The dimensions of the protein import channels in the outer and inner mitochondrial membranes

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Most mitochondrial proteins are imported into mitochondria through transmembrane channels composed largely, and perhaps exclusively, of proteins. We have determined the effective internal diameter of the protein import channel in the mitochondrial outer membrane to be between 20 Å and 26 Å during translocation. The diameter of the import channel in the inner membrane is smaller than the diameter of the outer membrane import channel. These results were obtained by measuring the effect of rigid steric bulk introduced into precursor proteins on import.

Precursor proteins are imported into mitochondria by a macromolecular machine that spans two membranes and contains at least nine different proteins (1). This import machinery transports the precursors through a channel in the membranes that is composed largely, and perhaps exclusively, of proteins. Import requires the hydrolysis of ATP and an electrochemical potential across the inner mitochondrial membrane. Here, we determine the internal dimensions of the protein import pore. This information is necessary to understand the mechanism of protein translocation. The protein import machinery may also be used to import macromolecules other than proteins. For example, some tRNAs are imported into mitochondria from the cytosol, and their uptake requires components of the mitochondrial protein translocation machinery (2). It has been suggested that the tRNAs are imported in association with adapter proteins, possibly their cognate amino acyl tRNA synthetases. The feasibility of this mechanism depends on the size limitations imposed on the imported molecules by the translocation machinery.

The amount of steric bulk that can be imported into mitochondria is limited by the internal diameters of the import channels. The protein import machinery permits the passage of some bulky groups such as branched polypeptides (3) and precursors with single- or double-stranded oligonucleotides attached to their C termini (4). The diameter of double-stranded DNA is ≈20 Å and thus is similar to the dimensions of folded protein domains. These observations imply that large structures can be imported through the protein import machinery. In particular, the dimensions of tRNA are similar to those of double-stranded DNA. tRNA forms an L-shaped molecule that consist largely of double-stranded RNA. The diameter of the molecule varies from ≈20 Å to ≈30 Å at the knee of the L. Precursor proteins are normally fully unfolded during import into mitochondria and do not retain residual structure (5). Bovine pancreatic trypsin inhibitor (BPTI) has the shape of a 30-Å-long cylindrical particle with a diameter of 12–19 Å; however, when a targeting sequence is attached to one end of the cylinder, BPTI cannot be imported without prior unfolding (6). Thus, the requirement of protein unfolding during import seems to contradict the conclusions based on the import of DNA and suggests that large structures such as tRNAs cannot be transported through the protein import machinery.

Some direct experimental information on the size of the protein import channel in the outer membrane has been obtained. Electron micrographs of detergent-solubilized protein import channels from the outer mitochondrial membrane (Tom complex) suggest that the channel has an internal diameter of ≈20 Å at the membrane surface (7). However, these measurements provide information only about the size of the channel's entrance. Also, if the import channel is gated, its diameter might be affected by the presence of a targeting peptide. Probing the size of the import channel of the outer mitochondrial membrane reconstituted into lipid vesicles by electrophysiological measurements yielded an average diameter of 22 Å (8). This value relies on assumptions of the length and shape of the channel as well as the interactions of the size probes with the lining of the channel. In addition, the experiments were conducted with a channel complex that was reconstituted in vesicles.

The diameter of the protein import channel in the inner membrane is expected to be different from that in the outer membrane, because the two membranes have very different ion permeabilities. Molecules of up to 1 kDa can diffuse freely across the outer mitochondrial membrane, whereas the inner mitochondrial membrane is impermeable to most ions (9). Similarly, precursor proteins can passively diffuse across the outer mitochondrial membrane in the absence of ATP, but import across the inner membrane is an active process and strictly requires ATP (10–12). No direct experimental information on the size of the protein import channel in the inner membrane has been obtained thus far.

We assessed the diameters of the import channels in the outer and inner membranes of purified yeast mitochondria directly by crosslinking rigid compounds of specific dimensions to the C termini of precursor proteins. The effects of these modifications on translocation across the outer and inner mitochondrial membranes were then determined. Our results show that the internal diameter of the protein import channel of the outer membrane is between 20 Å and 26 Å. The internal diameter of the inner membrane import channel is smaller than that in the outer membrane.

Materials and Methods

Precursor Proteins and Mitochondria. Mitochondrial precursor proteins, consisting of a presequence fused to the N terminus of a passenger protein, were constructed by using standard molecular biology techniques in the plasmid pGEM-3Zf(+) (Promega) and verified by DNA sequencing. The presequence was derived from the first 95 amino acids of yeast cytochrome b_2 (13), starting at the initiator methionine, with the following mutations: a Cys → Ser mutation at position 14 to prevent disulfide bond formation between targeting sequences, an Arg → Gly mutation at position 30 to prevent processing by the mitochondrial matrix processing protease (14), and a Leu → Pro mutation at position 62 to target the precursor to the mitochondrial matrix (15). The passenger protein was barnase, a ribonuclease from *Bacillus amyloliquefaciens* (16). A single Cys residue was added to the C terminus of barnase through a linker of the sequence Gly-Gly-Gly-Cys. Barnase contained two other mutations: one changing His-102 of the authentic barnase sequence to Ala to inactivate its

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ribonuclease activity (17) and one mutating Gln-2 of barnase to Met to allow radioactive labeling.

Radioactive precursors were expressed from a T7 promoter by *in vitro* transcription and translation in a rabbit reticulocyte lysate supplemented with [^{35}S]methionine (Promega). Ribosomes and associated incompletely translated polypeptide chains were removed by centrifugation at 150,000 × *g* for 15 min. Precursor proteins were then partially purified by precipitation with 50% (vol/vol) saturated ammonium sulfate for at least 30 min on ice, pelleted by centrifugation at 20,800 × *g* for 15 min, and resuspended in import buffer (50 mM Hepes-KOH, pH 7.4/50 mM KCl/10 mM $MgCl_2$/2 mM KH_2PO_4/5 mM unlabeled methionine/1 mg/ml fatty acid-free BSA).

Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B [MATα, ATCC 25657] (18) and purified by centrifugation through a Nycodenz gradient (19).

Size Probes and Their Attachment to Precursor Proteins. Monomaleimido Nanogold and monomaleimido Undecagold were obtained from Nanoprobes (Stony Brook, NY). Maleimide derivatives of fluorescein and Texas Red were obtained from Molecular Probes. To attach these probes to precursor proteins, barnase precursors containing a single Cys residue at their C termini were prepared as described above and resuspended in 80 μl of import buffer containing 100 μM of the modification compounds per 50 μl of translated protein. After incubation for 2 h at room temperature, modified precursors were used directly in the import experiments. Modification efficiency was assessed through a change in mobility of the modified precursor protein in SDS/PAGE.

Import Assays. Kinetics of precursor protein import into purified yeast mitochondria were measured as described (5). Briefly, 30 μl of resuspended radiolabeled precursor were prewarmed and incubated with 570 μl of mitochondrial suspension at 0.5 mg/ml mitochondrial protein in import buffer containing 4 mM ATP, 10 mM creatine phosphate, and 0.15 mg/ml creatine kinase at 20°C. At indicated time points, 50-μl samples were transferred to 100 μl of ice-cold stop buffer (0.6 M sorbitol/20 mM Hepes-KOH, pH 7.4/2 μM valinomycin/0.2 mg/ml proteinase K). After 10 min, proteinase K was inhibited with 1 mM PMSF. Mitochondria were reisolated by centrifugation at 7,000 × *g* and resuspended in SDS/PAGE sample buffer containing 2 mM PMSF. Samples were analyzed by SDS/PAGE, and the amount of imported protein was quantified by electronic autoradiography (Instant Imager, Packard). The extent of import was plotted as a percentage of the total amount of modified precursor in the import reaction. The import kinetics were analyzed with KALEIDAGRAPH (Abelbeck Software, Reading, PA) by assuming a simple first-order process.

Import specifically into the matrix was determined by rupturing the outer mitochondrial membrane by hypoosmotic shock (ref. 20; "mitoplasting"). For this purpose, the stop buffer described above was replaced with 400 μl of mitoplasting buffer (20 mM Hepes-KOH, pH 7.4/1 mg/ml fatty acid free BSA/0.1 mg/ml proteinase K/25 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). Proteinase K was inhibited with 1 mM PMSF after a 25-min incubation on ice. Otherwise, import experiments were performed as described above. The efficiency of mitoplasting was determined by using quantitative Western blotting to measure the amounts of the intermembrane space protein cytochrome b_2 and the matrix protein α–ketoglutarate dehydrogenase present before and after mitoplasting (21). We found that mitoplasting ruptured the outer membrane in 95–99% of the sample and that the inner mitochondrial membrane remained intact in ≈90% of the sample (data not shown).

We tested whether proteins that were protease resistant in the assays described above were fully imported into the matrix or only partially translocated and buried in the inner membrane by using a modification of the method previously described for this purpose (22). Precursor was imported into mitochondria, and import was stopped as described above; however, after centrifugation, the mitochondria were resuspended in 50 mM Hepes, pH 7.4/1 mg/ml fatty acid free BSA/200 mM NaCl/1 mM PMSF. The samples were frozen in liquid nitrogen and then thawed in a 25°C water bath a total of three times. After the last freeze-thaw cycle, samples were centrifuged at 150,000 × *g* at 4°C for 15 min, and both the supernatant and pellet were analyzed by SDS/PAGE and autoradiography. This procedure efficiently separated membrane-associated proteins from soluble proteins, because more than 90% of the membrane protein porin was found in the high speed pellet as determined by quantitative Western blotting (data not shown).

Results

Size Probes. We assessed the internal diameters of the import channels in the outer and inner mitochondrial membranes by covalently crosslinking rigid compounds of defined sizes to the C termini of precursor proteins and measuring the effect of these size probes on the import kinetics of the modified precursors.

Size probes were attached to precursor proteins by using a thiol-reactive maleimide derivative of the modification compounds. The probes were a spherical cluster of ≈60 gold atoms with a diameter of 26 Å (Nanogold, Nanoprobes; ref. 23) or a cluster of 11 gold atoms with a diameter of 20 Å (Undecagold, Nanoprobes; ref. 24). The gold atoms are in a specific predetermined arrangement in the core of the clusters, and tris phosphine and halide ion ligands form a hydrophilic but uncharged surface. Because these compounds are metal-coordination complexes rather than colloidal particles, their sizes are defined and known precisely. Thiol-reactive maleimide derivatives of the gold clusters were covalently linked to barnase precursors through the thiol group of the single Cys residue at the C terminus of the protein.

To test whether precursor modification itself affects import directly, we also modified the proteins with two heterocyclic compounds: fluorescein-5-maleimide and Texas-Red-C_2-maleimide. The compounds were linked to barnase precursors by means of the same chemistry as was used with the gold clusters. These two compounds are rigid but, in contrast to the gold clusters, planar rather than spherical. Their widest dimensions are ≈13 Å × 10 Å for fluorescein and ≈16 Å × 13 Å for Texas Red. The heterocyclic compounds are less suited than the spherical gold clusters for use as size probes, because the dimensions of the heterocyclic compounds are not uniform and because the geometry of their linkage to the precursor protein is unknown to us.

Outer Membrane Import Channel. We measured import rates of precursor proteins under conditions in which import is not limited by the unfolding of the precursor protein but rather by the interaction of residual steric bulk in the unfolded precursor protein with the import channel (5, 13). Because derivatization is not always quantitative, a mixture of modified and unmodified precursor is present in the import experiments. However, modified precursor can be differentiated from unmodified precursor, because derivatization reduces mobility of the precursor in SDS/PAGE (Fig. 1 *A* and *C*). Precursors modified with gold clusters with a diameter of 26 Å did not pass through the outer membrane import channel, whereas precursors modified with gold clusters with a diameter of 20 Å were imported as efficiently as unmodified precursors through this channel (Fig. 1 *A* and *B*). The 20-Å gold-modified precursors were not simply buried in the import channel, because some of these precursors were imported all the way into the matrix (see below). Precursors derivatized with the heterocyclic compounds were imported as efficiently

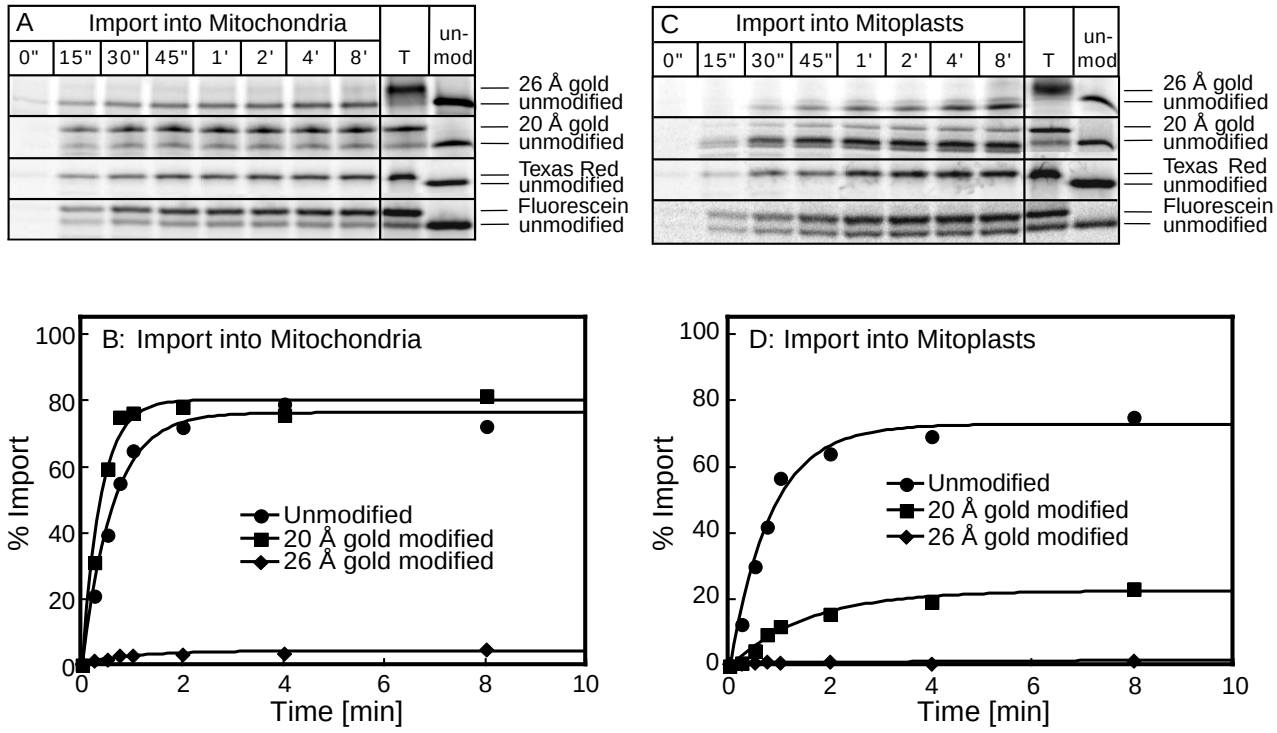

Fig. 1. Internal dimensions of the inner and outer membrane protein import channels. (*A*) Precursors containing a single Cys at their C termini were modified with monomaleimido Undecagold (20 Å gold), monomaleimido Nanogold (26 Å gold), Texas-Red-C_2-maleimide (Texas Red), or fluorescein-5-maleimide (Fluorescein) or were left unmodified (unmodified) and then imported into mitochondria at 20°C as described in *Materials and Methods*. Samples were taken at the indicated times [in seconds ('') or minutes (')], and import was stopped in normal stop buffer, which leaves the outer membrane intact. Mitochondria were reisolated, and the samples were analyzed for imported precursors by SDS/PAGE and autoradiography. For the total lanes, equivalent samples were taken before incubation with mitochondria. The autoradiograms show that precursor modification leads to decreased mobility in SDS/PAGE. Bands representing modified and unmodified precursor are labeled accordingly. The 20-Å gold-, fluorescein-, and Texas-Red-modified precursors are imported into mitochondria, whereas 26-Å gold-modified precursors are not. (*B*) To compare the import kinetics of the gold-modified and unmodified precursors, the import reactions described in *A* were quantified by electronic autoradiography. The amount of imported precursor is plotted as a percentage of the total amount of precursor presented to the mitochondria. (*C*) Conditions were the same as described for *A*, except that import was stopped in mitoplasting buffer to rupture the outer membrane by hypoosmotic shock. Mitoplasts were reisolated, and the samples were analyzed for imported precursors by SDS/PAGE and autoradiography. Bands represent protein imported across the inner mitochondrial membrane. The gels with 20-Å and 26-Å gold-modified precursors show additional bands that run faster than unmodified precursor and that presumably represent partially imported precursor from which the modification has been cleaved by the protease in the mitoplasting buffer. (*D*) A comparison was made in the same way described for *B*, except that import across the inner mitochondrial membrane is plotted.

and as rapidly as unmodified precursor (Fig. 1*A* and Table 1). When precursors that contain no Cys residues were treated with any of the modification reagents, import was not affected. Therefore, the luminal diameter of the outer membrane protein import channel is between 20 Å and 26 Å at its narrowest point.

Inner Membrane Import Channel. To assess the diameter of the inner membrane protein import pore, we ruptured the outer membrane by hypoosmotic shock before assaying for import (ref. 20; mitoplasting). After mitoplasting, the majority of precursors crosslinked to the 20-Å diameter particles that had been imported across the outer membrane became accessible to protease. Only ≈20% of the modified precursor presented to mitochondria appeared to be imported across the inner membrane (Fig. 1 *C* and *D*). Most of this protein was fully imported into the matrix rather than stuck in the import channel. We separated soluble proteins from membrane-bound proteins after import by freeze thawing followed by high-speed centrifugation and found 50–70% of the mitochondria-associated precursor modified with 20-Å gold in the soluble fraction; 90% of unmodified barnase precursor was found in the soluble fraction in control experiments (data not shown). We conclude that at least 10% of precursor modified with 20-Å gold particles presented to mitochondria was imported completely across both mitochondrial membranes. No precursors crosslinked to 26-Å-diameter particles were imported across the inner membrane. Precursors modified with the heterocyclic compounds were imported all the way into the matrix as efficiently as unmodified precursor, although the import rates were slightly reduced by the larger Texas Red compound (Table 1). Therefore, the internal diameter of the inner membrane import channel must be smaller than the internal diameter of the outer membrane import channel.

The gels for 20-Å and 26-Å gold-modified precursors show an additional band that runs faster than unmodified precursor (Fig. 1*C*). The band is presumably caused by a partially imported precursor from which the modification was cleaved by the protease in the mitoplasting buffer. Because the gold clusters are fused to the C termini of precursors, the gold-modified proteins are able to import up to the gold cluster. When the mitochondria are treated with protease, the gold clusters together with some of the connecting amino acids are removed, but the partially imported precursors remain associated with the mitochondria and form bands below those representing unmodified precursor.

Discussion

To determine the inner diameter of the import channels, we crosslinked compounds of known dimensions to a single Cys residue at the C terminus of barnase precursors. Precursors with 26-Å-diameter gold clusters crosslinked to their C termini are not imported into mitochondria, whereas precursors with 20-Å-

Table 1. Rate constants and relative inhibitions for import of modified barnase precursor proteins across the outer and inner membranes

Precursor modification	Outer membrane		Inner membrane	
	Import rate constant, min^{-1}	Import inhibition*	Import rate constant, min^{-1}	Import inhibition*
Unmodified	2.4 ± 0.1 (n = 4)	N/A	1.7 ± 0.1 (n = 5)	N/A
Fluorescein	2.1 ± 0.1 (n = 3)	1.2 ± 0.1	1.5 ± 0.1 (n = 3)	1.1 ± 0.1
Texas Red	2.0 ± 0.1 (n = 3)	1.2 ± 0.1	1.2 ± 0.1 (n = 4)	1.5 ± 0.1
Undecagold	2.2 ± 0.1 (n = 3)	1.1 ± 0.1	0.8 ± 0.1† (n = 4)	2.2 ± 0.2†
Nanogold	No import	Infinite	No import	Infinite

Import across the inner membrane was differentiated from import across the outer membrane by rupturing the outer membrane by osmotic shock before assaying for import. N/A, not applicable; n, number of repeat experiments.

*Import rate constant of unmodified precursor divided by import rate constant of modified precursor.

†The extent of import is greatly (≈4-fold) reduced, and therefore, a comparison of the import rate is problematic. Frequently, a decrease in the extent of import leads to an increase in the apparent rate constants, which here would lead to an underestimation of the inhibition.

diameter gold clusters are imported rapidly across the outer mitochondrial membrane. Therefore, the internal diameter of the protein import channel in the outer mitochondrial membrane is between 20 Å and 26 Å at its narrowest point. The sharp transition from uninhibited import to no import by a change in size of only 6 Å suggests that the import pore in the outer membrane is rigid. Our determination of the internal diameter of this import channel agrees well with measurements made with electrophysiological techniques on reconstituted channels that determined the diameter to be ≈22 Å (8) and electron micrographs of import channels detergent-solubilized from the outer membrane that suggested that the entrance to the channel has an internal diameter of ≈20 Å (7). The diameter of the outer membrane import channel is considerably smaller than the 40–60 Å diameter pore through which proteins are transported into the endoplasmic reticulum during cotranslational translocation (25).

Import across the inner membrane is partially inhibited by 20-Å gold particles. Therefore, the diameter of the inner membrane import channel is smaller than the diameter of the outer membrane channel. However, because of the partial inhibition, our experiments do not provide a definite pore size. Some of our previous experiments provide additional information on the size of the inner membrane import channel (5). We analyzed the effect of residual structure in precursor proteins on import rates by crosslinking strands of a β-sheet in a precursor protein with disulfide bridges. Depending on the number of crosslinks and their position, the crosslinks forced different numbers of polypeptide strands to be threaded through the import sites simultaneously. Table 2 shows that forcing increasing amounts of steric bulk through the import channel does not lead to a sharp decrease in translocation rates at a certain size cutoff but to a progressive inhibition of import. This finding suggests that the inner membrane channel does not have a well defined rigid pore but that it is flexible. The resting diameter of the channel seems to be small but able to expand to allow steric bulk to pass through it. This plasticity could provide a simple gating mechanism. The effects of the crosslinks on import across the outer membrane are much smaller than those across the inner membrane, in agreement with our conclusion that the diameter of the import channel in the inner membrane is smaller than the diameter of the import channel in the outer membrane. The inhibition of import across the outer membrane may result, because import across the outer membrane is coupled to import across the inner membrane. Formally, the data in Table 2 could also be explained by an ensemble of protein import channels of different sizes. However, this explanation is unlikely, because all specific membrane channels and transport systems characterized thus far were found to have well defined compositions (e.g., refs. 7 and 8). Additionally, precursor proteins may modify the flexibility of the channel as they interact with the wall of the channel during transport. In any case, the maximal diameter of the inner membrane import channel is near 20 Å. Finally, it is also possible that import of the modified precursors is inhibited, because a component of the intermembrane space, such as a chaperone, binds to the modification that was introduced. However, this possibility seems unlikely for two reasons. First, we observe differential effects on import by gold compounds that differ only by the number of gold atoms in their core. Second, import is inhibited in similar ways by very different types of steric bulk, i.e., the attachment of gold particles and internal crosslinking within a protein.

The different sizes of the protein import channels agree well with the biological demands on the two mitochondrial mem-

Table 2. The effect of residual structure in precursor proteins introduced by disulfide bridges on their import

Precursor protein					Import inhibition*	
No. of strands imported in parallel	Position of crosslink by linked amino acids†				Outer membrane	Inner membrane
3	43–80				1.1 ± 0.1	1.6 ± 0.1
3		70–92			1.2 ± 0.1	1.3 ± 0.1
3			85–102		1.1 ± 0.1	1.6 ± 0.1
3	43–80	70–92			1.4 ± 0.1	2.0 ± 0.1
3		70–92	85–102		1.6 ± 0.1	1.3 ± 0.1
5	43–80		85–102		2.3 ± 0.1	9 ± 1
5	43–80	70–92	85–102		3.7 ± 0.3	16 ± 3
5	43–80	70–92	85–102	96–110	8.5 ± 0.5	13 ± 1

Import across the inner membrane was differentiated from import across the outer membrane by rupturing the outer membrane by osmotic shock before assaying for import. Values are calculated from data in ref. 5 and are shown as means ± SEM from at least three repeat experiments.

*Import rate constant of wild-type precursor divided by import rate constant of precursors containing disulfide bridges.

†Disulfide bond formation was induced with 10 mM $K_3Fe(CN)_6$.

branes. Whereas the outer mitochondrial membrane contains porin and is permeable to compounds with molecular masses up to 1 kDa, mitochondria maintain an electrochemical potential across their inner membrane. A protein import channel in the inner membrane with a large pore such as that of the outer membrane could make maintaining the electrochemical potential difficult. At the same time, the import channel may need to accommodate larger structures. An example could be the import of RNAs bound to adapter proteins (2) discussed in the introduction.

The larger size of the outer membrane's protein import channel also agrees well with the observation that proteins can be imported across this membrane in the absence of ATP. Once a precursor is anchored in the inner membrane, the remainder of the protein can diffuse effortlessly through the large import channel in the outer membrane after unfolding (10–12). In contrast, precursors partially imported across the inner membrane are not observed to diffuse into the matrix in the absence of ATP (12). This finding could be due to the smaller import channel in the inner membrane fitting more tightly around a precursor, resulting in a drag force resembling friction created by interactions between the precursor protein and the translocation channel during import.

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