Protein unfolding by the mitochondrial membrane potential

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Mitochondria can unfold importing precursor proteins by unraveling them from their N-termini. However, how this unraveling is induced is not known. Two candidates for the unfolding activity are the electrical potential across the inner mitochondrial membrane and mitochondrial Hsp70 in the matrix. Here, we propose that many precursors are unfolded by the electrical potential acting directly on positively charged amino acid side chains in the targeting sequences. Only precursor proteins with targeting sequences that are long enough to reach the matrix at the initial interaction with the import machinery are unfolded by mitochondrial Hsp70, and this unfolding occurs even in the absence of a membrane potential.

Protein unfolding is a critical step in at least two processes in the cell: protein degradation by ATP-dependent proteases[1] and protein translocation across some membranes[2]. In both cases, unfolding can be catalyzed by the unraveling of the protein substrates from their targeting signals[3,4]. How these unfoldases unravel their substrates is unknown. Here we investigate how the mitochondrial import machinery unfolds precursor proteins targeted to the mitochondrial matrix.

Protein unfolding is intimately associated with import into mitochondria. Proteins are normally fully unfolded during translocation and are threaded through the import machinery amino acid by amino acid[5–7]. Nevertheless, import of native proteins can be many hundred times faster than their spontaneous unfolding, indicating that mitochondria can actively unfold proteins[3,8]. Mitochondria catalyze unfolding by unraveling precursor proteins from their N-terminal targeting sequences, thereby changing the unfolding pathways of some precursors[3]. The unfolding activity is not located at the mitochondrial surface[9] but seems to be associated with the inner membrane or the matrix[3,8]. Protein import requires ATP hydrolysis for the function of mitochondrial Hsp70 (mtHsp70) at the exit of the import channel in the matrix[10–13] and an electrical potential across the inner mitochondrial membrane[14–16]. mtHsp70 is involved in the unfolding of some precursor proteins[17–21]. However, many precursors will not be able to interact with mtHsp70 before unfolding because their targeting sequences are too short to reach across the two mitochondrial membranes. The shortest precursor found to be able to interact with mtHsp70 before its mature domain unravels at the mitochondrial surface has a 52-amino acid targeting sequence[22]; another precursor requires even longer targeting sequences to interact with mtHsp70 (ref. 8). A review of all the yeast proteins listed as mitochondrial in the yeast proteome database (YPD)[23,24] shows that the average length of the N-terminal part of precursor proteins preceding the processing site is 31 amino acids, with a standard deviation of 18 amino acids.

Because of its physical location, the membrane potential across the inner mitochondrial membrane would be able to act on precursors before they reach mtHsp70. The potential is required for all protein import into the matrix[14–16], and its elec-

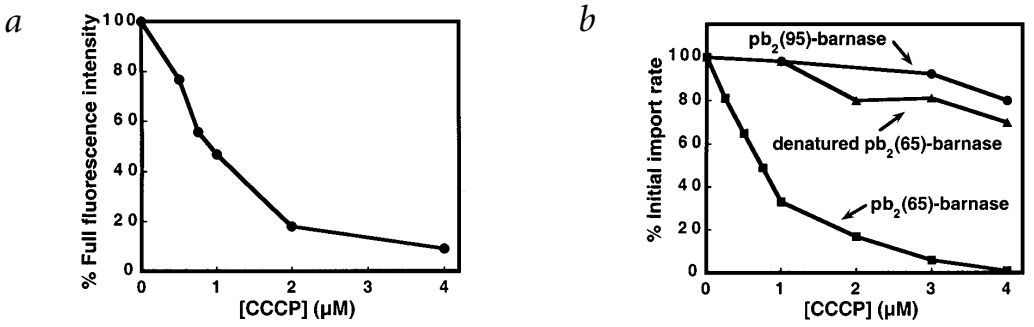

Fig. 1 Dependence of import on the membrane potential. ***a***, The electrical potential across the inner mitochondrial membrane was manipulated by the addition of varying amounts of CCCP. The magnitude of the potential was assessed by monitoring fluorescence of the dye JC-1. The y-axis shows the percentage change in fluorescence intensity at 595 nm upon addition of the uncoupler CCCP, standardized to the effect of completely dissipating the membrane potential by the addition of valinomycin and FCCP. The relationship between fluorescence and membrane potential is linear from 20% to 100% fluorescence, corresponding to ~50 mV–150 mV, as judged from standardization experiments with potassium diffusion gradients (data not shown). ***b***, Dependence of initial import rates of pb_2(65)–barnase, denatured pb_2(65)–barnase and pb_2(95)–barnase on the membrane potential. In the experiments with denatured pb_2(65)–barnase, the precursor contained the mutations I25A, I76V, I88V and I96V and was unfolded in 8 M urea before import. Relative initial import rates at different concentrations of CCCP were calculated as the percentage of the initial import rate in the absence of CCCP.

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Table 1 Import kinetics of the various precursor proteins[1]

Precursor	Rate constant[2] (min^{-1})	Extent of import[3] (%)	Initial rate[4] (% min^{-1})
pb_2(35)–barnase[5]	0.04 ± 0.002	21 ± 1	0.84 ± 0.06
pb_2(35)-T6A–barnase[5]	0.39 ± 0.02	86 ± 9	37 ± 4
pb_2(35; E15L)–barnase[5]	0.28 ± 0.01	35 ± 10	10 ± 3
pb_2(35; A16K)–barnase[5]	0.063 ± 0.005	25 ± 3	1.6 ± 0.2
pb_2(35+5)–barnase[5]	0.10 ± 0.01	44 ± 3	4.3 ± 0.7
pb_2(65)–barnase	0.13 ± 0.02	39 ± 4	5 ± 1
pb_2(95)–barnase	3.8 ± 0.4	95 ± 10	360 ± 50

[1]Import experiments were performed at 35 °C for all precursors except for pb_2(95)–barnase, where import was performed at 25 °C. Errors are standard errors calculated from at least three repeat measurements.
[2]Rate constants were obtained by fitting the import graphs to a single exponential equation.
[3]Extent of import as a percentage of the total amount of radioactive precursor presented to the mitochondria.
[4]Initial rates are calculated by multiplying the extent of import and the import rate constant.
[5]Precursors contained the mutations I76V/I88V/I96V.

trical component is sufficient for import because the proton gradient can be replaced with a potassium-diffusion potential without impairing protein translocation[16]. The membrane potential is required for an early step in import. Once translocation is initiated, the later ATP-dependent steps can progress in the absence of a membrane potential[25]. The potential has at least two functions in protein import. First, the potential enhances the dimerization of Tim23, a component of the import channel for matrix-targeted precursors in the inner membrane, perhaps to facilitate the interaction between targeting sequences and the import channel[26]. Second, the potential is thought to drive the insertion of targeting sequences into the inner membrane by an electrophoretic effect[27]. We propose that the membrane potential is the main factor in the unfolding of precursor proteins whose targeting sequences are too short to interact with mtHsp70 when bound to the mitochondrial surface.

The membrane potential and protein unfolding

If the membrane potential contributes to the catalysis of protein unfolding, reducing the potential should inhibit import when unfolding is rate determining. Protonophores, such as the uncoupler of respiration carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), reduce the electrical potential by allowing protons to leak across the membrane[27]. The magnitude of the membrane potential can then be monitored by following the fluorescence of dyes, such as 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), that aggregate in the membrane in a potential-dependent manner[28–30]. As expected, titrating limiting amounts of CCCP into our import reactions containing purified yeast mitochondria progressively reduced the electrical potential across the inner membrane (Fig. 1*a*). The potential is completely dissipated by the ionophore valinomycin, the protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or large amounts of CCCP (data not shown).

To test whether the membrane potential contributes to protein unfolding during import, we measured the effect of CCCP on the import rates of two precursor proteins: pb_2(65)–barnase and pb_2(95)–barnase. The precursors consisted of the first 65 (pb_2(65)–barnase) or 95 (pb_2(95)–barnase) amino acids of cytochrome b_2 attached to the N-terminus of the small ribonuclease barnase and differed only in the length of their targeting sequences[3,8]. The stability of the mature domain of the two precursors is unaffected by the targeting sequences[3,8]. For import of pb_2(65)–barnase into purified yeast mitochondria, unfolding of the barnase moiety at the mitochondrial surface is the rate-determining step[3,8]. In contrast, import of pb_2(95)–barnase is not limited by unfolding but rather by some later step, presumably turnover of the translocation machinery[3,8]. We found that reducing the membrane potential with CCCP leads to a progressive reduction in the initial import rates of pb_2(65)–barnase (Fig. 1*b*). At 4 μM CCCP, import of pb_2(65)–barnase is almost completely abolished. The effect of CCCP on import of pb_2(65)–barnase is not due to a general inhibition of the import machinery, because import of pb_2(95)–barnase is largely unaffected under the same conditions (Fig. 1*b*). The net charge of the pb_2(95)–barnase targeting sequence is less positive by one unit than the net charge of the pb_2(65)–barnase targeting sequence. Similarly, when the barnase domain in pb_2(65)–barnase is denatured by mutagenesis and urea before translocation, import of denatured pb_2(65)–barnase shows the same reduced dependence on membrane potential that is found with pb_2(95)–barnase (Fig. 1*b*).

The increased sensitivity of import of pb_2(65)–barnase to reductions in membrane potential compared to pb_2(95)–barnase is not simply due to import of pb_2(65)–barnase being only marginally efficient compared to that of pb_2(95)–barnase. Under our experimental conditions, the extent of import for pb_2(65)–barnase is ~40% of the total amount of precursor presented, only about two-fold lower than the extent of import for pb_2(95)–barnase (Table 1). Nevertheless, at 4 μM CCCP, import of pb_2(65)–barnase is almost completely inhibited, whereas import of pb_2(95)–barnase is nearly unaffected (Fig. 1*b*). We conclude that reducing the electrical potential inhibits the unfolding of barnase at the mitochondrial surface. These results suggest that the electrical potential at the inner mitochondrial membrane contributes to the unfolding of pb_2(65)–barnase during import.

The membrane potential and unfolding pathways

Mitochondria unfold barnase precursors by unraveling them from their N-termini[3]. Because barnase does not normally unfold by this pathway, mitochondria catalyze unfolding by changing the unfolding pathways of these precursors. This change in unfolding can be detected by several different experiments. For example, tightly binding ligands inhibit spontaneous unfolding of barnase *in vitro* and import of barnase precursors whose unfolding is not catalyzed. One such precursor is pb_2(35)–barnase[3,9], which consists of the first 35 amino acids of cytochrome b_2 attached to the N-terminus of barnase. In contrast, ligand binding has little or no effect on import of precursors that are actively unraveled by mitochondria, such as pb_2(65)–barnase and pb_2(95)–barnase[3]. To determine whether the membrane potential is required for the catalysis of precursor unfolding, we measured the effect of ligand binding on import of pb_2(65)–barnase in the presence and absence of the

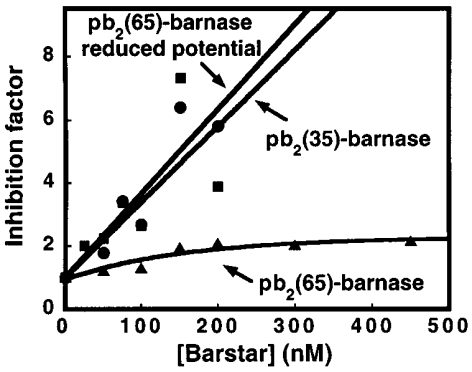

Fig. 2 Membrane potential and precursor unfolding. Reducing the membrane potential inhibits the mitochondrial unfolding activity. Ligand binding inhibits spontaneous unfolding during import of pb_2(35)–barnase and pb_2(65)–barnase in the presence of uncoupler (2 μM CCCP) but not catalyzed unfolding during import of pb_2(65)–barnase. The graph plots the inhibition of import of the indicated precursor proteins by increasing amounts of ligand. The y-axis is the initial import rate in the absence of ligand divided by the initial import rate in the presence of the indicated amount of ligand; and the x-axis is the barstar concentration in nM. pb_2(35)–barnase contains the mutation I76V/I88V/I96V in the mature domain; pb_2(35)–barnase lacking this mutation is not imported efficiently enough to allow ligand binding experiments.

protonophore CCCP. As expected, ligand binding has only a small effect on import of pb_2(65)–barnase when the full membrane potential is present (Fig. 2). However, when the electrical potential is reduced, ligand binding inhibits import of pb_2(65)–barnase as strongly as it inhibits import of pb_2(35)–barnase, demonstrating that pb_2(65)–barnase is no longer being unraveled by mitochondria[3,9] (Fig. 2).

Similarly, point mutations that remove interactions throughout barnase have different effects on spontaneous and catalyzed unfolding of barnase[3]. For example, mutations in a specific subdomain of barnase accelerate spontaneous unfolding but do not affect catalyzed unfolding. Therefore, these mutations accelerate import of pb_2(35)–barnase but not import of pb_2(65)–barnase[3] (Table 2). When the electrical potential across the inner membrane is reduced, the mutations regain most of their effect on import and unfolding of pb_2(65)–barnase, indicating that the unfolding pathway has changed back to that of spontaneous unfolding (Table 2). Together, these results demonstrate that the electrical potential is required to catalyze the unfolding of pb_2(65)–barnase.

Charged amino acids and precursor unfolding

N-terminal mitochondrial presequences are characterized by the presence of basic and the absence of acidic amino acids and, therefore, carry a net positive charge at physiological pH (ref. 31). In the simplest mechanism of membrane potential-driven unfolding, the potential would act directly on charges in the targeting sequence. If this mechanism applies, reducing the positive charge density in a targeting sequence should reduce the mitochondrial unfolding activity. We were unable to test this prediction because removing positive charges reduced the targeting efficiency of the presequences to an extent that it prevented reliable observation of import (data not shown). The explanation for this effect is presumably that the initial recognition of precursors at the mitochondrial surface involves electrostatic interactions[32]. However, a corollary prediction of the hypothesis is that increasing the positive charge density should increase the unfolding capacity of the membrane potential. The targeting sequence of pb_2(35)–barnase is unusual in that it contains a negatively charged amino acid. We tested our hypothesis that the membrane potential causes unfolding by acting on the positive charges in targeting sequences by removing the negative charge in the targeting sequence of pb_2(35)–barnase with the mutation E15L. Mitochondria do not catalyze unfolding of pb_2(35)–barnase without the mutation in the targeting sequence, and ligand binding to barnase inhibits precursor import[3,9] (Fig. 2). When the positive charge density of the targeting sequence was increased, the resultant pb_2(35; E15L)–barnase was imported into mitochondria ~10× faster than pb_2(35)–barnase (Table 1). As predicted, ligand binding no longer inhibited import or pb_2(35; E15L)–barnase (Fig. 3*a*), indicating that unfolding was now catalyzed.

To rule out the trivial explanation that the loss of import inhibition by ligand binding is due to the increased import efficiency of pb_2(35; E15L)–barnase, we enhanced import of pb_2(35)–barnase by destabilizing its mature domain with the mutation T6A. pb_2(35)-T6A–barnase is imported more efficiently than pb_2(35; E15L)–barnase (Table 1), yet barstar binding inhibits import of pb_2(35)-T6A–barnase effectively (Fig. 3*a*). Ligand binding does not affect import of pb_2(35; E15L)-T6A–barnase (data not shown). Similarly, we then studied the unfolding pathway of pb_2(35)–barnase by mutational analysis. Mutations within barnase that accelerated spontaneous unfolding of barnase and import of pb_2(35)–barnase had a much smaller effect on import of pb_2(35; E15L)–barnase (Table 2). Thus, the unfolding of pb_2(35; E15L)–barnase during import resembles that of pb_2(65)–barnase and is catalyzed by mitochondria. The effect of the E15L mutation on import is due to the increased positive charge, rather than some nonspecific effect of the amino acid

Table 2 Effect of mutations on mitochondrial import of different precursor proteins[1]

Mutation in barnase	pb_2(35)–barnase[2]	pb_2(35; E15L)–barnase[2]	pb_2(35; E15L)–barnase[2] in the presence of 4 μM CCCP	pb_2(65)–barnase	pb_2(65)–barnase in the presence of 4μM CCCP
N23A	9 ± 3	1.6 ± 0.5	8 ± 3	2.4 ± 0.4	9 ± 2
I25V	4 ± 1	1.1 ± 0.3	4 ± 1	0.8 ± 0.2	3 ± 1
V36A	3.2 ± 0.2	0.9 ± 0.2	3.6 ± 0.5	1.1 ± 0.2	2.0 ± 0.6
I51V	8 ± 1	2.3 ± 0.5	7 ± 2	1.1 ± 0.1	2.1 ± 0.4
D54A	29 ± 6	5.5 ± 0.5	13 ± 3	2.5 ± 0.4	14 ± 3
N77A	18 ± 8	2.1 ± 0.5	11 ± 2	1.8 ± 0.1	5.1 ± 0.2

[1]The effects of mutations on import are given as the ratio of the initial import rate of mutant divided by the initial import rate of wild type. Errors are standard errors calculated from at least three repeat measurements. Import experiments were performed at 35 °C.
[2]Mutations were generated in an I76V/I88V/I96V mutant background.

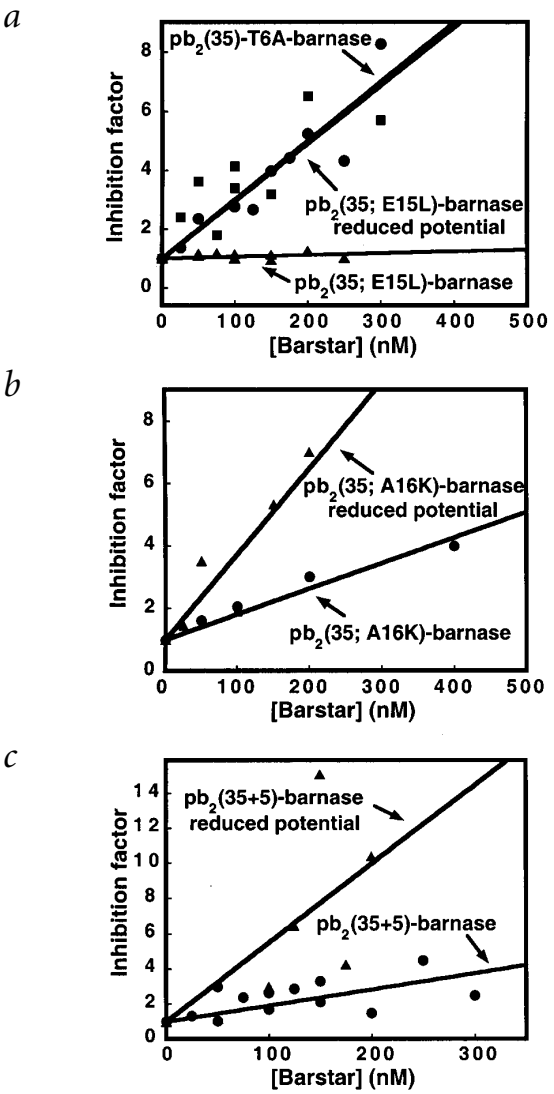

Fig. 3 Membrane potential and targeting sequence charge. The membrane potential exerts its unfolding activity through positively charged amino acids in the targeting sequences. Increasing the net positive charge of the targeting sequence increases the mitochondrial unfolding activity, and reducing the membrane potential reverses this effect. The graphs plot the inhibition of import of the indicated precursor protein by increasing amounts of ligand. The y-axis is initial rate in the absence of ligand divided by the initial import rate in the presence of the indicated amount of ligand; the x-axis is the barstar concentration in nM. ***a***, pb_2(35)-T6A–barnase and pb_2(35; E15L)–barnase in the absence and presence of 4 μM CCCP. ***b***, pb_2(35; A16K)–barnase in the absence and presence of 0.5 μM CCCP. ***c***, pb_2(35+5)–barnase in the absence and presence of 2 μM CCCP. Ligand binding has little effect on import of pb_2(35; E15L)–barnase, pb_2(35; A15K)–barnase and pb_2(35+5)–barnase, indicating that unfolding of these precursors is catalyzed. In contrast, ligand binding effectively inhibits import of pb_2(35)-T6A–barnase, as well as pb_2(35; E15L)–barnase, pb_2(35; A16K)–barnase and pb_2(35+5)–barnase at reduced membrane potential. All barnase precursors in this figure contain the mutation I76V/I88V/I96V in the mature domain.

change. A second, unrelated mutation that adds to the net positive charge in the targeting sequence, A16K, also induced the catalyzed unfolding in precursors during import (Fig. 3*b*).

To test whether the change in unfolding mechanism is due to the membrane potential rather than some other effect of the increased positive charge in the targeting sequences, we reduced the electrical potential with CCCP and again analyzed the unfolding pathway of pb_2(35; E15L)–barnase and pb_2(35; A16K)–barnase during import. At the reduced potential, barstar binding reacquired its inhibitory effect on import of both precursors (Fig. 3). Similarly, mutations in barnase regained their effect on import of pb_2(35; E15L)–barnase in the presence of CCCP (Table 2). Together, these results show that the electrical potential induces protein unfolding by acting directly on positive charges in targeting sequences.

Charge distribution in targeting sequences

The membrane potential is effectively limited to the portion of the import channel in the inner membrane, and only charges in the targeting sequence that are located in the inner membrane will feel the potential. Introducing positive charges in the targeting sequence at positions 18 or 19 did not lead to membrane potential driven unfolding of pb_2(35)–barnase (data not shown). The explanation for this observation is presumably that this part of the targeting sequence is not located in the inner membrane when the folded barnase domain resides at the entrance to the import channel at the mitochondrial surface. Amino acids 18 and 19 are adjacent to a cluster of positively charged amino acids consisting of Arg 20, Lys 23 and Arg 25. If our hypothesis was correct, lowering these charges into the inner mitochondrial membrane by inserting five amino acids between the targeting sequence and the mature domain should lead to membrane potential driven unfolding of the precursor. We tested this prediction by introducing five Gly residues at the end of the targeting sequence of pb_2(35)–barnase to create pb_2(35+5)–barnase. As predicted, advancing the charge cluster into the inner mitochondrial membrane leads to membrane potential-driven unraveling of the precursor. Ligand binding no longer inhibits import of this precursor indicating that unfolding of its mature domain is now catalyzed (Fig. 3*c*). This acceleration of unfolding is dependent on the membrane potential because the effect is abolished when an uncoupler reduces the membrane potential (Fig. 3*c*).

Targeting sequences have to bind to import receptors at the mitochondrial surface and possibly the intermembrane space, as well as to mtHsp70 in the matrix. Some of these interactions involve the burial of hydrophobic surface in the targeting sequence[33,34]. Charged amino acids in the interface between the targeting sequence and the different receptors would interfere

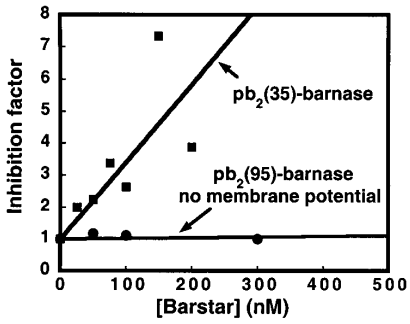

Fig. 4 Unfolding of precursors with long targeting sequences. Unfolding of pb_2(95)–barnase during import is catalyzed in the absence of a membrane potential. Ligand binding does not inhibit import of pb_2(95)–barnase in the absence of a membrane potential, showing that precursor unfolding is still catalyzed by mitochondria. The import experiments were performed in two steps. Precursors were preincubated with mitochondria at 4 °C without added ATP, and chased in the matrix by warming the reaction mixture to 35 °C and addition of ATP and uncoupler.

Table 3 Effect of mutations on mitochondrial import of precursors with long targeting sequences[1]

Mutation in barnase	pb_2(35)–barnase[2]	pb_2(95)–barnase[3]	pb_2(95)–barnase membrane potential dissipated[3,4]
L14A	140 ± 20	1.2 ± 0.3	1.3 ± 0.4
N23A	9 ± 3	0.9 ± 0.2	1.3 ± 0.3
I25V	4 ± 1	0.9 ± 0.2	0.8 ± 0.1
I51V	8 ± 1	1.3 ± 0.4	0.9 ± 0.2
D54A	29 ± 6	0.9 ± 0.2	1.2 ± 0.3
N77A	18 ± 8	1.1 ± 0.2	1.5 ± 0.5

[1]The effects of mutations on import are given as the ratio of the initial import rates of mutant divided by the initial import rate of wild type. Errors are standard errors calculated from at least three repeat measurements.
[2]Mutations were generated in an I76V/I88V/I96V mutant background. Import experiments were performed at 35 °C.
[3]Import experiments were performed at 25 °C.
[4]Import experiments were performed in two steps. An import intermediate was first accumulated at 0 °C. The precursor protein was then chased into mitochondria by adding ATP, dissipating the membrane potential and raising the temperature to 25 °C. To dissipate the membrane potential, 2 μM valinomycin and 25 μM FCCP were used.

with these interactions. Mutating residue 14 to Arg in the targeting sequence of pb_2(35)–barnase does not lead to membrane potential-driven unfolding but impairs import instead (data not shown). A probable explanation is that the charged residue interferes with an interaction of the targeting sequence with a component of the import machinery. In agreement with this interpretation, we find that mutating Cys 14 to the hydrophilic Ser leads to an ~10-fold reduction in the initial import rates of pb_2(65)–barnase, whereas mutating Cys 14 to the more hydrophobic Val does not impair import (data not shown).

Precursors with long targeting sequences

Import of a precursor with an exceptionally long targeting sequence, pb_2(95)–barnase, is considerably less sensitive to the reductions of the electrical potential than the shorter precursors (Fig. 1*b*). This observation indicates that precursor proteins with long targeting sequences may rely on factors other than the electrical potential for unfolding. We tested whether the electrical potential is required for the unfolding of these long precursors by performing a two-step import assay. In these experiments, the general requirement for the electrical potential for the insertion of targeting sequences into the import machinery was separated from the unfolding step[25]. Precursors were first preincubated with ice-cold mitochondria in the presence of the electrical potential. Under these conditions, the targeting sequence inserts into the import channel, but the mature domain remains at the mitochondrial surface. The mature domain remains folded, and treatment with the nonspecific protease proteinase K leads to the quantitative recovery of soluble and protease resistant mature protein (data not shown). The mitochondria were then reisolated and import was initiated at a physiological temperature by simultaneously adding strong uncouplers and ATP, so that the membrane potential was dissipated as the ATP-dependent component of the import machinery became activated. pb_2(95)–barnase was imported efficiently in the absence of the membrane potential (data not shown). Similarly, barstar binding did not inhibit import of pb_2(95)–barnase (Fig. 4), and destabilizing mutations in the barnase domain did not accelerate import (Table 3) in the absence of the membrane potential. These results demonstrate that unfolding was still catalyzed by mitochondria and did not become rate determining for import. Therefore, catalysis of unfolding of this long precursor protein does not depend on the electrical potential. In contrast, import of shorter precursor proteins, such as pb_2(65)–barnase and pb_2(35; E15L)–barnase, was completely abolished in equivalent experiments (data not shown), suggesting that the unfolding of shorter precursor proteins depends primarily on the membrane potential.

mtHsp70 and precursor unfolding

If unfolding of pb_2(95)–barnase is not catalyzed by the electrical potential, the most probable alternative candidate is mtHsp70. The role of mtHsp70 in protein unfolding is well established, primarily through experiments with the mutation *ssc1-2* in the peptide-binding domain of mtHsp70 (refs 17–21). The *ssc1-2* mutation seems to specifically affect an unfolding activity of mtHsp70 because it does not change import of a precursor protein containing a loosely folded domain[18,19]. We found that the *ssc1-2* mutation inhibited import of pb_2(95)–barnase precursors, as expected (Fig. 5*a*). This result suggests that mtHsp70 plays a major role in unfolding pb_2(95)–barnase during import, as was found for import of other precursors with long targeting sequences[17–21]. In contrast, import of pb_2(35; E15L)–barnase is not affected by the *ssc1-2* mutation (Fig. 5*b*). This result is expected because pb_2(35; E15L)–barnase cannot interact directly with mtHsp70 before unfolding. Therefore, we propose that the unfolding of pb_2(35; E15L)–barnase depends primarily on the membrane potential acting directly on the positive charges of targeting sequences.

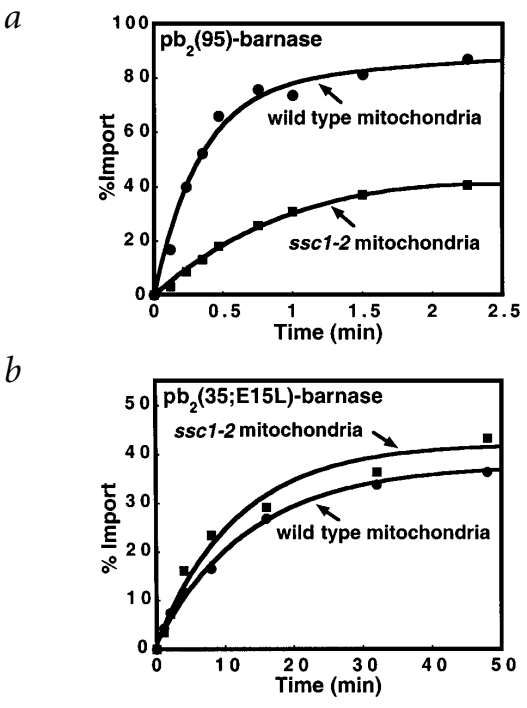

Fig. 5 Unfolding by mtHsp70. Import of pb_2(95)–barnase is more sensitive to impairment of the unfolding activity of mtHsp70 than pb_2(35; E15L)–barnase. Import graphs show the effects of *ssc1-2* mutation in mtHsp70 on the import of ***a***, pb_2(95)–barnase and ***b***, pb_2(35; E15L)–barnase. The mutant phenotype was induced by preincubating mitochondria at 37 °C for 15 min before import was initiated at 25 °C by the addition of precursor proteins.

Conclusions

Protein unfolding can be catalyzed by the mitochondrial import machinery[3,8]. The two candidates for this unfolding activity are the electrical potential across the inner mitochondrial membrane and mtHsp70 in the matrix. We propose that most precursor proteins cannot interact with mtHsp70 in their native conformation. These precursors are unraveled by the membrane potential, which acts directly on their positively charged targeting sequences. We suggest that the interaction between membrane potential and precursor biases the diffusion of the targeting sequence through the import channel. The polypeptide stretch linking the charged amino acids and the mature domain then transmits the effect of the membrane potential to the folded structure at the mitochondrial surface. There, spontaneous unfolding fluctuations at the N-terminus of the structure are trapped by the movement of the polypeptide chain into the import channel; the mature domain then unravels[3]. At the same time, the force generated on the targeting sequence by the membrane potential may also lower the activation barrier for the spontaneous local unfolding events in the mature domain or, less probable, induce new fluctuations. Regardless, the membrane potential unravels precursor proteins by pulling at their charged targeting sequences.

What is the function of mtHsp70 in protein unfolding? One function of mtHsp70 will be to arrest the movement of the targeting sequence induced by the membrane potential[35]. In addition, mtHsp70 itself acts as an unfoldase[17–21] and can unravel some proteins in the absence of the electrical potential (Fig. 4; Table 3). Unfolding by the electrical pulling mechanism described above requires the polypeptide link between the inner membrane and the folded domain to provide a tense connection. This requirement is not always satisfied for precursors with long targeting sequences. For example, import and unfolding of pb_2(95)–barnase, which has a long targeting sequence, depends on the unfolding activity of mtHsp70 (Fig. 5). A similar situation probably occurs for precursors that contain several independently folded domains. Presumably, the domains in these precursors will unfold sequentially during import so that the C-terminal domains will be separated from the targeting sequence by a long stretch of connecting polypeptide chain. In this situation, any pulling of electrical potential at the targeting sequence will not be able to contribute to the unfolding of C-terminal domains. mtHsp70 may provide the main unfolding activity in these situations because it binds all protein sequences with relatively small variations in specificity[36,37], and both mtHsp70 and precursor are present at high local concentrations at the exit of the import channel. Finally, mtHsp70 is strictly required for all protein import into the mitochondrial matrix, including import of proteins that lack three-dimensional structure[10]. One of the functions of mtHsp70 could be to overcome friction between the translocating polypeptide chain and the import channel[38]. Friction may occur because the inner membrane channel seems to close tightly around precursors[7,39].

Methods

Proteins and mitochondria. Precursor proteins, consisting of a mitochondrial-targeting sequence fused to the N-terminus of the ribonuclease barnase[40], were constructed in pGEM-3Zf(+) vectors (Promega)[8]. The targeting sequence was derived from cytochrome b_2 and contained two mutations: R30G to prevent processing by the mitochondrial matrix processing protease[41], and L62P to inactivate the intermembrane-space sorting signal and target attached proteins to the matrix[42]. The passenger protein barnase contained the mutation H102A to inactivate its ribonuclease activity[43], as well as the mutation Q2M to allow radioactive labeling[8]. In addition, the mature barnase domain in the pb_2(35)–precursors (pb_2(35)–, pb_2(35; E15L)–, pb_2(35; A16K)– and pb_2(35+5)–barnase) was destabilized by the mutations I76V/I88V/I96V to allow efficient import[3]. pb_2(35)–barnase lacking these mutations is not imported efficiently enough to allow satisfactory measurement of import inhibition. The mutations to probe protein structure were introduced by subcloning from the original vectors[44] or using the QuickChange kit (Stratagene). All mutations were verified by DNA sequencing. For the ligand binding experiments, the C40A/C82A double mutant of barstar[45] was purified from *Escherichia coli* as described[9].

Three yeast strains were used for isolation of mitochondria. Mitochondria were purified from *Saccharomyces cerevisiae* strain D273-10B (MATα, ATCC 25657) by centrifugation through a Nycodenz (Nycomed) gradient[46]. Mitochondria were purified from CMV2 and CMV3, carrying a plasmid expressing mutant *ssc1-2* and wild type *SSC1* (mtHsp70), respectively, using published protocols at the permissive temperature[18,19].

Import experiments. Import rates were measured as described[6]. Briefly, radioactive proteins were expressed from a T7 promoter by *in vitro* transcription and translation in rabbit reticulocyte lysate (Promega) supplemented with [^{35}S]Met, and partially purified by high-speed centrifugation and ammonium sulfate precipitation[8]. Precursors were then incubated with mitochondria at 0.4 mg ml^{-1} mitochondrial protein in import buffer (0.6 M sorbitol, 50 mM HEPES-KOH, pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mg ml^{-1} fatty acid-free bovine serum albumin (BSA) and 5 mM Met) containing 4 mM ATP, 10 mM creatine phosphate and 0.15 mg ml^{-1} creatine kinase. At designated times, aliquots were transferred to ice-cold stop buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4, 2 μM valinomycin and 0.2 mg ml^{-1} proteinase K). After 10 min, the protease was inhibited with 1 mM PMSF and mitochondria were re-isolated by centrifugation. Samples were separated by SDS-PAGE, and the amount of imported protein was quantified by electronic autoradiography. The import kinetics were analyzed using Kaleidagraph (Synergy Software), assuming first-order kinetics. Initial import rates were calculated by multiplying the import rate constants with the extent of import obtained by curve fitting.

Import into D273-10B mitochondria was performed at 35 °C for pb_2(35)–barnase, pb_2(35; E15L)–barnase, pb_2(35; A16K)–barnase and pb_2(65)–barnase, and at 25 °C for pb_2(95)–barnase, unless stated otherwise. Import into CMV2 and CMV3 mitochondria was performed at 25 °C after the mitochondria were incubated at 37 °C for 15 min to induce the mutant phenotype. In the ligand binding experiments, import rates were measured as described above except that barnase precursors and import mix were preincubated with barstar at the indicated concentrations for 5 min. In the import assay of pb_2(95)–precursors in the absence of an electrical potential, precursors were first incubated with 0.3 mg mitochondria for 1 min on ice. The mitochondria were re-isolated by centrifugation, and the mitochondrial pellet was resuspended in 50 μl ice-cold import buffer. Import was then initiated by adding 40 μl mitochondria into import buffer that contained 4 mM ATP, 10 mM creatine phosphate, 0.15 mg ml^{-1} creatine kinase, 2 μM valinomycin, 25 μM FCCP and 5 μg ml^{-1} oligomycin at 35 °C so that the potential is dissipated as mtHsp70 becomes activated. At designated times, aliquots were transferred to eight volumes of ice-cold mitoplasting stop buffer (20 mM HEPES-KOH, pH 7.4, 1 mg ml^{-1} fatty acid-free BSA and 0.2 mg ml^{-1} proteinase K). After 30 min, the protease was inhibited with 1 mM PMSF, and mitochondria were re-isolated by centrifugation.

CCCP titration in import experiments. The protonophore CCCP was used to reduce the mitochondrial membrane potential[27,47,48]. Before adding precursor proteins, 0.4 mM NADH and different amounts of CCCP were added to 0.4 mg ml^{-1} mitochondria in import mix. Oligomycin (5 μg ml^{-1}) was added to prevent the reverse action of F_oF_1-ATPase to generate membrane potential. The import mix

was then incubated at 25 °C for 10 min. Import experiments were performed as described above.

Assessment of the electrical potential of the inner mitochondrial membrane. The magnitude of the mitochondrial membrane potential can be determined by measuring fluorescence of J-aggregates of the dye JC-1 (Molecular Probes) because of its potential-dependent uptake into membranes and aggregation[28–30]. JC–1 J-aggregate fluorescence (emission maximum at 595 nm) increased linearly with increasing membrane potential over the range of 30 to 180 mV (ref. 29; data not shown). Experiments were performed in an ISS PC1 fluorimeter (excitation wavelength 490 nm, emission scan over a range of 505 to 620 nm and 4 nm slits) at 35 °C as described[28]. Mitochondria were incubated at 0.4 mg ml^{-1} in the presence of different amounts of CCCP in import buffer containing 4 mM ATP, 10 mM creatine phosphate, 0.15 mg ml^{-1} creatine kinase and 0.4 mM NADH. Fluorescence was measured after 5 μg ml^{-1} oligomycin, and 2.6 μM of JC-1 were added.

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Competing interests statement

The authors declare that they have no competing financial interests.

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