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Active unfolding of precursor proteins during mitochondrial protein import

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Precursor proteins made in the cytoplasm must be in an unfolded conformation during import into mitochondria. Some precursor proteins have tightly folded domains but are imported faster than they unfold spontaneously, implying that mitochondria can unfold proteins. We measured the import rates of artificial precursors containing presequences of varying length fused to either mouse dihydrofolate reductase or bacterial barnase, and found that unfolding of a precursor at the mitochondrial surface is dramatically accelerated when its presequence is long enough to span both membranes and to interact with mhsp70 in the mitochondrial matrix. If the presequence is too short, import is slow but can be strongly accelerated by urea-induced unfolding, suggesting that import of these 'short' precursors is limited by spontaneous unfolding at the mitochondrial surface. With precursors that have sufficiently long presequences, unfolding by the inner membrane import machinery can be orders of magnitude faster than spontaneous unfolding, suggesting that mhsp70 can act as an ATP-driven force-generating motor during protein import.

Keywords: heat shock protein 70/hsp70/import/motor proteins/protein translocation

Introduction

Protein precursors that are imported into mitochondria from the cytosol must be in an unfolded state in order to pass through the import machinery (Eilers and Schatz, 1986). Import appears to be driven by the ATP-dependent action of the mitochondrial hsp70 (mhsp70), a matrix-localized chaperone of the hsp70 family. Precursors interact with mhsp70 while still associated with the mitochondrial membranes (Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991) and mutations in mhsp70 lead to an accumulation of precursors stuck in the import channel (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Schneider *et al.*, 1994). Mhsp70 drives import in conjunction with Tim44, a component of the inner membrane import channel (Kronidou *et al.*, 1994; Rassow *et al.*, 1994), and mGrpE (also known as Mge1p or Yge1p), a nucleotide exchange factor related to bacterial GrpE (Bolliger *et al.*, 1994; Laloraya *et al.*, 1994; Voos *et al.*, 1994). However, the molecular mechanism by which mhsp70 functions in protein import remains unclear.

It is particularly puzzling that mitochondria can import some precursor proteins that fold stably prior to import. Examples are artificial precursors consisting of short mitochondrial presequences fused to mouse dihydrofolate reductase (DHFR). Import of these precursors occurs within tens of minutes, with import rates apparently determined by the spontaneous unfolding of the DHFR moiety (Vestweber and Schatz, 1988). For many other proteins the half-time of spontaneous unfolding is in the range of hours or even days (Creighton, 1993), yet mitochondria can import precursors with such stable domains (Glick *et al.*, 1993). Here we show that a component of the inner membrane import machinery, presumably mhsp70, causes the unfolding of a precursor protein at the outer surface of mitochondria while bound to the precursor's N-terminal presequence in the mitochondrial matrix. These results have a bearing on the question of how mhsp70 works as an import machine.

Results

Precursor proteins

In order to test whether the import rate is determined by a precursor's ability to interact with different parts of the import machinery before unfolding, we constructed a series of precursors with N-terminal presequences of varying lengths. The presequences were derived from the presequence of yeast cytochrome b_2, and contained between 35 and 95 amino acids in steps of 10 amino acids, starting from the initiator methionine. All presequences contained an Arg→Gly mutation at position 30 of the authentic yeast sequence, to prevent processing by the mitochondrial matrix processing protease (Arretz *et al.*, 1994), and a Leu→Pro mutation at position 62 to target the precursor to the mitochondrial matrix (Beasley *et al.*, 1993). The passenger protein was either mouse DHFR (Hurt *et al.*, 1984) or barnase, a small bacterial ribonuclease (Hartley, 1989).

There are two sharply different import rates for a given passenger protein, depending on the length of the presequence

For the precursors just described, we measured the rates of import into purified yeast mitochondria. Radioactively labeled precursors were synthesized *in vitro* by coupled transcription and translation, partially purified by ammonium sulfate precipitation, and incubated with purified yeast mitochondria in the presence of ATP. At different times, mitochondrial samples were treated with protease,

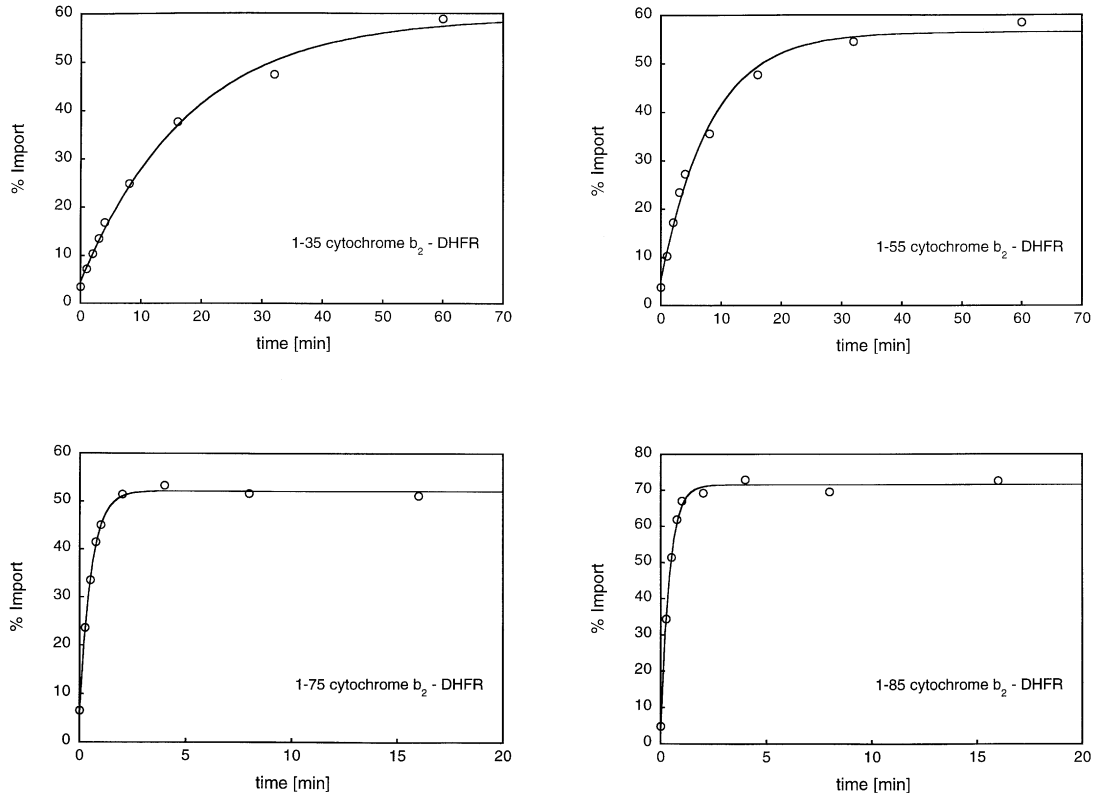

Fig. 1. Import kinetics of cytochrome b_2–DHFR precursors with presequences of 35, 55, 75 and 85 amino acids. *y*-axis: amount of precursor imported as a percentage of the total amount of precursor presented to the mitochondria. *x*-axis: import time in minutes. See Materials and methods for experimental details.

re-isolated and subjected to SDS–PAGE. Autoradiographs of the dried gels revealed a single radioactive band representing the imported protein. This band could be quantified with a phosphor-imager or an electronic autoradiography system. However, we found that it was much faster and equally accurate to acid-precipitate the re-isolated mitochondria and measure the imported protein by liquid scintillation counting. This new method allowed us to sample many points and determine import kinetics with reasonable precision. The extent of import was plotted as a percentage of the total amount of precursor added to the mitochondria at the beginning of the time course (Figure 1). The two methods of determining import yielded similar results.

A plot of the import rate constants versus the length of the presequence showed that import rates of DHFR-containing precursors depended on the length of the presequence, with a sharp increase at about 65 amino acids (Figure 2). The import rate constant of precursors with presequences of 75 or more amino acids was 40 times larger than that of precursors with presequences shorter than 55 amino acids. There was another increase in the rate constant when the presequence exceeded 85 amino acids, but this increase was only 2-fold, much less than the 40-fold increase seen at around 65 amino acids.

Folded precursors with presequences of different length interact with different parts of the import machinery

The substrate analog methotrexate stabilizes DHFR by binding to its active site (Eilers and Schatz, 1986; Kraut and Matthews, 1987). In the presence of methotrexate, a DHFR-containing precursor binds to the mitochondrial import receptor and inserts its presequence into the import machinery, but the methotrexate-stabilized DHFR moiety cannot be unfolded and remains at the mitochondrial surface (Eilers and Schatz, 1986; Haucke *et al.*, 1995). Experiments with such arrested precursors had shown that the presequence must be at least 55 amino acids long to

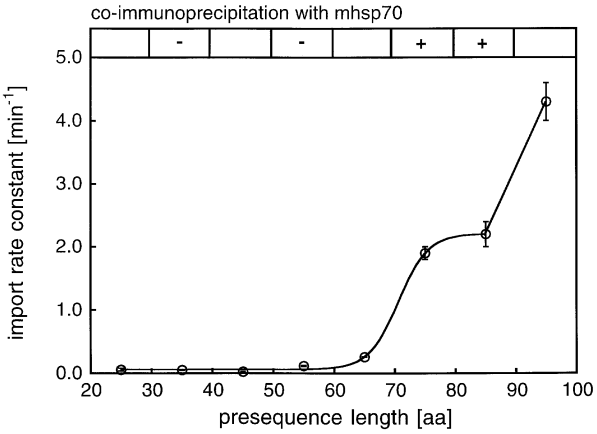

Fig. 2. Dependence of import rate constants of cytochrome b_2–DHFR precursors on presequence length. *y*-axis: import rate constants in min^{-1}. *x*-axis: presequence length in number of amino acids (aa). The import rate constants were measured at 30°C as described in Materials and methods. Error bars give the standard deviation of at least three repeat measurements. Precursors with presequences of 35, 55, 75 and 85 amino acids were also tested for co-immunoprecipitation with antibodies against mhsp70.

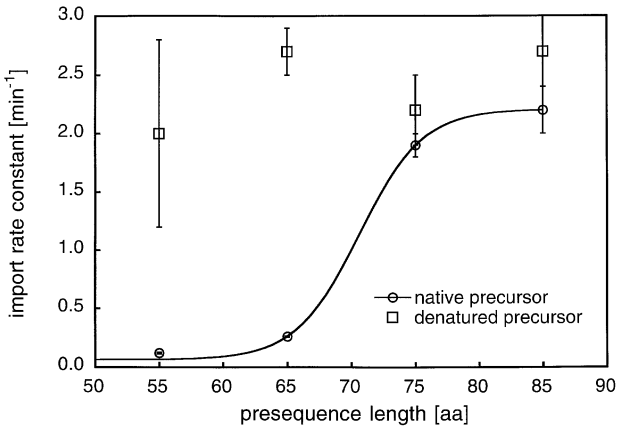

Fig. 3. Denaturation of cytochrome b_2–DHFR precursors accelerates import of short precursors, but not that of long precursors. After *in vitro* transcription/translation, precursors were precipitated with 66% saturated ammonium sulfate and redissolved in import buffer (native precursor) or in 8 M urea containing 25 mM Tris–HCl, pH 7.4 and 25 mM dithiothreitol (denatured precursor). Import kinetics were measured at 30°C. Error bars give the standard deviation of at least two repeat measurements.

span both mitochondrial membranes (Rassow *et al.*, 1990) and that it must be at least 60 amino acids long to interact with mhsp70 in the matrix (Ungermann *et al.*, 1994).

The DHFR-containing precursors used here behaved similarly: when they were presented to mitochondria in the presence of methotrexate, they bound to the mitochondria without being imported; and if their presequence was at least 75 amino acids long, they could be co-immunoprecipitated with antibodies against mhsp70 after solubilization of the mitochondria (Figure 2, top bar). Thus, once the critical presequence length of 75 amino acids is reached, the folded, translocation-arrested DHFR fusion protein can interact with mhsp70 in the matrix. This critical length agrees with that at which there is a sharp increase in the import rate of the folded precursor.

The two basic import rates represent two different rate-limiting steps in import

When precursors with presequences of 65 amino acids or less were denatured with urea, their import rates were drastically increased (Figure 3; see also Eilers *et al.*, 1988). For presequences of 55 and 65 amino acids, import of the denatured precursors became as fast as that of native precursors with presequences longer than 75 amino acids. The import rates of precursors with presequences of 75 or 85 amino acids were not affected (Figure 3). Denaturation of the DHFR domain thus abolishes the effect of critical sequence length at which the bound precursor can interact with mhsp70 and at which import of native precursors suddenly increases dramatically.

Import of folded precursors can also be accelerated by point mutations that destabilize the mature protein (Vestweber and Schatz, 1988). When we used such a destabilized DHFR variant (called 7/42/49) as the passenger protein in our series of precursors, import of precursors with presequences of 65 amino acids and less was accelerated, whereas import of precursors with presequences of 75 amino acids or more was unaffected (Table I).

We have also measured precursor unfolding rates during import using the observation that folded DHFR is protease-resistant whereas unfolded DHFR is protease-sensitive. Thus, when importing mitochondria are treated with protease, precursor that is not yet imported is released from the mitochondrial surface. If the precursor is folded it will appear as a protease-resistant fragment in the supernatant; if it is unfolded, it will be degraded to small peptides. In addition to the imported protein and the folded protein outside mitochondria, Figure 4 also shows the total amount of protease-resistant precursor. If the precursor unfolds as rapidly as it is imported, the total amount of protease-resistant precursor should remain constant throughout the experiment because the imported molecules become inaccessible to the added protease. If the precursor unfolds more rapidly than it is imported, the total amount of protease-resistant protein should decrease rapidly and then increase as the unfolded protein becomes protease-inaccessible upon import. Figure 4 shows that (1–35) cytochrome b_2–DHFR precursor unfolds at the same rate at which it is imported, suggesting that unfolding is rate determining. For the (1–85) cytochrome b_2–DHFR precursor this is not case. The import machinery unfolds the long precursor more rapidly than it imports the precursor. Although the unfolding of the precursor is too rapid to be measured by this method, the graph clearly shows the expected recovery in the total amount of protease-resistant and protease-inaccessible protein as the unfolded precursor is imported.

Table I. Import half-times for cytochrome b_2–DHFR precursors

Targeting sequence length (amino acids)	Import half-times (s)		Acceleration factor
	wt DHFR	Destabilized DHFR	
35	710 ± 40	44 ± 3	16 ± 2
55	350 ± 10	70 ± 6	5.0 ±0.5
65	160 ± 4	35 ± 6	4.6 ± 0.8
75	22 ± 0.3	18 ± 2	1.2 ± 0.1
85	19 ± 2	12 ± 1	1.6 ± 0.2

In summary, three independent experiments show that for precursors with short presequences, the import rate is limited by the unfolding of the precursor on the mitochondrial surface. Precursors with long presequences can interact with the import machinery before they unfold. In this case the import machinery unfolds the precursor rapidly and then imports it. The import rate is no longer limited by spontaneous unfolding, but is probably determined by the turnover rate of the import machinery.

The sudden increase in import rates occurs at shorter presequence lengths when precursors are imported across only a single membrane

Inner membrane vesicles consist of a single membrane, the mitochondrial inner membrane, which entraps diluted matrix. They are prepared from mitochondria by hypotonic shock, sonication and sucrose density gradient centrifugation (Jascur, 1991). Although these vesicles lack the import machinery of the outer membrane, they can import precursor proteins efficiently. With inner membrane vesicles, the sharp increase in import rates was already found when the length of the presequence reached 55 amino acids, which is 15 amino acids fewer than that for

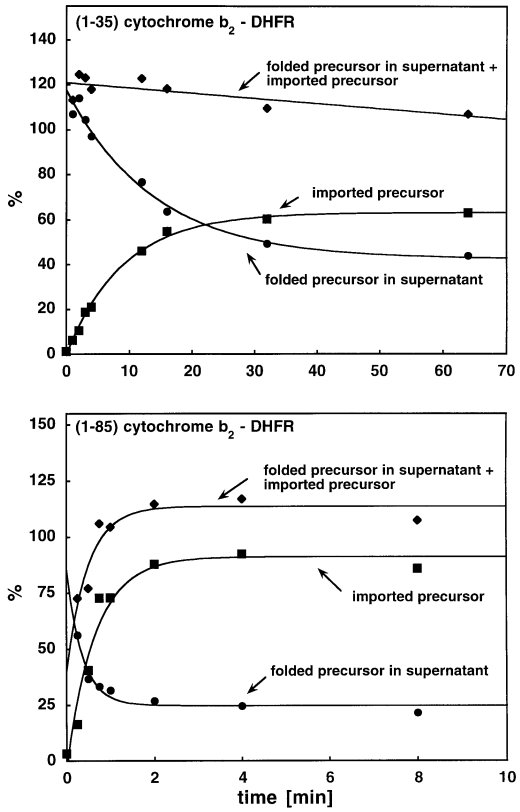

Fig. 4. Precursor unfolding is rate limiting for the (1–35) cytochrome b_2–DHFR precursors but not for the (1–85) cytochrome b_2–DHFR precursor. Import experiments were performed as described in Materials and methods at 30°C but the amount of folded precursor outside mitochondria was also determined by precipitating the supernatant after protease treatment of the import sample and quantifying the amount of radioactivity in protease-resistant DHFR fragment by SDS–PAGE and electronic autoradiography. The graphs also show the sum of imported precursor plus protease-resistant precursor outside mitochondria as the total amount of protease-resistant precursor.

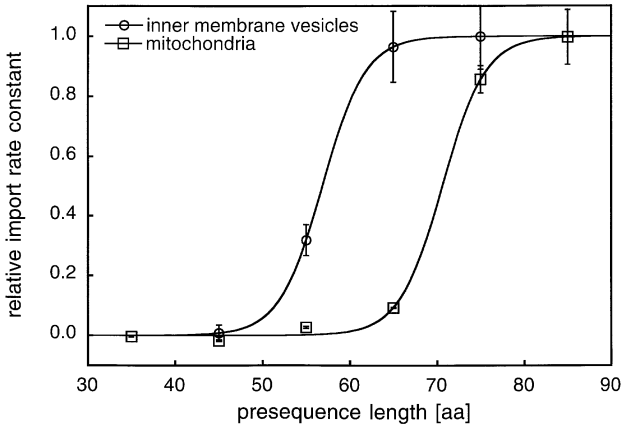

Fig. 5. Import into inner membrane vesicles decreases the critical length of the presequence at which import into intact mitochondria suddenly becomes much faster. To facilitate comparison of the critical length of the presequence for import into mitochondria and inner membrane vesicles, the import rates of the long precursors into both preparations were separately set to 1, and those of the short precursors were set to 0. *y*-axis: relative import rate constants. *x*-axis: length of the presequence in amino acids. The import rate constants were measured at 30°C. Error bars reflect the standard deviation of at least three measurements for import into mitochondria and the curve-fitting error estimated by the fitting program for import into inner membrane vesicles.

intact mitochondria (Figure 5). The import rate of the shortest precursor analyzed (a 45-amino-acid targeting sequence), for which spontaneous unfolding is rate-limiting, was identical to that seen with intact mitochondria. The import rate for the long precursors [i.e. (1–65) cytochrome b_2–DHFR and (1–75) cytochrome b_2–DHFR] was about six times lower than with intact mitochondria, probably because the matrix-localized components of the import machinery (such as mhsp70) were diluted when inner membrane vesicles were prepared. Nevertheless, import of the long precursors into inner membrane vesicles was still six times faster than that of the short precursors. Destabilization of the passenger protein by point mutation (see above) increased the rate of import of the shortest precursor tested (a 45-amino-acid presequence) 4-fold and that of the longest precursor tested (a 75-amino-acid presequence) only 2-fold. We do not know why the critical presequence length with inner membrane vesicles is not exactly half that seen with intact mitochondria; perhaps mhsp70 binding to the precursor imposes an additional length requirement beyond that imposed by the outer membrane. Nevertheless, these data confirm that it is the relative lengths of the presequence and the import channel, rather than interactions of the presequence with the DHFR domain, that determine the import rates of the fusion proteins.

Unfolding rates of precursors can be measured by protease resistance and by binding to GroEL

The experiments described above suggested that the import rate of DHFR-containing precursors with short presequences is limited by the spontaneous unfolding of the DHFR domain. Is the spontaneous unfolding of DHFR fast enough to explain the observed import rates? To answer this question, we measured DHFR unfolding by two different methods. The first method is based on the observation that folded DHFR is protease-resistant whereas unfolded DHFR is protease-sensitive; the spontaneous unfolding rate of DHFR can thus be estimated from the rate at which DHFR is degraded by added protease. However, since the protease digests the targeting sequence rapidly, this method can only measure the spontaneous unfolding of the passenger protein lacking the targeting sequence. The second method exploits the observation that *Escherichia coli* GroEL binds to unfolded, but not to folded, DHFR (Viitanen *et al.*, 1991); the time-dependent binding of DHFR to added GroEL thus measures the spontaneous unfolding rate of DHFR (Table II). For DHFR without a targeting sequence, the unfolding rates measured by the two methods agree within a factor of <2. The spontaneous unfolding at 30°C of a DHFR precursor with a presequence of 35 amino acids was ~7 times faster than import. The fact that spontaneous unfolding of the short precursor is faster than import suggests that not every unfolding transition leads to a productive interaction with the import machinery. In contrast, spontaneous unfolding in free solution of a DHFR precursor with a presequence of 85 amino acids was ~5 times slower than import at 30°C and 24 times slower than import at 10°C. Thus, DHFR precursors with long

Table II. Unfolding and import half-times for different precursor proteins

Passenger protein	Presequence length (amino acids)	Half-times (s)	
		Unfolding	Import
DHFR (30°C)	0	63 ± 8[a]	n/a
DHFR (30°C)	35	114 ± 13[b]	710 ± 40
DHFR (30°C)	85	102 ± 7[b]	19 ± 2
DHFR (10°C)	85	8000 ± 1600[b]	350 ± 60
barnase (30°C)	0	>1800[c]	n/a
barnase (30°C)	95	n.d.	11 ± 1

n.d.: not determined. n/a: not applicable.
[a]Measured by protease sensitivity.
[b]Measured by binding to GroEL.
[c]Measured by extrapolation of urea unfolding data obtained by monitoring by intrinsic tryptophan fluorescence (Matouschek *et al.*, 1994a). For experimental details see Materials and methods.

presequences are actively unfolded by the import machinery.

The slowly unfolding barnase domain is rapidly imported into mitochondria only when the attached presequence is long enough to interact with mhsp70

We constructed a second series of precursors that contained the same cytochrome b_2 presequences, but barnase as the passenger protein. Barnase is a small (12 382 Da) ribonuclease of *Bacillus amyloliquefaciens* (Hartley, 1989). Folding of heat- or urea-denatured barnase has been analyzed in some detail by measuring intrinsic tryptophan fluorescence, circular dichroism, deuterium–hydrogen exchange and nuclear magnetic resonance (Matouschek *et al.*, 1994b). Wild-type barnase in water at 30°C unfolds with a half-time of >30 min (Matouschek *et al.*, 1994a, 1995). Although these unfolding rates of barnase have been measured in the absence of an attached presequence, the effect of mitochondrial presequences on the stability of the attached mature domain is generally very small (Endo and Schatz, 1988; Schmid *et al.*, 1992) and no significant effect on the unfolding rates can be detected in the case of DHFR (Table II). Unfolding rates of barnase with targeting sequences cannot be measured by the GroEL binding assay because barnase refolds even while bound to GroEL (Gray and Fersht, 1993).

We measured import rates of barnase fusion proteins containing the same set of presequences that had been used for the DHFR constructs (Figure 6). Import of the barnase constructs with presequences shorter than 55 amino acids was too slow to be measured accurately, presumably because spontaneous unfolding of barnase is so slow. Precursors with presequences longer than 65 amino acids were imported as fast as, or even faster than, the corresponding DHFR-containing precursors. Import of barnase attached to a presequence of 75 amino acids or more was at least 200 times faster than the spontaneous unfolding rate of barnase in solution (Table II).

Even short precursors are largely associated with the mitochondrial outer membrane throughout the import experiment

To check whether import of a slowly importing (1–55) cytochrome b_2–DHFR precursor occurred from a pre-

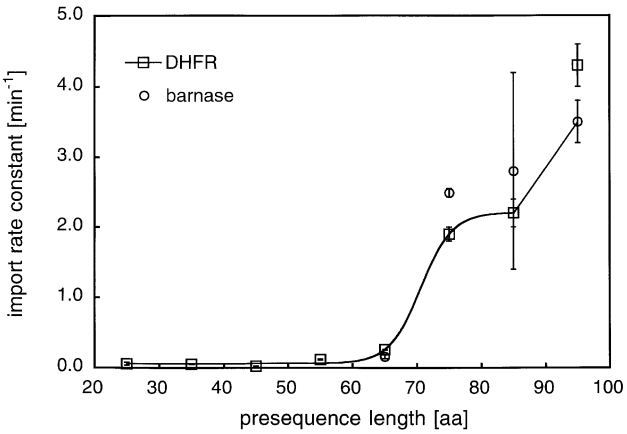

Fig. 6. Import rates of cytochrome b_2–barnase and DHFR precursors with presequences of different lengths. Import rates were measured as described in Materials and methods and in Figure 2. Error bars give the standard deviation of at least two repeat measurements.

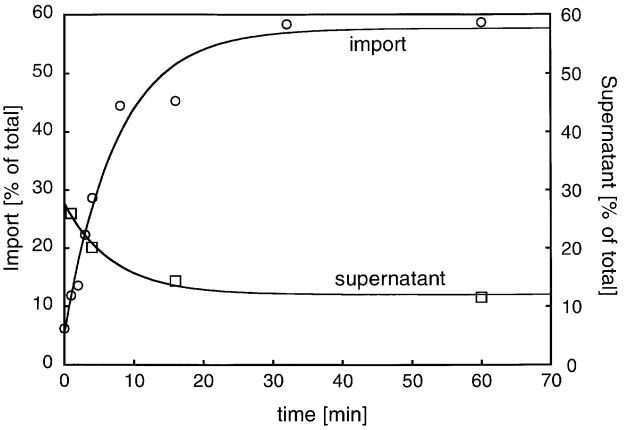

Fig. 7. Most of the 1–55 cytochrome b_2–DHFR precursor is already associated with mitochondria at the beginning of the import experiment. Import reactions were performed as described in Materials and methods. After stopping import, the mitochondria were sedimented by centrifugation and the amount of radioactive precursor in the supernatant was determined as described in Materials and methods. Mitochondria were then treated with protease, reisolated and analyzed for the amount of imported protein. The graph shows the amount of precursor in the supernatant and the amount of precursor imported as a percentage of the total amount of precursor added to the import reaction.

bound state or from free solution, we quantified the precursor in the supernatant and inside the mitochondria throughout an import experiment. After initiating import, samples were taken at different times and cooled to 4°C to block further import of any bound precursor. After removing mitochondria by centrifugation, the proteins of the supernatant were acid-precipitated and assayed for radioactive precursor as described above. The mitochondria were treated with protease to remove bound precursor, re-isolated and analyzed for imported precursor. Figure 7 shows that as much as two-thirds of the import-competent precursor was already associated with the mitochondria at the first time point, when only <5% of the precursor had been imported. Under our assay conditions, most of the import-competent precursor is thus bound to the mitochondrial surface at the beginning of the import assay.

(1–95) Cytochrome b_2–barnase and –DHFR precursors bound to the mitochondrial surface in the absence of ATP remain protease-resistant for longer than it takes to import them

When precursors containing a folded domain are incubated with ATP-depleted mitochondria that still maintain an electrochemical potential across the inner membrane, they form a productive import intermediate with their folded domain accessible to protease at the mitochondrial surface. On adding ATP, the precursor is chased into the mitochondria (Hwang *et al.*, 1991; Rospert *et al.*, 1996). Does the mitochondrial surface unfold the folded domain of these bound import intermediates? To check this point, mitochondria were allowed to accumulate the import intermediate of (1–95) cytochrome b_2–barnase. The mitochondria were then re-isolated and incubated at 30°C in import buffer lacking ATP. At different times aliquots were taken and analyzed by protease treatment to detect precursor remaining folded at that time point, or simply by re-isolating the mitochondria without protease treatment to detect how much protein remained associated with mitochondria. Figure 8A shows that after 4 min of incubation in the absence of ATP, around 90% of the precursor initially bound to the mitochondria remains bound and 70–80% of the bound precursor is still protease-resistant. For comparison, in the presence of ATP at 30°C, (1–95) cytochrome b_2–barnase is fully imported after 2 min (see Figure 6). When the experiment was repeated at 10°C, import was found to be ~200 times faster than outer membrane-mediated unfolding (Table III). Thus, any unfolding activity of the outer membrane is not strong enough to explain the rapid import of the barnase precursors.

With the (1–95) cytochrome b_2–DHFR precursor, a productive import intermediate can be generated by incubating the precursor with ATP-depleted mitochondria in low-salt import buffer in the absence of an electrochemical potential (Hines and Schatz, 1993; Haucke *et al.*, 1995). Under these conditions, unfolding of the DHFR domain of the (1–95) cytochrome b_2–DHFR precursor at 30°C was slightly faster than in free solution; nevertheless, precursor unfolding was 2–3 times slower than import in the presence of ATP and an electrochemical potential (Figure 8B). We also performed this experiment at 10°C. Again, the long DHFR precursor unfolded ~4 times more slowly than it was imported (Figure 8C).

Discussion

In this study we have fused two well-defined single-domain proteins of different stabilities to mitochondrial presequences of increasing lengths and studied the rates at which these constructs are imported into mitochondria. We found that when the presequence is long enough to span both mitochondrial membranes and to bind to mhsp70 in the matrix, the import rate increases dramatically and is no longer enhanced by denaturing the precursor. This critical presequence length is reached at around 65 amino acids. If the presequence is shorter, import of the precursors

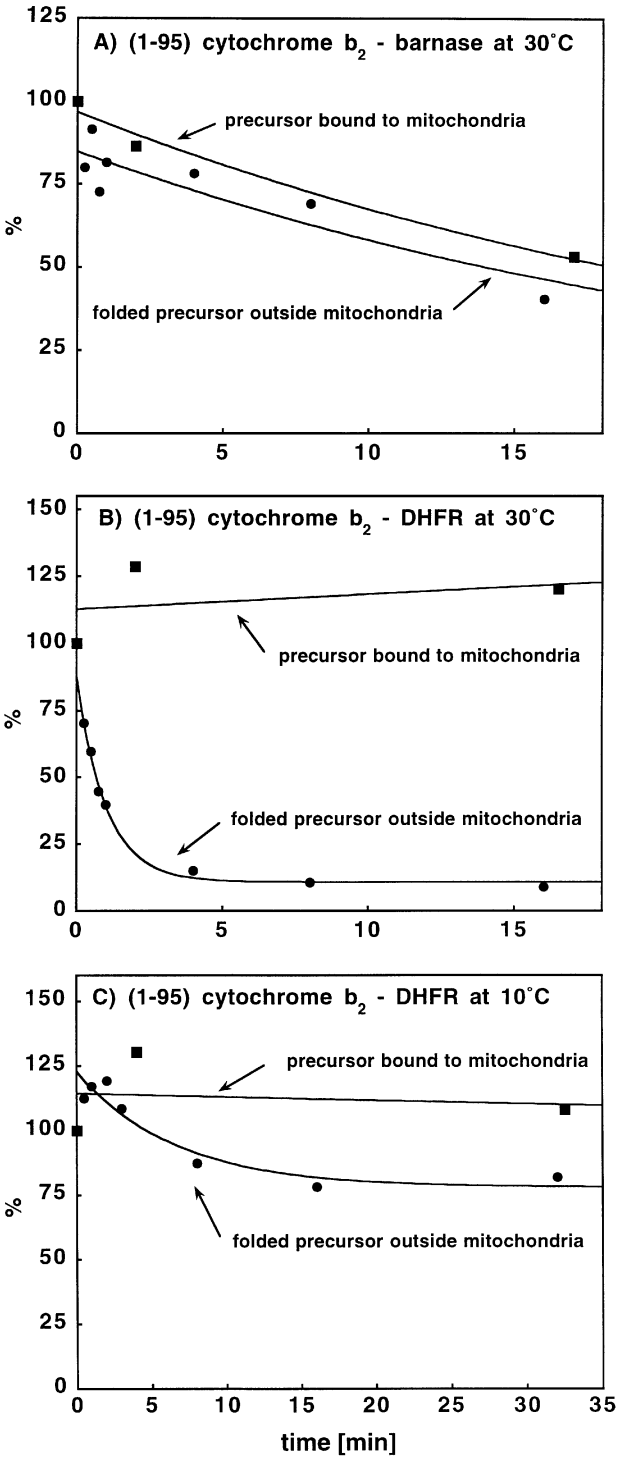

Fig. 8. The folded domain of the (1–95) cytochrome b_2–barnase is protease resistant at the mitochondrial surface. (**A**) (1–95) Cytochrome b_2–barnase bound to the mitochondrial surface in the absence of ATP was incubated at 30°C. Samples were taken at the indicated times and analyzed either for the amount of folded protein outside the mitochondria or the total amount of precursor bound to the mitochondria. The total amount of precursor associated with mitochondria at time zero was set to 100%. See Materials and methods for experimental details. (**B**) (1–95) Cytochrome b_2–DHFR bound to the mitochondrial surface was incubated at 30°C in low ionic strength import buffer in the absence of ATP and without an electrochemical potential across the inner mitochondrial membrane. Samples were taken at the indicated times and analyzed either for the amount of folded protein outside the mitochondria or the total amount of precursor bound to the mitochondria. The total amount of precursor associated with mitochondria at time zero was set to 100%. See Materials and methods for experimental details. (**C**) Experiment as in (B) but at 10°C.

Table III. Half-times of unfolding at the mitochondrial surface and of import into mitochondria for DHFR and barnase precursors with (1–95) cytochrome b_2 presequences

Passenger protein	Temperature (°C)	Half-times (s)		Ratio
		Unfolding[a]	Import[b]	
DHFR	30	42 ± 4	15 ± 2	2.8 ± 0.5
DHFR	10	2000 ± 700	500 ± 100	4 ± 2
Barnase	30	1000 ± 250	11 ± 1	90 ±20
Barnase	10	6000 ± 1700	25 ± 1	240 ± 70

[a]Unfolding half-times were estimated from Figure 8.
[b]Import half-times were measured as described in Materials and methods under the same conditions as unfolding. For further experimental details see Materials and methods.

is slow but can be stimulated by urea denaturation. These results suggest that unfolding of a precursor at the mitochondrial surface can be mediated by a factor on the *trans*-side of the double membrane barrier, presumably mhsp70, and that this effect reflects an unfoldase activity of mhsp70, which pulls the precursor into the matrix. Presequences of different lengths thus serve as yardsticks to probe the unfolding activities of different parts of the import machinery.

Spontaneous unfolding of the passenger protein limits the import rate only for precursors with presequences that are too short to span both membranes. Import of these precursors is slower than spontaneous unfolding in solution, probably because not all spontaneous unfolding events at the mitochondrial surface lead to a productive interaction of the presequence with mhsp70 in the matrix. Precursors with presequences of 75 amino acids or longer are imported faster than they unfold spontaneously in solution. With barnase, this acceleration is at least 200-fold, and with DHFR up to 24-fold. Presumably, import of the precursors with long presequences is limited not by unfolding, but by the turnover of the import machinery.

The strong acceleration of import by long presequences does not reflect a destabilizing effect of the presequence itself on the passenger protein: when the presequence has to reach only across one membrane, as in inner membrane vesicles, the critical presequence length is significantly reduced and the acceleration of import occurs with shorter presequences. We have also found that the length of the presequence does not significantly affect the spontaneous unfolding rate of an attached DHFR domain (Table II). Furthermore, the increase in import rates occurs at the same presequence length for two passenger proteins of drastically different structure and stability.

Where in mitochondria is the unfolding activity located? Previous studies reported that the mitochondrial outer membrane can unfold DHFR precursor proteins without the action of the import machinery of the inner membrane (Eilers *et al.*, 1988; Endo and Schatz, 1988; Mayer *et al.*, 1995). Indeed, we found that DHFR precursors bound to mitochondria in the absence of ATP appear to unfold more rapidly than in free solution (compare Table II and Table III). However, any unfolding activity of the mitochondrial surface is not sufficient to explain the rapid unfolding and import of precursors with long presequences. When (1–95) cytochrome b_2–DHFR or (1–95) cytochrome b_2–barnase is bound to the mitochondrial surface as an import-competent intermediate, the passenger protein remains folded longer than it would take to import the precursor in the presence of ATP (Figure 8, Table III). This difference is particularly clear for the barnase precursor. Indeed, indentification of the unfolding activity of the import machinery in the inner membrane requires the use of precursors whose passenger protein is significantly more stable than the commonly used DHFR as well as presequences long enough to interact with the inner membrane import machinery. Three further observations corroborate that the unfoldase activity is not exclusively located at the mitochondrial surface. First, mitochondrial inner membrane vesicles can unfold and import precursors efficiently even though the outer membrane is bypassed. The mitochondrial inner membrane has very different lipids and proteins than the outer membrane, lacking both outer membrane receptors and outer membrane channel proteins, yet short precursors are imported into inner membrane vesicles at roughly the same rate as into intact mitochondria. This result suggests that when import is limited by spontaneous unfolding, the import rate is not significantly affected by the membrane to which the precursor is bound. Second, when unfolding rates of a short precursor with a 35-amino-acid presequence are measured during ATP-driven import, the presence of mitochondrial membranes does not accelerate unfolding even though >60% of the precursor molecules are bound to the mitochondria from the beginning of the experiment (data not shown). Third, most of the precursor molecules are already associated with the mitochondria from the very beginning of an import experiment, even in the case of DHFR precursors with short presequences. Thus, if the outer membrane had an unfoldase activity, it would also act on these slowly importing short precursors. In summary, the combined results suggest to us that unfolding and import of the long precursors are mediated by a component of the import machinery of the inner membrane, presumably mhsp70, which acts on the transmembraneous precursor against the scaffold of the two apposed protein translocation channels.

How does mhsp70 unfold precursors? Barnase is very stable (the $\Delta G°$ for unfolding is ~10 kcal/mol in dilute buffer at 25°C) and its activation energy for unfolding is very high (>20 kcal/mol in dilute buffer at 25°C) (Matouschek *et al.*, 1994a). Just as enzymes can change the pathway of a reaction, mhsp70 may change the unfolding pathway of a precursor. Members of the 70 kDa heat shock protein family undergo ATP-dependent conformational changes (Liberek *et al.*, 1991; Wilbanks *et al.*, 1995; Shi *et al.*, 1996). Since mhsp70 binds both to the N-terminus of an incoming precursor and to the inner membrane protein Tim44, a conformational change in mhsp70 could pull a segment of the polypeptide chain out of a folded domain on the mitochondrial surface and, since protein folding is highly cooperative, cause a collapse of this domain. If binding of mhsp70 to the precursor and Tim44, followed by a structural change, were repeated in an ATP-dependent cycle, mhsp70 could function as a motor pulling a precursor into the matrix (Pelham, 1986; Glick, 1995; Pfanner and Meijer, 1995) in a mechanism reminiscent of the motor action of myosin in muscle contraction.

Some proteins are known to undergo partial unfolding

because subdomains unfold faster than the protein as a whole. Examples are RNase A (Mayo and Baldwin, 1993) and cytochrome *c* (Bai *et al.*, 1995). If such partial unfolding at the N-terminus were followed by rapid diffusion of the unfolded part into the import channel, mhsp70 could trap these fluctuations, either by binding to the sequences emerging at the inner mouth of the channel or by undergoing a conformational change if part of the precursor were already bound to mhsp70 before the unfolding fluctuation. Such a trapping action could change the unfolding pathway of the precursor and accelerate its global unfolding. Thus, mhsp70 would mediate an inwards movement of the precursor as proposed by the 'Brownian ratchet model' (Simon *et al.*, 1992; Stuart *et al.*, 1994), with the modification that mhsp70 now has a direct role in the unfolding. The 'motor model' of protein import does not necessarily disagree with the 'Brownian ratchet model', but may emphasize a different aspect of the same mechanism. Brownian ratchet-type models are also being discussed for the action of myosin in muscle contraction (Madox, 1994).

The ATP-dependent action of mhsp70 is not limited to unfolding a bound precursor, because even import of urea-denatured precursors requires ATP in the matrix (Eilers *et al.*, 1988). The mitochondrial import channel probably interacts with the precursor in different ways depending on the precursor's amino acid sequence and the chemical properties of the channel walls. Mhsp70 might be required to overcome the resulting friction and to move the precursor into the matrix (Glick, 1995).

There is little information on the folding of authentic mitochondrial precursor proteins in the cytosol *in vivo*. We do not know whether mitochondrial precursor proteins have been selected in evolution for rapid spontaneous unfolding or whether they are generally kept unfolded by binding to chaperones such as cytosolic hsp70 (Chen and Douglas, 1987; Deshaies *et al.*, 1988; Smith and Yaffe, 1991) or mitochondrial import stimulating factor (Hachiya *et al.*, 1993). The present results agree with earlier findings on the import of authentic cytochrome b_2 into isolated mitochondria. Unlike other proteins of the intermembrane space, authentic cytochrome b_2 requires the ATP-dependent action of mhsp70 for import. This requirement is caused by the folded heme-binding domain of cytochrome b_2, which is stable even at temperatures above 80°C (Cyr *et al.*, 1993; Glick *et al.*, 1993; Voos *et al.*, 1993). Import of this precursor also requires the correct spacing between the presequence and the heme-binding domain to allow unfolding of this domain by mhsp70 (Gärtner *et al.*, 1996).

In conclusion, we propose that mhsp70 can unfold a precursor at the mitochondrial surface by interacting with its presequence in the matrix.

Materials and methods

Precursors proteins and mitochondria

The precursor proteins consisted of presequences containing between 35 and 95 amino acids fused to either mouse dihydrofolate reductase (DHFR) (Chang and Cohn, 1978) or barnase (Paddon and Hartley, 1987). The presequence consisted of the first 35, 45, 55, 65, 75, 85 or 95 amino acids of yeast cytochrome b_2 (Guiard, 1985) which had been mutated twice: at position 30 (Arg→Gly) to prevent cleavage by the matrix processing peptidase (Arretz *et al.*, 1994), and at position 62 (Leu→Pro) to inactivate the intermembrane space sorting signal and to target attached proteins to the matrix (Beasley *et al.*, 1993). The genes were under control of the *tac* promoter and inserted into a pDS-derived plasmid (Bujard *et al.*, 1987). All experiments described here were performed with radioactive precursor expressed from the *tac* promoter by *in vitro* transcription and translation in a rabbit reticulocyte lysate containing [^{35}S]methionine (Stueber *et al.*, 1984).

Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B [MATα, ATCC 25657 (Hase *et al.*, 1984)] and purified by centrifugation through a Nycodenz gradient (Glick and Pon, 1995).

Import assays

Radioactive precursor protein was precipitated by adding saturated ammonium sulfate to 66%, pelleted by centrifugation at 14 000 *g* for 10 min and dissolved in the original volume of import buffer (final pH = 7.0; 0.6 M sorbitol; 50 mM HEPES; 50 mM KCl; 10 mM $MgCl_2$; 2 mM KH_2PO_4; 5 mM unlabeled methionine; 1 mg/ml fatty acid-free bovine serum albumin). For experiments with urea-denatured precursor, the ammonium sulfate precipitate was dissolved in 8 M urea containing 25 mM Tris–HCl pH = 7.4. It was shown previously that urea at the concentration obtained after dilution does not affect import (Eilers *et al.*, 1988). Sixty µl of precursor solution were incubated at 30°C with 1140 µl mitochondrial suspension at 0.5 mg mitochondrial protein per ml in import buffer containing 4 mM ATP, 10 mM creatine phosphate and 0.15 mg/ml creatine kinase, which had been prewarmed to 30°C. At the indicated time points, 100 µl samples were transferred to 100 µl of ice-cold resuspension buffer (0.6 M sorbitol; 20 mM HEPES–KOH pH 7.4) containing 2 µM valinomycin and 0.2 mg/ml proteinase K. After 10 min, proteinase K was inhibited with 1 mM phenylmethylsulfonyl fluoride (PMSF). The mitochondria were then re-isolated by centrifugation at 8000 *g* for 5 min and resuspended in ice-cold resuspension buffer (see above) containing 1 mM PMSF. Proteins were precipitated with 5% trichloroacetic acid, collected on glass fiber filters (Whatman GFC), washed extensively with 5% trichloroacetic acid and assayed for radioactivity by liquid scintillation counting. The extent of import was plotted as a percentage of the total amount of precursor in the import reaction at the end of the time course. The 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, A_0 is the total amount of import, k is the import rate constant and C is a constant offset due to background counts and unspecific binding of radioactivity to the filters.

Immunoprecipitation

Aliquots (15 µl) of precursors consisting of DHFR and presequences of 35, 55, 75 and 85 amino acids were incubated with 500 µg of yeast mitochondria in the presence of methotrexate for 10 min in a final volume of 0.5 ml at 25°C. To deplete ATP, the aliquots were incubated for 5 min on ice in import buffer with 10 units of apyrase per ml in the presence of the ATP synthase inhibitors oligomycin (5 µg/ml) and efrapeptin (2 µg/ml). NADH was then added to 2 mM and the mitochondria were incubated for a further 5 min on ice, pelleted at 8000 *g*, resuspended at 5 mg/ml in solubilization buffer (50 mM HEPES–KOH pH 7.4; 250 mM NaCl; 10% glycerol; 1% octyl polyoxyethylene; 5 mM PMSF; 5 µg/ml bestatin; 0.5 µg/ml pepstatin) and incubated for 10 min on ice. The mixture was then centrifuged for 10 min at 14 000 *g* and the supernatant incubated for 3 h with polyclonal anti-mhsp70 IgG covalently coupled to protein A–Sepharose beads. The beads were washed three times with solubilization buffer, boiled in SDS–PAGE loading buffer, and the eluate was analyzed by SDS–PAGE and scanning of the gels with a β-imager (Biospace Mésures, France).

Import into inner membrane vesicles

Inner membrane vesicles were prepared from mitochondria as described (Hwang *et al.*, 1989; Jascur, 1991). Import reactions into inner membrane vesicles were carried out in buffer containing 0.6 M sorbitol, 20 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM KCl, 0.3 mM EDTA, 0.6 mM DTT, 10 mM succinate, 10 mM L-malate, 1 mg/ml fatty acid-free bovine serum albumin, 0.625 mg/ml cytochrome *c*, 3.8 mM ascorbate, 4 mM ATP, 10 mM creatine phosphate and 0.15 mg/ml creatine kinase. The import assays were the same as described above for whole mitochondria with the difference that inner membrane vesicles were pelleted at 100 000 *g* in a tabletop ultracentrifuge after import.

Unfolding kinetics of precursor proteins

Precursors were synthesized as described above. To measure unfolding rates through the susceptibility of unfolded precursor to protease, 95 µl

of precursor were added to 1000 µl import buffer and a 95 µl sample was taken. One sample was treated with 0.5 mg/ml trypsin on ice for 10 min, then precipitated with trichloroacetic acid and taken as the 100% standard. The remaining reaction mixture was warmed to 30°C, trypsin was added to 0.5 mg/ml and 100 µl samples were taken at different time points and added to 100 µl of 10% ice-cold trichloroacetic acid. The precipitated proteins were analyzed by SDS–PAGE and quantification of the DHFR band by electronic autoradiography (β-imager, Biospace Mésures, France or Instantimager, Packard, USA). The same rates within experimental error were obtained using different concentrations of proteinase K (0.1, 0.2 and 0.4 mg/ml; data not shown) and trypsin (0.05, 0.1, 0.2 and 0.5 mg/ml; data not shown).

To measure unfolding rates on the basis of binding of unfolded DHFR to *E.coli* GroEL, 95 µl of precursor solution were added to 1000 µl import buffer containing 3 µM GroEL. The solution was incubated at 30°C, 100 µl samples were taken and added to the same volume of ice-cold resuspension buffer (see above) containing 200 µg/ml proteinase K. DHFR bound to GroEL is degraded by the protease, whereas folded DHFR in solutions is resistant against protease on ice. After 2 min, the protease was inhibited with 1 mM PMSF, the samples were precipitated with 5% trichloroacetic acid and the precipitated proteins analyzed by SDS–PAGE and electronic autoradiography (β-imager, Biospace Mésures, France or Instantimager, Packard, USA). Binding to GroEL was not rate determining in this assay because increasing the GroEL concentration 4-fold did not affect the measured unfolding rate (data not shown).

Protease-sensitivity at mitochondrial surface

For the unfolding experiments in the presence of an electrochemical potential, mitochondria were depleted of ATP by incubation for 5 min at 30°C at 0.5 mg mitochondrial protein/ml in 950 µl import buffer (see above) containing 10 units/ml apyrase, 5 µg/ml oligomycin and 2.5 µg/ml efrapeptin for 5 min on ice. In order to establish a membrane potential, NADH was added to 1 mM and incubation continued for 5 min at 20°C, with a further addition of NADH to a nominal final concentration of 6 mM, followed by an additional incubation for 5 min at 20°C. The mitochondria were than cooled on ice for 5 min, 50 µl of precursor were added and incubation was continued for 5 min on ice. Mitochondria were then pelleted at 7000 *g* for 5 min, resuspended in 100 µl ice-cold import buffer and added to 1000 µl of import buffer prewarmed to 30°C. This was set as time zero and 100 µl samples were taken immediately and after 2 min and 16 min, and added to ice-cold resuspension buffer (see above). Further samples were taken at 15 s, 30 s, 45 s, 1 min, 4 min, 8 min and 16 min and added to Stop-buffer (see above) and incubated with proteinase K at 0.1 mg/ml for 10 min on ice. The protease was then inhibited with 1 mM PMSF and the mitochondria were pelleted by centrifugation for 5 min at 8000 *g*. The proteins of the supernatant were precipitated with 5% trichloroacetic acid and both mitochondrial samples and supernatants were analyzed for ^{35}S-labeled precursor by SDS–PAGE and quantification of the labeled bands by electronic autoradiography (β-imager, Biospace Mésures, France or Instantimager, Packard, USA). The radioactive band in the mitochondrial fractions of samples not treated with protease represents the total amount of precursor associated with mitochondria and the radioactive band in the supernatant fraction of protease-treated samples represents the total amount of protease-resistant, and thus folded, precursor outside the mitochondria. The amount of radioactive precursor in these bands was quantified taking into account that two-thirds of the methionine residues in the (1–95) cytochrome b_2–barnase precursor are located in the presequence.

Experiments to measure unfolding in the absence of an electrochemical potential were performed as described above with three differences: (i) a low ionic strength import buffer was used (0.6 M sorbitol, 25 mM HEPES–KOH pH 7.4, 12 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA); (ii) the mitochondria were not incubated with NADH but the import buffer contained 25 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); and (iii) mitochondria–precursor complex was isolated immediately after addition of precursor by centrifugation at 20 000 *g* for 90 s.

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