The mitochondrial inner membrane contains a large number of polytopic proteins that are derived from prokaryotic ancestors of mitochondria. Little is known about the intramitochondrial sorting of these proteins. We chose two proteins of known topology as examples to study the pathway of insertion into the inner membrane: Mrs2 and Yta10 are bitopic proteins that expose negatively charged loops of different complexity into the intermembrane space. Here we show that both Mrs2 and Yta10 transiently accumulate as sorting intermediates in the matrix before they integrate into the inner membrane. The sorting pathway of both proteins can be separated into two sequential reactions: (i) import into the matrix and (ii) insertion from the matrix into the inner membrane. The latter process was found to depend on the membrane potential and, in this respect, is similar to the insertion of membrane proteins in bacteria. A comparison of the charge distribution of intermembrane space loops in a variety of mitochondrial inner membrane proteins suggests that this mode of “conservative sorting” might be the typical insertion route for polytopic inner membrane proteins that originated from bacterial ancestors.

The phylogenetic separation of mitochondria and bacteria took place about 1.5–2 billion years ago. Although the specific needs of an intracellular organelle caused massive adaptations of the mitochondria over this time period, mitochondria still resemble their prokaryotic ancestors in many respects. About half of the mitochondrial proteome shows significant homology to bacterial proteins (1). Many of these conserved proteins fulfill housekeeping functions, such as energy metabolism, DNA replication, or protein synthesis. During evolution, regulatory or accessory components were added together with proteins that mediate the exchange of metabolites and polypeptides with the hosting cell. Upon transition from endocellular bacteria to organelles, most genes were transferred to the nucleus genome. As a consequence, these proteins have to be imported into mitochondria following synthesis in the cytosol and directed to their respective subcompartment: the outer membrane, the intermembrane space (IMS),1 the inner membrane, or the matrix.

Proteins destined for the matrix typically carry N-terminal extensions, called presequences, which are proteolytically removed in the matrix following translocation. Presequences have the potential to form amphipathic helices with one face being positively charged and the other being hydrophobic. Import of these proteins is achieved by the translocase in the outer membrane, the TOM complex, and a translocase of the inner membrane, the TIM23 complex (for a review, see Refs. 2 and 3). Integral inner membrane proteins can be imported on the same pathway and are inserted into the membrane following arrest at the level of the TIM23 complex (4, 5). This was referred to as stop-transfer pathway, and all substrates identified so far are monotopic proteins. Either these proteins carry typical presequences that direct the N terminus into the matrix (6), or alternatively, amphipathic helices directly C-terminal to transmembrane domains can function as internal signals that allow import and membrane insertion of loop-like protein structures. Upon arrest of the transmembrane domain in the inner membrane, these proteins acquire an \( N_{\text{out}}C_{\text{in}} \) topology in the membrane (7).

A second group of inner membrane proteins is inserted from the mitochondrial matrix. These proteins carry presequences that direct them across both mitochondrial membranes. Because protein integration occurs in the same direction as in bacteria, this route was called the conservative sorting pathway (8). Examples are both monotopic proteins (9, 10) and the polytopic inner membrane protein Oxa1 (11). The insertion of the few examples studied so far was found to depend on the function of Oxa1 (12). Oxa1 belongs to a ubiquitous protein family that is involved in protein insertion processes into membranes of bacteria, mitochondria, and chloroplasts (for a review, see Ref. 13). Recently, a second component, Mba1, that cooperates with Oxa1 in this process (14) was identified in yeast mitochondria. Obviously, membrane proteins synthesized on mitochondrial ribosomes also have to be inserted from the matrix. The membrane integration of several, but not all, of these proteins also depends on Oxa1 and Mba1 function (14–18).

A third class of inner membrane proteins is inserted by a second TIM translocase, the TIM22 complex (19, 20). Substrates of this pathway are members of the solute carrier family and components of the TIM complexes, all proteins for which bacterial homologues were not found. In contrast to the proteins sorted by the two former pathways, TIM22 substrates do not contain amphipathic targeting sequences but instead contain more complex internal signals.

Very little is known about the insertion pathways of polytopic inner membrane proteins of prokaryotic origin. Oxa1 is the only example whose import pathway has been studied in some detail (11). It was suggested that proteins with two membrane-spanning domains of \( N_{\text{in}}C_{\text{in}} \) topology might be integrated into the inner membrane from the IMS side (21). According to this
hypothesis, a series of import and stop-transfer signals in polytopic proteins might initiate and halt their translocation into mitochondria at the level of the inner membrane, thereby inserting transmembrane domains from the intermembrane space. Thus, bitopic proteins should contain two independent signals: a presequence inserting the N terminus and a loop-forming segment inserting the C terminus of the protein. Such a situation was found for cytochrome c₁, which is proteolytically cleaved in the IMS following insertion into the inner membrane resulting in a monopartite N Cec topology (21).

In this study, we analyzed the insertion pathways of two bitopic proteins, Mrs2 and Yta10. Both of them have close relatives in bacteria. Mrs2 is a Mg²⁺ transporter that exposes only eight amino acid residues into the IMS (22). Yta10 is an inner membrane protease containing a more complex IMS loop of 89 amino acid residues. Both proteins were found to be first completely imported into the matrix, where they form soluble intermediates that are subsequently integrated into the inner membrane. The efficiency of the membrane insertion of both proteins was influenced by the membrane potential; however, translocation of the longer IMS loop of Yta10 was significantly more dependent on the energetic state of the inner membrane.

**Experimental Procedures**

**Recombinant DNA Techniques—** In vitro transcription and translation of Mrs2, the MRS2 open reading frame was amplified by PCR from genomic yeast DNA using primers MRS2N1 (5′-GGGGAATTCGCGACCATGAATCGCCGCTCCTGG-3′) and MRS2C1 (5′-GGGGTTCGACGATTTTTCTTGCTTC-3′) and was used for the generation of Yta10(1-265)-DHFR mut. The Yta10 expression plasmid was described before (24). The MRS2 sequence was amplified from the pGEM4 constructs described above with primers MRS2M3 (5′-GGGGATATCAATTTTAATTCTTTAAGATCCGTG-3′) and MRS2M4 (5′-GGGGGATATCGGTGATATAAAGGGCA-3′) and used for expression of DHFR-Mrs2(337-470), the corresponding sequence of MRS2 was amplified using primers MRS2N2 (5′-GGGTCGACGATTTTTCTTGTCTTC-3′) and MRS2C3 (5′-GGGAGACTTCCTCAATTTTCTCTTCTCTACAC-3′) subcloned into the EcoRI and BamHI sites of pGEM-DHFR mut (25). For expression of DHFR-Mrs2(337-470), the corresponding sequence of MRS2 was amplified using primers MRS2N2 (5′-GGGTCGACGATTTTTCTTGTCTTC-3′) and MRS2C3 (5′-GGGAGACTTCCTCAATTTTCTCTTCTCTACAC-3′) and inserted into pGEM4-DHFR (25). A plasmid was used as template with primers MRS2M3 (5′-GGGGATATCAATTTTAATTCTTTAAGATCCGTG-3′) and MRS2M4 (5′-GGGGGATATCGGTGATATAAAGGGCA-3′). The resulting product was digested with EcoRV, re-ligated, and used for expression for DHFR-Mrs2(337-470)/K/R→D1. For expression of full-length Mrs2 harboring the K36E and K364L mutations (MRS2mut), two parts of the MRS2 sequence were amplified by the primer pairs MRS2N1/MRS2C2 and MRS2N3 (5′-GGGGATATCGGTGATATAAAGGGCA-3′) and MRS2M1 (5′-GGGGGATATCAATTTTAATTCTTTAAGATCCGTG-3′) and MRS2M4 (5′-GGGGGATATCGGTGATATAAAGGGCA-3′). The resulting product was digested with EcoRV, re-ligated, and used for expression of DHFR-Mrs2(337-470)/K/R→D1. For expression of full-length Mrs2 harboring the K36E and K364L mutations (MRS2mut), two parts of the MRS2 sequence were amplified by the primer pairs MRS2N1/MRS2C2 and MRS2N3 (5′-GGGGATATCGGTGATATAAAGGGCA-3′) and MRS2M1 (5′-GGGGGATATCAATTTTAATTCTTTAAGATCCGTG-3′) and MRS2M4 (5′-GGGGGATATCGGTGATATAAAGGGCA-3′). The resulting product was digested with EcoRV, re-ligated, and used for expression of DHFR-Mrs2(337-470)/K/R→D1. For expression of full-length Mrs2 harboring the K36E and K364L mutations (MRS2mut), two parts of the MRS2 sequence were amplified by the primer pairs MRS2N1/MRS2C2 and MRS2N3 (5′-GGGGATATCGGTGATATAAAGGGCA-3′) and MRS2M1 (5′-GGGGGATATCAATTTTAATTCTTTAAGATCCGTG-3′) and MRS2M4 (5′-GGGGGATATCGGTGATATAAAGGGCA-3′). The resulting product was digested with EcoRV, re-ligated, and used for expression of DHFR-Mrs2(337-470)/K/R→D1.

In Vitro Protein Import and Mitochondrial Subfractionation—Import into isolated mitochondria of in vitro synthesized proteins was according to published procedures (11). Standard import reactions were carried out in the presence of 2 mM NADH and 2 mM ATP. For some experiments, an ATP-regenerating system containing 2.5 mM malate, 2.5 mM succinate, 1 mM creatine phosphate, and 0.1 mg/ml creatine kinase was used as well. The procedures used for subfractionation of mitochondria were described before (29). Protease treatment was generally performed at 0 °C with the exception of the experiment shown in Fig. 1B, in which the samples were treated with 25 °C. Efficient swelling and protease K treatment were controlled by Western blotting using cytochrome c peroxidase, Ddi1, Oxa1, and Mge1 as marker proteins.

For carbonate extraction, mitochondria were resuspended in 0.1 M Na₂CO₃ (pH 11.5) containing 1.5 mM phenylmethylsulfonyl fluoride. For 40 min at 4 °C, soluble and membrane fractions were separated by centrifugation for 30 min at 45,000 rpm at 4 °C in a Beckman TL44 rotor. Soluble proteins in the supernatants were precipitated following addition of 12% trichloroacetic acid.

**Results**

**Mrs2 Is a Conserved Inner Membrane Protein with an N Ctopology—** We chose Mrs2 of the yeast *Saccharomyces cerevisiae* as a model to study the topogenesis of a polytopic membrane protein because of its simple and well-characterized topology (22). Mrs2 is a Mg²⁺ transporter that belongs to a conserved family of bacterial and mitochondrial proteins (22). The topology of both Mrs2 and its bacterial homologues is known, and both contain a short negatively charged loop facing the IMS flanked by N- and C-terminal domains in the matrix or the cytoplasm, respectively (Fig. 1A). The N terminus of Mrs2 contains a typical mitochondrial targeting sequence. Directly C-terminal of the second transmembrane domain, Mrs2 contains a further segment with the characteristics of an amphipathic helix with one hydrophobic and one positively charged face. Such motifs have been shown to serve as internal mitochondrial targeting signals that mediate the insertion of the hydrophobic segment into the inner membrane and the subsequent translocation of the C-terminal domain into the matrix (7). This makes two alternative models possible for the topogenesis of Mrs2, both of which are depicted in Fig. 1B. Mrs2 might be first completely imported into the matrix from where it integrates into the inner membrane. In this case, an internal import signal would not be required. Alternatively, the first transmembrane domain of Mrs2 might be arrested at the level of the inner membrane. Subsequently, the C-terminal part of the protein would be inserted from the IMS employing the second targeting signal.

**Mrs2 Contains Two Independent Import Signals—** To identify the targeting signals in Mrs2, we analyzed the import competence of in vitro synthesized radiolabeled Mrs2 and derivatives thereof into isolated yeast mitochondria. As shown in Fig. 2A, full-length Mrs2 precursor (pre) was found to be efficiently imported into mitochondria resulting in a proteolytically processed protein (mature (m)). This processed form was hardly sensitive to externally added protease even after the rupturing of the outer membrane. At high protease concentrations, a minor fraction of the imported Mrs2 was cleaved, giving rise to two fragments (indicated with arrowheads). This would be consistent with a cleavage in the short intermembrane space loop between the first and second transmembrane domain of Mrs2. Most of the protein, however, was not cleaved by the protease, most likely due to the close proximity of the loop to the inner membrane. Upon carbonate extraction, most of the imported Mrs2 was found in the membrane fraction. This protease-resistant localization of Mrs2 in the inner membrane was described before for the endogenous protein (22). Thus, the Mrs2 precursor was sorted to its proper location in an in vitro import reaction.

Does the first transmembrane domain of Mrs2 serve as a stop signal that leads to an arrest of the imported polypeptide in the inner membrane? To test this, we replaced the second transmembrane domain and the C-terminal matrix stretch of
Mrs2 with a folding-defective version of mouse dihydrofolate reductase domain (DHFRmut). In *in vitro* import experiments, this fusion protein was imported completely into the mitochondrial matrix and remained in a carbonate-extractable location (Fig. 2B). The absence of an arrested, membrane-inserted species argues against the presence of an arresting signal around the first transmembrane domain of Mrs2.

To test whether the C-terminal part of Mrs2 contains a second import signal, we created a chimeric protein comprising amino acid residues 337–470 of Mrs2 fused to the C terminus of a wild-type DHFR sequence (Fig. 2C, left panel). This protein was not imported into mitochondria (lane 4), most likely due to the close proximity of the folded DHFR domain to the import signal, which may prevent access of the signal to the import machinery in the inner membrane (30). This fusion protein, however, integrated into the inner membrane when the outer membrane was opened by hypotonic swelling. Protease treatment revealed a 15-kDa fragment (lane 5), which matched the expected size of the Mrs2 C terminus (14.7 kDa). The accumulation of this fragment suggests that the DHFR-Mrs2(337–470) fusion protein acquired an Nout-Cin topology in the inner membrane, indicating that the C-terminal domain of Mrs2 has the potential to translocate across the inner membrane into the matrix independently of the Mrs2 N terminus. To verify that the positively charged segment following the second transmembrane domain is part of this insertion signal, we replaced the two lysine residues directly flanking the hydrophobic domain with aspartate and isoleucine (Fig. 2C, right panel). The corresponding protein was not inserted into the inner membrane, and no fragment was formed upon protease treatment (lane 10). From this we conclude that Mrs2 contains two targeting signals, a typical N-terminal presequence and an internal insertion signal similar to those in Bcs1 and cytochrome *c* (7, 21).
The Internal Import Signal Is Not Required for Sorting of Mrs2—The observed unarrested translocation of the first transmembrane domain of Mrs2 into the mitochondrial matrix raised the question of the relevance of the internal insertion signal for the sorting of the complete Mrs2 protein. To address this, we performed import experiments with an Mrs2 derivative in which the internal signal was eliminated (Mrs2mut). This protein was efficiently imported into the mitochondrial matrix, and no protease-accessible part remained exposed to the matrix. B, the internal signal is not required for Mrs2 function in vivo. ΔMrs2 cells were transformed with plasmids encoding for Mrs2 (wt) or Mrs2mut (mut), or with an empty plasmid for control, and grown for 3 days at 30°C on minimal medium containing glucose (left) or glycerol (right) as carbon source.

FIG. 3. The internal import signal is not essential for Mrs2 sorting in vitro and in vivo. A, Mrs2mut acquires an Nc-Cm topology after in vitro import. Radiolabeled full-length Mrs2 and Mrs2mut, in which the positive charges in residues Lys-363 and Lys-364 were mutated, were incubated with mitochondria for 30 min and further treated as described in the legend for Fig. 2B. M, mitochondria; MP, mitoplasts; P, pellet; S, supernatant; PK, proteinase K, pre, precursor; m, mature. B, the internal signal is not required for Mrs2 function in vitro. ΔMrs2 cells were transformed with plasmids encoding for Mrs2 (wt) or Mrs2mut (mut), or with an empty plasmid for control, and grown for 3 days at 30°C on minimal medium containing glucose (left) or glycerol (right) as carbon source.

Next we asked whether the internal signal of Mrs2 is required in vivo. Therefore, we tested whether Mrs2mut is able to rescue a mrs2 deletion mutant (Fig. 3B). Mrs2 functions as a transmembrane Mg2+ transporter that is required for growth on non-fermentable carbon sources such as glycerol. Transformation of plasmids encoding either the wild type Mrs2 or the Mrs2mut sequence did fully restore growth on glycerol, indicating that the internal sorting signal is dispensable for Mrs2 biogenesis and function in vivo. Western blotting revealed slightly reduced levels of Mrs2mut as compared with Mrs2 in the mutants (data not shown), which might be due to the reduced insertion efficiency of the protein (see above). From this, we conclude that the internal import signal of Mrs2 is not required for Mrs2 sorting into the inner membrane, consistent with a conservative insertion pathway of this bitopic protein.

Mrs2 Forms an Extractable Sorting Intermediate in the Matrix—The sorting of Mrs2 via the matrix might lead to a soluble translocation intermediate dependent on the kinetics of the import and export reactions. To identify such a soluble matrix intermediate, wild type Mrs2 was imported into isolated mitochondria using an import buffer lacking ATP and NADH to slow down the kinetics of import and sorting. After different time periods, aliquots were taken, and non-imported material was removed by protease digestion. Then the reactions were split, and mitochondrial proteins were either directly applied to SDS-PAGE or subfractionated by carbonate treatment into a soluble and a membrane fraction (Fig. 4A). Fractionation was controlled by Western blotting using antibodies against the inner membrane protein Aac2 and the soluble matrix protein Mge1. A, radiolabeled Mrs2 full-length protein was imported into mitoplasts in the presence of NADH and an ATP-regenerating system. After 3 and 30 min, aliquots were taken and incubated in the absence or presence of 100 μg/ml proteinase K (PK) at 25°C for 30 min. Protease digestion and integrity of mitoplasts were controlled by Western blotting using antisera against the matrix protein Mge1 and the inner membrane protein Dld1. The generated Mrs2 fragments are indicated by arrowheads. Positions of the molecular size standards are shown on the left, pre, precursor; m, mature.

The mutant proteins were either directly applied to SDS-PAGE or subfractionated by carbonate treatment into a soluble and a membrane fraction (Fig. 4A). Fractionation was controlled by Western blotting using antibodies against the inner membrane protein ATP/ADP carrier (Aac2) and the soluble matrix protein Mge1. After 5 min of import, the majority of the mature Mrs2 protein could be extracted with carbonate, and only a minor fraction was found in the membrane fraction. Thus, at this early stage of import, most of the protease-
inaccessible Mrs2 species was not stably integrated into the inner membrane. Because this species was matured by the mitochondrial processing peptidase, it most likely resides in the matrix. Over time, the carbonate-resistant fraction of the Mrs2 protein increased, and most of the imported protein fractionated with the membranes after a 60-min incubation. The sorting kinetics of Mrs2 was analyzed in six independent experiments, and the ratio of carbonate-resistant to total Mrs2 was quantified. As shown in Fig. 4B, at early time points of the import reaction, only minor fractions of Mrs2 were obtained in the membrane fraction, whereas after 60 min, most of the Mrs2 consistently cofractionated with membrane proteins. This suggests that Mrs2 transiently forms a sorting intermediate in the matrix, which is integrated into the inner membrane in an export step as depicted in Fig. 1B, left panel.

As shown above (Fig. 2A), a part of the imported Mrs2 can be converted by protease digestion into two fragments most likely representing the N- and C-terminal domains of Mrs2. As an independent proof for the sorting of Mrs2 via a matrix-localized sorting intermediate, we imported Mrs2 precursor into mitochondria and assessed the generation of protease fragments after 3 and 30 min. After 3 min, hardly any fragments of Mrs2 were detectable. In contrast, after 30 min of incubation, a significant amount of Mrs2 could be converted into the fragments indicated in Fig. 4C by the arrowheads. Taking into account the lower methionine content of the fragments relative to the mature Mrs2 form (12 for the mature protein and 8 and 4 for the N- and C-terminal fragments, respectively), more than half of the imported Mrs2 was accessible at the later time point. This strongly supports our conclusion that Mrs2 reaches its final topology after insertion from the matrix into the inner membrane.

Membrane Insertion of Mrs2 Is Not Dependent on Oxa1 Function—Oxa1 and Mba1 are components that play central roles in the translocation process of protein domains from the matrix into the inner membrane (12, 14, 17). Are these proteins required for the membrane integration of Mrs2? To test this, we imported radiolabeled Mrs2 precursor into mitochondria isolated from wild type, Δoaxa1Δmba1, and Δyta10 cells. Δyta10 mitochondria were used as a control because Yta10, like Oxa1, is required for respiratory activity but is not involved in protein export. Following incubation for 30 min and proteolytic removal of non-imported material, mitochondria were subfractionated by carbonate treatment, and the distribution of the imported Mrs2 was assessed following SDS-PAGE. The fractionation of the membrane protein Aac2 and the soluble matrix protein Mge1 was analyzed by Western blotting and used for control (Fig. 5A). In wild type mitochondria, 62% of the imported Mrs2 was found in the membrane fraction. In both Δoaxa1Δmba1 and Δyta10 mitochondria, slightly lower amounts (45%) acquired a carbonate-resistant location that might be explained by the lower membrane potential of respiratory-deficient mitochondria (Fig. 5B). Similar results were obtained with Δoaxa1 mitochondria (not shown). To differentiate whether the lower membrane potential in the mutant strains or a missing insertion-promoting function of Oxa1 caused the reduced insertion efficiency, Mrs2 was imported into mitochondria of a temperature-sensitive oxa1Δ mutant under restrictive conditions. In this strain, Oxa1 function can be compromised by a preexposure of the mitochondria to 37 °C for 10 min without affecting the membrane potential level of the mitochondria (16). Following import, comparable amounts of Mrs2 were found in the membrane fractions of wild type and temperature-sensitive oxa1Δ mitochondria (Fig. 5C). Thus, integration of Mrs2 into the inner membrane appears not to be dependent on Oxa1 and Mba1 function but on the energetic state of the mitochondria.

Yta10 Is a Bitopic Inner Membrane Protein with a Large IMS Loop—Is a conserved sorting pathway restricted to proteins with short IMS loops such as Mrs2 so that proteins with longer loops have to be inserted from the IMS? To answer this question, we chose Yta10 as a model protein that has a well established topology in the inner membrane (31, 32). As depicted in Fig. 6A, Yta10 has an N-terminal presequence and two membrane-spanning domains flanking a highly charged IMS domain of 89 amino acid residues. It was suggested that Yta10 could be inserted from the IMS (21); however, the region directly following the second transmembrane domain does not show the characteristics of an amphipathic helix. Yta10 precursor can be imported into isolated mitochondria and is processed to its mature form (Fig. 6B, lane 2). Opening of the outer membrane, this imported protein is largely accessible to protease, resulting in a C-terminal fragment of an apparent molecular size of 58 kDa (lane 3, fragment f). This matches the calculated molecular size of the second transmembrane and C-terminal matrix domains (58.7 kDa). It was reported before that the IMS loop of assembled Yta10 is not accessible to protease (91). We found that although treatment of mitoplasts mainly produced a fragment of about 62 kDa (indicated in Fig. 6 as f*), incubation with high amounts of protease at 25 °C converted endogenous Yta10 at least partially to the 58-kDa fragment that was detected by immunoblotting with an antisera raised against the C terminus of Yta10 (Fig. 6B, lane 6). Thus, the in vitro synthesized Yta10 was sorted into its proper location in isolated mitochondria, allowing us to monitor the insertion of the IMS loop by protease treatment.

Yta10 Forms a Matrix Intermediate That Inserts into the Inner Membrane—To investigate the sorting pathway of Yta10, we performed import experiments with a chimeric protein containing the N-terminal 265 amino acid residues of Yta10 fused to a DHFRmut domain (Yta10(1–265)-DHFRmut, Fig. 7A). This
protein comprised both transmembrane domains of Yta10, and it allowed us to easily distinguish between matrix-localized and membrane-inserted protein species since the latter can be converted into N- and C-terminal fragments by protease treatment. This fusion protein was imported faster than full-length Yta10 (not shown), which made it easier to technically dissect the sorting process into import and membrane insertion reactions. This fusion protein was incubated with isolated mitochondria, and aliquots were taken after different time points. Mitochondria were converted to mitoplasts and treated with protease. After short import periods, a mature species of this fusion protein was incubated with isolated mitochondria, and aliquots were taken after different time points.

To test whether the insertion of Yta10(1–265)-DHFRmut into the inner membrane depends on the Oxa1 translocase, we performed import experiments with mitochondria of an oxa1 deletion mutant. The fusion protein was efficiently imported into Δoxa1 mitochondria. In contrast to wild type mitochondria, however, membrane integration was drastically reduced, and the IMS loop of Yta10(1–265)-DHFRmut remained protease-inaccessible in the matrix (Fig. 7A, lanes 8–13). Mitochondria lacking Oxa1 have severely reduced membrane potential levels due to the absence of cytochrome oxidase and reduced cytochrome reductase and F1F0-ATPase activities (33). The observed defect in membrane integration might therefore be either due to a dependence on the Oxa1 protein insertion machinery or due to a certain membrane potential threshold that is not reached in Δoxa1 mitochondria. To discriminate between both possibilities, we monitored the sorting kinetics of Yta10(1–265)-DHFRmut in mitochondria harboring a temperature-sensitive oxa1 allele (34). In contrast to the Δoxa1 mitochondria, membrane integration was not affected in mitochondria of the temperature-sensitive mutant (Fig. 7, C and D), whereas the insertion of the conservatively sorted protein Oxa1 was strongly compromised under these conditions (Fig. 7E). This indicates either that Oxa1 function is not critical for the translocation of the Yta10 loop into the IMS or that the mutated Oxa1 protein is still able to play a role in the insertion of loop domains.

Since the strong integration defect in Δoxa1 mitochondria might point at a strong dependence of this process on the energetic state of the inner membrane, we performed insertion kinetics in wild type mitochondria in the presence or absence of NADH. In the latter case, we added oligomycin to the mitochondria to prevent the generation of a membrane potential by the F1F0-ATPase upon hydrolysis of the added ATP. In the absence of NADH, the efficiency of the membrane integration of Yta10(1–265)-DHFRmut was strongly reduced (Fig. 7F). To make sure that the matrix intermediate of the fusion protein was not a mislocalized dead end product, we re-established a membrane potential after 15 min of the reaction by addition of NADH. This allowed again the translocation of the IMS loop across the inner membrane (dotted line, triangles), indicating that the matrix population of this Yta10 fusion protein represents a sorting intermediate that is able to insert as the membrane potential is rising above a certain threshold.

**DISCUSSION**

We have analyzed the intramitochondrial sorting of two bitopic inner membrane proteins: Mrs2, which exposes a short loop of eight residues into the IMS, and Yta10, which contains a much larger IMS loop of 89 residues. Both proteins are synthesized in the cytosol with N-terminal mitochondrial presequences mediating their import into the mitochondrial matrix. We found for both Mrs2 and Yta10 a transient accumulation of the sorting intermediates in the matrix. After longer import periods, the levels of these intermediates declined, and instead, membrane-embedded species appeared. This indicates that the sorting of both bitopic inner membrane proteins can be subdivided into two sequential reactions: (i) import into the matrix and (ii) the insertion of transmembrane segments into and translocation of IMS domains across the inner membrane. Thus, both proteins follow a conservative sorting pathway in which the integration into the inner membrane occurs in the same direction as in bacteria. This is in contrast to what was proposed before, which is an insertion mode from the IMS of bitopic proteins mediated by internal signals (21).

A conservative sorting mode of precursors of inner membrane proteins has important implications on the evolution of mitochondrial proteins from bacterial ancestors. The addition of a mitochondrial import signal onto the N terminus of the proteins is the only prerequisite for correct protein sorting after gene transfer to the nucleus. This notion is supported by the
observation that the fusion of a mitochondrial targeting signal onto CorA, the bacterial homologue of Mrs2, led to proper sorting and function in eukaryotic cells (22). In this case, the insertion machinery inherited from bacteria could be used and gradually adapted to the specific needs of an intracellular organelle. In contrast, an insertion mode from the IMS would have required the development of additional internal insertion signals within each sorted polypeptide and the addition of specific components facilitating membrane integration and translocation of matrix domains.

What are the components that mediate translocation of loop domains across the inner membrane? It was shown that the inner membrane protein Oxa1 is required for efficient export into the IMS of the N-terminal domain of the mitochondrial encoded Cox2 protein (10) as well as of conservatively sorted proteins (9, 11, 12). In contrast, the export of loop domains of mitochondrial translation products occurs even in the absence of Oxa1, albeit with reduced efficiency (17). Our observations suggest that the function of Oxa1 is dispensable for membrane insertion of the loop domains of Mrs2 and Yta10. Thus, either Oxa1 seems not to be involved in the translocation of loops, or other components functionally overlap with Oxa1 and can take over its function. On the other hand, we cannot completely exclude that in the temperature-sensitive oxa1 strain, Oxa1 is still able to fulfill a role in the insertion of loop domains but is defective for the insertion of N termini.

What are the components that mediate Oxa1-independent membrane insertion? It was recently reported that the inner membrane protein Mba1 plays an important role in the Oxa1-independent insertion of mitochondrial encoded proteins (14). However, Mrs2 was found to insert in the absence of both Oxa1 and Mba1, and similar steady state levels of Yta10 were found in wild type and Δoxa1 mito-
Which energy source provides the driving force for the translocation of loops into the IMS? It has been suggested for bacterial inner membrane proteins that membrane insertion is mainly driven by an electrophoretic effect that allows the vectorial movement of negatively charged loops to the positively charged periplasmic face of the membrane. (38). This effect might also be the basis for the “positive-inside” rule according to which the charge distribution on both sides of transmembrane domains specifies their orientation. This appears to apply to both bacterial and mitochondrial proteins (39–41). The loops facing the IMS of both Mrs2 and Yta10 are negatively charged (cf. Figs. 1 and 6), and an electrophoretic effect might therefore serve as a driving force for their membrane translocation. In agreement with this assumption, we observed a reduced insertion efficiency at lower energization of mitochondria. The dependence on the membrane potential was by far more pronounced with Yta10, suggesting that larger and more complex loops need a higher membrane potential. We were, however, not able to study the membrane potential dependence of the insertion process in more detail because this reaction could not be experimentally discriminated from the process of mitochondrial import.

Is the insertion of negatively charged loops from the matrix a general principle in the biogenesis of inner membrane proteins, or are Mrs2 and Yta10 exceptions? If a conservative pathway is the typical sorting route for inner membrane proteins that are sorted via the TIM23 translocase, IMS loops of these proteins would be predicted to be generally acidic. As shown in Fig. 8, IMS loops of multispanning proteins that are synthesized with an N-terminal presequence consistently show a strong bias toward negative charges (upper row). In contrast, no charge preference was found for IMS loops of inner membrane proteins that are sorted via the TIM22 pathway (lower row). This suggests that the insertion of IMS loops from the matrix is a general principle in the topogenesis of polytopic inner membrane proteins of mitochondria.

Although Mrs2 was found to be sorted in a conservative manner, it contains an internal import signal that is able to promote the insertion of the C terminus from the IMS into the matrix. This signal was dispensable for the sorting and function of Mrs2 both in vivo and in vitro. We still cannot exclude that a minor fraction of the Mrs2 precursor might be arrested in the inner membrane upon import and that the internal signal might serve as a substitute that ensures a correct topology of a missedorted species. This situation of a presequence that has the potential to insert into the inner membrane both from the matrix and from the IMS might represent an evolutionary transition state. It seems conceivable that during evolution, pathways were established such as that described for cytochrome c1 (21), which was reported to insert into the inner membrane from the IMS.

In conclusion, mitochondrial polypotent inner membrane proteins can be subdivided into two groups: proteins that were newly added to the organellar proteome such as carriers and transporters that mediate the exchange of components with the cytosol. These proteins developed internal signals and are inserted from the IMS by a specialized inner membrane translocase, the TIM22 complex. In contrast, proteins that originate from bacterial ancestors obtained a presequence that enabled their targeting to the matrix from where they inserted into the inner membrane on a pathway that existed in bacteria. The biogenesis of these inner membrane proteins is surprisingly similar to that of bacterial proteins, and their membrane integration appears to adhere to the same principles such as the positive-inside rule and the translocation of negatively charged domains by an electrophoretic effect. The identification and characterization of components that assist this membrane integration process will be a major goal in the future.

Acknowledgments—We thank Benedikt Westermann for critical reading of the manuscript, Klaus Leonhard for providing the Yta10(1–265)-DHFRmut expression plasmid, and Silvia Hiesel and Elke Kolmann for help with some experiments.

REFERENCES

1. Karlberg, O., Canback, B., Karlund, C. G., and Andersson, S. G. (2000) Yeast 17, 170–187
2. Neupert, W. (1997) Annu. Rev. Biochem. 66, 863–917
3. Herrmann, J. M., and Neupert, W. (2000) Curr. Opin. Microbiol. 3, 210–214
4. Van Loon, A. P., and Schatz, G. (1987) EMBO J. 6, 2441–2448
5. Kirsch, V., Goltz, S., and Blobel, G. (1982) J. Biol. Chem. 257, 15654–15658
6. Rojo, E. E., Guiard, B., Neupert, W., and Stuart, R. A. (1998) J. Biol. Chem. 273, 8040–8047
7. Fölsch, H., Guiard, B., Neupert, W., and Stuart, R. A. (1996) EMBO J. 15, 247–257
8. Hartl, F. U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986) Cell 47, 939–951
9. Rojo, E. E., Stuart, R. A., and Neupert, W. (1995) EMBO J. 14, 3445–3451
10. Herrmann, J. M., Koll, H., Cook, R. A., Neupert, W., and Stuart, R. A. (1995) J. Biol. Chem. 270, 27079–27086
11. Herrmann, J. M., Neupert, W., and Stuart, R. A. (1997) EMBO J. 16, 2217–2226
12. Hell, K., Herrmann, J. M., Pratte, E., Neupert, W., and Stuart, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2530–2535
13. Luftig, J., Samuelsson, T., and de Gier, J. W. (2001) FEBS Lett. 501, 1–5
14. Preuss, M., Leonhard, K., Hell, K., Stuart, R. A., Neupert, W., and Herrmann, J. M. (2001) J. Cell Biol. 153, 1085–1096
15. Ii, M., and Mihara, K. (2003) J. Bio. Chem. 278, 24704–24712
16. Hell, K., Herrmann, J. M., Pratte, E., Neupert, W., and Stuart, R. A. (1997) FEBS Lett. 418, 367–370
17. Hell, K., Neupert, W., and Stuart, R. A. (2001) EMBO J. 20, 1281–1288
18. He, S., and Fox, T. D. (1997) Mol. Biol. Cell 8, 1449–1460
19. Sirenren, C., Bauer, M. F., Guiard, B., Neupert, W., and Brunner, M. (1996) Nature 384, 582–585
20. Kümper, O., Holder, J., Srinivasan, M., Leung, R. S., and Jensen, R. E. (1997) J. Cell Biol. 139, 1663–1675
21. Arnold, I., Fölsch, H., Neupert, W., and Stuart, R. A. (1998) J. Biol. Chem. 273, 1469–1476
22. Bui, D. M., Gregan, J., Jarosch, E., Ragnini, A., and Schweyen, R. J. (1999) J. Bio. Chem. 274, 20438–20443
23. Lee, C. M., Sedman, J., Neupert, W., and Stuart, R. A. (1999) J. Biol. Chem. 274, 20967–20972
24. Arlt, H., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. (1996) Cell 83, 875–885
25. Sekerski, R. S., and Hieter, P. (1989) Genetics 123, 19–27
26. Sherman, F., Finn, G. R., and Hicks, J. (1986) Methods in Yeast Genetics: A Laboratory Course, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Arlt, H., Steglich, G., Perryman, R., Guiard, B., Neupert, W., and Langer, T. (1998) EMBO J. 17, 4837–4847
28. Herrmann, J. M., Fölsch, H., Neupert, W., and Stuart, R. A. (1994) in Cell Biology: A Laboratory Handbook (Celis, J. E., ed) Vol. 1, pp. 538–544, Academic Press, San Diego
29. Leonhard, K., Guiard, B., Pellecchia, G., Tzagoloff, A., Neupert, W., and Langer, T. (2000) Mol. Cell 5, 629–638
30. Ungermann, C., Neupert, W., and Cvr, D. M. (1994) Science 266, 1250–1253
31. Pajic, A., Tauer, R., Feldmann, H., Neupert, W., and Langer, T. (1994) FEBS Lett. 353, 201–206
32. Guelin, E., Rep, M., and Grivell, L. A. (1994) Yeast 10, 1389–1394
33. Altamura, N., Capitanio, N., Bonnefoy, N., Papa, S., and Dujardin, G. (1996) *FEBS Lett.* **382**, 111–115
34. Bauer, M., Behrens, M., Eiser, K., Michaelis, G., and Pratje, E. (1994) *Mol. Gen. Genet.* **245**, 272–278
35. Woolhead, C. A., Thompson, S. J., Moore, M., Tissier, C., Mant, A., Rodger, A., Henry, R., and Robinson, C. (2001) *J. Biol. Chem.* **276**, 40841–40846
36. Samuelson, J. C., Jiang, F., Vi, L., Chen, M., de Gier, J. W., Kuhn, A., and Dalbey, R. E. (2001) *J. Biol. Chem.* **276**, 34847–34852
37. Saint-Georges, Y., Hamel, P., Lemaire, C., and Dujardin, G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13814–13819
38. Schuenemann, T. A., Delgado-Nixon, V. M., and Dalbey, R. E. (1999) *J. Biol. Chem.* **274**, 6855–6864
39. Andersson, H., and von Heijne, G. (1994) *EMBO J.* **13**, 2267–2272
40. Gavel, Y., and von Heijne, G. (1992) *Eur. J. Biochem.* **205**, 1207–1215
41. Rojo, E. E., Guiard, B., Neupert, W., and Stuart, R. A. (1999) *J. Biol. Chem.* **274**, 19617–19622
