An Amphiphilic Lipid-Binding Domain Influences the Topology of a Signal-Anchor Sequence in the Mitochondrial Outer Membrane[†]

Nancy A. E. Steenaart,[‡] John R. Silvius, and Gordon C. Shore*

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

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ABSTRACT: Mas70p is targeted and inserted into the mitochondrial outer membrane in the N_{in}-C_{cyto} orientation, via an NH_2-terminal signal-anchor sequence. The signal-anchor is comprised of two domains: an NH_2-terminal hydrophilic region which is positively charged (amino acids 1−10), followed by the predicted transmembrane segment (amino acids 11−29). Substitution of the NH_2-terminal hydrophilic domain with a matrix-targeting signal caused the signal-anchor to adopt the reverse orientation in the membrane (N_{cyto}-C_{in}). This substitution resulted in an increase in the net positive charge of the hydrophilic region, from +4 to +8. In contrast to the endoplasmic reticulum and the bacterial inner membrane, where the net positive charge is an important determinant in conferring protein topology in the lipid bilayer, we show here that the reversal of the Mas70p signal-anchor was not due to differences in the number and positions of basic amino acids in the hydrophilic domain. However, a reduction in the hydrophobic moment of predicted amphiphilic helices containing an arginine, obtained by converting the apolar amino acids flanking the arginine to polar residues, caused the otherwise N_{cyto}-C_{in} signal-anchor to re-adopt the original N_{in}-C_{cyto} orientation of Mas70p. The reduced hydrophobic moment at the NH_2-terminus significantly reduced the ability of this domain to bind to synthetic liposomes whose lipid composition reflected that of the outer membrane. These results identify amphiphilicity as an important determinant in causing retention of the NH_2-terminus of a mitochondrial signal-anchor on the cytosolic side of the outer membrane. In addition to potential interactions between this domain and cytosolic-exposed components of the import machinery, this retention may result as well from interaction of the NH_2-terminus with the surrounding membrane surface.

The topology of integral membrane proteins is largely determined during their biogenesis (Blobel, 1980; Singer, 1990). In particular, the first domain that is inserted into the lipid bilayer is of paramount importance, since the orientation of this segment will predetermine the orientation of all subsequent insertion events. In cases where the transmembrane segment is hydrophobic and conforms to a predicted α-helix, it can be classified into two types, according to its function during polypeptide chain translocation (Blobel, 1980; Wickner & Lodish, 1985; Singer, 1990): *stop-transfer sequences*, which do not carry intrinsic membrane-selective targeting information, but rather abrogate polypeptide translocation across the membrane that has been initiated by a proximal signal sequence; and *signal-anchor sequences*, in which the targeting and membrane-anchor (stop-transfer) functions are combined into one sequence. Signal-anchor sequences also carry information that determines their orientation in the membrane.

For the endoplasmic reticulum (ER),[1] the determinants within the signal-anchor sequence that specify transbilayer orientation have been extensively investigated, by both experimental and statistical analyses. And although exceptions do exist (Andrews et al., 1992), it is likely that net charge flanking the hydrophobic core of the signal anchor is of primary importance (von Heijne & Gavel, 1988; Hartmann et al., 1989; Beltzer et al., 1991), especially as this relates to the number and distribution of positively-charged residues on the NH_2-terminal side (Parks & Lamb, 1991, 1993). Likewise, positively-charged amino acids are an important topological determinant for integral membrane proteins of the bacterial inner membrane (von Heijne, 1986; Boyd & Beckwith, 1990), with the distribution of these positively-charged residues within the polypeptide conforming to the "positive inside (cytosolic) rule" (von Heijne, 1986). Indeed, mutations that violate this rule in polytopic constructs can be so disruptive that insertion of an otherwise transmembrane segment is bypassed in order to reestablish the positive-inside constraint (Gafvelin & von Heijne, 1994). For both the ER and bacterial inner membrane, these determinants of transbilayer orientation may function as retention signals (von Heijne, 1986; von Heijne & Gavel, 1988; Hartmann et al., 1989; Boyd & Beckwith, 1990; Beltzer et al., 1991; Parks & Lamb, 1991, 1993; Gafvelin & von Heijne, 1994). In bacteria, this is due in part to the electrochemical potential across the inner membrane (inside negative) (Andersson & von Heijne, 1994), and for the ER perhaps due to a potential and/or a specific binding site for the retention determinants on the *cis* side of the membrane (Audigier et al., 1987; Hartmann et al., 1989; Parks & Lamb, 1993).

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* Corresponding author. Telephone: (514) 398-7282. Fax: (514) 398-7384.

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[1] Abbreviations: ER, endoplasmic reticulum; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DHFR, dihydrofolate reductase; pOCT, pre-ornithine carbamyl transferase.

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In contrast to the ER and bacterial inner membrane, the available information on assembly of integral proteins into mitochondrial membranes is rudimentary. However, the principles that have emerged for the ER and bacterial inner membrane are likely to apply here as well (Blobel, 1980; Singer, 1990; Singer & Yaffe, 1990; Shore et al., 1995), including the existence of domains that have been characterized as stop-transfer (Hase et al., 1984; Nguyen & Shore, 1987; Nguyen et al., 1988; Glaser et al., 1990; Miller & Cumsky, 1993) and signal-anchor (Li & Shore, 1992a; McBride et al., 1992) sequences. Details of the latter have emerged from studies of yeast Mas70p, a bitopic protein of the mitochondrial outer membrane that adopts an N_{in}-C_{cyto} orientation (Hase et al., 1984), leaving the bulk of the polypeptide facing the cytosol where it functions as a protein import receptor (Hines et al., 1990). Characteristics of the Mas70p topogenic domain are strikingly analogous, both structurally and functionally, to ER signal-anchor sequences (Li & Shore, 1992a; McBride et al., 1992; Shore et al., 1995), and for this reason the nomenclature was retained (McBride et al., 1992). In the context of mitochondria, a signal-anchor sequence guarantees selection of the outer membrane during import because it is predicted to trigger the release of the translocating polypeptide into the surrounding lipid bilayer prior to any possibility for commitment of translocation into the interior of the organelle (McBride et al., 1992). Additionally, the Mas70p signal-anchor controls the orientation of insertion (Li & Shore, 1992a) and contributes to the formation of protein oligomers (Millar & Shore, 1993, 1994).

The Mas70p signal-anchor sequence contains a positively-charged hydrophilic domain (amino acids 1−10) followed by the predicted transmembrane segment (amino acids 11−29) (Hase et al., 1984). The transmembrane segment is required for targeting and insertion, whereas the hydrophilic domain cooperates with the transmembrane segment to increase the rate of import (McBride et al., 1992). Substitution of the hydrophilic NH_2-terminus with one containing the matrix-targeting signal of pre-ornithine carbamyl transferase (pOCT) resulted in the signal-anchor adopting the reverse orientation in the outer membrane (*i.e.*, N_{cyto}-C_{in}) (Li & Shore, 1992a). Similarly, introduction of a heterologous stop-transfer sequence immediately downstream of the matrix-targeting signal in pOCT created the functional equivalent of a signal-anchor sequence, and caused this otherwise matrix-destined protein to insert into the outer membrane, again in the N_{cyto}-C_{in} orientation (Nguyen et al., 1988). Together, these results imply that the matrix-targeting signal contributed a retention function to the NH_2-terminus of the signal-anchor during protein translocation.

In the present study, we have analyzed the molecular determinants that specify this retention function and demonstrate that it is the amphiphilic character of this retention sequence, rather than the number and positions of positively-charged amino acid residues, which results in the N_{cyto}-C_{in} orientation of the signal-anchor. The amphiphilic positively-charged sequence has lipid-binding characteristics, implying that the membrane surface could contribute in the retention of the N-terminus on the cytosolic side of the membrane.

MATERIALS AND METHODS

General Procedures. Previous articles describe the routine procedures used in this study [Li and Shore (1992a), McBride et al. (1992), and Millar and Shore (1993) and references cited therein). These include *in vitro* transcription of pSP64 plasmids, translation of the resulting mRNA in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, purification of mitochondria from rat heart, protein import *in vitro*, and analysis of import products by SDS−PAGE and fluorography.

Mitochondrial Import. Reaction mixtures contained 10% (v/v) rabbit reticulocyte lysate transcription−translation products labeled with [^{35}S]methionine, mitochondria (0.5 mg of protein/mL), 0.125 mM sucrose, 32 mM KCl, 0.8 mM magnesium acetate, 9.0 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM ATP, 2.5 mM sodium succinate, 0.04 mM ADP, and 1.0 mM potassium phosphate, pH 7.5 (MRM/KMH). Some reaction mixtures also contained 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) as indicated in the figure legends. After incubation at 30 °C for 30 min, the mitochondria were collected by centrifugation for 3 min at 12000*g* and resuspended to 0.5 mg of protein/mL in MRM/KMH ± 1 μM CCCP. For post-trypsin treatment, these mitochondria were incubated with trypsin (0.125 mg/mL) for 20 min on ice after which soybean trypsin inhibitor (1.25 mg/mL) was added and the incubation continued for 10 min. Mitochondria were recovered by layering 50 μL aliquots over a 750 μL sucrose cushion (0.25 mM sucrose, 10 mM Hepes, pH 7.5, and 1.0 mM dithiothreitol) and centrifuging at 12000*g* for 6 min. Pellets were prepared for SDS−PAGE either directly or after extracting in alkali. For the latter, the mitochondria were resuspended in freshly prepared 0.1 M Na_2CO_3, pH 11.5, to a final 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 in a Beckman airfuge (Beckman Instruments, Carlsbad, CA).

Plasmids. The plasmids, pSP(pOMD29) (Li & Shore, 1992b) and pSP(pO-OMD) (Li & Shore, 1992a), were manipulated by standard PCR techniques to create the 13 other constructs employed in this study. The corresponding amino acid changes to the various topogenic domains are described in the figure legends. The authenticity of all DNA constructs was verified by nucleotide sequencing.

RESULTS AND DISCUSSION

The hybrid proteins, pOMD29 and pO-OMD, are described in Figure 1. pOMD29 (Li & Shore, 1992b) contains the signal-anchor sequence of yeast Mas70p (amino acids 1−29) fused through a glycine to amino acids 4−186 of dihydrofolate reductase (DHFR). pO-OMD (Li & Shore, 1992a), on the other hand, was created by replacing amino acids 1−11 of the Mas70p signal-anchor sequence in pOMD29 with amino acids 1−38 of rat liver pOCT, a domain which contains a potent matrix-targeting signal (Nguyen et al., 1986). Targeting and insertion of the two hybrid proteins into the outer membrane of intact mitochondria *in vitro* have been previously characterized (Li & Shore, 1992a,b; McBride et al., 1992; Millar & Shore, 1993, 1994). In the case of pOMD29, the bulk of the protein was accessible to external protease following import, indicating an N_{in}-C_{cyto} orientation (Li & Shore, 1992a,b; McBride et al., 1992) (Figure 1B). In contrast, pO-OMD adopted the reverse orientation in the outer membrane (*i.e.*, N_{cyto}-C_{in}, Figure 1B) (Li & Shore, 1992a). Treatment with external

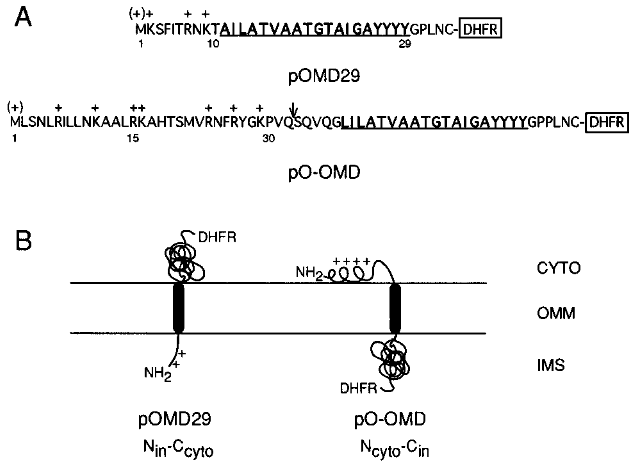

FIGURE 1: pOMD29 and pO-OMD. (A) pO-OMD was derived from pOMD29 by replacing amino acids 1−11 of pOMD29 (*i.e.*, corresponding to amino acids 1−11 of Mas70p; Hase et al., 1984) with amino acids 1−38 of pOCT (Nguyen et al., 1986). The predicted transmembrane segment is in boldface type and underlined. Arrow, the site in the pOCT signal sequence where proteolytic processing would otherwise take place in the matrix; DHFR, amino acids 8−186 of mouse dihydrofolate reductase (see Materials and Methods); pluses, positively-charged amino acids; numbers, amino acid positions relative to the initiator methionine. (B) Topology of pOMD29 and pO-OMD in the mitochondrial outer membrane (Li & Shore, 1992a; McBride et al., 1992). CYTO, cytosol; OMM, outer mitochondrial membrane; IMS, intermembrane space. See text for further details.

protease following import of pO-OMD resulted in clipping and removal of an ∼2 kDa fragment from the protein. The fragment corresponded to the NH_2-terminal pOCT matrix-targeting signal (Li & Shore, 1992a). Upon disruption of the outer membrane by osmotic shock, the external protease gained access to the intermembrane space and degraded the remainder of the pO-OMD polypeptide (Li & Shore, 1992a).

Import of pOMD29 and pO-OMD *in vitro* was ATP-dependent and temperature-sensitive (Li & Shore, 1992a,b; McBride et al., 1992). Moreover, similar to all outer membrane proteins analyzed to date and in contrast to most inner membrane and matrix proteins (Attardi & Schatz, 1988; Hartl et al., 1989), insertion of pOMD29 and pO-OMD into the mitochondrial outer membrane occurred in the presence of the uncoupler, CCCP and, therefore, did not require cooperation of the electrochemical potential across the inner membrane during import (Li & Shore, 1992a,b). In the absence of CCCP, however, a small fraction of the pO-OMD that was associated with the mitochondria had the pOCT signal sequence removed by the matrix processing machinery, indicating that at least the NH_2-terminus of these molecules had reached the matrix. This may result from the mitochondrial preparation containing a small percentage of organelles with a ruptured outer membrane. Exposed regions of the inner membrane in such damaged mitochondria have the ability to directly import pOCT at an efficiency which is in fact greater than that of intact mitochondria (Li & Shore, 1992b; unpublished). It is also possible that the potent matrix-targeting signal can partially override the signal-anchor and deliver a fraction of the molecules to the interior of the organelle (Hahne et al., 1993). To avoid this problem, all measurements of import into the outer membrane in both this and the previous study (Li & Shore, 1992a) were performed in the presence of CCCP, which abolishes this alternative pathway.

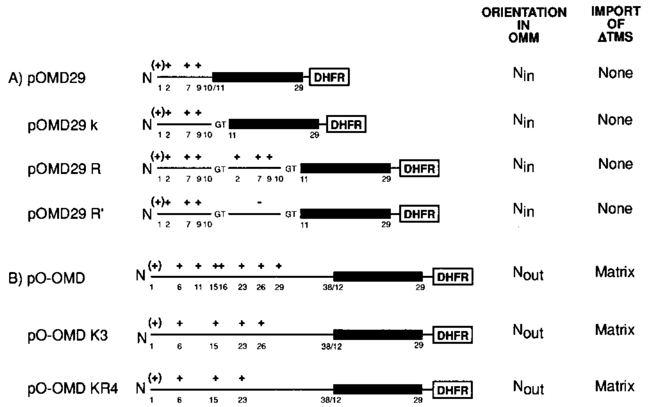

FIGURE 2: Schematic illustration of structural alterations to the NH_2-terminal hydrophilic domain of the signal-anchor sequence of pOMD29 and pO-OMD. In series A, the following amino acid sequences were introduced between residues 10 and 11 of pOMD29: glycine-threonine (pOMD29 k); amino acids 2−10 of pOMD29 flanked on either side by glycine-threonine (pOMD29 R); a sequence specified by codons for amino acids 2−10 of pOMD29 read in the reverse direction (CLVPCNEAL) and flanked on either side by glycine-threonine (pOMD29 R′). In series B, site-directed mutagenesis of pO-OMD was performed to convert lysines at positions 11 and 16 to asparagines and at position 29 to glutamine (pO-OMD K3). Additionally, the arginine at position 26 in pO-OMD K3 was converted to glutamine (pO-OMD KR4). Black rectangle, predicted transmembrane segment of the pOMD29 signal-anchor sequence (amino acids 11−29); pluses and minuses, positively and negatively charged residues, respectively; numbers, amino acid positions relative to the initiator methionine for sequences that derive from Mas70p (pOMD29 and pO-OMD) and pOCT (pO-OMD); ORIENTATION IN OMM, summarizes results of Figure 3 in which the signal-anchor sequence inserted into the outer mitochondrial membrane (OMM) in either the N_{in}-C_{cyto} (N_{in}) or the N_{cyto}-C_{in} (N_{out}) orientation; IMPORT OF ΔTMS, summarizes the results of Figure 4, in which the transmembrane segment (TMS) was deleted (Δ) from each construct, and the fate of the polypeptide following import into intact mitochondria was determined. Matrix, import to the matrix; None, no observable import (see Figure 4).

In converting pOMD29 to pO-OMD, both structural and functional alterations were made to the NH_2-terminal hydrophilic domain of the Mas70p signal-anchor sequence (Figure 1). These included an increase in length (from 10 to 37 amino acids), an increase in net charge (from +4 to +8), and a change from a domain that by itself exhibits negligible, or weak, targeting information (Mas70p amino acids 1−10 in pOMD29) with whole intact mitochondria (Hurt et al., 1984; Li & Shore, 1992b; McBride et al., 1992) to one that contains a potent matrix-targeting signal (pOCT amino acids 1−32 in pO-OMD) (Nguyen et al., 1986).

To investigate the contribution of net charge on the orientation of the protein in the mitochondrial outer membrane following import, a series of changes were made to the hydrophilic NH_2-terminus of pOMD29 that lengthened this domain and increased the number of positively-charged residues. This was achieved by duplicating the codons that specify the hydrophilic domain, either as a direct DNA repeat (pOMD29 R, in which the net charge of the encoded hydrophilic NH_2-terminal domain was increased from +4 to +7, Figure 2A) or as an inverted DNA repeat (pOMD29 R′, in which the number of encoded positively-charged residues was left unchanged, and a single negatively-charged residue was introduced into the second repeat, Figure 2A). Conversely, site-directed mutagenesis was performed on the NH_2-terminal hydrophilic domain of pO-OMD to convert the three lysine residues at positions 11, 16, and 29 to either

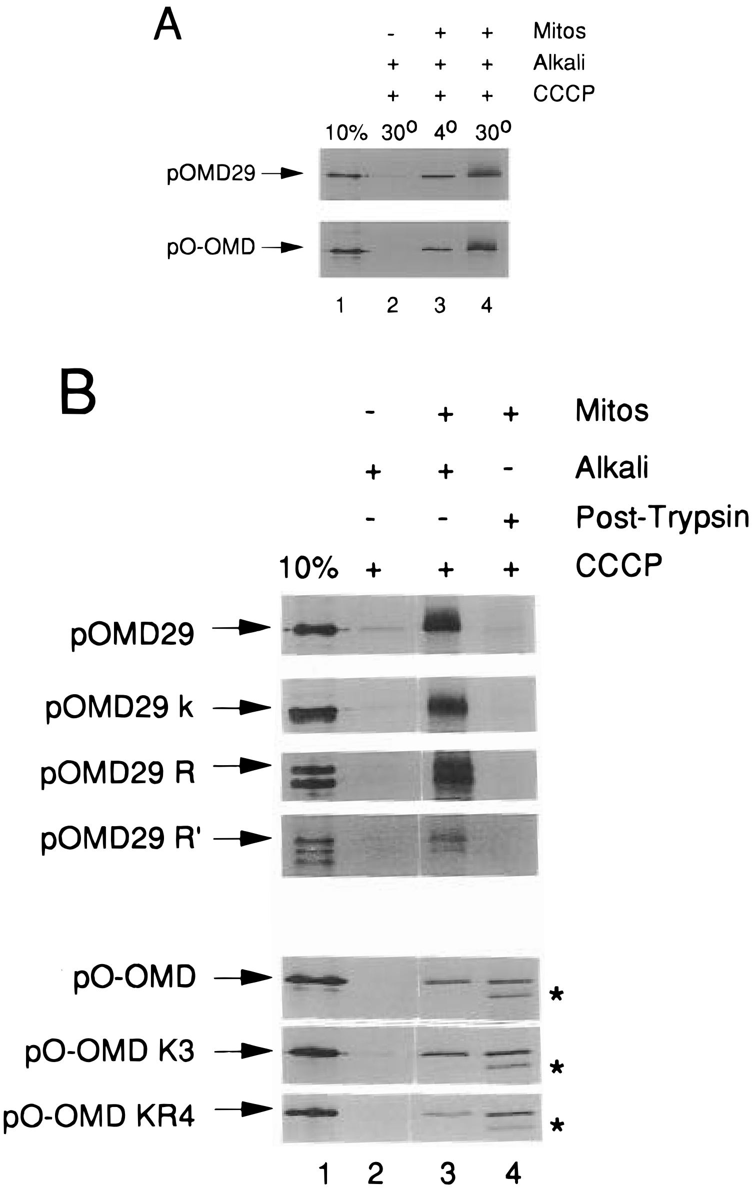

FIGURE 3: Insertion of pOMD29 and pO-OMD into the mitochondrial outer membrane. (A) Standard import reactions contained [^{35}S]-pOMD29 (upper) or [^{35}S]pO-OMD (lower) and were carried out at 4 °C (lane 3) or 30 °C (lanes 2 and 4) in the presence (lanes 3 and 4) or absence of mitochondria (Mitos) (lane 2) and in the presence of 1.0 μM CCCP (lanes 2−4). Following recovery by centrifugation, pellets were subjected to extraction with 0.1 M Na_2CO_3, pH 11.5 (Alkali) (lanes 2−4), and the insoluble material was analyzed by SDS−PAGE and fluorography. Lane 1, 10% of input radioactive precursor protein. (B) As in (A) except that the indicated [^{35}S]precursor proteins (described in Figure 2) were examined. After import, mitochondria were either extracted with alkali (lane 3) or subjected to proteolysis with trypsin (0.125 mg/mL) (Post-Trypsin) (lane 4). The asterisk denotes trypsin-clipped precursor protein.

asparagine or glutamine (pO-OMD K3), and, additionally, to convert the arginine residue at position 26 to glutamine (pO-OMD KR4) (Figure 2B). In this way, the net charge of the NH_2-terminal hydrophilic domain was reduced from +8 (pO-OMD) to either +5 (pO-OMD K3) or +4 (pO-OMD KR4).

Orientation of Mutant Proteins in the Mitochondrial Outer Membrane. The various protein constructs are described schematically in Figure 2 and their import into the mitochondrial outer membrane examined in Figure 3. Import *in vitro* was assayed by the acquisition of resistance to extraction at alkaline pH which, for both pOMD29 and pO-OMD, was dependent on the presence of mitochondria in the import reaction (Figure 3A, compare lanes 2 and 4), and was temperature-sensitive (Figure 3A, compare lanes 3 and 4). Orientation of the membrane-inserted polypeptides was determined based on their accessibility to exogenous trypsin, as previously documented (Li & Shore, 1992a). For pOMD29, proteolysis resulted in degradation of the polypeptide (Figure 3B, top panel, lane 4), consistent with its N_{in}-C_{cyto} topology (Figure 1B; Li & Shore, 1992a,b; McBride et al., 1992). In contrast, trypsin treatment of imported pO-OMD resulted in the removal of an ~2 kDa fragment from the NH_2-terminus of the protein (Figure 3B, lower panel, lane 4), due to the N_{cyto}-C_{in} orientation of the protein (Figure 1B; Li & Shore, 1992a). In Figure 3B, the clipped form is denoted by an asterisk. In the absence of mitochondria, pO-OMD was completely degraded by trypsin [not shown; see also Li and Shore (1992a)]. In both this (Figure 3) and the previous study (Li & Shore, 1992a), however, clipping of imported pO-OMD was incomplete, and a population of full-length pO-OMD was observed. It is possible that this constitutes pO-OMD in the N_{cyto}-C_{in} orientation, but where the NH_2-terminus is shielded from protease.

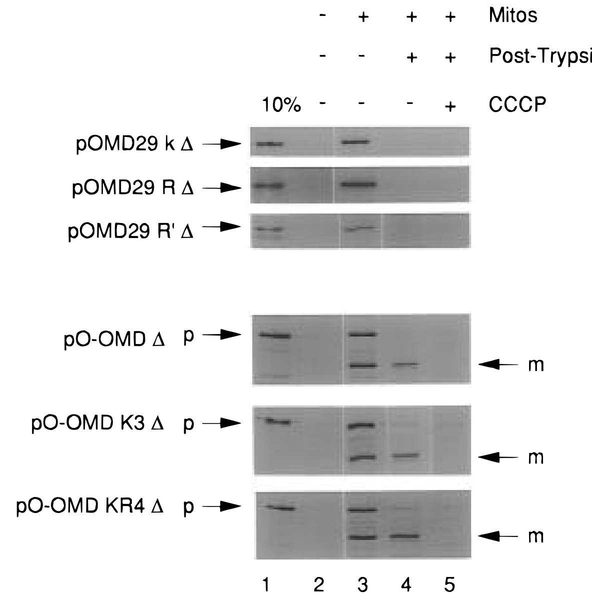

FIGURE 4: Import of pOMD29 and pO-OMD constructs lacking a transmembrane segment. All of the constructs listed in Figure 2 were manipulated to delete the 19 amino acid Mas70p transmembrane segment (indicated in the nomenclature as Δ), and import was performed. Conditions and analyses were as described in Figure 3 and under Materials and Methods. p and m denote the precursor and processed forms, respectively, of the pO-OMD Δ constructs.

The pattern of import of the mutant pOMD29 polypeptides (*i.e.*, pOMD29 k, pOMD29 R, and pOMD29 R′) was similar to that of wild-type pOMD29 (Figure 3B, upper panel). Acquisition of resistance to extraction by alkali was dependent on the presence of mitochondria (Figure 3B, lanes 2 and 3), and following membrane insertion, the bulk of the polypeptides were accessible to exogenous trypsin (Figure 3B, lane 4), indicating an N_{in}-C_{cyto} orientation. Increasing the net charge of the hydrophilic NH_2-terminus of the Mas70p signal-anchor sequence from +4 (pOMD29) to +7 (pOMD29 R), therefore, had no effect on the orientation of the protein in the outer membrane.

Likewise, decreasing the net charge within the NH_2-terminus of pO-OMD from +8 to +5 (pO-OMD K3) or to +4 (pO-OMD KR4) did not alter import of the protein, or the nature of its accessibility to exogenous trypsin (Figure 3B, lower panel); *i.e.*, the protein retained the N_{cyto}-C_{in} orientation in the outer membrane.

Deletion of the Transmembrane Domain from the pOMD29 and pO-OMD Constructs. To examine the effects of the various charge mutations on the independent ability of the hydrophilic NH_2-terminal domains of pOMD29 and pO-OMD to import the protein to the mitochondrial matrix, the 19 amino acid transmembrane domain was deleted from all constructs (see Figure 2).

It had previously been established that amino acids 1−15 of the Mas70p signal-anchor, when fused directly to DHFR, exhibited negligible activity as a matrix import signal (McBride et al., 1992), and this was also true using a mature matrix protein (OCT) as the passenger molecule, in place of DHFR (unpublished). Likewise, removal of the transmembrane domain from pOMD29 k, pOMD29 R, and pOMD29 R′ resulted in proteins (each designated by a Δ symbol) that were capable of binding to the surface of mitochondria (Figure 4, upper panels, lane 3), but did not cross into the interior of the organelle (Figure 4, compare lanes 3 and 4); i.e., they remained accessible to trypsin. None of the constructs were resistant to extraction by alkali (not shown). Increasing the net charge of the NH_2-terminal hydrophilic domain of the Mas70p signal-anchor, therefore, did not convert this domain to a matrix-targeting signal.

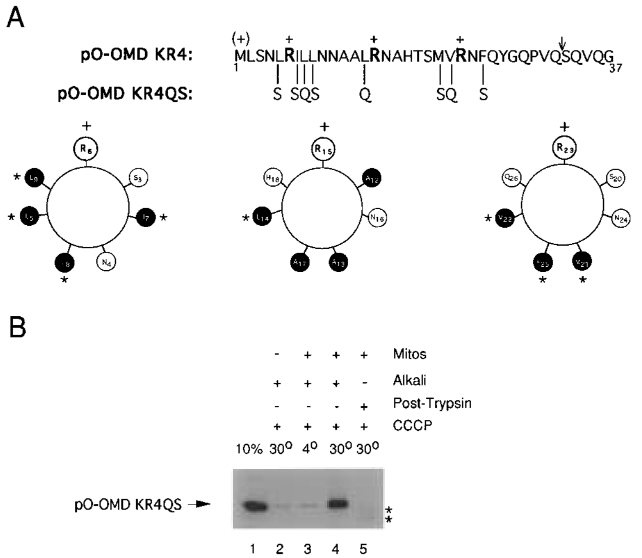

FIGURE 5: pO-OMD KR4QS. (A) Amino acids 1−37 of pO-OMD KR4, showing the amino acid substitutions giving rise to pO-OMD KR4QS. The pOCT signal processing site is indicated by an arrow. Helical wheel projections show the seven amino acids that surround each of the three arginine residues in pO-OMD KR4. Hydrophobic amino acids are indicated by black-filled circles. The asterisks denote amino acids converted to glutamine or serine. (B) Import of pO-OMD KR4QS. Conditions and analyses were as described in Figure 3 and under Materials and Methods. Asterisks denote trypsin-clipped precursor protein.

Correspondingly, a reduction in net charge of the NH_2-terminal domain of pO-OMD (*i.e.*, the pOCT signal sequence) from +8 to +4 did not prevent its ability to target DHFR to the matrix in the absence of a transmembrane domain (Figure 4, lower panels). Following import, both wild-type and mutant proteins were processed (Figure 4, lane 3), the processed products but not the full-length precursor polypeptides were protected from exogenous trypsin (Figure 4, lane 4), and this protection was dependent on the electrochemical potential across the inner membrane (Figure 4, lane 5). All of these findings are consistent with import to the matrix. Compared to pO-OMD Δ, however, the initial rates of import of the proteins to the matrix were decreased by ∼1.5- and ∼2.5-fold for pO-OMD K3 Δ and pO-OMD KR4 Δ, respectively (not shown).

Amphiphilic Domain Determines the N_{cyto}-C_{in} Orientation of the Signal-Anchor. Taken together, the results for pOMD29 and pO-OMD and their various mutant derivatives indicate that retention of the NH_2-terminus of the signal-anchor on the cytosolic side of the outer membrane during import correlates, unexpectedly, with the ability of this domain to function independently as a matrix-targeting sequence. A common property of matrix-targeting signals that might relate to a retention function, however, is their ability to adopt an amphiphilic helix. To examine this, the NH_2-terminus of pO-OMD KR4 was manipulated to convert apolar residues flanking the three arginines in this sequence (positions 6, 15, and 23) to polar amino acids (glutamine or serine, Figure 5A). While such changes are compatible with an α-helix (Chou & Fasman, 1974), they would be expected

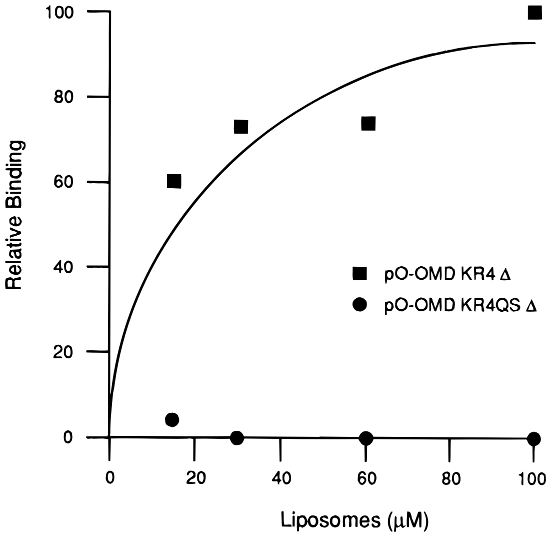

FIGURE 6: Liposome binding of pO-OMD KR4 Δ and pO-OMD KR4QS Δ. Liposomes were prepared by extrusion (Skerjanc et al., 1987; MacDonald et al., 1991) from bovine heart phosphatidylcholine, phosphatidylethanolamine, and cardiolipin and bovine liver phosphatidylinositol plus *N*-(biotinylaminocaproyl) phosphatidylethanolamine (Avanti, Polar Lipids, Alabaster, AL) in a molar ratio (based on phosphorus) of 48:31:9:11:2, respectively. Varying concentrations of liposomes were incubated with equivalent amounts of ^{35}S-labeled translation products corresponding to pO-OMD KR4 Δ and pO-OMD KR4QS Δ, for 10 min at 30 °C in a total volume of 50 μL containing 0.125 M sucrose, 10 mM Hepes, pH 7.5, 0.05 mM dithiothreitol, 40 mM KCl, and 1 mM magnesium acetate. Avidin (0.1 nmol/nmol of biotinyl groups) was added and the incubation continued for 30 min on ice. The aggregated liposomes were recovered by centrifugation for 10 min through a 500 μL sucrose cushion (as described for whole mitochondria under Materials and Methods). The pellets were analyzed by SDS−PAGE and the radioactive bands corresponding to pO-OMD KR4 Δ and pO-OMD KR4QS Δ quantified using a Fuji BAS-2000 Bio-Image Analyzer and expressed relative to the value obtained for pO-OMD KR4 Δ with 100 μM lipid present, which was set to 100 after subtracting the value obtained in the absence of lipid.

to reduce the hydrophobic moment of the predicted α-helix centered about each of the arginines (Eisenberg, 1984). As shown in Figure 5B, the resulting mutant, pO-OMD KR4QS, was efficiently imported and inserted into the mitochondrial outer membrane (Figure 5B, compare lanes 2 and 4), and the import was temperature-sensitive (compare lanes 3 and 4). In contrast to pO-OMD KR4 (Figure 3B), however, pO-OMD KR4QS was readily accessible to external trypsin and was degraded (Figure 5B, compare lanes 4 and 5); only minor levels of trypsin-protected fragments were detected (Figure 5B, asterisks). The majority of pO-OMD KR4QS, therefore, had the opposite orientation (N_{in}-C_{cyto}) compared to that of pO-OMD KR4 (N_{cyto}-C_{in}). As expected, deletion of the transmembrane segment from pO-OMD KR4QS yielded a protein (pO-OMD KR4QS Δ) that exhibited negligible import into mitochondria (not shown).

A reduction in the hydrophobic moment at the NH_2-terminus of the signal-anchor sequence might influence the ability of this domain to interact with a phospholipid bilayer surface. To examine this possibility, the NH_2-terminal domains of the pO-OMD KR4 and pO-OMD KR4QS signal-anchors (Figure 5) were fused directly to DHFR, thereby eliminating the transmembrane domain. The resulting proteins (pO-OMD KR4 Δ and pO-OMD KR4QS Δ) were incubated with liposomes under standard import conditions (Figure 6). The phospholipid composition of the liposomes reflected that of the outer mitochondrial membrane (Hovius et al., 1990) and included 2 mol % of a biotinyl-derivatized phosphatidylethanolamine to facilitate rapid recovery of the liposomes following cross-linking with avidin, as previously described (Skerjanc et al., 1988). As shown in Figure 6, binding of the pO-OMD KR4QS Δ mutant protein to the liposomes was dramatically reduced compared to the pO-OMD KR4 Δ protein. Thus, the presence of an amphiphilic lipid-binding domain at the NH_2-terminus of the signal-anchor sequence correlates with insertion of the signal-anchor into the outer membrane in the N_{cyto}-C_{in} orientation.

Conclusions. Results from both this and previous studies (Nguyen et al., 1988; Li & Shore, 1992a) suggest that properties associated with the NH_2-terminal region of a signal-anchor sequence selective for the mitochondrial outer membrane are responsible for its retention on the cytosolic side of the membrane during import, and cause the signal-anchor to adopt an N_{cyto}-C_{in} orientation in the membrane. This influence of the NH_2-terminus has been observed for protein constructs that contain different transmembrane segments and different downstream passenger proteins (Nguyen et al., 1988; Li & Shore, 1992a). Here, we have employed the Mas70p signal-anchor as a model to investigate characteristics of the NH_2-terminus that contribute to the retention function. Our findings indicate that net positive-charge alone is not the sole determinant that specifies orientation, but rather the retention function of the NH_2-terminus appears to correlate with its ability to function independently as a matrix-targeting signal.

This somewhat paradoxical finding, however, suggested an important attribute that might contribute to the retention function. Genetic analyses have revealed that numerous, diverse sequences can function as promiscuous matrix-targeting signals when located at the NH_2-terminus of a protein (Allison & Schatz, 1986; Baker & Schatz, 1987; Roise et al., 1988). These sequences have in common the potential to adopt a positively-charged amphiphilic helix with predicted anionic lipid-binding properties. Relatively tight membrane-surface-binding properties of the matrix-targeting signal used in this study, that of pOCT, have also been observed, both for a synthetic signal peptide (Epand et al., 1986; Skerjanc et al., 1987) and for the intact precursor protein (Skerjanc et al., 1988). Furthermore, reduction in the amphiphilicity of the pOCT matrix-targeting signal, due to substitution of leucine residues at positions 2, 7, and 9 with alanine, resulted in an ∼5-fold increase in the apparent K_d for the binding of the precursor to liposomes whose phospholipid composition was similar to that of the mitochondrial outer membrane (Skerjanc et al., 1988). Here, a more extensive substitution of apolar amino acids was made to reduce the amphiphilicity of predicted helices containing arginines at positions 6, 15, and 23 of the pOCT signal sequence in pO-OMD KR4. This alteration caused the otherwise N_{cyto}-C_{in} orientation of the signal-anchor to re-adopt the N_{in}-C_{cyto} orientation of the Mas70p signal-anchor. The re-adoption of the N_{in}-C_{cyto} orientation correlated with loss of lipid-binding properties of the NH_2-terminus of the signal-anchor sequence.

We suggest that a positively-charged amphiphilic domain at the NH_2-terminus of a mitochondrial outer membrane signal-anchor sequence can interact both with cytosolic-exposed acidic domains (Kiebler et al., 1993a; Lithgow et al., 1994; Hachiya et al., 1995; Haucke et al., 1995;

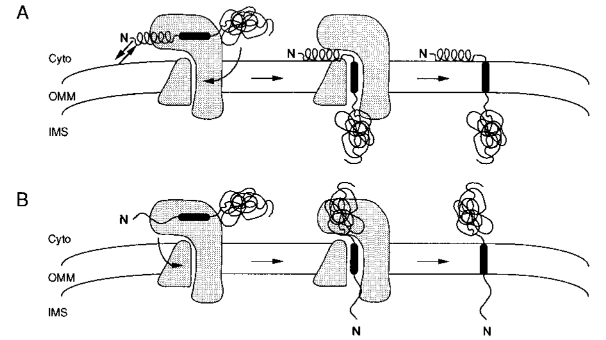

FIGURE 7: Orientation of signal-anchor sequences in the mitochondrial outer membrane. The import machinery is depicted as stippled shapes in the outer membrane, with the regions exposed to the cytosolic side representing the import receptor complex [for reviews, see Hannavy et al. (1993) and Kiebler et al. (1993b)]. The model predicts that orientation of an NH_2-terminal signal-anchor sequence in the membrane is determined by the presence or absence of a retention signal upstream of the transmembrane segment (black cylinders). (A) A slow rate of dissociation (arrows) of the retention signal from its protein- and lipid-binding sites on the *cis* side of the membrane, relative to the rate of unfolding of the polypeptide on the other side of the transmembrane segment, results in insertion across the bilayer in the N_{cyto}-C_{in} orientation. (B) In the absence of an NH_2-terminal retention signal, insertion is in the opposite N_{in}-C_{cyto} orientation. See text for discussion. Coil, amphiphilic helix; Cyto, cytosol; OMM, outer mitochondrial membrane; IMS, intermembrane space.

Honlinger et al., 1995) within the import receptor complex [for reviews, see Hannavy et al. (1993) and Kiebler et al. (1993b)] and with the surrounding membrane surface, resulting in retention of this domain on the cytosolic side of the membrane during import. Moreover, interaction of this domain with the membrane surface would likely be manifested, as well, after import is completed, and might explain why about half of the pO-OMD molecules following import were shielded from trypsin (Figure 3). This is represented schematically in Figure 7. Because of the interactions of the NH_2-terminal domain with protein- and lipid-binding sites on the *cis* side of the membrane, import favors unfolding and delivery of the COOH-terminal region of the protein into the translocation pore [for reviews, see Kubrich et al. (1995) and Lithgow et al. (1995)], resulting in insertion of the protein into the bilayer in the N_{cyto}-C_{in} orientation (Figure 7A). In the absence of strong interactions between the NH_2-terminus of the signal-anchor and the binding site on the *cis* side of the membrane, however, import favors the NH_2-terminal domain entering the translocation pore, rather than unfolding and translocation of the COOH-terminal region of the protein, and the protein adopts an N_{in}-C_{cyto} orientation (Figure 7B). Furthermore, we have recently demonstrated that pOMD29 Δ (*i.e.*, containing the Mas70p signal-anchor lacking the transmembrane segment) binds to yeast mitochondria bearing the Mas20p import receptor from either yeast (Ramage et al., 1993) or human (Goping et al., 1995) sources, but does not bind to the lipid bilayer surface of mitochondria specifically deleted of this receptor (H. M. McBride, I. S. Goping, and G. C. Shore, unpublished results). Despite the interaction of pOMD29 with this receptor, however, such binding is insufficient to retain the NH_2-terminus on the cytosolic side of the membrane following import (Figure 7B).

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