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The Sorting Route of Cytochrome \( b_2 \) Branches from the General Mitochondrial Import Pathway at the Preprotein Translocase of the Inner Membrane*

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Cytochrome \( b_2 \) is synthesized in the cytosol with a bipartite presequence. The first part of the presequence targets the protein to mitochondria and mediates translocation into the mitochondrial matrix compartment; the second part contains the sorting signal that is required for delivery of the protein to the intermembrane space. The localization of the structures that recognize the sorting signal is unclear. Here we show that upon import in vivo, the sorting signal of cytochrome \( b_2 \) causes an early divergence from the general matrix import pathway and thereby prevents translocation of a folded C-terminal domain into mitochondria. By co-immunoprecipitations we find that translocation intermediates of cytochrome \( b_2 \) are associated with Tim23, a component of the inner membrane protein import machinery. Cytochrome \( b_2 \) constructs with an alteration in the sorting signal are mistargeted to the matrix of wild-type mitochondria. In mitochondria containing a mutant form of Tim23, however, the translocation of the altered sorting signal across the inner membrane is inhibited, and cytochrome \( b_2 \) is correctly sorted to the intermembrane space. We suggest that the sorting signal of cytochrome \( b_2 \) is recognized within the inner membrane in close vicinity to Tim23.

Nearly all mitochondrial proteins are synthesized by cytosolic ribosomes and posttranslationally imported into the organelle (1–3). Translocation across the outer membrane requires transport through a general import pore that is formed by a complex of Tom proteins. Translocation across the inner membrane involves a common step for all preproteins carrying a presequence and is mediated by a complex of the translocase proteins Tim17 and Tim23 (Refs. 4–6; for the uniform nomenclature see Ref. 7). The initial entry of preproteins into the inner membrane requires the membrane potential (\( \Delta \phi \)). The matrix heat shock protein 70 (mtHsp70) then binds to segments of the preproteins emerging on the matrix side and drives their further translocation in an ATP-dependent reaction. In addition, mtHsp70 promotes the unfolding of preprotein domains during entry into the mitochondrial membranes. This is thought to occur by generation of an inward-directed force by preprotein-bound mtHsp70 (pulling). MtHsp70 transiently binds to the inner membrane, to the peripheral membrane protein Tim44 (8–10), and also directly to the import channel (Tim17-Tim23) (11); therefore, conformational changes of mtHsp70 are directional and can exert a force on the preprotein in transit.

Cytochrome \( b_2 \) of yeast mitochondria has for many years served as a paradigm in elucidating the more complex import pathways of proteins that have a final location in the intermembrane space. In this compartment, mature cytochrome \( b_2 \) \((\alpha+\gamma\text{-}lactate\text{ cytochrome } c\text{ oxidoreductase})\) is a soluble tetrameric protein (12–14). The preprotein contains a bipartite presequence. The first part of the presequence (residues 1–31) mediates translocation across both mitochondrial membranes and is cleaved in the matrix. The second part (residues 32–80) contains the information that is required for sorting of the protein to the intermembrane space. The inner membrane peptidase I, which carries the catalytic site on the intermembrane space side (15), removes the second part of the presequence, yielding the mature protein.

The sorting signal of cytochrome \( b_2 \) resembles the targeting signals of secretory bacterial proteins, suggesting a conservation of targeting mechanisms in mitochondria in line with the endo-symbiotic theory of organelle evolution (16). The sequencing project of the yeast genome, however, has recently demonstrated that a possible conservation of the prokaryotic targeting system does not include a conservation of the Sec machinery of the prokaryotic translocase; homologs of sec genes have not been found in the yeast genome (17). This does not exclude a conservation of mechanistic principles. In a series of studies it has been shown that the sorting signal of cytochrome \( b_2 \) seems to be imported into the matrix in tandem with the first part of the presequence (18–23). These experiments suggest that the sorting signal is subsequently recognized by a separate system besides the import machinery and re-exported to the intermembrane space. Other data have suggested that the sorting signal serves as a stop signal in the import machinery.

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§ The abbreviations used are: Tim, component of the preprotein translocase of the mitochondrial inner membrane of molecular weight \( x \); Tomx, component of the preprotein translocase of the mitochondrial outer membrane of molecular weight \( x \); Tricine, \( N\)-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; DHFR, dihydrofolate reductase; FeS protein, Rieske iron/sulfur protein of respiratory chain complex III; HB, heme-binding domain of cytochrome \( b_2 \); MOPS, 1-morpholinopropanesulfonic acid; mtHsp70, mitochondrial heat shock protein of 70 kDa; pb2-DHFR, precursor of cytochrome \( b_2 \)-DHFR fusion protein; PAGE, polyacrylamide gel electrophoresis; SP, spheroplasts; PCR, polymerase chain reaction.
ery and does not proceed into the matrix (24–28). In this concept, the sorting signal leaves the import machinery in the inner membrane laterally, without involvement of a separate reexport system.

In a previous study we used different constructs of cytochrome \( b_2 \) as a molecular ruler to localize the functional sorting signal within the mitochondrion (29). By studying \textit{in vitro} import into isolated mitochondria, we found that the sorting signal functions already during insertion into the inner membrane. mtHsp70 could exert an unfolding (pulling) activity only on folded domains that were located close to the cytochrome \( b_2 \) presequence. A folded domain located at the C terminus of a cytochrome \( b_2 \) fusion protein was not unfolded and therefore not translocated across the outer membrane, suggesting that the sorting signal directed an early divergence of the preprotein from the mtHsp70-driven matrix import pathway. We now asked whether these \textit{in vitro} findings were of relevance for protein sorting \textit{in vivo}. We indeed observed that the sorting signal of cytochrome \( b_2 \) strongly restricts the unfolding activity of the mitochondrial import machinery in intact cells. It prevents import of a folded C-terminal domain and finally leads to retrograde translocation and accumulation of the mature sized protein in the cytosol. To further localize the submitochondrial site of sorting of cytochrome \( b_2 \), we characterized a mutant form of the channel protein Tim23 and found that the \textit{tim23-2} mutation suppressed the mis-sorting of a cytochrome \( b_2 \) construct containing an altered sorting signal. Our results indicate a branching site of import pathways in the inner membrane Tim machinery, separating the import pathway of cytochrome \( b_2 \) from the general transport channel of matrix targeted proteins. We suggest that the sorting signal of cytochrome \( b_2 \) is recognized in close vicinity to Tim23.

Materials and Methods

\textit{Saccharomyces cerevisiae} Strains and Construction of Plasmids—For expression in yeast, the DNA constructs were cut from pGEM4 plasmids (29, 30) and transferred to the yeast expression plasmid pYE DP 1/8–10 (31). The pGEM4 plasmids were cut by HindIII at the 3′-end of the inserts and filled by Klenow fragment, and a BglII linker was inserted. The inserts were subsequently released from the plasmids by cutting with EcoRI and BglII and inserted between the EcoRI and BamHI sites of the vector pYE DP 1/8–10. The plasmids were propagated in the yeast strain 334 (32). This strain has no glucose repression of GAL expression and cannot utilize galactose.

Cytochrome \( b_2 \) constructs were amplified by oligonucleotide-directed mutagenesis. Two subsequent PCRs were performed on pb2(1–84)-DHFR in pGEM4 (30). In the first reaction, a 5′-primer corresponding to the SP6 promoter was combined with the 3′-mutagenesis primer. The resulting PCR product was used as 5′-primer in a second PCR with a 3′-primer corresponding to the TT primer. An EcoRI-XmaI fragment of the PCR product was inserted between the EcoRI and XmaI sites of the corresponding cytochrome \( b_2 \) constructs to introduce the mutations. The presences of the resulting constructs were sequenced. All DNA manipulations were carried out according to standard procedures (33–34).

Expression and Localization of Cytochrome \( b_2 \) Constructs in \textit{S. cerevisiae}—Yeast cells were grown at 30 °C in selective synthetic complete medium containing 3% glycerol to an \( A_{600} \) of 0.8–1.2. Galactose was added to a final concentration of 2% to induce the expression of the cytochrome \( b_2 \) fusion proteins. After 4 h of induction, −20 mg of cells were collected by centrifugation and converted to spheroplasts by lysozyme treatment as described previously (12). The suspension was divided into 5 aliquots, and the spheroplasts were resolated by centrifugation (1 min, 16,000 \( \times g \)). Two aliquots were resuspended in 350 \( \mu l \) of cold permeabilization buffer (0.1 M potassium acetate, 0.2 M sorbitol, 2 mM MgCl\(_2\), supplemented with 0.02 volumes of 1 M HEPES-KOH, pH 7.2) to open the plasma membrane (“perm. SP”) (35). The permeabilized spheroplasts were resolated by centrifugation (10 min, 16,000 \( \times g \)). Pellet and supernatant were separated and the proteins released to the supernatant were precipitated by 10% trichloroacetic acid in the presence of 0.05% sodium deoxycholate. One aliquot of the suspensions of spheroplasts was resuspended in 350 \( \mu l \) of EM buffer (1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) to open the plasma membrane and the outer mitochondrial membrane (“swelling”). The spheroplasts of an additional aliquot were resuspended in EM buffer containing 1% Triton X-100 to lyse all organelles. The latter two samples and one of the samples with permeabilized spheroplasts were treated with proteinase K (50 \( \mu g \)/ml) to digest the cytochrome \( b_2 \) proteins not protected by membranes. The fifth aliquot of spheroplasts was taken as a control and analyzed directly (“total SP”). Samples were resuspended in 100 \( \mu l \) of sample buffer, and portions of 40 \( \mu l \) were analyzed by SDS-PAGE and immunoblotting with antibodies directed against DHFR. Antibodies directed against the mitochondrial outer membrane protein Tom20 were used to control the fractionation procedure.

To compare the growth rate of different yeast strains (as shown in Fig. 2), the cells were first grown in liquid selective minimal medium at 28 °C containing 2% glucose. The cultures were diluted with Ringer’s solution and spotted on selective minimal medium plates containing 2% glucose or 2% glucose and 2% galactose. After 5 days at 28 °C, cells were scraped from the plates and again diluted with Ringer’s solution and spotted onto plates. Plates were incubated for 5 days at 28 °C.

Import of Proteins into Isolated Mitochondria—Yeast cells were grown in YPG medium (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol), and mitochondria were prepared according to published procedures (12). We used the strains PK82 (36), MB3 (WT, Ref. 37), and MB3–46 (tim23-2; Ref. 38). Radiolabeled preproteins were synthesized in rabbit reticulocyte lysate in the presence of [\(^{14}C\)]cytochrome \( b_2 \) (32). The resulting protein was incubated with radiolabeled preprotein (3–10% (v/v)) in import buffer (3% bovine serum albumin (w/v), 250 mM sucrose, 60 mM KCl, 5 mM MgCl\(_2\), 5 mM sodium malate, 2 mM ATP, 20 mM potassium phosphate, 10 mM MOPS-KOH, pH 7.2) at 37 °C. Reactions were stopped by addition of 1 μl valinomycin and cooling on ice. Samples with a dissipated mitochondrial potential received valinomycin prior to incubation with preprotein. For generation of mitoplasts, import reactions were diluted with 9 volumes of EM buffer and left on ice for 30 min. Control mitochondria were diluted with isonicotic SEM buffer (EM buffer supplemented with 250 mM sucrose). Proteinase K treatment, resolation of mitochondria, and separation by SDS-PAGE or Tricine-SDS-PAGE have been described previously (39, 40). Autoradiographs were obtained and quantified using a storage PhosphorImaging system (Molecular Dynamics).

Co-immunoprecipitations—Specific antibodies were pre-bound to protein A-Sepharose (10 μl wet volume, Pharmacia Biotech Inc.) for 1 h in 480 μl of lysis buffer (1% digitonin (Merck, 1 × recrystallized from ethanol), 10% glycerol (w/v), 50 mM NaCl, 2 mM EDTA, 30 mM HEPES-KOH, pH 7.4). Import reactions were performed for 20 min with 25 μg of mitochondrial protein per lane and 20% (v/v) reticulocyte lysate. After resololation and washing with SEM buffer, mitochondria were resuspended in lysis buffer supplemented with protease inhibitors (2 μg/ml aprotinin, 5 μg/ml aprotinin, 0.25 μg/ml chymostatin, 1.25 μg/ml leupeptin, 2.5 μg/ml pepstatin A, 0.5 μg/ml pepstatin A) and shaken end-over-end for 10 min at 8 °C. Unsolubilized material was removed by ultracentrifugation (30 min at 100,000 \( \times g \)). The supernatants were incubated for 1 h at 8 °C by end-over-end shaking with antibodies prebound to protein A-Sepharose. After three washing cycles with lysis buffer, the protein A-Sepharose pellets were heated in sample buffer and applied to SDS-PAGE.

Results

Localization of Different Cytochrome \( b_2 \) Constructs after Import in Vivo—Gärnert et al. (29) used three building blocks to monitor the sorting and unfolding pathway of cytochrome \( b_2 \) in isolated mitochondria (Fig. 1A) as follows: the bipartite presequence; the ~100-residue non-covalent heme binding domain (HB) of cytochrome \( b_2 \) that forms a tightly folded domain and strictly depends on the function of mtHsp70 for unfolding and translocation across the mitochondrial membranes (26, 41); since the mitochondrial matrix enzyme dihydrofolate reductase (DHFR) as passenger protein, the unfolding of DHFR requires only a low energy input and thus does not depend on functional mtHsp70 (26, 41). In the \textit{in vitro} experiments, a heme binding domain located at the C terminus (pb2-DHFR-HB) was not imported but got stuck in the mitochondrial import sites. When the intramitochondrial sorting signal was inactivated by deletion
of a 19-residue segment, the resulting preprotein pb\textsubscript{2-\Delta}-DHFR-HB was completely imported (29).

We now expressed the hybrid proteins (Fig. 1A) from a galactose-inducible vector in intact \textit{S. cerevisiae} cells. To avoid indirect effects of the induction by galactose, a yeast strain with \textit{gal1} mutation was employed that cannot utilize galactose as carbon source and lacks a glucose repression of \textit{GAL} expression (32). After a 4-h induction, cells were isolated and converted to permeabilized spheroplasts (Fig. 1B). We first analyzed two hybrid proteins with a complete presequence and DHFR at the C terminus; the heme binding domain was either directly adjacent to the presequence (pb\textsubscript{2-\Delta}-DHFR-HB) or not included (pb\textsubscript{2}-DHFR). Both proteins were found in the organelar pellet of permeabilized spheroplasts (Fig. 1B, lanes 2 and 8) and protected against proteasome K (Fig. 1B, lanes 6 and 12) unless the membranes were disrupted with detergent (Fig. 1B, lanes 5 and 11). In a hypotonic buffer (to swell mitochondria and open the intermembrane space), both proteins became accessible to added protease (Fig. 1B, lanes 4 and 10), pb\textsubscript{2}-DHFR-HB was efficiently processed to the mature sized form (Fig. 1B, lane 1), whereas pb\textsubscript{2}-DHFR was observed in both the intermediate sized form and the mature sized form (Fig. 1B, lane 7). A slower processing of pb\textsubscript{2}-DHFR has also observed in \textit{in vivo} import experiments (29) and may be attributed to a lack of most sequences of mature cytochrome \textit{b}_{2}. No precursor sized forms of the hybrid proteins were observed. These results indicate an efficient import of the proteins into mitochondria and a correct sorting to the intermembrane space in \textit{in vivo}.

We then studied the localization of the construct pb\textsubscript{2-\Delta}-DHFR-HB (Fig. 1B, lanes 13–18). This protein was efficiently processed to the mature sized form; only minute amounts of precursor or intermediate sized forms were observed (Fig. 1B, lane 13). However, the processed protein was mainly found in the cytosolic fraction (Fig. 1B, lane 15) and not protected against proteinase K in permeabilized spheroplasts (Fig. 1B, lane 18). A placement of the heme binding domain at the C terminus of a hybrid protein thus reveals a striking effect on its cellular sorting and prevents a complete import into mitochondria. We will address below (see Fig. 3) the mechanism how a processed protein can be found in the cytosolic fraction.

When the cytochrome \textit{b}_{2} sorting signal was inactivated, the resulting hybrid protein pb\textsubscript{2-\Delta}-DHFR-HB was found in the organelar fraction (Fig. 1B, lanes 20 and 24). It remained protease-protected also in swollen mitochondria (Fig. 1B, lane 22), demonstrating that it was transported across the inner membrane.

These results indicate that pb\textsubscript{2}-DHFR, pb\textsubscript{2}-DHFR, and pb\textsubscript{2-\Delta}-DHFR-HB were correctly sorted into mitochondria in \textit{in vivo} comparable to the situation \textit{in vitro}. Mis-sorting of the hybrid protein depends on both the presence of the heme binding domain at the C terminus and an intact sorting signal (pb\textsubscript{2-\Delta}-DHFR-HB). To determine the effect on the viability of yeast cells, we expressed the hybrid proteins permanently by grow-
Fig. 2. Growth of yeast strains expressing different \( \text{b}_2 \)-DHFR constructs. Yeast cells containing the empty vector pYE DP 1/8–10 (−) or the vector with an inserted cytochrome \( \text{b}_2 \) construct (\( \text{b}_2 \)-DHFR-HB, \( \text{b}_2 \)-DHFR) were grown on selective minimal medium plates containing 2% glucose (Glucose) or 2% glucose and 2% galactose (Galactose) for 5 days at 28 °C. Expression of the hybrid proteins is induced in the presence of galactose.

Sorting of \( \text{b}_2 \)-DHFR-HB is thus accompanied by an inhibitory effect on cellular viability.

Chase of Cytochrome \( \text{b}_2 \) Hybrid Proteins after Accumulation in Translocation Sites—The results obtained with \( \text{b}_2 \)-DHFR-HB \( \text{in vivo} \) (this study) and \( \text{in vitro} \) (29) are on the one hand comparable; under both conditions, the hybrid protein was not completely imported into mitochondria, indicating that the presence of an intact sorting signal prevents import of the heme binding domain located at the C terminus. On the other hand, the processing and the final location of \( \text{b}_2 \)-DHFR-HB were different between the \( \text{in vivo} \) and the \( \text{in vitro} \) situation; \( \text{in vitro} \) the hybrid protein was processed to the mature form and found in the cytosolic fraction; \( \text{in vivo} \) processing was mainly to the intermediate sized form that got stuck in mitochondrial import sites (29). This difference can be attributed to the slow kinetics of the second processing step. Since the \( \text{in vitro} \) experiments have to be performed within a time range of \( \sim \)1 h due to the limited stability of the isolated mitochondria, the time range used for the \( \text{in vivo} \) experiments (4 h) is not possible.

For further characterization of the \( \text{b}_2 \)-DHFR-HB transport intermediates, we again isolated mitochondria and imported the preprotein from reticulocyte lysate. To complete the processing of \( \text{b}_2 \)-DHFR-HB \( \text{in vitro} \), a short term import incubation was followed by a reisolation of the mitochondria and a second incubation (chase). \( \text{b}_2 \)-DHFR-HB and, as control, \( \text{b}_2 \)-DHFR were synthesized in rabbit reticulocyte lysates in the presence of \( [\text{35S}] \)methionine and incubated with isolated mitochondria for 10 min at 12 °C for accumulation in import sites of the mitochondrial membranes \( \text{Import} \). After reisolation, the mitochondria were resuspended in import buffer and incubated for 15 min at 30 °C \( \text{Chase} \). Samples were divided into halves, and one-half was treated with protease K \( \text{Prot. K} \). After reisolation of the mitochondria by centrifugation, the mitochondrial pellets (Pel.) and the supernatants (Sup.) were analyzed by SDS-PAGE and autoradiography. \( \text{i} \), processing intermediate; \( \text{m} \), mature protein. \( \text{B} \), time course of the release of \( \text{b}_2 \)-DHFR-HB from mitochondria. The experiment was performed as in A, varying the time of chase. The total amount of \( \text{m-b}_2 \)-DHFR-HB \( \text{pellet + supernatant} \) present after 60 min of chase was set to 100% (control).

Fig. 3. Import of \( \text{b}_2 \)-DHFR hybrid proteins into isolated mitochondria. A, \( \text{b}_2 \)-DHFR-HB is released into the supernatant after processing inside mitochondria. Mitochondria were isolated from a yeast wild-type strain. The hybrid proteins \( \text{pb}_2 \)-DHFR-HB and \( \text{pb}_2 \)-DHFR (see Fig. 1A) were synthesized in reticulocyte lysate in the presence of \( [\text{35S}] \)methionine and incubated with isolated mitochondria for 10 min at 12 °C for accumulation in import sites of the mitochondrial membranes \( \text{Import} \). After reisolation, the mitochondria were resuspended in import buffer and incubated for 15 min at 30 °C \( \text{Chase} \). Samples were divided into halves, and one-half was treated with protease K \( \text{Prot. K} \). After reisolation of the mitochondria by centrifugation, the mitochondrial pellets (Pel.) and the supernatants (Sup.) were analyzed by SDS-PAGE and autoradiography. \( \text{i} \), processing intermediate; \( \text{m} \), mature protein. \( \text{B} \), time course of the release of \( \text{b}_2 \)-DHFR-HB from mitochondria. The experiment was performed as in A, varying the time of chase. The total amount of \( \text{m-b}_2 \)-DHFR-HB \( \text{pellet + supernatant} \) present after 60 min of chase was set to 100% (control).
Association of Cytochrome $b_2$ Fusion Proteins with the Inner Membrane Translocase Protein Tim23—If the cytochrome $b_2$ sorting signal is arrested in the mitochondrial inner membrane, it should be possible to demonstrate interactions of translocation intermediates with distinct components of the inner membrane protein import machinery. By co-immunoprecipitation, we first tested for association of preproteins with Tim23. The following hybrid proteins were used: $b_2$-DHFR with a fully functional cytochrome $b_2$ presequence; $b_2^*$-DHFR with a partial inactivation of the sorting signal by replacing two positively charged residues (lysine 48 and arginine 49) by uncharged residues (Fig. 4A), the resulting hybrid protein is mistargeted into the matrix space (see below, Fig. 6B) (20, 27); and $b_2\Delta$-DHFR-HB with a complete inactivation of the sorting signal (due to the 19-residue deletion). The hybrid proteins were accumulated in mitochondrial import sites and processed to the intermediate sized form. All three hybrid proteins were found in association with Tim23 (Fig. 4B, lanes 2, 7, and 12), suggesting that cytochrome $b_2$ remains in contact with Tim23 irrespective of an alteration ($b_2^*$-DHFR) or partial deletion ($b_2\Delta$-DHFR-HB) of the sorting sequence. At least in this stage of translocation, cytochrome $b_2$ seems to be imported by the general import channel of matrix targeted proteins. Tim23 is suggested to participate in the constitution of this protein translocation channel (38, 42–46).

Association of Cytochrome $b_2$ Fusion Proteins with mtHsp70—In parallel we tested for the interaction of the fusion proteins with mtHsp70 (Fig. 4B, lanes 4, 9, and 14). Of the three constructs tested, only $b_2^*$-DHFR and $b_2\Delta$-DHFR-HB were co-immunoprecipitated in significant amounts. $b_2$-DHFR was only precipitated in minor amounts that were close to the level observed with preimmune serum (Fig. 4B, lane 4 versus lane 1). We conclude that in $b_2$-DHFR the intact sorting signal of cytochrome $b_2$ prevents stable binding of the translocation intermediate to mtHsp70. With $b_2^*$-DHFR and $b_2\Delta$-DHFR-HB, mtHsp70 can gain access to additional segments of the polypeptide chains (Fig. 4B, lanes 9 and 14) that are not accessible when the authentic sorting sequence is present (Fig. 4B, lane 4). Tim44 serves as a membrane anchor for mtHsp70 (49–51). No significant association with Tim44 was observed with either of the three constructs (Fig. 4B, lanes 3, 8, and 13). This result is in agreement with our recent observation that Tim44 is a component of a separate subcomplex of the import machinery that only transiently interacts with the Tim23-Tim17 core complex (11).

Preprotein Sorting in tim23-2 Mutant Mitochondria—The yeast mutant tim23-2 is impaired in preprotein translocation into the mitochondrial matrix in vivo and in vitro (38, 52). We used tim23-2 mitochondria to characterize preprotein sorting to the intermembrane space in dependence of the function of the inner membrane Tim machinery. We first tested the stability of the Tim23-Tim17 core complex (44, 46, 47) by co-immunoprecipitations with antibodies against Tim23. The total amounts of Tim23 and Tim17 were comparable between wild-type and mutant mitochondria; however, the stability of the complex with Tim17 was reduced in the tim23-2 mutant (Fig. 5A). The membrane potential of the mutant mitochondria was intact (not shown). The amounts of Tim44 and mtHsp70 were the same in the mitochondria of both strains (Fig. 5A, samples 5 and 6).

We next analyzed the import kinetics of two mitochondrial preproteins, cytochrome $b_2$ and the iron/sulfur protein of complex III of the respiratory chain (Rieske Fe/S protein), which are “classical” preproteins in the analysis of mitochondrial sorting pathways. The Fe/S protein is completely imported into the matrix and then exported across the inner membrane to its functional destination at the intermembrane space side of the inner membrane (53, 54). As shown in Fig. 5B, cytochrome $b_2$ was imported into tim23-2 mitochondria nearly with the same kinetics as into wild-type mitochondria. Processing to the mature protein and translocation to a protease-protected position within the mitochondria were only slightly retarded. The result was different with the Fe/S protein. Processing of the protein was drastically delayed (Fig. 5B). Interestingly, the import reaction was already delayed in the step from the preprotein to the processing intermediate. The step from the intermediate to
the mature protein was not inhibited. In the context of the conservative sorting pathway of the Fe/S protein (53), this implies that it is the import of the Fe/S protein into the matrix that is reduced in the tim23-2 mutant.

We then used two cytochrome $b_2$ hybrid proteins with a partial inactivation of the sorting signal due to the replacement of the positively charged residues 48 and 49 by uncharged ones, b$_2^*$-DHFR (Fig. 4A) and b$_2^*$-HB-DHFR. With wild-type mitochondria, both hybrid proteins were efficiently processed to the intermediate forms but not or only very poorly to the mature forms (Fig. 6A, lanes 1–3). The intermediate forms were protected against proteinase K added to intact mitochondria (Fig. 6A, lanes 1–3) and to swollen mitochondria (Fig. 6B, columns 3 and 6). The swelling opened the mitochondrial intermembrane space, as evidenced by the decrease of the content of cytochrome $b_2$ (Fig. 6B, column 2), but left the matrix space intact, shown with the level of mtHsp70 (Fig. 6B, column 1). Imported b$_2^*$-DHFR (Fig. 6B, column 3) and b$_2^*$-HB-DHFR (Fig. 6B, column 6) behaved like mtHsp70, indicating that they were mistargeted into the matrix.

With tim23-2 mitochondria, however, both constructs were processed further to the mature sized forms (Fig. 6A, lanes 5–7). 30–40% of the imported proteins were processed to the mature forms. The mature forms were protected against proteinase K in intact mitochondria (Fig. 6A, lanes 5–7) but became accessible to the protease after opening of the outer membrane (Fig. 6B, columns 5 and 8). mb$_2^*$-DHFR and mb$_2^*$-HB-DHFR thus fractionated like mature cytochrome $b_2$, indicating that they were correctly sorted to the intermembrane space in the mutant mitochondria. The processing to the mature forms was abolished by a dissipation of the inner membrane potential ($\Delta \psi$), demonstrating that it strictly depended on the insertion of the preproteins into the inner membrane. We conclude that the defect in Tim23 suppresses the sorting defect caused by the amino acid alteration in the presence of cytochrome $b_2$.

**DISCUSSION**

We have characterized the sorting of cytochrome $b_2$ in vivo and in organello and present evidence that its import route...
branched early from the general matrix targeting pathway.

A cytochrome b₂ construct with a C-terminal heme binding domain, which is able to form a tightly folded structure (26, 29, 41), cannot be completely imported into mitochondria in vitro. It is processed to the intermediate sized form and, after longer import times, to the mature form. The mature form is then released from the mitochondria and found in the cytosol. Due to the long accumulation in mitochondrial import sites, expression of this preprotein causes a growth defect of intact yeast cells. To translocate the heme binding domain across the mitochondrial membranes, the domain either has to be placed close to the presequence or the sorting signal in the presequence has to be inactivated. In the first case, an interaction of mtHsp70 with the N-terminal portion of the presequence generates a pulling force on the heme binding domain during its entry into/across the outer membrane (29). In the latter case, the preprotein lacking the sorting information is completely imported into the matrix and thus is continuously pulled in by the mtHsp70-driving system. The in vivo experiments shown here strongly support the validity of the in vitro import studies published previously (29) which demonstrated that a C-terminal heme-binding domain cannot be imported, indicating that the sorting pathway of cytochrome b₂ diverges early from the matrix import pathway (and the mtHsp70-dependent unfolding machinery).

The in vivo import studies bear an important implication on the role of preprotein folding in the cytosol during mitochondrial protein import. Since the heme binding domain located at the C terminus prevents mitochondrial import of a cytochrome b₂ construct with a high efficiency (more than 80%), it is most likely that the domain is folded in intact cells as has been observed in the in vitro import system (29). This implies that the 100-residue domain is completely synthesized before translocation into mitochondria, i.e. its import occurs by a post-translational mechanism. Wienhues et al. (55) used the cytosolic enzyme DHFR as passenger protein and a stabilization of its folding by an externally added ligand (aminopterin) to demonstrate a post-translational import mechanism for ~75% of the passenger proteins. With the heme binding domain, we present the first case of an authentic mitochondrial domain that is apparently folded in the cytosol. It has been discussed that mitochondrial protein import occurs mainly co-translationally in vivo (56), and thus preprotein folding should not be relevant. Moreover, cytosolic chaperone proteins could interfere with and prevent folding of protein domains (57, 58). The results of Wienhues et al. (55) and this study, however, provide strong evidence for a post-translational mechanism of mitochondrial protein import.

It has been discussed for many years whether the sorting signal of cytochrome b₂ functions during the import reaction by arrest in the inner membrane or whether it requires complete transport into the matrix and becomes functional in a subsequent re-export step (59, 60). In addition to the in vivo results, we show that a mutation affecting a component of the central core of the preprotein translocase in the inner mitochondrial membrane (Tim23) facilitates the recognition of the cytochrome b₂ sorting signal. If the cytochrome b₂ sorting signal had already left the inner membrane and moved into the matrix to engage a separate export machinery, a further movement of the polypeptide should not be facilitated by structures that had been left behind. If the sorting function were carried out by an independent export system, it is difficult to explain why a mutation of TIM23 should facilitate a subsequent sorting step. The findings reported here thus favor an early recognition of the sorting signal during the entry into the inner membrane. They do not necessarily imply that Tim23 itself recognizes the sorting signal, but Tim23 seems to mark the location where the sorting signal leaves the general matrix import route and moves into a separate branch directed toward the intermembrane space. The mutant Tim23 protein may delay the movement of the modified sorting signal across the inner membrane and thereby reestablish the translocation arrest of cytochrome b₂ constructs with a partially inactivated sorting signal. The weakened stability of the Tim23-Tim17 interaction in the tim23-2 mutant mitochondria may facilitate lateral release of the sorting signal from the translocation channel.

An inner membrane localization of the branching site of import pathways is further corroborated by the characterization of translocation intermediates. We find that, after processing to the intermediate sized form, cytochrome b₂ is stably inserted in the inner membrane and associates with Tim23 without involvement of mtHsp70 (30, 61; data not shown), in contrast to other proteins that require mtHsp70 to prevent release from the import machinery (62). Tokatlidis et al. (63) recently identified Tim11 as an inner membrane protein that is in close contact to the cytochrome b₂ sorting signal during its import and sorting. The authors similarly concluded that the branching of import pathways occurs at the inner membrane.

However, the precise function of Tim11 in this process is not yet clear (64). It has been proposed that the biogenesis of cytochrome b₂ involves an interaction of the mature part of the protein with the Tim machinery (23, 29, 65). The efficient release of the mature sized cytochrome b₂ construct into the cytosol in vivo suggests that such an interaction with the Tim machinery is only transient and of limited stability.

The principle of conservative sorting via the matrix space was originally described for the Rieske Fe/S protein (55). If cytochrome b₂ followed a different import pathway, both proteins should reveal differences in the import into mitochondria of the tim23-2 mutant. In fact, the results of our import experiments are in agreement with this prediction. Processing of cytochrome b₂ to the intermediate and to the mature protein was not affected in tim23-2 mutant mitochondria. In contrast, the Fe/S protein was only poorly processed to the intermediate form in tim23-2 mitochondria. We conclude that the biogenesis of the Fe/S protein requires transport of the preprotein into the mitochondrial matrix that depends on the intact Tim23-Tim17 complex. The tim23-2 mutation thus reveals a striking difference between the sorting pathways of cytochrome b₂ and the Fe/S protein.

REFERENCES


