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A novel intermediate on the import pathway of cytochrome b_2 into mitochondria: evidence for conservative sorting

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Cytochrome b_2 is sorted into the intermembrane space of mitochondria by a bipartite N-terminal targeting and sorting presequence. In an attempt to define the sorting pathway we have identified an as yet unknown import intermediate. Cytochrome b_2–dihydrofolate reductase (DHFR) fusion proteins were arrested in the presence of methotrexate (MTX) so that the DHFR domain was at the surface of the outer membrane while the N-terminus reached into the intermembrane space where the sorting signal was removed. This membrane-spanning, mature-sized species was efficiently chased into the mitochondria upon removal of MTX. Thus, an intermediate was generated which was exposed to the intermembrane space but was still associated with the inner membrane. This intermediate was also found upon direct import of cytochrome b_2 and derived fusion proteins. These membrane-bound mature-sized cytochrome b_2 species loop through the matrix and could be recovered in a complex with mt-Hsp70 and the inner membrane MIM44/ISP45, a component of the inner membrane import apparatus. This novel sorting intermediate can only be explained by a pathway in which cytochrome b_2 passes through the matrix. The existence of such an intermediate is inconsistent with a pathway by which entrance of the mature part of cytochrome b_2 into the matrix is stopped by the sorting sequence; however, its presence is fully consistent with the conservative sorting pathway.

Key words: cytochrome b_2/intramitochondrial sorting/mitochondria/*Saccharomyces cerevisiae*

Introduction

One of the unsolved questions of mitochondrial biogenesis concerns the sorting process of proteins to the mitochondrial intermembrane space. This process is particularly complex because a number of distinct pathways exist (for reviews see Glick *et al.*, 1992a; Lill *et al.*, 1992a; Stuart *et al.*, 1994c). It now seems clear that those precursors synthesized without a cleavable presequence destined for this subcompartment are imported directly across the outer membrane with no apparent involvement of the inner membrane (Stuart and Neupert, 1990; Lill *et al.*, 1992b). Many precursors are, however, synthesized with cleavable presequences, e.g. cytochrome b_2, cytochrome c_1 and the Rieske FeS protein. Precytochromes b_2 and c_1 contain unusually long presequences which are processed in two steps and which can be divided into two functional domains. The initial N-terminal hydrophilic domain represents a matrix-targeting signal. This domain is proteolytically cleaved off in the matrix by the mitochondrial processing peptidase (MPP), thus generating an intermediate-sized form. The remaining C-terminal hydrophobic portion of the presequence encodes the information necessary for sorting to the intermembrane space, where it is removed by the Imp1p–Imp2p protease complex which is located peripherally on the external face of the inner membrane (Schneider *et al.*, 1991; Jensen *et al.*, 1992; Beasley, 1993; Beasley *et al.*, 1993; Nunnari *et al.*, 1993; Schwarz *et al.*, 1993). Currently two mechanisms for how this sorting sequence operates are being discussed: the 'stop-transfer' pathway and the 'conservative sorting' pathway.

The term 'stop transfer' was introduced to describe how integral membrane proteins become anchored in the lipid bilayer (Blobel, 1980). It was subsequently adopted to describe the sorting of other intermembrane space proteins, such as cytochrome *c* peroxidase, cytochrome b_2 and cytochrome c_1 (Kaput *et al.*, 1982; van Loon *et al.*, 1986, 1987; van Loon and Schatz, 1987; Glick *et al.*, 1992a, 1993). The stop-transfer model for the sorting of mitochondrial proteins proposes that such precursors initiate import along the general import pathway used by matrix-targeted proteins. Complete translocation across the inner membrane of these intermembrane space precursors, however, is prevented due to an interaction of the sorting signal with the inner membrane import machinery; this results in the arrest of mitochondrial proteins in the import channel. Consequently only the extreme N-terminal portion of the presequence becomes exposed to the matrix, where it undergoes cleavage by MPP (Figure 1A, stage 1). The mature portion of the precursor would then be selectively translocated across the outer membrane only, a process proposed to be supported by the dissociation of the import channels of the two mitochondrial membranes (Figure 1A, stage 2). The arrested precursor is suggested to leave the import site by a lateral diffusion process, and would be freed from anchorage to the inner membrane following its proteolytic processing by one of the IMP proteases (Glick *et al.*, 1992b, 1993). This would result in the release of a soluble, mature-sized protein in the intermembrane space which could then fold and assemble into a functional enzyme (Figure 1A, stage 3).

The alternative view of intermembrane space sorting takes into account evolutionary aspects of organelles, such as mitochondria and chloroplasts which are thought to

have prokaryotic ancestors (Hartl *et al.*, 1986, 1987; Hartl and Neupert, 1990). It is clear that throughout evolution many prokaryotic features have been conserved in mitochondria, e.g. the translation system including its sensitivity with respect to antibiotics, many aspects of its electron transport system, ATP synthesis and the heat shock/chaperone system. The conservative sorting hypothesis stresses that along with these prokaryotic attributes, the process of prokaryotic protein secretion has also been maintained in mitochondria. It is proposed that the sorting signals of mitochondrial intermembrane space proteins, together with their mechanism of operation, have been conserved from the prokaryotic protein secretory pathway. Consequently, such intermembrane space proteins would be imported along the general import pathway into the matrix (a compartment analogous to bacterial cytoplasm; Figure 1B, stage 1), from where they would embark on an export pathway (analogous to the bacterial secretory pathway) which is directed by the signal sequence and which uses features/components conserved from their ancestral secretory pathway (Figure 1B, stages 2a and b). Experimental evidence in favour of such a pathway has been presented (Hartl *et al.*, 1986, 1987; Koll *et al.*, 1992; H.Ono, A.Gruhler, B.Guiard, R.A.Stuart, E.M.Schwartz and W.Neupert, manuscript in preparation).

We have shown previously that cytochrome b_2–dihydrofolate reductase fusion precursors (pb_2–DHFR) could be arrested as translocation intermediates spanning contact sites when the unfolding of the DHFR moiety is inhibited following methotrexate (MTX) binding (Rassow *et al.*, 1990). Those pb_2–DHFR precursors, which contained ≥260 residues of precytochrome b_2, were found to undergo both MPP and Imp1p processing reactions, giving rise to mb_2–DHFR fusion proteins despite the MTX block of the DHFR translocation (Koll *et al.*, 1992). These observations can be explained by both models.

First, according to the stop-transfer model, the mature-sized fusion protein would be arrested with the DHFR moiety outside the mitochondria, while the N-terminus would be free in the intermembrane space following Imp1p processing (Figure 1A, stage 4→5). No segment of this protein should therefore be associated with the inner membrane or matrix (Figure 1A, stage 5). The stop-transfer model proposes that cytochrome b_2 is imported across the outer membrane as an intermediate-sized species (Figure 1A, stage 2). Hence, cleavage by Imp1p prior to complete outer membrane translocation, as is the case for the MTX-arrested species, would result in the removal of the inner membrane anchorage. Further, it has been suggested that this arrested species should therefore not be able to complete its translocation into the intermembrane space (Figure 1A, stage 5; Glick *et al.*, 1992b, 1993).

On the other hand, according to the conservative sorting hypothesis the MTX-arrested species would be predicted to be spanning three membranes, looping through the matrix between the sites of import and export (Figure 1B, stage 4→5). Resumption of import following MTX removal would be expected according to the conservative sorting hypothesis (Figure 1B, stage 2a→3b). The chase would require the coordinated action of both the outer and inner import machineries, together with that of the export process, to correctly deliver the chased protein to

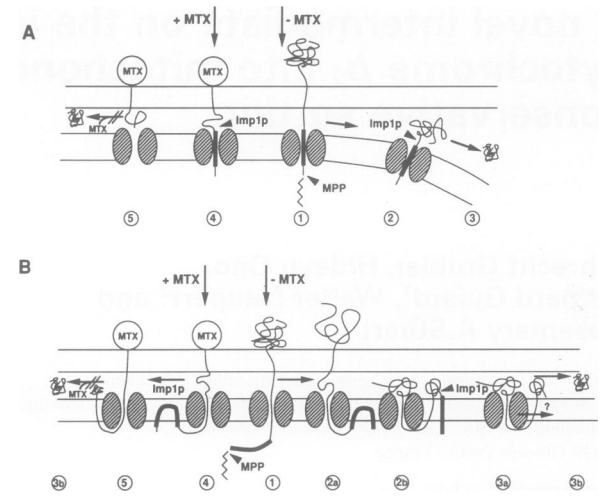

Fig. 1. Import and sorting of cytochrome b_2–DHFR fusion proteins: alternative views. (**A**) Stop transfer hypothesis. In the absence of MTX (–MTX), precytochrome b_2–DHFR initiates import along the general import pathway. However, complete translocation across the inner membrane is prevented due to an interaction of the sorting signal with the inner membrane import machinery (stage 1). Complete translocation across the outer membrane precedes the Imp1p processing of the protein (stage 2), thus causing the release of the soluble mature-sized species in the intermembrane space (stage 3). In the presence of MTX (+MTX), the translocation intermediate (stage 4) undergoes Imp1p processing prior to complete translocation, releasing a free N-terminus in the intermembrane space and freeing anchorage to the inner membrane (stage 5). As outlined in the text, this should result in a dead-end species which cannot be chased further into mitochondria. (**B**) Conservative sorting hypothesis. In the absence of MTX (–MTX), precytochrome b_2–DHFR initiates import along the general import pathway, thus exposing the sorting signal to the matrix (stage 1) where it undergoes an insertion step back into the inner membrane (stage 2a). When this occurs concomitantly with import, the protease-protected intermediate- (stage 2b) and mature-sized species (stage 3a) can still be in contact with the inner membrane import machinery while spanning through the matrix. Exit from this import channel, either further into the matrix or in a lateral manner, combined with completion of export, results in the generation of a soluble mature-sized species in the intermembrane space (stage 3b). In the presence of MTX (+MTX) complete translocation is prevented. Following MPP processing (stage 4), if the precursor is of sufficient length, the signal sequence undergoes insertion into the inner membrane and subsequent Imp1p processing (stage 5). Hence such a translocation intermediate remains in contact with the inner membrane as the processes of import and sorting are not completed. Segments of the MTX-arrested intermediate span through the matrix from the sites of import to those of export. Upon removal of the MTX block, chase to a protease-protected species can occur by the resumption of the import process across the outer membrane. This would result in a soluble intermembrane space species only if the export event is efficiently resumed.

the intermembrane space where it should accumulate as a soluble species (Figure 1B, stage 3b). Following insertion of the sorting signal (Figure 1B, stage 2b), exit of the mature part of the protein from the inner membrane import channel could occur either by further import into the matrix or possibly in a lateral manner directly into the membrane. This alternative sorting mechanism may prevail under conditions of retarded import over the inner membrane, i.e. limiting mt-Hsp70 action.

Here we report that MTX-arrested Imp1p-processed cytochrome b_2–DHFR fusion proteins can indeed be further translocated and accumulated, but largely as a membrane-bound species in mitoplasts. This membrane-

associated mature-sized cytochrome b_2–DHFR (mb_2–DHFR) species was found in a complex with mt-Hsp70 and with MIM44 (also termed Mpi1p or ISP45), a component of the inner membrane import apparatus (Maarse *et al.*, 1992; Blom *et al.*, 1993; Horst *et al.*, 1993), indicating that it is looping through the matrix. Furthermore, we demonstrate that the formation of this sorting intermediate is independent of the MTX arrest, because it can be observed also upon direct import of authentic precytochrome b_2 and other cytochrome b_2–DHFR fusion proteins. Like the above-mentioned mb_2–DHFR species, mature-sized cytochrome b_2 can also be found in a complex with mt-Hsp70. The existence of this novel sorting intermediate strongly supports the conservative sorting hypothesis.

Results

MTX-arrested cytochrome b_2–DHFR fusion proteins which are Imp1p processed can be chased into mitochondria

The precursor protein pb_2(260)–DHFR, consisting of the initial 260 amino acid residues from the cytochrome b_2 precursor fused in-frame to DHFR, was synthesized in reticulocyte lysate and incubated in either the absence (Figure 2A, lanes 1 and 2) or presence of MTX and NADPH (Figure 2A, lanes 3–12) prior to incubation with mitochondria ('1st incubation' in Figure 2A). Mitochondria were then re-isolated, resuspended in fresh import buffer and subjected to a further incubation ('chase') for 30 min at either 0 or 25°C, as indicated. Import efficiency was assessed following protease treatment of one half of each sample. When both incubations were performed in the absence of MTX, pb_2(260)–DHFR was processed efficiently to its mature form (Figure 2A, lane 1) which was imported into the mitochondria, as judged by its resistance to exogenously added trypsin (Figure 2A, lane 2). When MTX was present during both steps, pb_2(260)–DHFR was also processed efficiently to its mature form (Figure 2A, lanes 3 and 5). Under these conditions, however, the DHFR domain was arrested on the mitochondrial outer surface and remained protease-sensitive during the second incubation, at both 0 and 25°C (Figure 2A, lanes 4 and 6). When the MTX arrest was relieved by omitting the MTX during the re-isolation and second incubation, a significant proportion of this accumulated mature-sized species was chased into a protease-resistant location (Figure 2A, lane 8).

To analyse the energetic requirements of the chase reaction, valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was included to dissipate the membrane potential (Figure 2A, lanes 9–12). No significant inhibitory effect on the efficiency of chase to a protease-protected location was observed (Figure 2A, lanes 10 and 12). In addition, matrix ATP depletion did not block the chase, nor was the process inhibited in mitochondria from the *ssc1-3* mutant containing temperature-sensitive defective mt-Hsp70 (results not shown). In summary, the chase reaction measured here does not have any apparent energy requirement. It can be speculated that the partial reaction assessed here might be driven by the folding process.

Thus, the mature-sized MTX-arrested and surface-exposed cytochrome b_2–DHFR protein is not a dead-end species because it could be transported further into mitochondria. The efficiency of this reaction was high (50–60% of the import control in the absence of MTX), but the kinetics were relatively slow compared with direct import (Figure 2B). In the chase reaction the half-time was ~30–40 min; upon direct import it was <10 min (results not shown).

Taking all these results together we conclude that a

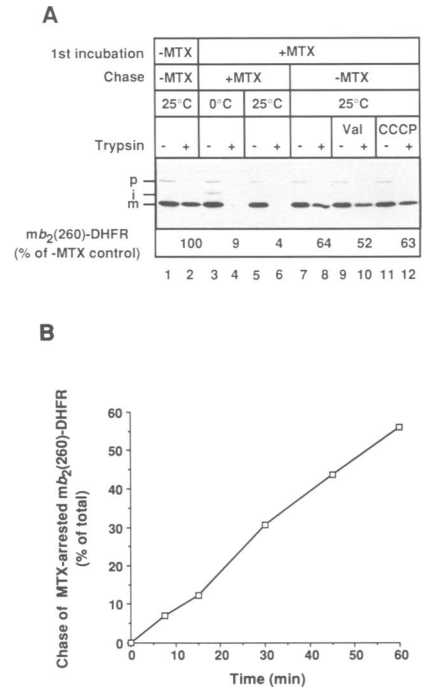

Fig. 2. Chase of MTX-arrested import intermediate of mb_2(260)–DHFR. (**A**) Reticulocyte lysate containing [^{35}S]methionine-labelled pb_2(260)–DHFR was incubated in P80 import buffer containing NADPH and MTX for 10 min at 0°C (lanes 3–12), as described in Materials and methods. MTX was omitted in the import controls (lanes 1 and 2). Mitochondria were then added and import was performed for 20 min at 25°C. The MTX-containing sample was divided into equal aliquots and received no further additions (lanes 3–8), valinomycin (1 μM; lanes 9 and 10) or CCCP (100 μM; lanes 11 and 12). Mitochondria were re-isolated by centrifugation from all samples. They were washed with SEMK buffer (lanes 1, 2 and 7–12) or SEMK buffer containing NADPH and MTX for the +MTX controls (lanes 3–6). Mitochondria were resuspended in P80 import buffer and were supplemented with NADPH and MTX (lanes 3–6), valinomycin (1 μM; lanes 9 and 10) or CCCP (100 μM; lanes 11 and 12); the remaining samples received no further additions (lanes 1, 2, 7 and 8). Samples were either kept on ice (lane 3 and 4) or returned to 25°C (lanes 1, 2 and 5–12) and incubated for a further 40 min. After this period, all samples were placed on ice and one half was mock-treated (all odd-numbered samples), while the other was trypsin-treated (50 μg/ml; all even-numbered samples) for 15 min on ice. Protease activity was stopped by the addition of soybean trypsin inhibitor (1 mg/ml). Mitochondria were re-isolated by centrifugation and lysed in SDS sample buffer. All samples were analysed by SDS–PAGE and the resulting fluorograph is presented here. (**B**) The MTX-arrested import intermediate of mb_2(260)–DHFR was generated as described above, prior to the re-isolation of mitochondria. Mitochondria were resuspended in fresh P80 import buffer and the translocation intermediate was chased at 25°C for the times indicated. All samples were subsequently treated with trypsin and processed further as described in (A). (A and B) Protease-protected mb_2(260)–DHFR was quantified by laser densitometry of the resulting fluorograph and expressed as a percentage of mb_2(260)–DHFR directly imported in the absence of MTX. Abbreviations: p, precursor; i, intermediate; m, mature; Val, valinomycin.

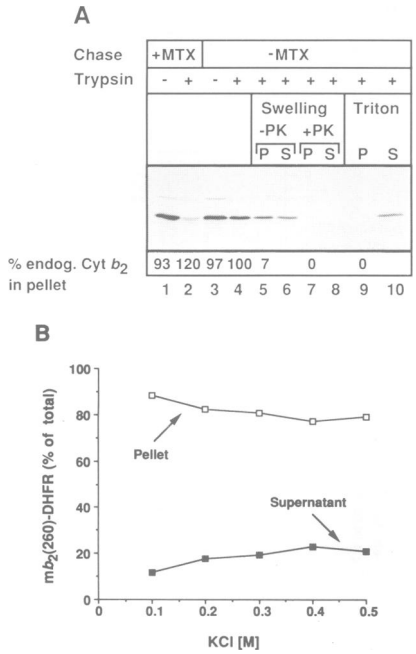

Fig. 3. Chased mb_2(260)−DHFR re-isolates with the mitoplasts. (**A**) Radiolabelled pb_2(260)−DHFR was incubated in the presence of MTX and NADPH with mitochondria for 15 min at 25°C. Mitochondria were re-isolated, washed and resuspended in fresh import buffer and divided. One aliquot was chased in the presence of MTX (lanes 1 and 2), while the rest was chased in the absence of MTX (lanes 3–10) at 25°C for a further 40 min. The +MTX sample was divided and was either mock- (lane 1) or trypsin-treated (lane 2), as described in the legend to Figure 2, to determine the efficiency of the MTX arrest. The −MTX sample was also divided. One aliquot was mock-treated (lane 3), while the rest (two thirds of the sample) was treated with trypsin (lanes 4–10). Mitochondria from both samples were re-isolated, and either lysed directly in SDS sample buffer (lanes 3 and 4) or converted to mitoplasts by hypotonic swelling in either the absence (lanes 5 and 6) or presence of proteinase K (100 μg/ml; lanes 7 and 8). The mitoplasts were re-isolated by centrifugation and the resulting pellets (lanes 5 and 7) and supernatant fractions (TCA-precipitated) are presented (lanes 6 and 8). One aliquot of mitochondria was lysed with 0.2% Triton X-100 buffer for 30 min on ice. Subsequent centrifugation resulted in a pellet (lane 9) and a supernatant fraction which was TCA-precipitated (lane 10). All samples were analysed by SDS−PAGE and blotted onto nitrocellulose. The resulting autoradiograph is presented. Immunoblotting was performed and the levels of the endogenous b_2 present in each pellet fraction were expressed as a percentage of the total (lane 4). (**B**) MTX-arrested mb_2(260)−DHFR was chased into mitochondria upon removal of MTX. Trypsin-treated mitochondria were then converted to mitoplasts. Re-isolated mitoplasts were incubated for 20 min at 0°C in SEM buffer containing varying concentrations of KCl, as indicated. Following centrifugation, the supernatant was TCA-precipitated and all samples were analysed by SDS−PAGE. The distribution of mb_2(260)−DHFR between the pellet (□) and supernatant (■) fractions is expressed as a percentage of the sum of the two. Abbreviations are as in the legend to Figure 2. Pel, pellet; Sup, supernatant.

cytochrome b_2-derived fusion protein arrested as a mature-sized species spanning the outer membrane can be efficiently transported further into a protease-protected location in mitochondria.

A novel mature-sized intermediate of cytochrome b_2 that is associated with mitoplasts

MTX-arrested mb_2(260)−DHFR (Figure 3A, lanes 1 and 2), upon removal of the MTX, was chased into mitochondria where it became inaccessible to exogenously added trypsin (Figure 3A, lanes 3 and 4). Mitochondria were then subjected to an osmotic swelling to rupture the outer membrane. The resulting mitoplasts were separated from soluble intermembrane space proteins by centrifugation. A significant fraction (~50%) of the chased species was recovered with the mitoplasts (Figure 3A, lane 5). Formation of the mitoplasts in the presence of protease resulted in the degradation of a large proportion of the imported species (Figure 3A, lanes 7 and 8). Furthermore, the mb_2(260)−DHFR recovered with mitoplasts could be solubilized with low levels of Triton X-100, indicating that the mitoplast-associated species did not represent an aggregated form of mb_2(260)−DHFR (Figure 3A, lanes 9 and 10).

Thus following chase into mitochondria, the mb_2(260)−DHFR becomes exposed to the intermembrane space. However, a certain proportion fails to reach the state of a soluble species and hence remains associated with the membrane.

The membrane-associated species was analysed further to determine its manner of association with the inner membrane. After the arrest of mb_2(260)−DHFR, chase and trypsin treatment of mitochondria, mitoplasts were formed and exposed to increasing concentrations of salt to remove peripherally associated proteins (Figure 3B). The membrane association of mb_2(260)−DHFR was unaffected under these conditions, suggesting a tight association with the membrane. Furthermore, this membrane-bound intermediate was also largely resistant to exposure to high levels of urea (up to 3 M; results not shown).

To demonstrate the membrane-associated species as an intermediate also of direct import (i.e. not only of the arrest−chase procedure), authentic precytochrome b_2 and a series of pb_2−DHFR fusion proteins, which differed in the lengths of their cytochrome b_2 portions, were imported into mitochondria (Figure 4A). Samples were protease-treated. Mitochondria were re-isolated and converted to mitoplasts. In all cases a substantial amount of mature-sized species was recovered with the mitoplasts (Figure 4A), with the proportion of membrane-bound species increasing with the increasing lengths of the preproteins. This length dependence correlated with the likelihood for an Imp1p processed species to span both import and export sites (Koll *et al.*, 1992).

To demonstrate that the membrane-associated species is a kinetic intermediate of the sorting pathway, authentic precytochrome b_2 was imported into wild-type mitochondria for up to 60 min. Mitochondria were treated with trypsin to remove all precursor exposed to the outer surface, and then mitoplasts were prepared. The relative amounts of mature-sized species soluble in the intermembrane space and associated with the mitoplast membrane were determined. Import of precytochrome b_2 occurred during the first 10 min of incubation. The proportion of soluble species in the intermembrane space increased over a time period of 60 min at the expense of imported membrane-bound species (Figure 4B). However, the release from the inner membrane was not complete, suggesting that the retranslocation process was limiting. This would be in agreement with the slow chase kinetics of the MTX-arrested species observed in Figure 2B. The sorting step appears to be labile and once stalled is

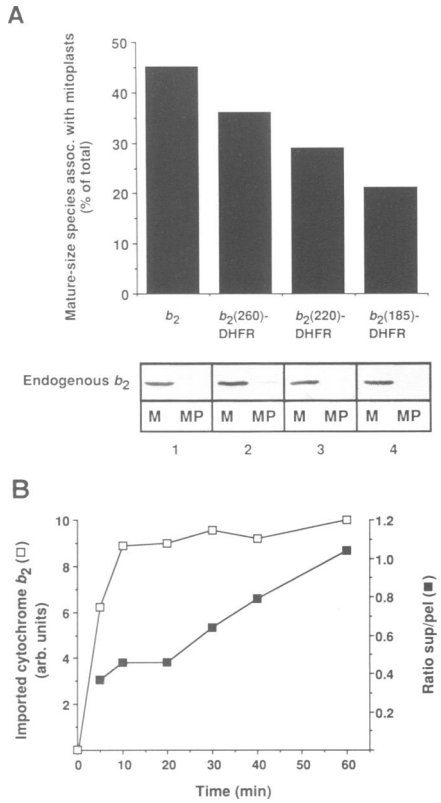

Fig. 4. Membrane-bound species is a sorting intermediate of the direct import process. (**A**) Radiolabelled precytochrome b_2, pb_2(260)−DHFR, pb_2(220)−DHFR and pb_2(185)−DHFR were imported directly into mitochondria for 15 min at 25°C. All samples were then treated with trypsin. Samples were divided and mitochondria from one aliquot were converted to mitoplasts by hypotonic swelling. Mitochondria and mitoplasts were re-isolated from both sets of samples. They were analysed by SDS−PAGE and blotted onto nitrocellulose. The resulting autoradiograph was quantified by laser densitometry. Values of the membrane-bound mature-sized form of each of the cytochrome b_2-derived species are given as a percentage of the unswollen total (upper panel). Immunoblotting of the endogenous marker protein cytochrome b_2 in mitochondrial and mitoplast fractions was performed (lower panel). (**B**) Radiolabelled precytochrome b_2 was imported into mitochondria at 25°C. After each indicated time point an aliquot of the sample was removed and placed on ice. All samples were trypsin-treated and divided into two. Mitochondria from one aliquot were converted to mitoplasts by hypotonic swelling. The resulting mitoplasts were re-isolated by centrifugation to yield a pellet and a supernatant fraction (TCA-precipitated). The mitochondria from the remaining half of the sample, the mitoplast pellet (pel) and the supernatant (sup) fractions were analysed by SDS−PAGE and blotted onto nitrocellulose. The resulting autoradiograph was quantified by laser densitometry. The total imported signal is expressed as arbitrary units (□), while the distribution of this species between mitoplast and supernatant fractions is expressed as a ratio of the signal supernatant:pellet (sup/pel; ■). Immunoblotting of the endogenous marker protein cytochrome b_2 was performed and swelling was >95% efficient in each case (results not shown). Abbreviations: b_2, cytochrome b_2; M, mitochondria; MP, mitoplasts.

inefficiently resumed (H.Ono, A.Gruhler, B.Guiard, R.A.Stuart, E.M.Schwartz and W.Neupert, manuscript in preparation). Furthermore, it seems possible that a factor necessary for translocation, e.g. $\Delta\Psi$, or a factor required for folding in the intermembrane space, may become limiting during such a prolonged incubation period and hence result in the premature cessation of sorting.

Thus, the membrane-bound Imp1p-processed species represents a precursor of the soluble m-cytochrome b_2 in the intermembrane space.

Membrane-associated species loops through the matrix

In an attempt to demonstrate that the membrane-associated m-cytochrome b_2 and mb_2(260)−DHFR span through the matrix, the interaction with the matrix-localized mt-Hsp70 was investigated. For these experiments mitochondria from the yeast strain *ssc1-2* were used which harbour a temperature-sensitive mt-Hsp70. Mt-Hsp70 of *ssc1-2* cells exposed to 37°C binds substrate proteins but does not release them efficiently; thus the complex formed is more stable. This is reflected in a higher efficiency of co-immunoprecipitation of mt-Hsp70 with unfolded polypeptide chains in transit (Gambill *et al.*, 1993; Herrmann *et al.*, 1994).

Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria at 25°C and samples were either maintained at this temperature or shifted to the non-permissive temperature of 37°C to induce the phenotype of the mutant (Figure 5A). Mitochondria were treated with trypsin and lysed with detergent. Immunoprecipitation was performed with antibodies raised against purified mt-Hsp70 and against *Escherichia coli* DnaK (which also recognizes mt-Hsp70). Co-immunoprecipitation of mb_2(260)−DHFR with mt-Hsp70 following import at both temperatures was observed. However, the efficiency was much higher in the samples which had been exposed to 37°C, where ~10% of the total solubilized signal could be recovered as a mt-Hsp70 co-immunoprecipitate (Figure 5A). Under these conditions ~60% of the imported material was re-isolated with the mitoplast fraction. Since only this fraction of the total detergent-solubilized species was a substrate for mt-Hsp70, the efficiency of the co-immunoprecipitation was therefore even higher. When mitochondria were incubated at 37°C to induce the phenotype prior to the import reaction at 25°C, the efficiency of co-immunoprecipitation was in the range of 2–4% of total solubilized material (results not shown).

These data indicate that the membrane-bound species is spanning the inner membrane, whereby the matrix-exposed loop can be a substrate for mt-Hsp70. Although post-lysis binding would not expected to occur here because lysis was performed in the absence of Mg-ATP and in the presence of EDTA, it was essential to exclude this possibility. Therefore we performed a series of relevant control experiments.

Radiolabelled pb_2(260)−DHFR was imported into mitochondria isolated from either the *ssc1-2* (Figure 5B, lane 1) or wild-type cells (Figure 5B, lanes 2 and 3). Import was performed initially at 25°C and then the temperature was shifted to 37°C. Following protease treatment, mitochondria were re-isolated and lysed in the presence of either additional wild-type mitochondria (Figure 5B, lanes 1 and 2) or *ssc1-2* mitochondria which had also been exposed to 37°C (Figure 5B, lane 3). The efficiency of co-immunoprecipitation with mt-Hsp70 was significantly higher when pb_2(260)−DHFR was imported into the *ssc1-2* mitochondria than that observed following import into the wild-type mitochondria (Figure 5B, lanes 1 and 2). The low level of co-immunoprecipitation in the wild-type mitochondria was observed irrespective of whether

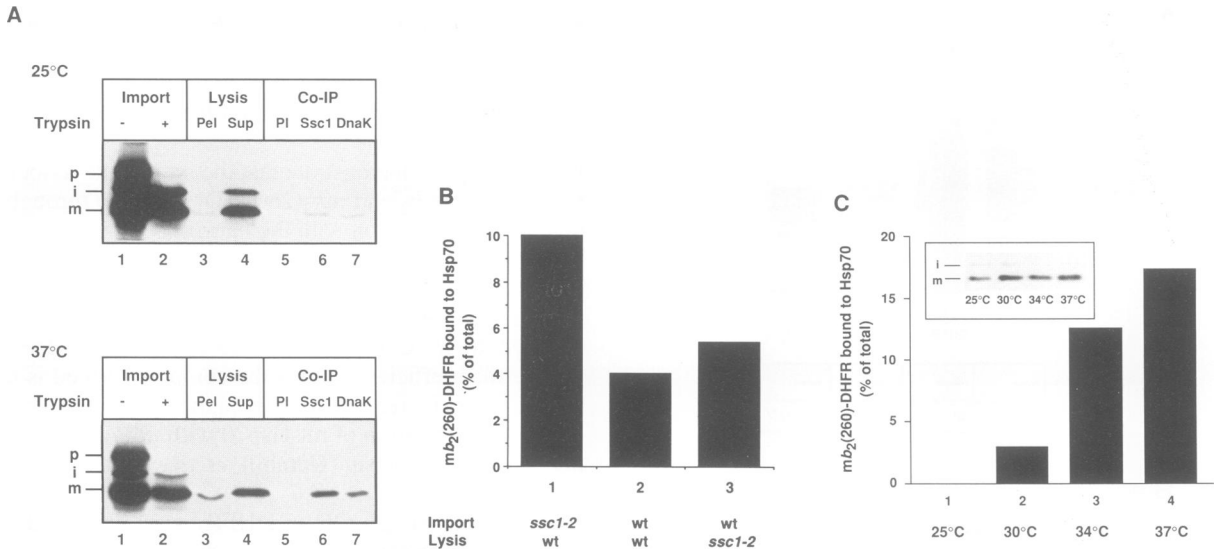

Fig. 5. mb_2(260)−DHFR forms a complex with mt-Hsp70. (**A**) Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria for either 15 min at 25°C (upper panel) or 5 min at 25°C, and then shifted to 37°C for 10 min (lower panel). One aliquot of each sample was removed (lane 1) and the remainder was trypsin-treated. The mitochondria were then re-isolated by centrifugation. One aliquot was removed (lane 2); the rest was lysed in 0.2% Triton X-100 lysis buffer for 10 min on ice and then subjected to a clarifying spin. The resulting pellets were resuspended in SDS sample buffer (lane 3) and the supernatants were divided further into four parts. One part was TCA-precipitated (lane 4) and the others were used for co-immunoprecipitation with preimmune serum (lane 5), Ssc1p (lane 6) or DnaK antiserum (lane 7), as described in Materials and methods. All samples were analysed by SDS−PAGE and fluorography. The amount of label depicted in the supernatant samples corresponds to 20% of that used for the co-immunoprecipitation. A total of 20% of the pellet fraction was loaded. (**B**) Radiolabelled pb_2(260)−DHFR was imported into either *ssc1-2* (20 μg; lane 1) or the corresponding wild-type mitochondria (20 μg; lanes 2 and 3) for 5 min at 25°C and then shifted to 37°C for a further 10 min. Samples were then trypsin-treated and mitochondria re-isolated and lysed in the presence of an equivalent amount of either wild-type (lanes 1 and 2) or *ssc1-2* mitochondria (lane 3; also treated at 37°C). The supernatant following the clarifying spin was used for co-immunoprecipitation analysis with antiserum raised against Ssc1p. Samples were analysed by SDS−PAGE and the resulting fluorograph was quantified by laser densitometry. The level of mb_2(260)−DHFR found associated with mt-Hsp70 was expressed as a percentage of the total present in the lysis supernatant fraction. (**C**) Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria for 5 min at 25°C and was followed by 10 min at 25 (lane 1), 30 (lane 2), 34 (lane 3) or 37°C (lane 4). Following trypsin treatment samples were divided. The mitochondria from one aliquot were re-isolated and lysed in SDS sample buffer. They were subjected to SDS−PAGE and fluorography to assess import efficiency (inset). Mitochondria re-isolated from the remaining aliquot were lysed in 0.2% Triton X-100 buffer and complex formation with mt-Hsp70 was analysed by co-immunoprecipitation as described in Figure 5A. The level of mb_2(260)−DHFR found associated with mt-Hsp70 was expressed as a percentage of the total present in the lysis supernatant fraction. Abbreviations: Pel, pellet; Sup, supernatant; PI, preimmune serum; Ssc1, antiserum raised against Ssc1p; DnaK, antiserum raised against *E.coli* DnaK protein; wt, wild-type mitochondria; *ssc1-2*, *ssc1-2* mitochondria.

the mitochondria were lysed in the presence of additional wild-type or *ssc1-2* mitochondria (Figure 5B, lanes 2 and 3). This result shows that the co-immunoprecipitation of mb_2(260)−DHFR reflects a complex formed *in situ* with mt-Hsp70 and not after lysis of the mitochondria.

In summary, it appears that this membrane-spanning m-cytochrome b_2 derivative is tightly associated with mt-Hsp70 in *ssc1-2* mitochondria following exposure at 37°C, but not at 25°C. The temperature dependence of the association of the membrane-bound mb_2(260)−DHFR with mt-Hsp70 was then analysed. Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mutant mitochondria initially at 25°C. Samples were then exposed to temperatures varying between 25 and 37°C, as indicated (Figure 5C). Efficient co-immunoprecipitation of mb_2(260)−DHFR was observed at temperatures between 30 and 37°C. These data demonstrate that the membrane-spanning intermediate upon exposure to temperatures of ≥30°C undergoes an alteration that renders it a substrate for mt-Hsp70. Thus, the loop exposed in the matrix may be extended by the increased movement of segments into the matrix or by association of the spanning loop with a matrix component. The loop may be disturbed at temperatures of ≥30°C. Furthermore, to exclude the possibility that the high temperature of 37°C would have some adverse effect on the structure of mitochondria, leading to an artifactual interaction of mb_2(260)−DHFR with mt-Hsp70, it was proved that mitochondria which had been exposed to temperatures ranging between 30 and 37°C were normal in terms of import of both a matrix- and an intermembrane space-targeted preprotein; thus the integrities of the inner membrane and intermembrane space were demonstrated (results not shown).

The following set of experiments was aimed at independently excluding the possibility of post-lysis binding of mb_2(260)−DHFR to mt-Hsp70. The first test was a competition experiment whereby lysis was performed in the presence of excess substrate for mt-Hsp70 (Figure 6A). The addition of reduced carboxymethylated lactalbumin (RCMLA), a permanently unfolded protein known to bind efficiently to Hsp70s, did not affect the extent of co-immunoprecipitation. Lysis in the presence of a molar excess of a fragment of cytochrome b_2, which had been denatured in 8 M urea prior to the addition, did not reduce the level of co-immunoprecipitation of mb_2(260)−DHFR (Figure 6B). In addition, precursor accumulated on the outer surface of mitochondria was not found in a complex with mt-Hsp70 (Figure 6C).

Notably, the efficiency of co-immunoprecipitation of mb_2(260)−DHFR was similar from both mitochondrial and mitoplast detergent extracts, despite the fact that ~60–70% of the imported species was found to be associated

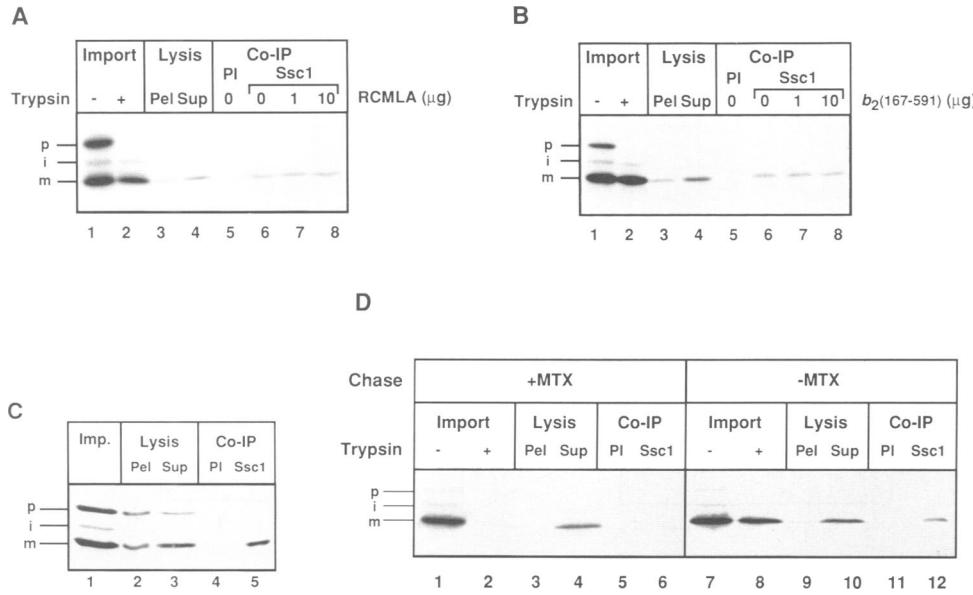

Fig. 6. Complex formation of mb_2(260)−DHFR with mt-Hsp70 occurs *in situ*. (**A**) Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria for 5 min at 25°C and then shifted to 37°C for 10 min. An aliquot was removed (lane 1) and the rest was trypsin-treated. The mitochondria were re-isolated by centrifugation. One aliquot was removed (lane 2) and mitochondria from the rest were resuspended and lysed in the presence of no further additions (lanes 5 and 6), 1 μg RCMLA (lane 7) or 10 μg RCMLA (lane 8). Samples were subjected to a clarifying spin and the resulting pellets were resuspended in SDS sample buffer (lane 3). The supernatants were divided further into three parts. One part was TCA-precipitated (lane 4) and the others were used for co-immunoprecipitation with either preimmune serum (lane 5) or serum raised against Ssc1p (lanes 6–8). Samples were processed further as described in the legend to Figure 5A. The lysis samples (pellet and supernatant) and preimmune lanes were identical for all concentrations of RCMLA. Therefore only those of the −RCMLA are presented. (**B**) Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria as described in the legend to Figure 5B. Samples were processed further in a similar manner as that described in (A), with the exception that the mitochondria were subsequently lysed in the presence of no further additions or 1 (lane 7) or 10 μg (lane 8) of a fragment of cytochrome b_2 (corresponding to amino acids 167–591 of the precursor) which had been over expressed in *E.coli*, purified and denatured in 8 M urea. (**C**) Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria as described in the legend to Figure 5B. The trypsin treatment was omitted and mitochondria were re-isolated directly. Samples were processed as described in the legend to Figure 5A, with the exception that the DnaK co-immunoprecipitation was omitted. (**D**) Radiolabelled pb_2(260)−DHFR was imported into *ssc1-2* mitochondria in the presence of MTX and NADPH for 15 min at 25°C. Mitochondria were re-isolated and resuspended in the presence of fresh P80 import buffer, in either the presence (lanes 1–6) or absence (lanes 7–12) of NADPH/MTX, and chased by incubating for a further 6 min at 37°C. Two aliquots from each sample were removed and were either mock- (lanes 1 and 7) or trypsin-treated (lanes 2 and 8). The mitochondria from the remaining parts of the sample were lysed in the presence of 0.2% Triton X-100 lysis buffer. They were treated further as described in the legend to Figure 5A, with the exception that the DnaK co-immunoprecipitation was omitted. For abbreviations see the legend to Figure 5.

with the mitoplast fraction (results not shown). We conclude that the soluble intermembrane space species is not binding to mt-Hsp70, and only the membrane-bound species is a substrate for the chaperone as it loops through the matrix.

We then compared the efficiency of co-immunoprecipitation of mb_2(260)−DHFR which had been arrested with MTX as a translocation intermediate in the *ssc1-2* mitochondria, with that which had been chased into the mitochondria in a subsequent step (Figure 6D). Only the mature-sized species chased into the mitochondria upon removal of MTX was found in a complex with mt-Hsp70; the MTX-arrested form was not present in such a complex (Figure 6D, lanes 6 and 12). It appears that in the case of the MTX-arrested preprotein, the cytochrome b_2 portion of the preprotein extends through both the outer and inner membrane import machinery. Thus the segment spanning the matrix is either of insufficient length for mt-Hsp70 binding or else not exposed to the chaperone. On the other hand, in the absence of the restraint of a tightly folded DHFR, a larger portion of the protein most probably becomes exposed to the matrix whereby it associates with mt-Hsp70. Post-lysis binding was excluded again by the following observations. If mitochondria containing imported mb_2(260)−DHFR were subsequently incubated with MTX and then lysed, co-immunoprecipitation with mt-Hsp70 was still observed despite the presence of a tightly folded DHFR (results not shown). Furthermore, imported authentic precytochrome b_2 was also found in a complex with mt-Hsp70, showing that the co-immunoprecipitation did not depend on the presence of an unfolded DHFR domain (results not shown).

Taking these observations together, the membrane-bound mature-sized cytochrome b_2 and mb_2(260)−DHFR reflect a stalled sorting intermediate which is exposed to the mitochondrial matrix. While looping through the matrix, spanning from the site of import to that of export, segments of the sorting intermediate can present binding sites for mt-Hsp70, especially under conditions where the length of the loop or its state of unfolding is increased at temperatures >25°C.

Membrane-spanning mature-sized cytochrome b_2 is in contact with components of the inner membrane import apparatus

Radiolabelled pb_2(260)−DHFR was imported into wild-type mitochondria. Following protease treatment, mitochondria were lysed with detergent. Complex formation with MIM44, a component of the inner membrane import machinery, was analysed by co-immunoprecipitation

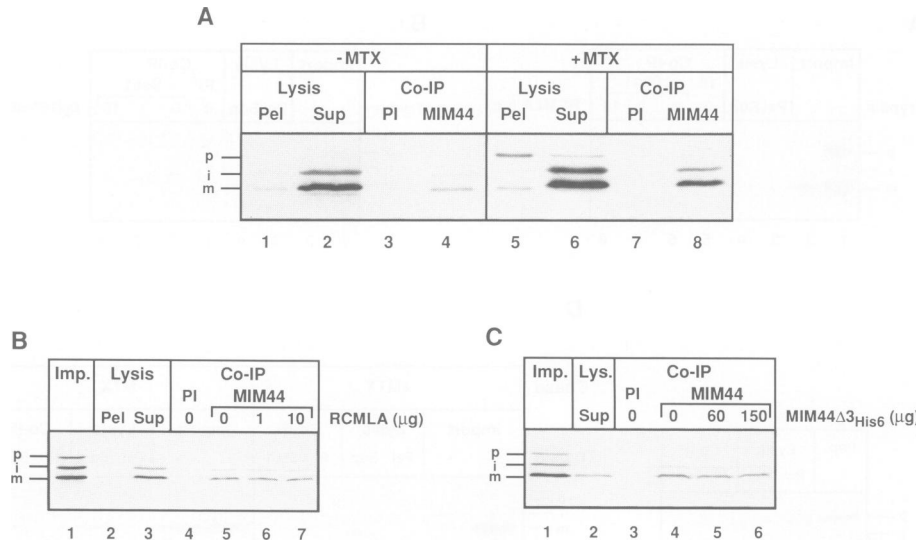

Fig. 7. Membrane-bound mb_2(260)−DHFR is in association with MIM44. (**A**) Radiolabelled pb_2(260)−DHFR was imported into wild-type mitochondria in either (i) the absence of MTX/NADPH and then trypsin-treated (−MTX, lanes 1–4) or (ii) the presence of MTX/NADPH and mock-treated (+MTX, lanes 5–8). Mitochondria were re-isolated and lysed in 0.2% Triton X-100 lysis buffer. Samples were subjected to a clarifying spin and the resulting pellets were resuspended in SDS sample buffer (lanes 1 and 5). The supernatants were divided further into three parts. One part was TCA-precipitated (lanes 2 and 6); the others were used for co-immunoprecipitation using either preimmune serum (lanes 3 and 7) or MIM44 serum (lanes 4 and 8). Samples were processed further as described in the legend to Figure 5A. (**B**) Radiolabelled pb_2(260)−DHFR was imported into wild-type mitochondria in the presence of MTX and NADPH. Mitochondria were re-isolated by centrifugation. One aliquot was removed (lane 1) and mitochondria from the rest were resuspended and lysed in the presence of no further additions (lanes 4 and 5) or 1 (lane 6) or 10 μg RCMLA (lane 7). Samples were subjected to a clarifying spin and the resulting pellets were resuspended in SDS sample buffer (lane 2). The supernatants were divided further into five parts. One part was TCA-precipitated (lane 3) and the others were used for co-immunoprecipitation using either preimmune serum (lane 4) or MIM44 serum (lanes 5–7). Samples were processed further as described in the legend to Figure 5A. The lysis samples (pellet and supernatant) and preimmune lanes were identical for all concentrations of RCMLA. Therefore only those of the −RCMLA are presented. (**C**) Radiolabelled pb_2(260)−DHFR was imported into wild-type mitochondria (30 μg) in the presence of MTX and NADPH. Following re-isolation, the mitochondria were incubated with apyrase (40 U/ml) and oligomycin (40 μM) for 5 min at 30°C in the presence of either no further additions (lanes 2 and 3) or with 60 (lane 5) or 150 μg (lane 6) of mitochondria harbouring the MIM44Δ3$_{His6}$. Mitochondria were lysed with 0.2% Triton X-100 lysis buffer (no EDTA present) and a clarifying spin was performed. The supernatants were subjected to Ni-NTA chromatography to selectively deplete the MIM44Δ3$_{His6}$ and any mb_2(260)−DHFR which could have bound in a post-lysis manner. The resulting eluate from each of the samples was divided into two aliquots. One part was TCA-precipitated (lane 2) and the others were used for co-immunoprecipitation with either preimmune serum (lane 3) or MIM44 serum (lanes 4–6). Samples were processed further as described in the legend to Figure 5A. The lysis sample (Sup, 10% of that used for the co-immunoprecipitation) and preimmune lanes were identical for all conditions. Therefore only those of the −MIM44Δ3$_{His6}$ mitochondria (lane 4) are presented. Abbreviations: Imp., import; Pel, pellet; Sup, supernatant; p, precursor; i, intermediate; m, mature; Co-IP, co-immunoprecipitation; PI, preimmune serum; MIM44, antiserum raised against MIM44 protein.

(Figure 7A). Both ib_2(260)−DHFR and mb_2(260)−DHFR were observed to be in a complex with MIM44. This suggests that both of these species were in association with the inner membrane import channel. When import was performed in the presence of MTX, thereby blocking complete import, the efficiency of co-immunoprecipitation of both b_2(260)−DHFR forms was elevated (>10% of total solubilized material). This result is consistent with sorting via the inner membrane translocation apparatus into and through the matrix, as such MTX−translocation intermediates should remain in the import channel when the completion of import is blocked. The efficiency of co-immunoprecipitation was significantly lower if Mg-ATP was present during the lysis (results not shown), suggesting that the complex may become dissociated by ATP hydrolysis, as has been shown previously for preprotein−MIM44 interactions (Schneider *et al.*, 1994).

The increase of co-immunoprecipitation observed with the MTX-arrested species would argue against that the binding observed results from a post-lysis complex formation. Furthermore, saturating amounts of RCMLA, a substrate for MIM44 (Schneider *et al.*, 1994), were unable to compete for b_2(260)−DHFR−MIM44 complex formation (Figure 7B).

In a further experiment to control for the specificity of interaction with MIM44, pb_2(260)−DHFR was imported into mitochondria in the presence of MTX. The mitochondria were lysed in the presence of added mitochondria which contained a modified form of MIM44 with six histidine (His) residues at the C-terminus (MIM44Δ3$_{His6}$; Figure 7C). This MIM44Δ3$_{His6}$ is functional as it complements a mim44 deletion strain (Schneider *et al.*, 1994). Following lysis, the detergent extract was passed over a Ni-NTA column to specifically deplete the extract of the MIM44Δ3$_{His6}$ (Schneider *et al.*, 1994). The level of b_2(260)−DHFR−MIM44 complex formation was then assessed in the eluate which contains only the wild-type form of the MIM44. The efficiencies of recovery of the ib_2(260)−DHFR− and mb_2(260)−DHFR−MIM44 complexes were similar irrespective of whether the additional mitochondria containing the MIM44Δ3$_{His6}$ were present (Figure 7C, lanes 5 and 6) during the detergent lysis or not (Figure 7C, lane 4). If pb_2(260)−DHFR was imported in the presence of MTX into mitochondria containing this modified form of MIM44 (MIM44Δ3$_{His6}$), a similar level of co-immunoprecipitation with MIM44 was observed as in wild-type mitochondria (results not shown), thus excluding the trivial possibility that this form

is incompetent for binding to the intermediate- and mature-sized forms of b_2(260)−DHFR. Furthermore, if following import into wild-type mitochondria lysis was performed in the presence of chemical amounts of purified MIM44Δ3$_{His6}$ and subjected to Ni-NTA chromatography, no decrease in the level of co-immunoprecipitation from the eluate was observed (results not shown).

In summary, these results show that Imp1p-processed cytochrome b_2 species on their way into the intermembrane space are still located in the import channel of the inner membrane. Together with the mt-Hsp70 co-immunoprecipitation data, these results provide compelling evidence for the looping of these intermediates through the matrix from the site of import in the inner membrane presumably to the site of export.

Discussion

Defining an intracellular sorting process as complex as that of cytochrome b_2 is limited essentially by two kinds of experimental problems. First, it is not always easy to unequivocally determine the precise submitochondrial location of an intermediate, in particular because these intermediates are large molecules of undefined conformation. Second, a certain species may occur in a successive manner in different compartments. To discriminate between the suggested sorting pathways for cytochrome b_2, we decided to characterize an intermediate which would not be prone to these two methodological problems. We have described previously mature-sized species of cytochrome b_2-derived DHFR fusion proteins which accumulate as translocation intermediates in the presence of MTX (Koll *et al.*, 1992). The N-terminus of such MTX-arrested species was extended into the intermembrane space as it had undergone processing to its mature form by the Imp1p protease. The C-terminal DHFR remained outside the mitochondria due to its tightly folded state and was thus accessible to proteases.

According to the stop-transfer model, the MTX-arrested mature-sized cytochrome b_2 species should be a dead-end intermediate (Glick *et al.*, 1992a, 1993) and most importantly should be neither in contact with the inner membrane nor exposed to the matrix. Rather, the stop-transfer model proposes that the intermediate-sized species whilst anchored to the inner membrane traverses the outer membrane, driven by the dissociation of the two import channels in the outer and inner membranes. Imp1p processing prior to complete translocation should thus prevent subsequent import of the rest of the precursor, as the polypeptide would no longer be tied to the inner membrane and therefore could not be able to avail of the 'pulling effect' caused by the separation of the membranes (Glick *et al.*, 1992a, 1993). However, the translocation of the MTX-arrested intermediate observed here is consistent with the conservative sorting model. In support of this observation, import of authentic precytochrome b_2 when performed at low temperatures results in an efficient Imp1p processing event prior to the completion of translocation across the outer membrane (H.Ono, A.Gruhler, B.Guiard, R.A.Stuart, E.M.Schwartz and W.Neupert, manuscript in preparation).

Following the completion of import into mitochondria, a proportion of the mature-sized cytochrome b_2 and b_2−DHFR derivatives were found as membrane-bound species. Such an observation is inconsistent with the stop-transfer model of protein sorting, where Imp1p processing relieves the anchorage to the inner membrane (Figure 1A, stage 2→3). On the other hand, the finding is consistent with a sorting mechanism through the matrix, where retardation of the translocation through the import site of the inner membrane and/or the export site would result in the prolonged membrane interaction of an Imp1p-processed species (Figure 1B, stage 3a). The membrane-bound sorting intermediates remain in contact with the inner membrane import channel, as verified by complex formation with MIM44.

Therefore it was essential to unambiguously determine the topology of this intermediate. Most importantly, segments of the intermediate were found to be exposed to the mitochondrial matrix as they interacted with mt-Hsp70 in the matrix. Mature-sized cytochrome b_2, according to the stop-transfer pathway, would never be found in or exposed to the matrix space. In contrast, upon sorting through the matrix this species would be exposed to the matrix space in a transient fashion. By definition, a mature-sized species can never be completely in the matrix because processing of the sorting sequence takes place at the outer surface of the inner membrane (Schneider *et al.*, 1991). Thus, the localization of mature-sized cytochrome b_2 with segments exposed to the matrix can be expected to provide a reliable criterion for the pathway through the matrix.

Interestingly, our data show that the bound mt-Hsp70 is not one that is actively promoting import into the matrix at the inner face of the inner membrane (i.e. outlet of import channel), but one that is bound to unfolded segments that are present in the matrix. This is entirely consistent with recent reports that the import of cytochrome b_2 (after mt-Hsp70-dependent unfolding of the N-terminal heme binding domain) is not dependent on mt-Hsp70 (Glick *et al.*, 1993; Voos *et al.*, 1993; Stuart *et al.*, 1994a,b). As proposed previously, the driving force for import of cytochrome b_2 into the membrane space does not rely (at least not obligatorily) on ATP hydrolysis driving the mt-Hsp70−MIM44 cycle. Rather, mt-Hsp70 binds to unfolded segments of the cytochrome b_2 intermediates (Stuart *et al.*, 1994b).

Altogether our findings strongly favour the conservative sorting process, and support a pathway by which translocation from the matrix across the inner membrane can occur concomitantly with the import process (Koll *et al.*, 1992). The export process can be initiated prior to the completion of import, causing a looping of segments of the precursor through the matrix. This appears to be the preferred mode of sorting of cytochromes b_2 and c_1, but not of the Rieske FeS protein. On the other hand, a kinetic analysis has provided clear evidence for a passage of intermediate-sized species through the matrix (H.Ono, A.Gruhler, B.Guiard, R.A.Stuart, E.M.Schwartz and W.Neupert, manuscript in preparation). Thus, a certain fraction of ib_2 may be almost completely in the matrix in a transient manner. The amount of this fraction would be increased when the export step is retarded over the import one. This may occur either at low temperatures or when the sorting signal of the precursor has been mutated so that it no longer operates efficiently. Furthermore, these species

probably represent those which were observed in association with hsp60. It seems possible that *in vitro* part of this species in the matrix does not efficiently leave the matrix space, as the export reaction appears to be labile (H.Ono, A.Gruhler, B.Guiard, R.A.Stuart, E.M.Schwartz and W.Neupert, manuscript in preparation).

Immediately following matrix exposure, the sorting signal sequence could interact with a putative mitochondrial signal binding protein (mt-SBP) and/or, depending on charges flanking the hydrophobic core of the sorting signal, insert into the inner membrane (Schwarz *et al.*, 1993). This would serve to channel the incoming protein along the export pathway back across the inner membrane. If, however, the sorting signal fails to rapidly initiate export, e.g. due to mutations in the sorting signal, the protein becomes imported along the default pathway into the matrix in a mt-Hsp70-dependent manner. Intermediates which accumulate in the matrix are subsequently inefficiently exported (H.Ono, A.Gruhler, B.Guiard, R.A.Stuart, E.M.Schwartz and W.Neupert, manuscript in preparation). Further compelling evidence for the movement of the mature part of cytochrome b_2 through the inner membrane import apparatus comes from the observation of its interaction with components of this apparatus, in particular with MIM44, a protein peripherally associated with the inner face of the inner membrane.

This sorting process is quite similar to that described for protein translocation in bacteria and in the endoplasmic reticulum (ER), where polypeptide translation is largely but not always obligatorily coupled to protein translocation. Upon emergence from the ribosome, interaction with the signal recognition particle (SRP) is thought to prevent further translation until the partially synthesized polypeptide has reached the ER translocation machinery, which leads to a resumption of translation. By doing so, the polypeptide is assured of correct targeting and is also prevented from adopting a folded conformation incompatible with subsequent membrane translocation. We envisage a similar reaction in the sorting of intermembrane space proteins such as cytochromes b_2 and c_1. The possibility of intermediates accumulating in the matrix where they would fold/misfold and thus be rendered translocation-incompetent is thereby minimized.

Materials and methods

Isolation of mitochondria

Saccharomyces cerevisiae wild-type (D273-10B) cells were grown on lactate medium (Daum *et al.*, 1982) at 30°C and harvested at an OD_{578} of ~1. Mitochondria were isolated as described previously (Daum *et al.*, 1982) and were resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA and 10 mM MOPS/KOH, pH 7.2) at a protein concentration of 10 mg/ml. The mt-Hsp70 mutant *ssc1-2* (PK81) and its corresponding wild-type strain (PK82; Gambill *et al.*, 1993) were grown in the same medium but at 24°C.

Import of precursor proteins into mitochondria

The cytochrome b_2 precursor protein and DHFR derivatives thereof have been described previously (Guiard, 1985; Rassow *et al.*, 1990; Koll *et al.*, 1992; Stuart *et al.*, 1994b). Precursor proteins were synthesized in rabbit reticulocyte lysate (Promega Corporation, Madison, WI) in the presence of [^{35}S]methionine as described previously (Pelham and Jackson, 1976). Unless otherwise indicated, import was performed as follows: mitochondria (60–100 μg) were incubated in a mixture (final volume 200 μl) of P80 buffer [3% BSA (w/v), 80 mM KCl, 250 mM sucrose, 10 mM MOPS–KOH, 5 mM $MgCl_2$, pH 7.2], supplemented with 3 μM hemin, 2 mM NADH, 2 mM dithiothreitol (DTT) and 3–5% reticulocyte lysate which contained the radiolabelled precursor protein. Samples were incubated at 25°C for the time periods indicated and were then placed on ice. Samples were divided and one half was treated with trypsin (50 μg/ml) for 15 min and then soybean trypsin inhibitor was added (1 mg/ml). Mitochondria were re-isolated by centrifugation and either lysed immediately in electrophoresis sample buffer or when swelling was performed they were resuspended in SEMK buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, 80 mM KCl, pH 7.2).

Osmotic swelling of mitochondria

For localization experiments following import, mitochondria were subjected to a swelling procedure by exposure to a hypotonic buffer. Following the trypsin treatment, re-isolated mitochondria (40 μg) were initially resuspended in 100 μl SEMK buffer. For non-swelling (control) conditions the suspension was diluted 10-fold with SEMK. To generate mitoplasts, the suspension was diluted to the same extent in 10 mM MOPS, pH 7.2. Samples were left on ice for 30 min and then mitochondria or mitoplasts were recovered by centrifugation and lysed directly in an electrophoresis sample buffer. Samples were analysed by SDS–PAGE and immunoblotting to nitrocellulose was performed. The extent of swelling achieved upon exposure to the hypotonic buffer was assessed following immunodecoration of the blot with antisera against endogenous cytochrome b_2, a soluble intermembrane space marker.

MTX arrest of cytochrome b_2–DHFR fusion proteins and chase reaction

The import of the precytochrome b_2–DHFR fusion proteins was arrested with MTX/NADPH in the following manner. Reticulocyte lysate (3%) containing the radiolabelled precursor protein was initially incubated in P80 buffer supplemented with 1 mM NADPH, 0.25 μM MTX, 2 mM DTT and 3 μM hemin, for 10 min on ice. Isolated mitochondria were then added (0.3 mg/ml) together with 2 mM NADH. Samples were incubated for 15 min at 25°C. The MTX was omitted in the control samples. The extent of the MTX/NADPH block of import was assessed by treating part of the sample following the import with trypsin and by comparing the protected signal obtained with that of a trypsin-treated control sample.

The MTX-arrested translocation intermediates were chased into mitochondria in a second reaction as follows. Following the initial import reaction in the presence of MTX and NADPH, samples were not subjected to a trypsin treatment but rather the mitochondria were re-isolated directly by centrifugation for 4 min at 9200 *g* in a Sigma rotor 12 154 at 2°C. The mitochondria were washed once in P80 buffer and then resuspended in fresh P80 buffer containing 2 mM DTT at a final concentration of 0.3 mg/ml. In addition, samples that were chased in the presence of MTX received 1 mM NADPH and 0.25 μM MTX during both wash and chase treatments. Samples were incubated further at 25°C for 40 min, unless otherwise indicated, and the import of the arrested species was monitored by trypsin treatment.

Co-immunoprecipitation experiments

Following the import reaction and trypsin treatment, mitochondria were re-isolated by centrifugation and lysed for 15 min at 4°C in 200 μl of 0.2% Triton X-100 lysis buffer [0.2% Triton X-100 (w/v), 150 mM NaCl, 10 mM MOPS–KOH, 5 mM EDTA, 1 mM PMSF, pH 7.2]. In the case of wild-type mitochondria, samples were incubated for 5 min at 25°C in the presence of 20 μM oligomycin and 40 U/ml apyrase prior to lysis. This oligomycin/apyrase treatment was omitted in the case of MIM44 complex analysis and respective controls, unless otherwise indicated. After a clarifying spin for 15 min at 36 000 *g* in a Sigma rotor 12 154 at 2°C, the supernatant was divided and one aliquot was TCA-precipitated; the rest was added to 2 mg protein A–Sepharose (Pharmacia) to which the immunoglobulin fraction from 30 μl of either preimmune or specific antiserum raised against Ssc1p, MIM44p or the *E.coli* DnaK had been bound, as indicated. The suspension was gently shaken for 1 h at 4°C. The Sepharose beads were collected by centrifugation in an Eppendorf centrifuge, washed twice with 0.2% Triton X-100 lysis buffer and once with 10 mM MOPS–KOH, pH 7.2. The sample was finally resuspended in an electrophoresis sample buffer and subjected to SDS–PAGE and fluorography.

Miscellaneous

The following procedures were performed according to published methods: protein determination (Bradford, 1976), SDS–PAGE (Laemmli, 1970) and fluorography (Hartl *et al.*, 1986).

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References

Beasley,E.M. (1993) In Brown,A.J.P., Tuite,M.F. and McCarthy,J.E.G. (eds), NATO ASI Series, Vol. 71, pp. 223–233.
Beasley,E.M., Müller,S. and Schatz,G. (1993) *EMBO J.*, **12**, 2303–2311.
Blobel,G. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 1496–1500.
Blom,J., Kübrich,M., Rassow,J., Voos,W., Dekker,P.J.T., Maarse,A.C., Meijer,M. and Pfanner,N. (1993) *Mol. Cell. Biol.*, **13**, 7364–7371.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Daum,G., Böhni,P.C. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028–13033.
Gambill,B.D., Voos,W., Kang,P.J., Miao,B., Langer,T., Craig,E.A. and Pfanner,N. (1993) *J. Cell Biol.*, **123**, 109–118.
Glick,B.S., Beasley,E.M. and Schatz,G. (1992a) *Trends Biochem. Sci.*, **17**, 453–459.
Glick,B.S., Brandt,A., Cunningham,K., Müller,S. and Schatz,G. (1992b) *Cell*, **69**, 809–822.
Glick,B.S., Wachter,C., Reid,G.A. and Schatz,G. (1993) *Protein Sci.*, **2**, 1901–1917.
Guiard,B. (1985) *EMBO J.*, **4**, 3265–3272.
Hartl,F.-U. and Neupert,W. (1990) *Science*, **247**, 930–938.
Hartl,F.-U., Schmidt,B., Wachter,E., Weiss,H. and Neupert,W. (1986) *Cell*, **47**, 939–951.
Hartl,F.-U., Ostermann,J., Guiard,B. and Neupert,W. (1987) *Cell*, **51**, 1027–1037.
Herrmann,J., Stuart,R.A., Craig,E.A. and Neupert,W. (1994) *J. Cell Biol.*, **127**, 893–902.
Horst,M., Jenö,P., Kronidou,N.G., Bolliger,L., Oppliger,W., Scherer,P., Manning-Krieg,U., Jascur,T. and Schatz,G. (1993) *EMBO J.*, **12**, 3035–3041.
Jensen,R.E., Schmidt,S. and Mark,R.J. (1992) *Mol. Cell. Biol.*, **12**, 4677–4686.
Kaput,J., Goltz,S. and Blobel,G. (1982) *J. Biol. Chem.*, **257**, 15054–15058.
Koll,H., Guiard,B., Rassow,J., Ostermann,J., Horwich,A.L., Neupert,W. and Hartl.,F.-U. (1992) *Cell*, **68**, 1163–1175.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lill,R., Hergersberg,C., Schneider,H., Söllner,T., Stuart,R.A. and Neupert,W. (1992a) In Neupert,W. and Lill,R. (eds), *Membrane Biogenesis and Protein Targeting*. Elsevier Science Publishers, Amsterdam, The Netherlands.
Lill,R., Stuart,R.A., Drygas,M., Nargang,F.E. and Neupert,W. (1992b) *EMBO J.*, **11**, 449–456.
Maarse,A.C., Blom,J., Grivell,L.A. and Meijer,M. (1992) *EMBO J.*, **11**, 3619–3628.
Nunnari,J., Fox,T.D. and Walter,P. (1993) *Science*, **262**,1997–2003.
Pelham,H.R.B. and Jackson,R.J. (1976) *Eur. J. Biochem.*, **64**, 247–256.
Rassow,J., Hartl,F.-U., Guiard,B., Pfanner,N. and Neupert,W. (1990) *FEBS Lett.*, **275**, 190–194.
Schneider,A., Behrens,M., Scherer,P.E., Pratje,E., Michaelis,G. and Schatz,G. (1991) *EMBO J.*, **10**, 247–254.
Schneider,H.-C., Berthold,J., Bauer,M.F., Dietmeier,K., Guiard,B., Brunner,M. and Neupert,W. (1994) *Nature*, **371**, 768–774.
Schwarz,E., Seytter,T., Guiard,B. and Neupert,W. (1993) *EMBO J.*, **10**, 2295–2302.
Stuart,R.A. and Neupert,W. (1990) *Biochimie*, **72**, 115–121.
Stuart,R.A., Cyr,D.M., Craig,E.A. and Neupert,W. (1994a) *Trends Biochem. Sci.*, **19**, 87–92.
Stuart,R.A., Gruhler,A., van der Klei,I.J., Guiard,B., Koll,H. and Neupert,W. (1994b) *Eur. J. Biochem.*, **220**, 9–18.
Stuart,R.A., Fölsch,H., Gruhler,A. and Neupert,W. (1994c) In Hartl,F.-U. (ed.), *Advances in Molecular and Cellular Biology*. Academic Press, New York, Vol. 43, in press.
van Loon,A.P.G.M. and Schatz,G. (1987) *EMBO J.*, **6**, 2441–2448.
van Loon,A.P.G.M., Brändli,A.W. and Schatz,G. (1986) *Cell*, **44**, 801–812.
van Loon,A.P.G.M., Brändli,A.W., Pesold-Hurt,B., Blank,D. and Schatz,G. (1987) *EMBO J.*, **8**, 2433–2439.
Voos,W., Gambill,B.D., Guiard,B., Pfanner,N. and Craig,E.A. (1993) *J. Cell Biol.*, **123**, 119–126.

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