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Membrane Topology of the 60-kDa Oxa1p Homologue from *Escherichia coli**

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We have characterized the membrane topology of a 60-kDa inner membrane protein from *Escherichia coli* that is homologous to the recently identified Oxa1p protein in *Saccharomyces cerevisiae* mitochondria implicated in the assembly of mitochondrial inner membrane proteins. Hydrophobicity and alkaline phosphatase fusion analyses suggest a membrane topology with six transmembrane segments, including an N-terminal signal-anchor sequence not present in mitochondrial Oxa1p. In contrast to partial N-terminal fusion protein constructs, the full-length protein folds into a protease-resistant conformation, suggesting that important folding determinants are present in the C-terminal part of the molecule.

The nuclear gene *OXA1* was first isolated in *Saccharomyces cerevisiae* (1). Recent studies have shown that the Oxa1p protein is localized to mitochondria and is involved in the assembly of mitochondrial inner membrane proteins (2–5). Both nuclearly and mitochondrially encoded inner membrane proteins depend on Oxa1p for efficient export of their N- and C-terminal tails to the intermembrane space and have been shown to physically interact with Oxa1p (2, 4, 6). Oxa1p is believed to represent a component of a novel export machinery in the mitochondrial inner membrane that may also be present in bacteria (2, 4).

Oxa1p is synthesized as a precursor with an N-terminal presequence. After import into the mitochondrial matrix, the presequence is cleaved off by the mitochondrial processing peptidase, and the 90-amino acid-long N-terminal tail is translocated to the inter-membrane space in a process dependent on pre-existing Oxa1p (4, 7). Based on hydrophobicity analysis and limited proteolysis data, Oxa1p is thought to span the mitochondrial inner membrane five times with the C terminus in the matrix (7), although this model remains to be experimentally verified.

Oxa1p homologues have been found in both Gram-positive and Gram-negative bacteria (1, 8). A homologue of Oxa1p, ALB3, is present in chloroplasts and is involved in chloroplast biogenesis (8), and a human homologue has also been cloned (9).

To further characterize this potentially important family of proteins, we have determined the membrane topology of the *Escherichia coli* Oxa1p homologue ($Oxa1^{Ec}$), a 60-kDa inner membrane protein encoded by the *yidC* gene (10). Hydrophobicity and PhoA fusion analyses suggest a topology with six transmembrane (TM)[1] segments. In contrast to mitochondrial Oxa1p, $Oxa1^{Ec}$ has an uncleaved, N-terminal signal-anchor sequence. Interestingly, although most $Oxa1^{Ec}$-PhoA fusions are degraded by periplasmically added proteinase K, only a short N-terminal piece is removed from the wild-type and full-length fusion proteins. C-terminal parts of the protein are thus essential for folding and/or oligomerization into a protease-resistant form.

MATERIALS AND METHODS

Enzymes and Chemicals—Unless otherwise stated, all enzymes were from Promega. T7 DNA polymerase was from Amersham Pharmacia Biotech. Proteinase K was from Life Technologies, Inc. [^{35}S]Met was from Amersham Pharmacia Biotech. Oligonucleotides were from Kebo Lab (Stockholm, Sweden). PhoA antiserum was from 5 Prime → 3 Prime, Inc. (Boulder, CO). Hen egg white lysozyme, CHAPS (3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate), phenylmethylsulfonyl flouride, and the alkaline phosphatase chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate were from Sigma.

Strains and Plasmids—Experiments were performed in *E. coli* strains MC1061 (Δ*lacX74, araD139,* Δ*(ara,leu)7697, galU, galK, hsr, hsm, strA*) (11), CC118 (Δ*(ara-leu)7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(am) *recA1*) (12) and TOP10F′ (F′(tet^r) (*mrr-hsdRMS-mcrBC*) *lacZ*Δ*M15 rpsL* (Sm^r) *endA1*) (Stratagene). Cloning of the $Oxa1^{Ec}$ gene was performed using the pGEM-T Easy Vector System 1 (Promega). All constructs were expressed in *E. coli* from the pING1 plasmid (13) by induction with arabinose.

DNA Techniques—All plasmid constructs were confirmed by DNA sequencing using T7 DNA polymerase. The *yidC* gene encoding $Oxa1^{Ec}$ was amplified by PCR from *E. coli* TOP10F′ chromosomal DNA. The naturally occurring *Kpn*I site was removed by introducing a silent mutation using the "double PCR" approach and a 5′ *Xho*I and a 3′ *Kpn*I site were introduced in the regions flanking the *yidC* open reading frame. The PCR product was first cloned into the pGEM-T Easy Vector System 1, excised using *Xho*I and *Kpn*I, and cloned behind the *ara* promoter in a *Xho*I-*Kpn*I restricted plasmid derived from pING1 containing a *lep* gene with a 5′ *Xho*I site just upstream of the initiator ATG and a *Kpn*I site in codon 78. Relevant parts of the *yidC* gene were amplified by PCR from the pING1 plasmid with a 5′ *Sal*I and a 3′ *Kpn*I site encoded in the primers. Finally, the PCR *Sal*I-*Kpn*I fragment carrying the *lep* upstream region and the relevant *yidC* segment were cloned into a previously constructed plasmid (14) carrying a *phoA* gene lacking the 5′ segment coding for the signal sequence and the first 5 residues of the mature protein and immediately preceded by a *Kpn*I site. In all constructs, an 18-amino acid linker (VPDSYTQVASWTEPF-PFC) was present between the $Oxa1^{Ec}$ and PhoA moieties.

Protease Protection Assay—*E. coli* strain MC1061 transformed with the pING1 vector carrying the relevant constructs under control of the arabinose promoter was grown at 37 °C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, 100 μg/ml thiamin, and all amino acids (50 μg/ml each) except methionine. An overnight culture was diluted 1:25 in fresh medium, shaken for 3.5 h at 37 °C, induced with arabinose (0.2%) for 5 min, and labeled with [^{35}S]methionine (75 μCi/ml). After 1 min, nonradioactive methionine was added (final concentration 500 μg/ml) and stopped by chilling on ice. For the

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[1] The abbreviations used are: TM, transmembrane; CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate; PCR, polymerase chain reaction.

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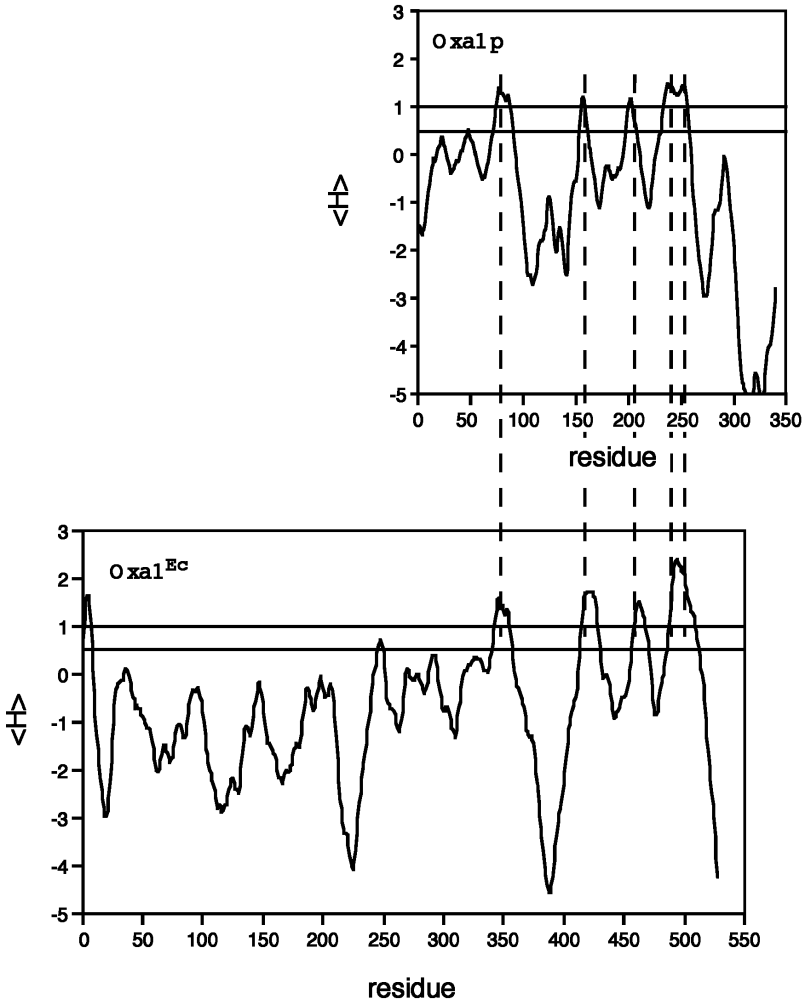

FIG. 1. **Hydrophobicity plots for *S. cerevisiae* Oxa1p (*top*) and *E. coli* Oxa1^{Ec} (*bottom*).** The two plots have been aligned on the region homologous between the two proteins. Corresponding TMs are indicated by *dashed lines*. Plots were made using TOPPRED II (25) with default parameter settings and using the Engelman-Steitz hydrophobicity scale (26). The *two horizontal lines* in each plot indicate the cutoffs for predicting "certain" (*upper line*) and "putative" (*lower line*) transmembrane segments.

experiment in Fig. 4*C*, pulse labeling was done for 15 s. Cells were spun in an Eppendorf bench-top centrifuge at 14,000 rpm for 2 min, resuspended in ice-cold buffer (40% w/v sucrose, 33 mM Tris-HCl, pH 8.0), and incubated with lysozyme (5 mg/ml) and 1 mM EDTA for 15 min on ice. Aliquots of the cell suspension were incubated 1 h on ice, either with no additions or with the addition of 400 μg/ml proteinase K (15). After addition of phenylmethylsulfonyl flouride, samples were acid-precipitated (trichloroacetic acid, 10% final concentration), resuspended in 10 mM Tris, pH 7.5, 2% SDS, immunoprecipitated with antisera to PhoA, Lep, or Oxa1^{Ec} as required, washed, and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were scanned in a FUJIX Bas 1000 phosphoimager and analyzed using the MacBAS software (version 2.31).

PhoA Activity Assay—Alkaline phosphatase activity was measured by growing strain CC118 transformed with the appropriate pING1-derived plasmids in liquid culture in the presence of 0.2% arabinose (16). Mean activity values were obtained from two independent measurements and were normalized by the rate of synthesis of the fusion protein determined by pulse labeling of arabinose-induced CC118 cells for 2 min followed by immunoprecipitation and quantitation by phosphoimager analysis. Normalized activities were calculated as: $A = (A_0 \times A_{600} \times n_{Met})$/counts/min, where A_0 is the measured activity, A_{600} is the cell density at the time of pulse labeling, n_{Met} is the number of Met residues in the fusion protein, and counts/min is the intensity of the relevant band measured on the phosphoimager.

Preparation of Oxa1^{Ec} Antiserum—A rabbit antiserum was raised by Agrisera AB, Umeå, Sweden, against a synthetic peptide comprising the last 17 amino acids of Oxa1^{Ec} (YRGLEKRGLHSREKKKS).

RESULTS

Hydrophobicity Analysis Predicts Five to Seven Transmembrane Segments—The C-terminal membrane domain of Oxa1p is conserved from prokaryotes to eukaryotes (1, 8), and is thus very likely to have the same topology in different organisms.

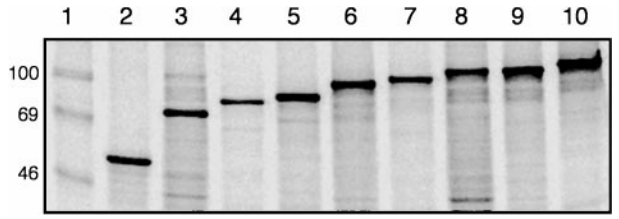

FIG. 2. **Expression of Oxa1^{Ec}-PhoA fusions in strain CC118.** Cells were pulse-labeled for 2 min with [^{35}S]Met, and the fusion proteins were immunoprecipitated by a PhoA antiserum. Molecular masses in kilodaltons are indicated on the *left*. *Lane 1*, molecular mass markers; *lane 2*, Oxa1^{Ec}(40)-PhoA; *lane 3*, Oxa1^{Ec}(225)-PhoA; *lane 4*, Oxa1^{Ec}(290)-PhoA; *lane 5*, Oxa1^{Ec}(340)-PhoA; *lane 6*, Oxa1^{Ec}(415)-PhoA; *lane 7*, Oxa1^{Ec}(458)-PhoA; *lane 8*, Oxa1^{Ec}(494)-PhoA; *lane 9*, Oxa1^{Ec}(512)-PhoA; *lane 10*, Oxa1^{Ec}(548)-PhoA.

Hydrophobicity analysis of Oxa1p and Oxa1^{Ec} does not give a clear-cut prediction of the number of transmembrane (TM) segments, however, and models with five to seven TMs are possible, Fig. 1. In particular, it cannot be determined whether the weakly predicted TM around residue 250 is real and whether the C-terminal hydrophobic region around residue 500 corresponds to one or two TMs (although the Oxa1p hydrophobicity plot would suggest two closely spaced TMs in this region).

PhoA Fusions Analysis Supports a Topology with Six Transmembrane Segments—To experimentally determine the membrane topology of Oxa1^{Ec}, we made a series of Oxa1^{Ec}-PhoA fusions (17). As two critical disulfide bonds are necessary for PhoA activity (18), PhoA will only be active when located in the

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oxidizing environment of the periplasm but not when located in the cytoplasm. To retain topological information in the cytoplasmic and periplasmic loops (19), PhoA fusions were made near the C-terminal end of predicted loops in Oxa1^{Ec}. The fusion proteins were expressed in the PhoA^{-} strain CC118, Fig. 2, and alkaline phosphatase activities were measured in liquid culture.

TABLE I
Alkaline phosphatase activity of Oxa^{Ec}-PhoA fusions

Fusion (fusion-joint codon indicated)	Alkaline phosphatase activity	Rate of synthesis	Normalized activity
	units	*arbitrary units*	
Oxa^{Ec}(40)-PhoA	758	11.4	66
Oxa^{Ec}(225)-PhoA	690	7.5	92
Oxa^{Ec}(290)-PhoA	1064	5.4	197
Oxa^{Ec}(340)-PhoA	691	9.9	70
Oxa^{Ec}(415)-PhoA	64	10.7	6
Oxa^{Ec}(458)-PhoA	267	3.6	74
Oxa^{Ec}(494)-PhoA	17	3.7	6
Oxa^{Ec}(509)-PhoA	282	1.7	160
Oxa^{Ec}(512)-PhoA	254	5.9	42
Oxa^{Ec}(515)-PhoA	225	5.4	42
Oxa^{Ec}(538)-PhoA	84	9.3	9
Oxa^{Ec}(548)-PhoA	163	9.8	17

Alkaline phosphatase activities and relative expression levels measured for the various fusions are given in Table I. Fusions in the region between the first hydrophobic domain and residue 340 all have high normalized activities, suggesting a periplasmic location. Two additional periplasmic loops are identified by the high activity fusions in positions 458 and 512. Low activity fusions in positions 415, 494, and at the C terminus (positions 548) identify cytoplasmic parts. To confirm the topology in the C-terminal part of the protein, two additional fusions were made in the last periplasmic loop (positions 509 and 515; both have high activity) and in the C-terminal tail (position 538; low activity); these fusion proteins are not shown in Fig. 2.

These data strongly suggest a topology with an N-terminal TM, a large periplasmic domain, and five additional, closely spaced C-terminal TMs.

TM1 Is an Uncleaved Signal-Anchor Sequence—Since TM1 of Oxa1^{Ec} is not present in mitochondrial Oxa1p, we wanted to

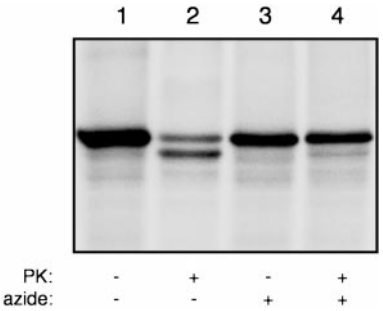

FIG. 3. **TM1 is an uncleaved signal-anchor sequence.** Fusion Oxa1^{Ec}(40)-PhoA was expressed in strain MC1061 in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of sodium azide. Spheroplasts were treated with proteinase K (*PK*), and the fusion protein was immunoprecipitated by a PhoA antiserum.

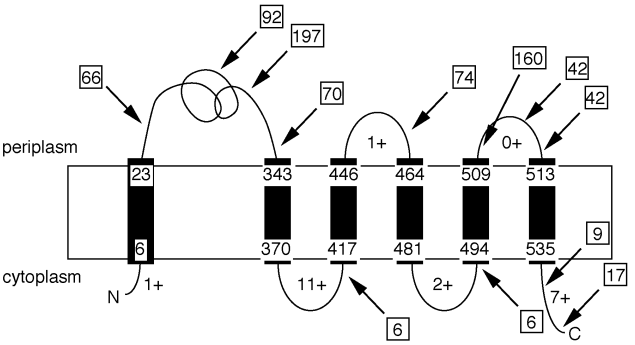

FIG. 5. **Topology model for Oxa1^{Ec}.** PhoA fusions are indicated by *arrows*, together with their respective normalized alkaline phosphatase activities. Approximate ends of the transmembrane segments as well as the number of basic residues (Lys + Arg) in the tails and loops are indicated. Note that the loop between TM1 and TM2 is too long to be relevant in a charge-bias calculation (24).

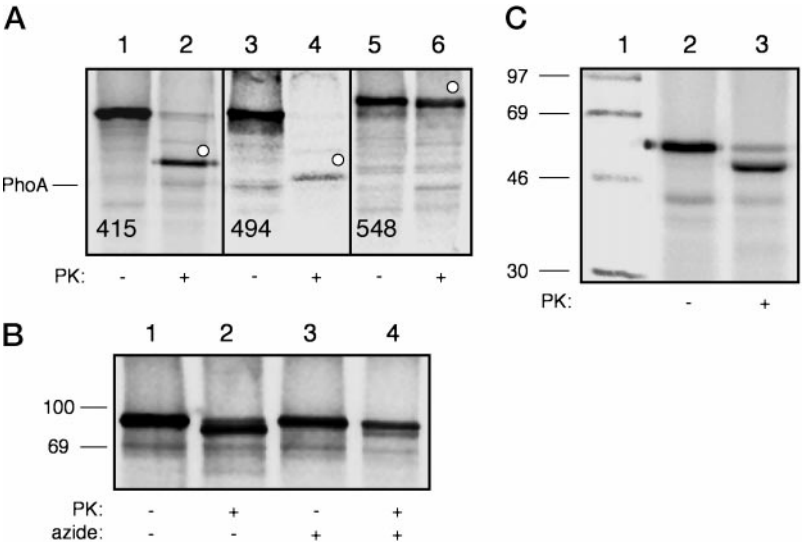

FIG. 4. **Full-length Oxa1^{Ec} is protease-resistant.** *A*, the indicated Oxa1^{Ec}-PhoA fusions were expressed in strain MC1061 and pulse-labeled with [^{35}S]Met for 1 min. Spheroplasts were treated with proteinase K (*lanes 2*, *4*, and *6*), and fusion proteins were immunoprecipitated with a PhoA antiserum. Protease-resistant fragments are indicated by a *white dot*, and the position of the mature PhoA domain (47 kDa) is indicated on the *left*. *B*, the Oxa1^{Ec}(548)-Lep(P2) fusion was expressed in strain MC1061 in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of sodium azide and pulse-labeled with [^{35}S]Met for 1 min. Spheroplasts were then treated with proteinase K (*lanes 2* and *4*), and the fusion protein was immunoprecipitated with a Lep antiserum. Molecular masses (in kilodaltons) are indicated on the *left*. *C*, wild-type Oxa1^{Ec} was expressed in strain TOP10F′ and pulse-labeled with [^{35}S]Met for 15 s. Spheroplasts were either not treated or treated with proteinase K (*lanes 2* and *3*), and the protein was immunoprecipitated with an antiserum raised against a C-terminal Oxa1^{Ec} peptide. Molecular mass standards (in kilodaltons) are shown in *lane 1*.

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determine whether it is a cleavable signal peptide or an uncleaved signal-anchor. To this end, the shortest PhoA fusion (at residue 40) was expressed in the absence and presence of sodium azide, an inhibitor of the SecA ATPase activity (20) that blocks the translocation of large periplasmic domains in inner membrane proteins and thus also prevents cleavage of signal peptides by the periplasmically exposed leader peptidase enzyme (21). As shown in Fig. 3, there is no difference in size of the protein expressed in the absence (*lane 1*) or presence (*lane 3*) of azide, indicating that TM1 is not cleaved by leader peptidase during or after translocation. Proteinase K treatment of spheroplasts confirms that the PhoA domain is translocated to the periplasm in the absence (*lane 2*) but not in the presence (*lane 4*) of azide. Note that periplasmic, properly folded PhoA is intrinsically protease-resistant (15), and that proteinase K thus only removes the TM1 segment from the fusion protein (lane 2). As expected, the Oxa1^{Ec}(40)-PhoA is found in the membrane fraction after sonication of the cells (data not shown).

Full-length Oxa1^{Ec} Folds into a Protease-resistant Conformation—Interestingly, only two of the cytoplasmic Oxa1^{Ec}-PhoA fusions are sensitive to proteinase K treatment of spheroplasts and give rise to protease-protected fragments of sizes expected for cleavage in the N-terminal periplasmic domain and in the periplasmic loop between TM3 and TM4, respectively, Fig. 4*A* (*lanes 1-4*). In contrast, the full-length fusion (at residue 548) is largely resistant to proteinase K (*lanes 5* and *6*).

To confirm the unexpected protease resistance of Oxa1^{Ec}, we carried out further protease protection experiments both on the wild-type protein and on an Oxa1^{Ec}-Lep(P2) fusion where the C-terminal, periplasmic P2 domain of the *E. coli* inner membrane protein leader peptidase (Lep) has been fused to residue 548 in Oxa1^{Ec}. As shown in Fig. 4*B*, only a small N-terminal fragment (roughly corresponding to TM1) is removed from the Oxa1^{Ec}-Lep(P2) fusion (*lanes 1* and *2*), and the protein is almost completely protease-resistant when expressed in the presence of azide (*lanes 3* and *4*); incidentally, the protease resistance of the Lep P2 domain further supports the cytoplasmic localization of the C terminus of Oxa1^{Ec}, since the P2 domain is readily digested by proteinase K when located in the periplasm (22). Similarly, only a small N-terminal fragment of ~7 kDa (corresponding to ~65 residues) is removed from the 59-kDa wild-type protein upon proteinase K treatment of spheroplasts, Fig. 4*C* (note that the antibody used was raised against a C-terminal Oxa1^{Ec} peptide). Obviously, the periplasmically exposed parts of Oxa1^{Ec} fold into a protease-resistant conformation, but only in the full-length protein. Possibly, this reflects assembly into an oligomeric complex, as has been suggested to occur for mitochondrial Oxa1p (4).

DISCUSSION

Mitochondrial Oxa1p has been implicated in the translocation of N- and C-terminal tails of mitochondrial inner membrane proteins from the matrix to the intermembrane space (2, 4, 6). Since *S. cerevisiae* mitochondria do not contain a Sec-type protein translocation machinery (23), Oxa1p is thought to be a component of a new protein translocation pathway present also in bacteria, possibly specialized in the assembly of inner membrane proteins.

We have determined the membrane topology of the 60-kDa Oxa1p homologue present in *E. coli* (Oxa1^{Ec}). Hydrophobicity and PhoA fusion analyses support a N_{cyt}-C_{cyt} topology with six transmembrane segments, Fig. 5. The distribution of positively charged residues (Arg, Lys) conforms to the "positive inside" rule (24), lending further support to the proposed topology.

Mitochondrial Oxa1p lacks a TM domain at the N terminus but otherwise has a similar hydrophobicity profile as Oxa1^{Ec} (Fig. 1). In agreement with the topology model proposed here for Oxa1^{Ec}, previous work has shown that the large N-terminal domain and the loop between the second and third TM in Oxa1p is exposed to the intermembrane space (7). It is thus likely that Oxa1p and Oxa1^{Ec} have the same topology beyond the N-terminal TM present only in Oxa^{Ec}.

Interestingly, despite having most of its mass exposed to the periplasm, Oxa1^{Ec} folds into a protease-resistant conformation where only the most N-terminal TM can be removed by proteinase K treatment of spheroplasts. Protease resistance is only seen in the full-length protein, suggesting that important folding determinants are present in the C-terminal ~50 residues and that full-length Oxa1^{Ec} might be part of an oligomeric complex, as has been proposed for the mitochondrial Oxa1p (4).

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