Proline residues of transmembrane domains determine the sorting of inner membrane proteins in mitochondria

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Most inner membrane proteins of mitochondria are synthesized in the cytosol and reach the inner membrane using one of two alternative sorting pathways. On the stop transfer route, proteins are arrested during import at the level of the inner membrane. The conservative sorting pathway involves translocation through the inner membrane and insertion from the matrix. It is unclear how the translocase of the inner membrane 23 protein translocation machinery differentiates between the two classes of proteins. Here we show that proline residues in hydrophobic stretches strongly disfavor the translocation arrest of transmembrane domains (TMDs) and favor the transfer of preproteins to the matrix. We propose that proline residues, together with the hydrophobicity of the TMD and the presence of charged residues COOH-terminally flanking the TMD, are determinants of the intramitochondrial sorting of inner membrane proteins.

Introduction

The vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes as precursor proteins. Translocation complexes in the outer membrane, and the inner membrane—the translocase of the inner membrane (TIM) 23 complex—mediate the transport of proteins into the matrix in an ATP- and membrane potential–dependent process (for review see Koehler, 2000; Pfanner and Geissler, 2001; Jensen and Dunn, 2002).

Proteins that are destined for the inner membrane use two different pathways: proteins with one transmembrane domain (TMD) can be arrested at the level of the TIM23 complex and be integrated laterally into the inner membrane. This sorting route is referred to as "stop transfer" pathway (Van Loon et al., 1986; Glaser et al., 1990; Glick et al., 1992). Alternatively, proteins can reach the inner membrane after transport into the matrix in an export-like insertion reaction. Proteins that embark on this 'conservative sorting' pathway (Hartl et al., 1987) are predominantly polytopic proteins of bacterial origin (Herrmann et al., 1997; Herrmann and Neupert, 2003). Hence, the TIM23 translocase has to meet the challenge of discriminating two types of hydrophobic segments: those that have to be arrested and laterally inserted into the lipid bilayer, and those that have to be imported further into the mitochondrial matrix.

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Abbreviations used in this paper: TIM, translocase of the inner membrane; TMD, transmembrane domain.

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Little is known about the sorting signals by which the two groups of inner membrane proteins are distinguished. Charged amino acid residues COOH-terminally flanking the hydrophobic domains played a critical role in the translocation arrest of the inner membrane protein *D*-lactate dehydrogenase (Dld1) (Rojo et al., 1998). Here we present evidence that at least two characteristic properties of the hydrophobic stretches are critical determinants for the sorting of inner membrane proteins: a strong hydrophobic character, and the absence of proline residues support sorting by the stop transfer mechanism. Conversely, moderate hydrophobicity and the presence of proline residues disfavor a translocation arrest at the level of the TIM23 translocase, and thus, are characteristic for TMDs of conservatively sorted inner membrane proteins.

Results and discussion

Arrested TMDs typically lack proline residues

For several proteins of the mitochondrial inner membrane of baker's yeast, the topology is known and the intramitochondrial sorting route has been verified experimentally. The hydrophobic segments of these membrane proteins are depicted in Fig. 1 A, and have been classified into sequences which are arrested and laterally inserted (left panel) or transferred into the matrix by the TIM23 translocase (right panel). TMDs that are arrested at the level of the inner membrane tend to be of higher

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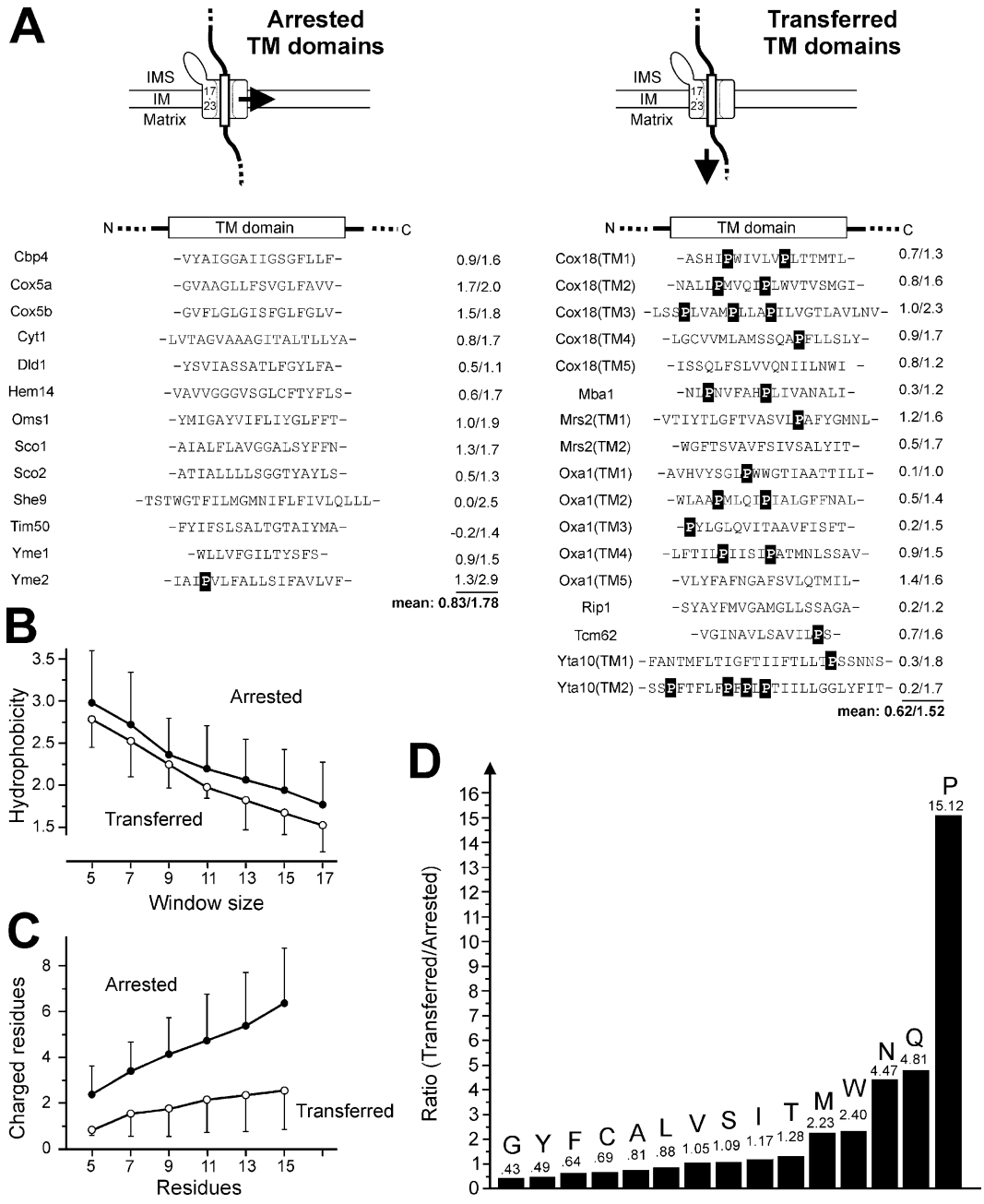

Figure 1. **TMDs of stop transferred and conservatively sorted proteins differ in their content of proline residues.** (A) Inner membrane proteins of known topogenesis are listed. The proteins were classified according to their sorting by the TIM23 translocase as sketched on the top. Monotopic proteins, which are sorted by the stop transfer pathway, are shown on the left. Import along the conservative sorting pathway was verified experimentally for the proteins shown on the right. Sequences depict the putative TMDs of the proteins. Proline residues are highlighted by black boxes. The minimal and maximal hydrophobicity scores of the segments were calculated as described in the Materials and methods section and are indicated beside the sequences. IM, inner membrane; IMS, intermembrane space. (B) The mean maximal hydrophobicity scores of arrested and transferred TMDs were calculated using window sizes from 5 to 17 residues. The standard deviations are indicated. (C) The numbers of charged residues in a sequence of 5 to 15 residues COOH-terminal of the TMDs were counted. The mean values and standard deviations are depicted. (D) The graph depicts the ratio of the frequencies of specific amino acid residues in the two groups of proteins. For the calculation, the frequency of a given residue (i.e., the number of a given residue divided by the number of all residues) in the transferred TMDs was divided by the frequency of the same residue in arrested domains.

hydrophobicity. When the hydrophobicity of a 17-residue wide frame within these sequences was calculated, arrested sequences showed mean lower and upper values of 0.83 and 1.78, respectively, compared with 0.62 and 1.52 for the transferred segments (Fig. 1 A). The same bias of arrested sequences toward an increased hydrophobicity was observed for smaller window sizes (Fig. 1 B). In addition, arrested sequences often were COOH-terminally followed by several charged residues (Fig. 1 C). However, there was a significant variability with respect to hydrophobicity and flanking charges within both groups of sequences. Some arrested TMDs, like those of Dld1 or Sco2, are less hydrophobic than many transferred sequences. This suggests that additional features of the sequences contribute as sorting determinants. Interestingly, arrested and trans-

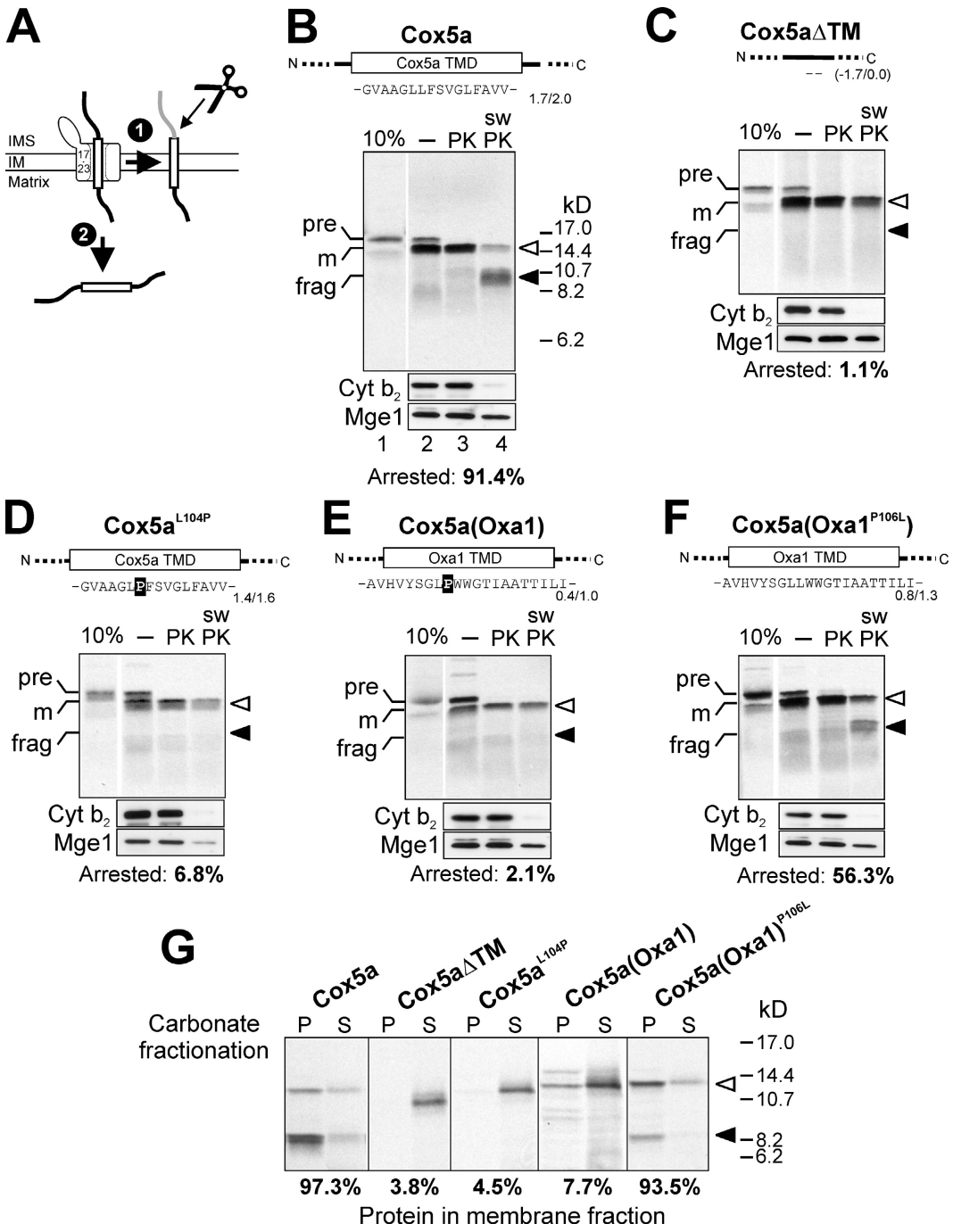

Figure 2. **The introduction of proline residues inactivates the stop transfer function of the TMD of Cox5a.** (A) Sorting pathway of wild type Cox5a (1) and of mutants that are missorted to the matrix (2). IM, inner membrane; IMS, intermembrane space. (B) Radiolabeled Cox5a precursor protein (pre) was incubated for 5 min at 25°C with isolated mitochondria. The mitochondria were exposed to proteinase K (PK) without or after hypotonic swelling (sw) as indicated. The radioactive bands of the mature form (m) and the protease fragment (frag) generated are depicted by white and black arrowheads, respectively. Radioactive signals were quantified by densitometry and corrected for their specific methionine content. The percentage of arrested (i.e., membrane-inserted) protein was calculated from the ratio of protease-accessible/total imported protein. For comparison, lane 1 shows 10% of the precursor protein used per import reaction. Efficiency of swelling was controlled by Western blotting using antibodies against a protein of the intermembrane space (cytochrome b_2, Cyt b_2) and of the matrix (Mge1). (C–F) Import of the Cox5a derivatives Cox5aΔTM, Cox5a^{L104P}, Cox5a(Oxa1), and Cox5a(Oxa1^{P106L}) were performed as described for B. The sequences and minimal and maximal hydrophobicity scores of the hydrophobic domains of the preproteins are shown on top of the panels. (G) The fusion proteins used for B to F were imported into mitochondria. Following swelling and protease treatment, mitoplasts were fractionated by carbonate treatment into a membrane pellet (P) and a soluble fraction (S). The numbers represent the fraction of imported protein found in the membrane pellet.

ferred TMDs differed strikingly in their content of proline residues (Fig. 1 D). Proline residues were present in most of the transferred hydrophobic domains, but were almost entirely absent in arrested sequences. The only example of an arrested domain with a proline residue that we found, Yme2, showed an exceptionally high hydrophobicity score.

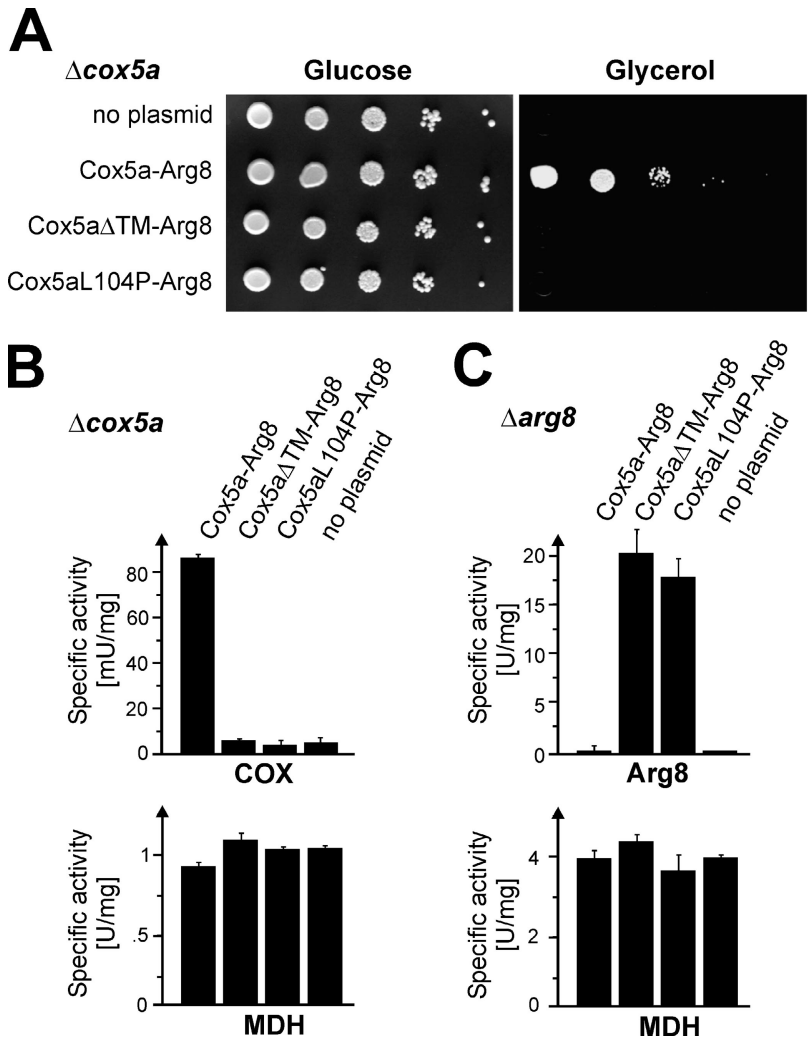

Figure 3. **The introduction of proline residues into the TMD of Cox5a leads to sorting into the matrix in vivo.** (A) *Δcox5a* mutant cells expressing the indicated proteins were grown to log phase. 10-fold serial dilutions were spotted on plates containing glucose or glycerol and incubated at 30°C for 2 and 3 d, respectively. (B) *Δcox5a* cells expressing the indicated fusion proteins were grown on galactose medium. Mitochondria were isolated and the enzymatic activities of cytochrome oxidase (COX) and malate dehydrogenase (MDH) were measured. (C) The indicated fusion proteins were expressed in *Δarg8* cells on lactate medium. Mitochondria were purified and the activities of Arg8 and MDH were measured.

Proline residues compromise stop transfer

To assess the significance of proline residues for the intramitochondrial sorting of inner membrane proteins experimentally, we used an in vitro import assay with radiolabeled precursor proteins. We chose Cox5a as a reporter protein that follows a stop transfer route to reach its final topology in the inner membrane (Fig. 2 A, pathway 1). The precursor form of Cox5a (Fig. 2 B, pre) was imported into isolated mitochondria and converted to the mature Cox5a protein, which was inaccessible to externally added protease (Fig. 2 B, m). Upon hypotonic rupturing of the outer membrane ("swelling"), the imported protein was converted almost entirely to a fragment of an apparent size of ~10.5 kD (Fig. 2 B, lane 4). This fragment comprises the matrix and inner membrane part of Cox5a and originates from the membrane-embedded protein species (Glaser et al., 1990). Because this fragment contains only two of the four methionine residues that are present in the mature Cox5a protein, the amount of arrested protein is underestimated by autoradiography. Quantification and correction for the different methionine content revealed that >90% of Cox5a were arrested during import at the level of the inner membrane. The small amounts of nondigested mature Cox5a protein likely are due to incomplete swelling of the mitochondria.

As control for a matrix-targeted protein, we used a Cox5a mutant lacking the TMD. As expected, this protein was completely targeted to the matrix and protected from added protease after swelling (Fig. 2 C).

Remarkably, the introduction of a single proline residue into the TMD of Cox5a led to the mistargeting of >90% of Cox5a to the matrix (Fig. 2 D). From this we conclude that introduction of a single proline residue is sufficient to overrule the stop transfer signal of Cox5a.

Is the exchange of the TMD of Cox5a for that of the conservatively sorted protein Oxa1 sufficient to target Cox5a to the matrix? As shown in Fig. 2 E, this chimeric protein (Cox5a(Oxa1)) was imported into the matrix completely. Thus, the critical sorting information for this precursor protein apparently is encoded in its TMD. However, when the proline residue present in the TMD of Oxa1 was replaced by a leucine residue (Cox5a(Oxa1^{P106L})), the protein was arrested partially (Fig. 2 F). Notably, this latter fusion protein has a significantly lower hydrophobicity score than the arrested construct

$Cox5a^{L104P}$ (Fig. 2 D). Thus, the hydrophobicity of a TMD may influence the intramitochondrial sorting of proteins, but does not seem to be the major discriminating factor.

As an additional assay to monitor the intramitochondrial sorting of the various Cox5a variants, we fractionated the protease-treated mitoplasts after the import reaction by alkaline extraction (Fig. 2 G). Cox5a and $Cox5a(Oxa1^{P106L})$ were found largely in the membrane fraction, consistent with the results of the protease-protection experiment. In contrast, Cox5aΔTM and the proline-containing construct $Cox5a^{L104P}$ fractionated with soluble proteins, again indicating that proline residues in the TMDs disfavor membrane integration.

Proline residues influence protein sorting in vivo

To assess the intramitochondrial sorting of the Cox5a mutants in vivo, we constructed fusion proteins of Cox5a variants with acetylornithine aminotransferase (Arg8). Arg8 is a matrix enzyme that was used to assess the sorting of protein domains in mitochondria in vivo (Steele et al., 1996). Upon expression in a *Δcox5a* strain, only the fusion protein containing the wild-type Cox5a protein allowed for growth on nonfermentable carbon sources (Fig. 3 A) and resulted in considerable levels of cytochrome oxidase activity (Fig. 3 B). To verify that this is due to missorting of the proline-containing Cox5a variant, we measured the Arg8 activities after expression in a *Δarg8* strain (Fig. 3 C). No Arg8 activity was found with wild-type Cox5a-Arg8 fusion protein because the Arg8 domain was unstable and degraded (unpublished data). In contrast, upon expression of Cox5aΔTM-Arg8 and Cox5a(L104P)-Arg8, considerable levels of Arg8 activity were detected as Arg8 was localized to the matrix. This suggests that, in vivo like in vitro, the insertion of a proline into the TMD of Cox5a prevented the efficient arrest of the Cox5a(L104P)-Arg8 fusion protein.

Proline residues determine the sorting route

We then tested the impact of proline residues present in the TMD of the conservatively sorted protein Oxa1 into isolated mitochondria. Oxa1 is imported first into the matrix from where it inserts into the inner membrane (Fig. 4 A, pathway 1) (Herrmann et al., 1997). Following its import into mitochondria, Oxa1 resided partially in the matrix and mainly was inserted into the inner membrane. The latter species could be converted into specific COOH-terminal fragments of ~27 kD by protease treatment of mitoplasts (Fig. 4 B, f_{27}).

To test whether this protein can be redirected to the stop transfer pathway, we replaced the second TMD of Oxa1 by the TMD of Cox5a. We used the second TMD because mature Oxa1 does not contain methionine residues NH_2-terminal of its first TMD, so that NH_2-terminal fragments of arrested Oxa1 proteins would not be detected by autoradiography. The Oxa1(Cox5a) preprotein was imported efficiently into mitochondria. Upon swelling and protease treatment, a fragment of 19 kD appeared (Fig. 4 C, black arrowhead). The size corresponded well to the calculated 18.6 kD that was expected for an arrested mature Oxa1(Cox5a) species (compare Fig. 4 A, f_{19}). This fragment contains 4 out of the 10 methionine residues that are present in mature Oxa1. Quantification revealed that >70% of the imported Oxa1(Cox5a) precursor was arrested at the level of the inner membrane. The insertion of proline residues, but not of glutamine residues, into this chimeric fusion protein restored the conservative sorting of the protein. The insertion of two proline residues reduced the amount of arrested protein from 72.1% to 27.1%, whereas upon insertion of two glutamine residues, 70.1% were arrested at the level of the inner membrane (Fig. 4, E and F). Thus, the presence or absence of proline residues determined the intramitochondrial sorting of this membrane protein.

The bacterial homologue of Oxa1, YidC, was shown to tolerate significant changes in its primary sequence without affecting its function. Even mutants in which complete TMDs were replaced remained functional (Jiang et al., 2003). This inspired us to test whether the different Oxa1 mutants can complement an *oxa1* deletion strain. The replacement of the second TMD of Oxa1 by that of Cox5a did not lead to a functional protein. However, the introduction of one proline residue into the hydrophobic segment restored the function of Oxa1 partially, and that of two proline residues led to almost wild-type growth (Fig. 4 G). This suggests that the addition of proline residues also redirects Oxa1(Cox5a) onto a conservative sorting pathway in vivo, and thereby, reestablishes the respiration-competent phenotype (Fig. 4 G). In contrast, the insertion of alanine or glutamine at the same position did not restore respiratory activity. The lack of growth of the Oxa1(Cox5a)-expressing mutant most likely is due to a sorting deficiency of the protein; this is indicated by the lack of a 27-kD proteolytic fragment that is characteristic for the properly inserted protein. However, this fragment was regained by introduction of the proline residues into the TMD of Oxa1(Cox5a) (Fig. 4 H).

Conclusion

Our findings suggest that the presence of proline residues in TMDs is necessary and sufficient to disfavor lateral insertion of proteins into the inner membrane. The presence of proline residues together with the hydrophobicity of the TMD and the presence of following charges serve as crucial sorting information that is deciphered by the TIM23 translocase. The identification of these signals should help to improve predictions on the intramitochondrial sorting routes of individual proteins (see Supplemental material). Consistent with our results, the insertion of proline residues into the hydrophobic sorting sequence of cytochrome b_2 compromised its translocation arrest and caused its transfer into the matrix (Beasley et al., 1993). Interestingly, in an extensive screen in which the impact of individual amino acid residues on the insertion of TMDs into the ER membrane was studied in a systematic way, proline residues counteracted membrane insertion significantly more than it was expected from their hydrophobicity (Hessa et al., 2005). Thus, the impact of proline residues on the transfer of hydrophobic stretches does not seem to be limited to mitochondria. However, mitochondria apparently use the effect of proline residues in a physiologic reaction to direct inner membrane proteins to two different sorting pathways.

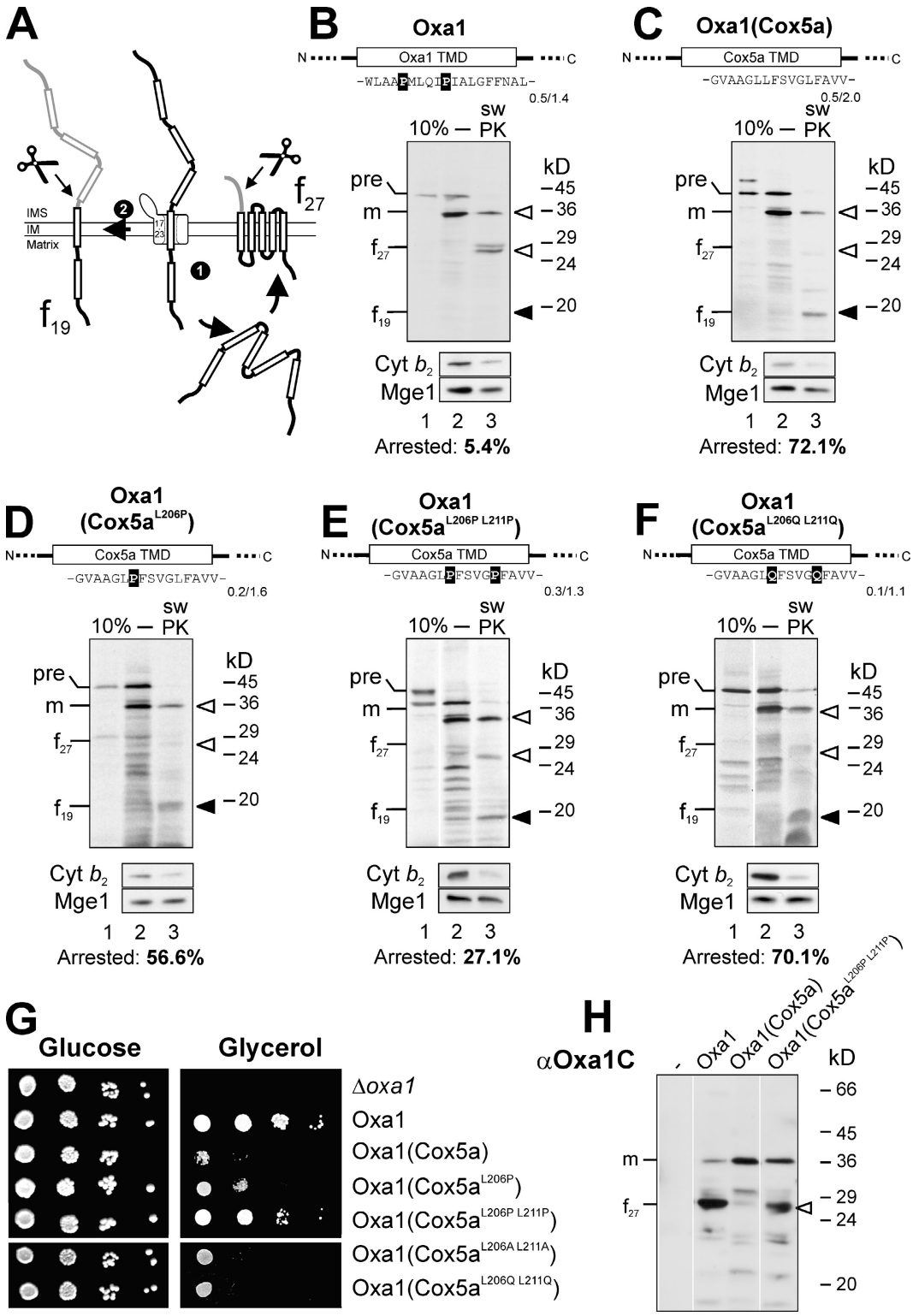

Figure 4. **The exchange of one hydrophobic domain leads to the arrest of the normally conservatively sorted protein Oxa1.** (A) Conservative sorting of Oxa1, a protein containing five TMDs (1). Correct insertion of Oxa1 allows proteolytic digestion to a COOH-terminal fragment of 27 kD (f_{27}). Translocation arrest at the second TMD and lateral insertion into the membrane is expected to lead to a fragment of ~19 kD (f_{19}, 2). IM, inner membrane; IMS, intermembrane space. (B–F) Radiolabeled Oxa1, Oxa1(Cox5a), Oxa1(Cox5a^{L206P}), Oxa1(Cox5a$^{L206P\ L211P}$), and Oxa1(Cox5a$^{L206Q\ L211Q}$) were imported for 10 min at 25°C into mitochondria and further processed as described for Fig. 2. White arrowheads depict the two protein species that represent products of the conservative sorting pathway. An f_{19} fragment derived from arrested preprotein is indicated by black arrowheads. (G) Oxa1-deficient yeast mutants were transformed with plasmids expressing the indicated Oxa1 variants. Growth of the cells on fermentable and nonfermentable carbon source was assessed as described for Fig. 3A. (H) Mitochondria were isolated from *Δoxa1* mutants expressing the proteins indicated. The outer membrane of the mitochondria was opened by hypotonic swelling, and the resulting mitoplasts were incubated with 100 μg proteinase K on ice. The full-length protein and COOH-terminal fragments (white arrowhead) of Oxa1 were detected by Western blotting of the resulting samples.

The presence of a proline residue in the first TMD of Oxa1 was critical for its transfer across the inner membrane into the matrix. Interestingly, this proline residue is well-conserved among animal and fungal members of the Oxa1 family (see Table SI). On the contrary, no proline residues are found in most of the first TMDs of bacterial homologues of Oxa1. Possibly, these proline residues appeared during evolution of mitochondria and have been maintained since that time. Proline residues also are present frequently in the first, and always present in the second, TMDs of mitochondrial members of the Yta10/Yta12 family (see Table SII). The strict conservation of the proline residue in the second TMD of Yta10/Yta12 proteins in fungi, animals, and plants indicates its general relevance. However, it is unclear whether this is due to the impact of proline residues on the intramitochondrial sorting, to functional constraints, or both.

The recently resolved three-dimensional structure of the Sec translocase provided exciting insights into the mechanisms by which a protein translocase can catalyze the membrane insertion of hydrophobic segments (Van den Berg et al., 2004). Further studies will elucidate how the TIM23 translocase deciphers the sorting signals in hydrophobic segments, and manages to function as a channel allowing passage of TMDs and as an insertase that mediates the transfer of such segments into the lipid phase of the inner membrane of mitochondria.

Materials and methods

Plasmid construction

For in vitro transcription/translation, the coding sequence of *COX5a* of *Saccharomyces cerevisiae* was amplified by PCR using the primers SM56 (5′-GGGAATTCACAATGTTACGTAACACTTTTAC-3′) and SM57 (5′-GGGTCGACATTGTATTGGGCGGATG-3′), and cloned into the EcoRI and SalI sites of the vector pGEM4 (Promega). For synthesis of Cox5aΔTM, the sequences encoding residues 1 through 97 and 114 through 153 were amplified by the primer pairs SM56/SM65 (5′-GCGGCGTCTAGAACCTTTGGCAATAAAACTGG-3′) and SM66 (5′-GCGGCGTCTAGAGACGCAAAGACCATGAATAAGG-3′)/SM57. The PCR products were digested with EcoRI, XbaI, and SalI and subcloned into pGEM4. For construction of the Cox5a(Oxa1) plasmid, the primer pairs SM56/SM75 (5′-CTAGAGTTTACTCTGGGTTGCCTTGGTGGGGAACTATCGCGGCCACCACCATCCTCATTGTTCGAATGGCGGGTGGCCAAG-3′), and SM76 (5′-CTAGCTTGGCCACCCGCCATTCGAACAATGAGGATGGTGGTGGCCGCGATAGTTCCCCACCAAGGCAACCCAGAGTAAACT-3′)/SM57 were used. The resulting fragments were digested with EcoRI, XbaI, and SalI and subcloned into pGEM4. Construction of a plasmid for in vitro transcription/translation of Oxa1 was described before (Herrmann et al., 1997).

For generation of the Oxa1(Cox5a) plasmid, the primer pairs Sp6/SM105 (5′-CGCGAGCTCAGCTACTCTATTCGGTTATTTGTTCGCTAACGCATTG-3′) and SM106 (5′-GTTGCTGCAGGCCTACTATTTTCAGTGGGACTTTTTGCTGTCGTCAACCATTGAGACACATGGCTAAC-3′)/T7 were used. The resulting fragments were cloned after digestion with BamHI, PstI, and KpnI into pGEM3 (Promega). For introduction of specific mutations, the Quickchange Site Directed Mutagenesis Kit was used (Stratagene). The wild-type *OXA1* gene, including 300 bp upstream and 300 bp downstream of the coding region, was cloned into the *Xho*I/*Not*I sites of the pRS314 vector. For expression of the different *oxa1* mutants in vivo, fragments of the pGEM3 vectors were released by cleavage with *Cla*I and *Eco*RI and subcloned into the *Cla*I/*Eco*RI sites of the *OXA1*-pRS314 vector. The resulting plasmids were transformed into an *oxa1* deletion mutant.

For generation of the Cox5a-Arg8 reporter constructs, the different Cox5a variants were amplified by PCR from the corresponding pGEM4 plasmids. The promoter region and the sequence encoding the mature form of Arg8 were amplified individually by PCR from genomic yeast DNA and cloned into pRS314 and pRS316 plasmids (Sikorski and Hieter, 1989) to allow expression of the Cox5a-Arg8 fusion proteins under control of the *ARG8* promoter. The resulting plasmids were transformed in *Δarg8* and *Δcox5a* yeast mutants. All constructs used were confirmed by DNA sequencing.

Protein import into isolated mitochondria

Mitochondria were isolated from the yeast wild-type strain W303A as described (Herrmann et al., 1994). Precursor proteins were synthesized in the presence of [^{35}S]methionine in reticulocyte lysate (Promega) according to the manufacturer's instructions. Import into isolated mitochondria of in vitro synthesized proteins was according to published procedures (Herrmann et al., 1997). Standard import reactions were performed in the presence of 2 mM NADH, 2 mM ATP, 10 mM creatine phosphate, and 0.1 mg/ml creatine kinase. Mitochondria were converted to mitoplasts by 10-fold dilution in ice-cold 20 mM Hepes, pH 7.4. Protease treatment was performed by addition of 50 μg/ml proteinase K to the reaction and incubation for 30 min at 0°C.

Enzyme assays

The activity of acetylornithine aminotransferase (Arg8) was assayed essentially as described (Vogel and Jones, 1970). 50 μg of purified mitochondria were incubated in 500 μl of 100 mM potassium phosphate, pH 8.0, 20 μM pyridoxal phosphate, 3.4 mM α-ketoglutarate, and 3 mM acetyl ornithine for 30 min at 30°C. After addition of 300 μl 6 M hydrochloric acid, the sample was boiled for 30 min in the dark, cooled to 25°C, and diluted with 1 ml 3.6 M sodium acetate and 200 μl 3.7 μM aminobenzaldehyde. After incubation for 15 min at 25°C, the absorption was measured at 440 nm, and the specific activity of acetylornithine aminotransferase was calculated using the extinction coefficient ε_{440} of pyrroline-5-carboxylate (1.163×10^6 cm^{-1} M^{-1}). For construction of plasmids and yeast strains see Supplemental material. All strains used in this study are derivatives of the wild-type strain W303-1A or BY4741. Yeast cultures were grown at 30°C in selective or full medium supplemented with 2% glucose, galactose, or glycerol (Herrmann et al., 1994).

Miscellaneous

Autoradiographic signals were quantified by densitometry using the Image Master 1D Elite software package (GE Healthcare). All signals were corrected for the respective methionine contents of the radiolabeled protein species. For calculation of the minimal and maximal hydrophobicity (Kyte and Doolittle, 1982) and helicity (Chou and Fasman, 1978) scores of the respective hydrophobic segments and the five NH_2-terminal flanking residues, the ProtScale tool (ExPASy, Swiss Institute of Bioinformatics) was used with a frame width of 17 amino acid residues.

Online supplemental material

Online supplemental material includes a genome-wide prediction of single-spanning mitochondrial inner membrane proteins lacking or containing proline residues in their TMDs. The presence of proline residues in the first TMD of mitochondrial Oxa1 homologues is shown. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200505126/DC1.

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