

Protein Sorting between Mitochondrial Membranes Specified by Position of the Stop-Transfer Domain

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Abstract. Recently, we fused a matrix-targeting signal to a large fragment of vesicular stomatitis virus G protein, which contains near its COOH-terminus a well-characterized endoplasmic reticulum (ER) stop-transfer sequence; the hybrid G protein was sorted to the inner mitochondrial membrane (Nguyen, M., and G. C. Shore. 1987. *J. Biol. Chem.* 262:3929–3931). Here, we show that the 19 amino acid G stop-transfer domain functions in an identical fashion when inserted toward the COOH-terminus of an otherwise normal matrix precursor protein, pre-ornithine carbamyl transferase; after import, the mutant protein was found anchored in

the inner membrane via the stop-transfer sequence, with its NH₂ terminus facing the matrix and its short COOH-terminal tail located in the intermembrane space. However, when the G stop-transfer sequence was placed near the NH₂ terminus, the protein was inserted into the outer membrane, in the reverse orientation (NH₂ terminus facing out, with a large COOH-terminal fragment located in the intermembrane space). These observations for mitochondrial topogenesis can be explained by a simple extension of existing models for ER sorting.

PROTEINS destined for translocation into mitochondria are then sorted between four subcompartments (outer and inner membrane, intermembrane space, and matrix). To achieve this, it has been proposed that two functionally distinct signals may be used: an NH₂-terminal matrix-targeting domain and a distal sorting domain (14, 15, 20, 27, 39). Precursor proteins destined for the matrix lack the sorting domain (14, 15, 20, 39) and, therefore, may follow a default pathway analogous to protein sorting in the secretory apparatus (21). Interestingly, however, the evidence to date suggests the existence of two types of sorting signals: sequences that function as putative stop-transfer domains (3) to arrest translocation of proteins during vectorial import (15, 27), and a domain found in the signal sequence of the Fe/S subunit of the cytochrome *bc₁* complex, which is responsible for targeting an intermediate form of the precursor from the matrix to the intermembrane space (14).

Here we have examined the problem of how proteins are selectively inserted during import into either the outer (OMM)¹ or inner (IMM) membrane of mitochondria, via a hydrophobic stop-transfer sequence. To do so, we have made the assumption that once proteins destined for different cellular compartments interact with their cognate receptors (in this case, the import receptor located on the surface of mito-

chondria), subsequent translocation and membrane insertion may follow basically similar mechanisms. As an experimental system, therefore, polypeptides were constructed, modeled on a prototypic protein for studying polypeptide insertion into the endoplasmic reticulum (ER) membrane, G protein of vesicular stomatitis virus. G is a simple transmembrane protein that contains toward its COOH-terminus a well characterized stop-transfer domain (3, 13, 32, 33). Here, we have used cassette mutagenesis to introduce the 19 amino acid G stop-transfer sequence at different positions within an otherwise normal matrix precursor polypeptide. The position of the stop-transfer sequence within the matrix precursor was found to influence both the membrane location and transbilayer orientation of the protein after import into mitochondria in vitro.

Materials and Methods

Earlier publications describe the procedures used for transcription-translation of recombinant plasmids (25), import of translation products by purified rat heart mitochondria in vitro (26), analysis of products by SDS-PAGE (25), and NH₂-terminal radiosequence analysis of electroeluted polypeptides (26). Additional details, as well as the procedures used for the construction of recombinant plasmids, are given in the figure legends.

Results

In an earlier study (27), it was shown that a hybrid protein, containing the signal sequence of rat pre-ornithine carbamyl transferase (pOCT) fused to a large COOH-terminal frag-

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; pOCT, pre-ornithine carbamyl transferase; pOCT-GST35, G stop-transfer sequence positioned after amino acid 35 in pOCT; pOCT-GST319, G stop-transfer sequence positioned after amino acid 319 in pOCT.

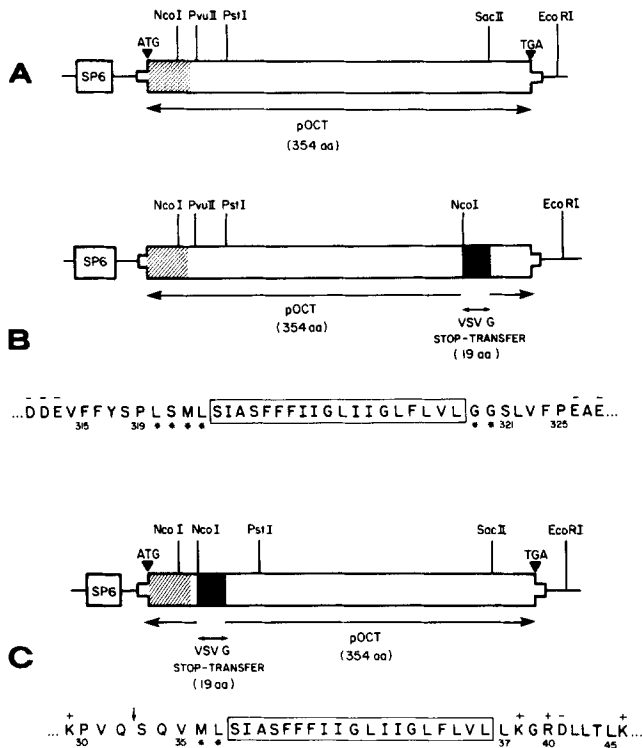


Figure 1. Construction of pSPOGS and pSPOGP encoding pOCT precursor derivatives carrying the VSV G stop-transfer sequence. (A) pSP019 is derived from pSP64 and contains a cDNA insert coding for a functional full length copy of rat liver pOCT(25). (B) pSPOGS encodes pOCT containing the VSV G stop-transfer sequence positioned between amino acids 319 and 321 of the pOCT sequence. The derived polypeptide precursor is designated pOCT-GST319. (C) pSPOGP encodes pOCT-GST35, which is pOCT containing the VSV G stop-transfer sequence positioned between amino acids 35 and 37 of pOCT. *Slashes*, pOCT signal sequence; *solid region*, VSV G stop-transfer sequence. Also shown is the single-letter amino acid code for the VSV G stop-transfer domain (*boxed*) and the flanking pOCT sequences; amino acids that were added as a consequence of DNA manipulation are indicated by an asterisk. The downward arrow in C denotes the pOCT signal processing site between amino acids 32 and 33. To construct pSPOGS (B), pSP019 was digested to completion with SacII and a single copy of a SacII adaptor composed of two complementary oligodeoxyribonucleotides, 5'-TTCCATGGATCCCGGGC-3' and 5'-CCGGGATCCATGGAAAGC-3', and containing an NcoI site and a unique SmaI site, was inserted and ligated. A portion of the adaptor was then deleted by cutting the plasmid to completion with SmaI followed by a partial digestion with NcoI; the deleted fragment was replaced with a second synthetic double-stranded DNA fragment composed of 5'-CATGGTCTCTATTGCCTCTTTTTTATTTATCATAGGGTTAATCATGGACTATTCTTGGTTCTC-3' and 5'-GAACCAAGAATAGTCCAATGATTAACCCATGATAAAGAA-AAAAGAGGCAATAGAGAC-3', which encodes the 19 amino acid VSV G stop-transfer sequence. For the construction of pSPOGP, the NcoI-PvuII fragment shown in A (corresponding to amino acids 22-37 of pOCT) was deleted from pSP019 and replaced with the synthetic VSV G adaptor. The plasmid was then digested to completion with NcoI, and amino acids 22-35 of pOCT were reconstructed by the insertion of an adaptor comprised of the two complementary oligodeoxyribonucleotides, 5'-CATGGTTCGAAATTTTCGGTATGGGAGGCCAGTCCAGAGTCAAGT-3' and 5'-CATGACCTGACTCTGGACTGGCTTCCCATAACGAAAATTCGAAC-3'. The various plasmid constructions were confirmed by DNA sequence analysis.

ment of vesicular stomatitis virus (VSV) G (pOCT-VSV G), was sorted to the mitochondrial inner membrane. The precursor was efficiently processed; the processed molecules were further reduced in size (by 2.0-2.5 kD) when mitochondria were supplied with exogenous protease under conditions (digitonin-treatment) where protease gained access to the intermembrane space, but not to the matrix. Subsequent NH₂-terminal radiosequence analysis (unpublished) revealed that processing occurred at the normal pOCT cleavage site (between gln³² and ser³³, reference 26) and that exogenous proteolysis after digitonin treatment removed exclusively COOH-terminal amino acids. Taken together, the results indicate that pOCT-VSV G was inserted into the inner membrane via the G stop-transfer domain, with its NH₂ terminus facing the matrix (where the pOCT processing enzyme is located, reference 5) and its short COOH-terminal tail located in the intermembrane space.

To examine whether the G stop-transfer sequence can function in a background composed exclusively of an intact matrix precursor molecule, we used synthetic oligonucleotide cassettes and inserted the 19 amino acid G stop-transfer sequence into pOCT at either of two positions (Fig. 1). In the first instance, the positional organization of the stop-transfer sequence in the pOCT-VSV G hybrid (27) was mimicked by placing the G stop-transfer toward the COOH terminus of pOCT, 319 amino acids from the NH₂ terminus (versus 260 in the hybrid) and 33 amino acids from the COOH terminus (versus 29 in the hybrid). The mutant polypeptide was designated pOCT-GST319 (Fig. 2 B). In the second case, the G stop-transfer sequence was placed three amino acids downstream of the pOCT signal (35 from the NH₂ terminus), and the mutant designated pOCT-GST35 (Fig. 2 C).

Import of pOCT-GST319

When added to intact mitochondria, pOCT-GST319 was imported and processed (Fig. 2, lanes 3-5), indicating that at least its NH₂ terminus gained access to the matrix where mitochondrial signal peptidase is located; the processed product was resistant to exogenous proteinase K, whereas residual precursor located on the surface of the organelle was degraded (Fig. 2, lane 5). Import was dependent on a transbilayer electrochemical potential across the IMM (Fig. 2, lane 6), a typical requirement for protein insertion into the inner membrane (16, 28). The processed product was largely resistant to solubilization by alkali extraction (Fig. 2, lane 4), suggesting a strong interaction with the lipid bilayer of the IMM (8); in contrast, precursor located at the surface of the organelle (Fig. 2, compare lanes 3 and 4), as well as the soluble processed form of pOCT located in the matrix (Fig. 2, lane 10), were both extracted by this procedure. When mitochondria were treated after import with proteinase K and 0.2 mg/ml digitonin (to selectively permeabilize the outer membrane, references 12 and 24), processed pOCT-GST319 was reduced in size by ~2.5 kD (Fig. 2, lane 12). The remainder of the polypeptide, however, was relatively resistant to degradation under these conditions (*m* and *m'*, lanes 11 and 12 in Fig. 2), to about the same extent as was processed pOCT located in the matrix (Fig. 3 B); 2 of the 11 methionine residues in processed pOCT (located at positions 17 and 20 from the COOH terminus, reference 22) would be expected to be removed during conversion of *m* to *m'*. At a higher con-

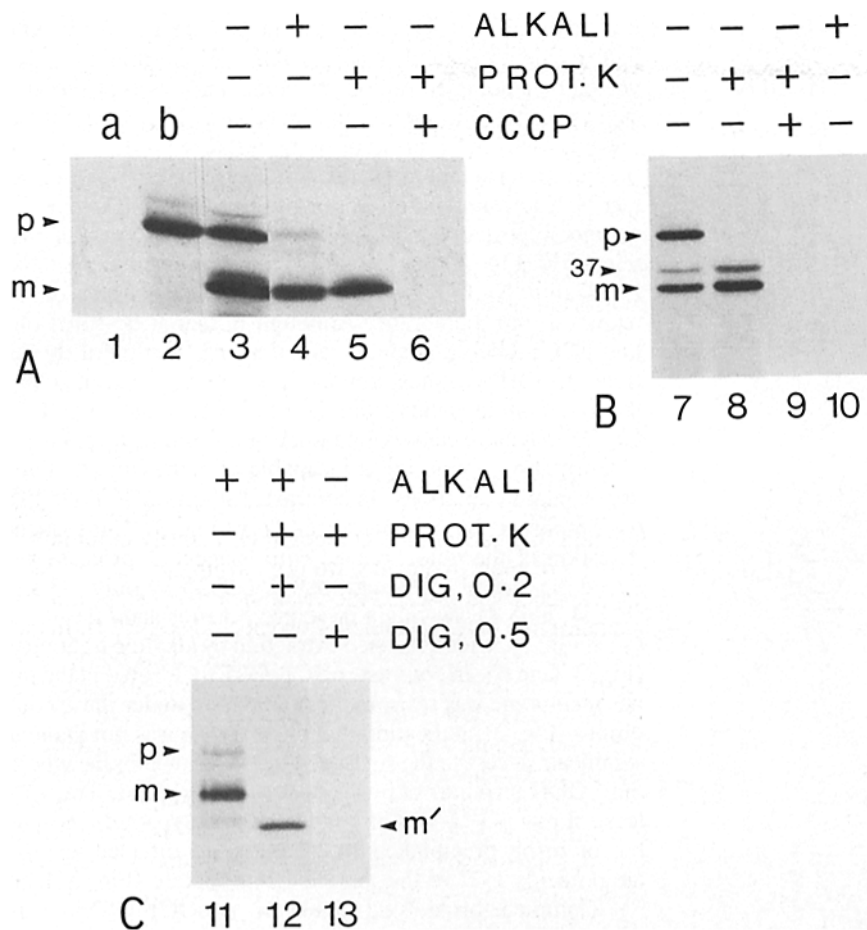


Figure 2. In vitro import and processing of pOCT and pOCT-GST319. pSP019 and pSPOGS were linearized with EcoRI, transcribed, and the resulting mRNAs translated in a rabbit reticulocyte lysate system containing [³⁵S]methionine, as described in reference 25. Translation products (pOCT and pOCT-GST319, respectively) were incubated with purified rat heart mitochondria (0.5 mg protein/ml) using the import conditions described in references 26 and 27; mitochondria were recovered after import by centrifugation at 1,200 g for 5 min, dissolved in SDS sample buffer, and analyzed by SDS-PAGE and fluorography. For protease treatments, import incubation was cooled to 4°C, maintained for 20 min in the presence of 100 µg/ml proteinase K, adjusted to 2 mM phenylmethylsulfonyl fluoride, and incubated an additional 10 min before recovering the mitochondria by centrifugation. For alkali extractions, mitochondria (50 µg protein) were recovered after import, resuspended in 150 µl of 0.1 M Na₂CO₃, pH 11.5, sonicated to disperse completely the pellet, and incubated on ice for 30 min (8); the insoluble membrane fraction was then recovered by centrifugation in an airfuge operating for 10 min at 100,000 g. (A) Lanes 1 and 2, translation products obtained with no mRNA added (lane 1) or pSPOGS mRNA (lane 2); lanes 3–6, incubation of pOCT-GST319 with mitochondria; lane 3, mitochondrial pellet; lane 4, mitochondrial membranes after

extraction of mitochondria with 0.1 M Na₂CO₃, pH 11.5 (ALKALI); lane 5, treatment of mitochondria after import with exogenous proteinase K (PROT. K); lane 6, as in lane 5 except that import was performed in the presence of 1.0 µM carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) to collapse the transbilayer electrochemical potential across the mitochondrial inner membrane. (B) Lanes 7–10, import of pOCT, with conditions the same as for lanes 3–6, as indicated in the figure; only half of the import reaction was analyzed in lane 7. (C) Lanes 11–13, mitochondrial pellets after import of pOCT-GST319; lane 11, alkali extraction as in lane 4; lane 12, after import, mitochondria were treated with exogenous proteinase K in the presence of 0.2 mg/ml digitonin (DIG, 0.2), followed by extraction with 0.1 M Na₂CO₃, pH 11.5; lane 13, proteinase K digestion was performed in the presence of 0.5 mg/ml digitonin (DIG, 0.5). Precursor and processed forms of pOCT and pOCT-GST319 are indicated by *p* and *m*, respectively; *m'*, protease-clipped *m*; 37, a 37-kD intermediate product, which arises during processing of pOCT in vitro.

centration of digitonin, proteinase K gained access to the matrix and degraded completely both the hybrid protein (Fig. 2, lane 13) and OCT (not shown, see also reference 27). Complete degradation of pOCT-GST319 by proteinase K was also observed in the absence of digitonin, but where import had been prevented by CCCP, thus indicating that the digitonin effect was at the level of membrane permeabilization rather than simply facilitating degradation of an otherwise resistant protein. We conclude from these observations, therefore, that processed pOCT-GST319 is anchored at the inner membrane in a transmembrane orientation via the G stop-transfer domain, with its NH₂ terminus facing the matrix and its COOH-terminal tail facing the intermembrane space. In all respects, pOCT-GST319 functions like the pOCT-VSV G hybrid described earlier (reference 27). Interestingly, however, the sequences flanking the G stop-transfer domain in the two molecules are rather different; whereas the stop-transfer in G is flanked immediately on either side with basic residues (13, 32), the flanking residues in pOCT-GST319 are

neutral (or at some distance away, negatively charged) (Fig. 1 B). Clearly, therefore, it is the stop-transfer sequence per se, rather than contributions from the background polypeptide, that specifies translocation-arrest at the inner membrane.

Additionally, we have found that pOCT-GST319 was anchored in the inner membrane after import even in the presence of an inhibitor (26) that prevents proteolytic cleavage of the signal sequence (not shown). Processing, therefore, does not appear to be required to activate the sorting domain (i.e., the stop-transfer sequence). This is in marked contrast to the situation for Rieske Fe/S-protein in *Neurospora*, where the matrix targeting signal must be removed to allow the contiguous sorting domain to direct translocation to the intermembrane space (14).

Import of pOCT-GST35

The fate of pOCT-GST35 after import is described in Fig. 3. After transcription-translation of the derived pSP64 plas-

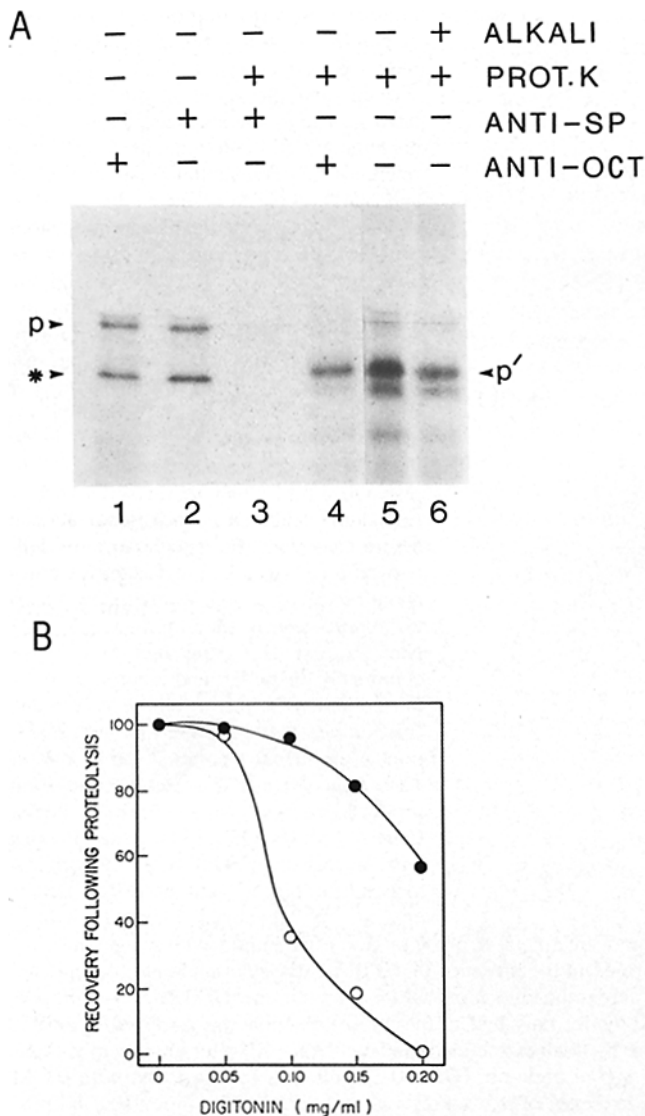


Figure 3. In vitro import of pOCT-GST35. Conditions for import, protease digestion, and alkaline (pH 11.5) extraction are given in Fig. 2. The procedure used for immunoprecipitation is described in references 1 and 25. Antiserum was raised (25) against a synthetic peptide corresponding to amino acids 1-27 of the pOCT signal sequence (10) coupled to hemocyanin by the glutaraldehyde method (31). (A) The two translation products of pSPOGP mRNA (*p* and ***, see text) were incubated with mitochondria under standard import conditions, and the mitochondria recovered by centrifugation. Lane 1, import products precipitated with monospecific antibody against OCT (*ANTI-OCT*); lane 2, import products precipitated with monospecific antibody against the pOCT signal peptide (*ANTI-SP*); lanes 3 and 4, before recovery of mitochondria, import mixtures were treated with proteinase K (*PROT. K*), the mitochondria were recovered, and import products precipitated with anti-SP (lane 3) or anti-OCT (lane 4); lane 5, total import products after digestion with exogenous proteinase K; lane 6, as in lane 5 except that mitochondria were extracted with 0.1 M Na₂CO₃, pH 11.5. *p*, full-length pOCT-GST35; ***, pOCT-GST35 with a COOH-terminal truncation; *p'*, protease-clipped *p*. (B) Import incubations were treated with exogenous proteinase K in the presence of the indicated concentrations of digitonin; mitochondria were recovered, analyzed by SDS-PAGE and fluorography, and the relative levels of pOCT-GST35 (*p* or *p'* in A) or of processed pOCT (*m* in Fig. 2 B) were determined by laser densitometric tracings of autoradiographs, and plotted against digitonin concentration. (●) processed pOCT; (○) pOCT-GST35.

mid, two major polypeptide products were obtained; both had an identical (and intact) pOCT NH₂ terminus, as revealed by automated radiosequencing analysis (not shown), suggesting that the smaller product arose either from breakdown at the COOH terminus of the full-size product or from premature termination of translation. Despite having an intact NH₂ terminus, the smaller product (asterisk in Fig. 3) was not imported; it was largely degraded by exogenous proteinase K (Fig. 3, lanes 1 and 4) and remained extractable by alkaline Na₂CO₃ (not shown), indicating its lack of integration into membrane. Although it cannot be ruled out that pOCT-GST35 contains targeting information at its extreme COOH terminus, a more probable explanation is that the loss of amino acids from the COOH terminus of pOCT-GST35 may have caused the molecule to assume an aberrant conformation, rendering it incapable of being imported under standard conditions. In contrast, full-size pOCT-GST35 was imported, but not processed (*p* in Fig. 3). Subsequent digestion of the mitochondria with exogenous protease reduced the size of full-length pOCT-GST35 by only ~3 kD (Fig. 4, lane 4), leaving a large protease-resistant fragment (*p'* in Fig. 3), which resisted extraction by alkaline treatment (Fig. 3, lane 6). In contrast, pOCT-GST319 located in the inner membrane was resistant to degradation under these conditions (Fig. 2), indicating that the protease was not gaining significant access to the surface of the inner membrane where the COOH terminus of pOCT-GST319 is exposed. The protease clip of pOCT-GST35 rendered the polypeptide incapable of being precipitated by an antibody directed against amino acids 1-27 of the pOCT signal peptide (Fig. 3, lane 3). Complete proteolytic digestion of pOCT-GST35 was achieved in the presence of low concentrations of digitonin (Fig. 3 B), conditions that selectively permeabilize the OMM; under these conditions (0.2 mg digitonin/ml), however, processed pOCT located in the matrix remained largely resistant to proteolysis (Fig. 3 B), indicating that the protease was not gaining access to the matrix. The effect of digitonin on the sensitivity of pOCT-GST35 to protease was likely due to OMM permeabilization (Fig. 3 B) rather than to a direct effect on the precursor itself because pOCT-GST35 was completely degraded in the presence of import-incompetent mitochondria minus digitonin (Fig. 4, lane 3). We conclude, therefore, that pOCT-GST35 was inserted into the outer membrane via the G stop-transfer sequence and assumed a transmembrane orientation in which its NH₂ terminus faced outside the organelle and a large COOH-terminal fragment extended into the intermembrane space. Consistent with observations for a variety of native OMM proteins (7, 9, 23), we found that insertion of pOCT-GST35 did not require a mitochondrial electrochemical potential (not shown). Moreover, the precursor did not insert into rat liver microsomes (not shown) and, importantly, it did not insert into mitochondria whose surface had been pretreated with trypsin to inactivate potential import receptors (Fig. 4). The trypsin pretreatment of mitochondria described in Fig. 4 also inhibits import of wild-type pOCT by ≥90% (1, 11).

Discussion

We have found in this study that an ER stop-transfer sequence derived from VSV G is functional when placed in the context of an otherwise normal and well-characterized mitochondrial matrix precursor protein, pOCT (17, 18, 25, 26). When

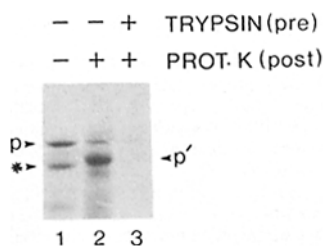


Figure 4. A protease-sensitive component on the surface of mitochondria is required for insertion of pOCT-GST35 into the OMM. Before import, mitochondria were treated with 40 μ g trypsin (lane 3) after which inhibitors were added to the trypsin sample or with 40 μ g

trypsin preinhibited with aprotinin and phenylmethylsulfonyl fluoride (lanes 1 and 2), exactly as described in reference 32 (*TRYP SIN, pre*). Import was carried out as described in Fig. 3, after which the import mixtures were treated with (lanes 2 and 3) or without (lane 1) proteinase K (*PROT. K, post*), as described in Fig. 2. Mitochondria were recovered and radioactive products visualized following SDS-PAGE and fluorography (only half of total product was applied to lane 1). *p*, *p'*, and * are defined in Fig. 3.

inserted toward the NH₂ terminus (pOCT-GST35), the protein was delivered to the OMM; near the COOH terminus (pOCT-GST319), the G stop-transfer sequence resulted in translocation arrest at the IMM. Because pOCT-GST319 and pOCT-VSV G were both targeted to the inner membrane where they assumed the same transmembrane disposition strongly implies that it is the stop-transfer domain per se rather than contributions from immediate flanking sequences (which are quite different in the two proteins) that functions as the sorting signal. That pOCT-GST319 and pOCT-GST35 are delivered to different membranes, yet contain identical matrix-targeting and stop-transfer sequences, suggests that position of the two signals relative to one another may be an important determinant in mitochondrial membrane sorting.

pOCT-GST35 displays import characteristics typical of outer membrane proteins (7, 9, 16, 23); it is inserted into the membrane without cleavage of the signal sequence and with-

out the requirement of an electrochemical potential. Furthermore, insertion was prevented by pretreatment of intact mitochondria with trypsin (Fig. 4), using the identical conditions that also prevented import of normal pOCT to the matrix (11). It would appear, therefore, that pOCT-GST35 follows a typical import pathway rather than simply partitioning into the membrane bilayer by an unrelated mechanism.

Because stop-transfer sequences presumably anchor proteins in membranes by simple hydrophobic interactions, a direct role of the lipid bilayer in decoding the G stop-transfer sequence as a specific sorting signal seems unlikely. Rather, our observations fulfill many of the predictions for a proteinaceous translocation apparatus carrying out such functions (3, 36), as do our observations that the pOCT signal peptide, though potentially amphiphilic (6), is incapable of penetrating membrane lipid bilayers (37). In further support of this notion is the finding that ADP/ATP translocase, an integral protein of the IMM, is initially imported via a water-accessible pathway (29). In Fig. 5, we have applied existing models developed for the ER to illustrate how simple positional information pertaining to stop-transfer signals might specify protein sorting between mitochondrial outer and inner membranes. The translocation machineries of the two membranes are shown as pore complexes (3, 36), components of which function in the recognition of matrix-targeting and stop-transfer signals. Two proposals are made. (a) When the protein translocation machinery encounters a stop-transfer domain, it releases this region of the polypeptide from the translocator into the surrounding lipid bilayer (3, 36) (perhaps at a gated interface between two subunits of the translocator, shown in face-view in the inset in A); if a distal strand of the polypeptide is simultaneously engaged in the translocator, it continues to be transported (e.g., A). (b) As the signal peptide emerges from the OMM translocator, it interacts with the translocator of the IMM and triggers physical interaction between the translocation machineries of the two

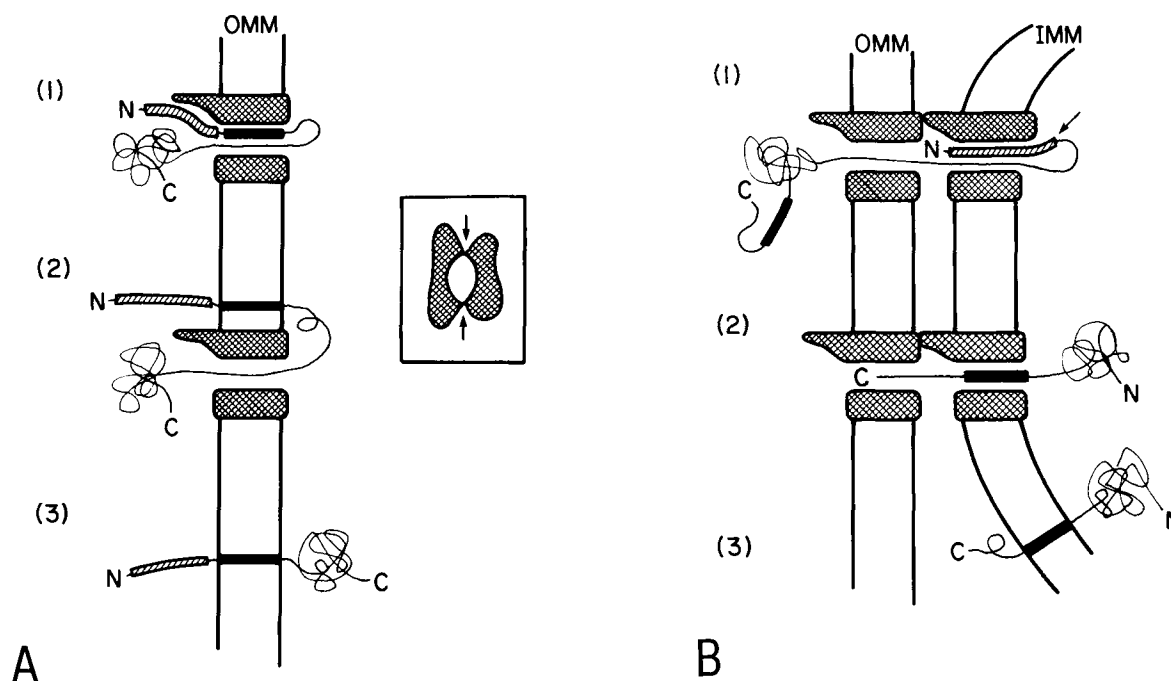


Figure 5. Protein sorting between mitochondrial membranes. (A) pOCT-GST35; (B) pOCT-GST319. Details are provided in the text.

membranes (presumably at a stable contact point which allows an incoming precursor polypeptide to span both membranes simultaneously, references 34 and 35); contact between the two renders the OMM translocator incapable of responding to a stop-transfer domain, while the IMM translocator is functional in this regard (B).

The available evidence suggests that precursor polypeptides are threaded into mitochondria NH₂-terminal first (34). In the case of pOCT-GST35, the signal sequence presumably engages the OMM translocator as an extended NH₂-terminal loop, thus leading to the observed orientation of the protein in the membrane (A). This is in distinct contrast to yeast OMM-70, which assumes a reverse orientation in the outer membrane (14, 30), even though it shares with pOCT-GST35 a similar overall organization of its topogenic domains (NH₂-terminal signal sequence followed immediately by a stop-transfer sequence; reference 15). Analogous situations have been described for the ER; asialoglycoprotein receptor (38) and Sindbis virus PE₂ (4), for example, both have internal uncleaved signal sequences, yet insert into the ER membrane in opposite transmembrane orientations. To account for these observations, together with findings for various mutants of opsin, Audigier et al. (2) have recently proposed that relatively strong interactions between the signal sequence and a *cis*-located signal-binding subunit of the ER translocation machinery prevents translocation of the NH₂ terminus (yielding an orientation in which the NH₂ terminus faces the cytoplasm), while a weaker interaction allows subsequent dissociation of the signal which can then transit (flip) across the membrane (2), yielding an orientation in which the NH₂ terminus faces the lumen. A similar explanation might account for the opposite orientations of pOCT-GST35 and OMM-70; according to such a model, the OMM-70 signal sequence disengages the signal-binding site at the surface of mitochondria and transits the OMM before insertion of the stop-transfer sequence into the translocation machinery, while in the case of pOCT-GST35 the signal sequence disengages the *cis* binding site after insertion of the stop-transfer sequence (Fig. 5 A), and thus remains outside the organelle. If this model (2) is correct, the retention time of the signal sequence on the *cis* side of the translocation machinery might likewise govern the minimum distance between the signal sequence and the stop-transfer domain that permits passage of proteins to the inner membrane (Fig. 5 B).

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