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Import into chloroplasts of a yeast mitochondrial protein directed by ferredoxin and plastocyanin transit peptides

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Abstract

Many chloroplast proteins are synthesized in the cytoplasm as precursors which contain an amino terminal transit peptide. These precursors are subsequently imported into chloroplasts and targeted to one of several organellar locations. This import is mediated by the transit peptide, which is cleaved off during import. We have used the transit peptides of ferredoxin (chloroplast stroma) and plastocyanin (thylakoid lumen) to study chloroplast protein import and intra-organellar routing toward different compartments. Chimeric genes were constructed that encode precursor proteins in which the transit peptides are linked to yeast mitochondrial manganese superoxide dismutase. Chloroplast protein import and localization experiments show that both chimeric proteins are imported into the chloroplast stroma and processed. The plastocyanin transit sequence did not direct superoxide dismutase to the thylakoids; this protein was found in the stroma as an intermediate that still contains part of the plastocyanin transit peptide. The organelle specificity of these chimeric precursors reflected the transit peptide parts of the molecules, because neither the ferredoxin and plastocyanin precursors nor the chimeric proteins were imported into isolated yeast mitochondria.

Introduction

Relatively few chloroplast proteins are encoded by the chloroplast genome itself [5]. Most chloroplast proteins are encoded by the nucleus, their mRNAs are translated on cytoplasmic ribosomes and the proteins are post-translationally imported into chloroplasts. These imported proteins are synthesized as precursor molecules that contain an amino terminal sequence not found in the mature protein, named the transit peptide. The transit peptide is removed by a stromal protease during or shortly after import [1, 21]. Four different studies have demonstrated that a chloroplast transit sequence contains targeting information [16, 22, 27, 30]. In all cases the transit sequence from the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase was employed to demonstrate the import into chloroplasts

of non-chloroplast proteins.

Several different compartments are present in chloroplasts. The most prominent are the stromal space and the continuous thylakoid membrane system that encloses the thylakoid lumen. Imported proteins are specifically distributed among the different chloroplast compartments and, therefore, must contain targeting information for the routing inside the chloroplast.

Two imported proteins that are localized differently in the chloroplast are ferredoxin and plastocyanin, two photosynthetic electron carrier proteins of low molecular weight (approx. 10 kDa). Ferredoxin is present in the stroma, whereas plastocyanin is present in the thylakoid lumen. Routing of the plastocyanin precursor toward the lumen is a two-step process [25]. This precursor is imported into the chloroplast stroma where part of the transit peptide is removed by a stromal processing enzyme. The stromal intermediate then traverses the thylakoid membrane and is processed to its mature size by a different, thylakoid associated enzyme [8].

Previously, we demonstrated that the ferredoxin and plastocyanin transit peptides contain routing information [25]. However, during analysis of two chimeric precursor proteins we found that the plastocyanin transit peptide could direct the transport of ferredoxin mature protein across the thylakoid membrane only very inefficiently, if at all. Two possible explanations were considered for this observation. First, possibly this lack of thylakoid transport was due to the deletion of the plastocyanin mature processing site from the transit peptide during the construction of the chimeric precursor. Alternatively, it was possible that ferredoxin was not a suitable passenger molecule.

We wished to distinguish between these two possibilities. We also wished to investigate whether the plastocyanin and ferredoxin transit peptides, like the small subunit transit peptide, are capable of importing a non-chloroplast protein into chloroplasts. Accordingly, we constructed and tested chimeric precursor proteins, that contain transit peptides of ferredoxin and plastocyanin linked to yeast mitochondrial manganese superoxide dismutase mature protein [18]. Our import and localization results show that these fusion proteins are imported into chloroplasts and processed, but that the fusion protein containing a lumen-specific transit peptide is not translocated across the thylakoid membrane. We further show that these chimeric precursor proteins demonstrate organelle specificity in that they are not imported into mitochondria.

Materials and methods

Cloning procedures, in vitro *expression*

The DNA manipulation techniques used were as described [17, 25]. An NcoI-PstI adaptor consisting of the 15-mer 5′-CATGGCCGCCCTGCA-3′ and the 7-mer 5′-GGGCGGC-3′ was chemically synthesized. The oligonucleotides were purified by gel electrophoresis on a non-denaturing 20% polyacrylamide gel and visualized by ethidium bromide staining and UV illumination. The oligomers were eluted by overnight incubation of the gel slices in water. Acrylamide was removed by centrifugation and the supernatant was butanol concentrated, ether extracted and dialyzed against water on a VSWP 01300 membrane disk (Millipore). The oligomers were annealed by combining equimolar amounts, heating to 65 °C and slow cooling to room temperature. Approx. 50 ng vector was combined with 40 ng SOD9 insert DNA and 5 pmol of the non-phosphorylated adaptor (to prevent adaptor concatenation) in a 0.01 ml volume, ligated overnight at 15 °C and *E. coli* JM101 cells were transformed with the ligation mixture.

For the SP6 transcription reaction the plasmids were linearized by EcoR1 digestion, phenol/chloroform extracted and ethanol precipitated. Unmethylated cap analog (Pharmacia-PL) was included in the transcription reaction. Reaction conditions and ribonucleotide concentrations were as described [19, 25], except that after 30 minutes the GTP concentration was adjusted to 0.1 mM. The transcribed RNA was phenol/chloroform extracted, ethanol precipitated and translated in a wheat germ system as described [4, 25] in the presence of ^{3}H-leucine (specific activity 167.5 Ci/mM, NEN).

Chloroplast import and fractionation

Intact pea chloroplasts were isolated from 9–11-day old pea seedlings by Percoll gradient centrifugation as reported previously [3, 4]. One import equivalent consisted of approx. 10^6 dpm of radiolabeled proteins and chloroplasts to an equivalent of 0.1 mg chlorophyll in a volume of 0.2 ml. This mixture was incubated for 10 minutes in the light at room temperature. Protease treatment and reisolation of intact chloroplasts by centrifugation through a 40% Percoll cushion were as described [4, 25]. The chloroplast fractionation and thylakoid protease treatment protocols used were as in [25]. The reisolated intact chloroplasts were lysed hypotonically in 50 mM Hepes-KOH pH 8.0 (Sigma) for 10 minutes on ice, in the dark. Next, an equal volume of two times concentrated import buffer was added and the thylakoids removed by centrifugation. The supernatant, containing stroma and envelopes, was centrifuged for 30 minutes at 40000 ***g*** to pellet the envelopes. The stroma was concentrated by TCA precipitation (10% final concentration). The thylakoid membranes were washed and divided into two equal aliquots, one of which was protease-treated for 30 minutes at 4 °C with 0.4 mg/ml thermolysin (dissolved in 10 mM $CaCl_2$) in import buffer. All fractions were solubilized in SDS containing sample buffer and analyzed on a 15% polyacrylamide gel [14, 15]. Reactions were assembled and terminated in dim green light.

The timecourse import was performed for the timepoints indicated. The import reaction was terminated by adding cold import buffer containing nigericin (50 nM) and transferring the reactions to the dark. Intact chloroplasts were reisolated as described, washed and solubilized in sample buffer.

Mitochondrial import

Mitochondria for *in vitro* import studies were isolated from *Saccharomyces carlsbergensis* strain S74 by a procedure described previously [29]. Import reactions were performed essentially as described [7, 11]. Capped RNAs were translated in a reticulocyte cell free system [20] and the translation products were subsequently incubated for 30 minutes at 30 °C with 0.2 mg purified mitochondria. Import of proteins was monitored by means of proteinase K protection (0.05 mg/ml) for 30 minutes at 0 °C. Protease digestions were stopped by the addition of PMSF (5 mM final concentration). Labeled polypeptides present after each incubation were analyzed on a 15% SDS-polyacrylamide gel [14] followed by fluorography [2]. The plasmid encoding the SOD-DHFR fusion protein (pDS5-SOD/DHFR) was constructed as described [29]. Transcripts formed under the control of the phage T5 promoter [26] encode a polypeptide consisting of the 34 NH_2-terminal amino acids of the yeast mitochondrial manganese superoxide dismutase [18] and 187 amino acids of the mouse dihydrofolate reductase. At the fusion site of the two genes three extra amino acids are encoded.

Results

Construction and in vitro *expression of chimeric genes*

The plasmids pSFTP81 and pSPPC74 [25] are pSP64 derivatives that contain the coding sequences for the *Silene pratensis* (white campion) ferredoxin (FD) and plastocyanin (PC) transit peptides, respectively [23, 24]. They both contain an NcoI restriction site in proximity of the precursor processing site. The plastocyanin mature coding region present in both plasmids was removed by NcoI and EcoR1 digestion and replaced by a DNA fragment encoding the mature sequence plus 3′ untranslated region of yeast manganese superoxide dismutase (SOD [18]) as indicated in Fig. 1A. For this purpose the mitochondria-specific presequence of the SOD precursor was removed using Bal31 exonuclease digestion and a M13mp10 clone (SOD9) was obtained that codes for amino acids 5–214 of the mature SOD protein. This fragment was excised by PstI-EcoR1 digestion and ligated into the ferredoxin and plastocyanin transit sequence containing vectors pSFTP81 and pSPPC74 with the use of a synthetic NcoI-PstI adaptor. The resulting constructs were named pFDSOD81 and pPCSOD74 and they code for chimeric proteins (FDSOD and PCSOD) consisting of

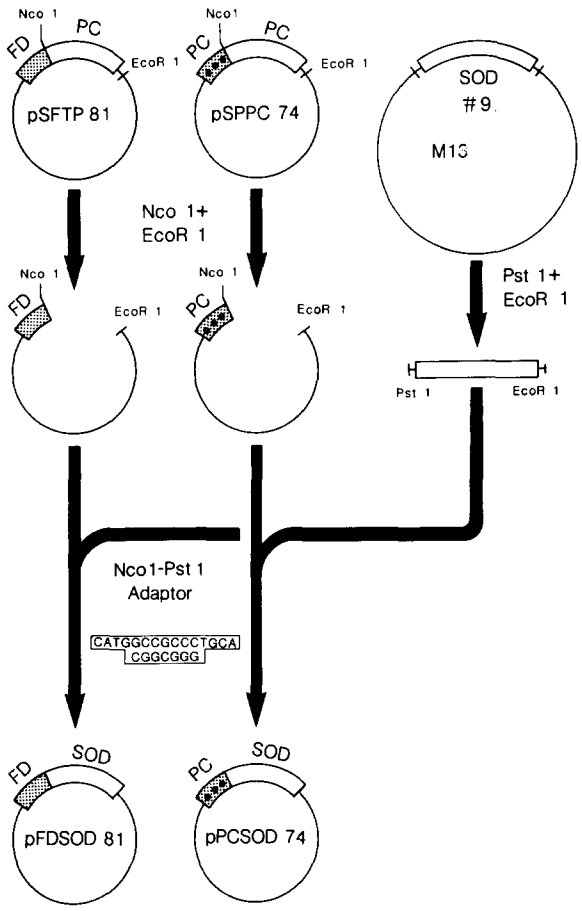

Fig. 1A. Construction of *in vitro* transcription plasmids pFDSOD81 and pPCSOD74. The construction of the transcription vectors pSFTP81 and pSPPC74 has been described previously [25]. The mature plastocyanin coding regions were removed from these vectors by NcoI-EcoR1 digestion. The yeast SOD gene was treated with Bal31 exonuclease to remove the mitochondrial specific presequence and the coding region for the mature protein, starting at nucleotide position 90 [18], was inserted into the SmaI site of M13mp10. The SOD mature coding region was excised by PstI-EcoR1 digestion and this fragment was ligated in the NcoI-EcoR1 sites of plasmids pSFTP81 and pSPPC74 using a chemically synthesized NcoI-PstI adaptor. The resulting construct contained the hybrid genes linked to the SP6 promoter and were named pFDSOD81 and pPCSOD74, respectively.

the ferredoxin and plastocyanin transit sequences fused to the SOD mature protein, respectively. The SOD9 PstI site was chosen so that part of the M13mp10 multiple cloning site was co-transferred to the hybrid construct. The NcoI-PstI adaptor restores the normal plastocyanin processing region, corrects the reading frame and recreates the NcoI and PstI restriction sites (Fig. 1B). In this way a "multiple cloning site" was introduced behind the ferredoxin and plastocyanin transit peptide coding regions (Fig. 1B). In FDSOD, the first 6 amino acids of mature ferredoxin are present fused to the amino acids encoded by the adaptor and part of the M13mp10 multiple cloning site (Fig. 1B). In PCSOD, only sequences encoded by the adaptor and multiple cloning site are present in between the transit peptide and the SOD mature protein.

A plasmid encoding the mature SOD protein (pSOD1) was constructed by deleting the fragment coding for the ferredoxin transit peptide from pFDSOD81. For this purpose pFDSOD81 was digested with Hind3 and BstE2, the ends repaired and the plasmid religated. Protein translation from this plasmid is initiated from the methionine codon in the NcoI site of the adaptor sequence (Fig. 1B). This protein contains the amino acid region encoded by the adaptor and the linker fused to the mature SOD sequence.

The resulting chimeric sequences were transcribed in an *in vitro* system using SP6 polymerase, the four ribonucleotides and unmethylated cap analog. The transcripts were translated in a wheat germ system in the presence of ^{3}H-leucine and the products analyzed by polyacrylamide gel electrophoresis and fluorography. The FDSOD and PCSOD chimeric proteins formed (Fig. 1C) had the predicted molecular weight (calculated M_r 29.4 kDa and 29.9 kDa, respectively) and could be immunoprecipitated by a SOD-specific antiserum (not shown). In both FDSOD and PCSOD identical in-frame internal translation initiation bands are present (Fig. 1C, arrow, M_r 23.8 kd). These proteins react with the SOD antiserum and comigrate with the protein encoded by the construct that lacks the transit peptide (pSOD1). Therefore, these proteins were generated by internal translation initiation at the methionine codon of the NcoI restriction site in the adaptor sequence. We consistently observe such internal translation initiation with wild type and fusion transcripts, mostly at methionine codons inside the transit peptide or near the processing site. The structure of the mRNAs for these proteins apparently enhances such initiation events, the extent of which de-

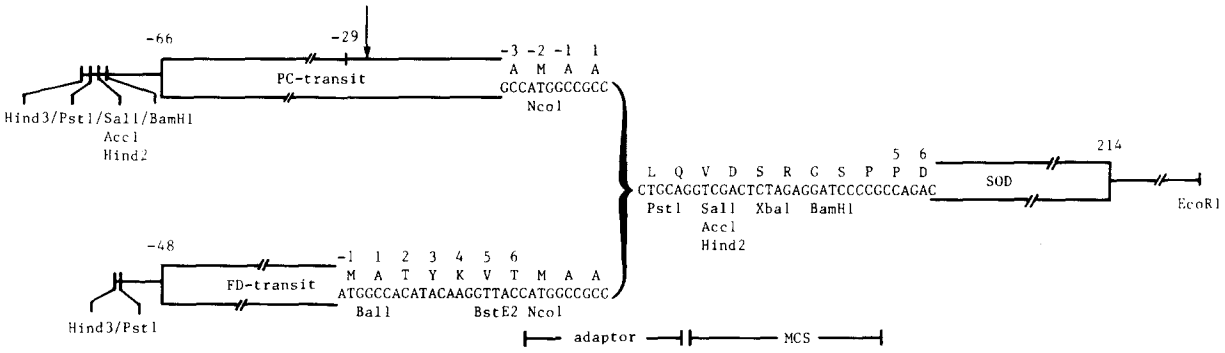

Fig. 1B. The effect of the fusion procedures on the amino acid sequences near the putative processing sites. The numbering above the sequence indicates the position relative to the putative processing sites. In this FDSOD fusion the first 6 amino acids of mature ferredoxin are present. The NcoI-PstI adaptor region was synthesized chemically, whereas the PstI-BamHI region is derived from the M13mp10 multiple cloning site (MCS). The mature SOD sequence starting from amino acid residue 5 (to 214) is indicated. In the plastocyanin transit peptide the intermediate processing site downstream but in close proximity of the only internal methionine residue (-29) is indicated by an arrow.

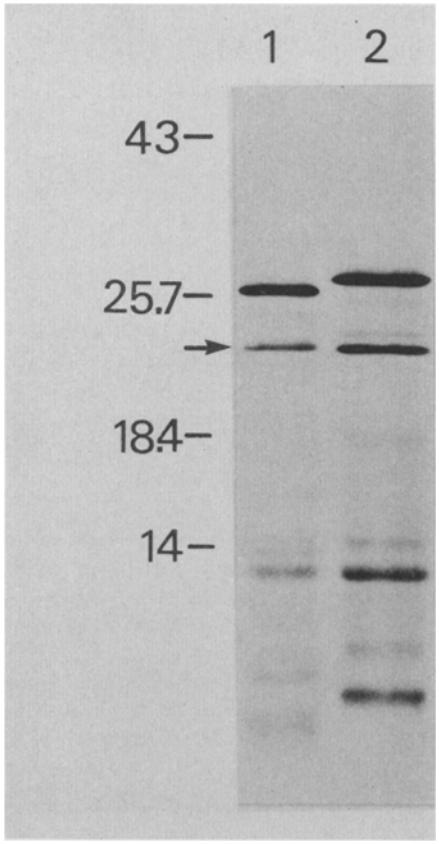

Fig. 1C. In vitro expression of pFDSOD81 (lane 1) and pPCSOD 74 (lane 2). The arrow indicates an in-frame internally initiated translation product, derived from translation initiation at the methionine codon in the NcoI site. Molecular weights are in kilodaltons.

pends to some degree on the batch of wheat germ used for the lysate preparation. For both FDSOD and PCSOD also low molecular weight (below 14 kd) translation products are observed.

Import and localization of the fusion proteins

The import into and distribution inside the chloroplast of the chimeric proteins was analyzed essentially as described previously [25]. The *in vitro* produced radiolabeled chimeric proteins were incubated for 10 minutes in the light at room temperature with isolated intact pea chloroplasts (Fig. 2, lanes 2). To discriminate between bound and imported molecules the chloroplasts were treated with a protease (thermolysin) that degrades proteins exposed at the chloroplast surface (Fig. 2, lanes 3). Next, intact chloroplasts were reisolated by centrifugation through a 40% Percoll cushion and the chloroplast location of imported products determined. The chloroplasts were lysed hypotonically and the stroma (lanes 4), envelope (lanes 5) and thylakoid (lanes 6) fractions separated by differential centrifugation. To test whether thylakoid-specific proteins are exposed on the outside of the thylakoid membranes or internalized, a protease treatment of isolated thylakoids was also performed (lanes 7).

The ferredoxin (FD) and plastocyanin (PC) wild-

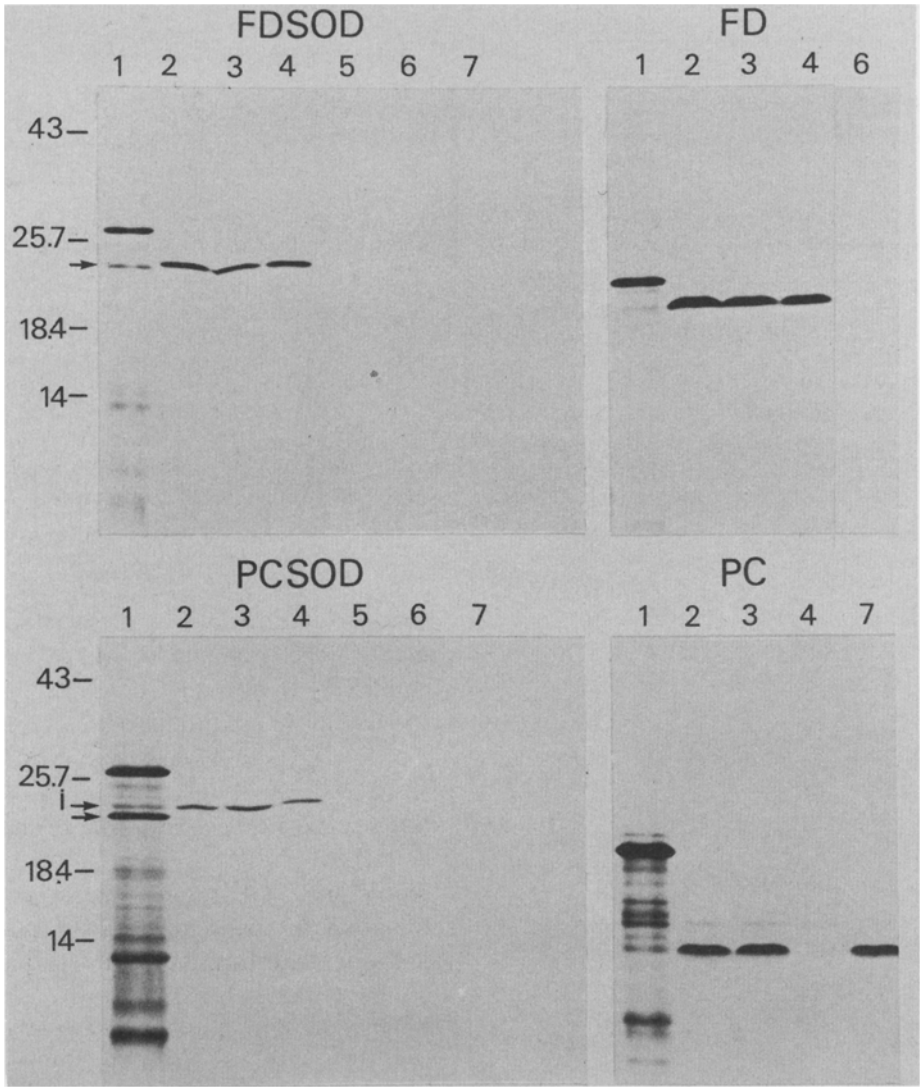

Fig. 2. Import into and distribution inside intact chloroplasts of the FDSOD and PCSOD fusion proteins and of the precursors for ferredoxin (FD) and plastocyanin (PC). Lane 1: a small aliquot of the *in vitro* translation mix, lanes 2: import into chloroplasts, lanes 3: import followed by protease treatment, lanes 4: stromal fraction, lanes 5: envelope fraction, lanes 6: thylakoid fraction, lanes 7: protease-treated thylakoid fraction. The arrows indicate the band generated by internal translation initiation at the NcoI AUG codon. The arrow marked i in the PCSOD panel denotes the putative internal initiation at the -29 methionine codon in the plastocyanin sequence [25]. In each lane the equivalent of one import reaction was electrophoresed (lanes 2–7). *Nota bene:* In the PC and FD panels the lanes are not numbered consecutively.

type precursor proteins are routed toward the stroma and thylakoid lumen, respectively. Imported ferredoxin (Fig. 2, FD, lane 2) is rendered protease-resistant (Fig. 2, FD, lane 3) and is present exclusively in the stroma (Fig. 2, FD, lane 4) in its processed mature form. Imported plastocyanin (Fig. 2, PC, lanes 2 and 3) is localized differently from ferredoxin in that all of the processed mature size plastocyanin is present in the protease-protected thylakoid fraction (Fig. 2, PC, lane 7). The plastocyanin intermediate that still contains the thylakoid transfer domain is present as a faint band in the stromal fraction

(Fig. 2, PC, lane 4, but more clearly in lanes 2 and 3). These results are in agreement with the expected distribution of ferredoxin and plastocyanin inside the chloroplast and with our previous work with these precursor proteins [25].

The FDSOD and PCSOD precursor proteins are imported into chloroplasts as shown by their resistance to added protease (Fig. 2, FDSOD, PCSOD, lanes 2 and 3). Both imported products are processed (see below) to a lower molecular weight form. Fractionation of the chloroplast into envelopes, stroma and thylakoids shows that both imported FDSOD and PCSOD are present exclusively in the chloroplast stroma (Fig. 2, FDSOD, PCSOD, lane 4). The stroma is the expected location for the imported FDSOD protein but for PCSOD an association with or transfer across the thylakoid membrane was anticipated. For the imported PCSOD protein no such association or transfer was found. Import of the yeast mitochondrial SOD protein into chloroplasts is transit peptide-dependent. The mature SOD protein, encoded by pSOD1, does not import into chloroplasts (not shown).

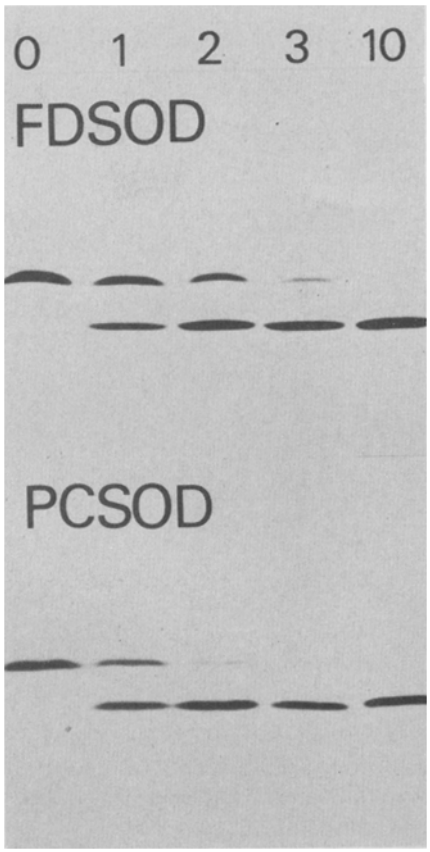

Fig. 3. Timecourse import experiment of FDSOD and PCSOD. Timepoints are in minutes.

Stromal PCSOD is present as the intermediate

Upon import, the fusion proteins are processed to a lower molecular weight form. Plastocyanin routing towards the thylakoid lumen involves two distinct processing events associated with both the import into the stroma and the thylakoid transfer process [25]. To investigate whether the chimeric proteins are also processed in one or more steps, an import timecourse experiment was performed. The FDSOD and PCSOD precursor proteins were incubated with chloroplasts for 0–10 minutes followed by reisolation of intact chloroplasts and gel analysis. As shown in Fig. 3 processing intermediates could not be detected for either precursor. The zero timepoint, which represents binding of precursor proteins to the chloroplasts, shows that only the precursor proteins bind to chloroplasts and not the internal initiation products (see Fig. 1C).

These results suggest that PCSOD is present in the stromal space as an intermediate. If this is true, the intermediate should contain part of the plastocyanin transit peptide and therefore migrate more slowly on a polyacrylamide gel than SOD alone. To test this prediction, we compared the relative electrophoretic mobilities of the precursor and processed proteins (Fig. 4). This comparison shows that processed PCSOD protein (Fig. 4, lane 3) has a higher molecular weight than either processed FDSOD or SOD alone (which migrates identically to the internally initiated SOD protein, Fig. 4, arrow). This confirms our prediction that the PCSOD protein is processed only to the intermediate form. The SOD protein and the internal initiation protein (Fig. 4, arrow) resulted from initiation at the methionine codon of the NcoI site (Fig. 1B). Six amino acids upstream of this methionine codon, the FDSOD precursor contains the normal ferredoxin processing site. The gel migration pattern supports the conclusion that this site is

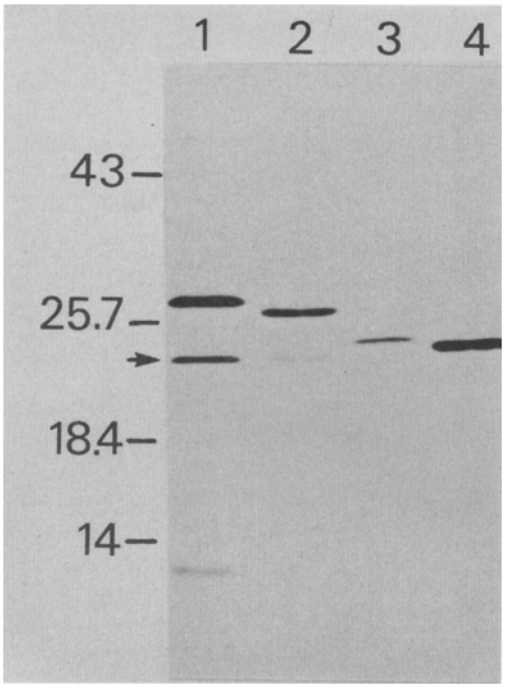

Fig. 4. Size comparison of precursors and imported products. Lanes 1 and 2: translation mixes of PCSOD and FDSOD, respectively. Lanes 3 and 4: stromal fractions of imported PCSOD and FDSOD, respectively.

used by the processing enzyme for processing of FDSOD.

Processing of PCSOD at the mature plastocyanin site would result in a protein of similar size as the internally initiated SOD protein (see Fig. 1B). The imported and processed PCSOD protein (Fig. 4 lane 3) has a higher molecular weight than the internally initiated SOD protein and is also larger than the processed FDSOD protein. Because this latter protein contains six amino acids of mature ferredoxin (Fig. 1B) the processed form of PCSOD must contain even more amino acids. Moreover, the processed PCSOD comigrates with a protein that most likely resulted from internal initiation at position -29 inside the plastocyanin transit peptide (Fig. 2, PCSOD, arrow marked i; [25]). Therefore, the stromalocated PCSOD contains a considerable part of the plastocyanin transit peptide, indicating that the thylakoid transfer domain [25] of the plastocyanin transit peptide is present in the stromal PCSOD. During import, only the chloroplast import domain is cleaved off by the stromal processing enzyme.

The wild-type and fusion proteins are not imported into yeast mitochondria

Plant cells synthesize precursors destined for both chloroplasts and mitochondria. Most likely these organelles will demonstrate specificity in the proteins that are imported. However, a recent report has demonstrated that part of a chloroplast transit sequence is capable of directing the import of a protein into mitochondria [12]. We sought to examine the organelle specificity of precursors containing plastocyanin and ferredoxin transit peptides. Radiolabeled ferredoxin, plastocyanin, FDSOD and PCSOD precursor proteins were incubated with isolated yeast mitochondria under conditions which allow import. The import reaction was followed by a protease treatment to discriminate between imported (protease-resistant) and bound (protease-sensitive) molecules.

As a positive control, a protein consisting of the presequence of the yeast SOD precursor and mouse cytosolic dihydrofolate reductase (SOD-DHFR; [29]) was also tested. This chimeric protein was imported and processed by the mitochondrial *in vitro* import system as shown by its resistance to added protease and its higher electrophoretic mobility (Fig. 5, lanes 2 and 3). However, no import was detected when the mitochondria system was used to test for import of the ferredoxin and plastocyanin wild-type and chimeric proteins (Fig. 5, lanes 6, 9, 12, 15). Some binding was observed for the precursors and internally initiated mature proteins, especially in the case of mature SOD containing proteins (Fig. 5, lanes 5, 8, 11, 14), but this affinity of the mature SOD protein for mitochondria was also noted in other mitochondrial import experiments (Van Steeg, unpublished observation).

Discussion

We have demonstrated that transit sequences from two low abundance chloroplast proteins, ferredoxin

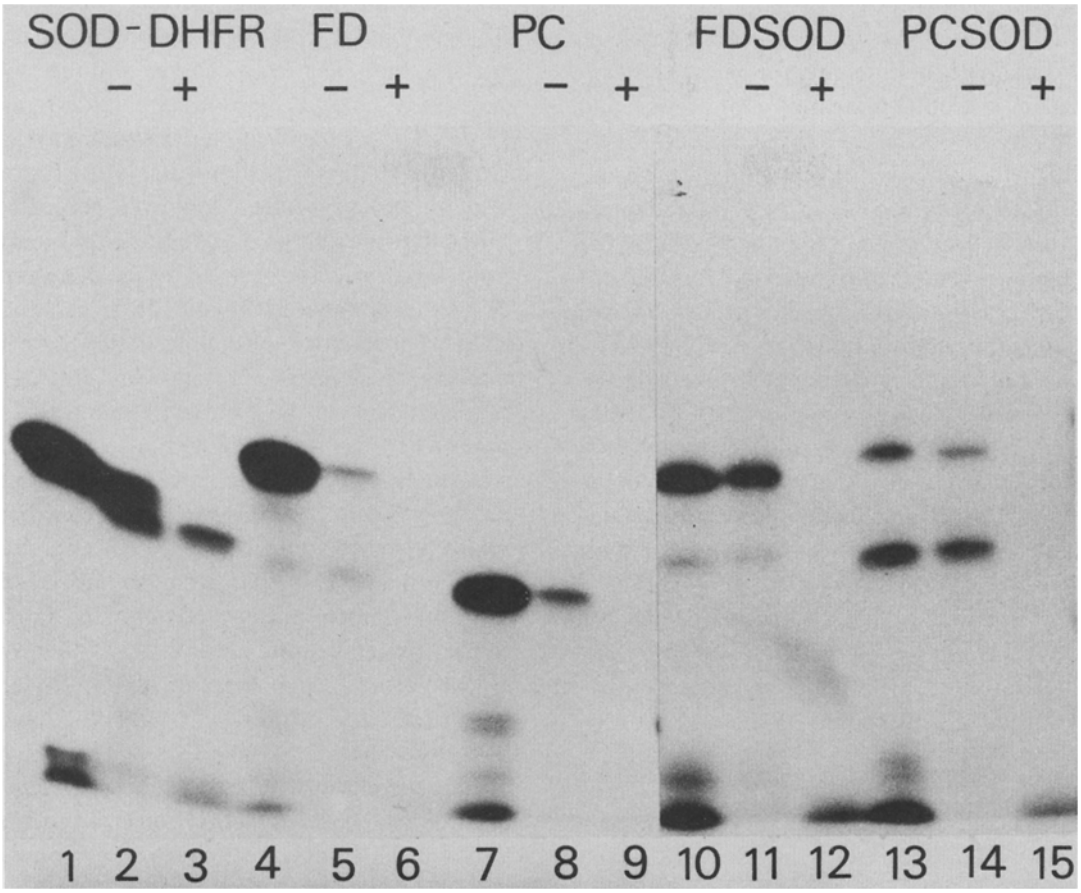

Fig. 5. Import into isolated yeast mitochondria of SOD-DHFR (lanes 1–3), ferredoxin (FD, lanes 4–6), plastocyanin (PC, lanes 7–9), FDSOD (lanes 10–12) and PCSOD (lanes 13–15). The first lanes of each series show an aliquot of the translation mixture, the second lanes (labeled −) show the import experiments, the third lanes (labeled +) show the import experiment followed by protease treatment.

and plastocyanin, can direct the import of yeast mitochondrial superoxide dismutase into chloroplasts. Previous work has demonstrated that the transit peptide of the small subunit of ribulose-bisphosphate carboxylase could direct the import of non-plastid proteins into chloroplasts both *in vitro* and *in vivo* [16, 22, 27, 30]. This ability to direct the import of non-plastid proteins into chloroplasts is most likely a general feature of chloroplast transit peptides. A similar conclusion was reached in studies of mitochondrial protein import where it has been demonstrated that pre-sequences from a variety of different precursor proteins can direct the import of non-mitochondrial proteins into mitochondria [6, 10, 11, 13, 28].

The organellar location of the imported FDSOD and PCSOD proteins was determined to investigate whether the intra-organellar targeting information of the ferredoxin and plastocyanin transit peptides was expressed in these chimeric proteins. The SOD imported by the ferredoxin transit peptide was found in the stromal compartment as expected (Fig. 2). The SOD imported by the plastocyanin transit peptide was also located in the stromal compartment rather than in the thylakoid lumen. Analysis of the stromally located PCSOD fusion protein demonstrated that it still contains part of the plastocyanin transit peptide. This is important because earlier import studies with the plastocyanin precursor protein showed that this protein is routed towards the thylak-

oid lumen in two distinct steps [25]. The first step results in the formation of an intermediate molecule in the stromal space after removal of part of the transit peptide by a stromal protease [8, 25]. The second step involves transport of the intermediate across the thylakoid membrane where it is processed to the mature size by a protease located in the thylakoid membrane or lumen [8]. Our analysis indicates that in the PCSOD fusion protein the thylakoid transfer domain of the plastocyanin transit peptide was still present; only the chloroplast import domain was removed by the stromal processing enzyme (Fig. 3). From this we conclude that the thylakoid transfer domain of plastocyanin is incapable of translocating mitochondrial SOD across the thylakoid membrane. Previously, a similar conclusion was reached for another chimeric protein consisting of the plastocyanin transit peptide and the ferredoxin mature sequence (PCFD; [25]). However, for this protein a significant portion of the imported intermediate was found associated with the outside of the thylakoids, suggesting that not the targeting to but, rather, the translocation across the thylakoid membrane was inhibited.

The results obtained with the two chimeric proteins (PCFD and PCSOD) suggest that transport across the thylakoids more critically depends on the nature of the passenger protein than does translocation across the chloroplast envelope. In both cases the plastocyanin transit peptide could direct import of the passenger proteins into chloroplasts but was not capable of transporting the proteins across the thylakoid membrane. This ability to block one process while allowing the other to occur, suggests that the two transport events involve significantly different mechanisms. These results also support the proposed two-step pathway for transport of imported proteins towards the lumen. Inhibition of the second step (thylakoid transfer) results in the appearance of an intermediate in the stroma. A similar result is obtained by deletion of the thylakoid transfer domain from the plastocyanin precursor protein. In this case plastocyanin is transported only to the stroma (J. Hageman et al., in preparation). Clearly, further work is needed to elucidate the as yet unidentified prerequisites for successful translocation across the thylakoid membranes of passenger proteins.

Targeting signals for import into chloroplasts and mitochondria share some general characteristics: both function post-translationally and both are rich in hydrophobic and hydroxy amino acids interspersed with positively charged residues, whereas negatively charged residues are underrepresented [9, 21]. These general similarities prompted us to investigate whether the chloroplast precursor proteins and chimeric proteins could be imported into mitochondria. Import of these proteins into mitochondria could not be detected using conditions that promoted import of a mitochondrial specific precursor. A possible inhibitory effect of mature chloroplast proteins on import into mitochondria can be excluded because the mature part of FDSOD and PCSOD is a yeast protein that is normally imported into mitochondria. If applicable to the plant cell, this result suggests that chloroplast-specific transit peptides differ sufficiently from mitochondrial targeting signals to prevent wasteful mitochondrial "mistargeting" of chloroplast proteins in the plant cell.

In a recent paper concerning this problem of cellular mistargeting of proteins [12], it was found that the transit peptide of the ribulosebisphosphate carboxylase small subunit (SS) from *Chlamydomonas* can direct proteins into yeast mitochondria, although with reduced efficiency when compared to yeast mitochondrial precursors. However, these authors used only the amino terminal 31 amino acids of the 45-residue long SS transit peptide. It cannot be excluded that specific targeting information in the second half of this transit peptide is important in proper localization. Mutagenesis experiments with the ferredoxin precursor transit peptide have demonstrated that chloroplast recognition is totally dependent on the presence of the carboxy-terminal part of the transit peptide (Smeekens *et al.*, submitted for publication).

We do not yet understand all the variables involved in targeting of proteins towards different cellular compartments. Especially only limited information is available onthe mechanism of post-translational transport of proteins into organelles and of the subsequent intraorganellar routing of these proteins. The chloroplast with its complex internal structure and its dependence on proteins imported from the cytoplasm, can serve as an excellent model system for studying these routing mechanism.

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