N-terminal Tail Export from the Mitochondrial Matrix

ADHERENCE TO THE PROKARYOTIC "POSITIVE-INSIDE" RULE OF MEMBRANE PROTEIN TOPOLOGY*

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Export of N-terminal tails of mitochondrial inner membrane proteins from the mitochondrial matrix is a membrane potential-dependent process, mediated by the Oxa1p translocation machinery. The hydrophilic segments of these membrane proteins, which undergo export, display a characteristic charge profile where intermembrane space-localized segments bear a net negative charge, whereas those remaining in the matrix have a net positive one. Using a model protein, preSu9(1–112)-dihydrofolate reductase (DHFR), which undergoes Oxa1p-mediated N-tail export, we demonstrate here that the net charge of N- and C-flanking regions of the transmembrane domain play a critical role in determining the orientation of the insertion process. The N-tail must bear a net negative charge to be exported to the intermembrane space. Furthermore, a net positive charge of the C-terminal region supports this N-tail export event. These data provide experimental evidence that protein export in mitochondria adheres to the "positive-inside" rule, described for sec-independent sorting of membrane proteins in prokaryotes. We propose here that the importance of a charge profile reflects a need for specific protein-protein interactions to occur in the export reaction, presumably at the level of the Oxa1p export machinery.

Proteins may be targeted to the mitochondrial inner membrane by one of a number of distinct sorting pathways. Many nuclear-encoded proteins become directly sorted to the inner membrane during the process of import into the mitochondria. A number of inner membrane proteins, however, achieve their correct orientation in the membrane via an insertion step from the matrix side of the membrane. This process involves the export to the intermembrane space of hydrophilic N- or C-terminal tails and often of hydrophilic loop structures between neighboring transmembrane domains.

We have focused previously in some depth on the process of export of N-terminal tails of nuclear and mitochondrial-encoded proteins from the matrix into the intermembrane space. This export pathway facilitates the insertion of membrane proteins encoded by the mitochondrial genome as well as a subset of nuclear-encoded proteins. The nuclear-encoded proteins sorted by this export pathway are initially targeted to the mitochondria by N-terminal presequences. These proteins are imported across the inner membrane and into the mitochondrial matrix in a membrane potential (Δψ)- and mt-Hsp70/ATP-dependent fashion. Following maturation by the mitochondrial-processing peptidase (MPP),1 insertion into the inner membrane leading to export of hydrophilic domains to the intermembrane space occurs. Export from the matrix requires a membrane potential (1–3) and, at least for those proteins that contain their N termini in the intermembrane space, is mediated by the Oxa1p export machinery (4–6).

Our observations on protein export in mitochondria were reminiscent of previous findings on N-terminal tail export in prokaryotic systems. There, it had been noted that the periplasmic N-tails of bacterial membrane proteins synthesized without leader sequences are exported in a sec-independent manner. These exported domains characteristically do not bear a net positive charge, but rather have a net negative one or are overall uncharged. This was found to be in agreement with the positive-inside rule of prokaryotic membrane protein topology, where positively charged amino acids tend to be more prevalent in the cytoplasmic than periplasmic regions flanking the transmembrane domains (7–11). Furthermore, introduction of positively charged amino acid residues into the N-tails of these bacterial proteins led to an inhibition of their export from the cytoplasm (12, 13).

It was previously noted that some proteins of the mitochondrial inner membrane display a charge distribution profile compatible with the "positive-inside rule"; their matrix-exposed segments contain with high frequency positively charged residues, whereas segments exposed to the intermembrane space bear a net negative or neutral charge (14). Indeed, all mitochondrial inner membrane proteins known to be sorted by the Oxa1p export pathway display this characteristic charge distribution profile. Does this charge profile determine the orientation of these proteins in the inner membrane? Does the sorting mechanism of these mitochondrial proteins therefore adhere to the positive-inside rule described for prokaryotes?

To study the importance of the charge distribution in the process of mitochondrial N-terminal tail export, we analyzed the sorting of a fusion protein, preSu9(1–112)-DHFR (2, 15). This protein consists of the N-terminal region of subunit 9 from the F,PO-ATPase (Su9) of Neurospora crassa encompassing the presequence and the first half of the mature part of the protein containing a single transmembrane segment. As previously shown, preSu9(1–112)-DHFR is initially imported into the mitochondrial matrix, where it is processed by MPP. The N-tail becomes exported across the inner membrane in a membrane

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1 The abbreviations used are: MPP, mitochondrial-processing peptidase; DHFR, dihydrofolate reductase; o-phe, o-phenanthroline; CCCP, carbonyl cyanide p-chlorophenylhydrazone.
potential and Oxa1p-dependent fashion to give rise to an Nterm-Cint topology (2, 6). The exported N-terminal tail of mSu9(1–112)-DHFR bears a net negative charge.

We demonstrate here that the nature of the charges flanking the transmembrane segment play a crucial role in determining the orientation the protein attains in the inner membrane. From our data, we conclude that mitochondrial protein export mediated by the Oxa1p machinery adheres to the positive-inside rule described for prokaryotic, sec-independent, protein export.

EXPERIMENTAL PROCEDURES
Recombinant DNA Techniques, Mutagenesis, and Plasmid Constructions
The recombinant DNA techniques applied were essentially as described previously (16). The precursor of the nuclear-encoded N. crassa subunit 9 was used in this study. preSu9(1–112)-DHFR and its mutant derivatives were obtained using a polymerase chain reaction site-directed mutagenesis procedure with one single mutated primer and two wild-type external primers, as described previously (17). Multiple point mutants were obtained by repeated rounds of mutagenesis. Final polymerase chain reaction products were subcloned in front of DHFR as EcoRI/SacI fragments in pGEM4 vectors and transcribed in vitro with SP6 RNA polymerase.

Isolation of Mitochondria and Protein Import
Standard procedures for cell growth (Saccharomyces cerevisiae wild-type, D273-10B) and mitochondrial isolation were used (18). preSu9(1–112)-DHFR and its mutant derivatives were synthesized in rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine, as described previously (19). Import was performed as described previously (2, 20). Import in the presence EDTA and o-phenanthroline and their subsequent chase by the addition of Mg2+/Mn2+ was performed as described previously (2). Following import, samples were treated when indicated with trypsin (40 μg/ml) for 15 min on ice. This reaction was stopped by the addition of (0.2 mg/ml) soybean trypsin inhibitor. Hypotonic swelling of mitochondria was performed to determine the extent of export of the N-terminal tail of mSu9(1–112)-DHFR (2). Mitoplasts/matrix mitochondria were re-isolated and then lysed directly in SDS-sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting to nitrocellulose. The efficiency of swelling (>95%) was assessed following immunodecoration of the blot with anti-trans against endogenous cytochrome c peroxidase (soluble intermembrane space protein) and Mge1p (matrix-located protein) (not shown).

Subfractionation of Mitochondria
Sonication—Mitochondria were converted to mitoplasts by hypotonic swelling and were sonicated in the presence of salt (300 mM KCl) (Branson Sonifier 250, microtip, step 1–2, 80% duty, 10 × 10-s pulse) on ice. Samples were divided; one-half was trichloroacetic acid-preincubated directly (total), and the other half was centrifuged for 30 min at 226,000 × g at 2 °C (TL-45, Beckman TL-100). Both pellet and supernatant fractions were also trichloroacetic acid-precipitated.

Lysis with Detergent—After import and trypsin treatment, mitochondria were lysed in 500 μl of 20 mM HEPES, pH 7.4, 0.1% (v/v) Triton X-100, 150 mM NaCl. One-half of the sample was trichloroacetic acid-precipitated (total), and the other half was centrifuged for 30 min at 226,000 × g at 2 °C (TL-45, Beckman TL-100). The resulting pellet and supernatant fractions were trichloroacetic acid-precipitated.

Miscellaneous
The following procedures were performed according to published methods: protein concentration determination (21), SDS-polyacrylamide gel electrophoresis (22), and Western blotting (23). The detection of endogenous proteins after blotting onto nitrocellulose was performed using the ECL detection system (Amersham Pharmacia Biotech).

RESULTS
Increasing the Negative Charge of N-terminal Tail Influences Both the Efficiency and Rate of Its Export across the Inner Membrane—The N-terminal tail of mSu9(1–112)-DHFR bears a net negative charge (−1), as it contains two glutamic acid residues (Glu-70 and Glu-77) and one lysine residue (Lys-80). To address the importance of the net negative charge in the

N-terminal Tail Export from the Mitochondrial Matrix

To address the role of the negatively charged amino acids in the sorting of the N-terminal tail, the glutamic acids at either position 70 or 77 were exchanged individually for a lysine (E70K or E77K, respectively, Fig. 2A). After import of the resulting fusion proteins into isolated mi.
Su9(1–112)-DHFR and Su9(1–112)K80N-DHFR: the intermembrane space.

A 100 mM preSu9(1–112)-DHFR and preSu9(1–112)K80N-DHFR followed by trypsin treatment was performed as described in

value obtained for the export of preSu9(1–112)K80N-DHFR.

converted to mitoplasts and treated with proteinase K as indicated.

mitochondria in the presence of EDTA and

f 

m

112)K80N-DHFR (Fig. 4 B) though, did inhibit export, as presented in the N-terminal tail to the intermembrane space is inhibited when it bears a net positive charge; rather, these mutated species remain in the matrix, in association with the inner side of the inner membrane.

The N-tail Must Bear a Net Negative Charge to Be Exported—Is a net negative charge required for export of the N-tail? To address this question, the charge of the N-terminal tail was altered to zero. To this end, the glutamic acid residue at position 77 was mutated to an asparagine (E77N) (Fig. 3 A). preSu9(1–112)K80N-DHFR was imported into isolated mitochondria, and export to an Nout-Cin orientation across the inner membrane was assessed by protease treatment of mitoplasts following import. In contrast to the corresponding wild-type protein, preSu9(1–112)K80N-DHFR did not become correctly sorted but remained protease-inaccessible in the matrix. In conclusion, a negatively charged N-terminal tail is essential for export from the matrix.

Role of the Net Positive Charge C-terminal to the Transmembrane Segment—The C-terminal region of the transmembrane domain of preSu9(1–112)-DHFR is flanked by amino acid residues with a net charge of +1. To determine the importance of this C-terminalcharged region, an arginine residue (Arg-111) was initially replaced with an aspartic acid residue, resulting in preSu9(1–112)K8111D-DHFR. Radiolabeled preSu9(1–112)R8111D-DHFR was imported into isolated mitochondria where it became sorted in a similar manner as its wild-type counterpart (Fig. 4 B). Thus the exchange of the single positively charged residue for a negative one C-terminal to the transmembrane segment did not influence the import and the sorting of the N terminus of Su9. The introduction of a second aspartic acid residue at position 112 (preSu9(1–112)R8111D,N112D-DHFR, Fig. 4 A) though, did inhibit export, as the resulting imported species remained in the matrix following import (Fig. 4 B). Residue 8 of the DHFR protein is an arginine and taking this into account, the net charge of the C-terminal region of the transmembrane domain from the wild-type protein is thus +2. Thus the C-terminal-flanking regions

tochondria, both proteins, preSu9(1–112)E70K-DHFR and preSu9(1–112)E77K-DHFR, accumulated as MPP-processed species in the matrix. They failed to undergo N-terminal tail export in contrast to the wild-type protein (Fig. 2B). mSu9(1–112)E70K-DHFR and mSu9(1–112)E77K-DHFR were membrane-associated, as they were recovered exclusively in the pellet fraction after sonication of the mitochondria and centrifugation (Fig. 2C). These proteins were not aggregated in the matrix because wild-type and mutant variants were recovered in the supernatant after lysis of the mitochondria with detergent (Fig. 2D).

In Escherichia coli, the membrane potential has been postulated to oppose the translocation of positive charges across the plasma membrane (24). In certain instances, the dissipation of the membrane potential before insertion made possible the translocation of a positively charged N-tail engineered by in vitro mutagenesis (24). Does a similar situation occur in mitochondria? To address this question, preSu9(1–112)E70K-DHFR and preSu9(1–112)E77K-DHFR were imported in the presence of EDTA/o-phe, where they accumulated as precursors in the matrix (Fig. 2E). Following the restoration of MPP activity by the addition of divalent cations, the proteins were chased either in the presence (plus NADH) or absence of a membrane potential (plus CCCP). The export of both mSu9(1–112)E70K-DHFR and mSu9(1–112)E77K-DHFR remained inhibited even in the absence of the proposed barrier of a membrane potential. The effectiveness of the CCCP treatment was indicated by the efficient inhibition of the export of mSu9(1–112)K80N-DHFR (data not shown).

Taken together, export of an N-terminal tail to the intermembrane space is inhibited when it bears a net positive charge; rather, these mutated species remain in the matrix, in association with the inner side of the inner membrane.

N-terminal Tail Export from the Mitochondrial Matrix

FIG.1. preSu9(112)K80N-DHFR is targeted more efficiently to the intermembrane space. A, schematic representation of mature Su9(1–112)-DHFR and Su9(1–112)K80N-DHFR: black box, transmembrane domain; flanking charged amino acid residues are indicated with their position in the protein sequence. For simplification, the presense was omitted. B, preSu9(1–112)-DHFR (WT) and preSu9(1–112)K80N-DHFR (K80N) were imported into isolated mitochondria at 25 °C for the times indicated. Mitoplasts were generated in the presence of proteinase K (PK, 100 μg/ml) (swelling + PK) to assess export of the N terminus by generation of the fragment (m). C, preSu9(1–112)-DHFR and preSu9(1–112)K80N-DHFR were imported into mitochondria in the presence of EDTA and o-phe. Following trypsin treatment, mitochondria were re-isolated and resuspended in fresh import buffer and subjected to a second incubation in the presence of Mg2+/Mn2+ (to reactivate MPP), NADH, and ATP. Mitochondria were converted to mitoplasts and treated with proteinase K as indicated. Export of the N terminus is expressed as the percentage of the maximal value obtained for the export of preSu9(1–112)K80N-DHFR. D, import of preSu9(1–112)-DHFR and preSu9(1–112)K80N-DHFR followed by trypsin treatment was performed as described in C. Chase in the presence of Mg2+/Mn2+ was performed either in the presence of 2 mM NADH or 100 μM CCCP or no additions, as indicated. N-terminal tail export in each case is given as percentage of total imported species.

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of preSu9(1–112)E70K,E77K,DHFR and preSu9(1–112)E70K,E77K,DHFR were imported into isolated mitochondria as described in Fig. 1B, F, proteinase K, C, preSu9(1–112)DHFR, preSu9(1–112)R111D,DHFR, and preSu9(1–112)R111D,N112D-DHFR were imported into isolated mitochondria at 25 °C as in C. Following trypsin treatment, mitochondria were lysed in Triton X-100 (0.1%)-containing buffer. One-half of each sample was trichloroacetic acid precipitated (total (T)), and the other half was centrifuged to give rise to pellet (P) and supernatant (S) fractions. Samples were further treated as described in Fig. 1C.

FIG. 3. A neutral N-terminal tail is not exported. A, schematic representation of mature Su9(1–112)E77N-DHFR. B, preSu9(1–112)-DHFR and preSu9(1–112)E77N-DHFR were imported in buffer A (see “Experimental Procedures”) into isolated mitochondria. Mitochondria were converted to mitoplasts (“swelling”) and, where indicated, samples were treated with proteinase K (PK). p, precursor form; m, mature form; f, fragment; WT, wild type.

FIG. 4. Role of the charges C-terminal to the transmembrane domain in the sorting of the N-terminal tail. A, schematic representation of mature Su9(1–112)-DHFR, Su9(1–112)R111D,DHFR, and Su9(1–112)R111D,N112D-DHFR. B, preSu9(1–112)-DHFR (WT), preSu9(1–112)R111D,DHFR (R111D), and preSu9(1–112)R111D,N112D-DHFR (R111D, N112D) were imported into isolated mitochondria. Samples were further treated as described in Fig. 2B, F, proteinase K, P, precursor form; m, mature form; f, fragment; WT, wild type.

the matrix (Nin,Cout), we performed import under conditions where MPP processing was inhibited by the addition of the chelators EDTA/phe. By doing so, an N-terminal fragment located in the matrix (Nin-Cout) would result in the attainment of a reverse orientation of the protein in the membrane. Samples were further treated as described in Fig. 1C.
imported into isolated mitochondria in the presence of EDTA/ 
{o}-phe for various times, after which the localization of imported 
species was ascertained by hypotonic swelling in the presence 
of added protease K (Fig. 5, left panel). Imported preSu9(1– 
112)-DHFR accumulated across the inner membrane 
with an N_{in}-C_{out} orientation, as indicated by the 
generation of the f_{12} fragment protease protected in the matrix, 
whereas the bulk of the protein was accessible to protease in 
mitoplasts. This topology was maintained throughout the 
etire import period, as the f_{12} fragment accumulated in a stable 
manner (Fig. 5, left panel). Complete import into the matrix 
of this protein was not observed. A similar fragment was also 
observed during import of preSu9(1–112)-DHFR, however, only 
at the early times of import (Fig. 5, right panel). Thus, the 
wild-type derivative appears to pause during translocation 
across the inner membrane but proceeds to complete import 
into the matrix at later time points.

We conclude that reversal of the charges flanking the transmembrane 
segment of preSu9(1–112)-DHFR results in a reverse topology across the 
mitochondrial inner membrane. The kinetic analysis of import suggests, though, that the reversed topology is achieved by translocation arrest during import 
rather than by export of the C-terminal domain from the matrix 
into the intermembrane space.

**DISCUSSION**

Export of N-terminal tails of proteins from the mitochondrial matrix 
to the intermembrane space is a process that is accompa-
nied by the insertion of a hydrophobic segment of amino 
acids (usually a transmembrane domain) into the inner mem-
brane. What determines the translocation of the N-terminal, 
rather than the C-terminal, flanking region?

Our results show that the charged amino acid residues flanking 
both sides of the hydrophobic domain play a critical role in 
the directionality of the insertion process. The N-terminal tail 
of our model protein, preSu9(1–112)-DHFR, bears a net nega-
tive charge, whereas the region C-terminal to the hydrophobic 
stretch is positively charged. Increasing the net negative 
charge of the N-terminal tail resulted in a more efficient and 
rapid export from the matrix. Altering the charge to a net 
positive one or neutralizing it resulted in export inhibition and 
retention in the matrix. Thus the sorting of N-terminal tails 
in mitochondria apparently adheres to a principle similar to that 
of N-tail export in prokaryotes, where the positive-inside rule 
has been defined to describe the underlying mechanism (7, 8).

What role do the negative charges in the N-terminal tail 
play? Export of N-tails across the inner mitochondrial mem-
brane and the bacterial periplasmic membrane, both, require a 
membrane potential (1–5, 24). An energized mitochondrial inner 
membrane (or bacterial plasma membrane) is polarized, 
whereby the intermembrane space side (the periplasmic side in 
prokaryotes) is more positively charged with respect to the 
matrix side (the bacterial cytosolic side). Thus an electrophore-
sis-like mechanism for the translocation of N-tails in pro-
karyotes was proposed whereby the export of negatively 
charged N-terminal tails would be selectively stimulated (24– 
26). Introduction of positive charges into the N-tail led to an 
inhibition of export, thus provoking the suggestion that the 
membrane potential opposes the translocation of positively 
charged residues (7, 11, 24–26). Consistent with this conclu-
sion, the disruption of the membrane potential in bacteria 
allowed in some instances a partial translocation of positively 
charged N-tails to the plasmid (24, 25).

In mitochondria, the export of N-terminal tails displays a 
more stringent requirement for net charge and \( \Delta \mu \text{H}^+ \). First, 
increasing the net negative charge of the N-tail did not dimin-
ish its dependence on the \( \Delta \mu \text{H}^+ \) for export. Second, export of 
neutral and positively charged tails was not observed, even in 
the absence of the proposed barrier of the energized membrane. 
Thus the electrophoretic effect of the membrane potential is 
unlikely to be the only explanation for the importance of 
the negative charges for N-tail export in mitochondria. The export 
of N-tails in mitochondria has recently been shown to be 
mediated by the Oxa1p machinery (4–6). We would propose that 
the negative charge of the N-tail may be important to promote 
an interaction with Oxa1p itself or other components of the 
Oxa1p complex. Protein-protein interactions based on interac-
tions of charged domains in this manner may form the basis 
for the specificity and directionality of the export event in mito-
chondria. The same may hold true for prokaryotes, where the 
bacterial Oxa1p homolog, the IM60 protein, has also been 
described (27).

What is the role of the charges flanking the C-terminal side of 
the transmembrane segment? Export inhibition of the N-tail 
was observed when the charge of the C-terminal region was 
altered from net positive to a net negative one. Thus, when both 
N- and C-terminal-flanking sequences bear a net negative 
charge, topological frustration occurs, as the protein remains 
in the matrix. Hence, the C-terminal positive charges may also 
be involved in binding to matrix-exposed elements of the export 
apparatus. These results differ from those observed with the
coat protein of *Pseudomonas aeruginosa* phage Pf3 in *E. coli*, where it was shown that, when both termini carried a negative net charge, the Pf3 protein was able to insert either the N or the C terminus into membranes, leading to a 50%/50% mixed topology (26). However, in this study, both N- and C termini of the Pf3 protein were short. In the case of Su9(1–112)-DHFR, both the length (262 residues) and the folded state of DHFR may prevent a possible C-terminal export, as an N\textsubscript{in}-C\textsubscript{out} topology was not obtained.

The reversal of the net charge between the N and C termini resulted in the imported protein adopting a reversed N\textsuperscript{in}-C\textsuperscript{out} orientation in the membrane. Attainment of this topology, however, did not appear to involve transit of the preprotein completely through the matrix and subsequent C-terminal export. Both the wild-type and reversed charge mutant protein probably pause during import over the inner membrane. During pausing, the TIM17–23 complex may scan incoming sorting signals to discriminate between those that should be arrested permanently in the membrane from those that stop transiently and then resume complete import into the matrix before export. Reversal of the charges flanking the transmembrane domain apparently resulted in an increased pausing of the mutant protein in the inner membrane, which was coupled with a premature and stable partitioning of the protein into the membrane.

In summary, the charge distribution flanking a hydrophobic sorting signal plays a crucial role in the attainment of the correct topology in the membrane. The charge distribution influences sorting both at the level of the inner membrane translocase during import as well as at the Oxa1p export machinery. The positive-inside rule described for the sec-independent sorting of inner membrane proteins is conserved throughout evolution and holds true for those mitochondrial proteins that undergo export from the matrix.

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