The Properties of the Positively Charged Loop Region in PSI-G Are Essential for Its “Spontaneous” Insertion into Thylakoids and Rapid Assembly into the Photosystem I Complex

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The PSI-G subunit of photosystem I (PSI) is an 11-kDa membrane protein that plays an important role in electron transport between plastocyanin and PSI and is involved in the stability of the PSI complex. Within the complex, the PSI-G subunit is bound to PSI-B and is in contact with Lhca1. PSI-G has two transmembrane spans connected by a positively charged stromal loop. The loop is inaccessible to proteases, indicating a tightly bound location within the PSI complex. Here, we have studied the insertion mechanism and assembly of PSI-G. We show that the protein inserts into thylakoids by a direct or “spontaneous” pathway that does not involve the activities of any known chloroplast protein-targeting machinery. Surprisingly, the positively charged stromal loop region plays a major role in this process. Mutagenesis or deletions within this region almost invariably lead to a marked lowering of insertion efficiency, strongly indicating a critical role for the loop in the organization of the transmembrane regions prior to or during membrane insertion. Finally, we have examined the assembly of newly inserted PSI-G into the PSI complex, since very little is known of the assembly pathway for this large multimeric complex. Interestingly, we find that inserted PSI-G can be found within the full PSI complex within the import assay time frame after insertion into thylakoids, strongly suggesting that PSI-G normally associates at the end of the assembly process. This is consistent with its location on the periphery of the complex.

Different pathways of translocation allow nucleus-encoded chloroplast proteins to be imported and sorted to their final destinations in the chloroplast. First, precursor proteins containing envelope transit peptides are recognized and translocated by the Toc (translocon at the outer thylakoid) pathway or the Sec machinery, utilizing ATP hydrolysis (3). Thylakoid membrane proteins are inserted by different mechanisms: the SRP (signal recognition particle) pathway or the apparently “spontaneous” pathway. The SRP pathway appears to be a specialized mechanism for the insertion of many members of the light-harvesting complex superfamily and requires binding of SRP to the substrate, GTP hydrolysis, and the assistance of proteins FtsY and Alb3 (4). By contrast, the “spontaneous” insertion pathway operates without detectable assistance from other proteins and in the absence of both the thylakoidal ΔpH or nucleotide triphosphate hydrolysis (5–8).

Here, we have studied the mechanism of insertion of PSI-G into the thylakoid membrane. PSI-G is an 11-kDa peripheral subunit of photosystem I (PSI) and is homologous to the PSI-K subunit; it has two transmembrane spans connected by a positively charged stromal loop (7, 9, 10). PSI-G is involved in the regulation of electron transport from plastocyanin to the reaction center P700, and it stabilizes the PSI complex (11). Study of the homologous PSI-K subunit, which has a similar structure, revealed that PSI-K inserts “spontaneously” into the thylakoid membrane (7). The topology of PSI-G was difficult to solve, because the stromal loop strongly resists degradation by proteases, unlike that of PSI-K (7, 12). However, study of the insertion mechanism and the role of the positive charges present in the loop suggest interesting features. Insertion of PSI-G does not require proteins present in stromal extract or hydrolysis of NTPs, suggesting that SRP and its partners FtsY and Alb3 are not involved. Furthermore, PSI-G inserts into trypsin-treated thylakoids, indicating that the insertion of PSI-G does not need stromally exposed protein factors such as those involved in the Sec and Tat pathways and strongly suggesting that PSI-G inserts directly into the thylakoid membrane.

Previous work on “spontaneously” inserting proteins did not investigate the influence of positive charges on the insertion in the membrane (5–7, 13). We investigated the influence of the number of positive charges present in the loop upon the insertion of PSI-G in the membrane. Altering the number and distribution of positive charges clearly

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2 The abbreviations used are: PSI, photosystem I; Chl, chlorophyll; SRP, signal recognition particle; TM, transmembrane; WT, wild type; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethyl]propane-1,3-diol.

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affected the topology and insertion of PSI-G. Correct insertion of PSI-G depends on intrinsic constraints on the cis and trans sides of the membrane. Furthermore, two-dimensional blue native gel analysis of thylakoid membranes after chloroplast import showed that the newly inserted, radiolabeled PSI-G is assembled into PSI immediately after insertion.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—A full-length cDNA clone encoding the precursor of Arabidopsis PSI-G (pPSI-G) and the mature size protein (PSI-G) were previously cloned in the Promega pGEM4Z vector (12). Mutagenesis of this construct was carried out using the QuikChange method (Stratagene, La Jolla, CA), resulting in the mutants shown in Fig. 4A. Each PCR product was digested with restriction enzyme DpnI before transformation of Escherichia coli in the presence of [3H]leucine (Amersham Biosciences) according to the protocol described (13). The plasmids were sequenced to verify the mutations and integrity of the PSI-G coding sequence.

**In Vitro Import Assays**—The various PSI-G constructs were transcribed in vitro using SP6 RNA polymerase and then translated in a wheat germ lysate system (Promega GmbH, Mannheim, Germany) in the presence of [3H]leucine (Amersham Biosciences) according to the manufacturer’s instructions. Intact pea chloroplasts were isolated from pea seedlings (var. Kelvedon Wonder), and assays for in vitro import of precursor proteins into intact pea chloroplasts and isolated thylakoid membranes were essentially as described (14) except that the light intensity during import was 300 μmol of photon/m²/s. For the nigericin treatment, the proton ionophore nigericin (Sigma) was dissolved in ethanol and used at a final concentration of 2 μM in the presence of 10 mM KCl; control samples were identical, except they contained an equivalent amount of ethanol instead of nigericin. Assays to measure the effect of apyrase (Sigma, type VI) were carried out as described (15). All of the samples were analyzed by SDS-PAGE and fluorography.

**Postimport Treatment of Thylakoid Membranes**—In order to test whether PSI-G was correctly inserted in the thylakoid membrane (either after import into intact chloroplasts or after insertion into isolated thylakoid membranes), thylakoid membranes (between 10 and 20 μg of chlorophyll (Chl) depending on the experiment) were washed with 0.5 ml of ice-cold 10 mM Hepes-KOH, pH 8, 5 mM MgCl₂ (HM) and resolated by centrifugation at 18,000 g for 5 min in a microcentrifuge. Next, the membranes were resuspended in HM and digested with 0.2 mg/ml trypsin (Sigma, type XIII) in a final volume of 100 μl for 30 min on ice. Trypsin digestions were stopped by the addition of 0.5 mg ml⁻¹ trypsin inhibitor (Sigma, type I-S), followed by centrifugation at 18,000 × g at 4 °C for 10 min in a microcentrifuge. Finally, the thylakoid membranes were resuspended in 15 μl of HM containing 5 μg of trypsin inhibitor and an equal volume 2× SDS-PAGE loading buffer and then immediately boiled for 5 min. Urea washes of thylakoids were performed using 6.8 M urea, according to a protocol adapted from Ref. 16 and described in detail (15). Insertion efficiencies were analyzed using fluorography and quantification of the resulting protein bands using gel scanning densitometry.

**Proteolytic Treatment of Thylakoid Membranes before Insertion Assays**—The protocol was as described (5) with the following modifications. Isolated thylakoids were resuspended in HM buffer containing 25 mM KCl and 10 mM dithiothreitol. The thylakoids were exposed to light of intensity 220 μmol of photon m⁻² s⁻¹ for 8 min at 4 °C in order to restore the ΔpH and then washed and resuspended in HM buffer for trypsin treatment. After treating the thylakoids with 60 μg/ml trypsin (Sigma, type XIII) for 10 min on ice, the digestion was stopped by the addition of 120 μg/ml trypsin inhibitor (Sigma, type I-S), and the thylakoids were resolated by centrifugation at 20,000 × g for 10 min at 4 °C. The thylakoids were washed twice in HM containing 60 μg/ml trypsin inhibitor, centrifuged at 20,000 × g for 5 min at 4 °C, and finally resuspended in stromal extract or HM buffer. Each sample contained thylakoids equivalent to 20 μg of Chl, 0.5 mM MgATP, and 10 μl of translation mixture, all buffered by HM (final volume, 50 μl). Stromal extract, when present, was at a concentration equivalent to 1.3 times the Chl concentration. The thylakoid import assay was carried out under a green safe light, for 30 min at 26 °C.

**Two-dimensional Blue Native Gel Analysis**—After import of pPSI-G into chloroplasts, the organelles were fractionated into stromal extract and thylakoid membranes. The thylakoid fraction was centrifuged at 1700 × g for 5 min in a cooled microcentrifuge. The pellet was resuspended in ACA buffer (750 mM aminocaproic acid, 50 mM bis-tris, pH 7, 0.5 mM EDTA) to a final concentration of 2 mg/ml. An equal volume of 2% (w/v) β-dodecyl maltoside in ACA buffer was added, resulting in a final concentration of 1% β-dodecyl maltoside. The sample was solubilized for 20 min on ice. After centrifugation at 20,000 × g for 30 min at 4 °C, the supernatant was transferred to a fresh tube, and one-quarter of the final solubilized volume of loading buffer (5% (w/v) Serva blue G, 750 mM aminocaproic acid, 35% sucrose) was added to the solubilized sample. To separate the thylakoid complexes according to size (first dimension gel), solubilized thylakoids corresponding to 20 μg of Chl were loaded per lane on a 6—13.5% blue native polyacrylamide gel (17). After the first dimension run, the lane was cut out of the gel and submerged in denaturing solution (2% (w/v) SDS, 2% (w/v) dithiothreitol) and denatured for 25 min at 60 °C. The denatured blue native PAGE lane was placed on top of a 12% SDS-polyacrylamide gel, and the proteins were separated according to size.

**RESULTS**

**ATP Requirement for PSI-G Insertion**—In general, membrane proteins are inserted into the thylakoid membrane by two mechanisms: an SRP-dependent mechanism that requires stromal extract containing SRP and NTPs required for the action of both SRP and FtsY or the “spontaneous” pathway (1). In order to investigate the mechanism of insertion of PSI-G, direct thylakoid insertion assays were performed under varying conditions. For these assays, we used a mature sized radiolabeled PSI-G, because the extensive N-terminal envelope transit peptide would presumably interfere with the translocation of the N-terminal region across the membrane.

Thylakoids and the wheat germ translation mixture were preincubated with apyrase in order to hydrolyze all NTPs present. Thylakoid insertion assays were performed in the presence of stroma (+S), in the absence of stroma (−S), and in the presence of stroma depleted of NTPs with apyrase (+S+Ap) (Fig. 1). As a positive control for apyrase activity, we analyzed the Sec-dependent import of the 33-kDa oxygen-evolving complex protein (33K), whose import into thylakoids is totally dependent on ATP hydrolysis (18). It is located in the lumen, so the protein is protected from the trypsin treatment of thylakoid membranes (Fig. 1C, T⁺ lanes). We used the intermediate form of 33K (i33K), which lacks the envelope transit peptide, for thylakoid imports in the presence of apyrase. During the import of i33K into thylakoids, a signal peptide was removed to yield the mature form of the protein, 33K (Fig. 1C, lane +S). The translocation of i33K through the membrane was blocked when stromal extract was treated with apyrase (Fig. 1C, lane +S+Ap), indicating that the apyrase had hydrolyzed the stromal NTPs. As a further control, we imported the Tat substrate p23K, a luminal subunit of the oxygen-evolving complex, which is transported in the complete absence of NTPs (18). Fig. 1B shows a trypsin-resistant, mature sized band in all
Membrane Insertion of PSI-G

FIGURE 1. Insertion of PSI-G is not dependent on nucleotide triphosphates. A, fluorography of SDS-polyacrylamide gels after thylakoid import of PSI-G. DP, the degradation product of PSI-G. B, p23K, which is known to be transported in the absence of NTPs. C, I33K, whose transport is known to be totally dependent on NTP hydrolysis. Thylakoid proteins corresponding to 30 μg of Chl were resuspended either in stroma (+ S) or HM buffer (− S) or in stroma containing 2 units of apyrase (+ S + Apy). Apyrase was added to the translation mixture separately before the thylakoid import assay. After incubation for 10 min on ice, the import mixtures were transferred to an illuminated water bath, and experiments were carried out as described under “Experimental Procedures.” Samples were analyzed directly (T) or after trypsin treatment of the thylakoid membrane (T +). The translation mixture (Tr) was digested by trypsin (Tr +) to distinguish the different degradation products between the inserted and noninserted proteins.

of the T + tracks, indicating that 23K was correctly processed and transported into the lumen. A similar pattern is observed in the case of PSI-G (Fig. 1A); a trypsin-resistant, mature sized band is visible in each of the T + tracks, suggesting that PSI-G can insert into the membrane in the presence or absence of stromal extract and in the absence of hydrolyzable NTPs. The stromal loop connecting the two transmembrane spans of PSI-G is unusually resistant to trypsin treatment (12), in contrast to the homologous PSI-K, for which trypsin digestion of the membrane-inserted protein results in two distinct degradation products (7). The decrease of signal between the T and T + lanes is due to the fact that the signal in the T lane is composed of both noninserted (peripherally bound) and inserted proteins. The noninserted fraction was associated with the membrane, probably due to the high hydrophobicity of PSI-G. This fraction was digested to a degradation product DP when the thylakoids were treated with trypsin. The remaining fraction of PSI-G in the T + lanes was the protein that was inserted in the thylakoid membrane. Overall, this experiment demonstrates that stromal proteins and NTPs are not required for the insertion of PSI-G into the thylakoid membrane.

Does PSI-G Insertion Require the Proton Gradient?—To further analyze the energetics of PSI-G targeting, we tested whether its thylakoid insertion is stimulated by the proton gradient across the thylakoid membrane. Intact chloroplast import assays were performed in the presence or absence of nigericin (Fig. 2), a proton ionophore that dissipates the proton gradient (∆pH). We compared the insertion of PSI-G with that of pHcb1 (the major light-harvesting complex apoprotein; Fig. 2B), which is a substrate for the chloroplast SRP pathway and whose insertion is partially inhibited when the ∆pH is dissipated (19). In the presence of nigericin, insertion of Lhcb1 into the thylakoid membrane (lane T +) was reduced by ~44% (as quantified by densitometry), in agreement with previously published data (19, 20). Reduction in the amount of thylakoid inserted protein was accompanied by an accumulation of Lhcb1 in the stromal fraction (Fig. 2B, + nigericin, lane S). By contrast, the insertion of PSI-G was unaffected by the presence of nigericin (compare A, + nigericin, lane T +). Similarly, nigericin had no inhibitory effect upon the insertion of PSI-G into isolated thylakoids (data not shown). This demonstrates that the insertion of PSI-G does not depend upon the proton gradient. The nigericin completely blocked insertion of the 23K standard Tat substrate (not shown) as found in previous work (19, 21).

Does PSI-G Use a Translocase for Its Insertion in the Thylakoid Membrane?—It has been shown previously that the Sec, Tat, and SRP pathways can all be blocked by pretreating isolated thylakoids with trypsin (7, 8, 15). As another means to test whether PSI-G utilizes any of these pathways, thylakoid membranes were treated with trypsin (lanes T +), washed, and resuspended in stroma containing trypsin inhibitor before the thylakoid import assay of mature PSI-G (Fig. 3A). As controls, pPsbW (Fig. 3B), p23K (Fig. 3C), and p33K (Fig. 3D) were also incubated with the trypsin-pretreated thylakoids. p23K is a substrate for the Tat translocase and as shown above requires the proton gradient (∆pH) for its translocation through the thylakoid membrane. Trypsin-pretreated thylakoids can generate a proton gradient in the dark if the chloroplast ATPase is first activated by bright illumination and reduction (by dithiothreitol) and then supplied ATP during the assay (14). This proton gradient supports the import of p23K into non-trypsin-treated thylakoids in the dark (Fig. 3C, Control, lane T +), but import is abolished by the trypsin pretreatment (Trypsin, lane T +). Similarly, transport of the Sec substrate, p33K, into the thylakoid lumen is prevented by trypsin pretreatment of thylakoids (Fig. 3D, Trypsin, lane T +). The well-characterized “spontaneous” pathway substrate, pPsbW (6, 8, 13, 21).
WT precursor PSI-G (pPSI-G) was imported, and all of the imported koid fraction was either digested with trypsin or washed with urea to plasts were fractionated into stroma and thylakoid samples. The thylakoid fraction was either digested with trypsin or washed with urea to determine the topology and insertion state of the different proteins. The WT precursor PSI-G (pPSI-G) was imported, and all of the imported protein was found in the thylakoid fraction (Fig. 4A, B–I, lane T), where it is resistant to proteolysis. In addition to the use of fractionation and proteolysis as criteria for efficient/correct insertion, we used urea washes, since these washes have previously been shown to effectively remove peripherally bound protein and membrane-bound protein containing a single transmembrane domain (7, 16) (Fig. 4B). It has been shown that single span proteins can be extracted by urea, and more than 50% of such proteins are recovered in the supernatant after the urea wash. However, proteins with two transmembrane (TM) spans are almost completely resistant to extraction (7).

The G1 mutant has two of the six positive charges removed; Arg\textsuperscript{88} and Lys\textsuperscript{93} were substituted by alanines. Arg\textsuperscript{88} and Lys\textsuperscript{93} are in the loop close to the first transmembrane span of PSI-G (Fig. 4A). This mutant was efficiently imported into the chloroplast and found in the thylakoid, like the WT protein (Fig. 4B, II). However, some mature protein was found in the stroma, suggesting that insertion was partially inhibited. Interestingly, the thylakoid-associated G1 was totally sensitive to trypsin (Fig. 4B, II, T + lane), and more than 50% of the protein was extractable by urea (US and UP lanes). This strongly suggests that some of the thylakoidal PSI-G had not inserted correctly into the membrane, since the presence of two TM spans in the WT proteins renders it essentially fully resistant to extraction. Among this population, it is not possible to determine whether the protein is peripherally bound or inserted by means of one of the two TM spans. The complete trypsin sensitivity of the stroma-resistant protein suggests that removal of the two positive charges has disrupted the protein-protein interactions that protect this region from digestion by trypsin.

The G2 mutant has one less positive charge compared with WT (Fig. 4A). The positive charge close to the second transmembrane span (Lys\textsuperscript{120}) was mutated into asparagine, and the Asp\textsuperscript{123} was mutated into an alanine, leading to an overall +5 charge in the loop of PSI-G. After import into chloroplast, the G2 protein in the membrane appeared totally sensitive to trypsin, which again suggests that changing the number of positive charges prevented some protein-protein interactions, as for the G1 mutant (Fig. 4B, III). Approximately half of the G2 protein was extracted by urea wash, suggesting that the protein was not fully inserted into the membrane, with perhaps just one of the transmembrane spans inserted.

In the G3 mutant, 12 amino acids in the loop were deleted (Δ102–114), generating a PSI-G mutant with only three positive charges in total in the loop region (Fig. 4A). The imported G3 protein was present in the

**FIGURE 4. Insertion of PSI-G depends on the positive charges present in the loop. A, schematic representation of PSI-G and the mutants of PSI-G. The transmembrane spans are represented by rectangles. The positive charges and their approximate location in the loop region and their approximate location in the loop region are indicated (\textcolor{red}{+}), 8, in vitro chloroplast import assays of pPSI-G and the various mutants. pPSI-G and the different pPSI-G mutants (pG1–pG5) were translated in vitro for chloroplast import assays (7). After the import assay, chloroplasts (C) were digested with thermolysin (C +) to degrade the nonimported proteins. Then chloroplasts were fractionated into stroma (S) and thylakoid membrane (T) fractions. The thylakoid membranes were divided into batches; one was treated with trypsin (T +), and the other was washed with 6.8 M urea as outlined under “Experimental Procedures.” C, summary table of results obtained in B.**
Membrane Insertion of PSI-G

thylakoids and was not found in the stroma, suggesting that deleting the most charged portion of the loop does not inhibit insertion (Fig. 4B, IV). The thylakoid-associated PSI-G was totally sensitive to trypsin, again suggesting that the protein-protein interactions that normally protect the loop from trypsin were disrupted. The G3 mutant was mostly resistant to urea extraction, which suggests that both helices are inserted in the thylakoid membrane.

The G4 mutant of PSI-G has one extra positive charge in the loop (Gly108 → Arg), leading to seven positive charges in total (Fig. 4A). This mutant has unexpected properties. First, a significant proportion of imported protein was found in the stroma, suggesting that insertion into thylakoids was less efficient (Fig. 4B, V). Second, more than half of the thylakoid-inserted protein was removed by urea, strongly indicating that these molecules have not inserted correctly. One possibility is that this protein population is only peripherally bound and thus easily extracted; the other main scenario is that only one of the two TM regions has inserted. In either case, the data clearly indicate that mutations in the stromal loop can severely affect insertion properties.

A mutant PSI-G protein with an extended C terminus of random 20 amino acids containing one positive charge (GDESTC9BHASLSPYSESY) (G5 mutant protein) was generated (Fig. 4A) and imported into chloroplasts. The G5 protein is trypsin-sensitive, and less than 50% was found in the supernatant after urea extraction, meaning that proper insertion into the membrane was restricted (Fig. 4B, VI). Presumably, one of the transmembrane spans was not properly inserted. This confirms the stromal location of the PSI-G loop, and the insertion of one TM in the G5 mutant shows that the transmembrane spans can insert independently.

To summarize the imports of different positive charge mutants of PSI-G, the chloroplast import results of mutants G1, G2, G4, and G5 are all sensitive to trypsin digestion and are extractable by urea (Fig. 4C). Additionally, G1 and G4 accumulate to some extent in the stromal fraction, suggesting that the rate of thylakoid insertion has been impeded. The summarized data in Fig. 4C provide strong evidence that one TM span can insert independently of the other. Only the G3 mutant protein has both TM spans inserted to the thylakoid membrane even if the loop is protease-sensitive (Fig. 4, B (IV) and C). We conclude from these data that the PSI-G loop is engaged in protein-protein interactions that involve the positive charges of the loop. The three positive charges of PSI-G (Arg98, Lys93, and Lys120), located close to the predicted transmembrane helices, seem to play a role in the insertion of the transmembrane spans in the thylakoid membrane.

Newly Imported PSI-G Is Assembled into the PSI Complex Immediately after Insertion into the Membrane—The loop of PSI-G is largely inaccessible to trypsin after import into the chloroplast or insertion into isolated thylakoids. This might indicate that PSI-G is immediately assembled into the PSI complex after insertion into the thylakoid membrane, and this in turn is of interest because very little is known of the PSI assembly pathway. In order to analyze this more closely, we performed chloroplast import assays and subsequently separated the stroma (not shown), thylakoid membranes (T), and trypsin-treated thylakoid membranes (T +) by two-dimensional blue native gel electrophoresis (Fig. 5). After a 20-min import assay, the thylakoid membrane complexes were gently solubilized and separated according to complex size in the first dimension. The first dimension lane was excised, denatured, and separated by SDS-PAGE, constituting the second dimension. No specific signal from PSI-G was detected in the stroma fraction (data not shown). However, the autoradiogram clearly shows two spots corresponding to PSI-G in the region of the gel where we expect to find the PSI-LHCl and the PSI core. Thus, the newly imported PSI-G was very rapidly assembled into the PSI complex, and the newly assembled protein was protected from trypsin treatment.

**DISCUSSION**

In this study, we are using a heterologous system where we import PSI-G from *Arabidopsis thaliana* into pea chloroplasts. PSI-G is a well conserved protein of PSI; within the dicots, PSI-G reveals 91–93% amino acid identity, and when the *Arabidopsis* sequence is compared with monocots, it reveals 80–85% identity (supplemental Fig. 1 and Table I). The available sequences of PSI subunits from pea are between 88 and 98% identical to the corresponding *Arabidopsis* PSI subunits (supplemental Table II). Thus, the PSI complex is extremely well conserved between species and justifies the use of pea chloroplasts for the study of topology and mechanism of insertion of the *Arabidopsis* PSI-G subunit.

The insertion of PSI-G in the thylakoid membrane in the absence of NTPs or stroma excludes an involvement by the Sec and SRP pathways, and the data strongly suggest that soluble stromal proteins do not assist the insertion process. Moreover, PSI-G can insert into thylakoid membranes when the ΔpH has been dissipated and when the known proteinaceous translocases have been digested by trypsin, which excludes the Tat pathway. The insertion mechanism of PSI-G thus seems similar to that used by its homolog PSI-K (7), and both proteins are strongly predicted to use the “spontaneous” insertion pathway. These are additional examples of “spontaneous” insertion of a thylakoid membrane protein. A number of single spanning membrane proteins were previously described to insert “spontaneously”: CFoII (5), PsbX (26), PsbW (6), and PsbY, a four-span precursor polyprotein, also use this pathway (15).

A similar mechanism of insertion has recently been described for the first time in mammalian cells (27). This study shows that the insertion of the cytochrome b5 into the endoplasmic reticulum is apparently “spontaneous.” The lack of requirement for an energetic driving force in the insertion of PSI-G and other previously described thylakoid membrane proteins is unexpected. PSI-K can insert into artificial liposomes in *vitro*, which suggests that there is no protein assistance for its inser-

2 C. Robinson, unpublished data.
tion into the membrane. One possible hint might be the lipid composition of the thylakoid membrane. The thylakoid membrane is exceptionally rich in galactolipids (80% of the total lipids), which could create a more fluid environment for the "spontaneous" insertion of proteins. In general, however, the targeting of these "spontaneously" inserted proteins to the correct membrane remains unclear.

In the case of PSI-G, the assembly of PSI-G into the PSI complex is extremely rapid in that the process occurs within the import assay time frame. However, very little is known about PSI assembly. The two-dimensional gel analysis reveals that the imported PSI-G in the chloroplast is probably all assembled into PSI, suggesting that PSI-G is assembled in the later phases of assembly of the PSI complex. A similar approach has been used before (28), where blue native gel electrophoresis after chloroplast import could reveal the location and assembly of two photosystem II subunits, PsbW and PsbS, which displayed slightly different routes into photosystem II. A study of photosystem II assembly was recently carried out by a pulse-chase approach (29). Under specific conditions consisting of a 2-h pulse on spinach leaf discs at low light intensity, the authors could detect significant turnover of photosystem II and also some turnover of PSI-G, PSI-K, and the LHca proteins but not the rest of the PSI core complex. The data obtained in Ref. 29 and the present study may imply that, under certain conditions, the PSI-G subunit is turned over faster than the rest of the PSI complex and that it is replaced more frequently than the rest of the core formed by the PSI-A, -B, -C, and -D subunits. It is also possible that the interaction between PSI and PSI-G drives the insertion of PSI-G into the thylakoid membrane. However, immunoblot analysis of the barley mutant *viridis* z*b63*, which has only 5–8% PSI compared with wild type, reveals that PSI-G is present in amounts corresponding to about 30% of wild type. This apparent surplus of PSI-G is present in the thylakoid and is possibly associated with LHCl, the peripheral light-harvesting complex of PSI, which accumulates in wild type amounts in the mutant thylakoids (30, 31).

One of the most interesting findings of this study relates to the positively charged stromal loop region. Whereas studies on membrane protein insertion generally highlight the role and importance of the TM spans, this connecting loop of PSI-G plays a major role in the insertion of the protein in the thylakoid membrane. All of the mutants of PSI-G generated in this study present an identical hydrophobicity profile when compared with WT PSI-G (data not shown). They are all predicted to have the loop on the stromal side of the membrane, in agreement with the positive inside rule (25). Nevertheless, there are clear indications that all of these mutants are dramatically affected in terms of insertion; all are urea-extractable to a significant extent, whereas the wild-type protein is fully resistant to extraction from the thylakoid membrane. These data strongly indicate that a population of each mutant protein is anchored by only one TM span, which in turn suggests that the mutations in the loop region have affected the ability of the other TM span to insert.

Whereas the wild-type protein is highly resistant to digestion by trypsin, it is notable that all of the mutant forms are completely sensitive to the same proteolysis regime. This provides further evidence for the idea that this loop in the wild type PSI-G subunit is tightly bound within the PSI complex. In the PSI crystal structure from pea, PSI-G has been assigned with a stromal loop that could possibly interact with PSI-B (10). However, a model based on a resolution of 4.4 Å does not provide structural information concerning the amino acid side chains and their possible interaction with PSI-B (10, 32). At present, we also cannot rule out that an unknown protein protects the loop of PSI-G from trypsin in *vitro*.

In the case of the G3 mutant, changing several of the positive charges (Δ102–114) does not affect the insertion of the protein into the thylakoid membrane. It strongly suggests that there is a strong electrostatic interaction between PSI-G loop and PSI-B subunit or other proteins, resulting in the protease resistance of the PSI-G loop (12). The G1, G2, G4, and G5 mutants cannot insert into the membrane like wild-type PSI-G. One or none of the transmembrane spans is inserted, showing that the transmembrane spans can insert independently as for the PSI-K subunit (7). These data suggest that the PSI-G loop might adopt a tight conformation before the insertion process. In the primary sequence of the loop region, there seems to be a tight balance between the positive and the negative charges. It is therefore possible that the loop forms a tight conformation by making ion bridges between negatively charged amino acid and positively charged amino acids, as indicated in Fig. 6. The G4 mutant is highly interesting, since one extra charge in the loop disturbs the insertion of the protein, suggesting that the G4 protein cannot form the tight conformation before the insertion process. These mutant study data are consistent with the import of tagged PSI-G (12). When a His or Strep tag is inserted in the loop of PSI-G, the protein inserts into the thylakoid membrane after import, but it is sensitive to trypsin (12), and it is partially extractable with urea, suggesting that insertion is affected by the presence of the tag in the loop. When the tags are present in the C terminus of PSI-G, the imported protein is trypsin-resistant and not extractable by urea washes, indicating that both of the TM are inserted into the membrane. The G5 mutant has a 20-amino acid extension on the C terminus containing one positive charge. The G5 mutant is urea-extractable, suggesting that only one transmembrane span is inserted. The C-terminal extension of the G5 protein contains one positive charge just like the Strep-tagged PSI-G, but it is 12 amino acids longer, suggesting that it is the length of the C-terminal extension that inhibits insertion of the last transmembrane span.

The data from the *in vitro* import experiments of the PSI-G constructs with mutated amino acids in the loop region suggest that the loop adopts a specific conformation stabilized by the alternating positive and negative amino acids in the loop, which facilitates its insertion into the membrane. We propose a model for the role of the different positive charges in the loop of PSI-G (Fig. 6). The three positive charges

![Model of the insertion of PSI-G into the thylakoid membrane](image-url)
present near the transmembrane spans (Arg\textsuperscript{88}, Lys\textsuperscript{93}, and Lys\textsuperscript{120}) and mutated in the G1 and G2 mutants (indicated by \textsuperscript{2} in Fig. 6A) seem to play a major role for the insertion of the transmembrane spans into the thylakoid membrane. This is consistent with previous observations (33) proposing that the side chains of lysine residues flanking the transmembrane spans may bury themselves in a membrane bilayer via a "snorkeling" process. This could explain why the G1 and G2 mutants cannot insert into the thylakoid membrane properly. The three positive charges absent in the G3 mutant (indicated as \textsuperscript{1} in Fig. 6A) could play a role in the protein-protein interaction between PSI-G and PSI-B and the proper interaction of PSI-G within the PSI complex. However, these three positive charges absent in G3 do not seem to be essential for the insertion of the protein itself into the thylakoid membrane.

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