Insertion of PsaK into the Thylakoid Membrane in a “Horseshoe” Conformation Occurs in the Absence of Signal Recognition Particle, Nucleoside Triphosphates, or Functional Albino3*

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The photosystem I subunit PsaK spans the thylakoid membrane twice, with the N and C termini both located in the lumen. The insertion mechanism of a thylakoid membrane protein adopting this type of topology has not been studied before, and we have used in vitro assays to determine the requirements for PsaK insertion into thylakoids. PsaK inserts with high efficiency and we show that one transmembrane span (the C-terminal region) can insert independently of the other, indicating that a “hairpin”-type mechanism is not essential. Insertion of PsaK does not require stromal extract, indicating that signal recognition particle (SRP) is not involved. Removal of nucleoside triphosphates inhibits insertion only slightly, both in the presence and absence of stroma, suggesting a mild stimulatory effect of a factor in the translation system and again ruling out an involvement of SRP or its partner protein, FtsY. We, furthermore, find no evidence for the involvement of known membrane-bound translocation apparatus; proteolysis of thylakoids destroys the Sec and Tat translocons but does not block PsaK insertion, and antibodies against the Oxa1/YidC homolog, Alb3, block the sec-dependent insertion but do not have an effect on PsaK insertion. Because YidC is required for the efficient insertion of every membrane protein tested in *Escherichia coli* (whether SRP-dependent or -independent), PsaK is the first protein identified as being independent of YidC/Alb3-type factors in either thylakoids or bacteria. The data raise the possibility of a wholly spontaneous insertion pathway.

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Studies in bacteria and plant thylakoids have demonstrated the operation of a complex signal recognition particle (SRP)*.

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Lhcb1 in thylakoids (13) and recent work has shown an E. coli homologue, YidC, to be essential for the insertion of at least some SRP-dependent membrane proteins in this organism (19).

Critically, YidC is also essential for the biogenesis of E. coli membrane proteins that do not require SRP or the Sec apparatus. The insertion mechanism of M13 procoat protein has been characterized in some detail (20) and this protein has been shown to insert spontaneously into the membrane, but depletion of YidC led to a rapid block in its insertion indicating a central role in their insertion. Thus, it appears that one pool of YidC may be associated with the Sec apparatus (21) while another pool may in effect represent a novel form of translocase dedicated for the insertion of some, if not all, SRP-independent membrane proteins. Like M13 procoat, a subset of thylakoid membrane proteins are synthesized with cleavable signal peptides but inserted in the absence of SRP (22, 23), but the possible involvement of the YidC homolog, Alb3, remains to be clarified.

All of the previous studies on thylakoid protein insertion have focused either on relatives of the well studied Lhcb1 (LHC proteins), or on proteins that bear cleavable N-terminal signal peptides. In this report we have sought to characterize the insertion of a different type of thylakoid membrane protein, PsaK, which is not synthesized with a cleavable signal peptide and which is unrelated to LHC proteins. We show that this protein inserts with high efficiency into thylakoids by a mechanism that does not involve SRP, NTP hydrolysis, or any of the known translocation machinery in the thylakoid membrane, including Alb3.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—A full-length cDNA clone encoding the precursor of barley PsaK (pPsaK) in the plasmid pBluescriptSK(−)− (gift from B. L. Moller (24)) was constructed as described in Ref. 24 and linearized with HindIII (30). Recent high-resolution crystallographic analyses of Synchococcus elongatus photosystem I that have confirmed these predictions for the cyanobacterial PsaK, which is highly similar to eukaryotic PsaK.2 This “horseshoe” configuration is illustrated in Fig. 1, which also indicates the locations of the methionine residues in the mature barley protein (two in the first transmembrane region, one in the second). The stroma-exposed loop region contains numerous positively charged residues and trypsin is therefore predicted to cleave in this region and generate two degradation fragments containing the indicated numbers of methionine residues. The amino acid sequence of pPsaK is also given in Fig. 1 together with the start site of a mature-size construct described below.

**Insertion of PsaK Does Not Require SRP or Nucleoside Triphosphates, but Is Stimulated by theDelta[PH]**—Fig. 2 shows a chloroplast import experiment conducted with [35S]methionine-labeled barley PsaK. The precursor protein (pPsaK) is imported into chloroplasts (lane C) where it is processed to the mature form and resistant to added protease (lane C+); the mature protein has an apparent mass of 7 kDa (in agreement with Ref. 24) and is found exclusively in the thylakoid fraction (lane T). The protein is highly resistant to urea extraction (lane Turea), which has been shown to be an effective means of identifying many membrane proteins (28). Trypsin treatment of the thylakoids from lane T (lane 1) leads to the generation of two fragments, one of which contains precisely twice as much [35S]radioactivity as the other (data not shown, from PhosphorImager analysis). On the basis of the data described above, the stronger, lower band most likely represents the first trans-
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tains essentially all of the SRP) cannot be omitted since it also contains almost all of the stromal peptidase required to generate the mature form of PsaK. The N terminus of the mature PsaK is located in the lumen, which means that processing to the mature size must precede insertion. We have found that the full precursor form does not insert correctly in the absence of stromal extract (see below), which is not surprising because the entire presequence would have to be translocated across the thylakoid membrane. Further experiments were therefore conducted with a mature size PsaK translation product synthesized from a truncated cDNA as described under “Experimental Procedures.”

Fig. 3B shows assays for the insertion of this construct (denoted PsaK) into isolated thylakoids. After each assay, samples of the thylakoids were either treated with trypsin (upper panel) or urea-extracted (lower panel). PsaK again inserts into isolated thylakoids and the inserted protein is again converted to the diagnostic degradation products TM1 and TM2 (“+ trypsin” panel). As a control, we incubated the translation product with trypsin in the absence of membranes (lane Tr+) and this procedure leads to an almost complete degradation of PsaK, with no evidence for either the TM1 or TM2 bands (providing further evidence that TM1 and TM2 represent membrane-integrated regions). In this assay, TM1 should contain three times as much [35S]Met as TM2 because of the initiation methionine introduced during the cloning procedure, which is located in the lumen after insertion and hence protected from proteolysis. In repeat assays such as those shown in Fig. 3B, the TM1:TM2 [35S]Met ratio was always near 3 (within 15% in each case) confirming correct insertion, and this is supported by the observation that the mature protein is equally resistant to urea extraction (“+ urea” panel). We have routinely found that PsaK inserts with very high efficiency in this assay system; 34% of translation product was found to be inserted in this particular experiment (based on the recovery of [35S]Met in TM1 and TM2) and insertion efficiencies of over 40% have been observed in other experiments (data not shown).

The left hand panel of Fig. 3B shows assays carried out in the presence of stromal extract (SE) or Hepes-magnesium buffer (HM), and in each of these cases insertion was assayed either with or without pretreatment of the assay mixture with apyrase (indicated as ± above the lanes). This enzyme hydrolyzes nucleoside triphosphates in the mixture and totally blocks insertion by the SRP route (9, 11). These lanes show that insertion is most efficient in the HM incubations, i.e. in the absence of stromal extract (and hence SRP). The presence of apyrase does, however, lead to a reduction in insertion efficiency (to 71% of the control value) but this is not due to SRP involvement because the presence of stromal extract also leads to a slight reduction in insertion efficiency (down to 81% of the HM value). Stromal extract contains essentially all of the SRP (see below) and this result rules out an SRP involvement.

In the same experiment we also assessed the effects of including GMP-PNP and AMP-PNP (non-hydrolyzable analogs of GTP and ATP, respectively) in an attempt to identify the cause of the reduction observed with apyrase. GMP-PNP, in particular, is an effective inhibitor of SRP (11). However, neither analog inhibits insertion to any marked extent, either in the absence or presence of stromal extract (right-hand panel in Fig. 3B).

As a control for these experiments we used insertion assays with Lhcb1, a known substrate for the SRP pathway. After insertion into thylakoids, Lhcb1 becomes highly resistant to trypsin digestion and a characteristic stable degradation product is generated (9, 11–13). Fig. 3C shows that insertion is completely dependent on stromal extract, as shown by the appearance of the degradation product in the SE panel. Insertion is also completely dependent on the presence of NTPs in the mixture after pretreatment with apyrase (lanes indicated by +) totally blocks insertion, as found previously (9, 11). On the basis of these data we conclude that PsaK does not require SRP for its insertion and, because insertion is not even stimulated by the presence of stroma, we propose that insertion is indeed completely independent of SRP. Insertion is, however, slightly inhibited by apyrase treatment even in the absence of stromal extract, which indicates a mild stimulatory influence of an ATP or GTP-hydrolyzing factor in the wheat germ translation system.

We believe the most likely explanation to be an anti-aggregation effect of chaperones in the wheat germ extract which may prevent some PsaK molecules from adopting an insertion-incompetent conformation.

The above studies rule out a central role for NTP hydrolysis in the insertion of PsaK but to obtain a more comprehensive picture of the energy requirements we tested whether the thylakoidal proton motive force stimulates insertion. Fig. 4A shows the effect of nigericin (a proton ionophore) on the import and sorting of pPsaK in intact chloroplasts. In the control assay, the protein is found exclusively in the thylakoid fraction, and only as the mature form. With nigericin present, however, some imported protein is found in the stromal fraction and it is notable that the primary stromal form is the full precursor protein. Some mature PsaK is also found in the stroma, together with a further, intermediate-size protein (iPsaK) that may represent a processing intermediate (alternatively, this may result from proteolysis of pPsaK). From these data it is evident that optimal insertion efficiency is dependent on the thylakoidal proton electrochemical gradient, $\Delta \mu_{H^+}$. The appearance of the full precursor protein, furthermore, suggests that iPsaK is not necessarily processed immediately upon entry into the stroma, and we suggest in addition that at least some molecules may be processed only during the later stages of the insertion pathway, since the appearance of iPsaK most likely stems from an inhibition of insertion.

To obtain further data on the $\Delta \mu_{H^+}$-dependence of insertion we conducted thylakoid insertion assays with PsaK in the presence of nigericin (Fig. 4B). The data show that the presence of nigericin (lanes N) reduces insertion efficiency to a moderate extent when compared with the control samples shown in lanes C (in this experiment by 20–25%) in both the presence and absence of stromal extract. Taken together, these data indicate that the $\Delta \mu_{H^+}$ stimulates PsaK insertion but is not essential.

**PsaK Insertion Does Not Require the Thylakoidal Sec Machinery or Alb3**—The data shown in Figs. 3 and 4 exclude the involvement of SRP or NTPs in PsaK insertion but it is equally important to understand whether membrane-bound translocation machinery is involved. The question of Alb3 involvement is critical because the homologous YidC protein is required for the efficient insertion of every E. coli membrane protein tested to date (19), and the SecYEG complex is also a prime candidate since this translocon is also used for some membrane proteins in bacteria (reviewed in Ref. 1). We addressed these possibilities in two ways. Previous studies (31) have shown that the insertion of Lhcb1 is totally inhibited by pretreatment of the thylakoids with trypsin, and the same study showed that translation of Sec-dependent luminal proteins is also completely blocked. This technique provides a simple means of destroying both the membrane-bound Sec apparatus and inhibiting integration by the SRP pathway. In previous studies using this approach we have maintained a $\Delta \mu_{H^+}$ by driving the ATP synthase in reverse in the dark (the synthase is highly resistant to trypsin) and we used the same method in this study since insertion of PsaK is stimulated by the $\Delta \mu_{H^+}$.
Fig. 4. Insertion of PsaK is stimulated by the thylakoidal ΔpH. A, intact pea chloroplasts equivalent to 50 μg of chlorophyll were incubated with 12.5 μl of in vitro translated pPsaK (lane Tr) in the absence (Control) and presence (+ Nigericin) of the proton ionophore nigericin (at 2 μM final concentration), for 20 min in the light. After the import incubation, the chloroplasts were washed, fractionated, and analyzed by SDS-PAGE and fluorography. Lanes C, total washed chloroplasts; lanes C+, thermolysin-treated chloroplasts; lanes S, stromal extract (prepared, as always, in the presence of 10 mM EDTA, to prevent residual thermolysin activity from degrading any stromal intermediates); lanes T, thylakoid membranes; lanes T+, trypsin-treated thylakoid membranes; pPsaK, precursor of PsaK; iPsaK, an intermediate form of PsaK; PsaK, mature protein; TM1 and TM2, degradation products corresponding to transmembrane spans 1 and 2, respectively. B, isolated pea thylakoids equivalent to 20 μg of chlorophyll were incubated with 5 μl of in vitro-translated PsaK (lane Tr), in the presence (SE) and absence (HM) of stromal extract, and in the presence (N) and absence (C) of 2 μM proton ionophore nigericin, for 20 min in the light. After the insertion incubation, the membranes were washed with HM and TB, before being extracted with urea (upper panel, + Urea). After urea extraction, the membranes were digested with trypsin (lower panel, + Trypsin). Urea-resistant mature protein is marked PsaK, while the protease degradation products corresponding to transmembrane spans 1 and 2 are marked TM1 and TM2, respectively. After the experiment the samples were analyzed by SDS-PAGE, and insertion efficiencies (shown below the lower panel, in %) measured by a PhosphorImager to calculate the amounts of urea-resistant mature PsaK relative to the control sample (HM, C).

Fig. 5. Predigestion of the thylakoid membranes with trypsin does not prevent insertion of PsaK. Thylakoid membranes were digested with 60 μg/ml trypsin (Trypsin) or buffer (Control) and the chloroplast ATPase was subsequently activated to generate a ΔpH in the dark, as described in detail in Ref. 31. The activated membranes (20 μg of chlorophyll) were incubated with 5 μl of in vitro translated PsaK, Plhcb1, or p23K (lanes Tr) in the dark for 30 min, with all manipulations being carried out under a dim, green, safe light. After the incubation, the membranes were washed with HM and reisolated. PsaK and Lhcb1 samples were washed further with TB, before being subjected to urea extraction (lanes T) and then trypsin digestion (lanes T+). 23K samples were analyzed directly after washing with HM (lanes T) or after digestion with 0.2 mg/ml thermolysin for 40 min on ice (lanes T+). All samples were analyzed by SDS-PAGE and fluorography. The insertion efficiencies of PsaK (values in %, relative to the control sample) were measured using a PhosphorImager. PsaK, mature protein; TM1 and TM2, trypsin degradation fragments corresponding to transmembrane helices 1 and 2 of PsaK; Plhcb1, precursor of Lhcb1; DP, trypsin degradation fragment of inserted Lhcb1; p23K, precursor of 23K; 23K, mature protein.

gradient (1), and the data show that in the absence of trypsin treatment this protein is indeed imported with high efficiency and processed to the mature size, in total darkness. This observation confirms the presence of a ΔpH, and import is abolished by trypsin treatment which has been shown previously to inactivate the Tat system (31). Insertion of Lhcb1 is also completely inhibited by this treatment; very little Lhcb1 is found associated with the thylakoids after the incubation (lane T of the “Trypsin” panel) and essentially no resistant degradation product is found after protease treatment (lane T+) and other
The specific question of Alb3 involvement was approached by more direct means. Preincubation of thylakoids with polyclonal anti-Alb3 antibodies almost blocks Lhcb1 insertion without affecting the Tat- or Sec-dependent pathways (13) and the same technique was used to test for its involvement in PsaK insertion. Fig. 6A shows a control assay using Lhcb1, in which insertion was monitored after incubation of the thylakoids with buffer (HM; as a control) with preimmune antibodies or anti-Alb3 antibodies. The preimmune serum causes a slight inhibition of insertion (to 89% of the control value) but the Alb3 antibodies reduce insertion efficiency to 27% of the control value. A similar level of inhibition was observed previously (13). In contrast, Fig. 6B shows that neither the preimmune nor the Alb3 antibodies affect insertion of PsaK into thylakoids, and the levels of urea-resistant protein or TM1 or TM2 degradation products remain undiminished. These data clearly indicate that PsaK is not dependent on Alb3 for insertion.

The C-terminal Transmembrane Span of PsaK Can Insert Independently—The above data show that PsaK inserts by a relatively simple mechanism that does not rely on any known translocation apparatus, including Alb3. This type of mechanism is highly unusual and we have sought to obtain further details on the overall insertion mechanism. One possibility, proposed for many membrane proteins (1), is that the two transmembrane spanning regions may form a "helical hairpin" that is able to insert with high efficiency due to the simultaneous partitioning of two hydrophobic regions. Several membrane proteins are known to form loop intermediates in which the loop region is on the trans side of the membrane, and similar principles may operate for those proteins, such as PsaK, where the loop remains on the cis side. We tested whether a single span of PsaK can insert independently into the thylakoid membrane, by simply using the full precursor protein instead of the mature PsaK construct, under conditions where cleavage by SPP is prevented. As explained above, the N terminus of mature PsaK lies in the lumen, hence cleavage of the large and highly charged presequence would appear to be essential before this N-terminal region can translocate across the membrane. We therefore carried out thylakoid import assays under two conditions: in the complete absence of stromal extract (using thylakoids that had been thoroughly washed to remove residual SPP), and after protease treating the thylakoids (to destroy any SPP on the membrane surface). Mature size PsaK was used as a control since this protein inserts under both sets of conditions as shown above.

The data (Fig. 7) show that the mature size PsaK (PsaK panel) behaves, as in experiments shown above, and the TM1 and TM2 fragments again appear with a labeling ratio which was calculated to be close to 3:1. Insertion occurs with both the washed and protease-treated thylakoids. However, very different results are obtained when the full precursor protein is used (pPsaK panel). The upper degradation fragment (TM2) is again observed after insertion into either washed or protease-treated thylakoids but the lower band (TM1) is now completely absent. A low-intensity smear of label is present below the TM2 band, presumably due to degradation of non-inserted PsaK regions, but no band is present in the TM1 region. We conclude from this result that the N-terminal transmembrane span is indeed unable to insert when the presequence is present, as predicted above, but the clear presence of TM2 is strong evidence that this region is able to insert independently under these conditions. These data also serve to reinforce the efficacy of the in vitro assay because they provide a third line of evidence that the bands denoted TM1 and TM2 do indeed represent inserted transmembrane regions; the N-terminal hydrophobic region is clearly highly susceptible to proteolysis (or is simply removed when the thylakoids are washed after the insertion reaction) when not inserted in the thylakoid membrane.

**DISCUSSION**

Several thylakoid membrane proteins have been previously analyzed in terms of insertion mechanism, and in this respect they fall into two broad categories. Lhcb1 follows a complex
pathway involving the input of numerous factors, both in the stroma and at the membrane surface, while several signal peptide-bearing proteins use an apparently simpler insertion mechanism that does not rely on any of the known protein machinery, although the issue of Alb3 involvement has yet to be addressed. PsaK is unlike any of the above proteins in that both the N and C termini are transported to the lumen, the protein is not synthesized with a signal-type peptide and it is not a member of the LHC superfamily of proteins.

The data from this study all point to a strictly SRP-independent insertion mechanism. Stromal extract contains essentially all of the SRP but is not required at any stage, and insertion does not depend at all on NTP hydrolysis. Both of these factors are critical for LhcB1 insertion. PsbY involvement can also be ruled out since this factor hydrolyzes GTP during its operating mechanism. We cannot rule out the possibility that other, as yet unidentified soluble factors may assist PsaK insertion, and the slight inhibitory effect of apyrase does raise the possibility that proteins in the soluble factors may assist PsaK insertion, and the slight inhibitory effect of apyrase does raise the possibility that proteins in the wheat germ translation system (e.g. chaperones) may aid insertion, but the data nevertheless indicate that the insertion of PsaK is fundamentally different from that of LhcB1. It is as yet unclear why LhcB1 is so dependent on SRP activity whereas other thylakoid membrane proteins studied to date are not.

We also find no evidence for the involvement of membrane-bound translocation machinery in PsaK insertion. Several studies have shown that trypsin treatment blocks the translocation of luminal Sec substrates, very strongly suggesting that the Sec apparatus is inactivated. This treatment slightly inhibits PsaK insertion (as indeed it does for PsbY (24)) but the effect is not marked and we conclude that the Sec translocon does not play a major role in this pathway. We also find no evidence for Alb3 involvement, since antibodies raised against Alb3 severely inhibit LhcB1 insertion yet have no effect on the insertion of PsaK. On the basis of these data alone we cannot exclude the possibility that PsaK may interact with Alb3 in a manner which is not affected by the antibodies used in this study. However, other ongoing studies in this laboratory (not shown) have demonstrated that Alb3 is completely degraded when thylakoids are treated with trypsin under the conditions used in this study (e.g. in Figs. 5 and 7) and, since this treatment barely affects PsaK insertion, we conclude that Alb3 is not required for PsaK biogenesis. This finding is significant because the homologous YidC protein plays a central role in the insertion of at least one SRP/Sec-independent membrane protein in E. coli (19). This factor has come to be regarded as a novel form of translocase in its own right, since the related Oxa1 protein also plays an important role in membrane protein biogenesis in yeast (17, 18) and there is no evidence as yet for additional subunits in the mitochrondrial Oxa1p complex. Our data thus indicate that PsaK is unique (to date) because it is the only Alb3/YidC-independent membrane protein among those analyzed in bacteria and plant thylakoids.

In general, the topology adopted by PsaK is consistent with the "positive-inside" rule (30) which states that positively-charged residues are found more frequently on the cis side of the membrane. The stroma-exposed loop region contains 4 basic residues whereas the C-terminal luminal tail contains only one and the N-terminal region does not contain any (24). However, it is presently unclear why insertion is stimulated to some extent by the thylakoidal \( \Delta \psi \), in bacteria and mitochondria, the extent of insertion of many membrane proteins is stimulated by the proton motive force (1, 17, 18) and the same applies to the Alb3-dependent insertion of LhcB1 in thylakoids (9, 11, 12). However, these effects probably reflect the harnessing of the \( \Delta \psi \) by the translocation machinery (the Sec and/or Oxa1-type apparatus) and these factors are not required for PsaK insertion. Possibly, there is a mildly stimulatory electrophoretic effect on the translocation of the C-terminal region of PsaK, which contains a single acidic residue. However, this point remains to be investigated in detail.

The Sec/Alb3-independent nature of the insertion mechanism raises the strong possibility that insertion of PsaK occurs spontaneously upon reaching the thylakoid membrane, and such mechanisms have been postulated before on theoretical grounds and following studies on membrane-interactive peptides and toxins (reviewed in Ref. 32). However, further work is required to address this possibility, and one argument against this possibility is that PsaK may then be able to interact with other membranes (e.g. the envelope) in the absence of a specific and dedicated targeting system. Possibly, PsaK is predisposed to insert only into thylakoid-type lipids, and the thylakoid membrane is indeed very unusual in terms of lipid composition, being composed primarily of galactolipids which are chemically very different to phospholipids (reviewed in Ref. 33). Further work is certainly required to determine whether such a lipid-based sorting process operates for PsaK insertion, or whether novel forms of translocation apparatus are involved.

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Insertion of PsaK into the Thylakoid Membrane in a "Horseshoe" Conformation Occurs in the Absence of Signal Recognition Particle, Nucleoside Triphosphates, or Functional Albino3
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