treated thylakoids. Incubation conditions were as in Fig. 6. The control activities were 476 (untreated) and 295 μmol O_2 /(mg Chl h) (pretreated). The protein concentration of the anti-PsaC containing IgG preparation was 10 mg/ml. The activities were related to the rates attained with control IgG preparation.

I polypeptide to be detached, addition of PsaE from *Synechococcus* (threefold excess over P700) fully restores methylviologen reduction. At higher NaSCN concentrations, the addition of PsaE leads to partial restoration of electron transport (Fig. 5). The PsaE protein from *Synechococcus* PCC 7002 was a gift of Prof. J.H. Golbeck. Specificity for PsaE is likely because no restoration of electron transport was observed by using the same concentrations of bovine serum albumin.

Effect of anti-PsaE and anti-PsaC on electron transport through PS I

A polyclonal antibody raised against PsaE (see above) was tested for its effect on electron transport of thylakoid membranes from an artificial electron donor to the artificial acceptor system methylviologen/O_2 or the natural acceptor $NADP^+$ catalyzed by externally added ferredoxin. The aforementioned cross-reactivity of our antiserum with the ribosomal protein L12 (see above) could be disregarded as we employed well-washed L12-free thylakoids. We found no effect of anti-PsaE on methylviologen reduction, but significant inhibition of $NADP^+$ reduction (Fig. 6). A similar result was recently reported by Andersen et al. [11].

Likewise, the influence of the antibody raised against PsaC on PS I electron transport was tested. Neither $NADP^+$ reduction (not shown) nor methylviologen reduction (Fig. 7) was affected in control thylakoids. However, when the thylakoids were pretreated with 0.5 M NaSCN, which removed 80% of PsaE (Fig. 2), the residual activity of methylviologen reduction (60% of control activity) was sensitive towards anti-PsaC (Fig. 7).

Discussion

Recently, significant progress in elucidation of the role of the subunit PsaE was made by analyzing dele-

tion mutants of cyanobacteria [13–16]. The observation that a psaE deletion mutant of *Synechococcus*-7002 cannot grow under photoheterotrophic conditions in the presence of glycerol with PS II being blocked by DCMU, suggested a defect in cyclic electron flow around PS I [15]. $P700^+$ re-reduction following a light flash was almost completely inhibited by DCMU in a mutant lacking PsaE when the electron pathway to $P700^+$ via NADH dehydrogenase was blocked. This block was achieved by introducing another mutation which led to deletion of the NADH dehydrogenase gene ndhF. $P700^+$ reduction could be restored by glycerol in the transformants lacking either psaE or ndhF, but not in the double deletion mutant [16]. These results are considered to establish the role of PsaE in cyclic PS I-mediated electron transport of cyanobacteria.

Irrespective of a possible role of PsaE in cyclic electron transport – whose occurrence in vivo is uncertain in chloroplasts – our biochemical and immunological studies suggest an important stabilizing role of this polypeptide on the acceptor side of spinach PS I, in particular on the functional interaction between the intrinsic PsaA/B and the extrinsic PsaC.

More or less this function was ascribed to subunit PsaD [31] which was shown to be an essential PS I protein in cyanobacteria [31]. The observation that a psaE deletion mutant produced a higher level of PsaD suggests that PsaD replaces PsaE in this transformant. A minimal PS I core can be reconstituted from the isolated heterodimer PsaA/B and isolated PsaC. For stable restoration of the F_A/F_B EPR signal and light-induced absorption changes, PsaD was also required [31]. The fact that the necessary amount of PsaD was twice as much as the amount of PsaC, suggests that two molecules of PsaD were incorporated in the complex. As the natural stoichiometry of PsaC:PsaD:PsaE is thought to be 1 [1,25], it should be interesting to know whether one PsaD can be replaced by PsaE in the reconstitution. Indeed, PsaE and PsaD exhibit similarities in their primary structures [26] which might explain partially similar function. The main function of subunit PsaD is considered to be the binding and correct orientation of ferredoxin to the PS I complex [8]. 1-Ethyl-3-(dimethylaminopropyl)carbodiimide (EDC) catalyzes crosslinking of ferredoxin with this PS I subunit [8,11] and apparently also with PsaE [11]. Since both PS I polypeptides have positive net charges and crosslinking is prevented in media of higher ionic strengths, binding of ferredoxin is thought to be caused by ionic interactions [8,11]. Our result that anti-PsaE inhibits $NADP^+$ reduction but not methylviologen reduction (Fig. 6), at the first sight also suggests participation of PsaE in ferredoxin binding. However, if PsaE were absolutely necessary for ferredoxin interaction, the activity of NADP reduction should vanish in parallel to the release of PsaE upon treatment with NaSCN. This, however, was not found (Fig. 4). The observed antibody effect therefore may be explained best by assuming sterical hindrance of either ferredoxin docking to PsaD or ferredoxin access to the F_A/F_B center due to the proximity of PsaE to both subunits. It is, however, also possible that the antibody, bound to PsaE, sterically inhibits the approach of reduced ferredoxin to FNR. Andersen et al. [12] recently reported isolation of a PS I complex which included LHCI and FNR (0.4 per P700). By crosslinking they detected a product consisting of FNR and PsaE and they concluded a role of PsaE in FNR binding to PS I. Our results demonstrating a much higher sensitivity of PsaE towards NaSCN washing than FNR (Fig. 2) exclude the possibility that PsaE is the main anchor for FNR in intact thylakoid membranes. Nevertheless, the results of Andersen et al. suggest proximity and surface interactions (which may be resolved by low NaSCN concentrations) between PsaE and FNR.

The NaSCN washing experiments show that PsaE is much less tightly bound to the PS I core than PsaD. Upon complete removal of PsaE about 50% of the electron transport activity to either methylviologen or NADP is still retained indicating that (i) resolution of PsaE is not the reason for inhibition of electron transfer per se, (ii) inhibition must be at a reaction step which is located before acceptance of electrons by methylviologen, i.e., before F_A/F_B. As we can exclude significant inhibition of primary charge separation (Fig. 3), it is likely that resolution of PsaE disturbs the correct orientation of the F_A/F_B iron-sulfur cluster with regard to the F_x iron-sulfur cluster so that electron transfer between these redox centers is impeded. This effect is reversed by reconstitution with *Synechococcus*-PsaE (Fig. 5). Although the cyanobacterial polypeptide differs from the chloroplast PsaE by the lack of 27 amino acids at the N-terminal end, restoration of electron transport by *Synechococcus*-PsaE is complete unless other subunits are also released.

An antibody against PsaC is effective only when PsaE is removed from PS I by NaSCN. Then the antibody is a strong inhibitor of methylviologen reduction (Fig. 7). This result suggests that PsaE shields PsaC or at least protects the antibody-reactive epitopes of PsaC. Obviously, binding of the antibody to PsaC, which is accessible and already dislocated to some extent in the PsaE-deficient PS I, effects either further displacement or a change of conformation of PsaC so that efficient electron transfer from F_x to F_A/F_B is prevented.

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