The Structure of Precursor Proteins during Import into Mitochondria*

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Precursor proteins must be at least partially unfolded during import into mitochondria, but their actual conformation during translocation is not known. Are proteins fully unfolded and threaded through the import machinery amino acid by amino acid, or do they retain some partial structure? The folding pathway of most proteins *in vitro* contains a partially folded intermediate known as the molten globule state, and it has been suggested that proteins are in the molten globule state during translocation across membranes. Here we show that precursors are normally fully unfolded during import into mitochondria. However, precursors containing residual structure can be imported, if less efficiently.

Approximately half of all proteins synthesized in a eukaryotic cell are transported into or across a membrane during their life cycle. Cells have developed elaborate protein translocation systems, which share several characteristic features (1). Protein unfolding is intimately associated with translocation, because proteins do not maintain their native state during translocation but can be folded both before and after translocation.

Here we determine the structure of precursor proteins during import into mitochondria. Precursor proteins are not in their native conformation during translocation into mitochondria (2), but it is not known how extensively they unfold. Are precursors fully unfolded and threaded through the import machinery amino acid by amino acid, or do they retain some partial structure during transport?

Although precursor proteins can be fully unfolded during translocation (2), the protein import machinery permits the passage of bulky groups such as branched polypeptides (3) and precursors with a single- or double-stranded oligonucleotide attached to the C terminus (4). Electron micrographs of the protein-conducting channel of the outer mitochondrial membrane reconstituted in lipid vesicles suggest that it has an internal diameter of ~20 Å at its entrance (5). Electrophysiological measurements estimate its internal diameter to be ~22 Å (6). Therefore, the mitochondrial import machinery may tolerate the passage of proteins that have retained some residual structure, such as secondary structural elements.

It has been proposed that proteins retain residual structure during translocation, as in the molten globule state (7, 8). The molten globule is an *in vitro* folding intermediate observed on the refolding pathways of most proteins (8). It is defined as a state in which the protein lacks defined tertiary structure but retains secondary structural elements. Many biological membranes contain negatively charged lipids, and these lipids are expected to increase the local proton and cation concentrations at the membrane surface (9, 10). The decreased pH at the surface of phospholipid vesicles induces the molten globule state in the protein toxin colicin A, which allows the toxin to undergo the conformational changes necessary to insert into the membrane (11). The outer mitochondrial membrane also contains negatively charged phospholipids (12), and it has been suggested that these charges contribute to the unfolding of precursors (13). Therefore, the surface of mitochondria may induce molten globule formation in precursors before import. Urea-denatured precursor proteins refolded in the presence of mitochondria are imported faster than native precursors, even after long incubations at low temperature (13). This acceleration of import is not observed when proteins are renatured in the absence of membranes. It appears that in the presence of mitochondrial membranes denatured precursors do not refold to their native state but into a translocation-competent folding intermediate, which may be the molten globule state (8). However, it is not known whether proteins are transported through membranes as folding intermediates or whether, before translocation, proteins only transiently form the intermediate and then unfold completely.

In this study we investigate whether precursor proteins retain residual structure during translocation into mitochondria by determining import kinetics for a series of precursors into which we introduced disulfide bridges that covalently cross-link two, three, four, or five of the strands of a β-sheet. Because mitochondria have no mechanism for reducing disulfides at their surface (14), disulfide bridges make it impossible for the import machinery to separate the cross-linked strands. If the β-sheet persisted during translocation, locking the structure with disulfide bridges would have no effect on import. On the other hand, if preventing β-strands from separating completely during import inhibits translocation relative to translocation of wild-type protein, the β-sheet is not normally present during import. We show that proteins are normally fully unfolded during import and not in the molten globule state. However, precursors containing residual structure can be imported, if less efficiently.

EXPERIMENTAL PROCEDURES

Precursor Proteins and Mitochondria—Mitochondrial precursor proteins, consisting of a presequence fused to the N terminus of a passenger protein, were constructed using standard molecular biology techniques in pGEM-3Zf(+) vectors (Promega, Madison, WI) and verified by DNA sequencing. The presequence was derived from the first 95 amino acids of yeast cytochrome b_2 (15), starting at the initiator methionine. The presequence contained a Cys^{14} → Ser mutation to prevent disulfide bond formation between targeting sequences, an Arg^{30} → Gly mutation

to prevent processing by the mitochondrial matrix processing protease (16), and a Leu[62] → Pro mutation to target the precursor to the mitochondrial matrix (17). The passenger protein was barnase, a ribonuclease from *Bacillus amyloliquefaciens* (18). In addition to the introduction of cysteine residues, barnase contained two further mutations: one changing His[102] of the authentic barnase sequence to Ala to inactivate barnase (19) 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 rabbit reticulocyte lysate (Promega) supplemented with [^{35}S]methionine and partially purified by high speed centrifugation and ammonium sulfate precipitation as described (15). Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B (*MATα*, ATCC 25657) (20) and purified by centrifugation through a Nycodenz gradient (21).

Selection of Sites for Cysteine Mutation—Three single disulfide bridge mutants and one double disulfide bridge mutant were described previously (22). To link β-strands 4 and 5 covalently, residues 96 and 110 of barnase were mutated to Cys. The positions of the cysteine mutations were selected by using the program EDPDB (23) and visual inspection of the barnase structure (24) using the program Xtalview (25).

Disulfide Bridge Formation—To induce disulfide bridge formation between Cys residues, precursors were oxidized with 10 mM $K_3Fe(CN)_6$ for 2 min at room temperature before precipitation with ammonium sulfate. In other experiments disulfide bridge formation was prevented by resuspending ammonium sulfate-precipitated precursors in import buffer containing 10 mM dithiothreitol (DTT).[1]

To test for disulfide bridge formation, unreacted cysteine residues were modified with 4-acetamido-4′-maleididylstilbene-2,2′ disulfonic acid (stilbene disulfonate maleimide (SDSM), Molecular Probes) and detected through a change in mobility of the modified proteins in SDS-PAGE. Precursors were oxidized with $K_3Fe(CN)_6$, partially purified by precipitation with ammonium sulfate, and reacted with 0.1 mM SDSM at 25 °C for 2 h in the dark. SDSM modification was detected through gel shift after autoradiography and *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine-SDS-PAGE (26).

Import Assays—Import kinetics of precursor proteins into purified yeast mitochondria were performed at 20 °C essentially as described previously (15). Briefly, radiolabeled precursors were treated with $K_3Fe(CN)_6$ or DTT to form or break disulfide bridges. 30 μl of precursor were prewarmed and then incubated with 570 μl of mitochondrial suspension at 0.5 mg of mitochondrial protein/ml in import buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4, 1 mg/ml fatty acid-free bovine serum albumin) containing 4 mM ATP, 10 mM creatine phosphate, and 0.15 mg/ml creatine kinase. For the import of precursors with reduced disulfides, the import reaction contained 10 mM DTT. 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 phenylmethylsulfonyl fluoride. The mitochondria were then reisolated by centrifugation at 7000 × *g* and resuspended in SDS-PAGE sample buffer containing 2 mM phenylmethylsulfonyl fluoride. Samples were analyzed by SDS-PAGE, and the amount of imported protein was quantified by electronic autoradiography. Analysis with reducing and nonreducing SDS-PAGE yields the same kinetic parameters. The extent of import was plotted as a percentage of the total amount of precursor in the import reaction, and import kinetics were analyzed using the software package Kaleidagraph (Abelbeck Software) by assuming a simple first-order process and fitting to the equation:

$$A = A_0[1 - \exp(-kt)] + C,$$

where *A* is the extent of import at any given time (*t*), A_0 is the extent of import at infinite time, *k* is the import rate constant, and *C* is a constant offset attributable to background.

Import specifically into the matrix space was measured by rupturing the outer mitochondrial membrane by hypo-osmotic shock (mitoplasting; Ref. 27) before assaying for import. The efficiency of mitoplasting was determined by quantifying the amount of the intermembrane space protein cytochrome b_2 and the matrix protein α-ketoglutarate dehydrogenase present before and after mitoplasting by quantitative Western blotting (28). Mitoplasting ruptured the outer membrane in 95–99% of the sample, whereas the inner mitochondrial membrane remains intact in ~90% of the sample (data not shown).
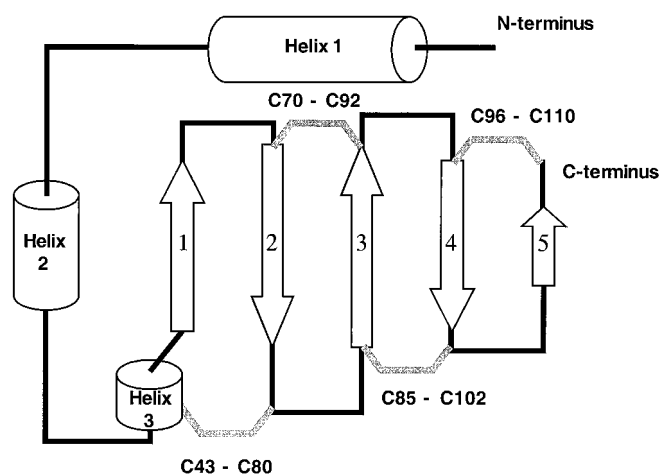

FIG. 1. **Wire diagram of barnase.** α-Helices are shown as *cylinders*, β-strands as *arrows*. Disulfide bridges are drawn in *gray* and labeled with the positions of the participating cysteine residues.

RESULTS

Precursor Proteins—We constructed a series of mitochondrial precursor proteins in which specific parts of the structure were prevented from unfolding by covalent cross-links. All precursor proteins consisted of a 95-amino acid mitochondrial presequence derived from cytochrome b_2 fused to the ribonuclease barnase (15, 29). Cross-links were introduced into barnase by mutating pairs of residues to cysteine at positions that allow disulfide bridge formation upon oxidation (Fig. 1). The disulfide bonds covalently link neighboring β-strands within the five-stranded antiparallel β-sheet of barnase and prevent their complete separation during import, because mitochondria have no mechanism for reducing disulfide bridges at their surface (see below and Ref. 14).

Three single disulfide bridge mutants were described previously (22) and cross-link β-strands 1 and 2 (Cys[43]-Cys[80]), β-strands 2 and 3 (Cys[70]-Cys[92]), and β-strands 3 and 4 (Cys[85]-Cys[102]) (Fig. 1). The structure of these proteins was determined by x-ray crystallography and found to be almost identical to that of wild-type barnase (30). To covalently link β-strands 4 and 5, we introduced an additional disulfide bridge by mutating residues 96 and 110 of barnase to cysteines. We constructed precursor proteins containing all single disulfide bridges and all combinations of two and three disulfide bridges between the first four β-strands, as well as a precursor with four disulfide bridges cross-linking all five β-strands.

Disulfide Bridges Are Formed by Oxidation—Neighboring Cys residues were induced to form disulfide bridges by oxidation with ferricyanide ($K_3Fe(CN)_6$). Disulfide bond formation was assessed by modification of free thiol groups with SDSM. After oxidation with ferricyanide and purification, none of the disulfide mutants could be modified with SDSM, whereas cysteine mutants that were not oxidized with ferricyanide before treatment with SDSM run slower in SDS-PAGE than ferricyanide-treated precursors (data not shown). Thus, all disulfide bonds form quantitatively after oxidation. Nonreducing SDS-PAGE after oxidation showed that <2% of precursors formed dimers or higher order multimers (data not shown).

Two results suggest that precursor proteins are in their native conformation after disulfide bridge formation. First, oxidized precursors are resistant to proteinase K to similar extents as wild-type protein (data not shown). Second, even precursors with eight Cys residues do not form dimers or multimers after oxidation, as judged by nonreducing SDS-PAGE, yet all Cys residues are involved in disulfide bridges. It is unlikely that the Cys residues could efficiently form non-

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[1] The abbreviations used are: DTT, dithiothreitol; SDSM, stilbene disulfonate maleimide; PAGE, polyacrylamide gel electrophoresis.
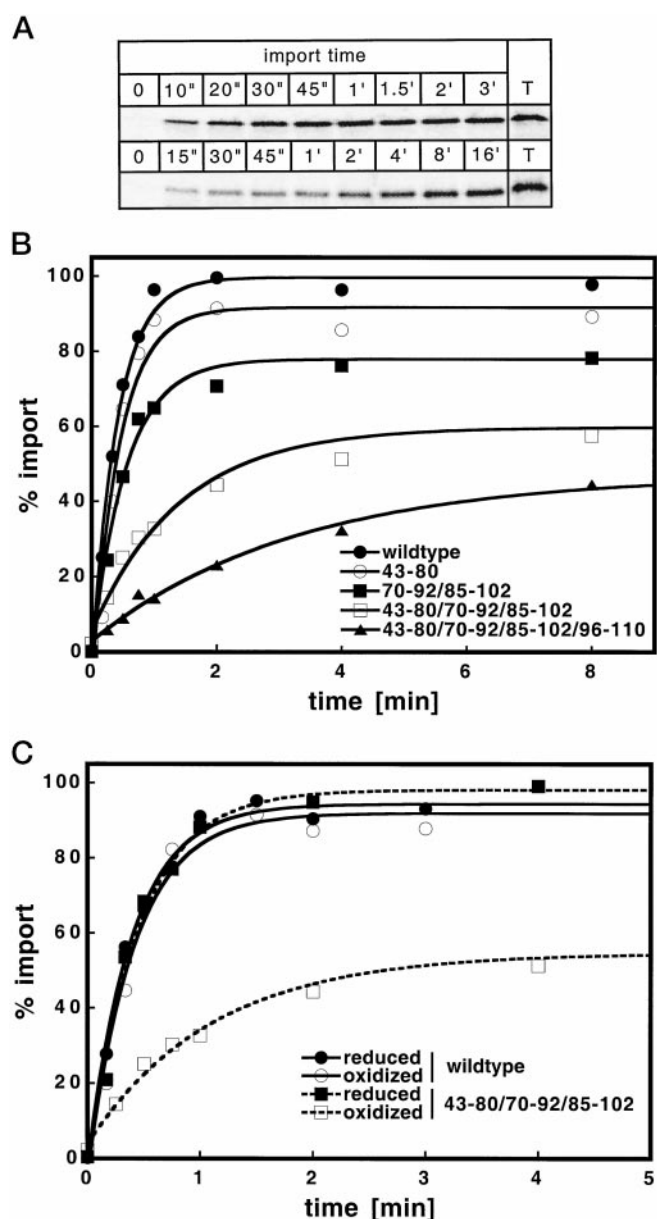
FIG. 2. **Import kinetics of oxidized and reduced cytochrome** b_2**-barnase precursor proteins.** *A*, autoradiograms of SDS-PAGE analysis of import experiments with wild-type and Cys[43]-Cys[80]/Cys[70]-Cys[92]/Cys[85]-Cys[102] precursors after oxidation with ferricyanide. Bands represent the amount of precursor associated with mitochondria after protease treatment and reisolation of mitochondria at the indicated times; *T*, total amount of precursor presented to mitochondria. *Top*, wild type; *bottom*, 43-80/70-92/85-102. *B*, import of precursor proteins with no, one, two, three, or four disulfide bridges after oxidation with ferricyanide. *C*, import of oxidized and reduced barnase precursors lacking disulfide bonds and precursors containing three disulfide bonds. The oxidized precursors were treated with ferricyanide, the reduced precursors were treated with DTT. *y*-axis, amount of precursor imported as a percentage of the total amount of precursor presented to the mitochondria.

native disulfide bridge pairs within a properly folded precursor protein.

Residual Structure in Precursors Inhibits Import—We measured the effect of increasing amounts of residual structure in barnase precursor proteins on import into purified yeast mitochondria under conditions where precursor unfolding is not rate limiting for import (15). Radiolabeled precursors were synthesized *in vitro* by coupled transcription and translation, treated with $K_3Fe(CN)_6$ or DTT to form or break disulfide bridges, partially purified, and incubated with purified yeast

mitochondria in import buffer. At designated time points samples were removed and analyzed for imported protein (Fig. 2*A*). The extent of import was plotted as a percentage of the total amount of precursor presented to the mitochondria (Fig. 2, *B* and *C*).

As precursors were forced to retain an increasing amount of residual structure during import by the cross-linking of β-strands, both the import rate constants and the extent of import decreased (Fig. 2*B* and Table I, oxidized). Import rates of precursors containing a single disulfide bridge were inhibited by a small but significant amount compared with import rates of precursors lacking disulfide bridges. With introduction of an additional disulfide bridge, import rates were inhibited further. Precursors containing three disulfide bridges (four cross-linked β-strands) were imported approximately four times more slowly than the wild-type precursor. In addition, the extent of import was reduced to ~50% of that of the precursor lacking disulfide bridges. Finally, precursors containing four disulfide bridges (five cross-linked β-strands) were imported eight to nine times more slowly than the wild-type precursor, and only ~30% of the cross-linked precursor could be imported. It is not immediately clear why residual structure in a precursor protein reduces the extent of import as well as the rates of import. However, it is commonly observed that the efficiency of import decreases with decreasing import rates. Two possible explanations are that aggregation of partially imported precursors competes with import, and that mitochondrial preparations deteriorate throughout import reactions.

The effect of oxidation on import was not attributable to the formation of precursor dimers or other multimers, because import kinetics analyzed by reducing or nonreducing SDS-PAGE yield identical kinetic parameters. Import of the cross-linked precursors was not attributable to slow reduction of the disulfide bridges, because disulfide bonds remained formed for at least 1 h when oxidized precursors were incubated with de-energized mitochondria in import buffer (data not shown). Import experiments conducted at 30 °C yielded import inhibitions equivalent to those performed at 20 °C.

The import inhibition of the oxidized precursors containing Cys residues is caused by the formation of disulfide bridges and not the presence of Cys residues themselves. 10 mM DTT fully reduces the previously characterized single disulfide barnase mutants (22). When import experiments were conducted in the presence of 10 mM DTT, all of the disulfide mutants showed import rates approximately equal to those of the wild-type precursor (Table I and Fig. 2*C*). The nondisulfide bridge containing wild-type precursor was imported at identical rates under oxidizing or reducing conditions (Table I and Fig. 2*C*).

Precursor Unfolding Is Not Rate Limiting for Import under the Chosen Conditions—Disulfide bridges may affect the stability of the fully folded precursor protein against global unfolding (22), and therefore, their effect on import rates could be caused by an effect on the stability of the native precursor rather than residual structure induced in the unfolded precursor. We have shown previously that import of barnase and dihydrofolate reductase precursors with the 95-amino acid presequence described here is not limited by unfolding of the passenger protein (15). When the length of the targeting sequence is reduced to 35 amino acids, unfolding of the passenger protein at the mitochondrial surface becomes rate limiting for import (15), and destabilizing mutations in barnase, such as the mutation of the Ile at position 25 in barnase to Ala, greatly accelerate import (>170-fold rate enhancement of a pseudo-wild-type barnase precursor).[2] The Ile[25] → Ala mutation did

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[2] M. P. Schwartz, S. Huang, and A. Matouschek, unpublished results.

TABLE I

Rate constants for import into mitochondria and import inhibitions for oxidized and reduced barnase precursor proteins

Precursor				Oxidized[a]		Reduced[b]	
				Import rate constant	Relative inhibition[c]	Import rate constant	Relative inhibition[c]
				min^{-1}		min^{-1}	
Wild type				2.48 ± 0.08 $(n = 3)^d$	NA	2.56 ± 0.05 $(n = 2)^d$	NA
43–80				2.29 ± 0.01 $(n = 2)$	1.08 ± 0.04	2.35 ± 0.02 $(n = 2)$	1.09 ± 0.02
	70–92			2.0 ± 0.2 $(n = 3)$	1.2 ± 0.1	2.44 ± 0.02 $(n = 2)$	1.05 ± 0.02
		85–102		2.20 ± 0.03 $(n = 3)$	1.10 ± 0.04	2.5 ± 0.1 $(n = 2)$	1.00 ± 0.04
43–80	70–92			1.81 ± 0.01 $(n = 3)$	1.37 ± 0.04	2.44 ± 0.01 $(n = 2)$	1.05 ± 0.02
43–80		85–102		1.07 ± 0.02 $(n = 5)$	2.3 ± 0.1	2.4 ± 0.1 $(n = 2)$	1.09 ± 0.04
	70–92	85–102		1.60 ± 0.05 $(n = 2)$	1.6 ± 0.1	2.51 ± 0.01 $(n = 2)$	1.02 ± 0.02
43–80	70–92	85–102		0.68 ± 0.05 $(n = 5)$	3.7 ± 0.3	2.63 ± 0.01 $(n = 2)$	0.97 ± 0.02
43–80	70–92	85–102	96–110	0.29 ± 0.01 $(n = 5)$	8.5 ± 0.5	2.3 ± 0.2 $(n = 2)$	1.1 ± 0.1

[a] Disulfide bond formation was induced with 10 mM $K_3Fe(CN)_6$.
[b] Disulfide bond reduction with 10 mM DTT.
[c] Import rate constant for wild-type precursor divided by import rate constant of precursors containing disulfide bridges. NA, not applicable.
[d] n, number of experimental measurements.

not accelerate import of any of the disulfide-containing precursors described here (Table II). This demonstrates that, under the conditions tested, global unfolding of the native precursor was never rate limiting for import. Therefore, the observed import rate inhibition is the result of the residual structure induced in the unfolded precursor protein by the disulfide bonds rather than any effect on the stability of the native conformation of barnase.

Import Across the Inner Mitochondrial Membrane—Import across both mitochondrial membranes into the matrix can be differentiated from import across only the outer membrane into the intermembrane space by rupturing the outer membrane by hypo-osmotic shock (mitoplasting) before assaying for import. When import of the precursors described here was analyzed by mitoplasting, no significant amounts of precursor could be detected between the two membranes, indicting that all of the precursors were imported completely into the matrix. Import rate constants for wild-type precursor and all the disulfide bridge mutants are shown in Table III together with the relative inhibition of import rates by the disulfide bridges. As for import across the outer membrane, introducing an additional Ile$^{25} \rightarrow$ Ala mutation in barnase did not affect inhibition by the disulfide bridges (Table III). Comparison of Tables I and III shows that import across the inner membrane appears somewhat slower than import across the outer membrane. Residual structure in the precursor protein has a stronger effect on import across the inner membrane than on import across the outer membrane.

DISCUSSION

To determine the structure of proteins during import, we forced increasing amounts of residual structure into a translocating precursor protein by cross-linking parts of the passenger protein barnase with disulfide bridges. The disulfide bridges cross-linked pairs of neighboring β-strands in the single antiparallel β-sheet of barnase. When two disulfide bridges were introduced simultaneously, they cross-linked either three neighboring β-strands, as in the mutants Cys^{43}-Cys^{80}/Cys^{70}-Cys^{92} and Cys^{70}-Cys^{92}/Cys^{85}-Cys^{102}, or two sets of two β-strands, as in the mutant Cys^{43}-Cys^{80}/Cys^{85}-Cys^{102} (Fig. 1). When three disulfide bridges were introduced simultaneously, they cross-linked four neighboring β-strands, and four disulfide bridges cross-linked all five β-strands of the β-sheet.

The largest amount of structure that any one disulfide bridge will stabilize is a pair of neighboring β-strands. However, the cross-linked parts of the protein need not remain in a β-sheet conformation during translocation but could rearrange to minimize steric bulk if the import channel imposed size constraints on the precursor. Tables I and III show that even single disulfide bridges inhibited import and that the inhibition becomes

stronger as more β-strands are cross-linked to each other. Therefore, the entire β-sheet in barnase is clearly not normally maintained during import and barnase precursors are not in the molten globule state. Furthermore, because even one and two disulfide bridges inhibited import, we conclude that barnase is normally fully unfolded during import into mitochondria.

The differential effects of the three combinations of two disulfide bridges we examined can be understood if one considers that precursors are imported from the N terminus to the C terminus. A single disulfide bridge in a precursor will introduce a loop and thus change the direction of the peptide chain. Therefore, if the directionality of import from N to C terminus is to be maintained, three strands of the precursor must pass through the import machinery at the same time. In the mutants Cys^{43}-Cys^{80}/Cys^{70}-Cys^{92} and Cys^{70}-Cys^{92}/Cys^{85}-Cys^{102} the second disulfide bridge cross-links the neighboring strand to the loop induced by the first disulfide bridge (Fig. 1). This second cross-link restricts the conformations allowed to all three neighboring strands and leads to a small increase in inhibition of import over that induced by a single disulfide bridge. In contrast, in the mutant Cys^{43}-Cys^{80}/Cys^{85}-Cys^{102} two pairs of two neighboring β-strands are cross-linked, presumably forcing four or five strands of protein to be imported next to each other simultaneously. As expected, this double disulfide bridge mutant shows a larger inhibition of import than the two double disulfide bridge mutants with cross-links between three neighboring β-strands. In the triple and quadruple disulfide bridge mutants, the conformation of the five protein strands during translocation is increasingly restricted, which leads to a correspondingly stronger inhibition of import.

The structure of proteins during import will be influenced by the internal diameter of the mitochondrial protein import channel. Small amounts of residual structure in precursor proteins, as induced by single disulfide bridges, slow import, but even precursors with large amounts of residual structure, as induced by several disulfide bridges, are imported. Import is progressively inhibited as the residual structure in precursors increases. Thus, the import channels do not show a clear size cutoff. Rather, they appear to be flexible and can accommodate structures considerably larger than a single strand of protein when required. However, the alternative explanation that mitochondria contain a range of import channels of different sizes cannot be ruled out.

Because import across the inner membrane is slower than across the outer membrane, and disulfide bridges inhibit import across the inner membrane more strongly than across the outer membrane, the import channel in the inner membrane appears to be tighter than that in the outer membrane.

TABLE II

Rate constants for import into mitochondria and accelerations through destabilization for oxidized barnase precursors

Precursor[a]				Import rate constant	Ile^{25} → Ala Import rate constant	Relative acceleration[b]
				min^{-1}	min^{-1}	
Wild type				2.48 ± 0.08 (n = 3)[c]	2.6 ± 0.3 (n = 2)[c]	1.0 ± 0.1
43–80				2.29 ± 0.01 (n = 2)	2.2 ± 0.1 (n = 2)	0.97 ± 0.06
	70–92			2.0 ± 0.2 (n = 3)	2.06 ± 0.06 (n = 2)	1.0 ± 0.1
		85–102		2.20 ± 0.03 (n = 3)	2.20 ± 0.01 (n = 2)	1.00 ± 0.01
43–80	70–92			1.81 ± 0.01 (n = 3)	1.84 ± 0.01 (n = 2)	1.02 ± 0.01
43–80		85–102		1.07 ± 0.02 (n = 5)	1.00 ± 0.04 (n = 2)	0.93 ± 0.04
	70–92	85–102		1.60 ± 0.05 (n = 2)	1.6 ± 0.1 (n = 2)	0.98 ± 0.09
43–80	70–92	85–102		0.68 ± 0.05 (n = 5)	0.67 ± 0.02 (n = 2)	0.99 ± 0.08
43–80	70–92	85–102	96–110	0.29 ± 0.01 (n = 5)	0.31 ± 0.01 (n = 3)	1.07 ± 0.05

[a] Disulfide bond formation was induced with 10 mM $K_3Fe(CN)_6$.
[b] Import rate constant for precursor with Ile^{25} → Ala mutation divided by import rate constant for corresponding precursor lacking the Ile^{25} → Ala mutation.
[c] n, number of experimental measurements.

TABLE III

Rate constants for import across the inner mitochondrial membrane, inhibitions by disulfide bridges, and accelerations by destabilization

Import rate constants for disulfide bridge-containing precursors and inhibition of import by the disulfide bridges relative to precursor lacking cross-links. 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. Disulfide bond formation was induced with 10 mM $K_3Fe(CN)_6$. The table also lists the effects of destabilizing cross-linked precursors by the mutation Ile^{25} → Ala of barnase.

Precursor				Import rate constant	Relative inhibition[a]	Ile^{25} → Ala	
						Import rate constant	Relative acceleration[b]
				min^{-1}		min^{-1}	
Wild type				1.93 ± 0.03 (n = 3)[c]	NA	1.91 ± 0.06 (n = 2)[c]	0.99 ± 0.03
43–80				1.20 ± 0.04 (n = 3)	1.61 ± 0.06	1.19 ± 0.02 (n = 2)	0.99 ± 0.03
	70–92			1.32 ± 0.04 (n = 4)	1.32 ± 0.04	1.5 ± 0.1 (n = 2)	0.99 ± 0.04
		85–102		1.19 ± 0.02 (n = 3)	1.63 ± 0.04	1.48 ± 0.04 (n = 2)	1.1 ± 0.1
43–80	70–92			0.99 ± 0.06 (n = 4)	2.0 ± 0.1	1.17 ± 0.04 (n = 2)	1.25 ± 0.04
43–80		85–102		0.23 ± 0.03 (n = 4)	9 ± 1	0.22 ± 0.01 (n = 2)	1.0 ± 0.1
	70–92	85–102		1.50 ± 0.09 (n = 4)	1.29 ± 0.08	1.54 ± 0.04 (n = 2)	1.00 ± 0.06
43–80	70–92	85–102		0.12 ± 0.02 (n = 3)	16 ± 3	0.09 ± 0.01 (n = 2)	0.8 ± 0.1
43–80	70–92	85–102	96–110	0.18 ± 0.02 (n = 4)	13 ± 1	0.18 ± 0.01 (n = 2)	1.0 ± 0.1

[a] Import rate constant for wild-type precursor divided by import rate constant of precursors containing disulfide bridges. NA, not applicable.
[b] Import rate constant for precursor with Ile^{25} → Ala mutation divided by import rate constant for corresponding precursor lacking the Ile^{25} → Ala mutation.
[c] n, number of experimental measurements.

The flexibility of the import channel may be biologically important. Mitochondria establish an electrochemical gradient across their inner membrane. Channels with large openings would make maintaining an electrochemical potential difficult. At the same time, the import channel may need to accommodate large structures. For example, some mitochondrial tRNAs are imported into mitochondria from the cytosol by the protein import machinery, and it has been suggested that they may be imported in association with their cognate aminoacyl tRNA synthetases (31). The task of maintaining ion gradients across the membrane would be facilitated by the import channel in the inner membrane having a small diameter and adjusting its size to expand around large structures that pass through it.

How do our conclusions apply to proteins other than barnase? Conclusions about the import channel itself, such as its size and flexibility, can be expected to be valid generally, because barnase was used simply as a probe to determine properties intrinsic to the channel. Conclusions about the structure of precursor proteins during import may or may not be general, depending on how good a model barnase is for the behavior of other proteins. The folding and unfolding of barnase have been studied extensively *in vitro* and appear typical when compared with the behavior of other proteins. Our results suggest that the structure of proteins during import is influenced by the properties of the channel. Size limitations imposed by the internal diameter of the import channel appear to favor minimal residual structure in precursors. Could α-helical structures persist in a precursor protein during import? The anhydrous diameter of an α-helix is 10–12 Å, and that of three extended protein strands is between 10 and 20 Å, depending on their arrangement. Therefore, our data cannot rule out that α-helices are maintained in a protein during import. However, this appears unlikely, because α-helices are unstable outside the context of other structural elements, and our results suggest that the structure of the import channel minimizes residual structure in precursors.

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