A Signal-Anchor Sequence Selective for the Mitochondrial Outer Membrane

Heidi M. McBride, Douglas G. Millar, Jian-Ming Li, and Gordon C. Shore

Department of Biochemistry, McIntyre Medical Sciences Building, McGill University, Montreal, Canada H3G 1Y6

Abstract. pOMD29 is a hybrid protein containing the NH_2-terminal topogenic sequence of a bitopic, integral protein of the outer mitochondrial membrane in yeast, OMM70, fused to dihydrofolate reductase. The topogenic sequence consists of two structural domains: an NH_2-terminal basic region (amino acids 1–10) and an apolar region which is the predicted transmembrane segment (amino acids 11–29). The transmembrane segment alone was capable of targeting and inserting the hybrid protein into the outer membrane of intact mitochondria from rat heart in vitro. The presence of amino acids 1–10 enhanced the rate of import, and this increased rate depended, in part, on the basic amino acids located at positions 2, 7, and 9. Deletion of a large portion of the transmembrane segment (amino acids 16–29) resulted in a protein that exhibited negligible import in vitro. Insertion of pOMD29 into the outer membrane was not competed by import of excess precursor protein destined for the mitochondrial matrix, indicating that the two proteins may have different rate-limiting steps during import. We propose that the structural domains within amino acids 1–29 of pOMD29 cooperate to form a signal-anchor sequence, the characteristics of which suggest a model for proper sorting to the mitochondrial outer membrane.

THE mechanism of protein insertion into mitochondrial membranes is not well understood, other than the observations that deletion of predicted transmembrane segments in a limited number of such proteins can lead to relocation of the protein to soluble mitochondrial compartments (Liu et al., 1988; Glaser et al., 1990) and, conversely, that introduction of a heterologous transmembrane stop-transfer segment can result in membrane insertion of an otherwise soluble matrix protein (Nguyen and Shore, 1987; Nguyen et al., 1988). Studies in this area have been complicated by a number of issues. For example, the mitochondrion contains two translocation-competent membranes; the problem of membrane insertion, therefore, is also a problem of protein sorting. Also, two pathways may have evolved for protein sorting to mitochondrial membranes: the conservative sorting pathway, in which cytoplasmically-synthesized precursor proteins may be routed first to the matrix and then exported to the inner membrane (Hartl et al., 1986; Hartl and Neupert, 1990; Mahlke et al., 1990), and the stop-transfer sorting pathway, in which proteins are inserted into either the outer or inner membrane during unidirectional import into the organelle (Blobel, 1980; Liu et al., 1990; Mahlke et al., 1990; Glick and Schatz, 1991). Finally, progress has been limited by the fact that some of the best-studied examples of mitochondrial membrane proteins exhibit relatively complex structures. For example, the major protein of the outer membrane, porin, is devoid of uniformly hydrophobic transmembrane segments but, like the bacterial porins, may intercalate into the membrane as a β barrel (Jap, 1989).

To avoid many of these complications, we have focused on a simple bitopic integral protein of the outer mitochondrial membrane in yeast, OMM70 (Hase et al., 1984) (also called MAS70, Hines et al., 1990). The topogenic information in OMM70 resides within a stretch of 29 amino acids at the NH_2 terminus, resulting in a protein that is anchored in the outer membrane via a predicted 19 amino acid transmembrane segment (amino acids 11–29) in the N_{in}-C_{cyto} orientation, leaving a large COOH-terminal fragment exposed to the cytosol (Hase et al., 1984; Nakai et al., 1989). Evidence has been obtained that the *Neurospora* homolog of OMM70, MOM72, employs the same import receptor as proteins destined for the matrix compartment (Söllner et al., 1990). However, proteins that are inserted into the mitochondrial outer membrane do not require an electrochemical potential across the inner membrane (Freitag et al., 1982; Mihara et al., 1982), indicating that routing to the outer membrane does not occur via a conservative sorting pathway.

Earlier studies suggested a model in which OMM70 is directed to mitochondria by a matrix-targeting signal located at the extreme NH_2-terminus (amino acids 1–12), with translocation to the matrix being arrested at the outer membrane by a stop-transfer sequence (amino acids 11–29) (Hurt et al., 1985). The efficiency of import to the matrix of reporter proteins carrying amino acids 1–12 of OMM70, however, was very weak and, as emphasized by Glick and Schatz (1991), such findings might arise indirectly from the fact that a high percentage of random, positively-charged sequences can function as weak matrix-targeting signals. Also, this region of OMM70 is replaced by a very different uncharged, proline-rich sequence in MOM72 (Steger et al., 1990).

Here, we present evidence that the basic NH_2-terminus of

OMM70 cooperates with the transmembrane segment to form the requisite topogenic sequence for selection of the mitochondrial outer membrane, which we term a signal-anchor sequence. Comparisons to the functionally analogous signal-anchor sequences of type II and type III proteins (von Heijne, 1988) that are inserted into the membrane of the endoplasmic reticulum (Blobel, 1980; Wickner and Lodish, 1985) suggest a mechanism for correct sorting to the mitochondrial outer membrane.

Materials and Methods

General

Previous articles describe the routine procedures employed for recombinant DNA manipulations (Skerjanc et al., 1990; Sheffield et al., 1990), transcription of pSP64 constructs (Nguyen and Shore, 1987), translation of pSP64-derived mRNA in a rabbit reticulocyte cell-free system in the presence of [^{35}S]methionine, and isolation of mitochondria from rat heart (Argan et al., 1983). Additional details are provided in the figure legends.

Recombinant Proteins

A 650-bp *Taq*I-*Bgl*II fragment was excised from pSV$_2$DHFR and inserted between the *Acc*I and *Bam*HI sites of pSP64 (Skerjanc et al., 1990). The resulting plasmid was digested with *Hin*dIII and *Pst*I, and two adaptors containing *Hin*dIII and *Pst*I overhangs were inserted between these sites (adaptor I: 5′-AGCTATGAAGAGCTTCATTACAAGGAACAAGACAGCCATTTTGGCTGCA and 3′-TACTTCTCGAAGTAATGTTCCTTGTTCTGTCGGTAAAACCG; adaptor II: 5′-GTTGCTGCTACAGGTACTGCCATCGGTGCCTACTATTATTACGCTGCA and 3′-ACGTCAACGACGATGTCCATGACGGTAGCCACGGATGATAATAATGCG). Part of the *Pst*I site was removed by standard PCR techniques. This created the plasmid pSP (pOMD29) which encodes amino acids 1–29 of yeast OMM70 (Hase et al., 1984) connected via a glycine (position 30) to amino acids 4–186 of dihydrofolate reductase (DHFR)[1], and in which amino acid 15 was changed from threonine to alanine. The plasmid pSP(pOMD29Δ16-29) was created by employing only adaptor I in the manipulations described above. pSP(pOMD29Δ2-10) was formed by deleting adaptor I from pSP(pOMD29) and replacing it with an adaptor (5′-AGCTATGGCCATTTTGGCTGCA and 3′-TACCGGTAAAACCG) that encodes amino acids 1 and 11–15 of pOMD29. Finally, adaptor I of pSP(pOMD29) was replaced with the adaptor, 5-AGCTTATGCAGAGCTTCATTCACAGAACCAGACAGCCATTTTGGCTGCA and 3′-ATACGTCTCGAAGTAATGTGTCTTGGTCTGTCGGTAAAACCG, to yield pSP(pOMD29KR2,7,9Q), in which amino acids 2, 7, and 9 of pOMD29 were replaced by glutamine.

Mitochondrial Import

Reaction mixtures contained 10% (v/v) rabbit reticulocyte lysate transcription-translation products labeled with [^{35}S]methionine, mitochondria (0.5 mg protein/ml), 0.125 M sucrose, 40 mM KCl, 1.0 mM $MgAc_2$, 10.0 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM ATP, 2.5 mM sodium succinate, 0.04 mM ADP, and 0.1 mM potassium phosphate, pH 7.5. After incubation at 30°C, aliquots (50 μl) were removed and layered over 500 μl 0.25 mM sucrose, 10 mM Hepes, pH 7.5, 1.0 mM dithiothreitol, and mitochondria were collected by centrifugation for 2 min at 12,000 *g*.

Alkali Extraction

After import, mitochondria were recovered by centrifugation, suspended in freshly prepared 0.1 M Na_2CO_3, pH 11.5, to a final protein concentration of 0.25 mg/ml, and incubated on ice for 30 min, with periodic vortexing. Membranes were collected by centrifugation at 30 psi. for 10 min at 4°C in a Beckman Airfuge (Beckman Instruments, Carlsbad, CA).

Results and Discussion

A hybrid protein, pOMD29, was created by fusing amino acids 1–29 of yeast OMM70 (Hase et al., 1984) through a glycine (residue 30) to amino acids 4–186 of dihydrofolate reductase (DHFR). The NH_2 terminal sequence of pOMD29 and its mutant derivatives are shown in Fig. 1. As documented elsewhere (Li and Shore, 1992*a*), pOMD29 was imported into the outer membrane of intact rat heart mitochondria in vitro by a process dependent on ATP and protease-sensitive mitochondrial surface components, and in which the orientation of the native OMM70 protein was retained (N_{in}-C_{cyto}). In common with all other outer membrane proteins examined to date, insertion of pOMD29 did not require the mitochondrial electrochemical potential and the protein was not proteolytically processed (Li and Shore, 1992*a*).

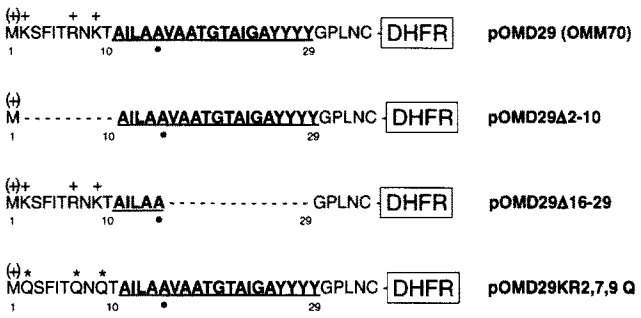

Figure 1. Recombinant proteins. The NH_2-terminal sequences of the proteins encoded by the various recombinant plasmids described in Materials and Methods are shown, using the single-letter code for amino acids. The predicted transmembrane segment (amino acids 11–29, Hase et al., 1984; Nakai et al., 1989) is underlined; •, alanine substitution for threonine at residue 15 of OMM70; (*dashes*) deletions; (*asterisks*) substitutions of lysine and arginine by glutamine; (*DHFR*) murine dihydrofolate reductase (see Materials and Methods).

Insertion of pOMD29 into the lipid bilayer of the mitochondrial outer membrane was assayed by its acquisition of resistance to extraction at pH 11.5, a property that is common to integral membrane proteins (Fujiki et al., 1982). Fig. 2 *A* demonstrates that recovery of the alkali-resistant form of pOMD29 was dependent on the presence of mitochondria during import (compare lanes *2* and *4*). However, appearance of the alkali-resistant form of pOMD29 occurred after binding of the protein to mitochondria at 30° (Fig. 2 *A*, compare lanes *3* and *4*) but much less so at 4° (Fig. 2 *A*, compare lanes *5* and *6*), suggesting that alkali extraction can distinguish between pOMD29 that is merely bound to the surface of the organelle (4°, Fig. 2 *A*) and that which is inserted into the bilayer (30°, Fig. 2 *A*). As expected, after import of a hybrid protein containing the matrix-targeting signal of preornithine carbamyl transferase fused to DHFR (i.e., pO-DHFR, Skerjanc et al., 1990), both the full-length precursor that was recovered with the organelle and the processed product, previously shown to be located exclusively in the matrix compartment (Skerjanc et al., 1990), were completely extracted at pH 11.5 (Fig. 3 *A*, *top*). As shown in Fig. 2 *B*, insertion of pOMD29 into the outer membrane and uptake of pO-DHFR to the matrix were both significantly reduced by pretreatment of the intact mitochondria with trypsin (see also Li and Shore, 1992*a*).

Targeting and Membrane-Anchor Domains of pOMD29

Deletions were introduced into pOMD29 to remove either

1. *Abbreviation used in this paper*: DHFR, dihydrofolate reductase.

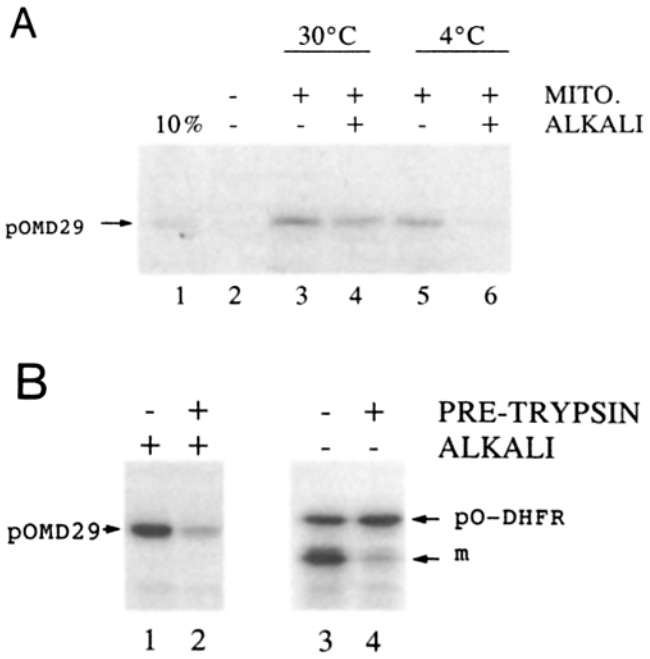

Figure 2. Temperature-dependent and protease-sensitive insertion of pOMD29. (*A*) pSP(pOMD29) was transcribed and translated in the presence of [^{35}S]methionine, and import of pOMD29 into purified rat heart mitochondria (MITO) was carried out at either 30 or 4°C. Aliquots from the reaction mixtures were removed and layered over a cushion of sucrose and centrifuged to recover mitochondria. Pellets were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) either directly (lanes *2*, *3*, and *5*) or after extraction with 0.1 M Na_2CO_3, pH 11.5 (*ALKALI*) (lanes *4* and *6*), and the products visualized by fluorography. (Lane *1*) 10% of input [^{35}S]pOMD29; (lane *2*) [^{35}S]-pOMD29 that sedimented in the absence of mitochondria. (*B*) Before import, mitochondria were treated with trypsin (*PRE-TRYPSIN*) in the presence (lanes *1* and *3*) or absence (lanes *2* and *4*) of excess soybean trypsin inhibitor, exactly as described (Li and Shore, 1992*a*). Import was carried out for [^{35}S]pOMD29 and [^{35}S]pO-DHFR as described in *A* and Fig. 3 *A*, respectively, and mitochondria were recovered and analyzed either directly (lanes *3* and *4*) or after extraction with alkali (lanes *1* and *2*) (*ALKALI*). The positions of pOMD29 and of the precursor and mature (*m*) forms of pO-DHFR are indicated.

the hydrophilic, positively charged NH_2-terminus of the protein (amino acids 2–10, Fig. 1) or a large portion of the predicted (Hase et al., 1984; Glick and Schatz, 1991) transmembrane segment (amino acids 16–29, Fig. 1). As shown in Fig. 3 *A*, pOMD29Δ2-10, which contains only the transmembrane segment at its NH_2-terminus, was capable of binding to intact mitochondria in vitro (compare lanes *2* and *3*) and, of the bound fraction, a significant amount was alkali-insoluble (lane *4*). Like pOMD29, pOMD29Δ2-10 required ATP for import (not shown) and was inserted in the N_{in}-C_{cyto} orientation, i.e., the bulk of the protein was accessible to externally-added trypsin (Fig. 3 *B*). Also, neither protein was imported into or across pancreatic ER microsomal membranes when mitochondria were replaced with these membranes in the import assay (Fig. 3 *C*), as judged by resistance to external trypsin (lane *4*) or resistance to extraction by alkali (lane *5*). These microsomes efficiently translocated and processed a major histocompatibility class 1 protein, HLA-2A; the predicted 3 kD cytoplasmic COOH-terminal tail of the polypeptide (Ennis et al., 1990) was accessible to external protease whereas the bulk of the polypeptide in the lumen was protected (Fig. 4, lane *2*), except in the presence of detergent (Fig. 4, lane *3*). In contrast to pOMD29Δ2-10, removal of a large portion of the transmembrane segment of pOMD29 resulted in a protein (pOMD29Δ16-19, Fig. 1) whose import was below the levels of detection in the heterologous system described here (mitochondria from rat heart) (data not shown). Taken together, therefore, these findings suggest that the transmembrane segment of pOMD29 makes an important contribution to targeting, as well as to membrane insertion.

Role of the Positively-Charged NH_2 Terminus of pOMD29

Despite the fact that amino acids 1–15 of pOMD29 (OMM70) on their own cannot support import of DHFR into rat heart mitochondria in vitro, the possibility that the hydrophilic NH_2-terminus of pOMD29 cooperates with the membrane-anchor segment to give optimal import was examined. A helical wheel projection of the NH_2 terminus of pOMD29 predicts that such a helix would be amphiphilic, with the basic residues at positions 2, 7, and 9 clustered on the hydrophilic face (Fig. 5). As a further consideration, therefore, the lysine residues at positions 2 and 9 and the arginine at position 7 were mutated to glutamine (Figs. 1 and 5). Like lysine and arginine, glutamine is compatible with an α-helix (Chou and Fasman, 1974), but its side chain amide is uncharged. The mutant was designated pOMD29KR2,7,9Q and was found to be competent for import (Fig. 3 *A*, *bottom*).

In Fig. 6, the rates of import and acquisition of alkali-insolubility for pOMD29, pOMD29Δ2-10, and pOMD29-KR2,7,9Q were analyzed by SDS-PAGE, and the results quantified by determining the radioactive content of gel slices containing the [^{35}S]-labeled proteins. The data show that the NH_2 terminus of pOMD29 makes a significant contribution to import, to the extent that pOMD29Δ2-10 was imported at a rate that was approximately five times slower than the rate of import of pOMD29. pOMD29KR2,7,9Q, on the other hand, exhibited a rate of insertion into the outer membrane that was approximately threefold lower than that of pOMD29 (Fig. 6). Thus, the positive charges at positions 2, 7, and 9 of pOMD29 contribute significantly, though not completely, to the optimal rate of import that is conferred by the hydrophilic NH_2 terminus of the protein. Like pOMD29 and pOMD29Δ2-10, pOMD29KR2,7,9Q was inserted into the outer membrane in the N_{in}-C_{cyto} orientation (Fig. 3 *B*).

Effects of a Bacterial-expressed Mitochondrial Matrix Precursor Protein on Import and Insertion of pOMD29 into the Outer Membrane

As reported earlier (Sheffield et al., 1990), the hybrid protein pO-DHFR (Fig. 3 *A*, top) has been expressed in bacteria and purified in a form that is efficiently imported into the matrix compartment of mitochondria in vitro. When the purified bacterial expression product was added to reticulocyte lysate containing [^{35}S]pO-DHFR produced by in vitro transcription-translation of the recombinant plasmid, pSP (pODHFR) (Skerjanc et al., 1990; Fig. 3 *A*), import of the radioactive precursor was effectively competed by bacterial pO-DHFR at concentrations of 1–6 μM (Fig. 7). This concentration range is very similar to the amount of a synthetic

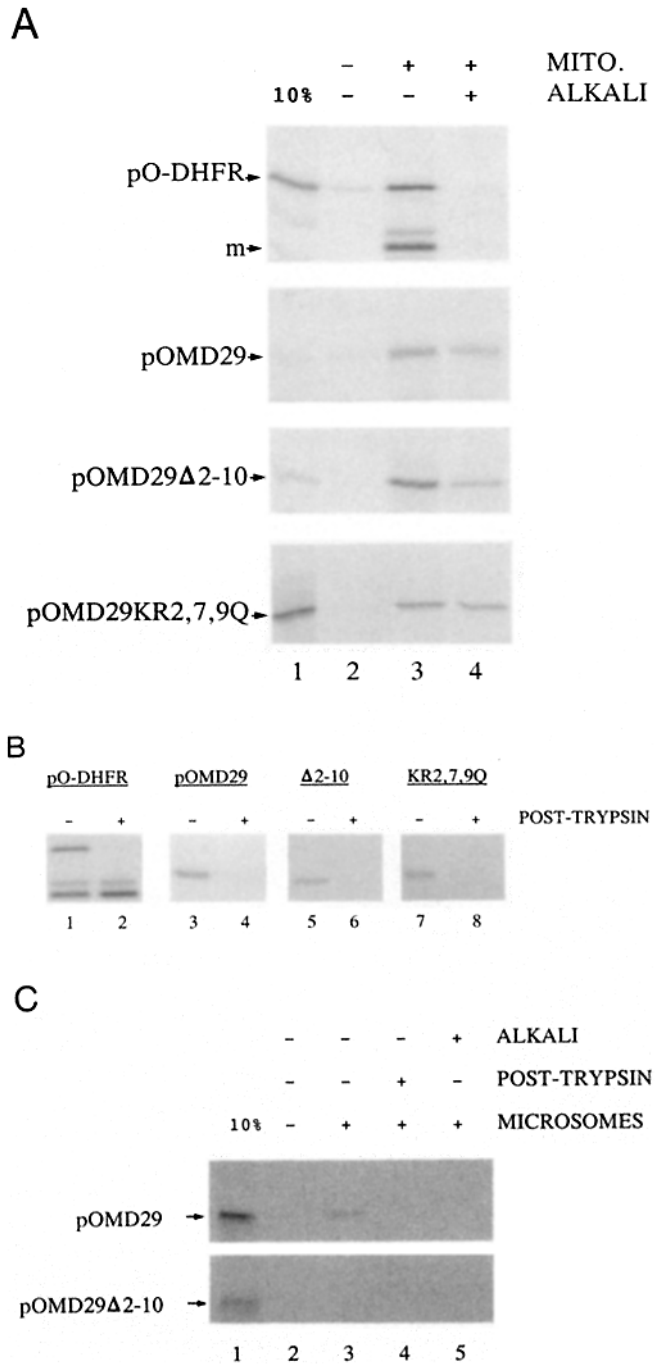

Figure 3. Import of pO-DHFR and normal and mutant forms of pOMD29. (*A*) Translation products containing [^{35}S]pO-DHFR (amino acids 1–36 of preornithine carbamyl transferase fused to DHFR, Skerjanc et al., 1990), [^{35}S]pOMD29, [^{35}S]pOMD29Δ2-10, and [^{35}S]pOMD29KR2,7,9Q were incubated with (lanes *3* and *4*) or without (lane *2*) mitochondria (*MITO*) under standard import conditions. Recovery of mitochondria, extraction with 0.1 M Na_2CO_3, pH 11.5 (lane *4*) (*ALKALI*), and analysis by SDS-PAGE and fluorography were as described in Fig. 2 and Materials and Methods. (Lane *1*) 10% of input [^{35}S]precursor protein; (lane *2*) [^{35}S]precursor protein that sedimented in the absence of mitochondria. (*B*) As in *A*, except that, after import, reaction mixtures were treated either with (lanes *2*, *4*, *6*, and *8*) or without (lanes *1*, *3*, *5*, and *7*) trypsin (*POST-TRYPSIN*) (Li and Shore, 1992*a*). Mitochondria were recovered and analyzed by SDS-PAGE and fluorography. (*C*) As in *A* except that mitochondria were substituted with dog pancreas microsomes (Walter and Blobel, 1981) in import reactions containing pOMD29 and pOMD29Δ2-10 and the reaction mixtures subsequently subjected to trypsin treatment as in (*A*) (lane *4*) or extraction with alkali (Materials and Methods) (lane *5*). The amount of ER membrane protein and mitochondrial outer membrane protein in *A* and *C* were the same (40 μg/ml).

peptide corresponding to the preornithine carbamyl transferase signal sequence of pO-DHFR that is required to block import of proteins to the matrix under similar conditions (Gillespie et al., 1985). Control experiments (data not shown) revealed that mitochondrial $\Delta\Psi$ was unaffected by the bacterial precursor, as judged by the $\Delta\Psi$-dependent uptake of [^{3}H]triphenylmethylphosphonium (iodine salt) (Bakker, 1978) into mitochondria in the import reaction. In contrast to the competition of import of pO-DHFR by itself, import and insertion of pOMD29 into the outer membrane was relatively unaffected by concentrations of bacterial pO-DHFR up to 6 μM, the highest concentration tested (Fig. 7). This was the case for import at 30° (Fig. 7), and as well for import after combining the two proteins with mitochondria at 4° for 10 min, followed by a chase at 30° (not shown). At higher levels, pO-DHFR tended to aggregate and, therefore, was not examined for its ability to compete for import of pOMD29.

Because the two proteins, pO-DHFR and pOMD29, differ only in their NH_2-terminal topogenic sequence (either 36 amino acids from pre-ornithine carbamyl transferase or 29 amino acids from OMM70 fused to DHFR, respectively), it is presumably these topogenic sequences alone that account for the differences in the competition profiles seen in Fig. 7. It is important to note, however, that the *Neurospora* homolog of OMM70 (MOM72) may employ the same master import receptor on the surface of mitochondria as do proteins that are imported to the matrix (Söllner et al., 1990). We conclude, therefore, that bacterial pO-DHFR at the concentrations examined competes for import of itself but not for pOMD29 because the rate-limiting step for import of pO-DHFR is at a point on the import pathway that is distal to translocation across the outer membrane. Such a distal rate-limiting step may explain why translocation intermediates in transit to the matrix can be detected that simultaneously span both the outer and inner mitochondrial membranes at translocation contact sites (Schleyer and Neupert 1985; Vestweber and Schatz, 1988). pOMD29, on the other hand, may insert into the outer membrane without penetrating into contact sites, a view that is compatible with the recent evidence that the translocation machineries of the two membranes are not permanently coupled (Glick et al., 1991; Pfanner et al., 1992).

Concluding Remarks

Our results indicate that the positively-charged NH_2 terminus of pOMD29 (OMM70) cooperates with the transmembrane segment to create the requisite topogenic domain for insertion of pOMD29 into the outer membrane. By analogy to the topogenic sequences of type II and type III proteins (von Heijne, 1988) inserted into the endoplasmic reticulum (Wickner and Lodish, 1985), we term this domain a signal-anchor sequence, in which the targeting domain is coinci-

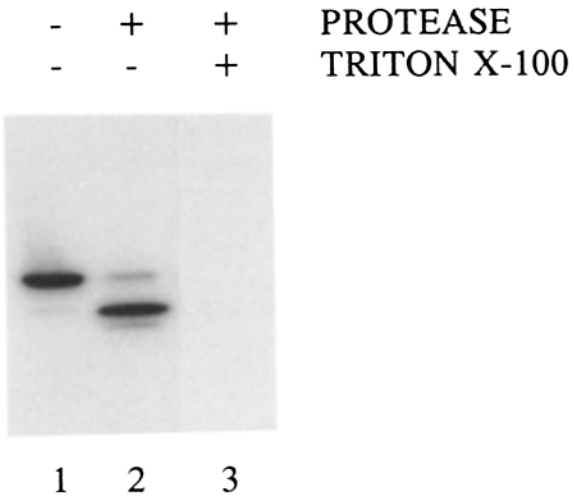

Figure 4. Insertion of HLA-2A into microsomal membrane. Human HLA-A2 cDNA (Ennis et al., 1990) was transcribed and translated in the presence of [^{35}S]methionine and dog pancreas stripped microsomes (Walter and Blobel, 1981). Microsomes were recovered, treated with (lanes *2* and *3*) or without (lane *1*) 100 μg proteinase K per ml in the presence (lane *3*) or absence (lanes *1* and *2*) of 1% (w/v) Triton X-100, and subjected to immunoprecipitation with W32 antibody (Barnstable et al., 1978). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

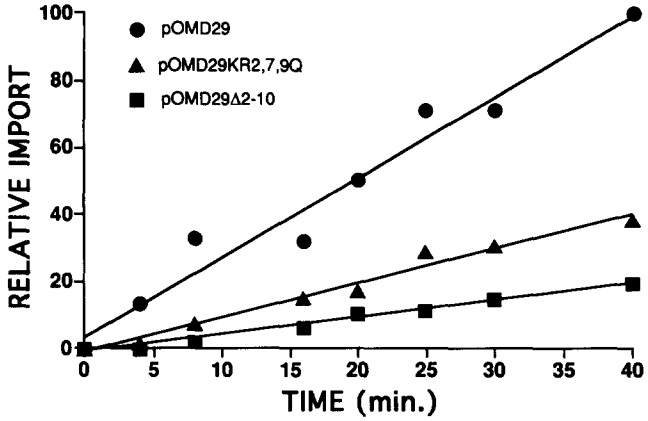

Figure 6. Rates of import and membrane insertion of pOMD29, pOMD29Δ2-10, and pOMD29KR2,7,9Q. Import reactions containing the various [^{35}S]-labeled precursor proteins were carried out for 4, 8, 16, 20, 25, 30, and 40 min., at which times mitochondria were recovered by centrifugation through a sucrose cushion, and alkali-insoluble protein obtained and subjected to SDS-PAGE and fluorography as described in Fig. 2 and Materials and Methods. Radioactive precursor proteins were located on the dried gel by aligning with an exposed x-ray film, and the bands were excised, dissolved in H_2O_2 and 0.7 M NH_4OH, and radioactivity determined by scintillation counting. The input amounts of the three [^{35}S]precursor proteins were normalized, and the results for each time point expressed as a percentage of the maximal import that was observed for pOMD29 (set at 100).

dent with, or overlaps, the membrane anchor domain. An important consequence of a signal-anchor function is that the domain that specifies targeting and initial translocation across the membrane is also the domain that abrogates this process and results in release of the segment into the surrounding lipid bilayer (Blobel, 1980; Singer, 1990). If these principles extend to mitochondrial membranes, the presence of a signal-anchor sequence may result in selection of the mitochondrial outer membrane for insertion, simply because this is the first membrane encountered by the incoming precursor protein. Similarly, the combination of a matrix-targeting signal followed immediately by a stop-transfer domain may also select the outer membrane for insertion if, again, the stop-transfer segment enters the outer membrane translocation machinery and abrogates translocation before the protein is committed for import into the interior of the organelle (Nguyen et al., 1988; Singer and Yaffe, 1990). It is interesting in this regard that the *Neurospora* homolog of OMM70, MOM72, may employ the same import receptor as do matrix-destined proteins (Söllner et al., 1990). What we have demonstrated, however, is that the transmembrane segment of pOMD29 specifies targeting and insertion, whereas the positively-charged NH_2-terminus affects only the efficiency of this process. When amino acids 1–10 of pOMD29 were replaced with a strong matrix-targeting signal (from preornithine carbamyl transferase), the protein was inserted into the outer membrane, but in an inverted orientation compared to pOMD29 (Li and Shore, 1992*b*). Both MOM72 (Steger et al., 1990) and MOM19 (Schneider et al., 1991), which are inserted into the outer membrane of *Neurospora* mitochondria with the same topology as pOMD29 (OMM70), lack a basic region upstream of the predicted transmembrane segment. It remains to be determined if the predicted transmembrane segments in these proteins, which are located at (MOM19) or toward (MOM72) the NH_2 terminus, function as signal-anchor sequences. Finally, certain proteins destined for the mitochondrial inter-

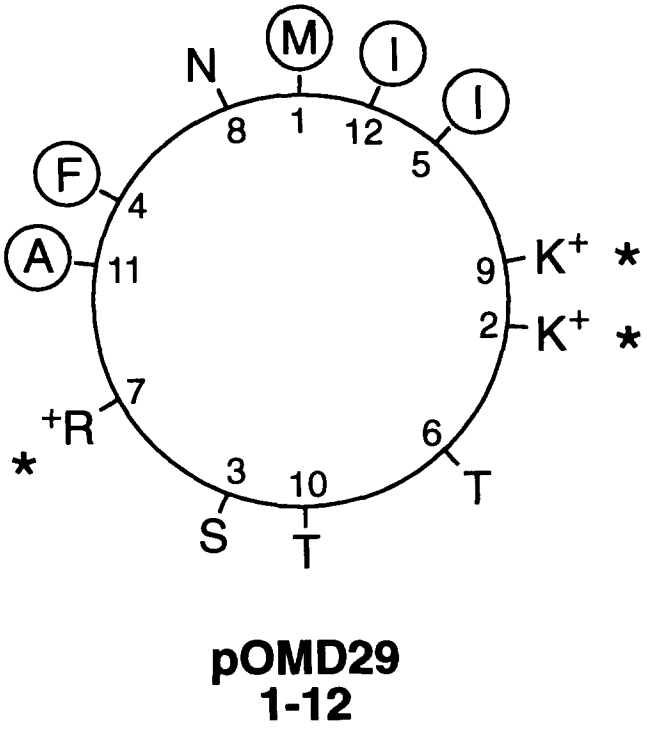

Figure 5. Helical wheel projection of amino acids 1–12 of pOMD29 (OMM70). Asterisks denote residues mutated to glutamine in pOMD29KR2,7,9Q. Hydrophobic amino acids are circled. Amino acids are designated by the single letter code.

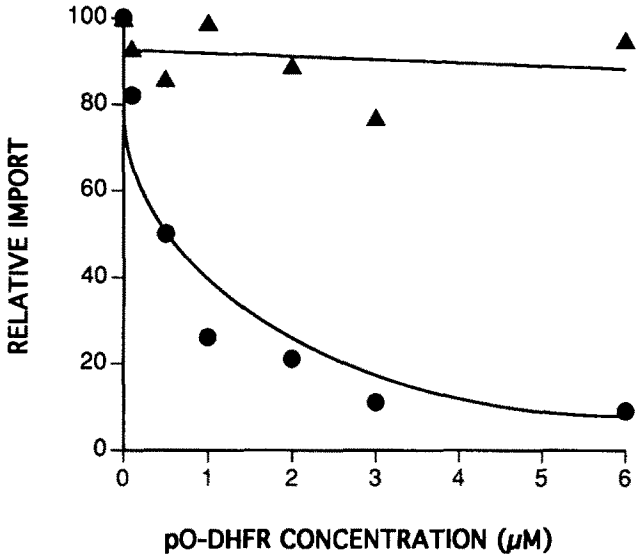

Figure 7. Insertion of pOMD29 into the mitochondrial outer membrane in the presence of bacterial-expressed pO-DHFR. pO-DHFR was expressed in bacteria, purified, and kept in 10 mM Hepes, pH 7.4, 7 M urea, and 1.0 mM dithiothreitol until use (Sheffield et al., 1990). Various concentrations of pO-DHFR in this mixture were rapidly diluted 50-fold into standard import reactions containing [^{35}S]pO-DHFR or [^{35}S]pOMD29 obtained by transcription-translation in reticulocyte lysate, and mitochondria were added to initiate import. After 10 min., reactions containing [^{35}S]pOMD29 were subjected to alkali extraction (Materials and Methods) and those containing [^{35}S]pO-DHFR were treated with trypsin (Li and Shore, 1992*a*). The products were recovered, analyzed by SDS-PAGE and fluorography, and the relative amounts of alkali-resistant pOMD29 and trypsin-resistant, processed pO-DHFR were quantified by laser densitometry (LKB2202 UltroScan) of bands on exposed x-ray film. Values obtained in the absence of bacterial pO-DHFR were arbitrarily set at 100. ●, [^{35}S]pO-DHFR; ▲, [^{35}S]pOMD29.

membrane space contain an apolar segment located toward the NH_2-terminus, immediately downstream of a matrix-targeting signal. However, whereas these sequences contribute to sorting, they probably do not function as membrane anchor sequences, i.e., these proteins do not appear to become embedded, even transiently, into the lipid bilayer (Glick et al., 1992; Koll et al., 1992). Characteristics other than simple hydrophobicity alone, therefore, contribute to the signals that specify sorting to the outer membrane and intermembrane space.

We are grateful to Dr. Weijia Ou in Dr. John Bergeron's laboratory for the gift of translocation-competent dog pancreatic microsomes and for performing the experiment in Fig. 4.

This work was supported by operating grants from the Medical Research Council and National Cancer Institute of Canada. D. G. Millar is the recipient of a Medical Research Council Studentship.

Received for publication 12 June 1992 and in revised form 31 July 1992.

References

Argan, C., C. J. Lusty, and G. C. Shore. 1983. Membrane and cytosolic components affecting transport of precursor for ornithine carbamyltransferase into mitochondria. *J. Biol. Chem.* 258:6667–6670.

Bakker, E. P. 1978. Accumulation of thallous ions (Tl^+) as a measure of the electrical potential difference across the cytoplasmic membrane of bacteria. *Biochemistry.* 17:2899–2904.

Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocyte, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell.* 14:9–20.

Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496–1499.

Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry.* 13:222–245.

Ennis, P. D., J. Zemmour, R. A. Salter, and P. Parham. 1990. Rapid cloning of HLA-A,B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. *Proc. Natl. Acad. Sci. USA.* 87:2833–2837.

Freitag, H., M. Jones, and W. Neupert. 1982. Biosynthesis of mitochondrial porin and insertion into the outer mitochondrial membrane of *Neurospora crassa*. *Eur. J. Biochem.* 126:197–202.

Fujiki, Y., S. Fowler, H. Shio, A. L. Hubbard, and P. Lazarow. 1982. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* 93:103–110.

Gillespie, L. L., C. Argan, A. K. Taneja, R. S. Hodges, K. B. Freeman, and G. C. Shore. 1985. A synthetic signal peptide blocks import of precursor proteins destined for the mitochondrial inner membrane or matrix. *J. Biol. Chem.* 260:16045–16048.

Glaser, S. M., B. R. Miller, and M. G. Cumskey. 1990. Removal of the hydrophobic domain within the native portion of a mitochondrial inner membrane protein causes its mislocation to the matrix. *Mol. Cell. Biol.* 10:1873–1881.

Glick, B., and G. Schatz. 1991. Import of proteins into mitochondria. *Annu. Rev. Genet.* 25:21–44.

Glick, B., C. Wachter, and G. Schatz. 1991. Protein import into mitochondria: Two systems acting in tandem? *Trends Cell Biol.* 1:99–103.

Glick, B. S., A. Brandt, K. Cunningham, S. Muller, R. L. Hellberg, and G. Schatz. 1992. Cytochromes c_1 and b_2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. *Cell.* 69:809–822.

Hartl, F.-U., and W. Neupert. 1990. Protein sorting to mitochondria. Evolutionary conservation of folding and assembly. *Science (Wash. DC).* 247: 930–938.

Hartl, F.-U., B. Schmidt, E. Wachter, H. Weiss, and W. Neupert. 1986. Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. *Cell.* 47:939–951.

Hase, T., U. Müller, H. Riezman, and G. Schatz. 1984. A 70-kD protein of the yeast mitochondrial outer membrane is targeted and anchored via its extreme amino terminus. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3157–3164.

Hines, V., A. Brandt, G. Griffiths, H. Horstmann, H. Brütsch, and G. Schatz. 1990. Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3191–3200.

Hurt, E. C., U. Müller, and G. Schatz. 1985. The first twelve amino acids of a yeast outer mitochondrial membrane protein can direct a nuclear-encoded cytochrome oxidase subunit to the mitochondrial inner membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3509–3518.

Jap, B. K. 1989. Molecular design of PhoE porin and its functional consequences. *J. Mol. Biol.* 205:407–419.

Koll, H., B. Guiard, J. Rassow, J. Ostermann, A. L. Horwich, W. Neupert, and F.-U. Hartl. 1992. Antifolding activity of hsp60 couples protein import into the mitochondrial matrix with export to the intermembrane space. *Cell.* 68:1163–1175.

Li, J.-M., and G. C. Shore. 1992*a*. Protein sorting between mitochondrial outer and inner membranes. Insertion of an outer membrane protein into the inner membrane. *Biochim. Biophys. Acta.* 1106:233–241.

Li, J.-M., and G. C. Shore. 1992*b*. Reversal of the orientation of an integral protein of the mitochondrial outer membrane. *Science (Wash. DC).* 256: 1815–1817.

Liu, X., A. W. Bell, K. B. Freeman, and G. C. Shore. 1988. Topogenesis of mitochondrial inner membrane uncoupling protein. Rerouting transmembrane segments to the soluble matrix compartment. *J. Cell Biol.* 107:503–509.

Liu, X., K. B. Freeman, and G. C. Shore. 1990. An amino terminal signal sequence abrogates the intrinsic membrane-targeting information of mitochondrial uncoupling protein. *J. Biol. Chem.* 265:9–12.

Mahlke, K., N. Pfanner, J. Martin, A. L. Horwich, F.-U. Hartl, and W. Neupert. 1990. Sorting pathways of mitochondrial inner membrane proteins. *Eur. J. Biochem.* 192:551–555.

Mihara, K., B. Blobel, and R. Sato. 1982. In vitro synthesis and integration into mitochondria of porin, a major protein of the outer mitochondrial membrane of *S. cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 79:7102–7106.

Nakai, M., T. Hase, and H. Matsubara. 1989. Precise determination of the mitochondrial import signal contained in the 70-kDa protein of yeast mitochondrial outer membrane. *J. Biochem.* 105:513–519.

Nguyen, M., and G. C. Shore. 1987. Import of hybrid vesicular stomatitis G protein to the mitochondrial inner membrane. *J. Biol. Chem.* 262:3929–3931.

Nguyen, M., A. W. Bell, and G. C. Shore. 1988. Protein sorting between mitochondrial membranes specified by position of the stop-transfer domain. *J. Cell Biol.* 106:1499–1505.

Pfanner, N., J. Rassow, I. J. Van der Klei, and W. Neupert. 1992. A dynamic model of the mitochondrial protein import machinery. *Cell.* 68:999–1002.
Schleyer, M., and W. Neupert. 1985. Transport of proteins into mitochondria: Translocation intermediates spanning contact sites between outer and inner membranes. *Cell.* 43:339–350.
Schneider, H., T. Söllner, K. Dietmeier, C. Eckerskorn, F. Lottspeich, B. Trülzsch, W. Neupert, and N. Pfanner. 1991. Targeting of the master receptor MOM19 to mitochondria. *Science (Wash. DC).* 254:1659–1662.
Sheffield, W. P., G. C. Shore, and S. K. Randall. 1990. Mitochondrial precursor protein. Effects of 70-kilodalton heat shock protein on polypeptide folding, aggregation, and import competence. *J. Biol. Chem.* 265:11069–11076.
Singer, S. J. 1990. The structure and insertion of integral proteins in membranes. *Annu. Rev. Cell Biol.* 6:247–296.
Singer, S. J., and M. P. Yaffe. 1990. Embedded or not. Hydrophobic sequences and membranes. *Trends Biochem. Sci.* 15:369–373.
Skerjanc, I. S., S. K. Randall, W. P. Sheffield, J. R. Silvius, and G. C. Shore. 1990. Import of proteins into mitochondria. Site of polypeptide unfolding. *J. Biol. Chem.* 265:9444–9451.
Söllner, T., R. Pfaller, G. Griffiths, N. Pfanner, and W. Neupert. 1990. A mitochondrial import receptor for the ADP/ATP carrier. *Cell.* 62:107–115.
Steger, H. F., T. Söllner, M. Kiebler, K. A. Dietmeier, R. Pfaller, W. Neupert, and N. Pfanner. 1990. Import of ADP/ATP carrier into mitochondria: Two receptors act in parallel. *J. Cell Biol.* 111:2353–2364.
Vestweber, D., and G. Schatz. 1988. A chimeric mitochondrial precursor protein with internal disulfide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. *J. Cell Biol.* 107:2037–2043.
von Heijne, G. 1988. Transcending the impenetrable: How proteins come to terms with membranes. *Biochim. Biophys. Acta.* 947:307–333.
Walter, P., and G. Blobel. 1981. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84–93.
Wickner, W. T., and H. F. Lodish. 1985. Multiple mechanisms of protein insertion into and across membranes. *Science (Wash. DC).* 230:400–407.