The Stable Assembly of Newly Synthesized PsaE into the Photosystem I Complex Occurring via the Exchange Mechanism Is Facilitated by Electrostatic Interactions†

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ABSTRACT: Photosystem I (PSI) is a photochemically active membrane protein complex that functions at the reducing site of the photosynthetic electron-transfer chain as plastocyanin−ferredoxin oxidoreductase. PsaE, a peripheral subunit of the PSI complex, plays an important role in the function of PSI. PsaE is involved in the docking of ferredoxin/flavodoxin to the PSI complex and also participates in the cyclic electron transfer around PSI. The molecular characterization of the assembly of newly synthesized PsaE in the thylakoid membranes or in isolated PSI complexes is the subject of the present study. For this purpose the *Mastigocladus laminosus psaE* gene was cloned and overexpressed in *Escherichia coli*, and the resulting PsaE protein was purified to homogeneity by affinity chromatography. The purified PsaE was then introduced into thylakoids isolated from *M. laminosus*, and the newly introduced PsaE subunit saturates the membrane. The solubilization and separation of the different thylakoid protein complexes indicated that PsaE accumulates specifically in its functional location, the PSI complex. A similar stable assembly was detected when PsaE was introduced into purified PSI complexes, i.e., in the absence of other thylakoid components. This strongly indicates that the information for the stable assembly of PsaE into PSI lies within the polypeptide itself and within other subunits of the PSI complex that interact with it. To determine the nature of these interactions, the assembly reaction was performed in conditions affecting the ionic/osmotic strength. We found that altering the ionic strength significantly affects the capability of PsaE to assemble into isolated thylakoids or PSI complexes, strongly supporting the fact that electrostatic interactions are formed between PsaE and other PSI subunits. Moreover, the data suggest that the formation of electrostatic interactions occurs concomitantly with an exchange step in which newly introduced PsaE replaces the subunit present in situ.

Photosystem I (PSI)[1] is a photochemically active membrane protein complex situated at the reducing site of the photosynthetic electron transfer chain. It functions as plastocyanin−ferredoxin oxidoreductase in the thylakoids of plants, algae, and cyanobacteria. In cyanobacteria, the PSI electron donor, plastocyanin, is occasionally replaced by a *c*-type cytochrome (cytochrome c_6), and during iron deficiency, the PSI electron acceptor, ferredoxin, is replaced by flavodoxin (*1*, *2*).

The PSI complex of cyanobacteria consists of 12 different subunits, namely, PsaA to PsaF, PsaI to PsaM, and PsaX, a transmembrane subunit that has recently been identified in the structure of the PSI complex (*3*). PsaA and PsaB, with 11 membrane-spanning helices each, form the hydrophobic core of the complex, to which all of the photosynthetic pigments and most of the electron carriers are bound (*4*, *5*). PsaI−PsaM and PsaX also contain hydrophobic region(s) that traverse(s) the thylakoid membrane. Apart from PsaL, all of these PSI subunits are of low molecular weight. PsaI has been shown to be involved in strengthening the binding of PsaL and PsaM to the PSI complex (*6*, *7*). PsaJ has been found to stabilize the binding of PsaE to the PSI complex (*8*). The *psaJ* gene is monocystronic with the *psaF* gene; hence deletion of the *psaJ* gene leads to 80% reduction in the PsaF content in PSI (*9*, *10*). PsaF, the only subunit located on the lumenal side of the cyanobacterial thylakoid membrane, has been shown to contain at least one transmembranal α helix (*11*, *12*). While in eukaryotes PsaF has been seen to be involved in the docking of plastocyanin to PSI (*13*, *14*), in cyanobacteria PsaF is not involved in the docking of cytochrome c_6 (*9*, *12*), and its specific function is still unknown. PsaL is the subunit responsible for PSI trimer formation in cyanobacteria (*6*, *15*) while the functions of PsaK and PsaM are not yet known.

Three stromal-facing subunits, PsaC, PsaD, and PsaE, comprise the reducing site of PSI. PsaC contains the two final electron carriers of PSI, the iron−sulfur (Fe-S) clusters, F_A and F_B, from where the electrons are transferred to ferredoxin (or to flavodoxin). PsaC is shielded by the two other peripheral subunits, PsaD and PsaE (*3*, *16*), both of which are involved in the docking of ferredoxin (*17*−*19*)

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[1] Abbreviations: PSI, photosystem I; Chl, chlorophyll; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DM, *n*-dodecyl β-D-maltoside.

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and flavodoxin (*20*) and in strengthening the binding of PsaC to PSI (*11*, *17*, *21*, *22*).

According to Barth et al. (*19*), PsaE is responsible for the dissociation of ferredoxin from the PSI complex. Another function attributed to PsaE is its involvement in the cyclic electron flow occurring around the PSI complex (*23*, *24*). This function is directly related to the process known in cyanobacteria as the CO_2-concentrating mechanism (CCM); thus the energy required for HCO_3^- uptake is supplied by the PsaE-mediated cyclic electron pathway (*25*).

The NMR-derived structure of PsaE (*26*, *27*), as well as the X-ray crystallographic structure of the reducing site of the PSI complex (*16*), found the protein to contain five antiparallel β-sheet strands connected by loops. From these structures, it is postulated that the loops are involved in the interactions of PsaE with other PSI subunits (mainly PsaA/B, PsaC, and PsaD), while the β strands βB and βC are involved in the association/dissociation of ferredoxin. The NMR-derived structure also revealed the similarity of PsaE to the SH3 domain known from proteins involved in signal transduction protein−protein interactions (β strands ending with a 3_{10} helix). This resemblance might suggest that PsaE plays a role in the assembly and stability of the entire PSI complex.

The peripheral subunits of PSI facing the cytosol/stroma (PsaD/E) have been shown to assemble into the thylakoids spontaneously without the need of any cytosolic factors or the presence of NTP's (*8*, *28*). Moreover, it has previously been shown that when PsaE is mutated on its N- or C-terminus, it loses its ability to assemble into thylakoids from the cyanobacterium *Synechocystis* sp. PCC 6803 (*18*), suggesting that both termini are involved in the assembly of PsaE into the PSI complex.

The present study aims to characterize the mechanism of the assembly of PsaE into thylakoids and/or isolated PSI complexes from the thermophilic cyanobacterium *Mastigocladus laminosus* at the molecular level. The results show that electrostatic interactions facilitate the stable assembly of PsaE within the PSI complex and that the newly introduced PsaE assembles into the PSI complex via an exchange mechanism.

MATERIALS AND METHODS

M. laminosus Growing Conditions. Cells were grown in medium D of Castenholz (*29*) at pH 8.2 with constant stirring at 55 °C. The culture was bubbled with water-saturated air supplemented with 5% CO_2. The cells were illuminated with white light at an intensity of 10^5 erg cm^{-2} s^{-1}.

Overexpression, Radiolabeling, and Purification of PsaE. The gene coding for PsaE was isolated and cloned in the commercial expression vector pET22b (Novagen). The vector bearing the *psaE* insert, containing a His tagging on its C-terminus, was transformed in HMS174(DE3) *Escherichia coli* cells. The plasmid DNA was sequenced in order to confirm the presence of *psaE*. The deduced amino acid sequence indicated a 71 amino acid polypeptide with an estimated p*I* value of ~9.5.

Bacteria were grown in A salts medium (*30*) containing 100 μg/mL ampicillin and 2.5 μg/mL thiamin at 37 °C until an OD value of ~0.6 was reached. IPTG (isopropyl β-D-thiogalactopyranoside, Sigma) was then added to a final concentration of 1 mM, and the culture was incubated for 3 h. [^{14}C]Valine was then added (0.5 μCi/mL), and the bacteria were incubated under the same conditions for an additional 45 min.

For PsaE purification, the cells were harvested by centrifugation at 6000 rpm for 5 min (SS-34 rotor). The pellet was suspended in a TN buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl; 3 mL of TN per pellet from a 50 mL culture) containing the following protease inhibitors: 1 mM PMSF, 0.1 mM leupeptin, and 1 μM pepstatin. The cells were then broken by sonication on ice (three cycles of 20 s each with 2 min intervals) and centrifuged at 13000 rpm for 20 min (SS-34 rotor). Following centrifugation the pellet was discarded, and the soluble fraction was filtered through a 0.2 μm filter. The filtrate was introduced to a Ni-NTA agarose column (QIAGEN; 1 mL of resin for 50 mL of supernatant) previously washed with 5 volumes of TN buffer containing 10 mM imidazole. The resin with the supernatant was shaken at 4 °C for 1 h, and the column was then built. The column was washed three times (5 column volumes per wash): once with TN buffer containing 10 mM imidazole and twice with the same buffer containing 25 mM imidazole. Elution was then performed with 3 column volumes of TN buffer containing 300 mM imidazole.

For assembly reactions, the purified PsaE was dialyzed against buffer containing 20 mM Tris-HCl, pH 7.9, and 50 mM NaCl to reduce the salt concentration and eliminate to the maximum the traces of imidazole. The pure protein was kept at −20 °C until used.

Isolation of Thylakoids from M. laminosus. M. laminosus cells (1−2 L) were harvested by centrifugation (6000 rpm for 5 min), and the pellet was washed with HMS buffer (30 mM Hepes−NaOH, pH 8.0, 5 mM $MgCl_2$, 0.4 M sucrose). Following the wash, the cells were suspended in HMS buffer containing PMSF, aminocaproic acid, and benzamidine (1 mM each) and broken at 4 °C for 20 min in a bead-beater chamber (Biospec Products) in the presence of prechilled 0.1 mm diameter glass beads. The breakage was carried out using four pulses of 20 s each with 5 min cooling intervals. The solution was then centrifuged (5 min at 3000 rpm) to remove unbroken cells. The thylakoids were pelleted from the supernatant by centrifugation (15000 rpm for 30 min using a SS-34 rotor) and washed twice more with HM buffer (30 mM Hepes−NaOH, pH 8.0, 1 mM $MgCl_2$).

The membranes were finally suspended in 1 mL of HM buffer, and the chlorophyll concentration was measured according to Arnon (*31*).

Purification of PSI Complex. The purification of *M. laminosus* PSI complexes was carried out as previously described (*32*). Obtaining homogeneous monomer PSI complexes from the highly purified trimer was performed as described before (*33*).

Insertion of PsaE into Isolated Thylakoid Membranes. A typical assembly reaction included isolated thylakoids (amount equivalent to 50 μg of Chl) and purified PsaE (200000 cpm). The reaction was carried out for 30 min at 4 °C, unless indicated otherwise. Following the assembly reaction, the thylakoids were washed once with HM buffer and then with high salt concentration; NaBr was added to a final concentration of 2 M, and incubation was carried out for 20 min at 4 °C. Following this treatment, the thylakoids were washed three times in HM buffer. The thylakoids were then extracted

with 1% DM (*n*-dodecyl β-D-maltoside) for 10 min at 25 °C. The extract was centrifuged (13000 rpm for 5 min), and the soluble supernatant was chromatographed either on SDS−PAGE or on a sucrose density gradient that was centrifuged for 4.5 h at 45000 rpm (SW50 rotor) or for 15 h at 33000 rpm (SW41 rotor). The different gradient fractions were collected, concentrated, and chromatographed on SDS−PAGE. All gels were exposed to a Fuji imaging plate for 12 h. The plates were then scanned using a FUJIX BAS 1000 Bio-imaging analyzer.

To determine the relative amounts of assembled PsaE, TINA version 2.10 g software was used. The net absorbance of each band was calculated by subtracting the background absorbance from it.

For the exchange mechanism experiments, isolated thylakoids (200 μg of Chl) were incubated with homogeneous labeled PsaE (300000 cpm; 10−20 μg of protein) for 30 min at 4 °C. The membranes were then washed three times with HM buffer, after which the sample was divided into two tubes. One sample was washed with NaBr and extracted with 1% DM, while the other was reincubated with nonlabeled PsaE (half the amount of the labeled subunit). Following incubation, this sample was washed with HM and treated with NaBr and DM as described above. The soluble fractions of each sample (labeled, following incubation with pure labeled PsaE; nonlabeled, following reincubation with homogeneous nonlabeled PsaE) were chromatographed on SDS−PAGE and exposed to a Fuji imaging plate.

For exchange in isolated monomeric PSI complexes, PSI containing 100 μg of Chl was incubated with pure labeled PsaE (100000 cpm) for 30 min at 4 °C. Following incubation, the sample was loaded on a 5%−25% sucrose density gradient. Following 4.5 h centrifugation at 45000 rpm (SW50 rotor), the green fraction containing monomeric PSI was collected, concentrated, and diluted with HM buffer containing 0.05% DM. The sample was divided into two tubes: nonlabeled PsaE was added to one, and reincubation was carried out as described above. The second tube was kept as the control. Following a period of 30 min incubation, the two samples were reloaded on another sucrose gradient identical to the first one. The green fractions were collected, concentrated, and denatured. Five micrograms of Chl of each sample (labeled and nonlabeled) was chromatographed on SDS−PAGE.

Miscellaneous Techniques. Chlorophyll concentration was determined following acetone extraction according to Arnon (*31*). Protein concentration was determined by measuring the absorbance of PsaE at 280 nm, taking the extinction coefficient of PsaE as 1.211 (from the Expasy site: www.expasy.ch/tools/protparam.html). SDS−PAGE was performed according to the method of Schagger and Von Jagow (*34*) with the addition of 6 M urea to all solutions. All gels contained 14% acrylamide and were 0.75 mm thick. Western blot analyses used polyclonal antibodies raised against PsaA/B of *Chlamydomonas reinhardtii* and PsaD of spinach (*35*).

RESULTS

The assembly of the peripheral, cytosol-facing subunits of PSI has been characterized mainly for PsaD from cyanobacteria and higher plants (*36*−*39*). For PsaE, previous studies followed the assembly of in vitro translated PsaE labeled with [^{35}S]Met (*8*, *28*). Those studies, in which minute amounts of PsaE were obtained and detected, did not permit detecting the assembly at a quantitative level. The present study aims at characterizing the assembly of PsaE in the thylakoid membranes quantitatively, i.e., with chemical amounts of labeled PsaE subunit (microgram quantities). The rationale behind this is that the introduction of relatively high amounts of the pure PsaE more closely resembles the in vivo situation. Moreover, the ability to follow the assembly on a quantitative level makes the characterization of the assembly mechanism possible.

The gene *psaE* from *M. laminosus* was subcloned into pET22b vector, and overexpression to high levels was achieved following induction with 1 mM IPTG for 3 h. The PsaE protein was then purified by Ni−agarose affinity chromatography. The concentration and radioactivity of the pure protein were measured; usually 3 μL of pure PsaE contained 10000 cpm in a concentration of 0.3−0.5 mg/mL of protein. The purified PsaE was used for assembly studies aimed at revealing its integration and its organization within the thylakoid membranes.

Integration of PsaE into Thylakoids Isolated from M. laminosus. Previous qualitative studies have indicated that the assembly of cyanobacterial PsaE into the thylakoids is spontaneous; i.e., there was no observed need for the presence of NTP's or for the assistance of cytosolic factors (*8*, *28*).

To quantitatively characterize the integration of PsaE into isolated thylakoids, increasing amounts of purified PsaE were introduced to isolated thylakoids, and the amounts incorporated within the membranes were determined.

Isolated thylakoids (equivalent to 50 μg of Chl) were incubated with purified labeled PsaE (10000−200000 cpm) at 4 °C for 30 min. Following incubation, the thylakoids were washed with HM buffer containing 2 M NaBr to remove PsaE associated with the thylakoids but not integrated within them. To further verify that the PsaE detected within the thylakoids was properly assembled, the NaBr-washed thylakoids were extracted with 1% *n*-dodecyl β-D-maltoside (DM), and only the soluble fraction was analyzed (Figure 1).

The results indicate that when low amounts of PsaE were introduced into the membranes, an increase in the amount of assembled PsaE could be detected (Figure 1). However, at higher amounts (70000−200000 cpm), the assembly reaction reached saturation, and no significant increase in the amount of integrated PsaE could be detected (Figure 1).

The finding that at lower concentrations the assembly of newly introduced PsaE increases linearly until saturation resembles the previously reported studies on another cyanobacterial peripheral subunit of PSI, PsaD (*39*).

Specificity of PsaE Assembly into the PSI Complex. To determine the final thylakoid location for the integration of PsaE, isolated thylakoids (200 μg of Chl) were incubated with 300000 cpm of PsaE for 30 min at 4 °C with constant stirring. Following the assembly reaction, the thylakoids were treated as described (Materials and Methods), and following centrifugation (5 min at 13000 rpm), the soluble fraction was loaded on a 5%−25% sucrose density gradient that was centrifuged for 15 h at 33000 rpm (SW41 rotor). The different gradient fractions were collected, concentrated, and

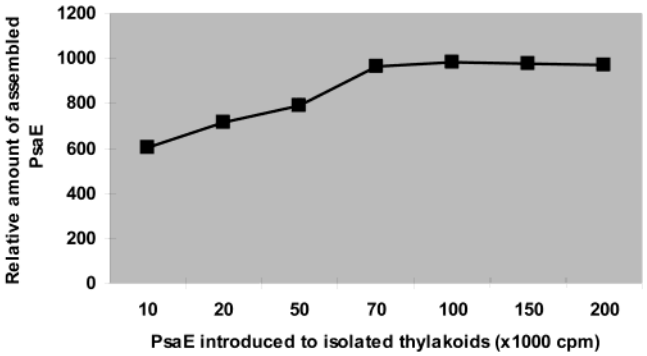

FIGURE 1: PsaE accumulates in the thylakoids. Isolated thylakoids (equivalent to 50 μg of Chl) were incubated for 30 min at 4 °C with increasing amounts of PsaE, as shown. Following the assembly reaction the samples were treated with 2 M NaBr and extracted with 1% DM. The soluble fractions that resulted from the DM extraction and microcentrifuge centrifugation (10 min at 13000 rpm) were chromatographed on SDS−PAGE (5 μg of Chl being loaded on each lane), and the gel was dried and autoradiographed by exposure to a Fuji imaging plate for 12 h. Following exposure, the gel was scanned using a FUJIX BAS 1000 Bio-imager analyzer. The amount of PsaE incorporated within the thylakoids was determined by calculating the relative amount of PsaE therein on the basis of the net absorbance of each band using the TINA version 2.10 g software.

analyzed on SDS−PAGE that was stained (Figure 2A) and autoradiographed (Figure 2B). The PsaE signal on the autoradiogram is the only signal shown since the recombinant PsaE is the only labeled component in this reaction. To confirm that PsaE was indeed assembled specifically into the PSI complex, we performed Western blot analyses with polyclonal antibodies raised against the PsaA/B and the PsaD subunits of PSI (Figure 2C).

The results indicate that the radioactive signal resulting from the PsaE assembly (Figure 2B) correlates perfectly with the signals from the two other PSI subunits (Figure 2C), thereby providing direct evidence that PsaE assembled into the proper thylakoid complex where it is functional—the PSI complex. This result corresponds to previous study on the assembly of PsaD (*40*).

The question that these findings raise is whether PsaE can assemble into isolated PSI complexes.

Assembly of PsaE into Purified PSI Complexes. To determine whether the specific assembly of PsaE into the PSI complex requires the presence of any thylakoid membrane components other than PSI, the assembly reaction was performed with purified PSI complexes. Purified monomeric PSI complexes (amount equivalent to 50 μg of Chl) were incubated with purified labeled PsaE (50000 cpm). The mixture was then loaded on a 5%−25% sucrose density gradient and centrifuged for 4.5 h at 45000 rpm (SW50 rotor).

To confirm that PsaE was properly assembled within the isolated PSI complexes, the PSI fraction obtained was washed and reloaded on a second sucrose gradient, identical to the first one. Following centrifugation, all fractions were concentrated and denatured. Samples were analyzed on SDS−PAGE, and the gel was stained (Figure 3A) and autoradiographed (Figure 3B). Since PsaE is the sole radioactive component in the experiment (see Figure 2B), only the portion where PsaE migrates is shown in the autoradiogram (Figure 3B).

The results show that PsaE is present only in the isolated PSI complexes, suggesting that the information for proper

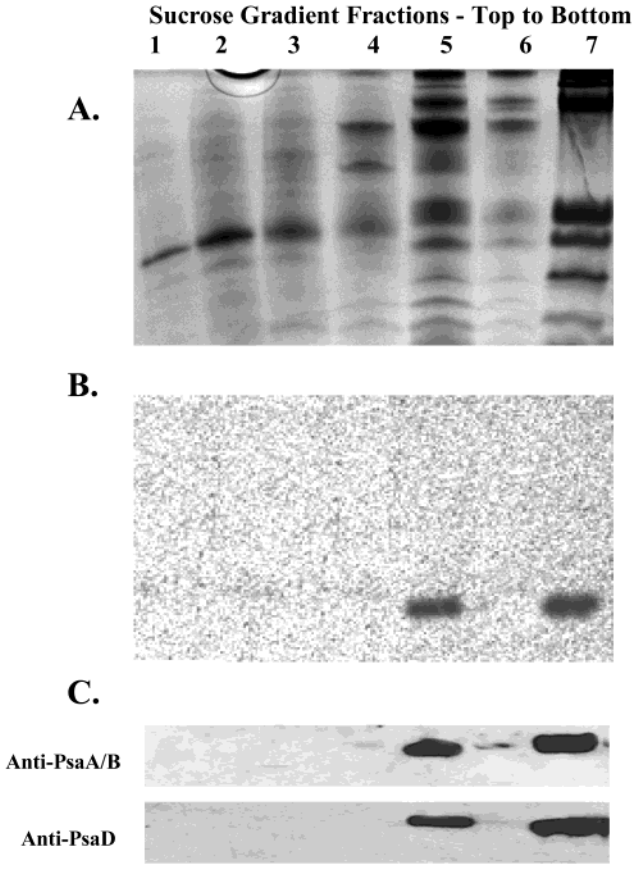

FIGURE 2: Specific assembly of PsaE into the PSI complex. The assembly reaction was performed in the presence of thylakoids (200 μg of Chl) and pure PsaE (300000 cpm) for 30 min at 4 °C. After the thylakoids were washed with 2 M NaBr and extracted with 1% DM, the soluble fraction was chromatographed on a 5%−25% sucrose density gradient (15 h, 33000 rpm using a SW41 rotor). The different gradient fractions were collected and concentrated before being analyzed on SDS−PAGE. The gel (5 μg of Chl of each fraction was loaded) was stained (A) and the dried gel was then exposed to a Fuji imaging plate for 12 h (B). Panel C presents the Western blot analyses performed for the different gradient fractions using antibodies raised against PsaA/B or PsaD.

assembly of PsaE lies within the PsaE polypeptide itself and/or within other PSI subunits with which it interacts. This finding strongly indicates that there is no requirement for any additional thylakoid membrane components for securing the stable and proper assembly of PsaE.

Stable Assembly of PsaE Is Dictated/Facilitated by Electrostatic Interactions. To understand the nature of the interactions formed between the newly assembled PsaE and other PSI components, the assembly reactions were performed in the presence of different salts or sorbitol at various concentrations. The interactions formed between different subunits of the complex may be hydrophobic or electrostatic in nature. PSI is embedded within a hydrophobic matrix, the thylakoid membrane. PsaE, on the other hand, is a basic peripheral membrane subunit (p*I* value ~9.5) that does not traverse the hydrophobic lipid phase, making it likely that it forms ionic/hydrophilic interactions with its surrounding subunits.

To examine this question, the assembly of PsaE into isolated thylakoids/PSI complexes was performed in the presence of different salts: monovalent or divalent.

Isolated thylakoids (50 μg of Chl) were incubated with 200000 cpm of PsaE in 30 mM Hepes−NaOH, pH 8.0, in

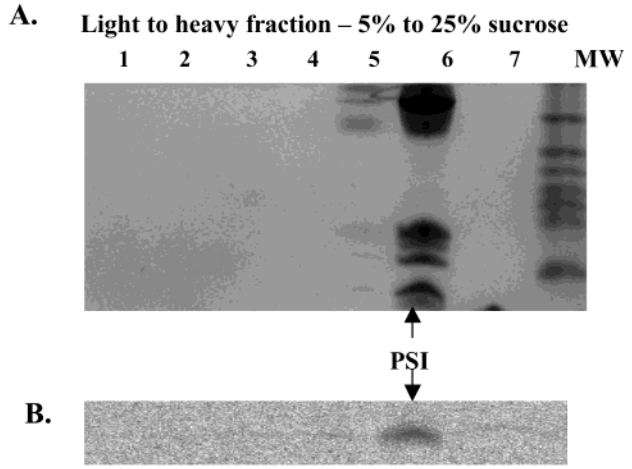

FIGURE 3: Stable assembly of PsaE requires only the PSI complex. Monomeric PSI (50 μg of Chl) was incubated with pure labeled PsaE (50000 cpm) for 30 min at 4 °C. Following incubation, the mixture was loaded on a 5%−25% sucrose density gradient that was centrifuged for 4.5 h at 45000 rpm (SW50 rotor). The PSI-containing fraction was washed with HM buffer and reloaded on a second gradient identical to the first to confirm that PsaE is stably assembled into the PSI complex. The different fractions from the second gradient were collected, concentrated, denatured, and analyzed on SDS−PAGE (20 μL of each fraction was loaded on the gel). The gel was stained (A) and autoradiographed as described above (B).

the presence of the different salts at various concentrations (specified in Figure 4). Figure 4A indicates that the presence of NaCl, KCl, or $CaCl_2$ strongly affected the proper assembly of PsaE, to a degree that only traces of the labeled subunit could be detected at high salt concentrations (500 mM for monovalent ions and 10 mM for divalent ions). Purified PsaE was found to have a tendency to aggregate after a few days when kept in low salt solution (lower than 50 mM NaCl), while at high salt concentration (50−500 mM NaCl), the pure PsaE is fully soluble (data not shown). This property of the pure PsaE protein eliminated the possibility of poor assembly of PsaE at high salt concentrations due to PsaE aggregation, thereby ensuring that the salts affect the assembly reaction itself.

The increased salt concentration may affect the membranes in two ways: it may increase the ionic strength and/or it may create an osmotic alteration in the membranes to a degree that causes osmotic shock. To define which of the two effects is responsible for the poor assembly of PsaE, we performed the assembly reaction with increasing concentrations of sorbitol. Unlike salts, sorbitol leads to osmotic changes but does not alter the ionic strength. The results clearly show that sorbitol had only a minor effect on the assembly of PsaE, and this could be detected only at very high concentrations of sorbitol (Figure 4A).

The results of PsaE assembly under different ionic/osmotic conditions strongly suggest that the interactions formed between PsaE and other PSI components are mainly of an ionic/electrostatic nature. These findings correlate previously reported studies showing that newly assembled PsaD forms electrostatic interactions with other PSI subunits (*38*).

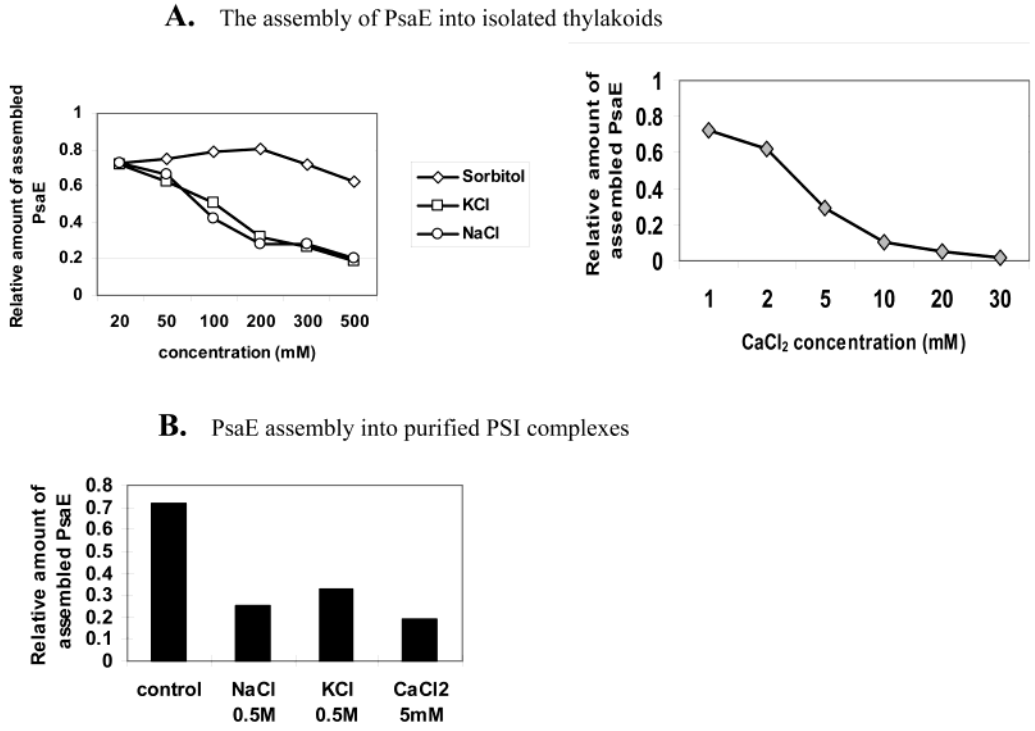

FIGURE 4: Stable assembly of PsaE is dictated/facilitated by the formation of electrostatic interactions. (A) The assembly of PsaE into isolated thylakoids was performed in the presence of different salts or sorbitol concentrations (0−500 mM KCl, NaCl, or sorbitol, 0−30 mM $CaCl_2$), as indicated. Following incubation, all of the thylakoid samples were washed with 2 M NaBr and extracted with 1% DM. The resulting soluble fractions were loaded on SDS−PAGE (5 μg of Chl per lane). (B) Isolated monomeric PSI complexes (50 μg of Chl) were incubated with purified PsaE (50000 cpm) for 30 min at 4 °C in the presence of 0.5 M NaCl, 0.5 M KCl, or 5 mM $CaCl_2$. Following incubation, the different reaction mixtures were loaded on sucrose density gradients that were then centrifuged for 4.5 h at 45000 rpm (SW50 rotor). The green fractions containing PSI were recentrifuged on an identical gradient. The fractions containing PSI were collected, concentrated, and denatured, after which the samples (5 μg of Chl each) were analyzed on SDS−PAGE. The gels were exposed to a Fuji imaging plate, and the amount of incorporated PsaE was calculated as described in Figure 1 (see also Materials and Methods).

To further confirm that electrostatic interactions formed between PsaE and other PSI components dictate the stable assembly of PsaE, an assembly reaction of PsaE into isolated PSI complexes was performed. Purified PSI complexes were incubated with recombinant PsaE in the presence of NaCl (0.5 M), KCl (0.5 M), or $CaCl_2$ (5 mM). The mixtures were then loaded on sucrose density gradients, and the green fractions were collected, concentrated, and loaded on SDS−PAGE. The results obtained are identical to those observed for PsaE assembly into thylakoids (Figure 4B): At high salt concentration the formation of electrostatic interactions between PsaE and other PSI subunits could not occur.

Mechanism for the Assembly of PsaE into PSI. The fact that a stable in vitro assembly of PsaE can be detected raises the following questions regarding the mechanism of this assembly: Does the newly introduced PsaE that assembles into a fully assembled PSI complex replace the subunit present in situ or does it accumulate in PSI in addition to the subunit present in situ? To answer these questions, the following experiment was performed: isolated thylakoids from *M. laminosus* (200 μg of Chl) were incubated with homogeneously labeled PsaE (300000 cpm equal to 10−20 μg) as described (Materials and Methods). Following incubation, the thylakoids were washed three times with HM buffer, and the sample was divided into two tubes. One tube was kept as a control while the other was reincubated with homogeneously nonlabeled ("cold") PsaE (5−10 μg, half the amount of the labeled PsaE). Both samples were treated as described above (see also Materials and Methods). The results in Figure 5A show that the radioactive PsaE signal was significantly reduced following reincubation with nonlabeled PsaE.

When the same experiment was performed with isolated monomeric PSI, identical results were obtained (Figure 5B). This reduction in the amount of labeled PsaE following incubation with nonlabeled protein is strongly indicative of the existence of an exchange mechanism. A newly introduced subunit replaces the native PsaE present in situ. Similar to the exchange observed for PsaD (*40*), the approximate molar ratio for recombinant PsaE:native PsaE was 1:1.8, and the replacement was calculated to be close to 1:1; i.e., for each molecule of recombinant PsaE that assembled in the PSI complex, one molecule of native PsaE left the complex. This exchange occurs without disrupting the entire PSI complex and with no need for the replacement of any additional PSI subunits. This exchange mechanism is similar to that recently described for PsaD (*40*).

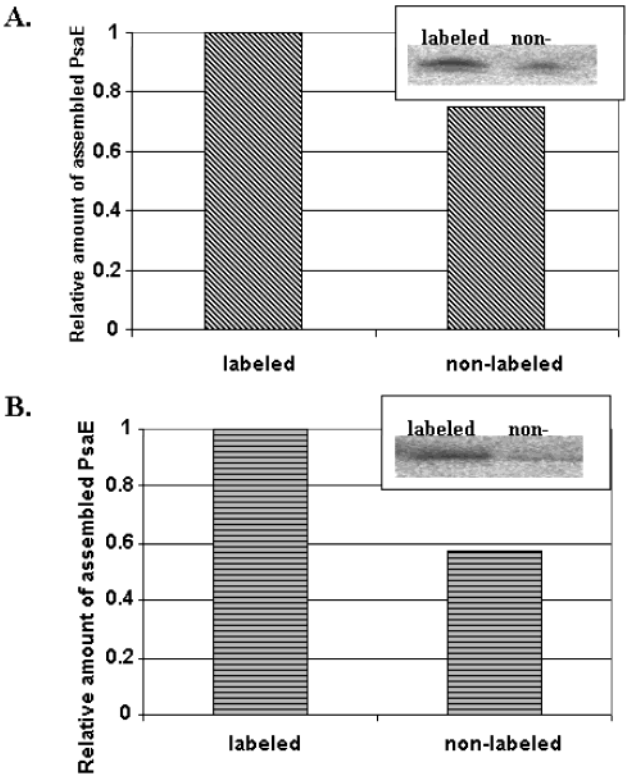

FIGURE 5: Stable assembly of newly introduced PsaE occurring via the replacement of the subunit present in situ. Assembly reactions were performed with isolated thylakoids (A) or with purified monomeric PSI complexes (B). (A) Isolated thylakoids were incubated with homogeneous labeled PsaE. Following assembly, the washed membranes were divided into two tubes. Reincubation with nonlabeled PsaE (half the amount of the labeled subunit) was carried out for one sample, while the other was kept as a control. The two samples were then treated as described (Materials and Methods). The soluble fractions of each sample (labeled, following incubation with pure labeled PsaE; nonlabeled, following reincubation with homogeneous nonlabeled PsaE) were chromatographed on SDS−PAGE (5 μg of Chl per lane). (B) Isolated monomeric PSI complexes purified from *M. laminosus* were incubated with pure labeled PsaE. Following incubation, the sample was loaded on a 5%−25% sucrose density gradient (see Figure 3). The green fraction containing monomeric PSI was collected, and nonlabeled PsaE was added to half of the sample. Reincubation was carried out as described. The two samples were reloaded on a second sucrose gradient. Following chromatography, the green fractions were collected, concentrated, and denatured. 5 μg of Chl of each sample was chromatographed on SDS−PAGE. In both experiments, the gels were treated as described in Figure 1. The graphs shown represent the quantitative measurements; the inserts show the autoradiogram of each experiment.

DISCUSSION

The PSI complex, a multisubunit membranal complex embedded within the thylakoids, served as our model system for studying the organization and the assembly of multisubunit membranal complexes. The assembly of its peripheral subunit, PsaE, was studied by introducing chemical amounts of homogeneous recombinant protein into the thylakoid membranes, as well as into isolated PSI complexes. This method, in which microgram amounts of protein are used, is more reflective of the in vivo situation than the introduction of picogram amounts of the in vitro translated product, which was common in previous studies (*28*, *36*−*39*).

The results obtained indicated that recombinant PsaE is capable of spontaneous assembly into the thylakoid membranes. The newly introduced subunit accumulated in the membranes until saturation (Figure 1). The recombinant PsaE was found to integrate specifically into the PSI complex (Figure 2). When introduced into isolated PSI complexes, PsaE was capable of stable assembly into a fully assembled isolated PSI complex (Figure 3). Taken together, these data indicate that the information required for the stable assembly of PsaE lies within the polypeptide itself and/or within other PSI subunits. The presence of other thylakoid components such as lipids, other multisubunit complexes (PSII, cytochrome b_6f, or the ATP synthase complex), or free pigments is not required for the assembly of the PsaE subunit; the pure protein integrates into the fully assembled isolated PSI

complex. Thus the assembly of recombinant, microgram amounts of PsaE is completely spontaneous and is independent of any of the thylakoid factors other than the PSI complex.

To further characterize the components facilitating this stable assembly, the interactions formed between PsaE and other PSI components were studied. The efficiency of the assembly of PsaE was detected under conditions in which the ionic and/or osmotic strength was altered. These changes provided an indication of the nature of the interactions formed between PsaE and other PSI components. Electrostatic interactions are usually disrupted by increasing ionic strength, while hydrophobic interactions would become stronger under the same conditions. The nature of the PSI complex (membranal complex) and the nature of its PsaE subunit (highly basic protein) allow both electrostatic and hydrophobic interactions to be formed. Therefore, the assembly reaction was performed in the presence of increasing concentrations of different salts as well as sorbitol. The results show that changing salt concentrations had a significant effect on the assembly of PsaE into the isolated thylakoids (Figure 4A). The same results were obtained when the assembly reaction was performed with isolated PSI complexes (Figure 4B). Increasing the salt concentration led to a decreased amount of assembled PsaE in the thylakoid membranes. The increased salt concentration may have a double impact on the membranal system: it leads to increased ionic strength but may also cause osmotic shock in the membrane. To confirm that the change of ionic strength is responsible for the decrease in the assembly of PsaE, we performed the assembly reaction in the presence of increasing concentrations of sorbitol. Unlike salts, sorbitol brings about osmotic shock, but it does not alter the ionic strength; therefore, sorbitol should have no effect on the formation of electrostatic interactions. The results clearly indicated that the osmotic effect of sorbitol did not interfere with the proper assembly of PsaE (Figure 4A). Hence, we conclude that the interactions formed by PsaE to allow its stable assembly are mainly electrostatic in nature.

The observations that recombinant PsaE assembles spontaneously into the fully assembled PSI complex and that the interactions stabilizing the subunit assembly within the complex are mainly ionic did not define the mechanism by which the assembly of PsaE occurs. For this, the thylakoids or the isolated PSI complexes that incorporated radiolabeled PsaE were reincubated with pure nonlabeled PsaE. The results (Figure 5) indicated that an exchange mechanism exists where the labeled PsaE replaced the native PsaE subunit, resulting in PsaE becoming radiolabeled. Furthermore, the nonlabeled protein added during reincubation replaced the labeled PsaE. This exchange mechanism of PsaE resembles the observed data for PsaD from cyanobacteria as well as from higher plants (*40*). The introduction of newly synthesized subunit induces the replacement of the subunit present in situ without causing any defect in the assembled PSI complex and with no requirement for the synthesis of other PSI subunits. Based on the obtained results, it may very well be that in vivo a damaged PsaE subunit in the PSI complex is replaced individually without the requirement of disassembling the entire PSI complex.

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