Sec-independent Insertion of Thylakoid Membrane Proteins

ANALYSIS OF INSERTION FORCES AND IDENTIFICATION OF A LOOP INTERMEDIATE INVOLVING THE SIGNAL PEPTIDE*

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A group of membrane proteins are synthesized with cleavable signal sequences but inserted into the thylakoid membrane by an unusual Sec/SRP-independent mechanism. In this report we describe a key intermediate in the insertion of one such protein, photosystem II subunit W (PSII-W). A single mutation in the terminal cleavage site partially blocks processing and leads to the formation of an intermediate-size protein in the thylakoid membrane during chloroplast import assays. This protein is in the form of a loop structure: the N and C termini are exposed on the stromal face, whereas the cleavage site has been translocated into the lumen. In this respect the insertion of this protein resembles that of M13 prococat, which also adopts a loop structure during insertion, and we present preliminary evidence that a similar mechanism is used by another thylakoid protein, PSII-X. However, whereas the negatively charged region of prococat is translocated by an apparently electrotransport mechanism using the $\Delta \mu H^+$, the corresponding region of PSII-W is equally acidic but insertion is $\Delta \mu H^+$ independent. We furthermore show that neutralization of this region has no apparent effect on the insertion process. We propose that a central element in this insertion mechanism is a loop structure whose formation is driven by hydrophobic interactions.

The biogenesis of biological membranes requires the synthesis of numerous hydrophobic proteins and their insertion into membrane bilayers, and intensive efforts have been made to understand the mechanisms involved. The majority of bacterial, chloroplast, and mitochondrial membrane proteins are inserted post-translationally, and interest has centered on the mechanisms by which these proteins are initially maintained in a soluble form, and how the hydrophobic regions are then transferred from an aqueous milieu into the apolar regions of the membrane bilayer. Some elements of the insertion events have been characterized in detail, one example being the influence of positive charges on overall topology, since these residues have a strong tendency to remain on the cis side of the membrane (1). It has also proved possible to recognize two broad categories of insertion mechanism, which can be regarded as “assisted” and “unassisted” according to whether protein translocation machinery is relied upon. In bacteria, for example, the secretion (Sec) apparatus used for the translocation of periplasmic proteins is also required for the insertion of some membrane proteins (2–5). In addition, another element of the export machinery, signal recognition particle (SRP),1 is involved in the insertion of a range of membrane proteins (6–8). Other proteins, however, are integrated by Sec-independent mechanisms and these may well insert spontaneously into the plasma membrane (9–12). However, whereas the overall requirements have been detailed for several membrane proteins, the actual insertion mechanisms are poorly understood in most cases. In vitro reconstitution assays favored for the study of protein translocation have been of limited use because most hydrophobic proteins are inherently unstable in aqueous solution. Few proteins have been examined in genuine mechanistic detail, although the coat proteins of the M13 and Pf3 phages are notable exceptions that have been shown to integrate into the Escherichia coli membrane by Sec-independent, possibly spontaneous mechanisms (reviewed in Ref. 9).

Our knowledge of membrane protein insertion is particularly vague in the case of thylakoid proteins. Most thylakoid proteins are imported after synthesis in the cytosol, and at least four distinct pathways have been identified for their subsequent targeting into and across the thylakoid membrane (reviewed in Refs. 13 and 14). Two of the pathways apply to thylakoid lumen proteins, which are imported by means of bipartite presequences containing envelope transit and thylakoid transfer signals in tandem. After import into the stroma, the transfer signal directs transport across the thylakoid membrane by either an ATP-dependent, Sec-related mechanism, or a very different mechanism that relies on the thylakoidal $\Delta \phi$H. After translocation, the stromal intermediates are processed to the mature forms by a lumen-facing thylakoidal processing peptidase, TPP (15). The thylakoid transfer signals of these proteins are all similar in overall structure to bacterial signal peptides, but specify translocation by only one of these mechanisms (16–18).

Two further pathways have been identified for integral thylakoid membrane proteins. The multispanning membrane protein, LHCP, is synthesized with an envelope transit signal only, and hence integrates into the thylakoid membrane by means of information contained in the mature protein (19, 20). The integration process requires GTP and a stromal homologue of the 54-kDa protein of signal recognition particles (21), and is thus likely to be similar in certain respects to the SRP-dependent pathway identified in bacteria. However, a very different mechanism has been demonstrated for three other thylakoid proteins: subunit II of the integral CF$_1$ component of the thylakoidal ATP synthase (CF$_{11}$II) and subunits X and W of photosystem II (PSII-X and PSII-W). These proteins are synthesized with bipartite presequences that strongly resemble typical Sec-type signal peptides (22–24), yet they integrate into thylakoid membranes in the absence of stromal factors, nucle-

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1 The abbreviations used are: SRP, signal recognition particle; TPP, thylakoidal processing peptidase; PSII, photosystem II.
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EXPERIMENTAL PROCEDURES

Synthesis of Precursor Proteins—Precursors of Arabidopsis thaliana PSII-W and PSII-X were synthesized by transcription in vitro of cDNA clones followed by translation of capped transcripts in the presence of \(^{3}H\)leucine as described (24). Mutated forms were prepared from leaves of 8–9-day-old pea seedlings (Pisum sativum, var. Feltham First) by Percoll-poly ethylene glycol centrifugation and used in import assays as described (24–26). Proteolysis of thylakoid membranes was carried out in 10 mM Hepes, pH 8.0, 5 mM MgCl\(_2\) (HM buffer) unless otherwise specified in the figure legends, using thylakoid suspensions at 1 mg/ml chlorophyll. Sonication involved the use of a sonicator water bath at 0 °C. Carbonate washing of thylakoids was carried out as detailed by (24–26). Mutated forms were prepared by oligonucleotide-directed site-specific mutagenesis of cDNAs using an Amersham International kit according to the manufacturer’s instructions.

Import Assays and Topological Analysis—Intact chloroplasts were prepared from leaves of 8–9-day-old pea seedlings (Pisum sativum, var. Feltham First) by Percoll gradient centrifugation and used in import assays as described (24–26). Proteolysis of thylakoid membranes was carried out in 10 mM Hepes, pH 8.0, 5 mM MgCl\(_2\) (HM buffer) unless otherwise specified in the figure legends, using thylakoid suspensions at 1 mg/ml chlorophyll. Sonication involved the use of a sonicator water bath at 0 °C. Carbonate washing of thylakoids was carried out as detailed by (24–26). Mutated forms were prepared by oligonucleotide-directed site-specific mutagenesis of cDNAs using an Amersham International kit according to the manufacturer’s instructions.

Sequence Analysis—The sequences of PSII-X proteins were extracted from data base entries for the plastid genomes of Porphyra purpurea (accession numbers Z67753, U30821, and U38804, respectively).

A. thal.
C. para.
F. purp.

\begin{align*}
\text{A. thal.:} & \quad \text{---DEALTSISAAALTASQWE} & \begin{array}{c}
\text{C. para.:} \\
\text{F. purp.:}
\end{array} \\
\text{C. para.:} & \quad \text{---MTLSQIAVLPQDYSW} & \begin{array}{c}
\text{F. purp.:}
\end{array} \\
\text{F. purp.:} & \quad \text{---MTLSQIAVLPQDYSW}
\end{align*}

\text{\textit{A. thal.} and \textit{C. para.} are shown in \textbf{bold}. The figure also shows (in \textit{bold}) the changes introduced into two pre-PSII-W mutants, \textit{W/A78T} and \textit{W/E88Q}, and the locations of the individual sections within iPSII-W (membrane, lumen, or stroma exposed) deduced in this study. Bottom panel, alignment of PSII-X sequences from Arabidopsis (\textit{A. thal}), O. sinensis (\textit{O. sinen}), C. paradoxa (\textit{C. para}), and \textit{P. purpurea} (\textit{P. purp}). The Arabidopsis sequence includes the signal peptide of the presequence region; the remaining sequences are given in full. Identical residues are denoted by \textit{asterisks}; conserved residues by dots.}

**RESULTS**

Cleavage by TPP Is Not Required for the Insertion of PSII-W—Pre-PSII-W and pre-PSII-X share overall structural similarities with M13 procoat and appear likewise to insert into their target membrane without the aid of translocation machinery. However, there are significant differences in terms of insertion requirements (see below) and we have set out to investigate the thylakoid proteins in greater detail. Our first aim was to determine whether the cleavable signal-type peptides of the thylakoid proteins are used for a similar purpose: the formation of a loop intermediate in which the hydrophobic region (H-domain) spans the membrane. This is probably the most important role of the M13 signal peptide and our priority was to determine whether such an intermediate is a core feature in the biogenesis of PSII-W and/or PSII-X. We addressed this question by inhibiting the cleavage step catalyzed by TPP (whose active site is in the thylakoid lumen; Ref. 15), in order to probe the location and topology of this protein immediately prior to the final maturation step. Fig. 1 shows the structures of the relevant sections of both precursors, including the signal peptide and the mature region of the mature protein. TPP is known to depend on the presence of short-chain residues at the −3 to −1 positions in the substrate, relative to the processing site, and Ala at the −1 position is essential with even Ser and Gly unable to support efficient cleavage (28). We therefore introduced Thr at this position in both pre-PSII-W and pre-PSII-X, in the expectation that processing would be drastically affected without substantially altering the characteristics of the translocated region.

The import characteristics of the PSII-W mutant (WA78T) are shown in Fig. 2. The wild-type precursor protein is imported into the chloroplasts, processed to the mature size, and fractionation tests confirm that this polypeptide is located exclusively in the membrane fraction (lane T). Protease treatment of the thylakoid membranes (lane T +) results in digestion of the exposed C-terminal region (as found in previous studies on PSII-W (29)), and the production of a slightly smaller degradation product. The mutant protein, on the other hand, is converted to a mixture of mature- and intermediate-size proteins, the latter form presumably resulting from the action of stromal processing peptidase, which cleaves the envelope transit domains from most bipartite presequences. Importantly, this iPSII-W form is also found only in the thylakoid lumen fraction (lane P). Since this procedure effectively removes extrinsic proteins from the thylakoid membrane (25), we can exclude the possibility that the action of TPP is required for the
insertion of this protein. Thermolysin treatment of the iPSII-W results in a mobility shift that is comparable to that of mature PSII-W, confirming that at least one section of the intermediate is exposed on the stromal face of the thylakoid membrane.

A Loop Intermediate in the Insertion of PSII-W—The topology of the iPSII-W polypeptide was mapped by determining the accessibility of sites for trypsin and thermolysin cleavage. Fig. 1 shows that the PSII-W mature protein contains only a single basic residue which is located in the luminal N-terminal region (Arg at position 5); the presequence contains many basic residues but these are all located prior to the H-domain in the signal peptide. In a loop structure these sites are predicted to be on the luminal and stromal sides of the membrane, respectively. Fig. 3A shows that trypsin cleaves iPSII-W to a smaller form that remains significantly larger than mature-size PSII-W; low concentrations of trypsin (e.g. 10 µg/ml) generate a mixture of iPSII-W and a smaller degradation product, whereas 25 µg/ml is sufficient to fully convert the iPSII-W to the degradation product. Trypsin therefore cleaves the N terminus of the iPSII-W signal peptide on the stromal surface of the membrane. A loop intermediate would also contain the C terminus of the mature protein on this face of the membrane, and Fig. 3B shows that this is the case. The C terminus of mature PSII-W is exposed on the stromal face and the data in Fig. 2 showed that it is cleaved by thermolysin; Fig. 3B shows that thermolysin also cleaves a small fragment from iPSII-W, generating a degradation product similar in mobility to that produced by trypsin (compare lanes Th and Tp). Significantly, incubation of iPSII-W with both thermolysin and trypsin generates a much smaller degradation product (lane Th/Tp) indicating that trypsin and thermolysin cleave at different ends of the molecule (the N and C termini, respectively). Lane Tp/son of Fig. 3B provides final confirmation of a loop structure: thylakoids containing iPSII-W were incubated with trypsin and the thylakoids were sonicated to allow access of the protease to the luminal side of the membrane. Both the mature PSII-W and the iPSII-W are almost quantitatively converted to a polypeptide that is smaller than mature PSII-W, indicating that cleavage has taken place after the Arg at position 5 in the mature protein, which must therefore be exposed on the luminal side of the membrane.

Evidence Favoring a Similar Insertion Process for PSII-X—A similar approach was also taken to investigate the insertion of PSII-X because it is as yet unclear whether the thylakoid proteins currently viewed as a group (CF, II, PSII-X, and PSII-W) actually insert by similar mechanisms. The cleavage site of pre-PSII-X was similarly mutated by the introduction of a Thr at the position and the effects examined as for PSII-W. Fig. 4 shows that this mutation likewise inhibits processing by TPP although the effects are in fact more drastic; analysis of the thylakoid fraction following an import reaction shows that hardly any mature PSII-X is detectable, indicating that the action of TPP is almost totally blocked. Once again, an intermediate-size form (int) accumulates which is presumed to result from the action of stromal processing peptidase. This intermediate form is located exclusively in the thylakoid membrane showing that TPP action is not required for the insertion of PSII-X.

Topology studies were carried out on the iPSII-X polypeptide but the results were not as clear-cut as those obtained with PSII-W. Thylakoids containing the imported, mature-size wild-type protein were incubated with thermolysin, Staphylococcus V8 protease (V8), a mixture of thermolysin/V8, and trypsin, but no cleavage was apparent (left-hand panel of Fig. 5). Apparently, only a small region of mature PSII-X protrudes into the stroma. Unfortunately, the membrane-bound intermediate form is also resistant to proteolysis as shown in the right-hand panel; thermolysin and trypsin fail to generate defined cleavage products, and only V8 of the proteases tested is able to cleave the protein. However, this takes place only at very high concentrations (200 µg/ml for 60 min) and some penetration into the lumen clearly occurs because the cleavage product (lane V) is similar in size to the mature protein. This presumably means that the V8 cleaves at one or both of the Gln residues in the C-terminal region of the signal peptide, just before the cleavage site (see Fig. 1). However, it is notable that this cleavage is much more efficient when the thylakoids are sonicated in the presence of V8 to allow complete access. Under these conditions the intermediate is quantitatively converted to the degradation product (lane Vs) providing evidence, albeit circumstantial, that this region is in the lumen. This would imply the formation of a loop intermediate as with PSII-W, but further tests will be required to confirm this point.
Evidence That Hydrophobic Forces Drive the Insertion of Pre-PSII-W—Studies in bacteria have shown that two types of force can be instrumental in driving protein insertion. Hydrophobic forces appear to be important in all cases, reflecting the loss of free energy achieved by moving hydrophobic regions into the lipid bilayer, but electrostatic forces are also vital in the insertion of both P39 coat protein and M13 prococat. In both cases, insertion depends on the ΔμH⁺, (positive outside) which is believed to drive the translocation of negatively charged residues across the membrane. The translocated regions of CF และ PSII-W, and pre-PSII-W are all similar to that of prococat in terms of charge: the N-terminal regions of mature coat protein, CFII, and PSII-W contain net charges of 

**DISCUSSION**

CF₂, II, PSII-W, and PSII-X represent an unusual group of membrane proteins in that no other membrane protein, with the exception of M13 prococat, has been found to rely on a cleavable signal peptide for membrane insertion by a Sec- and SRP-independent mechanism. As shown in Fig. 1, the three thylakoid proteins share additional common features with prococat: each contains a single transmembrane span in the mature protein and the translocated regions are all negatively charged. It was thus unsurprising that initial models for the Sec-independent insertion of CF₂II (25) were based on the prococat insertion model, and this study has confirmed that in one fundamental respect the insertion of PSII-W does indeed resemble that of prococat. Both proteins adopt a loop intermediate during the insertion process, strongly suggesting that the function of the PSII-W-signal peptide is to provide a second hydrophobic domain which, in concert with the transmembrane segment in the mature protein, is then able to drive the translocation of the hydrophilic intervening region into the thylakoid lumen. Interestingly, signal-type peptides appear also to form loop structures when directing soluble proteins across membranes by means of protein translocation systems (30, 31).

The experimental evidence favoring a Sec/SRP-independent insertion mechanism (23–27) is consistent with the unusual evolution of the signal peptides. Genes encoding PSII-X and CF₂II have been identified in cyanobacteria and also within the plastid genomes of several eukaryotic algae/diatoms, such as *O. sinensis* and *C. paradoxa*. None of these cyanobacterial or plastid-encoded proteins is synthesized with a signal-type peptide, strongly suggesting that the signal peptides were acquired after the transfer of these genes to the nucleus in higher plants. This situation contrasts starkly with “genuine” Sec-type signal peptides such as those found in the precursors of luminal proteins, because the cyanobacterial/plastid-encoded counterparts of these proteins are invariably synthesized with classical signal peptides. We therefore believe that the signal peptides of these membrane proteins differ from Sec-type signal peptides in terms of both function and origin. However, we can detect no large-scale structural differences among the mature proteins that might explain why nuclear-encoded, imported proteins are now synthesized with signal peptides. Mature PSII-X proteins, for example, are structurally very similar in higher plants and cyanobacteria (see Fig. 1). Given these similarities, it may be simplistic to suggest that the signal peptide functions simply by providing an additional hydrophobic segment, because the mature proteins in cyanobacteria and the above mentioned algae can clearly insert despite possessing only a single hydrophobic region. The only obvious difference concerns the charge distributions in the N-terminal regions; these regions are neutral in the cyanobacterial and plastid-encoded proteins whereas that of *Arabidopsis* PSII-X is basic due to the Lys at position 10. This could conceivably render the translocation process more difficult to the extent that the additional
hoosphobic region is required to aid insertion. However, it is also possible that this unusual insertion mechanism has been forced on these proteins as a consequence of their more complex insertion pathway; the proteins may insert co-translationally in cyanobacteria, in which case the protein may never be free in the aqueous phase and insertion may be more favorable. It is even possible that the signal peptide renders the precursor protein more stable in solution, perhaps by forming a “helical hairpin” in which the signal peptide partially masks the hydrophobic character of the transmembrane segment. Structural studies on this type of precursor protein should prove instrumental in resolving these points.

Although the insertion of these thylakoid proteins is reminiscent of M13 procoat insertion, there are equally significant differences in the mechanisms used. The initial concerns the insertion events in the insertion process. Gallusser and Kuhn (32) have shown that electrostatic interactions play a critical role in the insertion of procoat, in which basic residues in both the extreme N and C termini bind to the negatively charged membrane surface; removal of either set of basic residues renders procoat wholly insertion incompetent. Like procoat, pre-CFoII cated regions can be inserted even in the absence of a membrane insertion. It must be emphasized at this point that selectively charged regions through the membrane potential in driving the translocation of negatively charged regions are the primary driving force for membrane. In general, the actual insertion events are poorly characterized for those proteins that insert without the benefit of proteinaceous translocation apparatus, and structural studies are clearly required for an understanding of the conformational changes taking place.

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