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Protein sorting between mitochondrial outer and inner membranes. Insertion of an outer membrane protein into the inner membrane

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The amino terminal 29 amino acids of the outer mitochondrial membrane protein of yeast, OMM70 (MAS70), consisting of the targeting and membrane anchor domains, has been fused to a reporter protein, dihydrofolate reductase. The hybrid protein, designated pOMD29, was efficiently imported into the outer membrane of rat heart mitochondria by a process dependent on ATP and proteinase-sensitive components on the surface of the organelle, and in which the orientation of the native protein was retained. To determine if the protein translocation machinery of the inner membrane is also capable of recognizing and inserting pOMD29, direct access to the intermembrane space was provided to pOMD29 by selectively rupturing the mitochondrial outer membrane by osmotic shock. In this system, the outer membrane binding site for matrix-destined precursor proteins can be bypassed, and efficient import restored to proteinase-pretreated mitochondria. pOMD29 was imported into the inner membrane of osmotically-shocked mitochondria, mediated by protein components. The outer membrane orientation of pOMD29 was conserved when inserted into the inner membrane but, unlike the outer membrane, import into the inner membrane required $\Delta\psi$. We conclude that the protein translocation machinery of the mitochondrial inner membrane is capable of recognizing and inserting a protein whose topogenic information otherwise results in insertion of the protein to the outer membrane. The significance of these findings for sorting of proteins between the mitochondrial inner and outer membranes is discussed.

Introduction

Two pathways appear to have evolved for the delivery of nuclear-encoded proteins to mitochondrial membranes: the stop-transfer sorting pathway [1–7] and the conservative sorting pathway [7,8].

The conservative sorting pathway has been best documented for proteins that are delivered to the mitochondrial intermembrane space (for a review, see Ref. 8). Examples are the Rieske Fe-S protein [9], cytochrome b_2 [10], and cytochrome c_1 [10]. In all three cases, sorting involves initial translocation across both mitochondrial membranes to the soluble matrix compartment, removal of the N-terminal matrix-targeting signal sequence from the precursor protein, and export to the intermembrane space mediated by a second sorting signal in the precursor. An example of an inner membrane protein that follows the conservative sorting pathway is subunit 9 of *Neurospora crassa* F_0-ATPase, a small, highly hydrophobic protein [7]. A common feature of all proteins identified to date that follow the conservative pathway is their ancestral origins in bacteria. It has been proposed, therefore, that a bacterial export pathway from the matrix and into or across the inner membrane has been conserved during the endosymbiotic evolution of mitochondria in eukaryotic cells [8].

The stop-transfer sorting pathway describes those proteins that are integrated into either the outer or inner membrane via a unidirectional insertion mechanism rather than passing first to the matrix and then being redirected for export. Such a pathway presumably applies to all outer membrane proteins because these proteins do not require the mitochondrial electrochemical potential for insertion [13,14], i.e., they do not pass into or across the inner membrane. Addition-

Abbreviations: UCP, uncoupling protein; AAC, ADP/ATP carrier; SBTI, soybean trypsin inhibitor; MRM, mitochondrial resuspension medium; pOCT, pre-ornithine carbamoyltransferase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MTPP, methyltriphenylphosphonium; DHFR, dihydrofolate reductase; EDTA, ethylenediaminetetraacetate, sodium salt.

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ally, two proteins of the inner membrane that follow the stop-transfer pathway have been described, UCP [6] and AAC [7], neither of which have homologs in bacteria.

To date, little has been learned concerning the mechanisms of protein insertion into mitochondrial membranes, other than the findings that deletion of membrane anchor domains may result in translocation of a protein to the matrix compartment [5,15,16] and, conversely, insertion of a membrane anchor into an otherwise matrix-destined protein may result in insertion of the protein into either the outer or inner membrane [4]. An important question for developing models to explain sorting between the outer and inner membranes is whether the translocation machineries of the two membranes, when assayed independently, can execute insertion of the same protein into their respective lipid bilayers. In the present study, we have analyzed this question in a system [17,18] that allows a precursor to an outer membrane protein to gain direct access to the mitochondrial intermembrane space. Depending on the manipulations employed, this single protein is efficiently inserted into either the outer membrane in a $\Delta\psi$-independent manner or into the inner membrane by a mechanism dependent on $\Delta\psi$. In both cases, the orientation of the native protein is retained, and insertion is mediated by proteinase-sensitive components.

Experimental procedures

General. Previous articles [19–21] describe routine procedures employed for transcription in the pSP64 system, translation in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, isolation of rat heart mitochondria, import into mitochondria in vitro, and the plasmid construction encoding pOCT (pSP019). Additional details are provided in the figure legends.

Pretreatment of mitochondria with trypsin or apyrase. Following purification, mitochondria were suspended in mitochondrial resuspension medium (MRM, Ref. 20). Trypsin was added (8 μg trypsin per mg mitochondrial protein), the mixture was incubated on ice for 20 min, and a 5-fold weight excess of SBTI was added to stop proteinase digestion, and the mixture left on ice for an additional 10 min before using the mitochondria for import. For apyrase treatment, mitochondria were suspended in MRM lacking ATP and incubated with apyrase (1.3 units apyrase/mg mitochondrial protein) for 20 min on ice. Concurrently, reticulocyte lysate translation products were treated with 0.1 units apyrase/mg lysate protein.

Osmotic shock. Untreated or trypsin-pretreated mitochondria in MRM (15 mg protein/ml) were diluted 10-fold with 10 mM Na_2HPO_4-NaH_2PO_4 (pH 7.4) and incubated on ice for 20 min. As a control, mitochondria were also diluted in phosphate buffer containing 250 mM sucrose. The suspensions were adjusted to 1 × MRM medium and aliquots were added to a standard mitochondrial import assay (final concentration of mitochondrial protein: 0.5 mg/ml).

Alkaline extraction of mitochondria. After import, mitochondria were collected by centrifugation and suspended in 0.1 M Na_2CO_3 (pH 11.5) to a final protein concentration of 0.25 mg/ml. The suspensions were sonicated for 2 min with a Sonic Dismembrator at power setting 60 (Artek system Corporation) and incubated on ice for 30 min. Membranes were recovered after centrifugation in an airfuge operating at 30 psi for 10 min.

Electrochemical potential. $\Delta\psi$-dependent uptake of [^{3}H]methyltriphenylphosphonium (iodine salt) was measured by the procedure described in Ref. 22. The assay mixture contained rat heart mitochondria in MRM (0.75 mg protein/ml) with or without 1.0 μM CCCP and was incubated at 30°C for 10 min. Mitochondria were recovered by centrifugation and the radioactivity present in the pellet was determined by liquid scintillation counting.

Results

To provide access of an outer membrane precursor protein to the inner membrane, we have employed a system previously developed for yeast mitochondria in which the outer membrane is selectively ruptured by osmotic shock [18]. In preliminary experiments, the fidelity of the system employing rat heart mitochondria was first established for a well-characterized matrix precursor protein, pOCT [3,4,19–21].

Like yeast, the inner membrane of mammalian mitochondria is osmotically sensitive whereas the outer membrane is not. Fig. 1 and Table I establish that the outer membrane of rat heart mitochondria can be selectively ruptured by reversible swelling and shrinking of the inner membrane by osmotic shock, leaving the inner membrane intact. Breaking of the outer membrane was monitored by observing the reduction of externally-added cytochrome *c* by succinate–cytochrome-*c* oxidoreductase [23]. Rupturing the outer membrane by osmotic shock was as effective as digitonin treatment, a classic procedure for disrupting the outer membrane of mammalian mitochondria [24] (compare positions 6 and 10 in Fig. 1). Osmotic shock, however, avoided potential effects on protein integrity that may occur at higher concentrations of the detergent (a possible reason for reduced enzyme activity at positions 7 and 8 in Fig. 1). The proportion of mitochondria with a ruptured outer membrane was not determined.

Fig. 1 also established that pretreatment of intact mitochondria with trypsin did not affect the intermem-

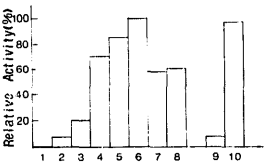

Fig. 1. Rupturing the outer membrane of rat heart mitochondria by digitonin or osmotic shock. Freshly isolated mitochondria were resuspended (3 mg protein/ml) in MRM medium (see Experimental procedures) and adjusted to 0, 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, or 4.0 mg of digitonin (twice recrystallized from ethanol)/mg protein. Alternatively, the mitochondria were treated with trypsin (8 μg/mg mitochondrial protein) and incubated on ice for 20 min, at which time a 20-fold weight excess of SBTI was added, and the mitochondria subjected to treatment with either isotonic (control) or hypotonic (osmotic shock) medium, as described in Experimental procedures. Aliquots from each treatment (45 μg protein) were assayed for succinate–cytochrome-c oxidoreductase activity as described in Ref. 23; maximal activity was arbitrarily assigned a value of 100% and corresponded to 260 nmol cytochrome c reduced /mg protein/min. Bar graph: positions 1–8 (treatment with 0–4 mg digitonin/mg mitochondrial protein, respectively); position 9, trypsin pretreatment, minus osmotic shock; position 10, trypsin pretreatment, plus osmotic shock.

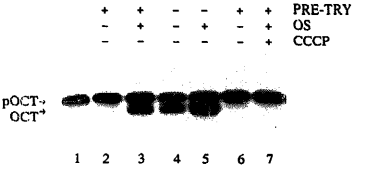

Fig. 2. Import of pOCT into trypsin-pretreated, osmotically shocked mitochondria. Freshly isolated mitochondria were treated with or without trypsin (8 μg/mg mitochondrial protein) and a portion of each subjected to subsequent osmotic shock (see Experimental procedures). Aliquots were incubated in a standard import reaction containing [^{35}S]pOCT for 30 min at 30°C, either in the presence or absence of 1 μM CCCP (lanes 2–7). Mitochondria were recovered by centrifugation and analyzed by SDS-PAGE and fluorography. Lane 1, 10% of input pOCT; lane 2, trypsin pretreatment; lane 3, trypsin pretreatment followed by osmotic shock; lane 4 untreated, intact mitochondria; lane 5, osmotic shock; lane 6, as in lane 3, but osmotic shock medium contained 250 mM sucrose (mock treatment); lane 7, as in lane 3, but including 1.0 μM CCCP. PRE-TRY pretreatment of mitochondria with trypsin; OS osmotic shock.

brane space enzyme and that subsequent treatment with trypsin inhibitor effectively inhibited the proteinase (Fig. 1, positions 9 and 10). Table I shows that trypsin pretreatment of mitochondria, followed by osmotic shock to selectively break the outer membrane, had little effect on mitochondrial $\Delta\psi$, a sensitive measure of the integrity of the inner membrane.

Import of pOCT

When intact mitochondria were digested with low concentrations of trypsin, subsequent import of pOCT was abolished (Fig. 2, compare lanes 2 and 4). Import was monitored by observing the processing of precursor to mature product (Fig. 2), a step that we have previously established takes place in the matrix compartment under the conditions employed here for import in vitro [3,4,19–21]. In contrast to import, however, pretreatment of mitochondria with trypsin did not reduce the amount of full-length pOCT recovered in

TABLE I

Mitochondria treated with trypsin and osmotic shock retain an electrochemical potential

Freshly isolated mitochondria were incubated on ice for 20 min in the presence of trypsin (8 μg/mg mitochondrial protein); soybean trypsin inhibitor (SBTI) was added either at the beginning (Control) or at the end (Trypsin) of the incubation. Aliquots were then subjected to osmotic shock. $\Delta\psi$-dependent uptake of ^{3}H-labelled methyltriphenylphosphonium (iodine salt) ([^{3}H]MTPP) into the mitochondria was determined as described in Ref. 22. Assays were performed in duplicate in the presence or absence of 1.0 μM CCCP. The results of two independent experiments are shown.

Treatment of mitochondria	[^{3}H]MTPP recovered in mitochondrial pellet (cpm)					
	Exp. I			Exp. II		
	−CCCP	+CCCP		−CCCP	+CCCP	
			% [a]			% [a]
Control	73000	3700	100	91000	6600	100
	79000	4500		100000	5800	
Trypsin	94000	4500	126	100000	5900	106
	96000	4700		100000	6200	
Trypsin + osmotic shock	96000	5800	124	92000	6700	91
	93000	4800		84000	7000	

[a] Relative uptake (cpm obtained in the presence of CCCP were subtracted as background).

mitochondrial pellets (Fig. 2). But because this amount of pelleted pOCT is seen in import reactions lacking mitochondria (e.g. see Fig. 5, left panel, lane 1), it presumably results from the formation of large aggregates of the precursor which co-sediment with the organelle. Such aggregation is variable but often occurs to an extent that masks the relatively low amount of input pOCT that would be expected to be specifically bound to translocation sites at any given time during import.

Efficient import into trypsin-pretreated mitochondria could be reestablished by a subsequent osmotic shock treatment to the organelle (Fig. 2, lane 3). Import into these osmotically-shocked mitochondria was dependent on an electrochemical gradient across the inner membrane (i.e., it was inhibited by CCCP, Fig. 2, lane 7) and was effected solely by the reversible change in osmolarity of the treatment (see the mock treatment, Fig. 2, lane 6). The pOCT processing enzyme located in the matrix did not leak from the organelle as a consequence of the osmotic shock because, again, processing was abolished by CCCP (Fig. 2, lane 7), i.e., processing took place as a consequence of $\Delta\psi$-dependent uptake of the precursor across the inner membrane. Taken together, therefore, the results indicate that the requirement for a proteinase-sensitive component on the outer membrane (presumably the pOCT import receptor, Refs. 25 and 26) can be bypassed in this system simply by exposing pOCT to the intermembrane space.

Interestingly, when osmotic shock was applied to mitochondria whose surface had *not* been pretreated with trypsin, an extent of import was achieved (Fig. 2, lane 5) that was considerably greater than that observed either for untreated (control) mitochondria (Fig. 2, lane 4) or for mitochondria that had been pretreated with trypsin and then osmotically shocked (Fig. 2, lane 3). One explanation for this observation is that pOCT is exposed to a net increase in high affinity import sites as a consequence of exposing the precursor to the inner membrane in osmotically-shocked mitochondria.

Finally, to examine if import of pOCT into trypsin-pretreated, osmotically-shocked mitochondria is mediated by proteinaceous components located beyond the surface of the organelle, an inhibitor of trypsin, SBTI, was added at the end, rather than at the beginning, of osmotic treatment, thereby allowing active trypsin to gain access to the intermembrane space. Under the conditions employed, SBTI effectively prevented the further action of trypsin (Fig. 1). Exposing the intermembrane space of osmotically-shocked mitochondria to trypsin inhibited subsequent import of pOCT (Fig. 3, compare lanes 6 and 8). Such treatment had a negligible effect on $\Delta\psi$ (as monitored by uptake of [^{3}H]MTPP, data not shown), indicating that the inner membrane remained intact.

Fig. 3. Reactivation of pOCT import by osmotic shock of trypsin-pretreated mitochondria is dependent on components sensitive to proteinase. Conditions and treatments were the same as those described in Fig. 2. Freshly isolated mitochondria were preincubated with (lanes 4–9) or without (lanes 2 and 3) trypsin (± PRE-TRY) and aliquots were then subjected to osmotic shock in the presence (lanes 6 and 7) or absence (lanes 8 and 9) of SBTI (OS and OS+TRY, respectively); inhibitor was added to the latter at the end of the osmotic shock treatment. Import of pOCT was carried out in the presence (lanes 3, 5, 7, 9) or absence (lanes 2, 4, 6, 8) of 1.0 μM CCCP. Lane 1 represents 10% of [^{35}S]pOCT added to each import assay.

Import of pOMD29 into the outer membrane of intact mitochondria

As described in Fig. 4, a recombinant pSP64 plasmid was constructed which encodes a hybrid protein consisting of the 29 N-terminal amino acids of yeast OMM70 fused in frame to amino acids 3–16 of murine dihydrofolate reductase (DHFR). The amino terminus contains the minimal mitochondrial targeting signal of OMM70 in yeast (amino acids 1–12, Refs. 5 and 15) as well as its hydrophobic outer membrane anchor domain (amino acids 11–29, Refs. 2 and 5; underlined in Fig. 4).

Transcription-translation of the recombinant plasmid yielded a single major product, designated pOMD29, of the expected size. When incubated with intact rat heart mitochondria under standard import condition in vitro, the majority of pOMD29 bound to the organelle (Figs. 4 and 6). Binding of pOMD29 to mitochondria was dependent on ATP (Fig. 5, right panel, lane 4) and was significantly (though not completely) inhibited by pretreatment of the mitochondria with low concentrations of trypsin (lane 3), suggesting that binding is mediated, as expected, by proteinase-sensitive outer membrane components.

A significant fraction of the pOMD29 that bound to mitochondria was resistant to extraction by 0.1 M Na_2CO_3 (pH 11.5) (see Fig. 10, compare lanes 3 and 5), indicative of a protein that is integrated into the lipid bilayer of a membrane [27]. In contrast, both the precursor and processed forms of imported pOCT were completely extracted by this treatment (see Fig. 10, lower panel). As predicted for a protein imported to the outer membrane [13,14], neither the binding of pOMD29 to intact mitochondria (Fig. 6, lane 5) nor

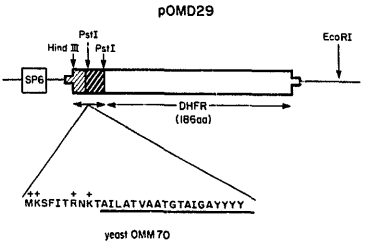

Fig. 4. Construction of pSP(pOMD29). Two DNA adaptors containing *Hin*dIII and *Pst*I overhangs were synthesized as follows. Adaptor I: 5′-AGCTATGAAGAGCTTCATTACAAGGAACAAGACAGCCATTTTGGCTGCA-3′ and 5′-TACTTCTCGAAGTAATGTTCCTTGTTCTGTCGGTAAAACCG-3′; adaptor II: 5′-GTTGCTGCTACAGGTACTGCCATCGGTGCCTACTATTATTACGCTGCA-3′ and 5′-ACGTCAACGACGATGTCCATGACGGTAGCCACCGGATGATAATAATGCG-3′. The plasmid, pSV$_2$DHFR [32] was digested with *Taq*I and *Bgl*II to generate a 650 bp fragment which was inserted between the *Acc*I and *Bam*HI sites of pSP64 [33]. A *Hin*dIII-*Pst*I fragment was removed from this construct and replaced with the two adaptors to yield pSP(pOMD29). It encodes amino acids 1–29 of yeast OMM70 fused in-frame to amino acids 3–186 of mouse DHFR, with a single glycine residue introduced between the two regions as a consequence of the recombinant manipulations [33]. Shown in the figure is the amino acid sequence (single letter code) of the amino terminus of OMM70 [34]; the underlined sequence (dark hatching) denotes the 19 amino acid membrane anchor domain of OMM70 [5]. pSP(pOMD29) was linearized with *Eco*RI prior to in vitro transcription-translation.

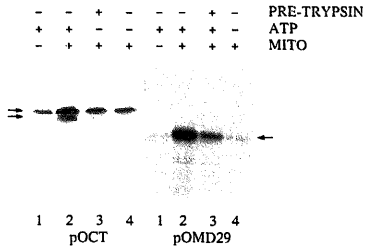

Fig. 5. Requirements for ATP and a mitochondrial surface protein for binding of pOMD29 to intact mitochondria. [^{35}S]pOMD29 and [^{35}S]pOCT were synthesized by transcription-translation in a rabbit reticulocyte lysate and incubated with or without purified mitochondria (MITO) under import conditions for 30 min at 30°C; alternatively, mitochondria either were pretreated with trypsin (PRE-TRYPSIN) or, together with reticulocyte lysate translation products, were treated with apyrase to remove ATP (see Experimental procedures). Following incubation, import mixtures were transferred to fresh tubes and layered over 1 ml of 30% (w/v) sucrose in MRM, and mitochondria recovered by centrifugation at 12000×*g* for 20 min. Left panel, pOCT; right panel, pOMD29. Lane 1, no mitochondria; lane 2, plus mitochondria; lane 3, mitochondria pretreated with trypsin; lane 4, pretreatment of import reactions with apyrase. Precursor and mature ornithine carbamoyltransferase are denoted by the upper and lower arrows, respectively, in the left panel. pOMD29 is indicated by the arrow in the right panel.

subsequent insertion into the lipid bilayer (see Fig. 10, lane 4) was inhibited by CCCP (i.e., required an electrochemical potential). And finally, following import into the outer membrane of intact mitochondria, pOMD29 was accessible to exogenous proteinase (Fig. 6, lanes 4 and 6) suggesting that, like OMM70, pOMD29 was anchored in the outer membrane with the bulk of the protein (i.e., the DHFR moiety) exposed to the outside of the organelle. In contrast, the imported and processed form of pOCT, but not the

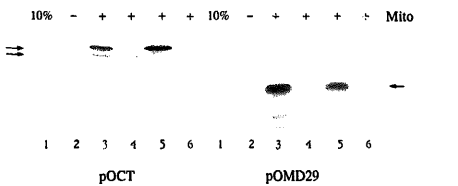

Fig. 6. Orientation and location of pOMD29 following import into intact mitochondria. Import of [^{35}S]pOCT or [^{35}S]pOMD29 was carried out as described in Fig. 5 and in Experimental procedures, either in the presence (lanes 5 and 6) or absence (lanes 2–4) of 1.0 μM CCCP. Following import, reaction mixtures containing lysate and mitochondria were treated with (lanes 4 and 6) or without (lanes 2,3 and 5) trypsin (0.2 mg) (POST-TRYPSIN) and the mitochondria recovered by centrifugation through a 30% (w/v) sucrose cushion, and mitochondrial proteins analyzed by SDS-PAGE and fluorography. Lane 1, 10% of input pOCT or pOMD29; lane 2, reactions lacking mitochondria. Arrows denote precursor (upper) and mature (lower) ornithine carbamoyltransferase and pOMD29.

exogenous precursor, was resistant to digestion under these conditions (Fig. 6, compare lanes 3 and 4). Likewise, the intermembrane space complex that channels cytochrome *c* to succinate–cytochrome-*c* oxidoreductase was resistant to trypsin under identical conditions (data not shown).

Insertion of pOMD29 into the inner membrane of osmotically shocked mitochondria

To date, all proteins examined that are delivered either into or across the mitochondrial inner membrane require $\Delta\psi$ whereas proteins delivered to the outer membrane, including pOMD29 (Fig. 6), do not. Delivery of pOMD29 to the inner membrane, therefore, was assayed by its acquisition of $\Delta\psi$-dependence. Mitochondria were not pretreated with trypsin because this treatment does not abolish completely import of pOMD29 to the outer membrane (Fig. 5). As shown in Figs. 7 and 8, a significant proportion of binding of pOMD29 to osmotically shocked mitochondria was $\Delta\psi$-dependent (compare lanes 2 and 3). The bulk of the protein was sensitive to exogenous trypsin (Fig. 7, lane 5), indicating that it was oriented toward the cytosolic side of the membrane. $\Delta\psi$-dependent binding of pOMD29 to osmotically shocked mitochondria was inhibited by pretreatment of the organelle with trypsin, under conditions where the proteinase gained access to the intermembrane space (Fig. 8, compare lanes 2 and 4), to an extent very similar to the proteinase sensitivity of pretreated intact mitochondria (Fig. 5). Because trypsin pretreatment of osmotically-shocked mitochondria did not affect $\Delta\psi$ (data not shown), the results of Fig. 8 suggest that $\Delta\psi$-dependent binding of pOMD29 was mediated by protein components.

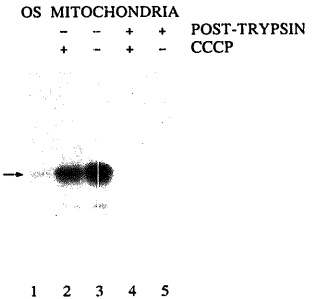

Fig. 7. $\Delta\psi$-dependent import of pOMD29 into osmotically-shocked mitochondria. Prior to standard import reactions, freshly-isolated mitochondria were subjected to osmotic shock (see Experimental Procedures). Import was performed in the presence (lanes 2 and 4) or absence (lanes 3 and 5) of 1.0 μM CCCP. Following import, reaction mixtures were treated either with (lanes 4 and 5) or without (lanes 2 and 3) trypsin (POST-TRYPSIN) (see Experimental Procedures), and the mitochondria recovered and analyzed by SDS-PAGE and fluorography. Lane 1, 10% of input [^{35}S]pOMD29. Arrow, pOMD29.

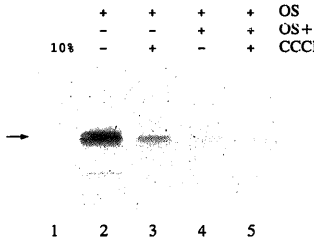

Fig. 8. $\Delta\psi$-dependent import of pOMD29 into osmotically-shocked mitochondria is dependent on components sensitive to trypsin. Freshly isolated mitochondria were subjected to osmotic-shock (OS) (lanes 2–5) either in the presence of trypsin (8 μg/ml) (OS+TRY) (lanes 4 and 5) or the presence of trypsin and SBTI (lanes 2 and 3). Import of [^{35}S]pOMD29 was carried out in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 1.0 μM CCCP, as described in Experimental procedures. Mitochondria were recovered and analyzed by SDS-PAGE and fluorography. Lane 1, 10% of input pOMD29. Arrow, pOMD29.

To confirm that pOMD29 was delivered to the inner membrane in osmotically-shocked mitochondria, mitochondria containing newly-imported pOMD29 were sonicated and subjected to sedimentation analysis in sucrose density gradients. As shown in Fig. 9, pOMD29 that was imported into intact mitochondria was subsequently recovered predominantly in the outer membrane fraction of intact mitochondria. However, in contrast to the situation for intact mitochondria, the majority of pOMD29 cosedimented with the inner membrane marker, UCP, following import into osmotically-shocked mitochondria (Fig. 9, upper right panel). It is not known, however, whether insertion into the inner membrane occurred at contact sites or at sites outside this region.

Finally, Fig. 10 demonstrates that a significant proportion of the pOMD29 that was recovered in association with the outer membrane in intact mitochondria and with the inner membrane in osmotically-shocked mitochondria was resistant to extraction by 0.1 M Na_2CO_3 (pH 11.5) indicating that it was integrated into the bilayer [27]. In contrast, both precursor and processed forms of pOCT following import into mitochondria were completely extracted by these procedures (Fig. 10).

Fate of pOMD29 lacking the transmembrane domain

A fragment of pSP(pOMD29) encoding a large portion of the transmembrane segment of pOMD29 (amino acids 16–29, Fig. 4) was removed by digestion with *Pst* I and religation of the plasmid (see Fig. 4). Following transcription-translation and incubation of the protein product with isolated mitochondria under standard import conditions, neither import nor binding to the surface of the organelle were observed. This was the case for both intact and osmotically shocked mitochondria (data not shown). Under the conditions of the present experiments, therefore, the transmembrane segment, presumably in cooperation with the positively-charged amino terminus of the protein (amino acids 1–9) makes an essential contribution to in vitro import of pOMD29 into rat heart mitochondria in vitro. Importantly, however, these results also indicate that stable binding of the precursor to the inner membrane of osmotically-shocked mitochondria is not mediated simply by the interaction of the positively-charged N-terminus of pOMD29 (amino acids 1–9) with negatively-charged lipids in the inner membrane, a finding that is consistent with the requirement for protein in the $\Delta\psi$-dependent import of pOMD29 by osmotically-shocked mitochondria (Fig. 8).

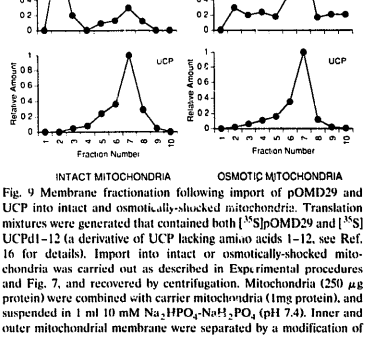

Fig. 9 Membrane fractionation following import of pOMD29 and UCP into intact and osmotically-shocked mitochondria. Translation mixtures were generated that contained both [^{35}S]pOMD29 and [^{35}S]UCPdl-12 (a derivative of UCP lacking amino acids 1–12, see Ref. 16 for details). Import into intact or osmotically-shocked mitochondria was carried out as described in Experimental procedures and Fig. 7, and recovered by centrifugation. Mitochondria (250 μg protein) were combined with carrier mitochondria (1mg protein), and suspended in 1 ml 10 mM Na_2HPO_4-NaH_2PO_4 (pH 7.4). Inner and outer mitochondrial membrane were separated by a modification of the procedures described in Refs. 35 and 36. Following incubation on ice for 10 min, the mixtures were given five strokes in a glass-teflon homogenizer, one-third volume of 1.8 M sucrose, 8 mM ATP and 8 mM $MgCl_2$ was added and an additional three strokes in a glass-teflon homogenizer was applied. The mixtures were incubated on ice for 5 min, and subjected to sonication with a Sonic Dismembrator (Artek System) at power setting 60, two times for 15 s each at 0°C. EDTA was added to a final concentration of 15 mM, and 1 ml aliquots loaded on a preformed 30–60% (w/v) sucrose density gradient in 10 mM sodium phosphate buffer. Centrifugation was carried out at 55000 × g for 15 h at 4°C. 1-ml fractions were collected, and protein precipitated in ice-cold trichloroacetic acid and analyzed by SDS-PAGE and fluorography. Bands corresponding to [^{35}S]pOMD29 and [^{35}S]UCP were quantitated by laser densitometry, with the peak fraction given an arbitrary value of 1.0. Outer membrane (OM) collected at the overlay/30% sucrose interface (fraction 2) while the inner membrane (IM) migrated into the gradient, as visualized by the marker protein, UCP.

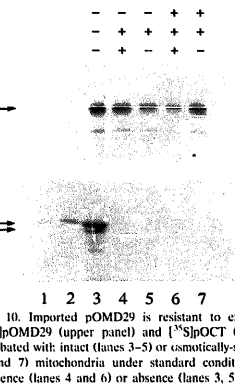

Fig. 10. Imported pOMD29 is resistant to extraction by alkali. [^{35}S]pOMD29 (upper panel) and [^{35}S]pOCT (lower panel) were incubated with: intact (lanes 3–5) or osmotically-shocked (OS) (lanes 6 and 7) mitochondria under standard conditions, either in the presence (lanes 4 and 6) or absence (lanes 3, 5, and 7) of 1.0 μM CCCP. Mitochondria were recovered by centrifugation through a 30% sucrose cushion and either analyzed directly (lane 3) or following extraction in 0.1 M Na_2CO_3 (pH 11.5) (lanes 4–7, see Experimental procedures). Lane 1, 10% of input [^{35}S]pOMD29 and [^{35}S]pOCT; lane 2, as in lane 3, except that mitochondria were not included in the import reaction. Arrows: pOMD29 (upper panel); precursor and processed forms of ornithine carbamoyltransferase (upper and lower arrows, respectively, in lower panel).

Discussion

In this study, we have examined the question of whether or not the protein translocation machinery of the mitochondrial inner membrane can recognize a protein that is normally destined for the outer membrane, i.e., a protein that otherwise is not encountered by the inner membrane under normal physiological conditions. Earlier studies in yeast provided the methodology to test this possibility by showing that the outer membrane translocation machinery could be bypassed in mitochondria whose outer membrane binding site for a matrix-destined protein had been inactivated by proteinase, and the mitochondrial outer membrane ruptured by osmotic shock [17,18]. We have obtained identical results for the delivery of pOCT into the

matrix compartment of osmotically-shocked mitochondria from rat heart. Import via this pathway is efficient and is dependent on proteinase-sensitive components. Although the assumption is made that such bypass import arises because the precursor gains direct access to the inner membrane protein translocator via the intermembrane space [17,18], it cannot be excluded that the precursor is somehow able to make contact with distal components of the outer membrane translocator (e.g., ISP42/MOM38, Refs. 25 and 26) as a consequence of osmotic shock, before moving on to the inner membrane. Import via the intermembrane space, however, is compatible with the observation that inner membrane vesicles free of detectable outer membrane are import competent [28,29]. The system is obviously not physiological since, in vivo, the process of import is a receptor-mediated event in which the first step is translocation through an intact outer membrane. The important point in terms of the present study, however, is that import into osmotically-shocked mitochondria in vitro can be employed to determine if a protein that is otherwise inserted into the outer membrane, can also be recognized and inserted into the lipid bilayer by the translocation machinery of the inner membrane, or whether the molecular signals in an outer membrane protein are not interpreted by the inner membrane translocator.

Of the limited number of mitochondrial membrane proteins whose import has been examined to date, most are polytopic proteins whose disposition and orientation in the bilayer have yet to be determined. An exception is the outer membrane protein of yeast, OMM70, a bitopic protein that is targeted to mitochondria and anchored in the outer membrane via its N-terminal 29 amino acids, leaving a large C-terminal fragment (> 60 kDa) exposed to the cytosol [2,5]. Evidence from studies employing fusion proteins in vivo and in vitro suggests that the requisite topogenic information for proper sorting and insertion of OMM70 lies within the N-terminal 29 amino acids [2,5,15]. In the present study, therefore, we have employed a hybrid protein, pOMD29, consisting of amino acids 1–29 of OMM70 fused to DHFR. Import into the outer membrane of rat heart mitochondria was efficient and depended on ATP and proteinase-sensitive mitochondrial components, and resulted in an orientation of pOMD29 in which the bulk of the protein was exposed to the exterior of the organelle. When a large segment of the membrane-anchor domain (amino acids 16–29) was deleted from pOMD29, no detectable import, or binding, of the precursor to mitochondria was observed (data not shown). Importantly, however, this was true for import into both the outer membrane of intact mitochondria and into the inner membrane of osmotically-shocked mitochondria. Earlier studies, in which amino acids 1–12 of OMM70 were fused to DHFR, resulted in import of the hybrid protein into the matrix compartment of yeast mitochondria in vitro, but the extent of such import was very low [5,15]. With rat heart mitochondria, such import was presumably below the limits of detection in this system. Our results suggest, therefore, that the membrane anchor domain of pOMD29 is required for efficient import, at least in vitro.

Employing osmotically-shocked mitochondria, a significant proportion of input pOMD29 was imported into the mitochondrial inner membrane, as determined by the acquisition of $\Delta\psi$-dependence for import and by membrane fractionation. The bulk of the protein was oriented to the outside of the inner membrane. Import to the inner membrane was sufficiently efficient in this system that prior inactivation of the outer membrane binding site for pOMD29 was not required. Import into the inner membrane depended on proteinase-sensitive components, and was not simply the result of electrostatic interactions between pOMD29 and the inner membrane since deletion of the neutral amino acids, 16–29 of pOMD29, abolished such interactions.

Taken together, these findings show that the protein translocation machinery of the mitochondrial inner membrane can import and insert an otherwise outer membrane protein. The results suggest that the import machineries of the outer and inner membrane interpret topogenic sequences and insert membrane proteins in a remarkably similar way. Retention of proteins in the outer membrane, therefore, presumably occurs because the membrane-anchor domain abrogates complete translocation of the polypeptide across the outer membrane. Such abrogation would be insured if, at least in part, a separate import pathway exists for outer membrane proteins, in which the outer membrane translocator remains unlinked to the inner membrane translocator; proteins destined for the inner membrane, on the other hand, might employ a different outer membrane translocator, one that does not respond to a stop-transfer domain. It is interesting in this regard that AAC and porin may be preferentially recognized by different receptors [30,31]. If, however, import of outer membrane proteins follows the same pathway used by proteins destined for the inner membrane, then mechanisms presumably exist to regulate whether or not the outer membrane translocator responds to a membrane-anchor domain [4,12]. If the outer membrane translocator is negatively regulated in this respect, the present data imply that the protein may by default be recognized by the inner membrane translocator.

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References

1 Blobel, G. (1980) Proc. Natl. Acad. Sci. USA 77, 1496–1499.
2 Hase, T., Müller, U., Reizman, H. and Schatz, G. (1984) EMBO J. 3, 3157–3164.
3 Nguyen, M. and Shore, G.C. (1987) J. Biol. Chem. 262, 3929–3931.
4 Nguyen, M., Bell, A.W. and Shore, G.C. (1988) J. Cell Biol. 106, 1499–1505.
5 Nakai, M., Hase, T. and Matsubara, H. (1989) J. Biochem. 105, 513–519.
6 Liu, X., Freeman, K.B. and Shore, G.C. (1990) J. Biol. Chem. 265, 9–12.
7 Mahlke, K., Pfanner, N., Martin, J. Horwich, A.L., Hartl, F.-U. and Neupert, W. (1990) Eur. J. Biochem. 192, 551–555.
8 Hartl, F.-U. and Neupert, W. (1990) Science 247, 930–938.
9 Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. and Neupert, W. (1986) Cell 47, 939–951.
10 Hartl, F.-U., Ostermann, J., Guiard, B. and Neupert, W. (1987) Cell 51, 1027–1037.
11 Attardi, G. and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333.
12 Singer, S.J. and Yaffe, M.P. (1990) Trends Biochem. Sci. 15, 369–373.
13 Freitag, H., Janes, M. and Neupert, W. (1982) Eur. J. Biochem. 126, 197–202.
14 Mihara, K., Blobel, G. and Sato, R. (1982) Proc. Natl. Acad. Sci. USA 79, 7102–7106.
15 Hurt, E.C., Müller, U. and Schatz, G. (1985) EMBO J. 4, 3509–3518.
16 Liu, X., Bell, A.W., Freeman, K.B. and Shore, G.C. (1988) J. Cell Biol. 107, 503–509.
17 Hwang, S.T., Wachter, C. and Schatz, G. (1991) J. Biol. Chem. 266, 21083–21089.
18 Ohba, M. and Schatz, G. (1989) EMBO J. 6, 2109–2115.
19 Argan, C., Lusty, C.J. and Shore, G.C. (1983) J. Biol. Chem. 258, 6667–6670.
20 Argan, C.A. and Shore, G.C. (1985) Biochem. Biophys. Res. Commun. 131, 289–298.
21 Nguyen, M., Argan, C., Lusty, C.J. and Shore, G.C. (1986) J. Biol. Chem. 261, 802–805.
22 Bakker, E.P. (1978) Biochemistry 17, 2899–2904.
23 Douce, R., Mannella, C.A. and Bonner, W.D., Jr. (1973) Biochim. Biophys. Acta 292, 105–116.
24 Greenawalt, J.W. (1974) Methods Enzymol. 31, 310–323.
25 Pfanner, N., Söllner, T. and Neupert, W. (1991) Trends Biochem. Sci. 16, 63–67.
26 Baker, K.P. and Schatz, G. (1991) Nature 349, 205–208.
27 Fujiki, Y., Fowler, S., Shio, H., Hubbard, A.L. and Lazarow, P. (1982) J. Cell Biol. 93, 103–110.
28 Hwang, S., Jascur, T., Vestweber, D., Pon, L. and Schatz, G. (1989) J. Cell Biol. 109, 487–493.
29 Pon, L., Moll, T., Vestweber, D., Marshallsay, B. and Schatz, G. (1989) J. Cell Biol. 109 2603–2616.
30 Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brütsch, H. and Schatz, G. (1990) EMBO J. 9, 3191–3200.
31 Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. and Neupert, W. (1990) Cell 62, 107–115.
32 Subramani, S., Mulligan, R. and Berg, P. (1981) Mol. Cell Biol. 1, 854–864.
33 Sheffield, W.P., Shore, G.C. and Randall, S.K. (1990) J. Biol. Chem. 265, 11069–11076.
34 Hase, T., Riezman, H., Suda, K. and Schatz, G. (1983) EMBO J. 2, 2169–2172.
35 Skerjanc, I.S., Sheffield, W.P., Randall, S.K., Silvius, J.R. and Shore, G.C. (1990) J. Biol. Chem. 265, 9444–9451.
36 Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Biochem. Biophys. Acta 860, 672–689.