Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6338–6342, June 1996
Plant Biology

The precursor of PsaD assembles into the photosystem I complex in two steps

(thylakoids/peripheral membrane proteins/protein folding)

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Communicated by George Feher, University of California at San Diego, La Jolla, CA, February 27, 1996 (received for review December 1, 1995)

ABSTRACT **The present study addresses the assembly in the chloroplast thylakoid membranes of PsaD, a peripheral membrane protein of the photosystem I complex. Located on the stromal side of the thylakoids, PsaD was found to assemble *in vitro* into the membranes in its precursor (pre-PsaD) and also in its mature (PsaD) form. Newly assembled unprocessed pre-PsaD was resistant to NaBr and alkaline wash. Yet it was sensitive to proteolytic digestion. In contradistinction, when the assembled precursor was processed, the resulting mature PsaD was resistant to proteases to the same extent as endogeneous PsaD. The accumulation of protease-resistant PsaD in the thylakoids correlated with the increase of mature-PsaD in the membranes. This protection of mature PsaD from proteolysis could not be observed when PsaD was in a soluble form—i.e. not assembled within the thylakoids. The data suggest that pre-PsaD assembles to the membranes and only in a second step processing takes place. The observation that the assembly of pre-PsaD is affected by salts to a much lesser extent than that of mature-PsaD supports a two-step assembly of pre-PsaD.**

Photosystem I (PSI) is one of two photochemically active complexes present in the thylakoid membranes of plants, algae, and cyanobacteria. It is a plastocyanin-ferredoxin oxidoreductase, with at least 12 polypeptide subunits (1, 2). In eukaryotes, these subunits are encoded in about equal proportions in the chloroplast and nuclear genomes. The nuclear-encoded subunits are synthesized in the cytoplasm as precursors, with a leader peptide in their amino terminus. After import into the chloroplast, the leader peptide is cleaved from the precursor, producing the mature form of the protein. The protein also integrates into the thylakoids within the plastids, where it assembles with cofactors and other subunits to form the active complex.

As with other membrane proteins, the sequence of steps in the assembly of thylakoid proteins in the membrane has been extensively studied (3–11). Nevertheless, the new insights into protein folding and organization within the bilayer should be complemented by information on the assembly of individual subunits into a membrane protein complex.

The PSI complex, with its integral hydrophobic core subunits and peripheral membrane proteins, provides a suitable and attractive system for such studies. The peripheral subunits of PSI are located on either the stromal side (PsaC, PsaD, PsaE, PsaH) or the lumenal side (PsaF, PsaN) of the thylakoids (1–2, 12–17). The two major components of the hydrophobic core of PSI differ in size: two large integral membrane proteins, PsaA and PsaB, each with 11 transmembranal α-helices and several low molecular weight subunits (PsaI, PsaJ, PsaK, PsaL and PsaM), which contain transmembranal segments (1, 2).

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The assembly pathways of integral and peripheral thylakoid membrane proteins exhibit different characteristics. The assembly of integral membrane proteins, such as the apoprotein of the light-harvesting chlorophyll protein (LHCP) antenna of photosystem II (PSII) and PSI, required energy in the form of ATP, GTP, and proton-motive force (7, 18–21). The membrane integration of integral proteins that posses trans membranal domains, like LHCPII, depends on the presence of the soluble stromal molecular chaperons, heat shock protein 70 (6), cpn60 (22), SecA analogue (9), and signal recognition particle-like components (23). Such proteins are found to facilitate transport of proteins across, and integration into membranes in other intracellular organelles, such as microsomes (24, 25) and mitochondria (26–28). The chaperons maintain an unfolded conformation of the protein they associate with in order to provide competence for integration into the membrane (11, 29, 30).

The assembly of peripheral stromal-facing membrane proteins, such as PSI subunits and the α and β subunits of the ATP synthase, is independent of any of the stromal assisting factors (31–33). PsaD, the peripheral subunit II of PSI, belongs to this class of proteins. Biochemical experiments (16, 34, 35) and analyses of the primary structure of PsaD suggest that it does not posses a transmembranal segment and that it faces the stromal side of the thylakoid membrane. The protein plays an important role in the assembly of the PSI complex; it is the first nuclear-encoded subunit to accumulate in the thylakoids during the light-triggered development of etiolated seedlings (36–38). In reconstitution experiments, the presence of PsaD in the PSI complex stabilized the binding of other subunits (PsaC, PsaE) to the PSI core (39). Moreover, a *Synechocystis sp.6803* mutant, in which the expression of PsaD is disrupted, loses the ability to grow autotrophically (40). Thylakoids of this mutant strain contain reduced amounts of other peripheral subunits of PSI (32, 41).

In spinach, PsaD is synthesized in the cytoplasm as a precursor of 23.2 kDa (42, 43) that is processed to produce the mature 17.9-kDa PasD. *In vitro* assembly assays indicated that both forms of the protein, pre-PsaD and PsaD, can assemble into the thylakoid membranes, specifically into the PSI complex (31, 44, 45).

The present study analyses the assembly of the precursor (pre-PsaD) *vis-a-vis* the mature form (PsaD). Attempts were made to identify specific characteristics of the assembly of each form in order to reveal the pathway of assembly and organization of the protein within the thylakoid membranes.

MATERIALS AND METHODS

Plant Material. Spinach plants (*Spinacea oleracea cv. Vyropholly*) were grown hydroponically for 3 weeks in light (150

Abbreviations: PSI, photosystem I; PSII, photosystem II; tp, translation products; LHCP, light harvesting chlorophyll protein.

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$\mu E/m^2$ per sec)/dark cycles of 14 and 10 h respectively (31). Pea (*Pisum sativum L. cv. Alaska*) seedlings were grown for 8 days under the same conditions. For import experiments, the seedlings were transferred after 8 days to dark for 2 days and re-illuminated for 30 min at 300 $\mu E/m^2$ per sec before grinding (31).

Construction of the Gene Encoding the Mature PsaD. Amplification of parts of the spinach *psaD* gene by PCR was used to omit the leader peptide from *psaD*. At the beginning and end of the gene the oligonucleotides for the PCR were designed to add recognition sites for the restriction endonucleases *Xba*I and *Pst*I to facilitate directional cloning of the product in pGem3Z vector.

***In Vitro* Transcription/Translation.** The genes coding for the pre-PsaD (*psaD*) as well as the mature protein were transcribed *in vitro* by using T7 RNA polymerase. The resulting mRNAs were then translated in a wheat germ extract in the presence of ^{35}S-methionine as described before for the apoprotein of the antenna of photosystem II (LHCP) (46, 47) and for PsaD (31).

Import into Intact Chloroplasts. Labeled translation products (10^6 cpm per reaction) were incubated with purified plastids equivalent to 100 μg chlorophyll for 30 min at 25°C (31). Where indicated, $HgCl_2$ was added to a final concentration of 3 mM to prevent processing (48). Those translation products not properly imported into the organelle were removed by incubating the plastids with thermolysin (0.1 mg/ml) for 30 min on ice. Following this treatment, intact plastids were re-isolated by centrifugation through a 33% percoll cushion. The plastids were then lysed, and their thylakoid membranes were recovered (31).

Assembly into Isolated Thylakoids. A typical assembly reaction contained translation products (400,000 cpm), 10 mM Mg-ATP, 10 mM methionine, and thylakoids (equivalent to 80 μg chlorophyll). The reaction was performed at 10–25°C for 5–30 min, with or without the chloroplast stromal fraction (31). In some experiments, processing of the precursor was inhibited by addition of 10 mM 8-hydroxyquinoline 5-sulfonic acid or a mixture of the following protease inhibitors: 3.3 mM 1,10-phenanthroline, 0.33 mM Na-EDTA (pH 8.0) and 0.33 mM phenylmethylsulfonyl fluoride (PMSF). To induce processing of the pre-PsaD, following the assembly reaction the thylakoids were washed with 100 mM Tricine-NaOH (pH 8.0) and incubated with the stromal fraction at 25°C for 10–60 min. The verification of the assembly of the protein (pre-PsaD/PsaD) into the membranes was performed following treatment of the thylakoids with 2 M NaBr or 0.1 M NaOH, or with the proteolytic enzymes thermolysin or trypsin.

Thermolysin Treatment. Thylakoids recovered from the import or assembly reactions were incubated with thermolysin (0.1 mg/ml) and 5 mM $CaCl_2$ for 30 min on ice. The reaction was terminated by washing the thylakoids with 0.1 M Tricine-NaOH (pH 8.0) and 10 mM Na-EDTA.

Trypsin Treatment. The thylakoids were incubated with trypsin (0.1 mg/ml) for 30 min at room temperature. The reactions were terminated by addition of 1 mM PMSF.

Miscellaneous Techniques. Chlorophyll concentration was determined after extraction of the pigments with 80% acetone (49). Protein concentrations were defined in Bio-Rad protein assay kit according to the manufacturer's instructions. Fully denaturing SDS/PAGE was performed according to the method of Schagger and von Jagow (50). Western blot analyses followed the method of Nelson (51). The immunodecoration was performed with polyclonal antibodies raised against spinach PsaD (36) and ^{125}I-labeled protein A.

RESULTS AND DISCUSSION

Previous studies indicated that the precursor of PsaD, synthesized *in vitro*, incorporates into the thylakoid membranes, specifically into the pigmented PSI complex (31). It was further demonstrated that the bound precursor can be processed to its mature form by a stromal peptidase (44). To characterize the assembly of pre-PsaD in the thylakoids in greater detail, its stability to chaotropic and alkali washes and to proteolytic digestion was tested. The results indicate that while newly assembled pre-PsaD is fully resistant to NaBr and NaOH washes, newly assembled precursor is degraded by both thermolysin and trypsin (Fig. 1).

To eliminate the possibility that the sensitivity of pre-PsaD to proteolysis results from the *in vitro* conditions of the assembly assay, we compared the stability of newly imported pre-PsaD and mature PsaD to that of PsaD present in the thylakoid membranes *in situ* (Fig. 2). Following incubation of pre-PsaD with intact pea plastids, the organelles were incubated with thermolysin to degrade un-imported proteins. Intact plastids were re-isolated and lysed and their thylakoids were purified. The membranes were then treated with NaBr (Fig. 2, lanes b and e), NaOH (Fig. 2, lane c), or thermolysin (Fig. 2, lanes d and f). When $HgCl_2$, which prevents processing of precursors to the mature forms (48) was present in the import assay, the pre-PsaD accumulated in the thylakoids (31). This pre-PsaD was resistant to NaBr wash (Fig. 2*A*, lane e), but sensitive to thermolysin degradation (Fig. 2*A*, lane f). It resembled the pre-PsaD incorporated into isolated thylakoids (Fig. 1, lane Therm). In the control import reaction—i.e. in the absence of $HgCl_2$—the precursor was processed to the mature form, which accumulated in the membranes (Fig. 2*A*, lane a). This PsaD was resistant to salt and alkali washes (Fig. 2*A*, lanes b and c). Unlike pre-PsaD, when thermolysin was applied, only a 2-kDa fragment was removed from the newly accumulated mature PsaD protein (Fig. 2*A*, lane d).

To find out whether this proteolytic pattern of the mature PsaD present in the thylakoids following an import reaction was similar to that of the mature protein present *in vivo*, we examined the effect of thermolysin on native PsaD. Thylakoids isolated from spinach leaves were treated with NaBr, NaOH, or thermolysin. Their proteins were separated by SDS/PAGE, transferred to nitrocellulose, and immunodetected with polyclonal antibodies raised against PsaD. The results (Fig. 2*B*) showed that the PsaD protein is resistant to NaBr and NaOH washes (Fig. 2*B*, lanes b and c). Thermolysin treatment of the thylakoids induced a cleavage of 2 kDa from the mature PsaD present *in situ* (Fig. 2*B*, lane d). This 2-kDa-depleted PsaD form, which resembled the thermolysin cleaved PsaD reported by Zilber and Malkin (16), is similar to the cleaved form observed after the import assay (Fig. 2*A*, lane d).

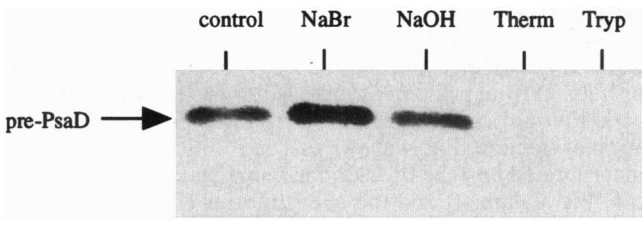

FIG. 1. Effects of NaBr, NaOH, thermolysin, and trypsin treatments on pre-PsaD assembled in the thylakoid membranes. Translation products (pre-PsaD) were incubated with thylakoids isolated from pea as described (control). The thylakoids were then incubated for 20 min on ice with 2 M NaBr (NaBr), 0.1 M NaOH (NaOH), thermolysin (0.1 mg/ml) (Therm), or trypsin (0.1 mg/ml) (Tryp). Following these treatments, the membranes were washed twice with 0.1M Tricine-NaOH (pH 8.0). For the thermolysin-treated membranes, the washing buffer contained 10 mM Na-EDTA. The buffer for washing the trypsin-treated membranes included 1 mM PMSF. Following the washes, the thylakoids (equivalent to 10 μg chlorophyll) were analyzed by SDS/PAGE. The gel was fluorographed and exposed for 3 days to a X-ray film.

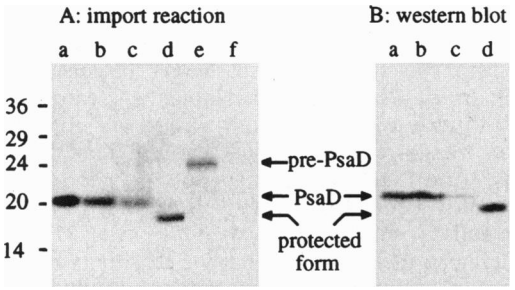

FIG. 2. Resistance of PsaD (mature and precursor forms) to removal from the thylakoid membranes by NaBr, NaOH, or thermolysin treatments. (*A*) Following import of pre-PsaD into intact chloroplasts. Intact plastids were isolated from spinach leaves and used for import reaction as described. The reactions were performed in the absence (lanes a–d) or presence (lanes e and f) of 3 mM $HgCl_2$. Following lysis of the plastids, their thylakoids were recovered (lane a) and treated with NaBr (lanes b and e), NaOH (lane c), or thermolysin (lanes d and f). Samples (containing 10 μg chlorophyll) were analyzed by SDS/PAGE. The gels were fluorographed and exposed to X-ray films. (*B*) Mature (PsaD) present *in situ* in the thylakoid membranes. Thylakoids isolated from spinach leaves were untreated (control, lane a), treated with 2 M NaBr (lane b), 0.1 M NaOH (lane c), or 0.1 mg/ml thermolysin (lane d) as described above. Following the treatments, the thylakoids were denatured and their proteins analyzed by SDS/PAGE. Following transfer to nitrocellulose, immunodecoration was performed as described.

Therefore, whereas the mature PsaD in the thylakoids is almost entirely protected from proteolytic digestion, the precursor form, pre-PsaD, when present in the membranes, is susceptible to proteolysis. Two explanations come to mind. (*i*) The removal of the transit peptide, after the precursor integrates into the membrane, induces conformational changes which assures the protein's resistance to proteolysis. This new conformation may enable and/or facilitate binding of PsaD to different domains of the PSI complex. Previously, interactions were shown to exist between PsaD, PsaE, PsaC, and PsaF or PsaJ (2, 17, 41). It may be that these subunits shield a large portion of the mature PsaD polypeptide, thereby protecting it from proteolysis. (*ii*) The mature form itself is of a conformation which assures its resistance to proteolytic digestion.

To eliminate one of these explanations, two different experiments were performed. Pre-PsaD assembled in thylakoid membranes was incubated with the chloroplast stromal fraction for different lengths of time in order to induce processing. Following processing, the membranes were treated with thermolysin. The results (Fig. 3) showed that the amount of the protected form of PsaD accumulated in the membranes correlated with the accumulation of the mature PsaD (produced by the processing). The second type of experiments aimed at examining whether the mature conformation itself confers protection from proteolysis: translation products were treated with thermolysin before and after their processing by the stromal fraction. Fig. 4 shows that in solution (i.e., when not incorporated into the thylakoid membranes), both the precursor (Fig. 4, lane 2) and the mature forms (Fig. 4 lane 4) are sensitive to the proteolytic digestion. While the data on pre-PsaD agree with the observations on pre-PsaD present in the thylakoids, the observed sensitivity to proteolysis of the soluble mature form contradicts the findings with PsaD assembled in the membranes. As seen in Fig. 2, most of the PsaD polypeptide in the thylakoids is protected from proteolysis. Taken together, both lines of evidence indicate that it is not the conformation of the mature form itself that assures resistance to proteolysis. It is probable that the interactions of the mature PsaD with different domains in the PSI complex, within the thylakoid membranes, shield PsaD from proteolysis.

To study and to better characterize the interactions formed between each form (precursor/mature) and the PSI complex,

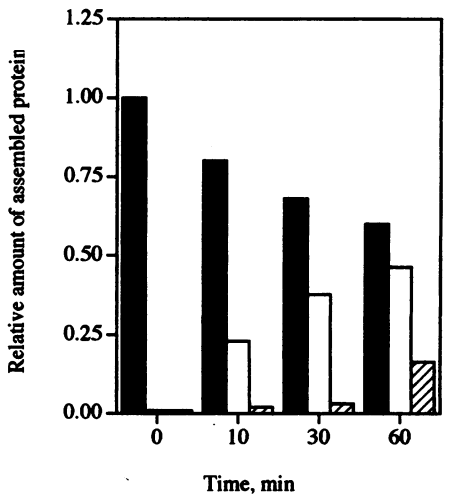

FIG. 3. The accumulation of the protease-resistant PsaD in the thylakoids correlated to the increase in processing of newly integrated pre-PsaD. The assembly reaction was performed as described in Fig. 1. Following the NaBr wash, thylakoids (equivalent to 40 μg chlorophyll) were incubated with the chloroplast stromal fraction (containing 900 μg protein) for increasing time periods (indicated in the figure). After the processing reaction, the thylakoids were washed twice with 0.1 M Tricine-NaOH (pH 8.0) and the membranes were treated with NaBr or thermolysin. Following treatments, samples containing 10 μg chlorophyll were denatured and analyzed by SDS/PAGE. The gel was fluorographed and exposed to a Fuji imaging plate for 4 h. Quantitation of the proteins present in the thylakoids was carried out by scanning the fluorogram in Bio-imaging analyzer (FUJIX BAS 1000). Pre-PsaD present in the membranes was calculated relative to pre-PsaD present in the thylakoids before their exposure to the stromal fraction (0 min of processing) (■). The mature form is presented as the relative amount of processed PsaD from the total labeled protein present in the membrane (pre-PsaD and PsaD) (□). The amount of thermolysin-resistant PsaD is presented relatively to the mature-PsaD (▨).

the assembly of pre-PsaD/PsaD into the thylakoid membranes was performed in increasing concentrations of NaCl (Fig. 5). The results indicated that while the assembly of pre-PsaD was only slightly affected by salt concentrations less than 500 mM (Fig. 5*A*), the ability of the mature form to properly assemble in the membranes was significantly decreased. At 0.75 M NaCl for example, <20% of PsaD was detected in the thylakoids, while at the same ionic strength >50% of pre-PsaD was found assembled in the membranes (Fig. 5*C*). Moreover, at salt concentration higher than 500 mM NaCl, the PsaD detected in the thylakoids was more sensitive to proteolysis (Fig. 5*B*, Therm lanes).

These observations strongly suggest that the mature PsaD mainly forms electrostatic interactions within the PSI complex.

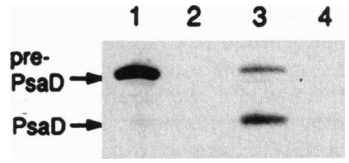

FIG. 4. Degradation by thermolysin of soluble PsaD, both precursor and mature forms. Translation products (pre-PsaD) (untreated, lane 1) were incubated with thermolysin (0.1 mg/ml) for 30 min on ice (lane 2). An equivalent sample of pre-PsaD was incubated at 25°C for 30 min with the chloroplast stromal fraction to induce processing (lane 3). Following this incubation, to half of the reaction thermolysin was added for additional 30 min incubation on ice (lane 4). The different samples were then analyzed by SDS/PAGE, and the gel was autoradiographed.

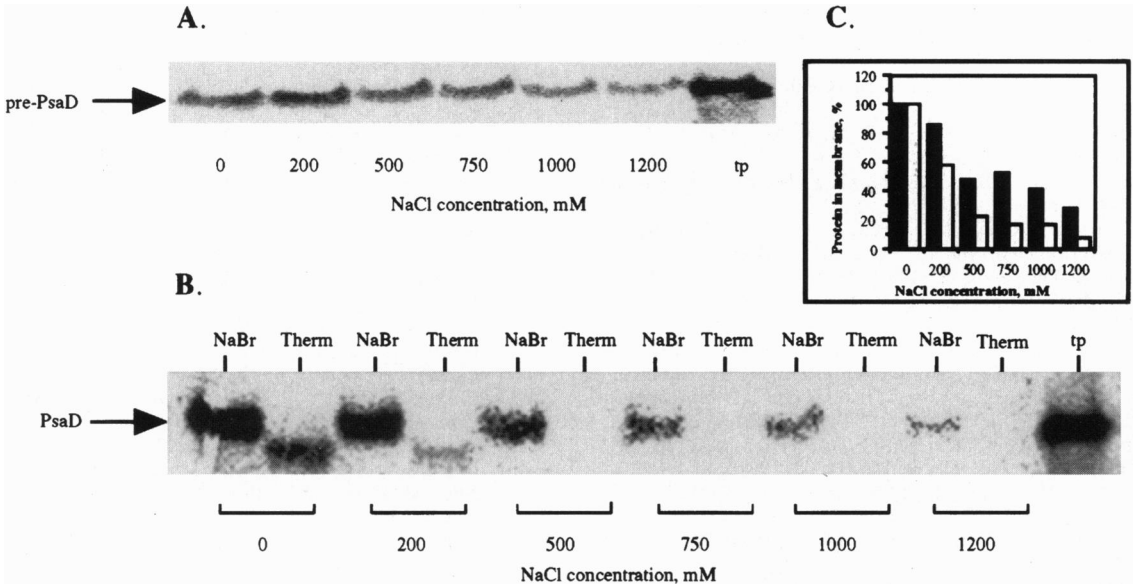

FIG. 5. NaCl affects the assembly of the precursor and mature forms of PsaD into the thylakoid membranes. The assembly reactions of pre-PsaD (*A*) and PsaD (*B*) were performed in the presence of increasing concentrations of NaCl. Following the reaction, the thylakoids were washed twice with 0.1 M Tricine-NaOH (pH 8.0) and treated with NaBr. In the assembly of the mature form, the membranes were also treated with thermolysin. Following the treatments, the thylakoids were denatured and analyzed by SDS/PAGE. The gels were fluorographed and exposed to Fuji imaging plate. (*C*) Quantitative analysis of the protein present in the membranes. The labeled protein present in the membranes after the assembly reactions in the absence of salt and following the NaBr wash was used as a reference (100% of proper assembly) for quantifying the amount of protein that assembled in the membrane in the presence of salt. Precursor, pre-PsaD (■); mature form, PsaD (□); tp, translation products for the *in vitro* synthesized pre-PsaD or PsaD.

The interactions formed by the precursor are somewhat different and are much less affected by increased ionic strength.

SUMMARY AND CONCLUSIONS

The aim of the present study was to elucidate the steps involved in the assembly into the thylakoid membranes of PsaD, a peripheral stromal-facing subunit of PSI. Previous work has shown that both the precursor, pre-PsaD, and the mature form, PsaD, could assemble into the thylakoid membranes (31). Here we demonstrated that the assembly of the two forms has different characteristics. While the assembly of the mature PsaD was found to be affected by salts, the increase in ionic strength influenced the integration of pre-PsaD into the thylakoids less dramatically. (For a more detailed discussion on the effect of salts on the assembly of the different forms of PsaD, see ref. 52.)

The assembled pre-PsaD in the thylakoids within the PSI complex was found to be susceptible to proteolytic digestion. However, following its processing to yield the mature PsaD, the protein becomes resistant to proteases; thermolysin removed only a 2-kDa fragment at the N terminus of the protein. This resistance to proteolysis of the newly processed mature PsaD is similar to that observed for PsaD present in the thylakoids *in situ*.

Taken together, the different observations suggest that at least two steps comprise the assembly of pre-PsaD in the thylakoid membranes. First, pre-PsaD binds to PSI probably in an extended conformation that provides its proteolytic susceptibility. The extended conformation, which may be similar to that of pre-PsaD in solution, is probably facilitated by the presence of the leader peptide. In bacterial systems it was proven that the leader peptide retards folding of the polypeptide (53).

Subsequent to the integration of pre-PsaD, in a second step, the processing to form the mature PsaD occurs. The latter is probably accompanied by a conformational change, which allows the formation of electrostatic interactions between PsaD and other subunits in PSI. These intracomplex interactions probably embed and stabilize the assembly of PsaD in the complex within the thylakoid membranes. They also shield the PsaD and protect it from proteolytic digestion. Mature PsaD is resistant to proteolysis only within the thylakoids.

This pathway of PsaD incorporation resembles the one reported for the CAB-7 protein, the apoprotein of the light harvesting complex of PSI. For this integral membrane protein, it was found that only after membrane integration is completed and CAB-7 binds to the holo-PSI, the protein becomes resistant to proteolytic digestion (7). Resistance to proteolysis upon completion of the assembly process—i.e., binding of the photosynthtic pigments, was also shown for LHCP (54). Another example where processing precedes completion of assembly in the membranes was recently shown for the peripheral lumenal cytochrome f protein. From cytochrome f crystalline structure determination it is evident that the removal of the leader peptide is necessary to allow the ligation of the heme by the N-terminal alpha amino group, which is the axial 6th ligand (55).

We wish to thank Prof. J. Lorch for helpful discussions and suggestions. This work was supported by the following Grant IS-2191-92RC from the United States–Israel Binational Agricultural Research and Development Fund, Grant I-259-153.03/92 from the German–Israeli Foundation for Scientific Research and Development, and Grant 470/93-1 from The Israel Science Foundation (administrated by The Israel Academy of Science and Humanities).

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