Accelerated Publications

The Assembly of the PsaD Subunit into the Membranal Photosystem I Complex Occurs via an Exchange Mechanism†

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ABSTRACT: PsaD is a peripheral stromal-facing subunit of photosystem I (PSI), a multisubunit complex of the thylakoid membranes. PsaD plays a major role in both the function and assembly of PSI. Past studies with radiolabeled PsaD indicated that PsaD is able to assemble in vitro specifically into the PSI complex. To unravel the mechanism by which this assembly takes place, the following steps were taken. (i) Mature PsaD of spinach and PsaD of the prokaryotic caynobacterium *Mastigocladus laminosus*, both bearing a six-histidine tag at their C-termini, were overexpressed in *Escherichia coli* and purified to homogeneity. (ii) The purified recombinant protein was introduced into the isolated PSI complex. (iii) Following incubation, the PsaD that assembled into PSI was separated from the nonassembled PsaD by a sucrose gradient. Differential Western blot analysis was used to determine whether the native and the recombinant PsaD were present as free or assembled proteins of the PSI complex. Antibodies that can recognize only the recombinant PsaD (anti-his) or both the native and recombinant PsaD (anti-PsaD) were used. The findings indicated that an exchange mechanism enables the assembly of a newly introduced PsaD into PSI. The latter replaces the PsaD subunit that is present in situ within the complex. In vivo studies that followed the assembly of PsaD in *Chlamydomonas reinhardtii* cells supported this in vitro-characterized exchange mechanism. In *C. reinhardtii*, in the absence of synthesis and assembly of new PSI complexes, newly synthesized PsaD assembled into pre-existing PSI complexes.

Photosystem I (PSI)[1] is one of two photochemically active complexes that take part in the photosynthetic electron transfer across the thylakoid membranes of oxygen-evolving organisms. During the course of this process, light energy is converted into chemical energy stored in the form of ATP and NADPH.

In higher plants and green algae, PSI, which functions as a plastocyanin-ferredoxin (Fd) oxidoreductase, comprises 13 subunits, of which eight are nuclear-encoded subunits (for a review, see ref *1*). One of these nuclear-encoded subunits is PsaD, a peripheral stromal-facing subunit of PSI. PsaD is synthesized in the cytoplasm as a precursor with a leader peptide at its N-terminus, which targets it to the chloroplast. There, the leader peptide is cleaved by a stromal processing peptidase (*2*), thus allowing PsaD to assemble properly into the PSI complex within the thylakoid membranes (*3*).

PsaD is a key subunit in the assembly, stability, and functionality of PSI. It is the first nuclear-encoded subunit to accumulate in the thylakoid membranes during the greening of etiolated seedlings (*4*−*6*). PsaD has been found to be essential for the stable assembly of PsaC and PsaE into the PSI complex (*7*−*9*). In the cyanobacteria *Synechocystis* sp. PCC 6803, the photoautotrophic growth of a mutant strain lacking PsaD was found to be much slower than that of wild-type cells (*10*). Electron microscopy (*11*, *12*), cross-linking experiments (*13*, *14*), and X-ray crystallography (*15*−*18*) have indicated that PsaD is a close neighbor of PsaC and PsaE. One of the loops connecting the four-stranded β-sheets of PsaD is attached to the stromally exposed sides of PsaC and PsaE (*18*). Together, these subunits form a wide ridge and are involved in the docking and reduction of the soluble electron acceptor of PSI, namely, Fd. These data confirm findings obtained by chemical cross-linking that have revealed a direct interaction between PsaD and Fd (*19*−*21*). PsaD has also been found to be in close contact with PsaL and PsaA/B (*13*, *18*).

The pathway of assembly of PsaD into the thylakoid membranes requires neither ATP nor the presence of a stromal fraction (*22*, *23*); i.e., PsaD assembles into the thylakoids spontaneously. Using a radiolabeled precursor and mature PsaD, it was shown that both forms of plant PsaD (precursor and mature) are able to assemble into isolated thylakoids, specifically into the PSI complex (*22*). These

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

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findings lead to the question of whether the native protein stays in the complex when the new subunit joins it, or whether it is replaced by the newly assembled protein; hence, the mole ratio of PsaD within PSI is constant.

This question could be addressed only when tools designed to follow PsaD quantitatively have been made available. For achieving that, the mature PsaD of spinach and PsaD of *Mastigocladus laminosus* have been overexpressed in *Escherichia coli*, purified to homogeneity, and introduced into the isolated PSI complex. Using different antibodies, native and recombinant PsaD proteins were detected differentially by Western blot analysis, enabling the determination of the precise amount of each PsaD protein present either in the PSI complex or as a free PsaD.

This study provides direct evidence that a new PsaD subunit replaces the PsaD present in situ within PSI by an exchange mechanism.

MATERIALS AND METHODS

Overexpression, Purification, and Radiolabeling of PsaD. The mature PsaD gene (*psaD*) of spinach was cloned into the pET20b vector at restriction sites *Nde*I and *Not*I. PsaD gene of *M. laminosus* was cloned as described in ref *24*.

Overexpression and purification of PsaD were performed as described in ref *25*, using two sequential columns: an anion exchange DEAE cellulose column followed by an Ni-affinity column (Ni−NTA agarose, Qiagen). Radiolabeling of the recombinant PsaD was carried out as described in ref *25*.

Photosynthetic Material. Pea seedlings (*Pisum sativum* L. cv. Alaska) and *M. laminosus* cells were grown as described in refs *22* and *25*. *Chlamydomonas reinhardtii* cells were grown on a Tris-acetate-phosphate (TAP) medium (pH 7.2) at 25 °C under dim light (40 μE m^{-2} s^{-1}).

In Vivo Labeling of C. reinhardtii Cells. Wild-type (WT) cells were grown on TAP medium until a cell density of 2×10^6 cells/mL was reached, and then washed with a minimal medium depleted of acetate. Growth was continued for 1.5 h. Chloramphenicol was added to a final concentration of 100 μg/mL for 15 min, and then [^{14}C]acetate was added to a final concentration of 13×10^{-6} M (7.5 μCi/mL), and growth was continued for 15 min. The pulse-labeling reaction was stopped by the addition of 1 volume of ice-cold TAP containing nonlabeled acetate at a concentration that is 3-fold higher than that in TAP.

In Vivo Labeling of M. laminosus Cells. Cells were grown until reaching an optical density of 0.75−0.85 at 730 nm. Sodium [^{14}C]carbonate ($Na_2{}^{14}CO_3$) was then added to a final concentration of 2 μCi/mL (53 mCi/mmol), and growth proceeded for an additional 18 h.

Thylakoid Isolation and PSI Purification. Pea thylakoids were isolated as described in ref *22*. *M. laminosus* thylakoids were isolated as described in ref *25*. *C. reinhardtii* thylakoids were isolated as described in ref *26*.

Pea and *C. reinhardtii* PSI complexes were extracted from isolated thylakoids as described in ref *2* and purified on an anion exchange DEAE cellulose column. *M. laminosus* PSI was isolated as described in ref *25*.

Assembly Reactions. Isolated PSI complexes containing 40 μg of Chl were incubated with 2−10 μg of homogeneous recombinant PsaD protein, for 10−60 min at 4 °C with constant swirling. Following incubation, the reaction mixture was loaded on a 5 to 30% sucrose gradient to separate free, nonassembled PsaD from PsaD that assembled into the PSI complex. The different fractions of the gradient were collected and analyzed by SDS−PAGE, which was either subjected to Western blot analysis or autoradiographed.

Quantification of the Amount of PsaD Protein. The amount of PsaD that was analyzed by Western blot was quantified as follows. (i) The blot was documented by the BIS 202-D camera of Fuji Film. (ii) The density of the bands (pixels count) was analyzed by the PCBAS software application (Fuji Film). Known amounts of recombinant PsaD, as determined by spectrophotometry, were analyzed alongside the different fractions of the sucrose gradient following assembly reactions. These were used by the software as standards by which pixels of the bands were converted to the actual amount (micrograms) of protein.

Determination of the Activity of PSI. Purified PSI complexes containing 0.1 mg/mL Chl were mixed with 2 mM ascorbate and increasing concentrations (0−0.1 mg/mL) of spinach Fd (Sigma). The photo-oxidation of PSI by Fd, following exposure to light (20 s, 4500 μE at 826 nm), was detected by the chlorophyll fluorescence measuring system (PAM-101).

Miscellaneous Techniques. Chlorophyll (Chl) concentrations were determined according to the method of Arnon (*27*). Protein concentrations were calculated according to the method of Coligan et al. (*28*) by measuring its absorption at 280 nm. SDS−PAGE was performed according to the procedure of Laemmli (*29*). Western blot analyses used polyclonal antibodies raised against PsaA−E of *C. reinhardtii* and PsaD of spinach (*30*) or monoclonal antibodies raised against a histidine tag (Babco).

RESULTS

It has previously been shown that radiolabeled PsaD is able to assemble into an isolated PSI complex (*2*). To determine whether chemical amounts (microgram quantities) of recombinant PsaD of spinach and *M. laminosus* are able to assemble into isolated PSI as well, the following steps were carried out. The PSI complexes were extracted from pea/*M. laminosus* thylakoid membranes and purified to homogeneity, and their ability to reduce Fd was determined using the PAM-101 system. The purified recombinant spinach PsaD (2 μg of protein) was incubated with the pea PSI complex (40 μg of Chl), at a recombinant PsaD:native PsaD mole ratio of ∼1:3.6. The reaction mixture was then separated on a sucrose gradient to discriminate between the PSI-assembled PsaD and free PsaD that did not integrate into the complex. In accordance with their mass, free PsaD floats in the upper fractions of the gradient, i.e., in the fractions containing 5−10% sucrose, whereas the PSI-assembled PsaD is found in the fractions containing PSI (segregated at ∼15% sucrose). Figure 1A clearly indicates that the recombinant PsaD, as detected by antibodies raised against the histidine tag, comigrated with the fractions containing PSI. In a similar experimental system, purified radiolabeled recombinant PsaD of *M. laminosus* was incubated with isolated PSI of *M. laminosus* in the amount of 100 μg of Chl. Following separation of the reaction mixture on a sucrose gradient, the PsaD protein was found in the fraction containing PSI (Figure 1B).

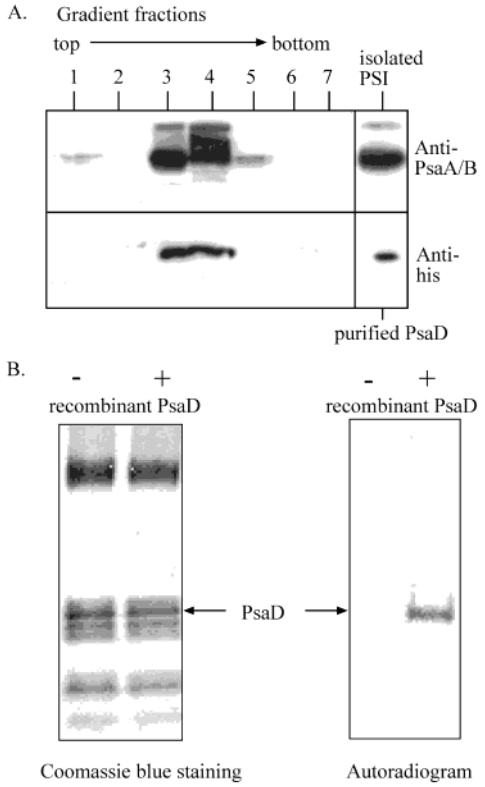

FIGURE 1: Recombinant PsaD assembles into isolated PSI complexes. (A) Pea PSI (40 μg of Chl) was incubated with the purified recombinant mature PsaD of spinach (2 μg) for 1 h at 4 °C. The assembly reaction mixture was then separated on a 5 to 30% sucrose gradient, which was centrifuged in a SW50 rotor for 4.5 h at 45 000 rpm. The different gradient fractions were collected and analyzed by Western blot analysis with antibodies raised against PsaA/B or a histidine tag. (B) *M. laminosus* PSI (100 μg of Chl) was (+) or was not (−) incubated with purified radiolabeled recombinant PsaD of *M. laminosus* (200 000 cpm) for 2 h at 4 °C. The assembly reaction mixture was separated on a sucrose gradient as described above, and the fractions containing PSI were collected. PSI equivalent to 5 μg of Chl was analyzed via SDS−PAGE, which was either stained with Coomassie blue (left) or autoradiographed (right).

A fundamental question regarding the mechanism by which the assembly of recombinant PsaD occurred needed to be answered. Did the recombinant PsaD compete with the native in situ PsaD subunit for the binding to PSI? To answer that question, 5 and 10 μg of recombinant spinach PsaD were incubated with isolated pea PSI (40 μg of Chl). This gave approximate mole ratios of 1.5:1 and 1:1.4 for the native and recombinant PsaD, respectively. The reaction mixture was then separated on a sucrose gradient whose different fractions were analyzed using Western blot analysis. The different bands on the blot were quantified using known amounts of recombinant PsaD as references.

Following this assembly reaction of recombinant PsaD into isolated PSI, and sucrose gradient chromatography, native PsaD, otherwise present only in the PSI fraction (Figure 2A), was detected as a free protein in the upper fractions of the sucrose gradient (Figure 2B). The amount of free native PsaD was calculated by subtracting the amount of free protein that reacted with the anti-his antibodies (recombinant PsaD) from the amount of free protein that reacted with the anti-PsaD antibodies (both native and recombinant PsaD). This observation indicated that only in the presence of recombinant

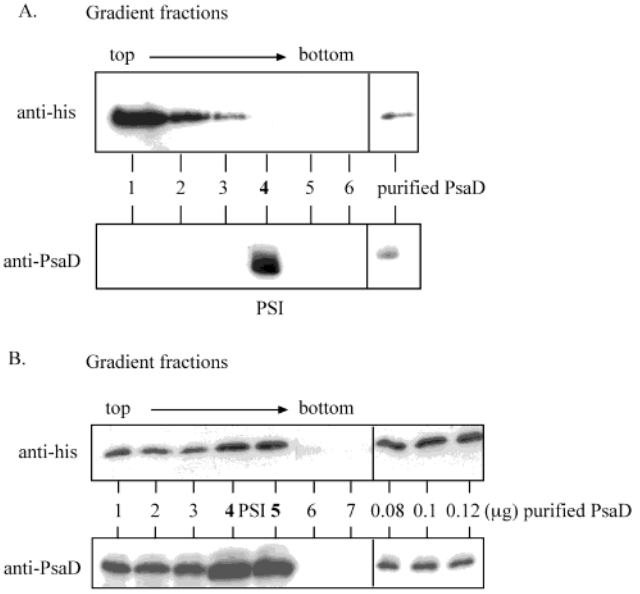

FIGURE 2: Native PsaD present in situ in PSI is replaced by newly introduced recombinant PsaD. (A) The purified recombinant PsaD (top panel) or the isolated PSI complex (bottom panel) was loaded on a sucrose gradient (see Figure 1). Following centrifugation, the different fractions were collected and analyzed using Western blot analysis with anti-his tag (top panel) or anti-PsaD (bottom panel) antibodies. (B) Purified recombinant PsaD (5 μg) was incubated with isolated PSI (40 μg of Chl) for 1 h at 4 °C. The reaction mixture was then separated on a sucrose gradient, and the different gradient fractions were collected and divided into two portions. One half was analyzed by Western blot analysis with anti-his-tag antibodies (top panel). The other half was analyzed by Western blot analysis with anti-PsaD antibodies (bottom panel). The gradient fractions containing PSI are highlighted in bold. In panel B, the known amounts of PsaD that were used as a reference for quantification of PsaD are indicated (right).

PsaD did the native PsaD leave the PSI complex and was the native PsaD replaced by recombinant PsaD (Figure 2B). Quantification of this replacement indicated that the average ratio between native PasD that left the PSI complex and recombinant PsaD that assembled into it was 1:1 (as calculated for 17 independent assembly reactions). These results were confirmed statistically by a two-tailed paired *t* test. On average, ∼50% of the native PsaD population was replaced by recombinant PsaD. Moreover, the ratio of recombinant PsaD in the general PsaD population was found to equal its ratio in the PSI-assembled PsaD population; this indicates that the native and recombinant PsaD had the same affinity for the PSI complex.

To ensure that the histidine tag itself does not force the native PsaD to leave PSI, two other proteins having a histidine tag at their C-termini were tested for their ability to replace PsaD. The PsaE subunit of PSI and the oncoprotein p38 were incubated with the isolated PSI complex in an assembly reaction mixture similar to that of the recombinant PsaD. Following incubation, no effect on the presence of native PsaD in the PSI complex was observed (data not shown).

To verify the generality of the exchange mechanism, the assembly of the cyanobacterial PsaD was followed. *M. laminosus* cells were grown in the presence of $Na_2{}^{14}CO_2$, and their proteins were radiolabeled. Thylakoids were isolated from these cells and incubated with non-radiolabeled purified recombinant PsaD. Following incubation, the PSI

Table 1: Recombinant PsaD of *M. laminosus* Replaced the Native PsaD Present in Situ within PSI[a]

PSI subunit	control PSI (cpm)	PSI isolated from radiolabeled thylakoids that were incubated with non-radiolabeled recombinant PsaD (cpm)
PsaA/B	62 401	62 166
PsaD	**15 589**	**10 990**
PsaF/L	23 313	24 127
PsaC/E	12 374	12 475

[a] Radiolabeled purified thylakoids of *M. laminosus* equivalent to 200 μg of Chl were incubated with 0.5 mg of recombinant PsaD at 25 °C for 30 min. As a control, no such incubation was performed. The thylakoids were then treated with 2 M NaBr for 20 min on ice and washed with 100 mM Hepes (pH 8.0). Their photosynthetic complexes were then extracted with 1% *n*-dodecyl β-D-maltoside and separated on a 5 to 25% sucrose gradient that was centrifuged in a SW50 rotor, for 4.5 h, at 45 000 rpm. The fractions containing PSI were collected, and equal amounts (5 μg of Chl) were analyzed on SDS−PAGE that was stained with Coomassie blue. The bands corresponding to the different subunits of PSI were excised from the gel and solubilized in 18% hydrogen peroxide and 4% SDS. Counts per minute were determined following the addition of 4 mL of a scintillation solution (Zinsser Analytic) using a scintillation counter (Beckman LS2800).

complex was isolated on a sucrose gradient and was analyzed by SDS−PAGE, which was stained with Coomassie blue. The profile of PSI before the addition of recombinant PsaD (control) and that following its addition were identical. The different subunits of PSI were extracted from the gel, and their radioactive intensity was determined. Table 1 shows that while the radioactive intensity of PsaA/B, PsaF/L, and PsaE/C remained relatively constant following the assembly reaction with recombinant PsaD, the native PsaD subunit showed a marked reduction in its level of radioactivity. These results strongly indicate that in PSI of *M. laminosus* the non-radiolabeled recombinant PsaD replaced the labeled native subunit; i.e., an exchange mechanism exists in cyanobacteria as well.

To confirm that the replacement of native PsaD is not a one-time event, i.e., a recombinant PsaD that assembled into PSI can be replaced further by introducing a newly synthesized recombinant PsaD into this PSI complex, the following steps were taken. Radiolabeled recombinant PsaD of *M. laminosus* (10 μg of protein) was incubated with purified trimeric PSI (200 μg of Chl), thus yielding a recombinant PsaD:native PsaD mole ratio of 1:3.6. The reaction mixture was then separated on a sucrose gradient, and the fraction containing PSI was collected. Excess sucrose was washed off the complex, and the washed fraction was divided into five portions. One-fifth was not treated further (control). The rest of the reaction mixture was incubated with increasing amounts of non-radiolabeled recombinant PsaD of *M. laminosus* or spinach (2−20 μg) to yield radiolabeled PsaD: non-radiolabeled PsaD mole ratios of 1:1, 1:2.5, 1:5, and 1:10. Each reaction mixture was then separated once more on a sucrose gradient, and the fraction containing PSI was collected and analyzed by SDS−PAGE and subjected to an autoradiogram. To gain further support for the finding that the exchange mechanism is a general phenomenon occurring in different phyla, we took advantage of the recent characterization of the assembly of PsaD into PSI of different organisms (*31*), showing that spinach PsaD is able to assemble into PSI isolated from *M. laminosus*. Figure 3 shows that in the presence of increasing amounts of non-

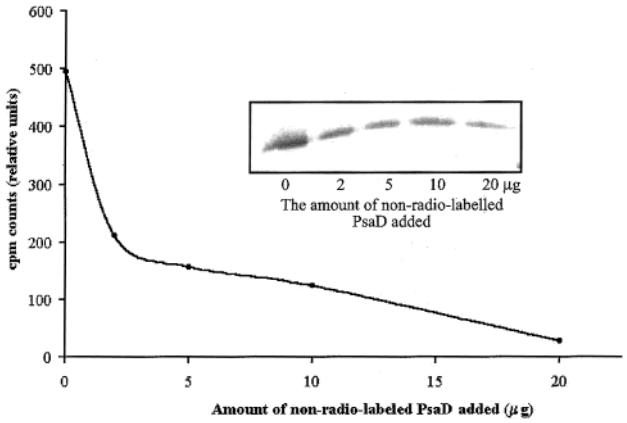

FIGURE 3: Recombinant radiolabeled PsaD, which is assembled into PSI, can be replaced further. Recombinant radiolabeled PsaD of *M. laminosus* (10 μg) was incubated with purified trimeric *M. laminosus* PSI (200 μg of Chl) for 10 min at 4 °C. Following assembly, the reaction mixture was loaded on a sucrose gradient (see Figure 1). The fraction containing trimeric PSI was collected and washed off the sucrose. It was then divided into five portions. One-fifth was kept as a control (0). The rest was incubated with increasing amounts of recombinant non-radiolabeled spinach PsaD for 10 min at 4 °C. The reaction mixtures were then separated again on a sucrose gradient, and the fractions containing trimeric PSI were collected and analyzed by SDS−PAGE that was autoradiographed. The different bands on the gel were quantified using the MacBAS software, and the graph was plotted using Microsoft Excel.

radiolabeled recombinant spinach PsaD, the observed amount of radioactivity of the radiolabeled recombinant *M. laminoisus* PsaD gradually decreases, suggesting that it leaves the PSI complex. The same results were obtained with non-radiolabeled *M. laminosus* PsaD (data not shown).

The above findings have brought up the question of the effect of this exchange on the activity of PSI. As mentioned above, PsaD has an important role in docking Fd to the PSI complex. To test whether the PSI complexes retained their functionality when approximately 50% of their PsaD subunits were replaced by recombinant proteins, both the native complexes and the complexes containing recombinant PsaD were tested for their ability to reduce Fd. The chlorophyll fluorescence measuring system (PAM-101) was used to follow the photo-oxidation of PSI. As can be seen in Figure 4A, both the native PSI and the complex containing recombinant PsaD were oxidized to the same extent by Fd. Figure 4B shows that the activity of both samples was at its height at the same concentration of Fd. These results indicate that the replacement of the native PsaD by the recombinant subunit did not alter the affinity of Fd for the complex. Thus, the PSI complex containing the recombinant PsaD subunit is fully active.

To relate the findings obtained in vitro to the assembly of PsaD occurring in vivo, we used the green alga *C. reinhardtii*. *C. reinhartii* cells were grown as described in Materials and Methods. Following transfer to a minimal medium, the culture was split into two portions. To one half was added chloramphenicol (CAP), an inhibitor of translation of chloroplast-encoded proteins; the other culture was not treated further (control). The cells were then pulse-labeled with [^{14}C]acetate, and PSI was isolated and analyzed by an autoradiogram. Comparison of the PSI profile of the CAP-treated cells with that of control cells clearly indicated that the level of synthesis of the core subunits PsaA and -B was

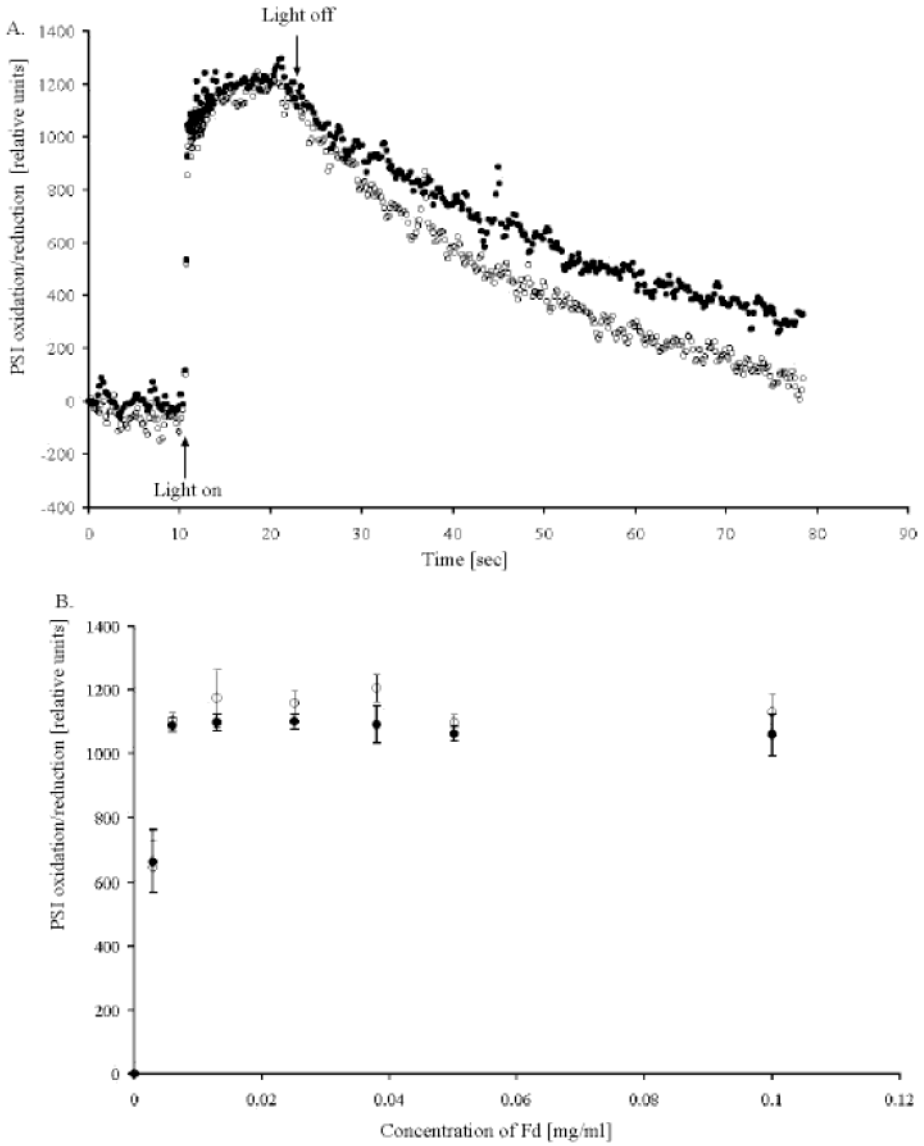

FIGURE 4: PSI containing recombinant PsaD (~50% replacement) is fully active in reducing its electron acceptor Fd. Using the PAM-101 system, the oxidation or reduction of the PSI complex (0.1 mg/mL Chl) was followed in the presence of 2 mM ascorbate and increasing concentrations (0−0.1 mg/mL) of Fd following illumination of the reaction center for 20 s. (A) The fluorescence pattern of P_{700} following illumination at 826 nm as a function of time. (B) The net fluorescence of the P_{700} reaction center obtained by subtracting the minimal fluorescence (in the dark) from the maximal fluorescence (following illumination) as a function of increasing Fd concentration: (○) native PSI and (●) PSI containing recombinant PsaD. Bars show the standard deviation of the mean.

considerably reduced in the presence of CAP. Quantification of the band corresponding to PsaA and -B revealed that in the presence of CAP only 7 ± 2.4% of the core subunits were synthesized as compared with normal synthesis in the absence of CAP. Yet, in the absence of the formation of new PSI complexes, the new, radiolabeled PsaD assembled into the pre-existing PSI complexes. In the presence of CAP, 72 ± 13% of the control amount of newly synthesized PsaD was detected within isolated PSI complexes. With regard to the other stromal-facing subunit of PSI, although the labeling of PsaE is hardly detectable in the presence of CAP, we do have evidence that *M. laminosus* PsaE labeled with [^{14}C]-valine assembles into isolated *M. laminosus* PSI in vitro (A. Lushy et al., manuscript in preparation). Figure 5 also indicates that assembly of newly synthesized LHCs is achieved, suggesting that the outer antenna proteins, like PsaD, join a pre-existing PSI complex.

DISCUSSION

Previously, in vitro assembly studies conducted to follow the biogenesis of PsaD have indicated that precursor, pre-PsaD, and mature PsaD are capable of assembling into thylakoid membranes, specifically into the PSI complex (*2*, *3*, *22*). Nevertheless, the mechanism by which this assembly takes place could not be unraveled due to the extremely small amounts of radiolabeled proteins that were used. Recently, overexpression of PsaD has been reported for both the plant and cyanobacterial PsaD proteins. Recombinant PsaD of spinach was used to follow the interactions between PsaD and Fd (*32*). Overexpressed PsaD of the cyanobacterium *M. laminosus* was used to examine the assembly of PsaD into mutants lacking the PsaD subunit (*24*), indicating a single binding site of PsaD per PSI.

This study aimed at revealing the mechanism of assembly of PsaD into PSI. To overcome the obstacle of small amounts

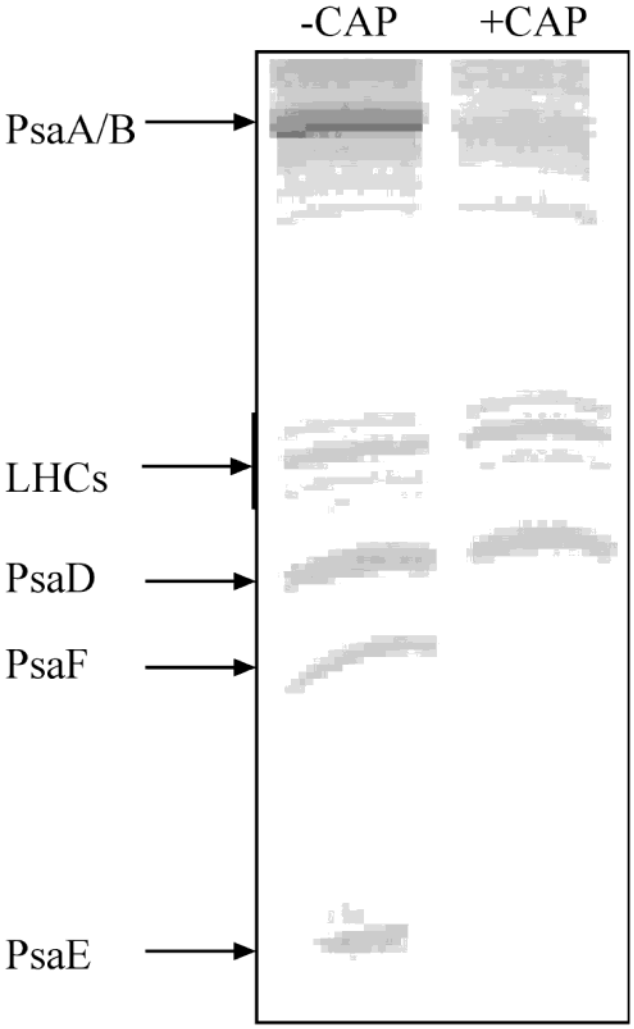

FIGURE 5: Newly synthesized PsaD subunit that can integrate in vivo into a pre-existing PSI complex. WT *C. reinhardtii* cells at a density of 2×10^6 cells/mL were pulse-labeled in the presence and absence of 100 μg/mL chloramphenicol (CAP). Following labeling with [^{14}C]acetate (13×10^{-6} M), the PSI complex was isolated and analyzed by SDS−PAGE that was autoradiographed. Quantification of PsaA/B and PsaD was carried out with the MacBAS software. PsaD was identified by antibodies that were raised against PsaD of *C. reinhardtii*.

of the protein, recombinant PsaD of spinach and recombinant PsaD of *M. laminosus* were overexpressed in *E. coli* cells and used for the assembly studies. The data indicated that the recombinant PsaDs of both species are able to assemble into the isolated PSI complex. This assembly of recombinant PsaD into PSI forced the native PsaD to leave the complex, and the amount of recombinant PsaD present in PSI equaled the amount of free native PsaD that left it. The quantitative results indicate that following replacement, the same mole ratio of PsaD is maintained within PSI regardless of the amount of PsaD that was introduced into PSI. These observations lead to the conclusion that a new PsaD is able to assemble into the fully assembled PSI by an exchange with the native PsaD subunit. Not only did recombinant PsaD replace the native one, but recombinant PsaD that had already replaced native PsaD also could be replaced further by a newly introduced recombinant PsaD, suggesting that PsaD is under constant maintenance. Since PsaD is essential for the assembly, stability, and functionality of the entire PSI, there is no question of the importance of keeping PsaD competent at all times. To analyze whether the exchange mechanism results from a fast turnover, the stability of PsaD was followed in *C. reinhardtii* cells grown in the presence of cycloheximide, an inhibitor of nuclear-encoded genes. Detection of PsaD with anti-PsaD antibodies showed that degradation of the protein started 24 h after the addition of cycloheximide (data not shown), suggesting that although PsaD is a relatively stable protein, its replacement by a newly synthesized PsaD subunit ensures its competency.

The determination of the activity of PSI in reducing Fd, mediated by PsaD, has indicated that the PSI complex fully retained its activity when approximately 50% of the native PsaD population was replaced by recombinant PsaD. The preservation of the activity of the PSI complex containing recombinant PsaD is indicative of the fact that the recombinant PsaD integrated within the PSI complex in an appropriate conformation, thus permitting the activity of PSI. These conclusions are strongly supported by the findings obtained in an in vivo system where newly synthesized PsaD was able to integrate into a pre-existing PSI.

Using dynamic light scattering, we found that in solution *M. laminosus* PsaD weighs 35 kDa, which is twice its weight as calculated according to its sequence. Size exclusion chromatography has shown the same phenomenon for spinach PsaD (data not shown). These results suggest that in solution, *M. laminosus* PsaD and spinach PsaD are present in the form of dimers as reported by Xia et al. for PsaD of the cyanobacterium *Nostoc* sp. PCC 8009 (*33*).

On the basis of the results described above and on the basis of the 2.5 Å resolution structure of PSI (*18*), showing the surface of PsaD to have two charged regions, one positive and one negative, we suggest the following mechanism for the assembly of PsaD into a preassembled PSI. When a new PsaD subunit approaches the PSI complex, it electrostatically interacts with the native PsaD subunit at its surface, probably forming a transient dimer of the two proteins. This interaction loosens the stable binding of the native PsaD within PSI and leaves the hydrophobic region of the newly synthesized PsaD exposed to an aqueous environment, thus providing an energetic incentive for the external PsaD subunit to rotate and replace the native subunit.

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