Mitochondria unfold precursor proteins by unraveling them from their N-termini

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Protein unfolding is a key step in the life cycle of many proteins, including certain proteins that are degraded by ATP-dependent proteases or translocated across membranes. The detailed mechanisms of these unfolding processes are not understood. Precursor proteins are unfolded and imported into mitochondria by a macromolecular machine that spans two membranes and contains at least nine different proteins. Here we examine import of a model precursor protein derived from the ribonuclease barnase and show that mitochondria unfold this protein by unraveling it from its N-terminus. Because barnase in free-solution unfolds by a different pathway, our results demonstrate that mitochondria catalyze unfolding in the way that enzymes catalyze reactions, namely by changing reaction pathways. The effectiveness of this mechanism depends on the structure of the N-terminal part of the precursor protein.

To be active, proteins must fold into well-defined three-dimensional structures. However, unfolding of proteins is also essential for several processes in the cell. One example is protein translocation across membranes[1]. Some precursor proteins fold before translocation, but must then be unfolded during import into mitochondria[2], chloroplasts[3] or the endoplasmic reticulum[4]. Proteins targeted to various organelles probably unfold in similar ways because the import processes share some common features: precursors are synthesized with N-terminal targeting sequences, travel across the membranes through specific proteinaceous channels, and are translocated in a nucleoside triphosphate-dependent manner with the help of Hsp70 homologs[1].

Similarly, ATP-dependent proteases, such as the proteasome[5,6] and the Clp [7], Lon[8] and AAA proteases, must unfold their substrate proteins to achieve degradation[9]. The unfolding reactions during degradation and translocation show several striking similarities. Both types of unfolding reactions involve ATP hydrolysis[10], N- or C-terminal targeting sequences can target proteins to the unfolding machineries, and substrate unfolding is apparently coupled to movement of the extended polypeptide chain through a channel[6,9]. None of these unfolding processes have been studied at the mechanistic level. Here, we determine the mechanism of protein unfolding during import into mitochondria.

Precursor proteins are imported into mitochondria by a macromolecular machine that spans two membranes and contains at least nine different polypeptides[1]. Import requires ATP and an electrochemical potential across the inner mitochondrial membrane[1]. Mitochondrial precursor proteins normally lack all structure during import[11], but some proteins assume their native form before translocation[12,13]. The import of these native precursor proteins can be many hundred times faster than their spontaneous unfolding, indicating that mitochondria can actively unfold proteins[14]. Using a model precursor protein derived from the ribonuclease barnase, we show that mitochondria change the unfolding pathway of this protein by unraveling the protein from its N-terminus. Three lines of evidence support this conclusion. (i) Whereas mutations throughout barnase affect its spontaneous unfolding, only mutations near the N-terminus of barnase affect its unfolding during import. (ii) Crosslinking the N-terminus of barnase accelerates its spontaneous unfolding but blocks its unfolding during import. (iii) Ligand binding stabilizes barnase against spontaneous unfolding but does not affect unfolding during import.

Unfolding of barnase in free-solution

The mitochondrial import machinery can be viewed as a large enzyme that catalyzes the unfolding and translocation of a substrate, the precursor protein. Some enzymes catalyze chemical reactions by changing the reaction pathways; by analogy, the

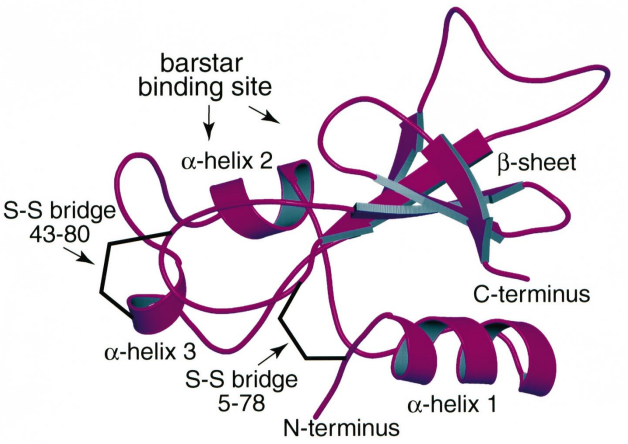

Fig. 1 The structure of barnase as determined by crystallography[38]. Shown are the locations of two disulfide bridges that were introduced into a subset of the precursors proteins. Also shown is the binding site of the protein barstar, which inhibits barnase activity. α-Helix 1 is formed by residues 6–18, α-helix 2 by residues 26–34, α-helix 3 by residues 41–46, and β-strands 1–5 by residues 50–55, 70–76, 85–91, 94–99 and 106–108 (ref. 17). This figure was prepared with the programs Molscript and Raster3D[39].

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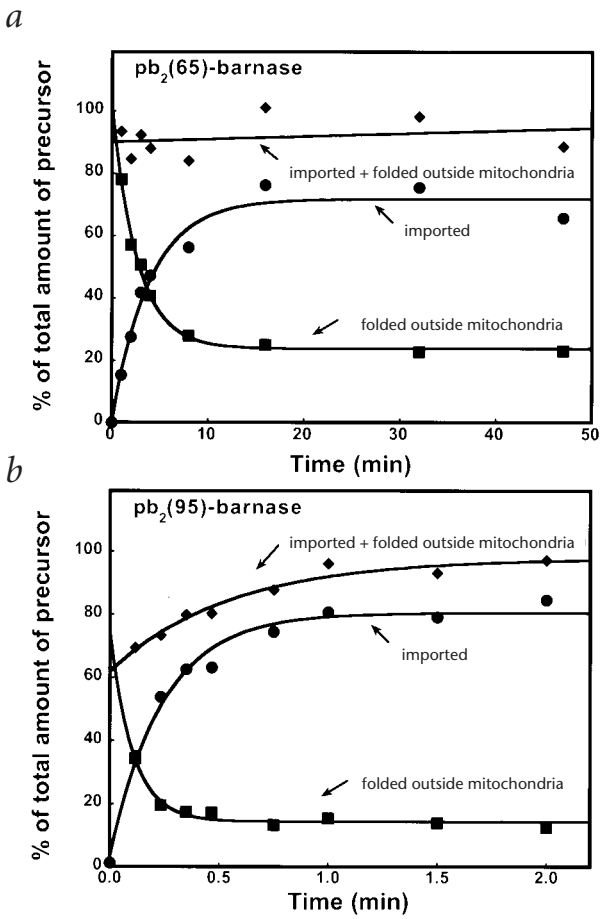

Fig. 2 Protein unfolding at the mitochondrial surface. Graphs show the amounts of protease-resistant precursor outside mitochondria, amounts of imported precursor and the sum of both throughout an import experiment as a percentage of the total amount of precursor presented to the mitochondria. The folding state of barnase can be assessed rapidly because native barnase is resistant to added proteases whereas denatured barnase is degraded[14]. ***a***, During import of pb_2(65)-barnase the amount of folded protein outside mitochondria decreases at the same rate that the protein appears inside mitochondria. As a consequence, the amounts of folded precursor outside the mitochondria plus imported precursor add up to ~100% of the amount of added precursor at all times. ***b***, When unfolding is not rate determining, as for pb_2(95)-barnase, the protein is unfolded at the mitochondrial surface faster than it is imported.

import machinery might catalyze precursor unfolding by changing the unfolding pathway. The pathways of spontaneous unfolding in free-solution have been described in detail for several proteins, one of which is barnase[15]. It is a single polypeptide chain of 110 amino acids that forms three α-helices followed by a five-stranded antiparallel β-sheet (Fig. 1). The spontaneous unfolding pathway of barnase was mapped by analyzing how the kinetics of unfolding are affected by mutations that specifically remove stabilizing interactions[15]. Certain regions of barnase denature early during unfolding so that they lack native-like structure in the rate-determining transition state. Mutations in these regions accelerate unfolding. In contrast, other regions of barnase lose their structure late during unfolding, and destabilizing mutations in these regions do not affect the unfolding rate.

These experiments revealed that unfolding of barnase in free-solution follows a defined pathway. In particular, a subdomain of barnase, formed by the second and third α-helices and several loops packing against the edge of the β-sheet, unfolds before the remainder of the protein[15].

Barnase precursor proteins

To investigate barnase unfolding during import, we converted barnase into a mitochondrial precursor protein by attaching targeting sequences of different lengths to its N-terminus. The targeting sequences consisted of the first 35, 65 or 95 amino acids of pre-cytochrome b_2, and the resulting precursors were called pb_2(35)-barnase, pb_2(65)-barnase, and pb_2(95)-barnase. We measured the equilibrium stability and unfolding and refolding kinetics of the precursor proteins (Table 1) using previously described methods[15–17]. It can be seen that attachment of these targeting sequences does not affect the stability or folding and unfolding kinetics of barnase *in vitro*.

These fusion proteins rapidly insert into the mitochondrial import channel until the folded barnase domain reaches the opening of the channel. Because key components of the import machinery are located in the inner mitochondrial membrane and matrix[14,18], the length of the targeting sequence determines how effectively the mitochondria unfold the barnase domain at their surface. The targeting sequence of pb_2(95)-barnase is long enough to reach into the matrix and fully engage the import machinery. As a consequence, the import rate of pb_2(95)-barnase is determined not by precursor unfolding but rather by the turnover of the import machinery[14]. In contrast, the targeting sequence of pb_2(35)-barnase is too short to fully engage the import machinery, and therefore the import rate of this precursor is determined by spontaneous unfolding of the barnase domain at the mitochondrial surface[14]. Finally, the most interesting precursor is pb_2(65)-barnase, because its targeting sequence reaches the inner membrane and begins to engage the associated

Table 1 *In vitro* folding and unfolding of barnase precursors[1]

	No targeting sequence[2]	35 aa targeting sequence	65 aa targeting sequence	95 aa targeting sequence
$[urea]_{50\%}$ (M)	4.57	4.4 ± 0.1	4.4 ± 0.1	4.3 ± 0.2
m_{equil} (kcal mol^{-1} M^{-1})	1.93	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.6
$log(k_u)$ (s^{-1})	-3.88	-3.89 ± 0.05	-3.6 ± 0.2	-3.4 ± 0.1
m_{ku} (s^{-1} M^{-1})	0.457	0.45 ± 0.01	0.42 ± 0.02	0.42 ± 0.02
k_f (s^{-1})	12.3	10 ± 1	9 ± 1	9 ± 1

[1]$[urea]_{50\%}$ is the urea concentration at which proteins are 50% unfolded; m_{equil} is the slope of a straight line fitted to a plot of the free energy of unfolding at a given urea concentration against the urea concentration; k_u is the unfolding rate constant in the absence of urea; m_{ku} is the slope of a straight line fitted to a plot of the logarithm of the unfolding rate constant at a given urea concentration against the urea concentration; k_f is the refolding rate constant in the absence of urea. Errors are standard errors calculated from repeat measurements. aa, Amino acid.
[2]Data are from refs 17 and 36.

Table 2 Effect of mutations on unfolding and mitochondrial import of barnase precursors[1]

Mutation in barnase	Acceleration of unfolding rates in solution[2]	Acceleration of import rates[3]		
	No targeting sequence	35 aa targeting sequence[4]	65 aa targeting sequence[5]	95 aa targeting sequence
(1)I4A	5	2.5 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
(2)N5A	13	3 ± 1	1.1 ± 0.1	1.1 ± 0.2
(3)T6A	13	23 ± 5	3.0 ± 0.6	1.0 ± 0.3
(4)V10A	17	14 ± 1	3 ± 1	1.2 ± 0.2
(5)Y13A	7	11 ± 1	3.5 ± 0.6	0.9 ± 0.1
(6)L14A	23	21 ± 7	5.6 ± 0.2	1.0 ± 0.2
(7)T16S	1.5	n.d.	1.1 ± 0.2	1.2 ± 0.2
(8)H18Q	1.1	3.3 ± 0.3	1.1 ± 0.1	1.2 ± 0.3
(9)N23A	20	9.3 ± 0.9	1.5 ± 0.2	1.1 ± 0.2
(10)I25V	6	3.0 ± 0.3	0.9 ± 0.1	0.9 ± 0.1
(11)T26A	17	5.8 ± 0.9	1.2 ± 0.2	0.9 ± 0.1
(12)V36A	7.6	2.6 ± 0.3	0.9 ± 0.1	0.8 ± 0.2
(13)I51V	19	7 ± 1	0.8 ± 0.1	1.1 ± 0.2
(14)D54A	273	13 ± 2	1.0 ± 0.3	1.1 ± 0.2
(15)N77A	14	5 ± 1	1.1 ± 0.1	1.0 ± 0.2
(16)N84A	23	n.d.	1.2 ± 0.1	1.0 ± 0.1
(17)I88A	1.7	n.d.	2.3 ± 0.2	1.3 ± 0.2
(18)I25A + I76V + I88V + I96V	n.d.	60 ± 10	6 ± 1	1.0 ± 0.2

[1]The effects of mutations on import and unfolding are given as the ratio of the mutant import or unfolding rate constant divided by the wild type import or unfolding rate constant. Errors are standard errors calculated from repeat measurements. The mutations have previously been described in detail[17]. aa, Amino acid; n.d., not determined.
[2]Determined at 25 °C, pH 6.3, 7.25 M urea, data from refs 15 and 37.
[3]Determined at pH 7.4, temperature of 35 °C for pb_2(35)-barnase and pb_2(65)-barnase precursors or 25 °C for pb_2(95)-barnase precursors.
[4]Mutations were generated in a I76V + I88V + I96V mutant background. For pb_2(65)-barnase, the effects of the displayed mutations are not changed significantly by adding the mutations I76V + I88V + I96V or by varying the temperature between 25 °C and 35 °C.
[5]All tested mutations have similar effects on import of pb_2(55)-barnase precursors.

components of the import machinery, yet unfolding remains rate determining for import[14].

Effect of mutations on barnase import

We then asked how the mutations used to analyze spontaneous unfolding in free-solution would affect import of barnase precursors with the three different targeting sequences (Table 2). The import rate constants of all three fusion proteins were independent of mitochondrial or precursor concentrations, indicating that binding of the precursors to mitochondria was rapid compared to unfolding and import (data not shown). As expected, none of the mutations affected import of pb_2(95)-barnase because unfolding is not rate determining for this precursor. With pb_2(35)-barnase, all of the mutations accelerated import, as predicted if spontaneous unfolding is rate determining for import. However, introducing mutations into pb_2(65)-barnase yielded novel results. For this precursor, some of the mutations had lost their effect on import (mutations 10–16 in Table 2), whereas other mutations continued to accelerate import significantly (mutations 3–6 and 17). This distinction does not correlate with the effectiveness of the mutations in accelerating spontaneous unfolding. Instead, the relevant factor seems to be the location of the mutated residues. Mutations 10–16 are in the subdomain at the edge of the central β-sheet that unfolds early in free-solution. Although these mutations increase spontaneous unfolding rates[15], in some cases dramatically so, they do not affect import of pb_2(65)-barnase. In contrast, mutations that affect the N-terminal portion of barnase (mutations 3–6 and 17) significantly accelerate import of pb_2(65)-barnase. This acceleration confirms that unfolding is rate determining for import of pb_2(65)-barnase. To test this critical point further, we simultaneously but independently monitored the unfolding and import of barnase precursors at various times during the import reaction[14]. The results demonstrated that for pb_2(65)-barnase, the unfolding and import rates were identical, whereas the longer pb_2(95)-barnase becomes unfolded at the mitochondrial surface before it is imported (Fig. 2).

The maximal enhancement of the pb_2(65)-barnase import rate was about six-fold, as exemplified by the quadruple mutation 18 in Table 2. This acceleration is not larger because the maximum import rate that can be achieved by mitochondria is only approximately 10–20 times larger than the import rate for wild type pb_2(65)-barnase, so that turnover of the translocation machinery quickly becomes rate determining for import of the most destabilized mutant precursors.

The mutations I4A and N5A (1 and 2 in Table 2) do not fall into the expected pattern because, despite being near the N-terminus of barnase and having relatively large effects on unfolding rates *in vitro*, they do not accelerate import of pb_2(65)-barnase. However, these two mutations are close to the fusion point of the targeting sequence and to the mutation Q2M, which was introduced into all precursors to allow radioactive labeling. Since the same two mutations (I4A and N5A) also have unexpectedly small effects on import of pb_2(35)-barnase, the most likely explanation is that the local structure in their vicinity is disturbed in all precursor proteins.

The very different effects of mutations on spontaneous unfolding of barnase versus import-driven unfolding of pb_2(65)-barnase show that these two reactions follow different pathways. Spontaneous unfolding in free-solution begins as a global process, with a large part of the structure unfolding early. In contrast,

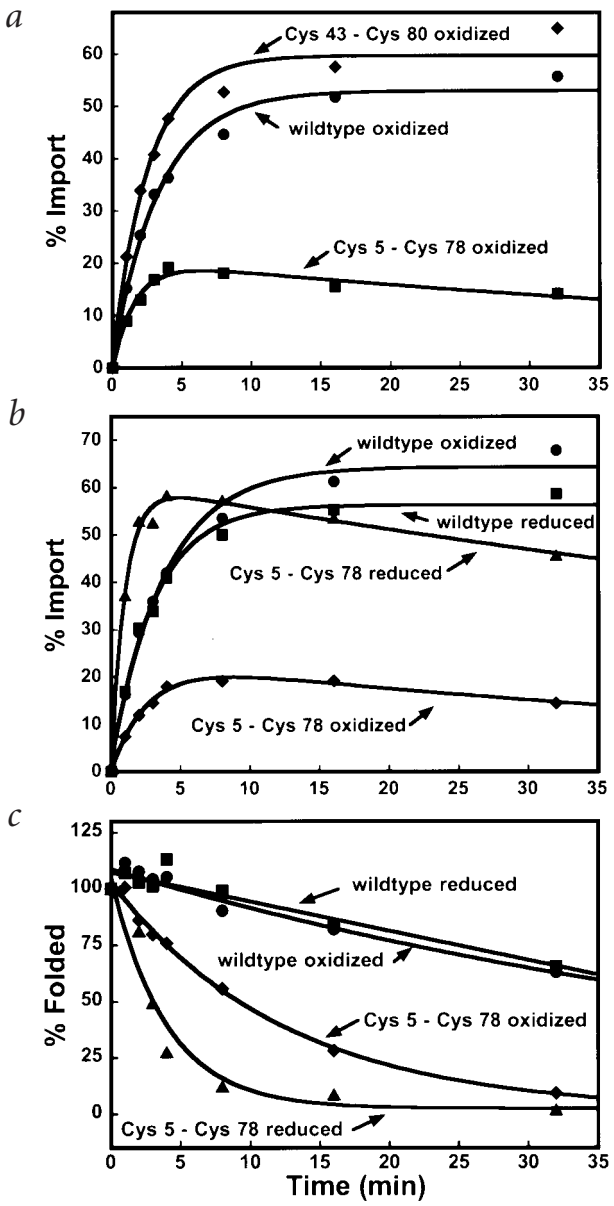

Fig. 3 Effects of disulfide bridges on import and precursor stability. ***a***, Locking the N-terminus of barnase onto the remainder of the protein inhibits import of pb_2(65)-barnase, whereas a crosslink elsewhere in the protein leaves import unaffected. ***b***, The cysteine mutations themselves do not affect translocation. Import experiments were performed as in (*a*) except that precursors were either oxidized or reduced as indicated. Note that precursors containing the Cys 5 and Cys 78 mutations are slowly degraded inside the mitochondria. ***c***, Protease resistance of wild type and crosslinked precursors. The degradation kinetics are independent of the protease concentration (data not shown).

import-driven unfolding begins at the N-terminus of the protein, and therefore only the mutations that destabilize this part of the protein accelerate unfolding. Once the N-terminal portion has been unraveled, the rest of the precursor protein denatures rapidly.

Crosslinking the N-terminus inhibits unfolding during import

If mitochondria unfold proteins by unraveling them from their N-termini, it should be possible to slow import of pb_2(65)-barnase by stabilizing the N-terminal portion of the barnase domain. To test this prediction, we introduced crosslinks into the barnase passenger protein by mutating pairs of residues to cysteine followed by oxidation[11]. The N-terminal portion of barnase was crosslinked to the body of the protein with a disulfide bridge between Cys 5 and Cys 78 (Fig. 1). A disulfide bridge between Cys 43 and Cys 80 (ref. 19) was used as a control. As expected, locking the N-terminus in place with the 5-78 crosslink severely inhibited import of pb_2(65)-barnase, whereas the 43-80 crosslink did not affect import (Fig. 3*a*). (The residual import of the 5-78 crosslinked precursor presumably reflects the very inefficient translocation of the crosslinked precursor; alternatively it could be due to molecules that failed either to fold or to form the crosslink.) This inhibitory effect is not due to the introduction of cysteines at positions 5 and 78, because the reduced form of the mutant pb_2(65)-barnase precursor actually imports faster than the wild type (Fig. 3*b*). It could be argued that the 5-78 disulfide bridge is inhibitory because this crosslinked form of barnase cannot pass through the import channel. We can exclude this possibility because the same crosslink does not significantly inhibit import of pb_2(95)-barnase (data not shown).

Interestingly, the inhibitory effect of the 5-78 crosslink is not due to an increase in the overall stability of the barnase domain free in solution. Both the reduced and the oxidized forms of the 5-78 mutant protein are more sensitive to protease than the wild type protein, indicating that the crosslink actually destabilizes the protein (Fig. 3*c*). *In vitro* unfolding studies using urea as the denaturant and following unfolding through intrinsic tryptophan fluorescence show that spontaneous unfolding of the oxidized 5-78 mutant is ~100 times faster than that of wild type pb_2(65)-barnase and that the equilibrium stability of the oxidized 5-78 mutant is decreased by ~3.6 kcal mol^{-1} (data not shown). In contrast, the 43-80 crosslink slows unfolding rates ~20-fold in solution and increases the equilibrium stability by more than 2 kcal mol^{-1} (ref. 19), but does not affect unfolding of pb_2(65)-barnase during import. These results suggest that the 5-78 disulfide bridge slows import of pb_2(65)-barnase specifically by preventing local unfolding near the N-terminus of barnase.

Stabilization of precursors by ligand binding

As a final test of our hypothesis that mitochondria change the unfolding pathway of precursor proteins, we stabilized the barnase passenger domain with a specific ligand. Barnase can be stabilized by binding of the substrate analog 3'-GMP[20] or the inhibitor protein barstar[21]. Interactions between 3'-GMP and barnase stabilize the native state more than they stabilize the transition state for unfolding, and therefore 3'-GMP reduces the unfolding rate[20]. We did not use 3'-GMP here because it binds barnase relatively weakly and is not very soluble in import buffer. However, because the barstar binding site contains the 3'-GMP site, barstar should also slow unfolding. Barstar binds to barnase on the side of the β-sheet opposite from the N-terminus, so barstar would be expected to inhibit spontaneous unfolding of barnase more strongly than it inhibits import-driven unfolding (Fig. 1). Indeed, we found that barstar greatly slowed import of pb_2(35)-barnase, but had much smaller effects on import of pb_2(65)-barnase and pb_2(95)-barnase (Fig. 4*a*).

Interestingly, pb_2(95)-barnase is imported efficiently even at high barstar concentrations (up to micromolar, data not shown), suggesting that there is no attainable stability of barnase that cannot be overcome by the import machinery. To determine whether this phenomenon is general, we investigated the effect of ligand binding on import of a second passenger protein, mouse

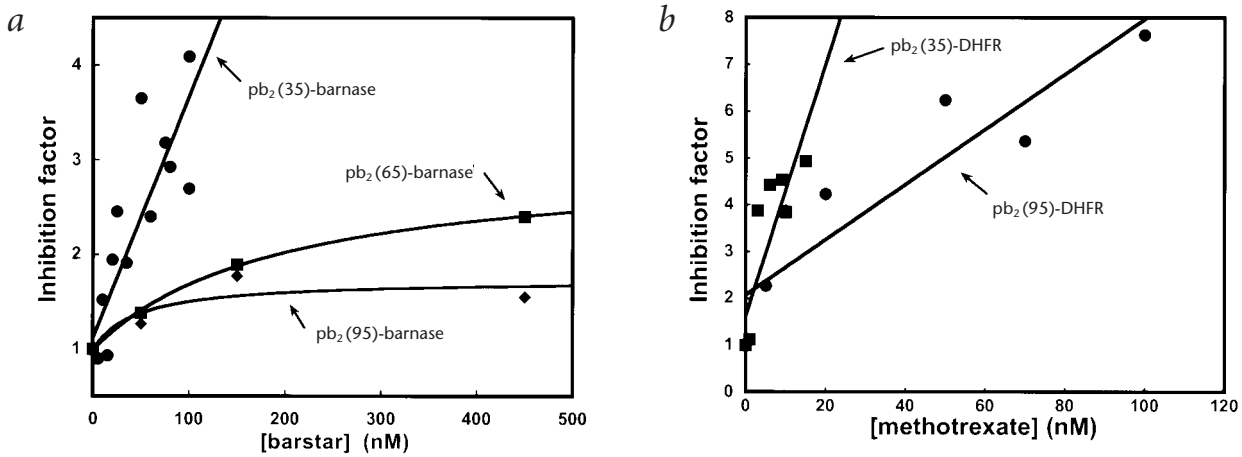

Fig. 4 Inhibition of import by ligand binding. ***a***, The graph shows the inverse of the measured import rate constants versus barstar concentration for pb_2(35)-barnase, pb_2(65)-barnase and pb_2(95)-barnase, standardized to the import rate constants for the unliganded precursors. The dissociation constant of the barstar–barnase complex at the mitochondrial surface is approximately 30 nM. ***b***, The graph shows the inverse of the total extent of import versus methotrexate concentration for pb_2(35)-DHFR and pb_2(95)-DHFR, standardized to the extent of import of the unliganded precursors. The extent of import is obtained as the amplitude of a single exponential fit of the import data. For DHFR precursors the extent of import decreases dramatically as import slows, presumably because slowly importing precursors associate with each other or with mitochondrial proteins (our unpublished observations). As a consequence, changes in the extent of import reflect inhibition of import better than changes in the rate constants. The dissociation constant of the methotrexate–DHFR complex at the mitochondrial surface is ~5 nM for pb_2(35)-DHFR and 30 nM for pb_2(95)-DHFR.

dihydrofolate reductase (DHFR). DHFR is stabilized against unfolding by the binding of methotrexate to its active site[2]. Although mitochondria catalyze unfolding of DHFR[14], stabilization of the DHFR domain with methotrexate prevents import into mitochondria for precursors with both long and short targeting sequences[18,22] (Fig. 4*b*). Methotrexate inhibits import of DHFR precursors directly because it does not affect import of barnase precursors (data not shown). Why does the efficiency of import-driven unfolding depend on the passenger domain? DHFR and barnase differ greatly in the structure near their N-termini. The N-terminal portion of barnase forms an α-helix at the surface of the protein, whereas the N-terminal portion of DHFR is buried in the core of the protein and forms an internal strand in a β-sheet that is sandwiched between α-helices[23]. As a consequence, the N-terminal portion of barnase can be unraveled by local unfolding, whereas unraveling the N-terminal portion of DHFR would require global unfolding of the protein. Therefore, the effectiveness of import-driven unfolding apparently depends on the structure of the precursor protein, particularly the structure near its N-terminus.

Conclusions

We present three lines of evidence that the unfolding pathway of a barnase precursor protein during mitochondrial import is distinct from its spontaneous unfolding pathway (Fig. 5). First, mutations at specific locations throughout barnase have markedly different effects on spontaneous unfolding in free-solution and unfolding during import. Second, crosslinking the N-terminal portion of barnase destabilizes the protein but inhibits its unfolding during import. Third, stabilizing barnase with ligands does not block import-driven unfolding. Therefore, the protein import machinery catalyzes protein unfolding by changing the unfolding pathway of its substrate protein, just as many enzymes catalyze chemical reactions by changing reaction pathways. Mitochondria unravel barnase from its N-terminus. The effectiveness of this unraveling mechanism appears to depend on the structure of the precursor protein. We predict that the import machinery will be able to unfold even the most stable proteins if the N-terminal portion of their folded domain lies at the surface of the protein. On the other hand, if the N-terminal portion of the folded domain is buried inside the protein, a precursor may be too stable to be unfolded and imported by the mitochondria. This hypothesis also agrees with the observation that the cytosolic isozyme of aspartate amino transferase can only be imported into mitochondria when its N-terminal 33 amino acids are replaced with those of the mitochondrial isozyme in addition to fusion to a targeting sequence[24].

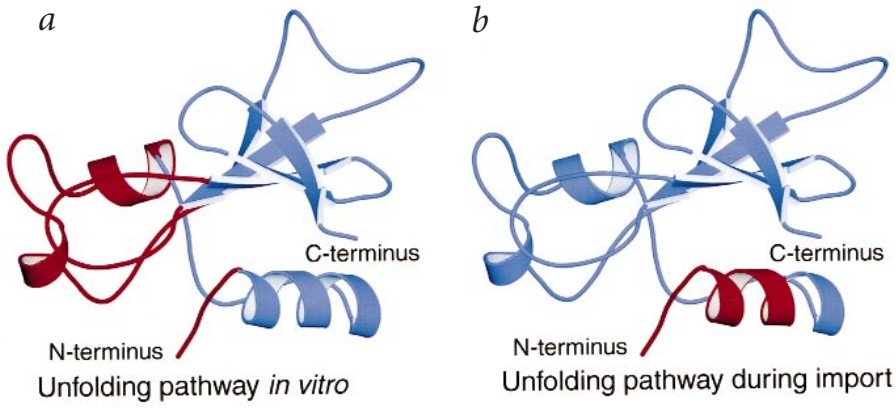

Fig. 5 Unfolding pathways of barnase. Sketches of the structure of barnase, color coded according to the order in which structure is lost, ***a***, during spontaneous unfolding in free-solution, and ***b***, during import into mitochondria. The parts of the structure shown in red unfold early, whereas those shown in blue unfold late.

The finding that the ease with which a protein can be unfolded depends on its structure is also interesting in the context of other biological unfolding processes. For example, in many neurodegenerative diseases, proteins undergo conformational changes and accumulate as aggregates[25]. It is possible that proteases are unable to degrade these aggregates because the proteins cannot be unfolded.

How could the observed change in unfolding pathway be induced by mitochondria? In the simplest mechanism the import machinery would trap spontaneous unfolding fluctuations near the protein's N-terminus, in effect pulling part of the polypeptide chain out of a folded protein domain. This action would collapse the structure because folding is generally highly cooperative[26]. What could be pulling at the N-terminus? Two obvious candidates are the electrochemical potential across the inner mitochondrial membrane[27] and mHsp70, a Hsp70 homolog in the mitochondrial matrix[28–31]. The magnitude of the electrical potential across the inner membrane of respiring yeast mitochondria is on the order of 100 mV, with the positive pole at the outer (intermembrane space) surface and the negative pole at the inner (matrix) surface. Since mitochondrial targeting sequences have a net positive charge under physiological conditions (+11 unit charges for the pb_2(65) targeting sequence), a targeting sequence in the import channel will experience a force directed toward the matrix.

Mitochondrial Hsp70 binds to components of the import channel as well as the translocating precursor protein in an ATP-dependent manner. By binding to parts of the precursor emerging from the import channel, mHsp70 could act like a ratchet. Since the ratchet would trap diffusion of the precursor in one direction only, it would in effect pull at the N-terminus of the protein[28–31]. Homologs of mHsp70 undergo ATP-dependent conformational changes[32,33]. If mHsp70 binding to precursors and import channel followed by a conformational change ('power-stroke') were repeated in an ATP-dependent cycle, mHsp70 could function in a mechanism reminiscent of the action of myosin on actin in muscle contraction[30,31].

Methods

Precursor proteins. Targeting sequences derived from the N-terminus of pre-cytochrome b_2 were fused to the N-terminus of barnase as described[14]. The targeting sequences were mutated to prevent processing and to inactivate the stop-transfer signal; barnase was mutated to inactivate its ribonuclease activity and to allow radioactive labeling[14]. The *in vitro* equilibrium stabilities and folding and unfolding kinetics of the precursor proteins purified from *Escherichia coli* were measured essentially as described[15–17]. To measure the equilibrium stabilities, 100 µl of ~0.5 mg ml^{-1} protein solution in 0.5 mM dithiothreitol (DTT), 450 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.0 were added to 900 µl urea solution, using ~40 different urea concentrations equally spaced between 0 and 10 M urea. After equilibration for 2–4 h at 25 °C, the intrinsic protein fluorescence was measured for all samples in an ISS PC1 fluorometer (excitation wavelength 290 nm, emission wavelength 320 nm, 8 nm bandpass). The fluorescence data were fitted assuming a two-state transition using the published equations[17]. Unfolding rates were measured by rapidly diluting ~0.3 mg ml^{-1} barnase into urea solutions of different concentrations at a 1:10 ratio to yield final urea concentrations between 5 and 9 M (all solutions contained 0.5 mM DTT, 41 mM MES, pH 6.0 after dilution). The intrinsic protein fluorescence was then followed in an SX.18MV Applied Photophysics stopped-flow apparatus at 25 °C using an excitation wavelength of 280 nm (9 nm bandpass) and a 348 nm glass cut-on filter in the emission window. The fluorescence traces were analyzed assuming first-order kinetics[15]. Refolding was measured by rapidly diluting ~0.7 mg ml^{-1} barnase at pH 1.8 into 50 mM MES at a 1:10 ratio to yield a final pH of 6.0. The intrinsic protein fluorescence was again followed in an SX.18MV Applied Photophysics stopped-flow apparatus at 25 °C and the fluorescence traces were fitted to three exponentials. The two slower processes are attributable to proline isomerizations; the fastest process, representing ~70% of the fluorescence change, is attributable to proline-independent folding and is analyzed further here[16].

Import experiments. Import rates were measured essentially as described[11,14]. Briefly, radioactive precursor protein was synthesized by coupled *in vitro* transcription and translation and partially purified; mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B[34]. Precursor protein was incubated with mitochondria at 0.5 mg mitochondrial protein per milliliter in import buffer (0.6 M sorbitol; 20 mM HEPES-KOH, pH 7.4; 1 mg ml^{-1} fatty acid free bovine serum albumin) containing 4 mM ATP, 10 mM creatine phosphate and 0.15 mg ml^{-1} creatine kinase. At different times, samples were transferred to ice-cold STOP buffer (0.6 M sorbitol; 20 mM HEPES-KOH, pH 7.4; 2 mM valinomycin; 0.2 mg ml^{-1} proteinase K). After 10 min, the protease was inhibited and mitochondria were reisolated by centrifugation. Samples were separated by SDS–PAGE and the amount of imported protein quantified by electronic autoradiography. 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) assuming first-order kinetics. The import rate constant for pb_2(95)-barnase was 5 ± 1 min^{-1} at 25 °C. The import rate constants at 35 °C were 0.25 ± 0.05 min^{-1} for pb_2(65)-barnase and 0.04 ± 0.01 min^{-1} for the I76V + I88V + I96V mutant of pb_2(35)-barnase. Wild type pb_2(35)-barnase is not imported efficiently.

To measure the amount of folded barnase domain outside mitochondria simultaneously with the amount of imported protein, import experiments were performed as described above except that after protease treatment in STOP buffer the whole sample was precipitated with 5% trichloroacetic acid and analyzed by SDS-PAGE and quantitative autoradiography. Imported precursor is resistant against the added protease, whereas unfolded precursor outside mitochondria is completely degraded and folded precursor outside mitochondria yields a protease-resistant barnase fragment[14]. These experiments were conducted at 25 °C for pb_2(65)-barnase or 20 °C for pb_2(95)-barnase.

For the ligand binding experiments, precursors and import mix were preincubated with barstar at the indicated concentrations for 5 min, and then import experiments were performed as described above at 35 °C for pb_2(35)-barnase and pb_2(65)-barnase and at 25 °C for pb_2(95)-barnase. To allow import of pb_2(35)-barnase, the barnase domain was destabilized by the mutations I76V + I88V + I96V; all barnase precursors contained the mutation T6A. The DHFR precursors have been described[14]. Methotrexate binding experiments were performed at 25 °C.

Crosslinking experiments. To lock the N-terminus of barnase onto the remainder of the protein, residues 5 and 78 were selected for mutation to cysteine using the program EDPDB[35]. The 43-80 disulfide bridge has been described[19]. Disulfide bridge formation was induced by oxidation with 10 mM $K_3Fe(CN)_6$ or prevented with 5 mM DTT[11]; import experiments were then performed as described above.

To assess the stability of crosslinked precursors under import conditions, radioactive precursor proteins were synthesized, oxidized or reduced as described above, and added to import buffer containing 0.5 mg ml^{-1} proteinase K. After incubation for the indicated times at 35 °C, the protease was inhibited with 1 mM phenylmethylsulfonyl fluoride and the sample was analyzed by SDS-PAGE and electronic autoradiography. The zero time point was obtained by treating precursor with proteinase K for 5 min on ice.

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