

# Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear-encoded Oxa1p

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**Oxa1p, a nuclear-encoded protein of the mitochondrial inner membrane with five predicted transmembrane (TM) segments is synthesized as a precursor (pOxa1p) with an N-terminal presequence. It becomes imported in a process requiring the membrane potential, matrix ATP, mt-Hsp70 and the mitochondrial processing peptidase (MPP). After processing, the negatively charged N-terminus of Oxa1p (~90 amino acid residues) is translocated back across the inner membrane into the intermembrane space and thereby attains its native N<sub>out</sub>-C<sub>in</sub> orientation. This export event is dependent on the membrane potential. Chimeric preproteins containing N-terminal stretches of increasing lengths of Oxa1p fused on mouse dehydrofolate reductase (DHFR) were imported into isolated mitochondria. In each case, their DHFR moieties crossed the inner membrane into the matrix. Thus Oxa1p apparently does not contain a stop transfer signal. Instead the TM segments are inserted into the membrane from the matrix side in a pairwise fashion. The sorting pathway of pOxa1p is suggested to combine the pathways of general import into the matrix with a bacterial-type export process. We postulate that at least two different sorting pathways exist in mitochondria for polytopic inner membrane proteins, the evolutionarily novel pathway for members of the ADP/ATP carrier family and a conserved Oxa1p-type pathway.**

**Keywords:** membrane insertion/mitochondria/Oxa1p/polytopic membrane protein/*Saccharomyces cerevisiae*

## Introduction

Proteins of the inner membrane of mitochondria display a diverse range of topological arrangements. There are proteins which span the inner membrane once, and others which contain two or more transmembrane (TM) segments. The N-termini of these proteins face either the matrix space or the intermembrane space. Independently of their topological arrangements, the proteins of the inner mitochondrial membrane may be classified according to their evolutionary origin and to the site of their synthesis within the cell. A first class of inner membrane proteins are synthesized within the mitochondria; they display a high degree of similarity to corresponding proteins in prokaryotes. Another class comprises mitochondrial inner

membrane proteins which are thought not to have any prokaryotic equivalents. This latter class includes, for example, components of the mitochondrial import machinery, several non-catalytic subunits of the respiratory chain complexes and also a number of nucleotide and substrate translocators. To date, sequence information has indicated that most, if not all, of these proteins are synthesized without N-terminal targeting signals; they are targeted to mitochondria by way of internal targeting signals. A third class of inner membrane proteins have prokaryotic counterparts, but are encoded within the nuclear genome, these proteins possess mitochondrial targeting signals, typically in the form of N-terminal cleavable presequences.

How are the members of these three classes of proteins sorted to the inner membrane? All of these proteins contain sequence determinants, so-called topogenic signals, which ensure the sorting of the protein to the inner membrane and determine the attainment of their orientation in the membrane (Stuart and Neupert, 1996). These topogenic signals comprise hydrophobic cores, usually TM segments which are flanked on both sides by charged hydrophilic amino acids. Of particular interest is the sorting and topogenesis of those proteins which span the inner membrane several times and hence contain a number of topogenic signals. Representatives of such proteins are found in all three classes described above.

Mitochondrially encoded proteins integrate into the inner membrane, obviously from the matrix side, in a fashion that bears similarities to the process of protein insertion into the plasma membrane in bacteria (Herrmann *et al.*, 1995; Stuart and Neupert, 1996). This insertion process, at least for a subset of these proteins, is not coupled to synthesis in an obligate manner (Herrmann *et al.*, 1995; Rojo *et al.*, 1995). These proteins show a typical charged amino acid distribution profile: the matrix-exposed segments contain, with high frequency, positively charged residues, whilst segments exposed to the intermembrane space mostly bear a net negative charge (Gavel and von Heijne, 1992). This reflects the charge distribution pattern observed with proteins of the bacterial cytoplasmic membrane, which has been termed the 'positive inside rule' (von Heijne, 1989; Boyd and Beckwith, 1990).

Proteins of the second class, such as the ATP/ADP carrier (AAC) do not show such a charge distribution (Gavel and von Heijne, 1992). Following synthesis in the cytosol, the AAC protein is imported into mitochondria in a  $\Delta\psi$ -dependent manner. After crossing over the outer membrane, AAC becomes inserted directly into the inner membrane without initial entry into the mitochondrial matrix. It has been proposed that the import of AAC and another member of this group, the phosphate carrier, is facilitated by a novel translocase of the inner membrane translocation, distinct from that used by matrix-targeted proteins (Sirrenberg *et al.*, 1996).

How do members of the third class become sorted to the inner membrane? Several nuclear-encoded inner membrane proteins have an amino acid charge distribution which is conserved from their bacterial counterpart, thus suggesting the 'positive inside rule' also holds true for them. This charge distribution is a determinant for membrane insertion in bacteria (von Heijne, 1989; Boyd and Beckwith, 1990; Cao *et al.*, 1995). Thus with these mitochondrial proteins, import across the inner membrane and export from the matrix may be envisaged as being key elements of their sorting process.

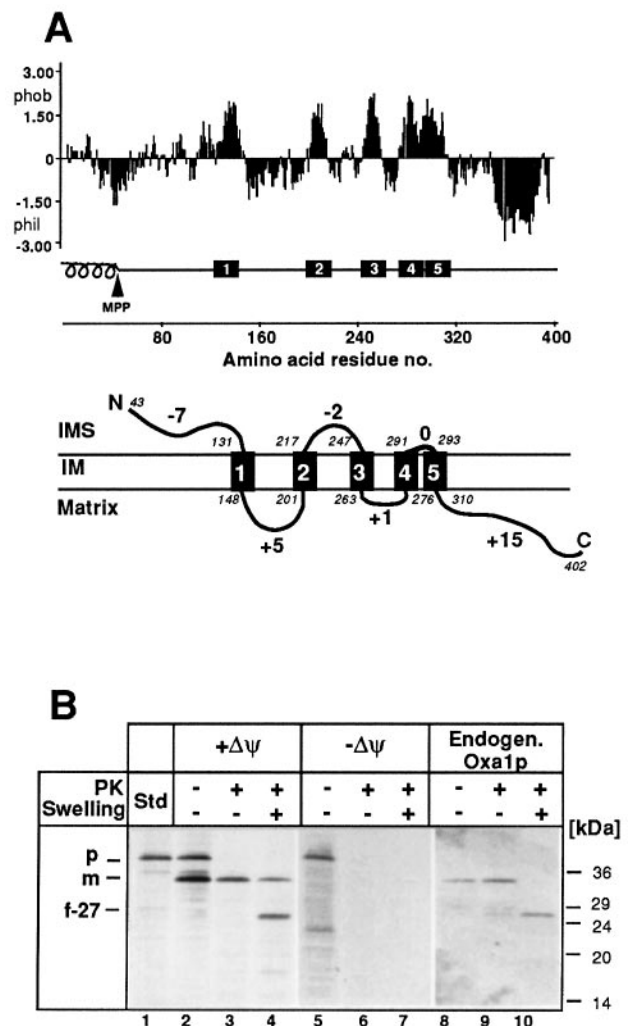
In the present study, we have analyzed the import and sorting of Oxa1p, a representative of the third class of proteins. Oxa1p is a nuclear-encoded protein from the yeast *Saccharomyces cerevisiae* which is conserved from prokaryotes throughout eukaryotes (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994a,b). Although the precise function of Oxa1p is not yet understood, it appears to be involved in the assembly of respiratory chain complexes in the inner membrane of mitochondria (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994a,b; Altamura *et al.*, 1996). Oxa1p has five predicted TM segments, and the intervening hydrophilic regions show an alternating net charge arrangement (Figure 1A). The sequence of the *OXA1* gene suggested the presence of a typical mitochondrial targeting sequence at the N-terminus of the Oxa1p.

We present evidence that Oxa1p is a polytopic inner membrane protein. Following import into the mitochondrial matrix where it is processed by the mitochondrial processing peptidase (MPP), mature-sized Oxa1p (mOxa1p) attains an N<sub>out</sub>-C<sub>in</sub> orientation across the inner membrane. Attainment of this orientation involves the export of the N-terminal segment of ~90 amino acids from the matrix. This export is accompanied or followed by the export of the hydrophilic loop of ~30 amino acid residues between the second and third TM segments. We demonstrate here that both of these processes can be monitored experimentally. Following short import times, mOxa1p is protease protected in mitoplasts and, only as a result of these subsequent export events, becomes accessible to added protease under hypotonic swelling conditions. These export events bear similarities to those of membrane insertion of mitochondrially encoded and bacterial membrane proteins. This suggests that during evolution not only the protein sequence of Oxa1p has been conserved between prokaryotes and eukaryotes, but also the pathway of its assembly into the membrane.

## Results

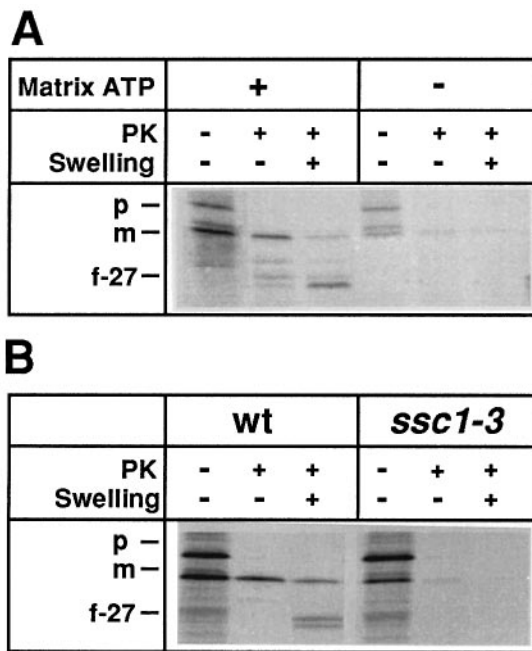
### Import and topology of the Oxa1p protein

Radiolabeled Oxa1p precursor, pOxa1p, of apparent M<sub>r</sub> 42 kDa (Figure 1B, lane 1), was imported into isolated mitochondria in the absence, but not in the presence of uncouplers of oxidative phosphorylation (Figure 1B, lanes 3 and 6). Import occurred into a protease-resistant location and was accompanied by efficient proteolytic processing to a 36 kDa protein, which had the same apparent molecular mass as the endogenous mature Oxa1p (Figure 1B, Endogen. Oxa1p). Following import, mitochondria were converted to mitoplasts so that added proteinase K had access to the inner membrane. Thereby the imported radiolabeled mOxa1p was degraded to a 27 kDa fragment



**Fig. 1.** Import of pOxa1p is dependent on  $\Delta\psi$  and results in an N<sub>out</sub>-C<sub>in</sub> topology in the inner mitochondrial membrane. (A) Upper panel: hydrophobicity plot of Oxa1p according to Kyle and Doolittle (1982). The positions of the presequence (curled structure), the predicted MPP cleavage site (an arrowhead) and the five TM segments (black boxes) of pOxa1p are depicted. Lower panel: the orientation of Oxa1p in the inner membrane, as concluded from this study. The net charges of the different hydrophilic segments of Oxa1p (larger numbers) are indicated together with amino acid positions bordering the TM segments and the first amino acid residue of mature Oxa1p (smaller numbers, italics). Abbreviations, IMS, intermembrane space; IM, inner membrane; N, N-terminus; C, C-terminus. (B) Radiolabeled pOxa1p was imported into mitochondria for 5 min at 25°C in the presence of either NADH (lanes 2–4, + $\Delta\psi$ ) or valinomycin (lanes 5–7, - $\Delta\psi$ ). After import, mitochondria were re-isolated and either mock treated (lanes 2 and 5) or proteinase K (PK) treated (50  $\mu$ g/ml) under non-swelling (lanes 3 and 6) or swelling conditions (lanes 4 and 7). All samples were analyzed by SDS-PAGE, blotted onto nitrocellulose and the resulting autoradiograph is presented. Immunoblotting of endogenous Oxa1p using a C-terminus-specific Oxa1p antiserum (Endogen. Oxa1p) was performed and is shown for the + $\Delta\psi$  panel (lanes 8–10). Immunodecoration of the intermembrane space marker protein CCPO and the matrix marker protein Mge1p was also performed to assess the swelling efficiency. Swelling was >95% efficient. Abbreviations: p, precursor, pOxa1p; m, mature, mOxa1p; f-27 denotes the C-terminal Oxa1p fragment generated by proteinase K treatment of mitoplasts. Std, 20% of the amount of radiolabeled precursor added to each sample.

(f-27) (Figure 1B, lane 4). This fragment could be immunoprecipitated with an antibody specific for the C-terminus of the Oxa1p (not shown). The endogenous Oxa1p species



**Fig. 2.** Import of pOxa1p requires the ATP-dependent mt-Hsp70 activity. Radiolabeled pOxa1p was imported for 5 min at 25°C into either (A) mock-treated (+matrix ATP) or matrix ATP-depleted mitochondria (-matrix ATP) in the presence of external ATP, or (B) into *ssc1-3* mitochondria (*ssc1-3*) or isogenic wild-type (wt) following their pre-incubation at the non-permissive temperature of 37°C. Mitochondria were re-isolated and subjected to proteinase K (PK) treatment either under non-swelling or swelling conditions, as indicated. Samples were analyzed by SDS-PAGE and blotted onto nitrocellulose. Immunoblotting of the marker proteins CCPO (intermembrane space) and Mge1p (matrix) was performed; swelling was >95% efficient. The mobility of the fragment of Oxa1p generated upon PK treatment of mitoplasts is indicated by f-27. p, pOxa1p; m, mOxa1p.

gave rise to the same characteristic 27 kDa fragment, as demonstrated by immunoblotting using the same C-terminus-specific Oxa1p antiserum (Figure 1B, lane 10). Rupturing of both mitochondrial membranes by sonication or by detergent lysis in the presence of protease led to the complete degradation of the endogenous and imported Oxa1p. Furthermore, the endogenous Oxa1p was resistant to carbonate extraction (see Figure 6), thus confirming that Oxa1p is an integral membrane protein.

In conclusion, pOxa1p is imported in a membrane potential ( $\Delta\mu\text{H}^+$ )-dependent manner. Following proteolytic maturation, the N-terminal segment of mOxa1p becomes sorted to the intermembrane space, whilst the C-terminal ~100 residues remain in the matrix, ( $\text{N}_{\text{out}}\text{-C}_{\text{in}}$ ) orientation (Figure 1A, lower panel).

#### Import of pOxa1p is dependent on matrix ATP and mt-Hsp70 activity

In order to analyze whether the import of pOxa1p to the inner membrane is facilitated by mitochondrial heat shock protein 70 (mt-Hsp70), the dependence on matrix ATP was tested. In matrix ATP-depleted mitochondria, the efficiency of both maturation and import of pOxa1p was strongly reduced as compared with matrix ATP-containing mitochondria (Figure 2A). A similar inhibition of import was obtained when pOxa1p was imported into mitochondria isolated from the mutant *ssc1-3* harboring a tempera-

ture-sensitive mt-Hsp70, following their exposure to 37°C (Figure 2B, wt versus *ssc1-3*). In both cases, matrix ATP-depleted and *ssc1-3* mitochondria, the degree of inhibition of Oxa1p import was similar to that of a control matrix-targeted protein (results not shown). Furthermore, neither in the case of matrix ATP-depleted mitochondria nor in *ssc1-3* mitochondria were proteolytic protected fragments of Oxa1p to be seen, suggesting that even partial import across the outer membrane had not occurred in the absence of mt-Hsp70 function. Thus the import of pOxa1p required the ATP-dependent action of mt-Hsp70.

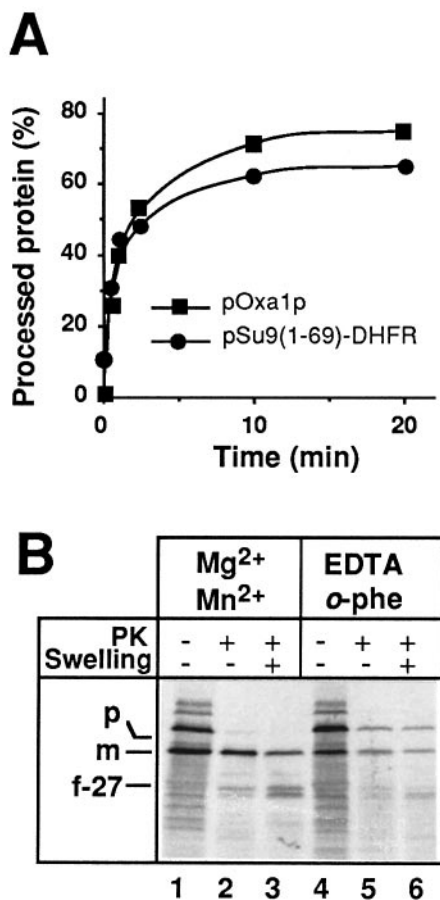
#### The mitochondrial targeting signal of pOxa1p is processed by MPP in the matrix

The N-terminal region of pOxa1p bears features of a typical cleavable mitochondrial matrix-targeting signal and a predicted MPP cleavage site between amino acid residues 42 and 43. Processing of pOxa1p at this position would correlate well with the shift in  $M_r$  from 42 to 36 kDa occurring upon import. To confirm that the N-terminal presequence is processed by MPP in the mitochondrial matrix, radiolabeled pOxa1p was incubated with purified MPP from *Neurospora crassa*. In a time-dependent manner, the 42 kDa precursor of Oxa1p was processed to a species of 36 kDa indistinguishable in size from the endogenous mature Oxa1p (Figure 3A). This reaction displayed similar kinetics to the maturation of pSu9(1-69)-DHFR (Figure 3A). In a second approach, pOxa1p was imported into mitochondria in the presence of EDTA/*o*-phenanthroline which inhibits the metal-dependent MPP activity. Non-processed pOxa1p accumulated in mitochondria (Figure 3B, lane 5). Upon conversion of these mitochondria to mitoplasts in the presence of proteinase K, this accumulated pOxa1p was not degraded. Thus, the N-terminal tail (N-tail) of this pOxa1p was not exposed to the intermembrane space, but rather was present in the matrix (Figure 3B, lane 6).

In conclusion, the N-terminal presequence of pOxa1p is imported initially into the matrix where it undergoes proteolytic processing by MPP. Maturation by MPP appears to be a prerequisite for subsequent sorting of the N-terminus from the matrix to the intermembrane space, as non-processed pOxa1p is inaccessible to added protease in mitoplasts.

#### Translocation of the N-tail of Oxa1p from the matrix to the intermembrane space

To study translocation of the N-tail out of the matrix, we established conditions under which import and export events could be dissected kinetically (Figure 4). Radiolabeled pOxa1p was imported into isolated mitochondria for various times, after which mitochondria were subjected to a proteinase K treatment under swelling conditions to assess the sublocalization of the imported species (Figure 4A). After short import times, the majority of the imported radiolabeled mOxa1p was protease protected in mitoplasts (Figure 4A). Only after longer periods was the N-tail of the imported mOxa1p accessible to added protease in mitoplasts, as monitored by the production of the 27 kDa fragment. The ability to generate the 27 kDa fragment of mOxa1p in mitoplasts with time was correlated with the loss of full-length mOxa1p protease protected in mitoplasts. This kinetic relationship suggested that the



**Fig. 3.** The Oxal1p precursor is processed by MPP. (A) Radiolabeled pOxa1p and control precursor pSu9(1-69)-DHFR were incubated with the purified subunits of MPP, as described in Materials and methods. After the times indicated, samples were removed and left on ice. Samples were analyzed subsequently by SDS-PAGE and the generation of the respective mature-size species was quantified by densitometry (Ultrascan XL, Pharmacia). The amounts of processed pOxa1p (■) and pSu9(1-69)-DHFR (●) are expressed as a percentage of total precursor added to the reaction. (B) Isolated wild-type mitochondria were pre-incubated either in the presence of divalent cations (Mg<sup>2+</sup>/Mn<sup>2+</sup>, lanes 1-3) or with EDTA (10 mM) and *o*-phenanthroline (2 mM) (EDTA/*o*-phe, lanes 4-6) for 10 min on ice. Import of radiolabeled pOxa1p at 12°C for 2 min followed by protease treatment and hypotonic swelling, when indicated, was performed as described in Figure 1A. Abbreviations: p, precursor, pOxa1p; m, mature, mOxa1p; f-27 denotes the C-terminal Oxal1p fragment generated by proteinase K treatment of mitoplasts (resolved as a doublet).

matrix-accumulated mOxa1p represented a kinetic precursor of the final sorted mOxa1p species.

In order to test whether the mOxa1p species accumulated in the matrix was a true sorting intermediate, radiolabeled pOxa1p was imported into mitochondria for a short time period. Mitochondria were re-isolated and subjected to a chase incubation (Figure 4B). After the initial import, radiolabeled mOxa1p was largely inaccessible to added protease when the outer membrane was disrupted by hypotonic swelling. Upon further incubation, the N-terminus of this mOxa1p species became translocated in a time-dependent manner to the intermembrane space, where it was accessible to added protease upon swelling (Figure 4B). Translocation required an energized inner membrane as it was completely inhibited if the chase reaction was performed in the presence of the uncoupler

CCCP (Figure 4B), or other inhibitors of  $\Delta\psi$  such as valinomycin, cyanide and azide (results not shown). This export process was only slightly stimulated in the presence of additional NADH, suggesting that the membrane potential established during the initial import reaction was sufficient for both the import and export steps (Figure 4B).

Does mt-Hsp70 interact with Oxal1p during its sorting? pOxa1p was imported into isolated mitochondria for a short period. Mitochondria were treated with trypsin, subjected to a chase incubation and converted to mitoplasts in the presence of proteinase K. Complex formation with mt-Hsp70 was monitored by co-immunoprecipitation analysis using antibodies against mt-Hsp70 (Figure 4C). After the initial import, mOxa1p was found associated with mt-Hsp70. Upon chase, the amount of mOxa1p bound to mt-Hsp70 decreased. Little or none of the C-terminal 27 kDa fragment generated upon protease treatment of the exported mOxa1p was present in a complex with mt-Hsp70 (Figure 4C).

In summary, pOxa1p can be accumulated efficiently in the matrix where it is complexed with mt-Hsp70. This species is a productive sorting intermediate, as its N-terminus is exported efficiently to the intermembrane space, in a membrane potential-dependent manner. Once the N-terminus is sorted correctly, however, mOxa1p is no longer a substrate for mt-Hsp70. Thus, the first TM segment (TM1) of pOxa1p appears to function as an insertion signal from the matrix side and to facilitate export of the N-terminal hydrophilic segment.

**The transmembrane segments of pOxa1p function as insertion signals rather than as translocation arrest signals**

Do the other four TM segments (TM2, TM3, TM4 and TM5) also operate as such insertion signals or do they serve to arrest the TM segment in the inner membrane during the import process? A series of chimeric proteins consisting of N-terminal regions of pOxa1p fused to mouse dihydrofolate reductase (DHFR) were constructed. The fusion proteins encompassed either the first two (pOxa1p-N1,2-DHFR), three (pOxa1p-N1,2,3-DHFR), four (pOxa1p-N1,2,3,4-DHFR) or all five TM segments (pOxa1p-N1,2,3,4,5-DHFR) (Figure 5A). If any of the TM segments acts as an import arrest signal, the DHFR domain should remain in the intermembrane space (as depicted for pOxa1p-N1,2-DHFR in Figure 5B, topology 1). On the other hand, if the TM segments serve as insertion signals from the matrix, the DHFR domains of all these different constructs should be imported into the matrix and thereby become protected against added protease in mitoplasts (Figure 5B, topologies 2 and 3).

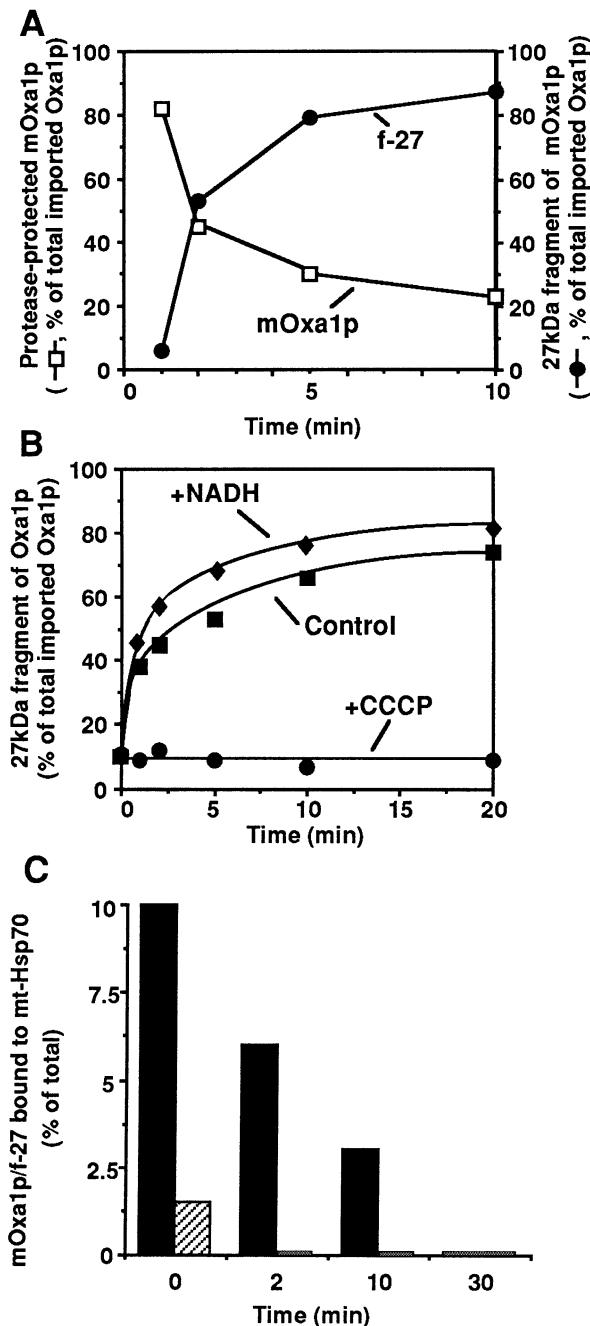
The fusion proteins were all imported efficiently into mitochondria where they were processed to their respective mature-size forms (Figure 5C). After conversion of mitochondria to mitoplasts, the mature-size species were accessible to added protease; in each case a predominant fragment (F1), resulting from the degradation of the exported 9 kDa N-tail, was generated. In addition, two smaller fragments (F2 and F3) were formed, as best seen with the pOxa1p-N1,2,3-DHFR (Figure 5C, lane 8). The larger of these two fragments (F2) is a C-terminal fragment containing DHFR; it arose by proteolytic cleavage in the loop between TM2 and TM3, which is exposed to the

intermembrane space in the correctly sorted protein (Figure 5C, right panel). The F2 fragment was progressively larger in each of the fusion proteins due to the presence of additional TM segments. The F1 and F2 fragments of the fusion proteins could be immunoprecipitated with a DHFR-specific antibody, confirming the presence of the DHFR moiety. The smaller fragment (F3) of ~12 kDa corresponds to the residual N-terminal segment of Oxa1p encompassing TM1 and TM2, linked by an ~50 residue long hydrophilic loop on the matrix side of the inner membrane (Figure 5C, right panel). The abundance of the F3 fragment for each fusion protein correlated with that of the F2 fragment. Moreover, the efficiency of production of the F2 and F3 fragments decreased with increasing numbers of TM segments present in the fusion protein [Figure 5C, compare pOxa1p-N<sub>1,2,3</sub>-DHFR (lane 8) with

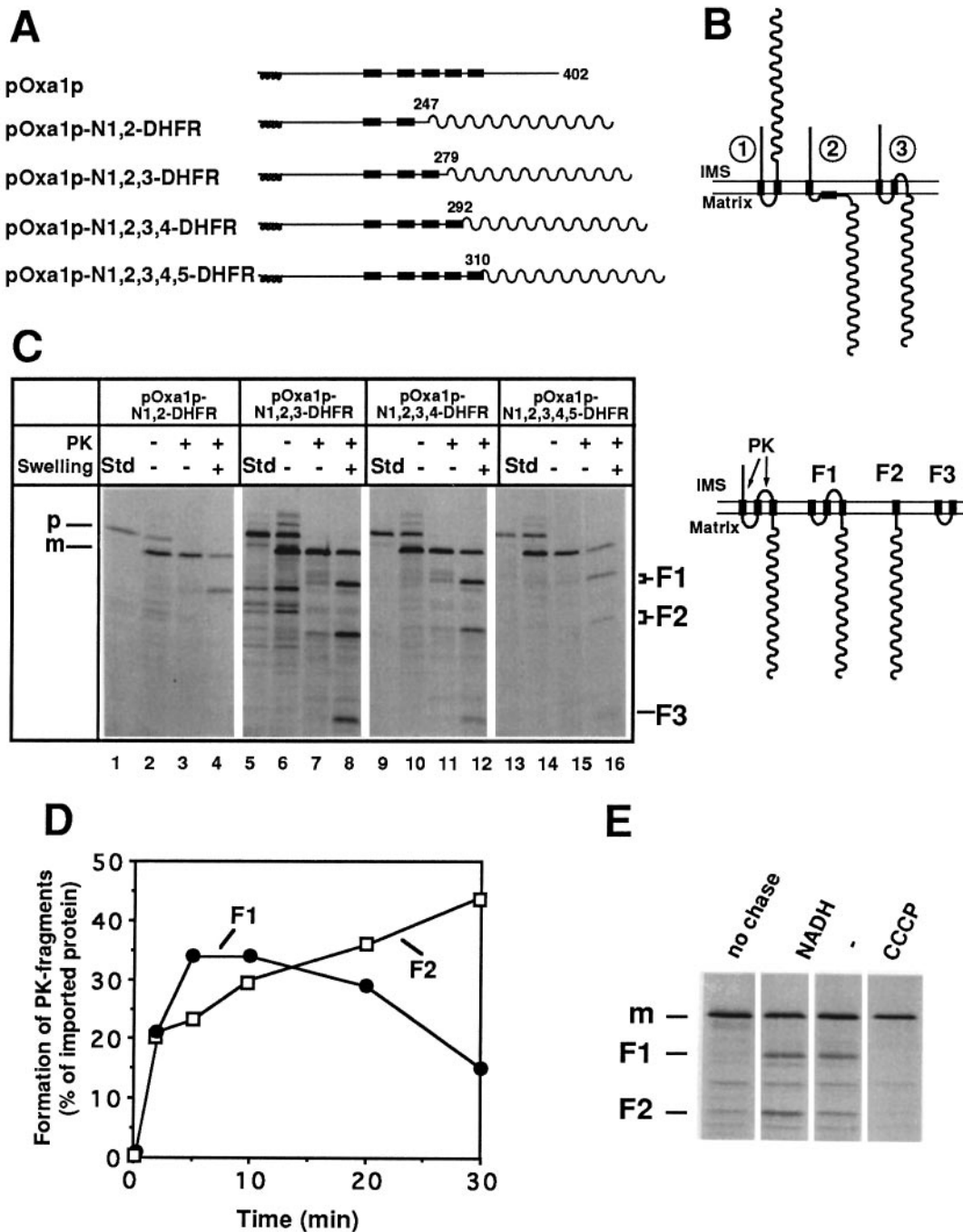
pOxa1p-N<sub>1,2,3,4</sub>-DHFR (lane 12) and pOxa1p-N<sub>1,2,3,4,5</sub>-DHFR (lane 16)]. These fragments were not observed with the imported authentic pOxa1p. Obviously, with increasing numbers of TM segments, the newly imported Oxa1p proteins attain an orientation in the inner membrane whereby the loop between TM2 and TM3 is no longer accessible to added protease in mitoplasts, as is the case for the endogenous Oxa1p species (see Figure 1B, Endogen. Oxa1p). In each case, the sum of the F1, F2 and F3 signals generated upon swelling, together with the remaining mOxa1p protected in mitoplasts, equalled that of the corresponding imported mature-size form. Thus, a significant fraction of the DHFR did not remain in the intermembrane space. Finally, F2 and F3 fragments were not observed with the pOxa1p-N<sub>1,2</sub>-DHFR (Figure 5C, lane 4); therefore, in the absence of TM3, the topology depicted in Figure 5B, (topology 3) cannot be attained. TM3 most likely acts in a co-operative manner with TM2 to achieve insertion into the membrane.

The insertion of TM2 and TM3 segments into the inner membrane and translocation of the loop between them could be monitored by the generation of the F2 fragment. Formation of the F1 fragment following import of pOxa1p-N<sub>1,2,3</sub>-DHFR occurred before the formation of F2 (Figure 5D). At no time point was a fragment observed that corresponded to an inserted TM2-TM3 loop and a non-exported N-tail. Formation of both F1 and F2 required a membrane potential; in the presence of the uncoupler CCCP the imported mature sized species remained in the matrix (Figure 5E, CCCP). Upon increase of the membrane potential, an increase of the F2 fragment was observed (Figure 5E, NADH).

In conclusion, TM segments of pOxa1p do not act as translocation arrest signals during import across the inner membrane. Once imported into the matrix, the TM seg-



**Fig. 4.** pOxa1p is sorted in an N<sub>out</sub>-C<sub>in</sub> orientation via the mitochondrial matrix. (A) Radiolabeled pOxa1p was imported into isolated mitochondria at 12°C. At the time points indicated, samples were removed and mitochondria were proteinase K treated under either non-swelling or swelling conditions. Swelling was >95% efficient as judged by monitoring marker proteins. The amount of mOxa1p protease protected in mitoplasts (□) and the accessibility of the N-terminus of mOxa1p to added protease in mitoplasts, as monitored by the generation of the 27 kDa fragment (f-27) (●), are expressed as a percentage of the total imported mOxa1p species. (B) Radiolabeled pOxa1p was imported into mitochondria for 2 min at 25°C. After this time, the total species imported was protease protected in mitoplasts and MPP processed to mOxa1p (not shown). Mitochondria were re-isolated through a sucrose cushion, as described in Materials and methods, resuspended in fresh import buffer and subjected to a second incubation at 25°C in the presence of either no further additions (■), 2 mM NADH (◆) or 100 μM CCCP (●). At the times indicated, samples were removed and the export of the N-tail of mOxa1p was monitored by generation of the 27 kDa fragment upon protease treatment of mitoplasts, and is expressed as a percentage of the total species imported during the first incubation. (C) Radiolabeled pOxa1p was imported at 25°C for 2 min after which mitochondria were trypsin treated and re-isolated. Following a second incubation for the times indicated, mitochondria were converted to mitoplasts in the presence of proteinase K. The resulting mitoplasts were lysed with Triton X-100-containing buffer and complex formation with mt-Hsp70 was monitored by co-immunoprecipitation analysis using a mt-Hsp70-specific antiserum (α mt-Hsp70), as described in Materials and methods. Samples were analyzed by SDS-PAGE and the resulting fluorographs were quantified by laser densitometry. The amounts of mOxa1p (black bars) and f-27 fragment (hatched bars) co-immunoprecipitated with mt-Hsp70 are expressed as a percentage of the total solubilized species, respectively.



**Fig. 5.** Import of pOxa1p-DHFR-derived fusion proteins. (A) pOxa1p-DHFR fusion proteins. Curled structures indicate the N-terminal presequence, black areas denote the TM segments, and the numbers denote the amino acid residue of pOxa1p after which the fusion to DHFR (wavy line) occurs. (B) Depiction of possible orientations in the inner membrane which could be achieved by pOxa1p-N1,2-DHFR following import into mitochondria. IMS, intermembrane space. (C) Left panel: the Oxa1p-DHFR-derived fusion proteins depicted were synthesized in a reticulocyte lysate in the presence of [<sup>35</sup>S]methionine and were imported into mitochondria for 10 min at 25°C. Mitochondria were proteinase K (PK) treated under either non-swelling or swelling conditions, as indicated. F1, F2 and F3 denote the fragments of the fusion proteins generated following protease treatment of the mitoplasts (see text for description of fragments). Std, 20% of the amount of radiolabeled precursor added to each sample. Right panel: proposed composition and orientation in the inner membrane of fragments F1, F2 and F3 of pOxa1p-N1,2,3-DHFR. (D) pOxa1p-N1,2,3-DHFR was imported into mitochondria for 2 min at 25°C. Mitochondria were then re-isolated through a sucrose cushion and subjected to a second incubation for the times indicated. They were then converted to mitoplasts in the presence of proteinase K (PK). Samples were analyzed by SDS-PAGE and blotting onto nitrocellulose. The resulting autoradiographs were quantified by densitometry. Export of the N-tail and insertion of TM2/TM3 were monitored by the generation of the F1 and F2 fragments, respectively, and are expressed as a percentage of the total species imported during the first incubation. (E) Import of pOxa1p-N1,2,3-DHFR was performed for 2 min at 25°C; following re-isolation of mitochondria as described in (D), samples were divided. One sample was left on ice and the others were incubated further at 25°C for 10 min, in the presence of either no further additions, 2 mM NADH or 100 μM CCCP, as indicated. Mitochondria were then converted to mitoplasts in the presence of proteinase K (PK). Samples were analyzed by SDS-PAGE and blotting onto nitrocellulose. The resulting autoradiographs are depicted.

ments act as insertion signals facilitating the membrane potential-dependent integration into the inner membrane and the translocation of hydrophilic segments into the intermembrane space. Following attainment of the  $N_{out}-C_{in}$  orientation across the inner membrane, Oxa1p apparently folds into a structure whereby the loop between TM2 and TM3 is no longer accessible to added proteases. This structure is only obtained in the presence of all five TM segments.

#### A carbonate-extractable intermediate of pOxa1p-N1,2-DHFR in the matrix

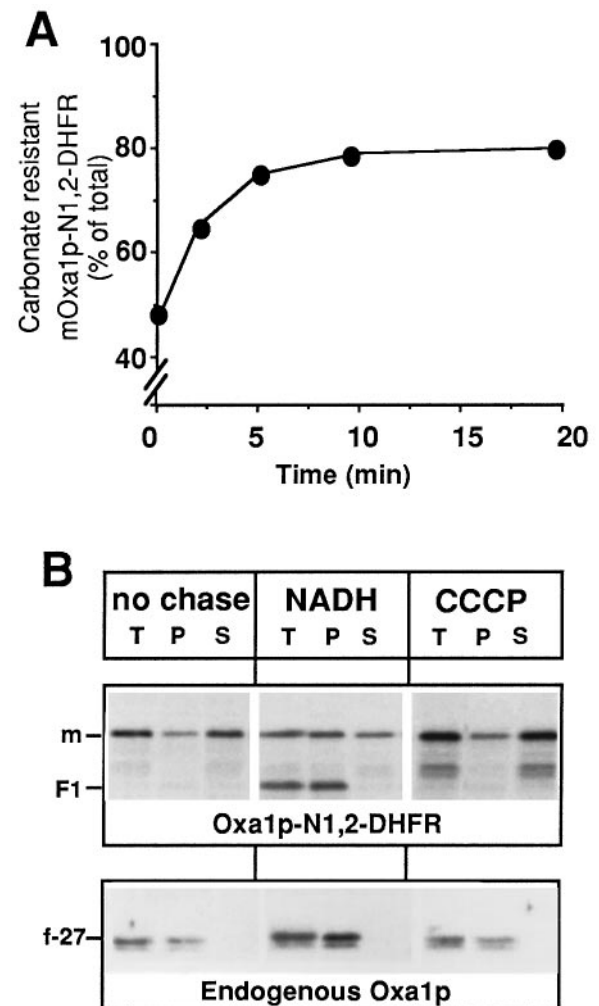
Can a sorting intermediate of Oxa1p which has not yet undergone membrane insertion be observed in the matrix? Resistance to alkaline extraction was taken as a criterion for membrane insertion. As the precursor form of the authentic Oxa1p displayed an intrinsic insolubility at alkaline pH, pOxa1p-N1,2-DHFR, whose precursor species was soluble at alkaline pH, was analyzed. After *in vitro* import, the matured form of Oxa1-N1,2-DHFR was largely extractable with carbonate (Figure 6A). This accumulated species could be chased to a carbonate-resistant form in a time-dependent manner (Figure 6A). Furthermore, this soluble mOxa1p-N1,2-DHFR species was located in the matrix (Figure 6B, lanes 1–3). Chase to the carbonate-resistant form in the presence of the membrane potential correlated with the attainment of the correct topology of  $N_{out}-C_{in}$ , as judged by the generation of the characteristic C-terminal F1 fragment (Figure 6B, +NADH). In the absence of a membrane potential, no export of the N-terminus was observed, and the accumulated mOxa1p-N1,2-DHFR species remained carbonate soluble in the matrix (Figure 6B, +CCCP).

In summary, a sorting intermediate of pOxa1p-N1,2-DHFR accumulates in the matrix as a soluble species which is not integrated into the membrane. Membrane insertion and export of the N-terminal domain occurs in a reaction dependent on a membrane potential, but independent from the import process.

## Discussion

Oxa1p is a polytopic protein of the mitochondrial inner membrane, spanning the membrane five times with an  $N_{out}-C_{in}$  orientation. This orientation is achieved as a result of initial import of the precursor, pOxa1p, into the matrix, followed by a number of distinct export events across the inner membrane. Import of pOxa1p into the matrix occurs in an mt-Hsp70-dependent manner. Once processed by MPP, mOxa1p undergoes membrane potential-dependent insertion into the inner membrane. Two distinct insertion events could be monitored experimentally, export of the ~90 amino acid N-tail and the insertion of TM2 and TM3, leading to export of the hydrophilic segment of ~30 amino acids between them. Whether the energy requirement observed is for the N-tail export step only, or also for TM2/TM3 insertion, is an open question, as the former event appears to precede the latter one.

We propose that the first TM segment constitutes an export signal (which at the same time facilitates the export of the N-tail), and that TM2 and TM3, as well as TM4 and TM5, act in a synergistic manner to mediate integration into the lipid bilayer from the matrix (Figure 7, left

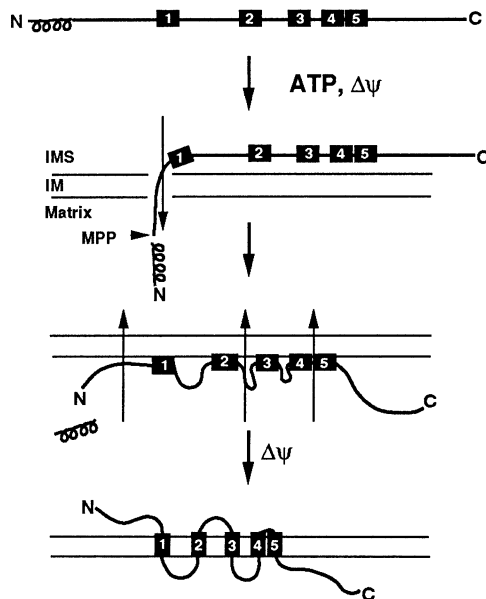


**Fig. 6.** pOxa1p-N1,2-DHFR is sorted via a carbonate-extractable intermediate in the matrix. (A) pOxa1p-N1,2-DHFR was imported into mitochondria for 2 min at 25°C and mitochondria were then re-isolated through a sucrose cushion and subjected to a second incubation for the times indicated. Mitochondria were then converted to mitoplasts in the presence of proteinase K (PK). Resulting mitoplasts were extracted by carbonate, as described in Materials and methods. Samples were analyzed by SDS-PAGE and blotting onto nitrocellulose. The resulting autoradiographs were quantified by laser densitometry. The amount of mOxa1p-N1,2-DHFR present in the carbonate-insoluble fraction is expressed as a percentage of the total species protease protected in mitoplasts after the initial import reaction. (B) Imported pOxa1p-N1,2-DHFR was subjected to a chase incubation for 10 min as described in (A), either on ice or at 25°C, in the presence of 2 mM NADH or 100 μM CCCP, as indicated. Mitochondria were re-isolated, converted to mitoplasts in the presence of proteinase K (PK) and were either lysed directly in SDS buffer (total, T) or were extracted by carbonate, as described in Materials and methods. The carbonate-treated samples were centrifuged to yield a pellet (P) and supernatant (S) fraction, which was TCA precipitated. Samples were analyzed by SDS-PAGE and blotting onto nitrocellulose, and the resulting autoradiographs are depicted. Immunodecoration of the nitrocellulose was performed with antibodies against Oxa1p, (Endogenous Oxa1p), lower panel.

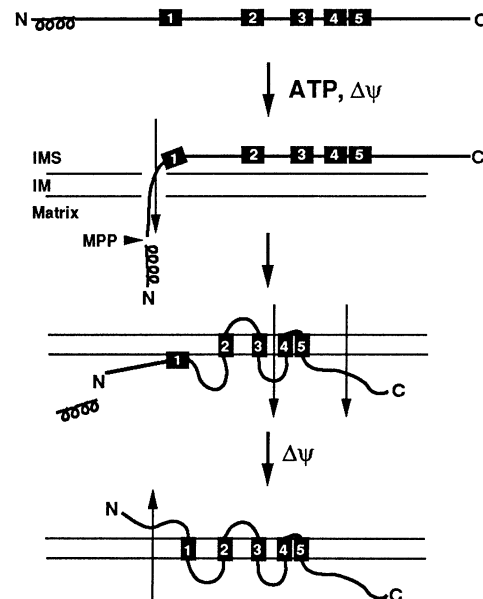
panel). Our data strongly argue against these hydrophobic segments functioning as arrest signals during the import process (Figure 7, right panel).

The sequences of the other eukaryotic homologs of Oxa1p also indicate an arrangement of five TM segments and a conservation of the distribution net charges between these segments (Waterston *et al.*, 1992; Bonnefoy *et al.*,

## Insertion from the matrix



## Insertion from the intermembrane space



**Fig. 7.** Model of import and sorting of pOxa1p across the mitochondrial inner membrane. See text for details. The presequence of pOxa1p is depicted by a curled line whilst the TM segments are indicated by black boxes and numbered 1–5. IMS, intermembrane space; IM, inner membrane.

1994b). In the bacterial homologs of Oxa1p, a signal sequence is found at the very N-terminus (Errington *et al.*, 1992; Ogasawara and Yoshikawa, 1992). This strongly argues that eukaryotic and prokaryotic members of the family are arranged in the membrane in the same manner, with the N-tail exposed to the intermembrane space and periplasm, respectively. The two intermembrane space-exposed hydrophilic regions of Oxa1p bear a net negative charge, whilst those facing the matrix are positively charged. The conservation of these charge distributions may, therefore, be of relevance for the topogenesis of the protein. Thus, Oxa1p is exported from the matrix in a fashion which resembles bacterial export in two important aspects, adherence to the positive inside rule and dependence on the proton motive force.

Is the insertion of mOxa1p from the matrix into the membrane mediated by a proteinaceous machinery? Participation of a bacterial Sec-type machinery appears to be unlikely as there is no indication, at least in yeast, of the existence of a mitochondrial Sec machinery (Glick and von Heijne, 1996) and, furthermore, the export of N-tails in *Escherichia coli* occurs in a Sec-independent manner (Cao and Dalbey, 1994; Dalbey *et al.*, 1995; Whitley *et al.*, 1995). Moreover, the synergistic insertion of neighboring hydrophobic segments into the lipid bilayer, consistent with the hairpin model proposed by Engelman and Steitz (1981), occurs independently of the Sec machinery in bacteria (Andersson and von Heijne, 1993; Cao *et al.*, 1994, 1995). Finally, the insertion of the polytopic protein, melibiose permease, into the cytoplasmic membrane of *E. coli* was shown recently to occur in a Sec-independent, but membrane potential-dependent manner (Bassilana and Gwizdek, 1996). It is not known whether, in bacteria, proteins mediate this Sec-independent membrane insertion

of polytopic proteins. If so, it will be interesting to see in the future whether these proteins have been conserved throughout evolution and are found in mitochondria.

Are other polytopic proteins of the mitochondrial inner membrane inserted in the same manner as pOxa1p? In the case of proteins encoded by mitochondrial DNA this may be true. Not only do these proteins adhere to the positive inside rule (Gavel and von Heijne, 1992), but several lines of evidence also suggests that at least some of them undergo membrane potential-dependent insertion into the inner membrane from the matrix (Herrmann *et al.*, 1995; Rojo *et al.*, 1995).

Those nuclear-encoded proteins which do not have prokaryotic equivalents apparently do not use the combined import–export sorting mechanism employed by pOxa1p. An analysis of the import of two members of this group, AAC and phosphate carrier, has indicated that they are inserted from the intermembrane space side of the inner membrane without prior import into the matrix (Mahlke *et al.*, 1990; Wachter *et al.*, 1992; Sirrenberg *et al.*, 1996). This insertion process is mediated by a novel translocase of the inner membrane rather than the general Tim17–Tim23–Tim44 machinery used by matrix-targeted proteins. One component of this novel translocase is Tim22, a component essential for the import of AAC and the phosphate carrier (Sirrenberg *et al.*, 1996). Import of pOxa1p does not require Tim22; this is consistent with it using the general matrix import pathway (C. Sirrenberg and J.M. Herrmann, unpublished results). We propose that the different modes of integration of pOxa1p and AAC into the inner membrane reflect the different evolutionary origins of both proteins. AAC seems to represent a ‘novel’ mitochondrial protein that, during evolution of mitochondria, became essential when metabolic processes



in the cytoplasm and the mitochondrial matrix needed to be connected. Oxa1p, on the other hand, following import into the matrix, embarks on an export pathway homologous to that used by its prokaryotic ancestors. Other nuclear-encoded polytopic proteins of mitochondria conserved from prokaryotic ancestors may also use such a conservative pathway.

## Materials and methods

### Recombinant DNA techniques and plasmid constructions

The recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). The *OXA1* gene was obtained by amplification of genomic DNA of strain D273-10B by PCR. The resulting DNA fragment was subcloned into the *Bam*HI and *Kpn*I sites of vector pGEM3 (Promega) yielding plasmid pOXA1p. To construct the plasmids for the expression of pOxa1p-N,1,2-DHFR, pOxa1p-N,1,2,3-DHFR, pOxa1p-N,1,2,3,4-DHFR, pOxa1p-N,1,2,3,4,5-DHFR and pOxa1p-DHFR, the relevant *OXA1* DNA fragments (see Figure 5A) were synthesized by PCR and subcloned into a *Bam*HI site in front of the DNA encoding mouse DHFR. A DHFR derivative was used which bears a number of point mutations, whereby Cys7, Ser42 and Asn49 were replaced by Ser, Cys and Cys, respectively. These mutations cause a destabilization in the folded structure of DHFR whereby, in contrast to its wild-type counterpart, the mutated DHFR remains sensitive to added proteases (Teichmann *et al.*, 1996). We chose this DHFR derivative as it was imperative to use a protease-sensitive passenger protein when analyzing the mitochondrial sublocalization of the pOxa1p-DHFR derivatives following import.

### Yeast strains

Yeast strains used in this study were wild-type D273-10B (ATCC No. 24657), the temperature-sensitive mutant of the *SSC1* gene product, mt-Hsp70, termed *ssc1-3* (PK83) and its isogenic wild-type (PK82) (Gambill *et al.*, 1993). The wild-type yeast D273-10B was grown on lactate medium at 30°C, the other strains at 24°C, and harvested at an OD<sub>578</sub> of ~1. Mitochondria were isolated as previously described (Herrmann *et al.*, 1994a), with the exception that zymolyase treatment was performed at 24°C in the case of the *ssc1-3* mutant and its isogenic wild-type.

### Import of precursor proteins into mitochondria

Precursor proteins were synthesized in the presence of [<sup>35</sup>S]methionine by coupled transcription-translation in reticulocyte lysate (Promega Incorp.) as described before (Pelham and Jackson, 1976). Import mixtures (100 µl) usually contained 1–3% reticulocyte lysate (v/v) in 3% bovine serum albumin (w/v), 220 mM sucrose, 10 mM MOPS-KOH, 80 mM KCl, 2 mM MgOAc, 40 mM potassium phosphate and 1 mM MnCl<sub>2</sub>, pH 7.2. The final concentration of mitochondria in the import reaction was 0.5 mg/ml. Protease treatment and mitoplasting were performed as described before (Fölsch *et al.*, 1996). Depletion of matrix ATP prior to import was as described by Stuart *et al.* (1994). Following import, when indicated, unbound precursor was separated from mitochondria by centrifugation for 7 min at 14 000 g and 4°C through a sucrose cushion (same composition as import buffer but with 0.5 M sucrose).

### Antibody production and immunoprecipitation

Antisera against the C-terminus of Oxa1p were raised in rabbits by injecting the chemically synthesized peptide CDNEKKLQESFKEKR, which had been coupled to activated ovalbumin (Pierce, IL, USA). Immunoprecipitations with Oxa1p antiserum and co-immunoprecipitations with mt-Hsp70 antiserum following Oxa1p import were done as described in Herrmann *et al.* (1994b).

### In vitro processing assay with MPP

Both subunits of *N.crassa* MPP were purified as described in Arretz *et al.* (1994). The subunits (0.4 µg of each) were incubated with 5% (v/v) reticulocyte lysate containing the pOxa1p or pSu9(1–69)-DHFR proteins in 0.1 mM MnCl<sub>2</sub>, 1% (w/v) Triton X-100, 30 mM HEPES, pH 7.8 for the times indicated at 25°C. Then proteins were precipitated by the addition of 12% (w/v) trichloroacetic acid (TCA), washed with acetone and resolved by SDS-PAGE.

### Miscellaneous

To extract proteins by alkaline treatment (Pfanter *et al.*, 1987), 40 µg of mitochondria were resuspended in 500 µl of 0.1 M Na<sub>2</sub>CO<sub>3</sub> and

incubated for 30 min on ice. Membranes and bound proteins were pelleted at 226 000 g for 1 h at 4°C in a Beckman Ti50 rotor and proteins from the resulting supernatant were precipitated with 12% (w/v) TCA. Proteins in both fractions were analyzed by SDS-PAGE. Following SDS-PAGE and fluorography, data were quantified by densitometry (Ultrascan XL, Pharmacia).

Protein determination and SDS-PAGE were performed according to the published methods of Bradford (1976) and Laemmli (1970), respectively. The detection of proteins after blotting onto nitrocellulose (Towbin *et al.*, 1979) was performed using the ECL detection system (Amersham).

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