

Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide

(ATP hydrolysis/import receptors/transit peptide/*Spinacea oleracea* L.)

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ABSTRACT A chloroplast outer envelope membrane protein was cloned and sequenced and from the sequence it was possible to deduce a polypeptide of 6.7 kDa. It has only one membrane-spanning region; the C terminus extends into the cytosol, whereas the N terminus is exposed to the space between the two envelope membranes. The protein was synthesized in an *in vitro* transcription-translation system to study its routing into isolated chloroplasts. The import studies revealed that the 6.7-kDa protein followed a different and heretofore undescribed translocation pathway in the respect that (i) it does not have a cleavable transit sequence, (ii) it does not require ATP hydrolysis for import, and (iii) protease-sensitive components that are responsible for recognition of precursor proteins destined for the inside of the chloroplasts are not involved in routing the 6.7-kDa polypeptide to the outer chloroplast envelope.

The chloroplast envelope membranes form a structural and functional barrier between the organelle and the cytoplasm. The inner membrane is the site of different metabolite translocators coordinating the metabolism in the stromal and cytosolic compartments (1). In addition, this membrane is the only site of galactolipid and prenylquinone synthesis in higher plants (2). Thus far, the only protein identified in the inner envelope membrane by primary sequence and function is the phosphate-3-phosphoglycerate-triosephosphate translocator (3). Much less is known about the structure and function of the outer envelope membrane. The outer membrane contains pores that have an exclusion limit for low molecular mass substances of about 10 kDa (4). Further activities present in the membrane include different types of protein kinases (5, 6) and at least one putative receptor polypeptide that is responsible for the recognition and translocation initiation of cytoplasmically synthesized proteins destined for the plastid (7, 8).

To gain access to study the structure and function of single proteins in the outer chloroplast envelope, cDNA libraries were screened using a polyclonal antibody directed against a mixture of envelope membrane proteins. We report here the cDNA sequence of an outer envelope membrane protein of chloroplasts.[§] Import studies into isolated chloroplasts revealed that the protein contains no cleavable transit peptide and is integrated into the membrane by a pathway distinct from that of other imported plastid proteins (9).

MATERIALS AND METHODS

Radiochemicals were obtained from Amersham-Buchler. Reagents and enzymes for recombinant DNA techniques were obtained from Pharmacia, Boehringer Mannheim, or GIBCO/BRL if not stated otherwise. Spinach plants (*Spinacea oler-*

acea L.) were obtained fresh from local fields or were grown in a hydroponic culture.

Construction and Immunoscreening of a cDNA Expression Library from Spinach. The spinach cDNA libraries used in this study either were a gift of R. G. Herrmann (University of Munich, described in ref. 10) or were constructed in the vector λ gt11 as described (3). Immunoscreening of the library was done as in ref. 11 using a polyspecific antibody raised in rabbits against a mixture of total envelope membrane proteins. The cDNA insertions obtained from purified phages after digestion with *Eco*RI were subcloned into the plasmid vector Bluescript (Genofit) and were sequenced directly in the vector as double-stranded DNA by the dideoxy chain-termination method using the Stratagene protocol (12).

***In Vitro* Transcription-Translation.** *In vitro* transcription of the linearized plasmid was done in the presence of m⁷GpppG using T7 polymerase according to the instructions of the manufacturer (Genofit, Geneva). Translation was carried out in a reaction volume of 100 μ l containing 25 mM Hepes/KOH (pH 7.2), 10 mM KCl, 50 μ M of all amino acids except methionine, 100 μ g of phosphocreatine, 3.8 μ g of phosphocreatine kinase, 33.5 mM potassium acetate, 270 μ Ci of [³⁵S]methionine (1 Ci = 37 GBq), and 33.5% (vol/vol) reticulocyte lysate (GIBCO/BRL). Alternatively, translation was performed in a wheatgerm cell-free system as in ref. 13, and the postribosomal supernatant was used for protein uptake studies.

Miscellaneous. Immunoprecipitation of translation products was done as in ref. 13 using the monospecific antibody E 10 (14). The clone containing pSSU cDNA is described in ref. 15. Transcription and translation were as above except that SP6 polymerase was used.

Protein Import into Isolated, Purified Spinach Chloroplasts. Chloroplasts were isolated by standard procedures (16) and further purified on Percoll gradients as described (17). Chlorophyll was determined as in ref. 18. Import studies into intact chloroplasts were performed in a medium containing 2% bovine serum albumin, 10 mM methionine, 20 mM potassium gluconate, 10 mM NaHCO₃, 3 mM MgSO₄, 330 mM sorbitol, 50 mM Hepes/KOH (pH 8.0), and intact, purified chloroplasts equivalent to 20 μ g of chlorophyll. Import was carried out for 15 min at 25°C in the dark under dim green safe light. Afterward, chloroplasts were washed twice in 50 mM Hepes/KOH (pH 8.0), 330 mM sorbitol, and 3 mM MgSO₄ (buffer A). Intact chloroplasts were purified by centrifugation through a 40% Percoll cushion (16) and washed in buffer A. Separation of inner and outer envelope mem-

Abbreviations: pSSU, precursor form of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; CCCP, carbonylcyanoide *m*-chlorophenylhydrazone.

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[§]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35665).

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branes of chloroplasts was achieved after hypertonic lysis as in ref. 19, and sucrose density centrifugation was as in ref. 20; see also ref. 3. Translation products and imported proteins were analyzed by SDS/PAGE as in ref. 21; fluorography was as in ref. 22 using an Agfa MR 800 intensifying screen and Kodak X-Omat AR x-ray film.

RESULTS

cDNA Cloning and Sequence Analysis of the 6.7-kDa Protein of Outer Envelope Membranes from Spinach Chloroplasts. A spinach cDNA library (10) constructed in the vector λ gt11 was screened with an antibody directed against total envelope membrane proteins (11). From 10^5 recombinant phage plaques examined, we obtained six independent positive clones. Sequencing revealed that five clones contained identical cDNA inserts of 479 base pairs (bp) (pSOE 1) in length. The cDNA contained a poly(A) tail and a putative poly(A) signal, AATAAA, 24 bp upstream from the poly(A) site (Fig. 1). The largest open reading frame starting with the only methionine present in the sequence began at nucleotide 137 and ended at nucleotide 322, coding for a protein of 62 amino acids and a calculated molecular mass of 6.7 kDa. A change of the AT/GC ratio was observed, which is 1.83, 0.87, and 1.89 in the leader, coding, and trailer regions, respectively. The remaining clone (pSOE 2a) had a length of 505 bp and was identical in the coding region with pSOE 1. Variations were found in the 5' and 3' nontranslated regions. Another cDNA clone (pSOE 2b, 548 bp) (Fig. 1, lane 2) with the same characteristics was obtained using a cDNA library and an antibody described in ref. 3. pSOE 2a and pSOE 2b had much longer 3' nontranslated regions of 261 bp and 288 bp, respectively. These additional nucleotides are marked by prominent palindromic sequence motifs. Whether the observed variations in the noncoding region are of functional importance is not known at the moment. Northern blot analysis using pSOE 1 and pSOE 2b as probes (23) revealed an identical size for the mRNA of about 600 nucleotides (not shown). *In vitro* transcription-translation of pSOE 1 and pSOE 2b in the

presence of [35 S]methionine also resulted in identical translation products of about 6–8 kDa.

Protein Structure Prediction. A computer search using the Protein Sequence Data Bank of the Martinsried Institute (MIPS; Martinsried, F.R.G.) revealed no significant homology to other published sequences. The amino acid composition showed a high percentage of nonpolar amino acids (60%) (Table 1), indicating the hydrophobic nature of this protein. The hydropathy profile calculated according to ref. 24 showed only one nonpolar stretch of about 22 amino acids from position 18 to 40 (Fig. 2). The length of this segment and its hydrophobic moment would be sufficient to span a membrane.

Identification of pSOE 1 Translation Product as an Outer Envelope Protein. The chloroplast envelope membranes from spinach contain two major proteins with molecular masses below 14 kDa. One prominent low molecular mass protein was described as E 10 (25) and serves as a marker protein for the outer envelope membrane. A monospecific antibody directed against this protein (characterized in detail in ref. 14) recognized the pSOE 1 translation product (Fig. 3, lane 2), whereas an antibody directed against the 37-kDa envelope membrane polypeptide (13) failed to precipitate the E 6.7 translation product (not shown). Outer envelope protein present in the immunoprecipitation assay competed with the radioactive translation product (Fig. 3, lane 3), further indicating that the pSOE 1 translation product represents an outer envelope membrane protein.

pSOE 1 Translation Product Is Imported into Chloroplast Membranes. The pSOE 1 translation product labeled with [35 S]methionine was incubated with intact, purified chloroplasts in a standard protein import assay. Membranes and soluble proteins were separated after hypotonic lysis by centrifugation ($100,000 \times g$, 30 min) and analyzed by SDS/PAGE (Fig. 4). Obviously, the label is recovered with the membrane fraction presumably representing envelope membranes. In a parallel experiment intact chloroplasts were treated with the protease thermolysin after import (Fig. 4, lanes 3 and 4). This protease specifically digests only outer envelope membrane proteins and has no access to the intermembrane space (26). Upon thermolysin treatment a lower molecular mass breakdown product appeared with an apparent molecular mass between 5 and 6 kDa. The molecular mass of this labeled polypeptide corresponds well with that obtained when isolated envelope membranes were treated with thermolysin (Fig. 4, lanes 5 and 6). This proteolytic pattern was also described as authentic proteolytic breakdown products of the outer membrane protein E 10 (14, 25).

Translocation of E 6.7 into the Outer Envelope. To gain information about the location of the imported E 6.7 protein within the envelope membrane, chloroplasts were reisolated after the import assay, incubated for 10 min at 4°C in 0.6 M

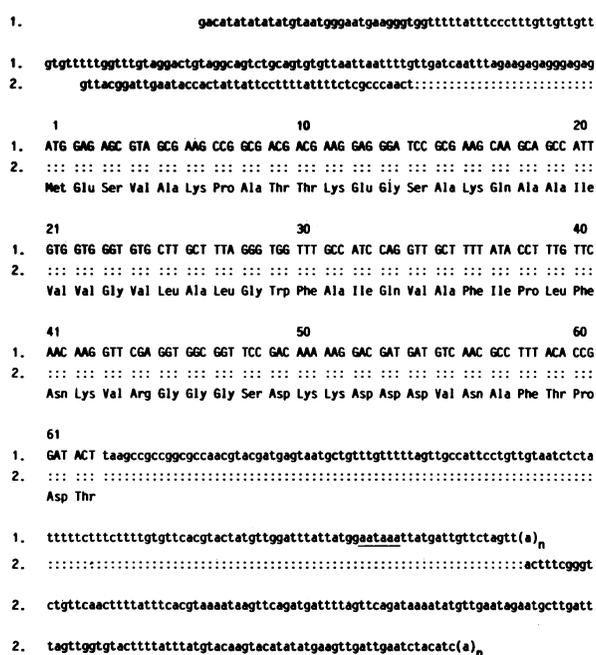


FIG. 1. Nucleotide sequence of the E 6.7 cDNA, its flanking regions, and the deduced amino acid sequence. The poly(A) signal is underlined. cDNA no. 1 (pSOE 1) was isolated and analyzed as described in the text. cDNA no. 2 (pSOE 2b) was isolated from a cDNA library using an antibody described in ref. 3.

Table 1. Amino acid composition of the 6.7-kDa outer envelope protein from spinach chloroplasts

Residue	Polar		Nonpolar		
	<i>n</i>	%	Residue	%	
Arg	1	1.6	Ala	9	14.6
Asn	2	3.2	Gly	6	9.6
Asp	5	8.0	Ile	3	4.8
Gln	2	3.2	Leu	3	4.8
Glu	2	3.2	Met	1	1.6
Lys	6	9.6	Phe	4	6.4
Ser	3	4.8	Pro	3	4.8
Thr	4	6.4	Tyr	1	1.6
			Val	7	11.2
Total	25	40.4		37	59.6

Calculated molecular mass = 6.682 kDa.

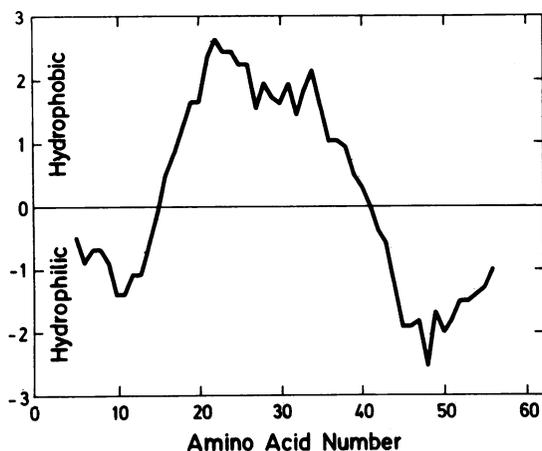


FIG. 2. Hydropathy profile of the predicted amino acid sequence of pSOE 1. The analysis was carried out according to ref. 24 using a span setting of 11 amino acids.

sucrose solution, and subjected to three freeze-thaw cycles (19). Separation of outer envelope membranes from inner envelope membranes and thylakoids was achieved by centrifugation on a sucrose density gradient (20). The translocated protein was located almost exclusively in the outer envelope; only very little label was present in the inner envelope or thylakoids (Fig. 5).

It has been shown earlier that E 6.7 is an integral membrane protein since it was recovered from the NaOH-insoluble fraction (14). Likewise, the *in vitro* synthesized and imported E 6.7 was recovered from the Na₂CO₃-insoluble fraction only, demonstrating its complete insertion into the outer envelope. E 6.7 that is bound to the surface of the chloroplasts but not integrated into the membrane was not detected in the Na₂CO₃-soluble fraction (not shown), indicating a high integration efficiency.

E 6.7 contains only the starting methionine as labeled amino acid (Fig. 1). After import the radioactive label was still detected, strongly indicating that this outer envelope protein did not possess a cleavable N-terminal transit peptide.

The E 6.7 breakdown product after thermolysin treatment still contains the starting methionine (Fig. 4, lane 4; see also Fig. 7). Therefore, the N terminus of E 6.7 is most likely located in the space between the two envelope membranes, where it is not accessible to externally added protease. The N terminus of E 6.7 is not protease resistant, as can be shown if membranes are solubilized by detergent prior to protease treatment. Additionally, E 6.7 translation product is completely digested by thermolysin treatment (not shown). Thus, that part of E 6.7 that is accessible to thermolysin should represent the C terminus, which appears to be exposed to the cytosolic side.

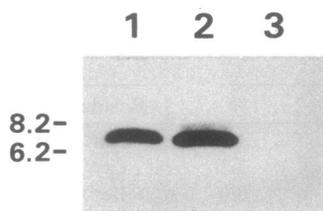


FIG. 3. Identification of pSOE 1 translation product. The protein was synthesized in an *in vitro* transcription-translation system in the presence of [³⁵S]methionine. Translation products were immunoprecipitated using a monospecific antibody directed against E 10 (14). The fluorogram shows the translation product (lane 1) and labeled proteins after immunoprecipitation (lane 2); in lane 3, 10 μg of outer envelope protein was added to the immunoprecipitation assay. Sizes are indicated in kDa.

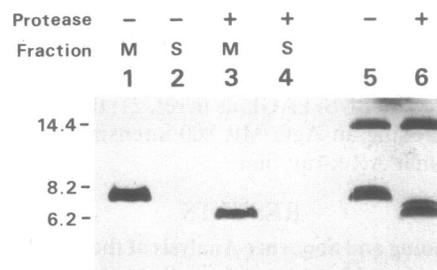


FIG. 4. *In vitro* synthesized E 6.7 is imported into intact spinach chloroplasts and recovered in the membrane fraction. Labeled E 6.7 protein was incubated with purified chloroplasts (100 μg of chlorophyll) under standard conditions. Chloroplasts were lysed in hypotonic buffer and separated into a membrane (M, lane 1) and a soluble fraction (S, lane 2) by centrifugation (100,000 × g, 20 min). Alternatively, repurified chloroplasts were treated with limited amounts of thermolysin (500 μg/mg of chlorophyll) for 45 min on ice and fractionated afterward (M, lane 3; S, lane 4). Lanes 1–4, fluorogram of a dried SDS/polyacrylamide gel; lanes 5 and 6, silver-stained SDS/polyacrylamide gel of outer envelope polypeptides from spinach (1 μg) before treatment with thermolysin (lane 5) or after protease treatment (3 μg) (lane 6). Sizes are indicated in kDa.

Comparison of E 6.7 Import to a Precursor Protein Destined to the Inside of the Chloroplast. We have compared the integration route of E 6.7 to the “normal” import pathway followed by precursor proteins destined for the stroma space—e.g., the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU) (Fig. 6). In accordance with published data (9), we demonstrated that pSSU could only be translocated in the presence of ATP in the dark (Fig. 6, lane 3). When ATP was removed from the translation mixture by incubation with apyrase (10 units/ml) for 10 min, no translocation of pSSU into chloroplasts was observed (Fig. 6, lane 2). The ATP levels in apyrase-treated translocation mixtures have been reported to be between 1 and 10 nM (27). In contrast to pSSU import, the efficiency of E 6.7 translocation into the outer membrane of chloroplasts was not altered if the translation mixture was ATP depleted by apyrase treatment. This indicates that ATP hydrolysis has no vital function in E 6.7 integration into the chloroplast outer envelope. In the light, photosynthetically made ATP can meet the energy requirements for pSSU translocation. If ATP synthesis is inhibited in the light by the presence of ionophores and protonophores (valinomycin, CCCP) import of pSSU is completely abolished. In the presence of ATP, however, the inclusion of valinomycin and CCCP in the import assay did not affect pSSU translocation. In contrast, E 6.7 integration into the outer membrane was not influenced either by dark or light or by the presence of valinomycin and

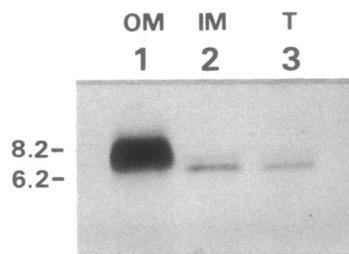


FIG. 5. Labeled E 6.7 is exclusively directed to the outer chloroplast envelope membranes. Import of E 6.7 into spinach chloroplasts (20 μg of chlorophyll) was carried out in five parallel assays. Chloroplasts were subsequently treated in hypertonic 0.6 M sucrose solution (19). Membranes were separated on a sucrose density gradient (20). Membrane fractions were analyzed by SDS/PAGE and fluorography. Lane 1, outer membranes (22 μg, OM); lane 2, inner membranes (22 μg, IM); lane 3, thylakoid membranes (40 μg, T). Sizes are indicated in kDa.

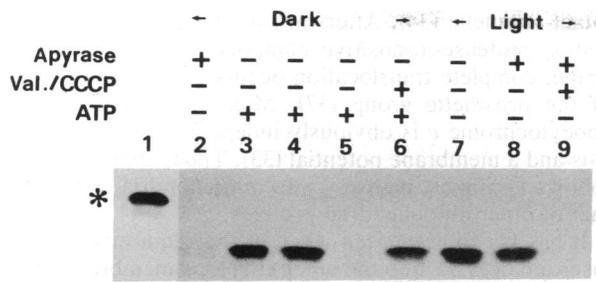


FIG. 6. Import of pSSU depends on ATP hydrolysis and protease-sensitive components on the surface of chloroplasts. Import was studied in the dark (lanes 2–6) or in the light (lanes 7–9) and in the presence of 10 units of apyrase per ml (lanes 2, 8, and 9), 2 mM ATP (lanes 3–6), and 3 μ M carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and 3 μ M valinomycin (Val.) (lanes 6 and 9). Lane 4, chloroplasts were treated with thermolysin (30 μ g/ml) for 15 min after import. Lane 5, chloroplasts were treated with thermolysin (50 μ g/ml) prior to import. The fluorogram of an SDS/polyacrylamide gel shows the imported and processed form in the soluble protein fraction. The asterisk denotes the position of the *in vitro* synthesized precursor protein (lane 1).

CCCP in the light (Fig. 7, lanes 5–6 and 11 and 12). The inclusion of 5-adenylymidophosphate (2 mM), a nonhydrolyzable ATP analog, during the import assay in the absence of exogenous ATP did not alter the yield of E 6.7 integration. The data suggest that neither a membrane potential nor ATP hydrolysis was necessary for E 6.7 integration. Pretreatment of the chloroplasts with protease generally prevents binding and import of chloroplast proteins (3, 8), suggesting that proteinaceous components of the outer envelope membranes are involved, which may function as protein receptor. Thus, translocation of pSSU was completely prevented by protease pretreatment of the chloroplasts (Fig. 6, lane 5); however, binding and insertion of E 6.7 are apparently not affected by this treatment [e.g., thermolysin, Fig. 7, lanes 13 and 14; proteinase K and chymotrypsin/trypsin (not shown)]. Taken together the results indicate that E 6.7 does follow a unique import pathway of proteins into chloroplasts.

Import of E 6.7 into Chloroplasts Is Temperature Dependent.

Intact purified spinach chloroplasts were incubated with labeled E 6.7 translation product at 25°C and 0°C. After completion of the import reaction and reisolation of the organelles, chloroplasts were treated with different concentrations of proteinase K. The results (Fig. 8) clearly demonstrated that at 25°C much more E 6.7 integrated into the membrane in a way that rendered it protease resistant than at 0°C.

DISCUSSION

E 6.7 is a prominent constituent of the outer chloroplast envelope from spinach and serves as a marker polypeptide for this membrane fraction (E 10 in refs. 14 and 28). Differ-

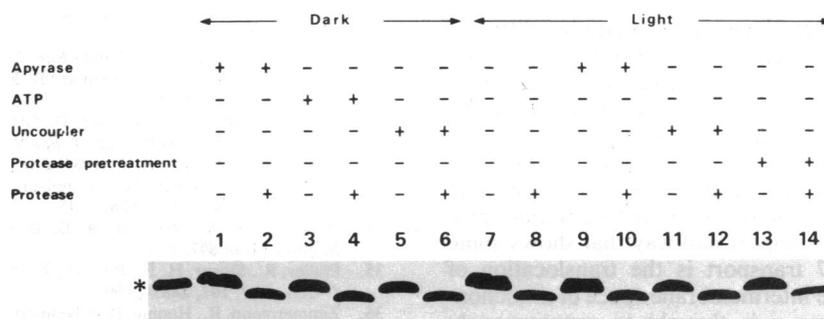


FIG. 7. Import of E 6.7 neither depends on ATP hydrolysis nor on protease-sensitive components on the surface of chloroplasts. Import of labeled E 6.7 was carried out in the dark (lanes 1–6) or in the light (lanes 7–14). The various manipulations are indicated at the top. Experimental conditions were identical to those described in the legend to Fig. 6. The asterisk indicates the migration position of the *in vitro* synthesized translation product. A fluorogram of the membrane fraction is shown.

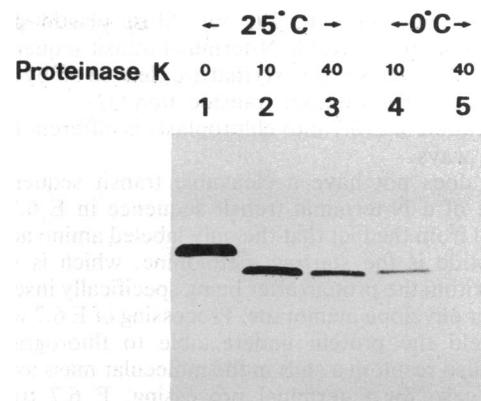


FIG. 8. Transport efficiency of E 6.7 is temperature dependent. Labeled E 6.7 translation product was imported into intact spinach chloroplasts at 25°C (lanes 1–3) or at 0°C (lanes 4 and 5). After import chloroplasts were treated with the indicated concentrations of proteinase K (μ g/ml) for 30 min at 0°C (lanes 2–5).

ences in the observed apparent molecular masses as revealed by SDS/PAGE could be due to the different systems applied (21, 25). The system used in this study allows an accurate separation of even low molecular mass proteins (21).

pSOE 1 was identified as coding for an outer envelope membrane protein by the following criteria: (i) pSOE 1 translation product comigrated in every case with authentic E 6.7 from spinach envelopes (Figs. 4 and 7); (ii) pSOE 1 translation product could be immunoprecipitated only using a monospecific antibody directed against the E 6.7 protein (E 10 in ref. 14), whereas another antibody (13) failed to recognize the protein; (iii) during protein import studies pSOE 1 translation product (E 6.7) is exclusively directed to and inserted into the outer chloroplast envelope.

The hydrophobicity distribution analysis suggests that E 6.7 contains only one membrane-spanning region, which means that N and C termini of the protein must be located on opposite sides of the outer envelope. Due to the proteolytic breakdown product of E 6.7 we assume that the N terminus of E 6.7 is located in the intermembrane space, being therefore protease protected, and that the C terminus represents the cytosolic part of the protein, which is accessible to exogenous proteases, as it is obviously the case also for the native protein *in situ*.

E 6.7 seems to follow a different and so far unobserved protein import pathway into chloroplasts. The general mechanism of protein translocation into chloroplasts comprises a number of steps common to all precursor proteins examined so far (9). The precursor proteins bind in an initial event to a protease-sensitive component, representing a putative receptor protein at the outer envelope membrane (7). Binding is followed by membrane translocation, which requires the

hydrolysis of ATP (16, 29, 30). Most plastid precursor proteins contain cleavable N-terminal transit sequences (one known exception, see ref. 31) that are cleaved off by a stromal processing peptidase after translocation (32).

Integration of E 6.7 into chloroplasts is different from that in three ways.

(i) It does not have a cleavable transit sequence. The absence of a N-terminal transit sequence in E 6.7 can be deduced from the fact that the only labeled amino acid in the polypeptide is the starting methionine, which is still contained within the protein after being specifically inserted into the outer envelope membrane. Processing of E 6.7 would not only yield the protein undetectable to fluorography but should also result in a shift in the molecular mass as it would be the case for C-terminal processing. E 6.7 translation product comigrated on a special SDS/PAGE system (21) routinely with the authentic protein from envelope membranes. Differences of 200 Da are detectable in this system.

(ii) Integration of E 6.7 into the outer envelope membrane is independent of protease-sensitive membrane components of the outer envelope membrane. Pretreatment of intact chloroplasts with thermolysin, trypsin/chymotrypsin, or proteinase K did not change the efficiency of E 6.7 integration into the membrane. This could indicate that E 6.7 does not interact with surface-exposed receptor proteins but reacts immediately with an envelope component that might be equivalent to the general insertion protein in mitochondria. This is much less sensitive to protease and is embedded in the outer mitochondrial membrane (33). Directing E 6.7 to the outer envelope could also be accomplished by interaction of E 6.7 with lipid constituents of the outer membrane (34). Remarkably, the outer chloroplast envelope has a unique composition that differs strikingly from other membranes exposed to the cytosol. Lipid constituents of the outer envelope are monogalactosyldiglyceride (17%), digalactosyldiglyceride (29%), both found exclusively in plastids, phosphatidylcholine (32%), phosphatidylglycerol (10%), and other minor components. On the other hand, they lack phosphatidylethanolamine, which is specific for mitochondrial membranes (24%) (28).

(iii) The insertion of E 6.7 into the outer envelope membrane seems to be independent of ATP hydrolysis. When ATP was depleted from the translation mixture containing labeled E 6.7 by the action of apyrase, the integration efficiency into the outer envelope membrane from intact chloroplasts was not altered. Apyrase treatment results in ATP levels in the 1–10 nM range (27). Although chloroplasts contain internal ATP in the dark this is most likely bound to thylakoid membranes and cannot be used by the organelle *in vitro* to drive protein translocation (29).

In conclusion, our results indicate that the energy requirement of E 6.7 is fundamentally different from other chloroplast proteins studied so far (30). Import of E 6.7 was also independent of the presence of a membrane potential, which is in accordance with other plastid import data but different from those obtained in mitochondria (9, 33). Porin, a major outer mitochondrial membrane protein, also does not possess a cleavable transit sequence but its import requires the hydrolysis of ATP. Furthermore, porin competes with other precursor proteins for proteinaceous receptors on the mitochondrial surface (35), indicating differences in the protein import pathways between these two outer membrane proteins. The only organellar import pathway that shows some resemblance with E 6.7 transport is the translocation of apocytochrome *c* into the intermembrane space of mitochondria (36). Apocytochrome *c* is thought to spontaneously interact with phospholipids of the outer membrane, since binding is not influenced by proteinase K treatment of the

intact organelle (34). Afterward it engages with a proteinaceous, protease-insensitive component of the outer membrane; complete translocation occurs only after attachment of the prosthetic group (37). Membrane translocation of apocytochrome *c* is obviously independent of ATP hydrolysis and a membrane potential (33). Thus, apocytochrome *c* follows an import pathway into mitochondria distinct from that of other mitochondrial proteins.

It has yet to be proven whether the requirements for the insertion of E 6.7 into the outer envelope membrane are also similar to other envelope membrane proteins. This will be possible as soon as further cDNA clones become available.

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