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Insertion of an uncharged polypeptide into the mitochondrial inner membrane does not require a trans-bilayer electrochemical potential: effects of positive charges

Heidi M. McBride, John R. Silvius, Gordon C. Shore *

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

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Abstract

Mitochondria with a ruptured outer membrane exhibited impaired import into this membrane of an outer membrane fusion protein containing the signal-anchor sequence of Mas70p. However, the Mas70p signal-anchor efficiently targeted and inserted the protein directly into exposed regions of the inner membrane. Import into the inner membrane was dependent on $\Delta\psi$ and this dependence was due to the presence of the positively-charged amino acids located at positions 2, 7, and 9 of the signal-anchor. In contrast to wild-type signal-anchor, mutants lacking the positively-charged residues mediated import into the inner membrane in both the presence and absence of $\Delta\psi$. The results suggest two conclusions: (1) $\Delta\psi$-dependent import of the signal-anchor sequence was due exclusively to an effect of $\Delta\psi$ on the positively-charged domain of the signal-anchor, rather than to an effect of $\Delta\psi$ on a property of the inner membrane import machinery; (2) in the absence of $\Delta\psi$, the positively-charged domain of the signal-anchor *prevented* the otherwise import-competent signal-anchor from inserting into the membrane. This suggests that the positively-charged domain leads import across the inner membrane, and that $\Delta\psi$ is required to vectorially clear this domain in order to allow the distal region of the signal-anchor to enter the translocation pathway. The implications of these findings on the mechanism of import into the mitochondrial inner membrane and matrix are discussed.

Keywords: Polypeptide; Import mechanism; Mitochondrial inner membrane; Electrochemical potential, transbilayer

1. Introduction

The step on the mitochondrial protein import pathway that involves translocation of the polypeptide chain into or across the inner membrane has been shown to require two primary sources of energy: matrix ATP [1–3] and the transmembrane electrochemical potential [4–6]. ATP is employed by mtHsp70 in the matrix to fuel a binding and release cycle that enables the chaperone to drive import of the incoming polypeptide from the *trans* side of the membrane [7–9]. The electrochemical potential, on the other hand, is required to initiate translocation of the polypeptide, but is no longer essential once the polypeptide establishes interaction with mtHsp70 [6,7]. Several studies have demonstrated that it is the positively-charged matrix-targeting signal of the precursor protein that first moves across the inner membrane [6,10,11], and that it does so by responding to the electrical component, $\Delta\psi$ (inside negative), of the total transmembrane proton motive force [12,13]. An obvious role for $\Delta\psi$, therefore, is to impose a voltage-driven electrophoretic motive force on the positively-charged signal sequence, thereby driving vectorial translocation of this domain across the membrane to the next step of the import pathway, which is the binding of the precursor polypeptide to mtHsp70. Consistent with this 'electrophoresis model' are the findings that different presequences exhibit different thresholds for inhibition of translocation by the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), i.e., $\Delta\psi$ did not have an all or nothing effect which might have been expected, for example, if $\Delta\psi$ controlled a voltage-gated import channel inde-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; pOCT, pre-ornithine carbamyl transferase; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis; RO-mitochondria, mitochondria with a ruptured outer membrane; TPMP, triphenylmethylphosphorium.

* Corresponding author. Fax: +1 (514) 3987384.

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pendently of its electrophoretic effect on the signal sequence [13]. These two possibilities, that $\Delta\psi$ acts either on the substrate or on the membrane machinery, however, are not mutually exclusive since $\Delta\psi$ could contribute to the function of one or more components of the inner membrane import machinery, but at a threshold which is below the level of $\Delta\psi$ that drives the movement of the signal sequence.

To further address the role of $\Delta\psi$ in protein import, we have taken advantage of the earlier findings in yeast that exposed regions of the inner membrane in mitoplasts are capable of direct and efficient import of proteins to the matrix [14,15]. This has also been observed in mammalian mitochondria, where it has been extended as well to direct insertion of integral proteins into the inner membrane [16]. Partial disruption of the outer mitochondrial membrane, therefore, allows for delivery to the inner membrane of a precursor protein which otherwise would not encounter the inner membrane during normal import into the intact organelle. One such protein is a fusion protein in which DHFR is linked to the signal-anchor sequence of the yeast outer mitochondrial membrane protein Mas70p. This topogenic domain is normally responsible for targeting and inserting Mas70p into the outer membrane in the N_{in}-C_{cyto} orientation, leaving the bulk of the polypeptide facing the cytosol [16,18]. The three positively-charged amino acids which are located in the hydrophilic NH_2-terminal region of the signal-anchor are not required for import, but rather they cooperate with the transmembrane portion (amino acids 11–29) of the signal-anchor to enhance the overall rate of import [19]. Here, we demonstrate that mitochondria with a ruptured outer membrane lose the ability to integrate the Mas70p signal-anchor into the outer membrane. Rather, the outer membrane is bypassed and this domain efficiently inserts into the inner membrane. Insertion is dependent on $\Delta\psi$ and this dependence is due to the positively-charged amino acids within the NH_2-terminal hydrophilic domain of the signal-anchor, rather than to an effect of $\Delta\psi$ on the inner membrane import machinery. We present evidence that the positively-charged residues prevent the *$\Delta\psi$-independent* insertion of the transmembrane domain, suggesting that the positively-charged residues must otherwise be vectorially cleared from the import machinery by $\Delta\psi$ to allow subsequent translocation of the distal portion of the signal-anchor.

2. Experimental procedures

2.1. General

Previous articles describe the standard procedures used in this study. These include in vitro transcription of recombinant plasmids [20], translation of the resulting mRNA in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine [20], import of radiolabelled recombinant proteins into mitochondria [21], and analysis of import products by SDS-PAGE and fluorography [21]. Additional details are provided in the figure legends.

2.2. Mitochondria

For isolation of intact mitochondria, livers from 200–250 g male Sprague-Dawley rats were minced and washed in ice-cold HIM (220 mM mannitol, 70 mM sucrose, 10 mM Hepes, pH 7.5, 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, and 2 mg/ml de-lipidated bovine serum albumin). The minced liver (3 g wet weight) was gently homogenized in 9 ml HIM in a Wheaten Dounce glass homogenizer using two complete up and down cycles of a glass 'B'-type pestle. The homogenate was diluted 2-fold with HIM and centrifuged at 4°C for 10 min at $600 \times g$ in a Sorval SS34 rotor. The supernatant was recovered, centrifuged at $7000 \times g$ for 15 min, and the pellet resuspended to the original homogenate volume in HIM minus albumin. After centrifuging at $600 \times g$, mitochondria were recovered from the supernatant by centrifuging at $7000 \times g$ for 15 min. The mitochondria were uniformly suspended in 0.5 ml MRM (250 mM sucrose, 10 mM Hepes, pH 7.4, 1 mM ATP, 5 mM Na succinate, 0.08 mM ADP, 1 mM dithiothreitol, and 2 mM K_2HPO_4, pH 7.4). Alternatively, mitochondria with a ruptured outer membrane were prepared as above, except that albumin was excluded at all stages and liver tissue was homogenized in a motorized Potter Elvehjem homogenizer using four up and down cycles with a tight-fitting teflon pestle operating at 400 rpm.

2.3. Liposomes

Liposomes were prepared by extrusion [38,39] from 40:45:12.5:2 (molar proportions, based on phosphorus) bovine heart phosphatidycholine, phosphatidylethanolamine and cardiolipin plus *N*-(biotinylaminocaproyl)phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL). For import, liposomes were diluted 1000-fold into MRM (10 μM phospholipid final concentration) and added to a standard import reaction, after which the liposomes were aggregated by addition of avidin (0.20 nmol/nmol biotinyl groups) and incubation for 30 min on ice. The reaction mixture was diluted 8-fold in 80 mM KCl, 10 mM Hepes, pH 7.4, and 2 mM Mg acetate and the cross-linked liposomes recovered by centrifugation at $12\,000 \times g$ for 10 min.

3. Results

As described in Experimental procedures, mitochondria from rat liver were isolated either with an intact outer membrane or with an outer membrane that was ruptured. The latter were designated RO-mitochondria (mitochondria

with a *R*uptured *O*uter membrane). In contrast to mitoplasts, in which the outer membrane has largely been stripped from the organelle [23], RO-mitochondria retained 60–80% of the outer membrane, as judged by immunoblot analysis employing an antibody against the outer membrane marker, monoamine oxidase A (not shown). Electron microscopy revealed that the remaining outer membrane was similar in morphology to that of intact mitochondria (i.e., a right-side out sheet), but contained gaps that left regions of the inner membrane exposed to the exterior of the organelle (not shown).

When analyzed for import of a matrix precursor protein, little difference was observed between intact mitochondria and RO-mitochondria. If anything, the latter were more effective than the intact organelle (Fig. 1). The precursor protein that was used in this analysis, pODHFR, contained the matrix-targeting signal of pre-ornithine carbamyl transferase (pOCT) fused to DHFR (Fig. 1). For both intact and RO-mitochondria, uptake and processing of pODHFR (Fig. 1, lanes 3 and 9) was abolished by CCCP (lanes 4 and 10) due to the dependence of this matrix precursor protein on $\Delta\psi$ for import across the inner membrane. In contrast to the full-length precursor outside the organelle, the processed product was protected against exogenous proteinase (not shown) (see Ref. [19]). Import of pODHFR was largely prevented by lowering the temperature of the import reaction to 4°C (lanes 5 and 11) or by pretreating the organelle with trypsin prior to import (lanes 6 and 12). Trypsinization of intact and RO-mitochondria had no effect on the electrochemical potential, as judged by the uptake of [^{3}H]TPMP (not shown, Ref. [24,25]). Although the route of import of pODHFR into RO-mitochondria was not examined in this study, earlier studies employing rat heart mitochondria revealed that pOCT was capable of being imported directly via exposed regions of the inner membrane [16].

In contrast to the results obtained for pODHFR, major differences between intact and RO-mitochondria were observed when examined for import of an integral protein of the outer membrane. This protein was constructed by

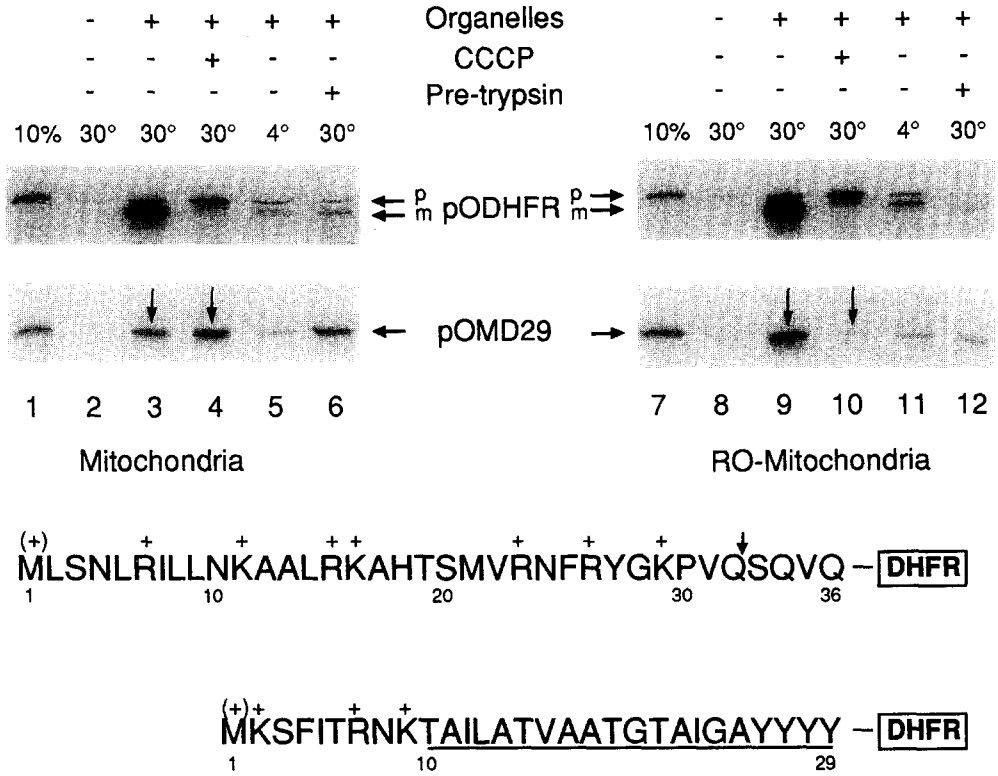

Fig. 1. Insertion of the outer membrane protein, pOMD29, into the inner membrane is dependent on $\Delta\psi$. Import reactions included either [^{35}S]pODHFR (upper panels) or [^{35}S]pOMD29 (lower panels) and were carried out in the presence of intact mitochondria (lanes 2–6) or mitochondria containing a ruptured outer membrane (RO-mitochondria) (lane 8–12) at a concentration of 0.5 mg organellar protein/ml, for 30 min at 4°C or 30°C, in the presence (lanes 4 and 10) or absence (lanes 2, 3, 5, 6 and lanes 8, 9, 11, 12) of 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In certain cases, the organelles were first treated with 0.125 mg/ml trypsin for 20 min. on ice, at which time 1.25 mg/ml soybean trypsin inhibitor [19] was added, and import performed (Pre-trypsin, lanes 6 and 12). After import, reaction mixtures (50 μl) were layered over 0.5 ml 250 mM sucrose in a 1.5 ml eppendorf tube, the organelles recovered by centrifugation and analyzed by SDS-PAGE and fluorography, either directly (pODHFR) or following extraction in 0.1 M Na_2CO_3, pH 11.5 [16,19] (pOMD29). Lanes 1 and 7, 10% of input radiolabelled protein. Arrows denote pOMD29 and the precursor (*p*) and processed (*m*) forms of pODHFR. At the bottom of the figure are shown the sequences (single letter amino acid code) of the NH_2-terminal topogenic domains of pODHFR (upper) and pOMD29 (lower), fused to amino acids 4–186 of mouse dihydrofolate reductase (DHFR). Arrow, processing site of pODHFR; underline, predicted transmembrane segment of pOMD29; plus signs, positively-charged amino acids.

replacing the pOCT matrix-targeting signal of pODHFR with the NH_2-terminal 29 amino acid signal-anchor sequence of yeast Mas70p (Fig. 1). Targeting and insertion of the resulting fusion protein, pOMD29 [16] into the outer membrane of intact mitochondria in vitro has been extensively characterized [16,19,26–28]. Like Mas70p [17,29], pOMD29 is inserted into the outer membrane in the N_{in}-C_{cyto} orientation via the predicted transmembrane domain of the signal-anchor (amino acids 11–29, Fig. 1), leaving the bulk of the protein exposed to the cytosol. Import of pOMD29 was assayed by the acquisition of resistance to extraction of the protein from the organelle at alkaline pH [16,19]. For both intact and RO-mitochondria, resistance to alkaline extraction was dependent on the presence of the organelle in the import reaction (Fig. 1, compare lanes 3 and 2, and lanes 9 and 8) and was temperature-sensitive (lanes 5 and 11). However, whereas import of pOMD29 into the outer membrane of intact mitochondria was, as expected, unaffected by the collapse of $\Delta\psi$ by CCCP (compare lanes 3 and 4, see also Refs. [16,19,26]), import into RO-mitochondria was reduced by CCCP to background levels (lanes 8–10, see also Ref. [16]). Moreover, pretreatment of RO-mitochondria with trypsin severely inhibited subsequent import of pOMD29 (lanes 9 and 12), whereas treatment of intact mitochondria at this concentration of trypsin had very little effect (lanes 4 and 6). This suggests that different receptors, exhibiting different availabilities to exogenous proteinase, were recognized by pOMD29 at the outer and inner membrane, respectively. Like import of pOMD29 into the outer membrane of intact mitochondria, however, pOMD29 was inserted into RO-mitochondria in the N_{in}-C_{cyto} orientation (not shown).

Based on the finding that CCCP prevented import of pOMD29 into RO-mitochondria, we conclude that the outer membrane was largely by-passed during import and that pOMD29 was inserted into the inner membrane. Import into the inner membrane is defined here operationally as $\Delta\psi$-dependent acquisition of resistance to extraction by alkali, since all proteins examined to date that enter or cross the inner membrane require $\Delta\psi$ for import whereas $\Delta\psi$ is not required for protein insertion into the outer membrane [30,31]. The fact that RO-mitochondria contain areas in which the outer membrane is intact, yet is largely incapable of importing pOMD29 into this membrane, suggests that the import capacity of the outer membrane has been severely compromised in RO-mitochondria. Similar observations have been made for impaired import of porin into ruptured outer membrane of yeast mitochondria. Failure to import into the ruptured outer membrane may have been due to loss of intermembrane space proteins during isolation of the organelle [32].

3.1. Import competition of pOMD29

The $\Delta\psi$-dependent import of pOMD29 into the inner membrane of RO-mitochondria was examined for competition by a synthetic peptide corresponding to amino acids 1–27 of the pOCT matrix-targeting signal [25] (Fig. 2). This peptide functions in a manner very similar to bacterially-expressed pODHFR in competing for import of both pOCT and an integral protein of the inner membrane, UCP, in intact mitochondria [33]. The rate-limiting step of import that is competed by matrix-targeting signal peptides, however, likely resides at the level of the inner membrane [34]. As shown in Fig. 2, the profile of competition of pOMD29 into RO-mitochondria was very similar to that exhibited by UCP and, therefore, the two proteins shared a common or overlapping pathway into the inner membrane. Competition for import of pODHFR was less than that observed for pOMD29 and UCP at relatively low concentrations of the synthetic pOCT signal peptide (Fig. 2), perhaps reflecting different translocation efficiencies for the different imported proteins. The electrochemical potential in RO-mitochondria was unaffected over the concentration range of peptide that was employed (0.2–2.0 μM).

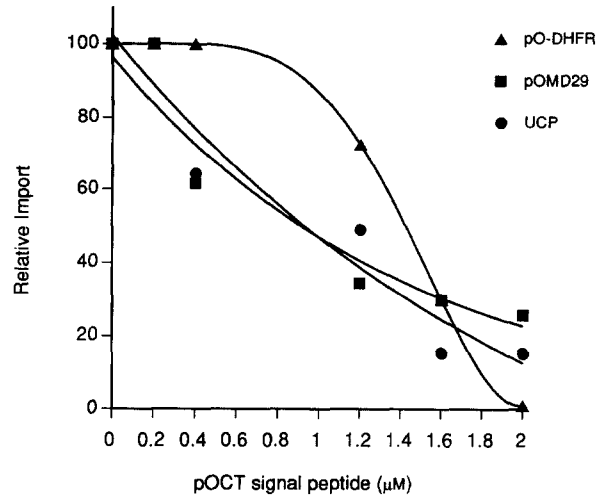

Fig. 2. Competition for pOMD29 import into the mitochondrial inner membrane by a synthetic matrix-targeting signal. Solution containing 7 M urea, 10 mM Hepes, pH 7.4, and various concentrations of the synthetic peptide, pO(1–27)cys [38] were flash diluted 25-fold into standard import reactions containing RO-mitochondria (0.5 mg protein/ml), and [^{35}S]pODHFR (▲), [^{35}S]pOMD29 (■), or [^{35}S]UCP (●). The final concentration of peptide (0–2.0 μM) is indicated. After 15 min at 30°C, RO-mitochondria were recovered and analyzed by SDS-PAGE and fluorography, either directly (pODHFR) or following alkali-extraction (pOMD29 and UCP) (see Fig. 1). Bands corresponding to alkali-resistant pOMD29 and UCP and to the processed form of pODHFR were quantified using a FUJI BAS 2000 bioimager, employing as baseline the product obtained for import in the presence of CCCP. Results obtained in the absence of peptide were arbitrarily set at 100.

3.2. Mutants of pOMD29

In Fig. 3, the effect of modifying charged amino acids in the pOMD29 signal-anchor sequence on $\Delta\psi$-dependent import of the protein into RO-mitochondria was examined. The charged residues were removed either by deleting

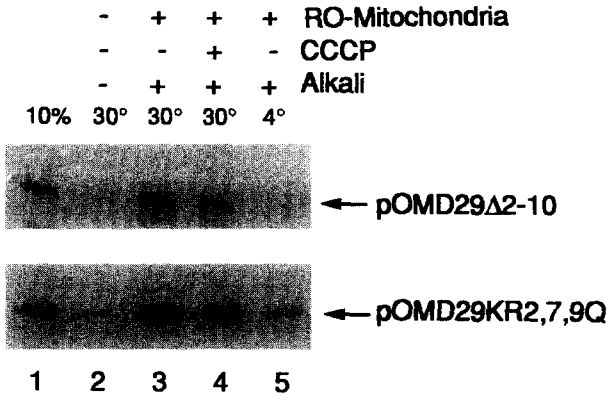

Fig. 3. Import of pOMD29Δ2–10 and pOMD29KR2,7,9Q into the inner membrane does not require $\Delta\psi$. Translation mixtures containing [^{35}S]pOMD29Δ2–10 (arrow, upper panel) or [^{35}S]pOMD29KR2,7,9Q (arrow, lower panel) were incubated with (lanes 3–5) or without (lane 2) RO-mitochondria, in the presence (lane 4) or absence (lanes 2, 3, 5) of CCCP, under standard import conditions for 30 min at the indicated temperature. Analysis was as described in Fig. 1. Lane 1, 10% of input radiolabelled protein.

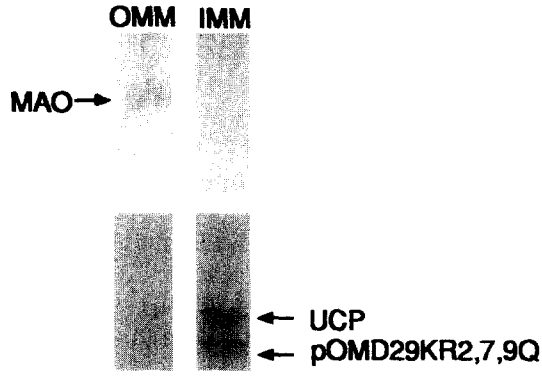

Fig. 4. Sucrose density gradient sedimentation. Reticulocyte lysate containing both [^{35}S]UCP and [^{35}S]pOMD29KR2,7,9Q was combined with RO-mitochondria, and a standard import reaction was conducted. The RO-mitochondria were recovered, subjected to sonication in hypo-osmotic medium, and outer (OMM) and inner (IMM) fractions obtained following separation in a sucrose density gradient, exactly as described in Ref. [16]. Aliquots of the fractions were subjected to SDS-PAGE and the gels analyzed either by immunoblotting with monospecific antibody against monoamine oxidase B (upper panels), or by fluorography (lower panels). OMM and IMM containing fractions are shown, and positions of monoamine oxidase A (MAO), UCP, and pOMD29KR2,7,9Q are indicated by arrows.

amino acids 2–10 (pOMD29Δ2–10) or by converting residues at positions 2, 7, and 9 to glutamine (pOMD29KR2,7,9Q) [19] (see Fig. 1). Earlier studies using intact mitochondria revealed that the positively-charged residues in the signal-anchor are not essential for protein targeting and insertion into the outer membrane, but rather that they cooperate with the transmembrane domain to enhance the overall rate of import [19]. For RO-mitochondria, import of pOMD29Δ2–10 and pOMD29-KR2,7,9Q was dependent on the presence of the organelle (Fig. 3, lanes 2 and 3) and was temperature-sensitive (lanes 4 and 5). However, in contrast to the wild-type signal-anchor (Fig. 1), the mutants forms of pOMD29 exhibited targeting and insertion that was no longer dependent on the electrochemical potential (Fig. 3, lanes 3 and 4). After import into RO-mitochondria, both mutant proteins were accessible to exogenous proteinase following import into RO-mitochondria (not shown), indicating that they had been inserted in the N_{in}-C_{cyto} orientation (not shown).

To examine the membrane location(s) of pOMD29KR2,7,9Q in RO-mitochondria, fractions enriched in outer or inner membranes were obtained by sonication of RO-mitochondria, and resolution by sucrose density gradient centrifugation [16]. As shown in Fig. 4, pOMD29KR2,7,9Q was recovered predominantly in the inner membrane fraction, together with the inner membrane marker, UCP. Conversely, pOMD29KR2,7,9Q and

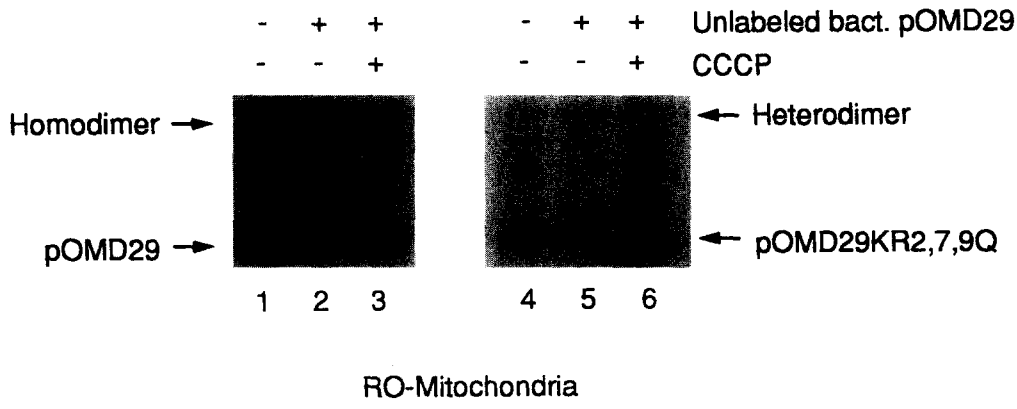

Fig. 5. $\Delta\psi$-dependent formation of pOMD29 homodimers and pOMD29-pOMD29KR2,7,9Q heterodimers in RO-mitochondria. Bacterial-expressed pOMD29 was purified in 7 M urea, 10 mM Hepes, pH 7.4 [33] and diluted 25-fold into import reactions (lanes 2, 3, 5, 6; final concentration = 0.5 μg protein/ml) containing RO-mitochondria and either [^{35}S]pOMD29 (arrow, lanes 1–3) or [^{35}S]pOMD29KR2,7,9Q (arrow, lanes 4–6). Lanes 1 and 4, urea solution minus bacterial pOMD29; lanes 3 and 6, import in the presence of CCCP. After 30 min at 30°C, RO-mitochondria were recovered, suspended and subjected to chemical cross-linking with bismaleimidohexane, as described in Ref. [27]. The products were analyzed as described in Fig. 1. Dimers are indicated by arrows.

UCP were barely detectable in the outer membrane fraction, whereas the outer membrane marker, monoamine oxidase, was enriched here (Fig. 4). The major location of pOMD29KR2,7,9Q in RO-mitochondria, therefore, was the inner membrane.

Earlier studies showed that pOMD29 is capable of forming homodimers following insertion into the outer membrane, mediated by specific helical packing between transmembrane segments [27,28]. When redirected to the inner membrane [16], pOMD29 is also capable of forming homodimers. Since insertion of wild-type pOMD29 into the inner membrane of RO-mitochondria depends on $\Delta\psi$ (Fig. 1), it follows that the formation of heterodimers between pOMD29 and pOMD29KR2,7,9Q in the inner membrane will also depend on $\Delta\psi$, despite the fact that insertion of the mutant is $\Delta\psi$-independent. To examine this possibility, [^{35}S]pOMD29KR2,7,9Q was co-imported into RO-mitochondria with non-radioactive pOMD29 purified from bacteria, at concentrations of the latter that stimulate the formation of dimers but are below the level required to compete for import (for details, see Refs. [27,28]). The formation of pOMD29 and [^{35}S]pOMD29 homodimers was sensitive to CCCP (Fig. 5, lanes 2 and 3), as was the formation of pOMD29 and [^{35}S]pOMD29KR2,7,9Q heterodimers (lanes 5 and 6). This suggested that pOMD29 and pOMD29KR2,7,9Q resided within the same $\Delta\psi$-dependent membrane compartment of RO-mitochondria. Similar results were observed for pOMD29Δ2–10 (not shown). Together with the results from sucrose gradient sedimentation (Fig. 4), these findings demonstrated that the mutant proteins were imported into the inner membrane, even though they lacked a requirement for $\Delta\psi$.

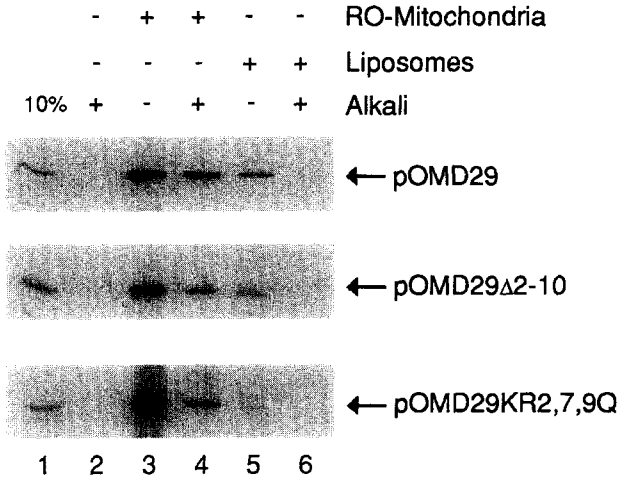

Fig. 6. Import into RO-mitochondria versus liposomes. Translation products containing [^{35}S]pOMD29 (top panel), [^{35}S]pOMD29Δ2–10 (middle panel), or [^{35}S]pOMD29KR2,7,9Q (bottom panel) were incubated without membranes (lane 2), with RO-mitochondria (0.5 mg/ml protein and 0.17 μM phospholipid) (lanes 3 and 4), or with Liposomes (2.5 μM phospholipid) (lanes 5 and 6), under standard import conditions for 30 min at 30°C. RO-mitochondria and liposomes were recovered and analyzed either directly (lanes 3 and 5) or following alkali extraction (lanes 4 and 6), as described in Fig. 1 and experimental procedures. Lane 1, 10% of input radiolabelled protein. Arrows denote the precursor proteins.

To rule out the possibility that the observed import of pOMD29Δ2–10 or pOMD29KR2,7,9Q into the inner membrane of RO-mitochondria was the result of non-specific insertion of the polypeptides directly into the lipid bilayer, the association of these mutant proteins was examined with liposomes whose phospholipid composition was similar to that of the inner membrane [35]. As documented previously, the liposomes contained biotinylated phosphatidylethanolamine which facilitated quantitative recovery of the liposomes from import reactions by cross-linking with avidin [22]. Liposomes were included in standard import reactions at a concentration of lipid that was about 15-times greater than the lipid content of the RO-mitochondria used in these assays. Compared to RO-mitochondria, the liposomes exhibited reduced binding of both the wild-type and mutant pOMD29 protein (Fig. 6, lanes 3 and 5). Moreover, virtually all of the protein that was recovered with the liposomes was extracted with alkali (Fig. 6, lanes 4 and 6), indicating that it had been associated peripherally with the liposome surface rather than integrating into the bilayer.

4. Discussion

We have found that a ruptured outer membrane severely compromised insertion of the Mas70p signal-anchor into the regions of this membrane that remained associated with the organelle and appeared otherwise normal morphologically. One explanation for this is that exposed regions of the inner membrane were far more efficient at importing pOMD29 than was the available outer membrane. A more likely possibility, however, may be that the integrity of the outer membrane import machinery was lost in RO-mitochondria, perhaps due to loss of a critical component during the isolation of the RO-mitochondria [11]. This interpretation is consistent with the findings that isolated outer membrane vesicles are relatively inefficient in integrating outer membrane proteins compared to the intact organelle (Refs. [36,37], and unpublished data). Nevertheless, properties of the Mas70p signal-anchor that relate both to its transmembrane segment and its positively-charged hydrophilic domain allowed the signal-anchor to by-pass the outer membrane of RO-mitochondria, and effectively target the inner membrane.

Insertion of the Mas70p signal-anchor into the inner membrane of RO-mitochondria followed the same import pathway as proteins native to the inner membrane and matrix, indicating that this otherwise outer membrane signal-anchor is recognized by the inner membrane translocation machinery, with a dependence on $\Delta\psi$ (inside negative). The charge-deficient mutants of the Mas70p signal-anchor were also integrated into the inner membrane, but

without a dependence on $\Delta\psi$. The requirement for $\Delta\psi$, therefore, was a property of the positively-charged domain of the signal-anchor sequence rather than an effect of $\Delta\psi$ on, for example, a component of the translocation machinery. This finding is consistent with the suggestion that $\Delta\psi$ exerts its effect by imposing an electrophoretic motive force on positively charged amino acids, resulting in vectorial conductance of the domain through the predicted translocation pore [13]. Once the translocating polypeptide chain establishes contact with mthsp70 on the *trans* side of the inner membrane, subsequent dependence on $\Delta\psi$ is overcome [7].

Despite the fact that the positive charge deficient mutants of the Mas70p signal-anchor were capable of insertion into the inner membrane in the absence of $\Delta\psi$, the wild-type signal-anchor was not. Thus, the positively-charged residues at positions 2, 7, and 9 of the signal-anchor *prevent* $\Delta\psi$-independent import, and this inhibition overrides the ability of the rest of the signal-anchor to independently insert into the membrane. One interpretation of this finding is that positively-charged residues within the signal-anchor sequence precede the transmembrane segment into the predicted translocation pore of the inner membrane, and that $\Delta\psi$ is required to vectorially clear these residues to the matrix, thereby allowing the adjacent transmembrane portion of the signal-anchor to enter the translocation pathway. This is consistent with the suggestions that an essential component of the inner membrane translocation machinery, MIM44, recognizes positively-charged targeting signals ([40], reviewed in [41]). $\Delta\psi$-dependent release of positively-charged residues from interactions with proteins such as MIM44 within the translocation pore might constitute a rate-limiting step during normal import into or across the inner membrane.

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References

[1] Hwang, S.T. and Schatz, G. (1989) Proc. Natl. Acad. Sci. USA 86, 8432–8436.
[2] Ostermann, J., Horwich, A.L., Neupert, W. and Hartl, F.-U. (1989) Nature 341, 125–130.
[3] Wachter, C., Schatz, G. and Glick, B.S. (1994) Mol. Biol. Cell 5, 465–474.
[4] Gasser, S.M., Daum, G. and Schatz, G. (1982) J. Biol. Chem. 257, 13034–13041.
[5] Schleyer, M., Schmidt, B. and Neupert, W. (1982) Eur. J. Biochem. 125, 109–116.
[6] Schleyer, M. and Neupert, W. (1985) Cell 43, 339–350.
[7] Neupert, W., Hartl, F.-U., Craig, E.A. and Pfanner, N. (1990) Cell 63, 447–450.
[8] Schatz, G. (1993) Prot. Sci. 2, 141–146.
[9] Stuart, R.A., Cyr, D.M., Craig, E.A. and Neupert, W. (1994) Trends Biochem. Sci. 19, 87–92.
[10] Chen, W.-J. and Douglas, M.G. (1987) Cell 49, 651–658.
[11] Schwaiger, M., Herzog, V. and Neupert, W. (1987) J. Cell Biol. 105, 235–246.
[12] Pfanner, N. and Neupert, W. (1985) EMBO J. 4, 2819–2825.
[13] Martin, J., Mahlke, K. and Pfanner, N. (1991) J. Biol. Chem. 266, 18051–18057.
[14] Hwang, S., Jascur, T., Vestwear, D., Pon, L. and Schatz, G. (1989) J. Cell Biol. 109, 487–493.
[15] Pon, L., Moll, T., Vestwear, D., Marshallsay, B. and Schatz, G. (1989) J. Cell Biol. 109, 2603–2616.
[16] Li, J.-M. and Shore, G.C. (1992) Biochim. Biophys. Acta 1106, 233–241.
[17] Hase, T., Müller, U., Reizman, H. and Schatz, G. (1984) EMBO J. 3, 3157–3164.
[18] Nakai, M., Hase, T. and Matsubara, H. (1989) J. Biochem. 105, 513–519.
[19] McBride, H.M., Millar, D.G., Li, J.-M. and Shore, G.C. (1992) J. Cell Biol. 119, 1451–1456.
[20] Nguyen, M., Argan, C., Lusty, C.J. and Shore, G.C. (1986) J. Biol. Chem. 261, 800–805.
[21] Argan, C., Lusty, C.A. and Shore, G.C. (1983) J. Biol. Chem. 258, 6667–6670.
[22] Skerjanc, I.S., Sheffield, W.P., Silvius, J.R. and Shore, G.C. (1988) J. Biol. Chem. 263, 17233–17236.
[23] Greenawalt, J.W. (1979) Methods Enzymol. 55, 88–98.
[24] Bakker, E.P. (1978) Biochemistry 17, 2899–2904.
[25] Gillespie, L.L., Argan, C., Taneja, A.T., Hodges, R.S., Freeman, K.B. and Shore, G.C. (1985) J. Biol. Chem. 260, 16045–16048.
[26] Li, J.-M. and Shore, G.C. (1992) Science 256, 1815–1817.
[27] Millar, D.G. and Shore, G.C. (1993) J. Biol. Chem. 268, 18403–18406.
[28] Millar, D.G. and Shore, G.C. (1994) J. Biol. Chem. 269, 12229–12232.
[29] Hines, V., Brandt, A., Griffith, G., Horstmann, H., Brültsch, H. and Schatz, G. (1990) EMBO J. 9, 3191–3200.
[30] Attardi, G. and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333.
[31] Hartl, F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45.
[32] Smith, M., Hicks, S., Baker, K. and McCauley, R. (1994) J. Biol. Chem. 269, 28460–28464.
[33] Sheffield, W.P., Shore, G.C. and Randall, S.K. (1990) J. Biol. Chem. 265, 11069–11076.
[34] Cyr, D.M. and Douglas, M.G. (1991) J. Biol. Chem. 266, 21700–21708.
[35] Hovius, R., Lambrechts, H., Nicolay, K. and De Kruijff, B. (1990) Biochim. Biophys. Acta 1021, 217–226.
[36] Mayer, A., Lill, R. and Neupert, W. (1993) J. Cell Biol. 121, 1233–1243.
[37] Iwahashi, J., Takaichi, S., Mihara, K. and Omura, T. (1994) J. Biochem. 116, 156–163.
[38] Skerjanc, I.S., Shore, G.C. and Silvius, J.R. (1987) EMBO J. 6, 3117-3123.
[39] MacDonald, R.L., MacDonald, R.I., Menco, P.Ph.M., Taskeshta, K., Subbarao, N.K. and Hu, L. (1991) Biochim. Biophys. Acta 1061, 297–303.
[40] Blom, J., Kubrick, M., Rassow, J., Voos, W., Dekker, P.J.T., Maarse, A.C., Meijer, M. and Pfanner, N. (1993) Mol. Cell Biol. 13, 7364–7371.
[41] Pfanner, N., Craig, E.A. and Meijer, M. (1994) Trends Biochem. Sci. 19, 368–372.