Two Distinct and Independent Mitochondrial Targeting Signals Function in the Sorting of an Inner Membrane Protein, Cytochrome $c_1$*

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Proteins of the mitochondrial inner membrane display a wide variety of orientations, many spanning the membrane more than once. Some of these proteins are synthesized with NH$_2$-terminal cleavable targeting sequences (presequences) whereas others are targeted to mitochondria via internal signals. Here we report that two distinct mitochondrial targeting signals can be present in precursors of inner membrane proteins, an NH$_2$-terminal one and a second, internal one. Using cytochrome $c_1$ as a model protein, we demonstrate that these two mitochondrial targeting signals operate independently of each other. The internal targeting signal, consisting of a transmembrane segment and a stretch of positively charged amino acid residues directly following it, initially directs the translocation of the preprotein into the intermembrane space. It then inserts into the inner membrane from the intermembrane space side in a $\Delta \psi$-dependent manner and thereby determines the orientation the protein attains in the inner membrane. Analysis of a number of other precursor-containing protein of the inner membrane suggest that they too contain such internal targeting signals.

The inner membrane of mitochondria harbors a multitude of proteins, which display a diverse range of orientations in the membrane. A few of these proteins are encoded by the mitochondrial genome, while the rest are encoded by the nucleus (1). The nuclear-encoded proteins contain mitochondrial targeting signals which ensure their targeting to mitochondria following their synthesis in the cytosol. Many of these proteins bear NH$_2$-terminal mitochondrial targeting signals (presequences) which, in addition to their targeting function, facilitate the early steps in the $\Delta \psi$-dependent translocation across the inner membrane (2, 3). Upon import into the matrix these signals are proteolytically removed by the mitochondrial processing peptidase (MPP)$^*$ (4, 5). A wide range of inner membrane proteins, however, do not have such cleavable presequences, but are targeted to mitochondria by means of internal signals. Very little is known about the nature of internal targeting signals, although recently one such signal has been described for the Bcs1p protein (6). A stretch of positively charged amino acid residues located directly after the single transmembrane segment of the protein serves to target Bcs1p to mitochondria, where it attains an N$_{\text{out}}$-C$_{\text{in}}$ orientation in the inner membrane. This stretch has the potential to form an amphipathic helix, displaying all positive charges on one side and a range of apolar ones on the other. It is thought that the apolar face of the helical structure interacts with the neighboring transmembrane segment to form a hairpin loop structure which penetrates the translocase of the inner membrane in a $\Delta \psi$-dependent fashion (6).

Other proteins of the inner membrane of mitochondria also contain such positively charged residues immediately following their transmembrane segments; from the topological arrangement of the proteins these residues are known to be localized on the matrix side of the inner membrane. Interestingly some of the proteins containing such putative internal mitochondrial targeting signals also bear NH$_2$-terminal cleavable presequences. This observation raises some important questions with regards to the role of internal mitochondrial targeting signals and intra-mitochondrial protein sorting. Can a preprotein contain more than one functional mitochondrial targeting signal? If so, do these signals operate independently of each other? What role do the internal signals play in the attainment of the final orientation of these proteins in the inner membrane?

To address these questions we analyzed the targeting and import mechanisms of cytochrome $c_1$. Cytochrome $c_1$, a subunit of the cytochrome $bc_1$ complex of the respiratory chain, is anchored to the inner membrane in an N$_{\text{out}}$-C$_{\text{in}}$ orientation via a single transmembrane segment near its COOH terminus (7). It is synthesized as a precursor protein, precytochrome $c_1$ which contains an NH$_2$-terminal cleavable bipartite presequence (8, 9). The first part of this presequence is a mitochondrial targeting signal which becomes proteolytically removed by MPP in the matrix to generate an intermediate size cytochrome $c_1$ (intermediate size Cyt$_{c_1}$). The second domain of the bipartite sequence, a hydrophobic sorting sequence, directs the protein to the inner membrane, whereby the NH$_2$ terminus of the intermediate size Cyt$_{c_1}$ is maintained in the matrix. Following the covalent addition of heme, intermediate size Cyt$_{c_1}$ undergoes a second processing event, catalyzed by the Imp2p protease 2 (10), thus resulting in the release of a free NH$_2$ terminus in the intermembrane space. The transmembrane segment of the COOH-terminal end of the protein serves to anchor the protein to the inner membrane with the carboxyl terminus exposed to the matrix. How this transmembrane segment becomes correctly sorted during the import pathway of cytochrome $c_1$ was not clear until now.

We demonstrate here that this COOH-terminal transmembrane segment of precytochrome $c_1$, together with a stretch of positively charged amino acids which directly follow it, constitute an internal mitochondrial targeting signal. Consequently...
precytochrome c₁ bears two distinct targeting signals, an NH₂-terminal cleavable one and a second internal one. We show here that these targeting signals operate independently from each other and are both essential to achieve the efficient import and attainment of the correct orientation of cytochrome c₁ in the inner membrane.

EXPERIMENTAL PROCEDURES

Isolation of Yeast Mitochondria—Saccharomyces cerevisiae wild-type strain (D273-1B) was grown in lactate medium (11) at 30 °C. The Tim22(Gal110) strain (12) was grown at 30 °C in minimal medium supplemented with 2% lactate, 0.1% glucose and either in the presence (Tim22 Δ) or absence (Tim22ΔI) of 1% galactose for five generations. The Tim23(fs) strain and its isogenic wild-type strain (12) were grown at 24 °C in minimal medium with 2% lactate, 0.1% glucose, and 1% galactose. Cells were harvested at an OD₆₅₀ of ~1 and mitochondria were isolated, as described previously (11). Isolated mitochondria were resuspended in 250 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EDTA (SEM buffer) at a protein concentration of 10 mg/ml.

Recombinant DNA Techniques and Plasmid Constructions—The recombinant DNA techniques applied were as described by Sambrook et al. (13). Yeast precytochrome c₁ was used in this analysis. All the cytochrome c₁-DHFR variants used were cloned by obtaining the required cytochrome c₁ regions through designed polymerase chain reaction and ligating them before DHFR as EcoRI/BamHI fragments. A DHFRmut derivative, the DHFR moiety of Cyt c₁-(TM-C)-DHFR, was used which bears a number of point mutations, whereby Cys-7, Ser-42, and Asn-49 were replaced by Ser, Cys, and Cys, respectively (14).

Precytochrome c₁ Contains Two Mitochondrial Targeting Signals

The COOH Terminus of Cytochrome c₁ Contains an Internal Mitochondrial Targeting Signal—Analysis of the amino acid sequence of cytochrome c₁ indicated the presence of a positively charged region immediately after the transmembrane segment at the COOH-terminal end of the protein. This sequence displayed the potential to form an amphipathic α-helical structure with positively charged residues on one side and an abundance of apolar amino acids on the opposite side (Fig. 1A). This arrangement of amino acid residues resembles the internal mitochondrial targeting signal recently described for the inner membrane protein, Bcs1p (6). To address whether this region could also function as such an internal import signal, a chimeric protein, Cyt c₁-(TM-C)-DHFR, consisting of the final 63 residues of the COOH-terminal region of cytochrome c₁ fused to a mutated form of DHFR was constructed (Fig. 1B). This region of cytochrome c₁ (amino acid 247–309) encompasses the transmembrane segment flanked by 26 residues at its NH₂ terminus and directly followed by the positively charged segment (amino acid residues 288–303), together with the final 6 residues of cytochrome c₁. The mutated form of DHFR bears a number of point mutations which cause a destabilization in the folded structure of the protein whereby in contrast to its wild-type counterpart, the mutated DHFR remains sensitive to added proteases (14).

If imported and sorted by the same mechanism as authentic cytochrome c₁, Cyt c₁-(TM-C)-DHFR (molecular mass 28 kDa) should be located in the inner membrane with an Nout-Cin orientation (Fig. 1C). Radiolabeled Cyt c₁-(TM-C)-DHFR was indeed imported in a membrane potential (Δψ)-dependent manner into both isolated mitochondria and mitoplasts (Fig. 1D). Hypotonic swelling of mitochondria following import combined with protease treatment resulted in the degradation of the N-tail exposed to the intermembrane space and protection of a larger fragment corresponding to DHFR plus the transmembrane segment (approximately 25 kDa). A similar fragment was observed upon protease treatment after import into mitoplasts. This result indicates that the COOH-terminal DHFR had been imported across the inner membrane and had attained the predicted Nout-Cin orientation.

It appeared necessary to exclude the possible existence of a cryptic targeting signal in the passenger protein used above, the mutated form of DHFR (DHFRmut). Furthermore, it was important to verify that the positively charged amino acid segment (residues 288–303) together with the neighboring transmembrane segment, were responsible for the mitochondrial targeting observed. To this end, the following set of chimeric proteins were constructed (Fig. 2A).

The first construct, DHFRmut, was not imported to a protease protected location, although it became associated with mitochondria (Fig. 2B). Thus this unfolded mutated derivative of DHFR does not contain a cryptic import signal. Second, a Cyt c₁-(TM-C)-DHFR derivative in which the transmembrane segment was deleted, Cyt c₁-(ATM-C)-DHFR was also not imported into isolated mitochondria (Fig. 2B). Third, placement of the positively charged residues (residues 288–303) of cytochrome c₁ at the NH₂ terminus of DHFR, Cyt c₁-(288–309)-DHFR resulted in the efficient import into mitochondria in a Δψ-dependent manner where it was located in the matrix (Fig. 2B). Thus this stretch of positively charged amino acids can act as an NH₂-terminal mitochondrial targeting sequence. In a further derivative, the DHFR moiety of Cyt c₁-(TM-C)-DHFR was exchanged for another passenger protein, the matrix domain of Bcs1p (ΔN-BCS1). This domain of Bcs1p does not contain any targeting information, as previously demonstrated (6). The resulting fusion protein, Cyt c₁-(TM-C)-(ΔN-BCS1), was efficiently imported into mitochondria (Fig. 2B).

Taken together these observations rule out the possibility that a cryptic import signal in DHFR becomes exposed when placed COOH-terminal to the cytochrome c₁ segment. Rather, the positively charged segment of residues 288–303 of predicted α-helical structure together with the transmembrane segment functions as an internal signal. We propose that this targeting signal forms a loop structure in which the apolar face of the amphipathic α-helix interacts with the hydrophobic transmembrane segment, similar to the targeting signal of the Bcs1p (6).

The Complete Bipartite Presequence of Cytochrome c₁ Together with the COOH-terminal Targeting Signal Leads to Attainment of Correct Orientation—Does this COOH-terminal internal mitochondrial targeting signal function as a second independent targeting signal in the cytochrome c₁ preprotein?
To address this question a series of fusion proteins were constructed where the COOH-terminal segment followed by the DHFR moiety (Cyt\(_c^1\)-(TM-C)-DHFR) was fused to NH\(_2\)-terminal regions of precytochrome \(c^1\) (Fig. 3A). When fused behind the complete bipartite presequence, pCyt\(_c^1\)(1–64)-(TM-C)-DHFR, efficient import and MPP processing of the cleavable matrix-targeting sequence was observed. Hypotonic swelling in the presence of added protease resulted in the degradation to a fragment (Fig. 3B, f) which could be immunoprecipitated with DHFR specific antiserum (results not shown). Thus the DHFR domain was located in the matrix, while the linker of hydrophilic amino acid residues between the bipartite presequence and the transmembrane segment remained in the intermembrane space, accessible to the added protease (depicted in Fig. 3C). A similar orientation was achieved after the import of pCyt\(_c^1\)(1–309)-DHFR, a chimeric protein of the complete presequence.

**FIG. 1.** The COOH-terminal region of cytochrome \(c^1\), contains an internal mitochondrial targeting signal. A, \(\alpha\)-helical plot of amino acid residues 297–304 of cytochrome \(c^1\), + denotes positively charged amino acids, apolar residues are circled. B, fusion protein Cyt\(_c^1\)-(TM-C)-DHFR. The black area denotes the transmembrane domain (amino acids 273–287), the zigzag line denotes the internal targeting sequence (amino acids 288–303), DHFR, mouse cytosolic dihydrofolate reductase (mutated derivative, see “Experimental Procedures”). C, schematic representation of the topology of Cyt\(_c^1\)-(TM-C)-DHFR in the mitochondrial inner membrane after import. OM, outer mitochondrial membrane; IM, inner mitochondrial membrane; IMS, intermembrane space. D, radiolabeled Cyt\(_c^1\)-(TM-C)-DHFR (28 kDa) was imported into isolated mitochondria (upper panel) or mitoplasts (lower panel) for 10 min either in the presence (+ NADH) or absence (+ Val) of a membrane potential. After import mitochondria and mitoplasts were reisolated, either mock-treated or proteinase K-treated under nonswelling or swelling conditions, as indicated. All samples were analyzed by SDS-PAGE and blotted onto nitrocellulose. Opening of the intermembrane space was >95% efficient while the integrity of the inner membrane was not perturbed (data not shown). Std, 30% of the amount of radiolabeled precursor added to each reaction.

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**FIG. 2.** Localization of the internal mitochondrial targeting signal. A, Cyt\(_c^1\)-(TM-C)-DHFR-derived fusion proteins. The black area denotes the transmembrane domain (amino acids 273–287), the zigzag line denotes the COOH-terminal targeting sequence (amino acids 288–303), DHFR, see Fig. 1B; ΔN-BCS1, amino acids 63–456 of the BCS1 protein. B, the radiolabeled fusion proteins depicted in A were imported into isolated mitochondria for 15 min at 25 °C either in the presence (+ NADH) or absence (+ Val) of a membrane potential. After import mitochondria were reisolated, either mock-treated or proteinase K-treated under nonswelling or swelling conditions, as indicated. All samples were analyzed by SDS-PAGE and blotted onto nitrocellulose. Opening of the intermembrane space was >95% efficient while the integrity of the inner membrane was not perturbed (data not shown). Std, 30% of the amount of radiolabeled precursor added to each reaction.
cytochrome \( c_1 \) protein fused to DHFR (Fig. 3, B and C). In contrast, when fused to only the matrix-targeting domain of the cytochrome \( c_1 \) bipartite presequence pCyt\(_c(1-35)-(TM-C)\)-DHFR, import into isolated mitochondria in a \( \Delta \psi \)-dependent fashion was observed together with maturation by MPP (Fig. 3B). This chimeric protein was, however, located completely in the matrix; the NH\(_2\)-terminal presequence had apparently overridden the internal targeting and sorting signals as the N\(_{\text{out}}\)-C\(_{\text{in}}\) orientation in the inner membrane had not been achieved (depicted in Fig. 3C).

Thus, the COOH-terminal transmembrane segment can operate as an internal targeting signal and become correctly sorted across the inner membrane in the presence of the complete NH\(_2\)-terminal bipartite presequence. In the absence of the hydrophobic sorting signal, i.e., in the presence of the NH\(_2\)-terminal matrix-targeting signal alone, the preprotein becomes targeted completely to the matrix. Thus the COOH-terminal transmembrane segment appears not to have the capacity to act as a reinsertion signal from the matrix side of the inner membrane.

**The Two Mitochondrial Targeting Signals of Cytochrome \( c_1 \) Act Independently of Each Other**—Can these two targeting signals operate independently of each other and sequentially, to achieve the correct orientation of cytochrome \( c_1 \) in the inner membrane?

In an attempt to dissect the operation of the two targeting signals, a kinetic analysis of the import and sorting of radiolabeled pCyt\(_c(1-64)-(TM-C)\)-DHFR into isolated mitoplasts was performed at 12 °C. Rapid processing by MPP was observed, indicating translocation of the NH\(_2\)-terminal region of pCyt\(_c(1-64)-(TM-C)\)-DHFR across the inner membrane (Fig. 4A). At these early times the DHFR moiety remained protease accessible, demonstrating it had not yet undergone translocation across the inner membrane. Completion of import of this COOH-terminal domain was observed at later times of incubation (depicted in Fig. 4B). A similar kinetic dissection of NH\(_2\)-terminal and COOH-terminal targeting and import events was also achieved during the import of pCyt\(_c(1-309)\)-DHFR (results not shown).

In conclusion, the internal mitochondrial targeting signal of cytochrome \( c_1 \) can act independently of the NH\(_2\)-terminal cleavable presequence. In addition, the COOH-terminal transmembrane segment of cytochrome \( c_1 \) does not operate as a signal for export following prior import into the matrix. Instead, it functions as an insertion signal from the intermembrane space side of the inner membrane, penetrating the inner membrane in a \( \Delta \psi \)-dependent fashion.

**Translocation of the COOH Terminus into the Matrix Requires the ATP-dependent Activity of mt-Hsps70**—Import of preproteins across the inner membrane into the matrix is facilitated by the ATP-dependent binding of mt-Hsps70. To address whether the import and sorting of the COOH-terminal region of cytochrome \( c_1 \) requires mt-Hsps70, the import of the DHFR-derived fusion proteins was analyzed in ATP-depleted mitochondria. Matrix ATP depletion did not have an adverse effect on the import of Cyt\(_c(1-64)-(TM-C)\)-DHFR, pCyt\(_c(1-64)-(TM-C)\)-DHFR, and pCyt\(_c(1-309)\)-DHFR; the efficiency of import was comparable to that in the control ATP-containing mitochondria (Fig. 5A). In contrast, hypotonic swelling of the mitochondria following import revealed that the translocation of the COOH-terminal DHFR into the matrix, facilitated by the internal targeting signal, was severely inhibited in the absence of the ATP-dependent activity of mt-Hsps70 (Fig. 5A, f).

Consequently in the presence of the bipartite presequence or when acting alone, the Cyt\(_c(1-35)\)-(TM-C)-DHFR-derived fusion proteins can be imported into mitochondria in a mt-Hsps70-independent manner, where their DHFR moiety accumulates in the intermembrane space. Although translocation of the NH\(_2\)-terminal presequence leading to MPP processing can occur inde-
independently of mt-Hsp70, passage of the COOH-terminal segments across the inner membrane requires productive mt-Hsp70.

Once accumulated in the intermembrane space, such “ATP-depletion intermediates” were further transported across the inner membrane upon restoration of matrix ATP levels (Fig. 5B). The efficiency of this chase reaction was not affected by the prior dissipation of the membrane potential by valinomycin (Fig. 5B). Thus the internal targeting signal can penetrate the inner membrane to beyond the Δψ-dependent step of import without requiring mt-Hsp70. Upon doing so it becomes stabilized and does not undergo retrograde translocation out of the inner membrane. This insertion step may be sufficient to ensure the translocation of the short COOH-terminal domain of the authentic cytochrome c1 (22 amino acids); import and sorting of cytochrome c1 has been reported to occur in a matrix-ATP-independent manner (21). On the other hand, further translocation of the larger DHFR moiety at the COOH terminus across the inner membrane requires the action of the ATP-driven mt-Hsp70, but no longer the Δψ.

The Role of the Tim Translocases of the Inner Membrane in the Translocation of the Internal Targeting Signal of Cytochrome c1—The mitochondrial inner membrane contains at least two distinct preprotein translocases, which differ in their substrate specificity. The Tim17/Tim23 translocase mediates the import of NH2-terminal presequence-targeted preproteins and its function is tightly coupled to that of the ATP-dependent chaperone, mt-Hsp70 (22–25). On the other hand, insertion of proteins of the carrier family into the inner membrane is facilitated by a second translocase, of which the recently identified Tim22 is a component (12). Does Tim22 translocase represent a general import site for proteins with internal targeting signals?

In the yeast strain Tim22(Gal10), the Gal10 promoter was

**Fig. 4.** Kinetic analysis of the import and sorting of pCytc1(1–64)-(TM-C)-DHFR into mitoplasts at 12 °C. A, radiolabeled pCytc1(1–64)-(TM-C)-DHFR was imported into mitoplasts at 12 °C for the times indicated. Samples were cooled on ice and were either mock-treated to detect MPP processing, or treated with proteinase K to remove non-inserted species or to generate the COOH-terminal 25-kDa fragment of the inserted species, respectively. Samples were analyzed by SDS-PAGE, fluorography, and laser densitometry. B, schematic representation of the insertion of the COOH terminus of pCytc1(1–64)-(TM-C)-DHFR into the inner membrane. Abbreviations as in Fig. 1C.

**Fig. 5.** Matrix ATP dependence of the insertion of the COOH terminus. A, isolated mitochondria were incubated in import buffer and were either depleted of matrix ATP (− ATP) or mock-treated (+ ATP), as described under “Experimental Procedures.” Samples were cooled on ice and radiolabeled preproteins (depicted in Fig. 3A) were imported for 5 min at 25 °C. After import, mitochondria were reisolated, either mock-treated or proteinase K-treated (depicted in Fig. 3A) were imported for 5 min at 25 °C. Following a trypsin treatment (100 μg/ml) to remove non-imported preprotein, mitochondria were reisolated, washed, resuspended in import buffer, and divided into three aliquots. Further incubation was performed for 15 min on ice (no chase) or at 25 °C after replenishing of matrix ATP levels in the presence (chase + Δψ) or absence (chase − Δψ) of the membrane potential. Mitochondria from all three samples were then reisolated and subjected to a proteinase K treatment either under nonswelling or swelling conditions. Samples were analyzed by SDS-PAGE, fluorography, and laser densitometry. “Fragment in mitoplasts” represents the amount of COOH-terminal 25-kDa fragment generated, expressed as % of total imported species.
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**DISCUSSION**

We describe here a novel mechanism of topogenesis of a precursor protein destined for the inner membrane of mitochondria which contains two distinct targeting signals, an NH₂-terminal cleavable one and an internal one. These signals mediate the Δφ-dependent insertion of different segments of the preprotein across the inner membrane from the intermembrane space and operate independently of each other. They play an essential role in the intramitochondrial sorting, as only together they ensure the attainment of the correct orientation of the protein in the mitochondrial inner membrane.

The internal signal of cytochrome c₁ is a composite one; it consists of the transmembrane segment and the positively charged stretch of amino acids directly following it. This signal integrated into the chromosome before the Tim22 gene, thus allowing regulated expression of Tim22 by galactose (12). Mitochondria were isolated from Tim22(Gal10) cells grown for five generations in the presence (Tim22↑) or absence of galactose (Tim22↓). Tim22↓ mitochondria display a strongly impaired ability to import the ADP/ATP carrier protein (Fig. 6A and Ref. 12). Import and correct sorting of Cyt c₁-(TM-C)-DHFR, like the control NH₂-terminal presequence-containing preprotein, pSu9(1-79)-DHFR, were unaffected in Tim22↓ mitochondria (Fig. 6A and Ref. 12). This result indicates that Tim22 is not involved in the recognition and translocation of the internal targeting signal of cytochrome c₁.

To address the role of the Tim23/Tim17 translocase in this event, Cyt c₁-(TM-C)-DHFR was imported into Tim23(fs) mitochondria. These mitochondria harbor a variant of Tim23, bearing a short COOH-terminal deletion due to a translation frameshift after methionine 178 (12). Import of NH₂-terminal presequence-targeted preproteins into Tim23(fs) mitochondria is drastically reduced, whereas the import of ADP/ATP carrier protein and other members of the carrier family is as efficient as in wild-type mitochondria (Fig. 6B and Ref. 12). Both import and sorting of Cyt c₁-(TM-C)-DHFR, however, were unaffected in mitochondria isolated from the Tim23(fs) mutant (Fig. 6B). Furthermore, treatment of mitoplasts prior to import to remove the NH₂-terminal hydrophilic domain of Tim23 which is exposed to the intermembrane space, results in the inhibition of both MPP processing and import of presequence-targeted preproteins; import of Cyt c₁-(TM-C)-DHFR in contrast was unaffected by this treatment (results not shown). Taken together, these results indicate that the import of the internal targeting signal of cytochrome c₁ does not display the same requirements for Tim23 as presequence-targeted preproteins.

The COOH-terminal Targeting Signal Alone Is Not Sufficient to Import Cytochrome c₁ into Mitochondria—It could be speculated that the internal mitochondrial targeting signal present at the COOH-terminal region is necessary and sufficient for sorting of cytochrome c₁ across both the outer and inner membrane. Indeed cytochrome c₁ from the non-pathogenic trypanosomatid Crithidia fasciculata and Bodo caudatus were reported to be synthesized without NH₂-terminal presequences (26). Is the NH₂-terminal presequence of cytochrome c₁ dispensable for import of yeast cytochrome c₁? We tested the ability of the internal mitochondrial targeting signal to import fusion proteins containing increasing lengths of the mature cytochrome c₁ sequence at their NH₂ terminus (Fig. 7A). The internal mitochondrial targeting signal of these fusion proteins conferred import of NH₂-terminal regions of cytochrome c₁, with low, but distinct efficiency, its capacity being reached at approximately 70 amino acid residues (Fig. 7B). Longer proteins become partially imported, whereby a segment corresponding to the DHFR moiety is found in the matrix (results not shown).

Thus the internal mitochondrial targeting signal can mediate the initial import into the intermembrane space of only a limited COOH-terminal region of cytochrome c₁. We predict the additional NH₂-terminal presequence is essential to achieve complete import of the NH₂-terminal region of yeast cytochrome c₁.
directs import of the COOH-terminal region of precytochrome $c_1$ initially into the intermembrane space (Fig. 8). In the presence of a $\Delta\psi$, the internal targeting sequence inserts across the inner membrane, presumably by adopting a hairpin loop structure. This is similar to the internal import signal described for the Bcs1p (6). The internal targeting signal operates from the intermembrane space side of the inner membrane to sort the COOH-terminal region of cytochrome $c_1$ to the matrix and to anchor the transmembrane segment in the inner membrane.

Our present evidence would speak against the transmembrane segment operating as an export signal from the matrix. Indeed if targeted to the matrix by an NH$_2$-terminal matrix targeting sequence, the protein remains in the matrix and appears incompetent for subsequent sorting. This is in contrast to a number of other inner membrane proteins whose transmembrane segments have been demonstrated to facilitate export of hydrophilic segments from the matrix to the intermembrane space (27–29).

The mechanisms of import and sorting of a number of presequence-targeted inner membrane proteins appear to adhere to the same principle. They contain potential internal mitochondrial targeting signals COOH-terminal to their second transmembrane segment, in addition to their NH$_2$-terminal presequences. These proteins include, for example, Yta10p (Afg3p), Yta12p (Rca1p) (30–33), and the recently described Shy1p protein (34). Interestingly, Shy1p which spans the inner membrane twice appears to be divided into two functional domains. Both domains can be separately expressed to form a functional protein (34). This finding implies that both of these domains contain their own independent mitochondrial targeting and submitochondrial sorting signals. We propose therefore that the positively charged stretch of amino acids located directly COOH-terminal to the second transmembrane segment of Shy1p forms an independent internal targeting signal, similar to that described here for cytochrome $c_1$.

Which protein translocase facilitates the passage of these internal targeting signals across the inner membrane? Insertion of the internal targeting signal of cytochrome $c_1$ occurs in mitochondria in which the Tim22 translocase was strongly
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Reduced such that members of carrier family were no longer imported; thus Tim22 does not operate as a general translocase for those preproteins containing internal targeting signals. Furthermore, the internal targeting signal of cytochrome c₁ does not display requirements for Tim23 which are similar to those of presequence-containing preproteins. The latter, in contrast, require the presence of the large NH₂-terminal hydrophilic domain of Tim23, suggesting it may act as a presequence receptor. Our data do not exclude the possibility that a membrane spanning domain of Tim23, which may be protected in the trypsin pretreated mitoplasts, may suffice for the passage of the internal targeting signal of cytochrome c₁. Alternatively, these data may suggest that Tim17 is involved in mediating the recognition and translocation across the inner membrane of these internal targeting signals in a manner independent of the Tim23.

Finally, how can the observation of cytochrome c₁ containing a second independent targeting signal be reconciled with earlier models of cytochrome c₁ sorting? Previously it had been suggested that the cytochrome c₁ was first imported via the matrix (“conservative sorting” model) (35). Together with the NH₂-terminal sorting signal, the transmembrane segment was postulated to reinsert into the inner membrane from the matrix resulting in the export of hydrophilic segment between them. We propose the following alterations to the model of cytochrome c₁ sorting to take account of the observations made here (Fig. 8). Cytochrome c₁ undergoes two distinct sorting events to gain its final orientation in the inner membrane. The COOH-terminal sorting event as described above occurs from the intermembrane space and hence does not require prior import into the matrix. The events in the sorting of the NH₂ terminus are presently unresolved (Fig. 8). A number of lines of evidence are in accordance with the NH₂-terminal cleavable sorting signal being imported into the matrix (35–38), however, it has also been argued that the sorting signal becomes arrested in the TIM machinery during the import step (“stop-transfer” model) (21, 39). Whereas the model of conservative sorting may adequately describe the sorting of a number of nuclear-encoded proteins (27–29), both it, and a stop-transfer type of model may be too simplistic to describe the complex reactions in the sorting process of cytochrome c₁. We favor a model where the NH₂-terminal sorting signal does not become arrested during import, but rather passes into the matrix by a default mechanism. Fig. 8 illustrates these alternative mechanisms which need to be addressed in further experiments to study the multistep processes of cytochrome c₁ topogenesis.

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