

# The N-Terminal Hydrophobic Region of the Mature Phosphate Translocator Is Sufficient for Targeting to the Chloroplast Inner Envelope Membrane

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To locate the sequence required for directing the phosphate translocator to the chloroplast inner envelope membrane, a series of chimeric proteins constituting parts of the phosphate translocator and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, which is normally located in the stroma, has been produced. Reciprocal exchanges of the presequences and mature sequences of the phosphate translocator and the small subunit indicated that the phosphate translocator presequence contains stromal targeting information and that the mature protein is responsible for inner envelope membrane targeting. Chimeric proteins containing the N-terminal 46 amino acid residues of the phosphate translocator were directed to the inner envelope membrane. Subdivision of this region into its composite hydrophilic and hydrophobic regions showed that the hydrophobic region alone, which consists of amino acid residues 24 to 45, was able to direct the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase to the inner envelope membrane.

## INTRODUCTION

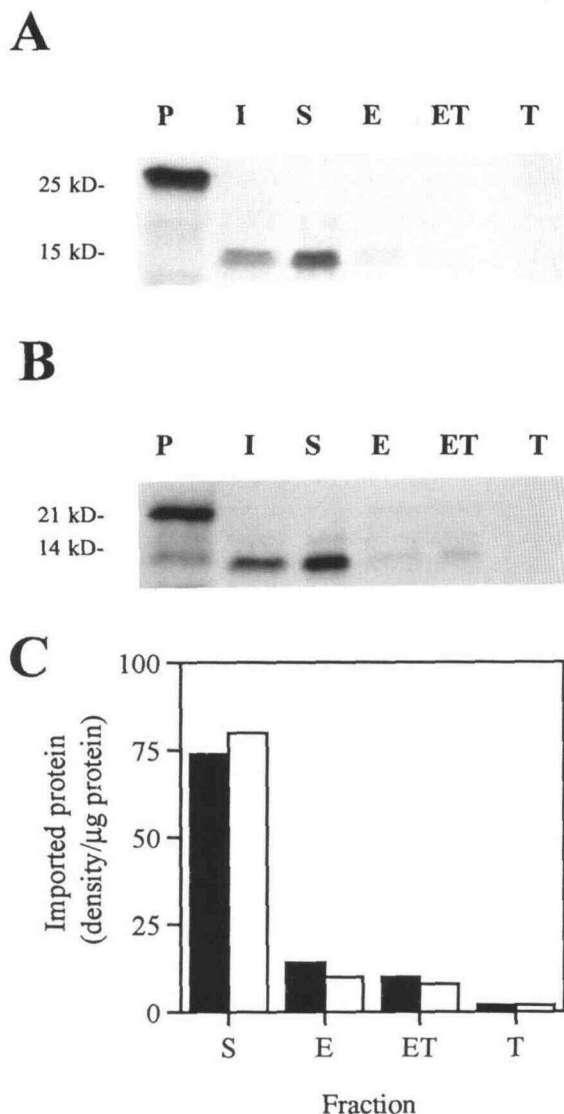
Chloroplasts are complex organelles consisting of six compartments, including the outer and inner envelope membranes, the intermembrane space, the stroma, the thylakoid membrane, and the thylakoid lumen. Consequently, information is required not only for the targeting of nuclear-encoded proteins to the chloroplast but also for sorting to the correct intraorganellar location. The majority of nuclear-encoded chloroplast proteins are synthesized as higher molecular mass precursors with an N-terminal presequence (Keegstra et al., 1989). The presequence directs the protein to the chloroplast surface, where it interacts with a proteinaceous receptor in an energy-dependent manner (Cline et al., 1985) and is subsequently translocated across the double envelope membrane. Two approaches, cross-linking of precursors to the import apparatus (Perry and Keegstra, 1994; Wu et al., 1994) and isolation of translocation complexes (Waegemann and Soll, 1991; Schnell et al., 1994), have enabled the identification of several of the components of the chloroplast import machinery. Maturation of imported protein occurs in the stroma and is catalyzed by the stromal processing peptidase (Oblong and Lamppa, 1992).

Targeting of proteins to the thylakoid membrane and to the thylakoid lumen has been well studied (see de Boer and Weisbeek, 1991), but there have been few studies of envelope membrane targeting. The first four chloroplast outer envelope membrane proteins to have been characterized are not synthesized with cleavable presequences, and membrane insertion

is not dependent on ATP or on a proteinaceous receptor (Salomon et al., 1990; Li et al., 1991; Ko et al., 1992; Fischer et al., 1994), suggesting that the targeting information must be contained in the mature protein. However, a recently identified outer envelope component of the import machinery has an N-terminal presequence (Hirsch et al., 1994). The targeting of three inner envelope membrane proteins has also been studied. These include a 37-kD protein of unknown function (Dreses-Werringloer et al., 1991), the maize *Brittle1* (*Bt1*) gene product identified as a putative amyloplast membrane metabolite translocator (Sullivan et al., 1991), and the phosphate translocator. The phosphate translocator is the most abundant chloroplast inner envelope membrane protein (Flügge and Heldt, 1979) and catalyzes the export of photosynthate across the inner envelope membrane (Fliege et al., 1978). The mature phosphate translocator protein is ~36 kD and is very hydrophobic, containing seven putative membrane-spanning regions. All three inner envelope proteins are synthesized with N-terminal presequences, and their import by chloroplasts is both energy dependent and receptor mediated (Flügge et al., 1989; Dreses-Werringloer et al., 1991; Li et al., 1992).

The phosphate translocator precursor proteins from spinach, pea, potato, and tobacco have presequences of 72 to 84 amino acid residues (Flügge et al., 1989; Willey et al., 1991; Schulz et al., 1993; Knight and Gray, 1994), whereas the presequence of the 37-kD precursor protein is 21 amino acid residues (Dreses-Werringloer et al., 1991). The presequences of both proteins are reported to contain features such as a higher hydroxyl amino acid and a lower arginine content as well as

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**Figure 1.** The Phosphate Translocator Presequence Contains Stromal Targeting Information.

**(A) and (B)** Precursor proteins pPTS and pSS, respectively. Precursor proteins were produced by transcription and translation *in vitro* and incubated with isolated pea chloroplasts in an import reaction to produce the mature-sized products. Chloroplasts were lysed hypotonically and fractionated by sucrose density gradient centrifugation; membrane fractions were washed with lysis buffer and recentrifuged to pellet the membranes. All procedures are described in Methods. Proteins (10 μg) from each fraction were electrophoresed on 15% SDS-polyacrylamide gels, and the radiolabeled protein was detected by fluorography. The numbers to the left of the gels give the molecular masses of the radiolabeled proteins.

**(C)** Imported protein in subchloroplast fractions was quantified by scanning the fluorographs. Closed bar, imported pPTS; open bar, imported pSS.

P, precursor protein; I, imported products; S, stromal fraction; E, envelope fraction; ET, mixed envelope-thylakoid fraction; T, thylakoid fraction.

the potential to form an amphiphilic  $\alpha$ -helix; these features distinguish them from other stromal or thylakoid-targeted proteins. These unusual presequences may be responsible for inner envelope membrane targeting as well as chloroplast targeting (Dreses-Werringloer et al., 1991; Willey et al., 1991).

Our goal in this study was to identify the residues required for the envelope targeting of the phosphate translocator. Our approach was to produce a number of chimeric proteins constituting portions of the inner envelope membrane phosphate translocator fused to the stromal small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco SSU) and to determine the location of these proteins after their import by isolated chloroplasts. The results indicated that the phosphate translocator presequence contains only stromal targeting information and that the mature phosphate translocator protein is responsible for inner envelope membrane targeting. Further refinement of the chimeric proteins showed that the N-terminal hydrophobic region of the mature phosphate translocator protein can direct a stromal protein to the inner envelope membrane, suggesting that targeting information is contained within this region.

## RESULTS

### The Phosphate Translocator Presequence Contains Stromal Targeting Information

To determine whether the presequence of the phosphate translocator contains information for targeting the mature protein to the chloroplast envelope, the chimeric protein pPTS was produced. This protein comprises the 73-amino acid residue presequence and the first four amino acid residues of the mature phosphate translocator protein, designated pPT, fused to the stromally located Rubisco SSU, designated S. A precursor protein of ~25 kD was produced in a wheat germ translation system primed with the pPTS transcript (Figure 1A, lane P). The precursor protein was imported by isolated pea chloroplasts and processed to a protein of ~15 kD (Figure 1A, lane I). These sizes are in close agreement with those calculated for the precursor (23 kD) and the mature (15 kD) protein, if the phosphate translocator cleavage site (P-C-P<sub>1</sub>A), which is retained during the production of this construct, were used. As a control, the authentic Rubisco SSU precursor (pSS) was imported by isolated pea chloroplasts (Figure 1B). The Rubisco SSU presequence is designated pS and the mature protein S. The precursor of pSS was 21 kD (Figure 1B, lane P) and was processed to a 14-kD protein after import (Figure 1B, lane I). Replacing the authentic Rubisco SSU presequence with that of the phosphate translocator only marginally reduced the efficiency of the import of pSS. Import of pPTS was 83% of pSS (Table 1).

After import of pPTS and pSS, chloroplasts were lysed hypotonically, and the stroma, a total envelope fraction, a mixed envelope-thylakoid fraction, and a thylakoid membrane fraction

**Table 1.** Efficiency of Import of Chimeric Proteins by Pea Chloroplasts

Control Protein	Protein	Import Efficiency <sup>a</sup> (% Control)
pSS <sup>b</sup>	pSS	100
	pPTS	83
pPTPT <sup>c</sup>	pPTPT	100
	pSPT	25
	pPT1-49S	100
	pPT1-93S	100
	pSPT3-49S	38
	pSPT3-93S	38
	pSPT7-45S	13
	pSPT24-49S <sup>d</sup>	16
	pSPT24-45S <sup>d</sup>	9

<sup>a</sup> The percentage of imported precursor protein in a protease-resistant location after import by pea chloroplasts over 30 min was assessed by quantitation of autoradiographs by laser scanning densitometry. Efficiency is shown relative to control protein import.

<sup>b</sup> The efficiency of pSS import was 18% of input precursor protein.

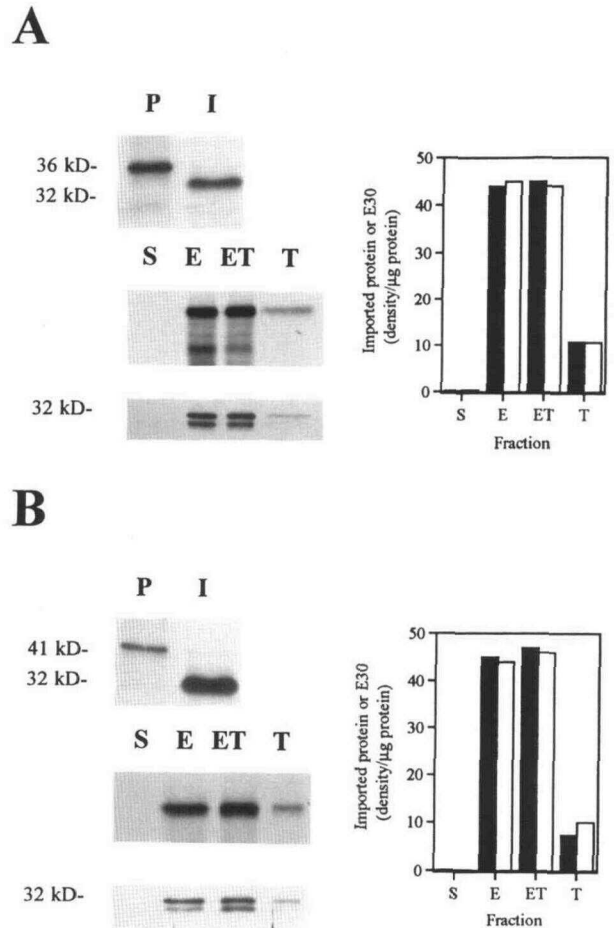
<sup>c</sup> The efficiency of pPTPT import was 8% of input precursor protein.

<sup>d</sup> All imported products were included in the quantitation.

were isolated on a discontinuous sucrose gradient (Figures 1A and 1B). The distribution of the imported pPTS was identical to pSS (Figure 1C). The quantification of imported protein in Figure 1C is plotted as density, equivalent to the amount of imported protein, in each fraction; as such, it indicates enrichment in a particular fraction. Subsequent quantifications are also shown on this basis (Figures 2 to 4). The majority of imported pPTS and pSS was in the stromal fraction, but low levels were also present in the envelope and envelope-thylakoid fractions. This is probably due to entrapment of stroma during chloroplast lysis and membrane vesiculation. Washing of membranes did not remove this contamination (see legend to Figure 1). These data suggest that the phosphate translocator presequence contains stromal targeting information and that the mature phosphate translocator is responsible for envelope membrane targeting.

### Envelope Targeting Information Resides in the Mature Phosphate Translocator Protein

If the mature phosphate translocator protein contains envelope targeting information, replacing the phosphate translocator presequence with that of the Rubisco SSU should not affect the localization of the imported phosphate translocator protein. To test this hypothesis, the chimeric protein pSPT was produced. This protein comprises the 57-amino acid residue Rubisco SSU presequence (pS) fused to amino acid residues 3 to 330 of the mature phosphate translocator protein, designated PT. Translation of the pSPT transcript produced a 36-kD precursor protein (Figure 2A, top gel, lane P) that was processed to a protein of ~32 kD after import by pea chloroplasts

**Figure 2.** Envelope Targeting Information Resides in the Mature Phosphate Translocator Protein.

(A) Precursor protein pSPT.

(B) Precursor protein pPTPT.

The top gels in (A) and (B) show precursor proteins imported by pea chloroplasts to produce imported products. Subchloroplast fractions isolated as described in Figure 1 are shown in the middle gels. Proteins (10 μg) from each fraction were electrophoresed on 15% SDS-polyacrylamide gels, and the imported protein was visualized by autoradiography (see Methods). Endogenous phosphate translocator protein in subchloroplast fractions was visualized by immune blotting with antibodies raised against the spinach E30 protein (see Methods); the results are shown in the bottom gels. The numbers to the left of the gels give the molecular masses of the radiolabeled proteins (top gels) or of the endogenous phosphate translocator protein (bottom gels). Imported protein and endogenous phosphate translocator protein in subchloroplast fractions (middle and bottom gels) was quantified by scanning densitometry. The results are shown in the graphs at right. Closed bars, imported protein; open bars, endogenous phosphate translocator protein. P, precursor protein; I, imported products; S, stromal fraction; E, envelope fraction; ET, mixed envelope-thylakoid fraction; T, thylakoid fraction.

(Figure 2A, top gel, lane I). As a control, the authentic phosphate translocator (pPTPT) was imported by isolated pea chloroplasts (Figure 2B). The phosphate translocator presequence is designated pPT and the mature protein PT. The precursor of pPTPT was 41 kD (Figure 2B, top gel, lane P) and was processed to a 32-kD protein after import (Figure 2B, top gel, lane I). The mature forms of both pSPT and pPTPT migrated faster than the expected size of 36 kD (Willey et al., 1991) in this gel system.

To determine the location of the imported chimeric protein (pSPT), chloroplasts were fractionated and the stroma, envelopes, mixed envelopes–thylakoids, and thylakoid membranes were isolated (Figure 2A, middle gel). As a control, an identical fractionation procedure was performed with chloroplasts after import of the phosphate translocator precursor protein (Figure 2B, middle gel). To assess the enrichment of the imported proteins, equal amounts of protein from each fraction were electrophoresed (Figures 2A and 2B, middle gels). Imported protein was enriched in envelope and mixed envelope–thylakoid fractions, whereas the enrichment was 4.5 times less in the thylakoid membrane fractions. No imported protein was found in the stromal fraction (Figure 2A, middle gel). The distribution of pSPT was identical to that of imported pPTPT (Figure 2B, middle gel) and the endogenous phosphate translocator protein (Figure 2A, bottom gel). After the fractionation, 60% of chloroplast protein was found in the stroma and 39% in the thylakoid membrane fraction, whereas only 0.5% of the total protein was in the envelope membrane fraction and 0.5% in the envelope–thylakoid membrane fraction (data not shown). The same distribution of protein was found for all subsequent fractionation experiments.

The enrichment of imported pSPT in the envelope membrane fractions strongly suggests that the mature phosphate translocator protein contains envelope targeting information. However, replacing the phosphate translocator presequence with that of the Rubisco SSU had a significant effect on the efficiency of the import of the chimeric precursor protein. The import of pSPT was 25% of the authentic phosphate translocator protein (pPTPT; Table 1). This suggests that the presequence, although not appearing to affect targeting (see Figure 2), does affect the efficiency of the import process.

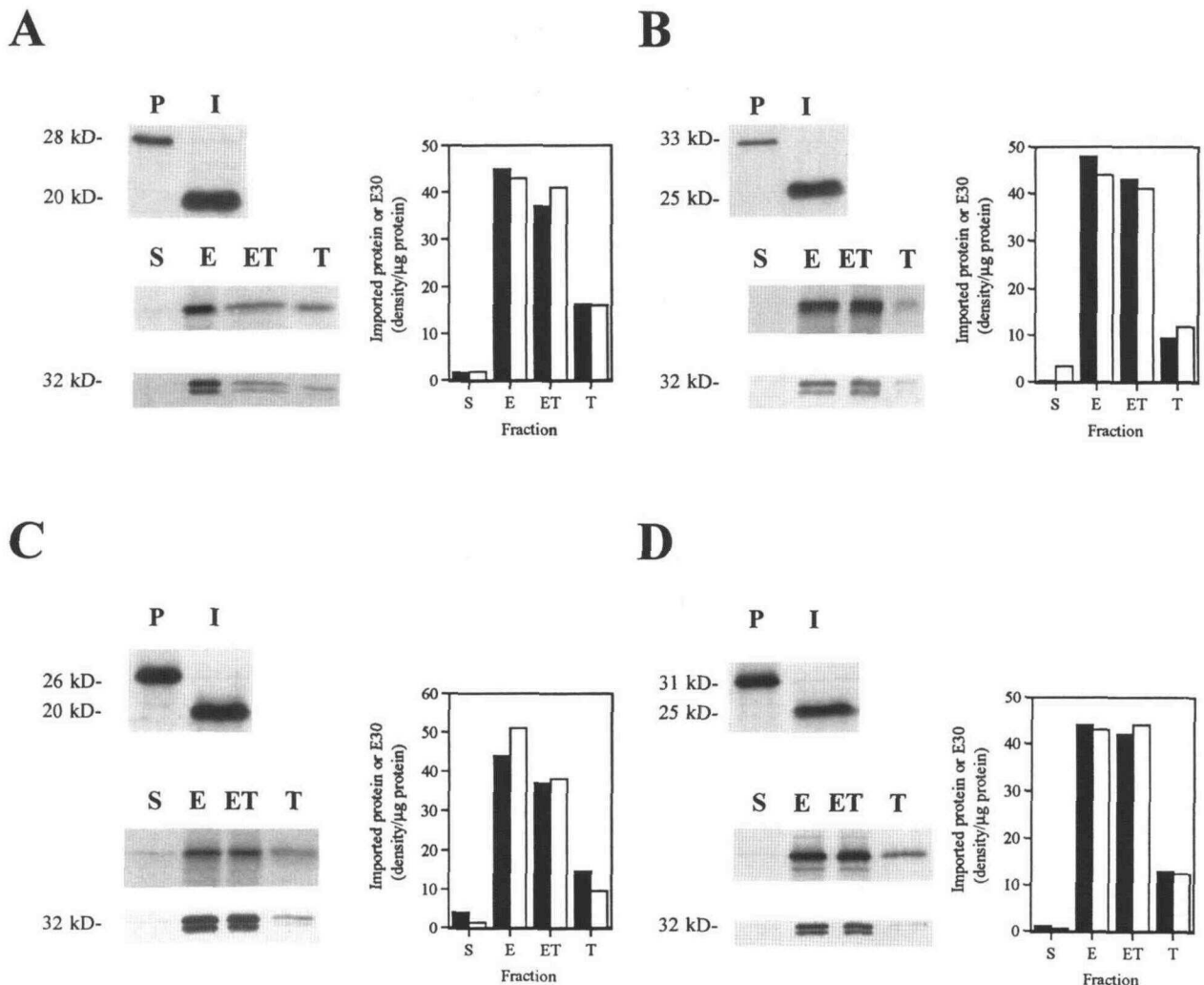
Endogenous phosphate translocator protein was assessed by the electrophoresis of fractions on SDS–polyacrylamide gels and blotting to nitrocellulose membranes; the same membranes were subjected to autoradiography to detect imported protein and probed with antibodies raised against the spinach phosphate translocator protein (E30; Joyard et al., 1982). The imported phosphate translocator comigrated with the upper band (32 kD) of the doublet of endogenous phosphate translocator protein in Figure 2B (bottom gel). A quantification of this and imported protein is shown in Figure 2B (graph). The assessment of endogenous phosphate translocator protein was deemed necessary because a large proportion (~85%) of the imported phosphate translocator protein was found in the thylakoid membrane fraction. This is due not to mistargeting of the phosphate translocator protein during import but to

contamination of the thylakoid fraction with envelope membranes. A similar level of contamination has been found by other researchers (Murakami and Strotmann, 1978; Andrews et al., 1985; Li et al., 1992) and was confirmed by an assessment of the distribution of galactosyl transferase, a marker for the envelope membrane (Douce and Joyard, 1979). Galactosyl transferase showed a distribution identical to that of the endogenous and the imported phosphate translocator proteins (data not shown). Endogenous phosphate translocator protein, determined by probing with E30 antibodies, was used subsequently as an internal control in chimeric construct import experiments.

### The N-Terminal Region of the Mature Phosphate Translocator Protein Contains Envelope Targeting Information

To define which region of the mature phosphate translocator protein contains envelope targeting information, four chimeric proteins containing the N-terminal region of the phosphate translocator were produced. The first two proteins contained the phosphate translocator presequence and either the first or the first and second N-terminal putative membrane-spanning regions of the mature phosphate translocator protein fused to the Rubisco SSU mature protein. The chimeric protein pPT1-49S contains the presequence and residues 1 to 49 of the mature phosphate translocator (Figure 3A), whereas the chimeric protein pPT1-93S contains the presequence and residues 1 to 93 of the mature phosphate translocator protein (Figure 3B). In the second set of constructs, either the first (PT3-49) or the first and second (PT3-93) N-terminal membrane spans of the mature phosphate translocator protein were placed between the Rubisco SSU presequence (pS) and mature protein (S) to produce pSPT3-49S (Figure 3C) and pSPT3-93S (Figure 3D). Translation of the transcripts in a wheat germ system produced precursor proteins of 28 kD for pPT1-49S, 33 kD for pPT1-93S, 26 kD for pSPT3-49S, and 31 kD for pSPT3-93S (Figures 3A to 3D, top gels, lane P). These are all close to their expected sizes. All four proteins were imported by isolated pea chloroplasts and processed to proteins of the expected sizes: 20 kD for pPT1-49S and pSPT3-49S (Figures 3A and 3C, top gels, lane I), and 25 kD for pPT1-93S and pSPT3-93S (Figures 3B and 3D, top gels, lane I). Again, the efficiency of import of these chimeric proteins appeared to be reduced when the authentic phosphate translocator presequence was replaced with that of the Rubisco SSU. The import of pPT1-49S and pPT1-93S was similar to that of pPTPT, whereas the import of pSPT3-49S and pSPT3-93S was 38% of the pPTPT level (Table 1).

Subchloroplast fractions were isolated after import of these chimeric proteins to determine whether envelope targeting had occurred. The distribution of imported protein (Figures 3A to 3D, middle gels) and endogenous phosphate translocator protein (Figures 3A to 3D, bottom gels) within the chloroplast subfractions indicated that all four imported proteins were associated with the chloroplast envelope membrane (Figures 3A



**Figure 3.** The N-Terminal Region of the Mature Phosphate Translocator Protein Contains Envelope Targeting Information.

(A) Precursor protein pPT1-49S.

(B) Precursor protein pPT1-93S.

(C) Precursor protein pSPT3-49S.

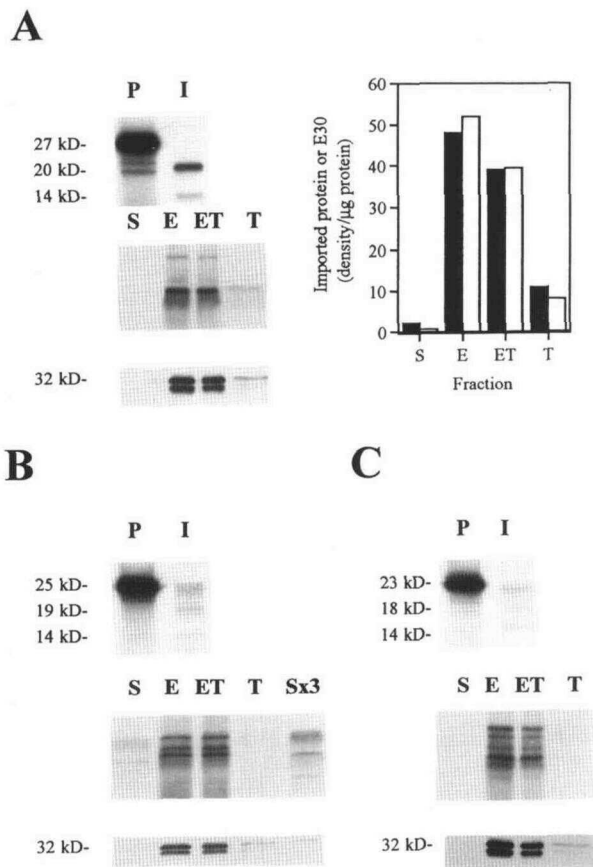
(D) Precursor protein pSPT3-93S.

Analyses in (A) to (D) are identical to those given in the legends to Figures 2A and 2B. The numbers to the left of the gels give the molecular masses of the radiolabeled proteins (top gels) or of the endogenous phosphate translocator protein (bottom gels). The bars and abbreviations are as given in the legend to Figure 2.

to 3D, graphs). However, a larger proportion (29%) of the total imported pSPT3-49S was found in the stromal fraction, compared with 5% of the total endogenous phosphate translocator protein. The enrichment of proteins in the membrane fraction confirmed our previous finding that the phosphate translocator presequence is not required for envelope targeting and, more importantly, suggests that the N-terminal region of the mature phosphate translocator protein (amino acid residues 3 to 49) can direct a protein normally targeted to the stroma to the chloroplast envelope.

#### Envelope Targeting Information Resides in the N-Terminal Hydrophobic Region of the Mature Phosphate Translocator Protein

The N-terminal region of the mature phosphate translocator protein, capable of targeting the Rubisco SSU to the chloroplast envelope membrane, comprises a potential  $\alpha$ -helical membrane-spanning region (amino acid residues 24 to 45) flanked by hydrophilic regions. The hydrophilic region N-proximal to the  $\alpha$ -helix contains three negatively charged (Asp-7, Glu-11,



**Figure 4.** The N-Terminal Hydrophobic Region of the Mature Phosphate Translocator Protein Contains Envelope Targeting Information.

- (A) Precursor protein pSPT7-45S.  
 (B) Precursor protein pSPT24-49S.  
 (C) Precursor protein pSPT24-45S.

Analyses in (A) to (C) are identical to those given in the legends to Figures 2A and 2B. The numbers to the left of the gels give the molecular masses of the radiolabeled proteins (top gels) or of the endogenous phosphate translocator (bottom gels). The bars and abbreviations are as given in the legend to Figure 2. Sx3, threefold loading of the stromal fraction.

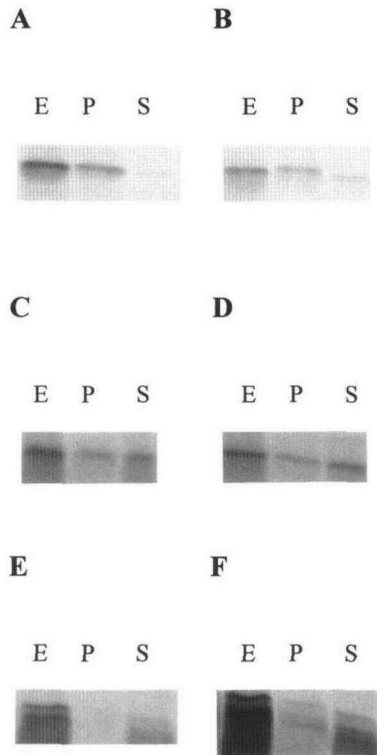
and Glu-12) and two positively charged (Lys-13 and Arg-22) amino acid residues, whereas two positively charged residues (Lys-47 and Lys-48) are located C-proximal to the putative membrane span. To define additional regions involved in targeting to the envelope membrane, chimeric proteins containing different regions from the phosphate translocator protein located between the presequence (pS) and mature (S) regions of the Rubisco SSU were produced. The chimeric protein, pSPT7-45S, contains amino acid residues 7 to 45 of the mature phosphate translocator. This region includes the putative membrane-spanning region and the 16 hydrophilic amino acid residues N-proximal to it. The pSPT7-45S precursor protein had a molecular mass of 27 kD (Figure 4A, top gel, lane P). This

precursor was imported by isolated pea chloroplasts and processed to a major 20-kD and a minor 14-kD product (Figure 4A, top gel, lane I). The precursor and the major processed product were close to their predicted sizes of 25 and 19 kD, respectively. The import of this chimeric protein was 13% of the authentic phosphate translocator protein (Table 1). After import, the 20-kD protein was associated with the envelope fraction (Figure 4A, middle gel). However, comparison with the distribution of the endogenous phosphate translocator protein suggests that some of the imported protein is found in the stromal fraction (Figure 4A, bottom gel and graph). Of the total imported 20-kD protein, 27%, compared with 10% of the total endogenous phosphate translocator protein, was found in this fraction. The enrichment of this chimera in the envelope membrane suggests that the hydrophilic region C-proximal to the hydrophobic membrane-spanning region was not required for targeting to the inner envelope membrane. However, this region may affect the efficiency of the import process.

The chimeric protein pSPT24-49S contains amino acid residues 24 to 49 of the mature phosphate translocator protein. This region includes the putative membrane-spanning region and the four amino acid residues C-proximal to it. The precursor of pSPT24-49S had a molecular mass of ~25 kD (Figure 4B, top gel, lane P). This is close to the predicted size of 24 kD. The precursor was imported, although rather inefficiently (16% of pTPT level; Table 1), by isolated pea chloroplasts (Figure 4B, top gel, lane I) to produce a number of products ranging in size from the precursor (25 kD) to ~19 kD. The latter is close to the predicted size of the mature protein. When chloroplasts were fractionated after import of pSPT24-49S, the precursor (25 kD) and proteins of ~21 and 20 kD were associated with the envelope membranes (Figure 4B, middle gel), whereas a group of proteins of ~23 kD and a 19-kD protein were found in the stromal fraction. These proteins can be seen more clearly with a higher loading of the stromal fraction (Figure 4B, middle gel, lane Sx3). These data suggest that after import of this chimeric protein, the subset of processed products associated with the envelope membrane is distinctly different from those found in the stromal fraction. This subset was subsequently shown not to be integrated into the envelope membrane (see Figure 5).

The chimeric protein pSPT24-45S contains only the putative hydrophobic membrane-spanning region (amino acid residues 24 to 45) without the flanking hydrophilic residues. This region is designated PT24-45. The precursor of pSPT24-45S had a molecular mass of 23 kD, which is identical to its predicted size (Figure 4C, top gel, lane P). This chimeric protein, like pSPT24-49S, was inefficiently imported by pea chloroplasts (9% of the pTPT level; Table 1) and resulted in the production of a number of protease-resistant products ranging in size from the unprocessed precursor (23 kD) to ~18 kD. After fractionation, all of these products were associated with the envelope membrane (Figure 4C, middle gel). This suggests that the N-terminal membrane span of the mature phosphate translocator protein is the only requirement for envelope targeting.





**Figure 5.** Extractability of Envelope-Associated Chimeric Proteins with 0.1 M NaOH.

- (A) Precursor protein pPTPT.  
 (B) Precursor protein pSPT.  
 (C) Precursor protein pSPT3-49S.  
 (D) Precursor protein pSPT7-45S.  
 (E) Precursor protein pSPT24-49S.  
 (F) Precursor protein pSPT24-45S.

Precursor proteins were imported by pea chloroplasts to produce imported products and an envelope fraction isolated as described in the legend to Figure 1. Envelopes were treated with 0.1 M NaOH as described in Methods. An equal volume (12.5  $\mu$ L) of unwashed envelopes (E) and envelopes washed with 0.1 M NaOH (P) and 40  $\mu$ L of the supernatant from the 0.1 M NaOH treatment (S) were electrophoresed on 15% SDS-polyacrylamide gels. Imported proteins were visualized by autoradiography (see Methods).

The multiple-processed products observed after the import of the chimeric proteins pSPT24-49S and pSPT24-45S suggest that some nonspecific processing occurred within the Rubisco SSU presequence. This has often been observed after import of fusion proteins into chloroplasts (Li et al., 1992), and the import of a Rubisco SSU mutant with an alteration at the C terminus of the presequence was shown to produce a number of products similar in size to those observed in this study (Ostrem et al., 1989). Because of these products, quantitation of the fractionation after import of pSPT24-49S and pSPT24-45S (Figures 4B and 4C) was not attempted.

These experiments suggest that the N-terminal hydrophobic region of the mature phosphate translocator protein can

target the Rubisco SSU to the chloroplast envelope. To determine whether targeting was to the inner envelope membrane, the sub-envelope location of five of the imported chimeric proteins (pSPT, pSPT3-49S, pSPT7-45S, pSPT24-49S, and pSPT24-45S) was determined (data not shown). There was an enrichment of all the chimeric proteins in the inner envelope membrane fraction, suggesting that the N-terminal hydrophobic region of the mature phosphate translocator protein can target the Rubisco SSU to the inner envelope membrane.

#### Imported Chimeric Proteins Are Not Stably Integrated into the Envelope Membrane

To test whether imported chimeric proteins were integrated into the envelope or merely associated with the envelope membrane, isolated envelope membranes were washed with 0.1 M NaOH. Resistance to NaOH treatment indicates that the polypeptide has penetrated the membrane's hydrophobic core (Steck and Yu, 1973). Comparison of imported authentic phosphate translocator protein in the pellet and supernatant fraction after treatment with 0.1 M NaOH shows that most of the protein is retained in the membrane fraction; only 13% of the imported protein was extracted by the alkali treatment (Figure 5A). For the chimera pSPT, slightly more protein (29%) was extracted by the same treatment, but most of the protein was retained in the membrane fraction (Figure 5B). A much smaller proportion (~20%) of the imported chimeric proteins pSPT3-49S, pSPT7-45S, and pSPT24-45S was retained in the envelope membrane by the alkali treatment (Figures 5C to 5F). However, none of the imported chimeric protein pSPT24-49S was detected in the membrane fraction after the alkali treatment (Figure 5E). These data suggest that although the N-terminal hydrophobic region of the mature phosphate translocator can target the Rubisco SSU to the inner envelope membrane, this region is not sufficient for the stable integration of the protein into the envelope membrane.

#### DISCUSSION

This study has demonstrated clearly that the presequence of the phosphate translocator is not responsible for targeting the mature protein to the envelope membrane. An analysis of the presequences of the phosphate translocator and a 37-kD inner envelope membrane protein was reported to indicate the presence of features distinguishing these from other stromal or thylakoid targeted proteins (Dreses-Werringloer et al., 1991; Willey et al., 1991). This analysis led these researchers to suggest that these presequences could be responsible for envelope targeting. But this is clearly not the case for the phosphate translocator presequence and, similarly, has been demonstrated for the maize amyloplast envelope *Bt1*-encoded protein (Li et al., 1992). Replacement of the phosphate translocator presequence, however, reduces the efficiency of import,

suggesting coevolution of the phosphate translocator presequence to suit this very hydrophobic passenger protein. Subunit 8 of *Neurospora crassa*  $F_1F_0$  ATPase, another very hydrophobic protein, could be targeted to the mitochondria using only the presequence of the equally hydrophobic subunit 9 of the ATPase (Gearing and Nagley, 1986). The data indicate that the phosphate translocator presequence functions as a chloroplast import and not as an envelope targeting signal. The information for targeting the phosphate translocator to the inner envelope membrane must therefore reside within the mature protein.

The N-terminal hydrophobic region of the mature phosphate translocator can target and cause integration of the Rubisco SSU into the inner envelope membrane. This cannot be a function purely of the hydrophobicity of the polypeptide chain, because nuclear-encoded thylakoid membrane proteins are efficiently translocated across the envelope membranes (Keegstra et al., 1989). Also, when the chloroplast-encoded integral thylakoid quinone binding protein D1 was fused to the SSU presequence and the protein was expressed in tobacco cells, it translocated across the envelope and was functional in the thylakoid membrane (Cheung et al., 1988). Considerable evidence exists for the involvement of hydrophobic domains in the targeting of proteins in other systems, including thylakoid (Kohorn and Tobin, 1989; Madueño et al., 1994) and mitochondrial (Pfanner et al., 1987; Jensen et al., 1992; Li and Shore, 1992; Beasley et al., 1993) membranes. However, the possibility that more than the first hydrophobic domain of the mature phosphate translocator protein is necessary for stable integration into the envelope membrane is suggested by the alkali extraction experiment (Figure 5). Another example of such a situation is the light-harvesting chlorophyll-protein complex (LHCP). The third membrane-spanning domain of LHCP can target the Rubisco SSU to the thylakoid membrane (Kohorn and Tobin, 1989), but other hydrophobic domains are required for the stable integration of the mature LHCP protein (Auchincloss et al., 1992).

The charged amino acid residues flanking the first membrane-spanning domain of the phosphate translocator are not essential for targeting in vitro but clearly play a role in the efficiency of the integration process. Even in the pSPT24-45S chimera, the importance of charged amino acid residues cannot be discounted totally, because two positively charged lysine residues at the N terminus of the mature Rubisco SSU protein are near the membrane-spanning domain. Charged amino acid residues close to hydrophobic signals have been implicated previously in targeting mechanisms, but there are also instances of hydrophobic signals alone being targeting competent, for example, cytochrome  $c_1$  of the inner mitochondrial membrane (Jensen et al., 1992). The function of a Golgi membrane protein retention signal correlates with hydrophobicity, and if this is sufficient, the charged residues are not an essential functional requirement (Kuroiwa et al., 1990).

The amphipathicity of the  $\alpha$ -helical hydrophobic region may be a feature of the membrane integration signal, as described for the retention of the coronavirus E1 protein in the Golgi

membrane (Swift and Machamer, 1991). Structural analysis of the first membrane-spanning region of the mature phosphate translocator protein by helical wheel projection suggests a tendency for this putative  $\alpha$ -helix to form an amphiphilic structure with threonine, asparagine, and tyrosine residues on one face and hydrophobic residues on the other. This type of structure is also present in the second, third, and fifth membrane-spanning regions (Willey et al., 1991). These authors have suggested that the hydrophilic side of these helices may form a translocation channel through the lipid bilayer, but these regions may have the capacity to act as envelope targeting signals. However, similar helical-wheel projection analysis of the putative membrane-spanning regions of two other inner envelope membrane proteins suggests that neither has the ability to form amphiphilic helices (37-kD protein, Dreses-Werringloer et al., 1991; *Bt1*-encoded protein, Sullivan et al., 1991). This suggests that an arrangement of specific amino acid residues rather than amphiphilicity may be involved in the targeting mechanism.

The two plausible mechanisms for inserting the phosphate translocator into the inner envelope membrane are stop transfer and re-export. Attempts to determine which of these mechanisms is responsible for the insertion of proteins into the analogous mitochondrial inner membrane have led to some controversy (Glick et al., 1992). A requirement for the stop-transfer mechanism is that neither precursor nor mature proteins are found in the stroma. Import of the phosphate translocator precursor gave no evidence of a stromal form, but stromal forms of the chimeric proteins pSPT3-49S, pSPT7-45S, pSPT24-49S, and pSPT24-45S were detected. However, the stromal form of the chimera pSPT7-45S could not be chased to the envelope membrane in extended incubations (data not shown).

If the stop-transfer mechanism is envisaged, some degree of mistargeting must be postulated to explain the location of the proteins. This appears unlikely because the entire phosphate translocator membrane-spanning domain, without alteration of hydrophobicity, and its surrounding charged amino acid residues are present in the chimeric protein pSPT3-49S. If this region has a stop-transfer function in the normal phosphate translocator protein, it is difficult to understand why it should not function as such in this chimeric protein, unless the other six membrane-spanning regions of the mature phosphate translocator protein also function as stop-transfer signals. Endoplasmic reticular stop-transfer signals were unable to retain the Rubisco SSU in the chloroplast envelope membrane (Lubben et al., 1987).

The accumulation of these chimeric proteins in the stroma could indicate that the stroma is the normal route of targeting to the inner envelope membrane (re-export) but reflect an inefficiency of their membrane insertion. The majority (~80%) of the envelope-associated chimeric proteins were not stably integrated into the envelope membrane. The Rieske FeS protein and LHCP have been shown to accumulate in the stroma when thylakoid membrane insertion is inhibited (Cline et al., 1989; Madueño et al., 1993). It is possible that the phosphate



translocator may associate with the stromal chaperonin, Cpn60. Tobacco plants with reduced levels of Cpn60 $\beta$  have reduced soluble carbohydrate and accumulate starch in their chloroplasts (Zabaleta et al., 1994). This phenotype is consistent with plants with reduced levels of the phosphate translocator (Barnes et al., 1994). Unfortunately, our experiments do not discriminate between the two possible mechanisms for inserting the phosphate translocator into the inner envelope membrane.

Determination of the orientation of the seven-membrane-spanning phosphate translocator protein in the inner envelope membrane could shed light on the membrane insertion mechanism. The topology of hydrophobic domains is probably determined by their surrounding charged amino acid residues (Beltzer et al., 1991; Li and Shore, 1992). Statistical analysis of integral membrane proteins of known topology in several membrane systems (von Heijne, 1986; von Heijne and Gavel, 1988; Gavel et al., 1991; Gavel and von Heijne, 1992) has suggested that the more positively charged domains may tend to exist in nontranslocated regions of the protein ("positive-inside" rule; von Heijne, 1986), whereas analysis of mitochondrial inner membrane proteins showed differences between mitochondrially and nuclear-encoded proteins. The former conforms to the "positive-inside" rule, but those encoded by the nuclear genome appear to have an increased proportion of glutamate residues in the matrix-located domains (Gavel and von Heijne, 1992). Analysis of the hydrophilic domains of the pea phosphate translocator protein suggests that one side of the membrane has a slight bias of positively charged amino acid residues (14 compared with nine) and a tendency for an increased proportion of glutamate residues (six compared with three). If this protein adheres to these rules, the N terminus of the phosphate translocator protein would exist in the intermembrane space, thereby lending support to the re-export route of membrane insertion.

## METHODS

### Materials

Radiochemicals were purchased from Amersham International, and enzymes for recombinant DNA techniques were obtained from either Amersham International or Boehringer Mannheim. Oligonucleotides were synthesized at the Cambridge Centre for Molecular Recognition facility. Pea (*Pisum sativum* var Feltham First) seeds were germinated and grown as described by Knight and Gray (1994).

### Construct Production

The plasmid pPPT8 is a derivative of pSP64 (Promega), with the pea phosphate translocator cDNA cloned into the EcoRI restriction site in the sense orientation with respect to the SP6 promoter (Willey et al., 1991). To produce the construct pPTS, a 260-bp EcoRI-PstI restriction fragment of pPPT8 encoding the 73-amino acid residue phosphate

translocator presequence and four amino acid residues of the phosphate translocator mature protein was cloned into EcoRI-PstI-digested pUBR. The latter (pUBR) was produced by cloning a 690-bp BamHI-PstI restriction fragment of pSMS58 (Anderson and Smith, 1986) encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco SSU) mature protein into similarly digested pUBS (multiple cloning site of pBluescript KS+ in pUC18). To produce the construct pSPT, a 260-bp HindIII-PstI restriction fragment encoding the phosphate translocator presequence and the first two amino acid residues of the phosphate translocator mature protein was excised from pPPT8 and replaced with a 210-bp HindIII-PstI restriction fragment from pSPTP19 (Anderson and Smith, 1986), encoding the 57-amino acid residue Rubisco SSU presequence.

To produce the construct pPT1-93S, polymerase chain reaction (PCR) was used to introduce an in-frame SphI restriction site 401 bp from the 5' end of the pea phosphate translocator cDNA. The sense primer was equivalent to the SP6 sequencing primer (Boehringer Mannheim), and the antisense primer was the oligonucleotide 5'-CGCGGCATGCAG-ATCTTCTTGTGAGGAT-3'. The underlined portion of the primer represents the restriction site used for cloning. The reaction contained 10 ng of template DNA (pPPT8), 1  $\mu$ M of each primer, 200  $\mu$ M each of dATP, dTTP, dCTP, and dGTP (Pharmacia Biosystems Ltd., Milton Keynes, UK), 2 units of AmpliTaq Taq polymerase (Perkin-Elmer Cetus), and 1  $\times$  Taq polymerase buffer (Perkin-Elmer Cetus). The reaction was conducted at 95°C for 2 min, 32°C for 1 min, and 72°C for 3 min for two rounds, followed by 10 rounds at 95°C for 2 min, 42°C for 1 min, and 72°C for 3 min. The 500-bp product, which encodes the phosphate translocator presequence and the first 49 amino acid residues of the phosphate translocator mature protein, was digested with SphI and EcoRI and cloned into SphI-EcoRI-digested pUBR. A 1.1-kb BamHI-EcoRI restriction fragment from this construct, encoding pPT1-49S, was then cloned into BamHI-EcoRI-digested pSP65 (Promega).

The construct pPT1-93S was produced using PCR to introduce an in-frame SphI restriction site 533 bp from the 5' end of the pea phosphate translocator cDNA. The same PCR conditions were used as those for the production of pPT1-49S, except that the antisense primer was the oligonucleotide 5'-CGCGGCATGCTCAGCAACTTCAGCAGGTT-3'. The underlined portion of the primer represents the restriction site used for cloning. The 632-bp product, which encodes the phosphate translocator presequence and the first 93 amino acid residues of the mature phosphate translocator protein, was digested with SphI and EcoRI and cloned into SphI-EcoRI-digested pUBR. A 1.2-kb BamHI-EcoRI restriction fragment, encoding pPT1-93S, from this construct was cloned into BamHI-EcoRI-digested pSP65.

To produce the construct pSPT3-49S, a 830-bp PstI-BamHI restriction fragment from the construct pPT1-49S, encoding amino acid residues 3 to 49 of the phosphate translocator mature protein, attached in frame to the 123-amino acid residue Rubisco SSU mature protein, was cloned into PstI-BamHI-digested pSPTP19. The construct pSPT3-93S was produced by digesting the construct pPT1-93S with PstI and BamHI to release a 958-bp restriction fragment, encoding amino acid residues 3 to 93 of the mature phosphate translocator protein attached in frame to the Rubisco SSU mature protein, which was cloned into PstI-BamHI-digested pSPTP19.

To produce the construct pSPT7-45S, PCR was used to introduce an in-frame SphI restriction site 271 bp and an in-frame PstI restriction site 388 bp from the 5' end of the pea phosphate translocator cDNA. The sense primer was the oligonucleotide 5'-GCGCGCATGCCCCGAT-TCCGCTGGTGAAG-3', and the antisense primer was the oligonucleotide 5'-GCCGCTGCGAGGATGTGAAAATCAC-3'. The underlined portions of the primers represent the restriction sites used for cloning. The reaction contained 10 ng of template DNA (pPPT8), 1  $\mu$ M of

each primer, 200  $\mu$ M each of dATP, dTTP, dGTP, and dCTP, 1  $\times$  *pfu* polymerase buffer (Stratagene), and 0.025 units per  $\mu$ L of cloned *pfu* DNA polymerase (Stratagene). The reaction was conducted at 95°C for 1 min, 32°C for 1 min, and 72°C for 5 min for three rounds, followed by 17 rounds at 95°C for 1 min, 60°C for 1 min, and 72°C for 5 min. The 137-bp product encoding amino acid residues 7 to 45 of the mature phosphate translocator protein was digested with *Sph*I and *Pst*I and cloned into *Sph*I-*Pst*I-digested pSPTP19 to produce pSPT7-45S.

To produce the construct pSPT24-49S, PCR was used to introduce in frame *Sph*I sites 323 bp and 401 bp from the 5' end of the pea phosphate translocator cDNA. The sense primer was the oligonucleotide 5'-GCGCGCATGCCAGCTCTTACTACC-3'; and the antisense primer was the oligonucleotide 5'-CGCGGCATGCAGATCTTCTTGTGAGGAT-3'. The underlined portion of the primers represents the restriction site used for cloning. The reaction conditions were identical to those used for the production of pSPT7-45S. The 98-bp PCR product encoding amino acid residues 24 to 49 of the mature phosphate translocator protein was digested with *Sph*I and cloned into *Sph*I-digested pSS19 to produce pSPT24-49S. pSS19 has a unique *Sph*I restriction site between the regions encoding the Rubisco SSU presequence and mature protein and was made by cloning a 690-bp *Sph*I-BamHI fragment of pSPT3-49S into *Sph*I-BamHI-digested pSPTP19.

To produce the construct pSPT24-45S, PCR was used to introduce an in-frame *Sph*I restriction site 323 bp and an in-frame *Pst*I site 388 bp from the 5' end of the pea phosphate translocator cDNA. The sense primer was the oligonucleotide 5'-GCGGGCATGCCAGCTCTTACTACC-3'; and the antisense primer was the oligonucleotide 5'-GCCGCTGCAGCGCAGGATGTTGAAATCAC-3'. The underlined portion of the primers represents the restriction sites used for cloning. The reaction conditions were identical to those used for the production of pSPT7-45S. The 87-bp PCR product encoding amino acid residues 24 to 45 of the mature phosphate translocator protein was digested with *Sph*I and *Pst*I and cloned into *Sph*I-*Pst*I-digested pSPTP19 to produce pSPT24-45. A 690-bp *Pst*I-BamHI fragment from pSMS58 encoding the Rubisco SSU mature protein was cloned into the *Pst*I-BamHI-digested pSPT24-45 to produce pSPT24-45S.

### Transcription and Translation in Vitro

Chimeric protein precursors were synthesized by transcription using SP6 RNA polymerase followed by translation in a wheat germ system in the presence of  $^{35}$ S-methionine. A typical reaction contained 8  $\mu$ g of plasmid DNA, 50 units of SP6 polymerase (Epicenter Technologies, Madison, WI), 0.5  $\times$  transcription salts (Promega), 80  $\mu$ g/mL BSA, 8 mM dithiothreitol, 40  $\mu$ M each of ATP, CTP, and UTP, 8  $\mu$ M GTP, 2 units of RNasin (Boehringer Mannheim), and 45  $\mu$ M P<sup>1</sup>-5'-7-methylguanosine-P<sup>3</sup>-5'-guanosine triphosphate (Cap; Boehringer Mannheim) in a volume of 22  $\mu$ L for 30 min at 42°C. Additional GTP (40  $\mu$ M) was added, and the reaction was incubated at 42°C for an additional 30 min. Typically, 20  $\mu$ L of the transcription reaction was translated in a wheat germ system (Amersham International) containing 20.7  $\mu$ L of wheat germ extract, 83 mM potassium acetate, 50  $\mu$ M of each amino acid (minus methionine), and 60  $\mu$ Ci (1000 Ci/mmol) of  $^{35}$ S-methionine in a volume of 110  $\mu$ L at 25°C for 30 min.

### Protein Import Reaction

Intact pea chloroplasts were prepared as described by Madueño et al. (1992). Routinely, import reactions were performed at 25°C for 30 min (Madueño et al., 1992) and contained intact pea chloroplasts equivalent to 200  $\mu$ g of chlorophyll, 109  $\mu$ L of the translation reaction, 1 mM

methionine, and 5 mM ATP in a final volume of 600  $\mu$ L of import buffer (50 mM Hepes-KOH, pH 8.0, 330 mM sorbitol). At the end of the incubation, thermolysin was added to 100  $\mu$ g/mL and incubated on ice for 30 min. Intact chloroplasts were reisolated (Madueño et al., 1992), and 5  $\mu$ g of chlorophyll was electrophoresed on a 15% SDS-polyacrylamide gel (Laemmli, 1970).

### Chloroplast Fractionation Procedures

To isolate total envelope membranes, the import reaction (given previously; 200  $\mu$ g of chlorophyll per 600  $\mu$ L of the reaction mixture) was treated with thermolysin (100  $\mu$ g/mL), after which intact chloroplasts were reisolated, washed once in 0.8 M sucrose in lysis buffer (10 mM Tricine-NaOH, pH 7.6, 2 mM EDTA), and finally resuspended at 8 mg/mL chlorophyll in 0.8 M sucrose in lysis buffer. Additional chloroplasts (100  $\mu$ g of chlorophyll) were added to the labeled chloroplasts, and chloroplasts were lysed by diluting to 0.3 mg/mL chlorophyll with lysis buffer. Chloroplasts (1.15 mL) were fractionated by centrifugation at 75,000g in an SW40 rotor (Beckman Instruments Ltd., High Wycombe, UK) for 60 min at 4°C through the discontinuous sucrose step gradient described by Douce et al. (1973), except that all sucrose solutions were in lysis buffer. Four fractions were collected. The stroma was in the top 1.15 mL of the gradient, the envelopes at the 0.6 M/0.93 M sucrose interface, mixed envelope-thylakoids at the 0.93 M/1.2 M sucrose interface, and the thylakoids at the 1.2 M/1.5 M sucrose interface. The membranes were diluted with lysis buffer, pelleted by centrifugation in an SW40 rotor at 100,000g for 60 min at 4°C, and resuspended in 0.3 M sucrose in lysis buffer.

For the alkali (0.1 M NaOH) treatment of envelope membranes, the isolated envelope membrane fraction was resuspended in 50  $\mu$ L of lysis buffer. Of this, 25  $\mu$ L was pelleted at 30 psi for 15 min in a Beckman Airfuge, resuspended in 0.1 M NaOH (100  $\mu$ L), and incubated on ice for 30 min. The membranes were then pelleted at 30 psi for 30 min. The resulting supernatant was adjusted to pH 8.5 by the addition of 25  $\mu$ L of 1 M Tris-HCl, pH 7.5 (total volume 125  $\mu$ L), whereas the pelleted membranes were washed once in lysis buffer and resuspended in 25  $\mu$ L of lysis buffer.

To separate inner and outer envelope membranes, the import reaction contained 100  $\mu$ g of chlorophyll and the same constituents as previously given in a total volume of 600  $\mu$ L. After thermolysin treatment (100  $\mu$ g/mL, 30 min, 4°C), intact chloroplasts were reisolated, washed once with import buffer, and resuspended at 1 mg/mL chlorophyll in 0.6 M sucrose in lysis buffer. Chloroplasts were lysed by a freeze-thaw procedure (Keegstra and Yousif, 1986), and similarly lysed nonradiolabeled chloroplasts (400  $\mu$ g of chlorophyll) were added. Chloroplasts were diluted to 0.3 M sucrose and 0.5 mg/mL of chlorophyll with lysis buffer, and 0.95 mL was layered onto a discontinuous sucrose step gradient (Keegstra and Yousif, 1986). The gradient was centrifuged in an SW40 rotor at 75,000g for 3 hr at 4°C, and four fractions were collected. The stroma was in the top 0.95 mL of the gradient, the outer envelopes were at the 0.46/0.8 M sucrose interface, the inner envelopes were at the 0.8/1.0 M sucrose interface, and the thylakoids were at the 1.0/1.5 M sucrose interface. The membranes were diluted with lysis buffer, pelleted by centrifugation in an SW40 rotor at 100,000g for 90 min at 4°C, and resuspended as given earlier.

### Gel Electrophoresis, Autoradiography, and Protein Gel Blotting

Proteins were quantified by a modified Lowry procedure (Peterson, 1977), and 10  $\mu$ g of protein was electrophoresed on 15% SDS-polyacryl-

amide gels (Laemmli, 1970). Proteins were either transferred (Towbin et al., 1979) to nitrocellulose membrane (Schleicher & Schuell) or dried onto filter paper after incubation in boiling 5% (w/v) trichloroacetic acid for 5 min, followed by a 15-min incubation in Amplify (Amersham International). Radiolabeled proteins were detected by exposure to Hyperfilm- $\beta$ max (Amersham International). After protein blotting, the same membrane was probed with antibodies raised against the 30-kD spinach inner envelope membrane protein (E30; Joyard et al., 1982). Bound antibodies were detected using enhanced chemiluminescence according to the manufacturer's instructions (Amersham International). The amounts of imported protein and endogenous phosphate translocator (E30) were quantified by scanning autoradiographs, using a Molecular Dynamics (Sunnyvale, CA) 300A laser scanning densitometer.

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