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Unfolding of preproteins upon import into mitochondria

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Unfolding of preproteins and translocation across the mitochondrial membranes requires their interaction with mt-Hsp70 and Tim44 at the inner face of the inner membrane and ATP as an energy source. We measured the temperature dependence of the rates of unfolding and import into the matrix of two folded passenger domains, the tightly folded heme-binding domain (HBD) of cytochrome b_2 and the loosely folded mouse dihydrofolate reductase (DHFR). Despite the stability of the HBD, its rates of thermal breathing were fast and the preprotein was imported rapidly at all temperatures. In contrast, rates of unfolding and import of DHFR were strongly temperature dependent and import was significantly slower than unfolding. In addition, import rates of DHFR were strongly dependent on the length of the presequence. We propose that the mitochondrial import motor does not exert a constant pulling force. Rather, mt-Hsp70 appears to release a translocating polypeptide chain such that the precursor can then slide back and refold on the surface of the mitochondria. Refolding competes with translocation, and passengers may undergo several rounds of unfolding and refolding prior to their import.
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Introduction

Translocation of mitochondrial preproteins across the mitochondrial inner membrane is driven energetically by hydrolysis of ATP, a process mediated by mt-Hsp70 in cooperation with Mge1p and Tim44 (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994, 1996). These components are parts of a molecular machinery which binds segments of the incoming polypeptide chain at the inner face of the inner membrane and prevents retrograde movement of the preprotein in the import channel (Schneider *et al*., 1994, 1996; Ungermann *et al*., 1994).

Different hypotheses have been proposed as to how this import motor might drive forward movement of preproteins into the mitochondrial matrix. On one hand, it has been suggested that the mt-Hsp70–Tim44 complex exerts a 'pulling force' on the incoming polypeptide chain ('swinging crossbridge') (Glick, 1995; Pfanner and Meijer, 1995; Schatz and Dobberstein, 1996; Voos *et al*., 1996; Matouscheck *et al*., 1997). On the other hand, it has been proposed that unidirectional movement of the translocating polypeptide chain would be the result of binding of Tim44 and mt-Hsp70 to the unfolded preprotein, controlled by ATP hydrolysis (Schneider *et al*., 1994, 1996; Ungermann *et al*., 1994; Berthold *et al*., 1995). This mechanism is essentially a 'Brownian ratchet' (Neupert *et al*., 1990; Simon *et al*., 1992) which combines spontaneous reversible movements of the translocating polypeptide chain with an energy-requiring trap that is located at the inner outlet of the import channel (Schneider *et al*., 1994, 1996).

A problem appears to arise when a folded domain of a preprotein reaches the translocation channel, which can accommodate only unfolded or largely unfolded structures (Eilers and Schatz, 1986). The 'ratchet model', a minimal model, predicts that unfolding relies on a spontaneous process determined by the rates of unfolding and refolding. Limited unfolding or breathing of a folded domain would allow movement of extended segments of the preprotein in the protein-conducting channel of the translocase. Binding of mt-Hsp70–Tim44 to the emerging preprotein would prevent retrograde movements and thereby shift the equilibrium to the unfolded state. The 'swinging crossbridge' model on the other hand, predicts that unfolding is promoted by a 'power stroke' of mt-Hsp70 which mechanically pulls on the preprotein and thereby unfolds a domain on the surface of the mitochondria. A 'power stroke' by mt-Hsp70 has been suggested to explain the efficient import of the tightly folded heme-binding domain (HBD) of yeast cytochrome b_2 (Glick *et al*., 1993; Glick, 1995).

To obtain insight into these complex reactions, we have analyzed unfolding and import of two chimeric preproteins which contain folded passenger domains, the cytochrome b_2 HBD and mouse dihydrofolate reductase (DHFR). We show that the overall stability of the domains correlates neither with the ability of the import machinery to unfold these protein domains upon import nor with the levels of ATP in the matrix that are required to unfold the preproteins at the surface of the mitochondria. Import rates of the loosely folded DHFR are strongly temperature-dependent while those of the tightly folded HBD are not. The import rates of the HBD correlate well with the rates of spontaneous thermal unfolding, indicating that unfolding is rate limiting. Import rates of DHFR are significantly slower than the rates of its spontaneous unfolding, indicating a rate-limiting step after unfolding. In addition, maximal rates of import of folded DHFR required presequences which were ~100 amino acid residues in length, though ~50 residues were sufficient to span both mitochondrial membranes and allow binding of mt-Hsp70 in the matrix. By contrast, the length of the presequence was

not crucial for import rates of the HBD. Our data are consistent with a mechanism by which folded passengers undergo several rounds of unfolding and refolding prior to their import. Refolding of the passenger on the surface of the mitochondria competes with translocation of the unfolded polypeptide chain. DHFR refolds efficiently while the HBD does not refold tightly due to the release of bound heme during the initial round of unfolding on the surface of the mitochondria.

Results

Stability of folded protein domains and import into mitochondria

The chimeric preproteins pSu9(1–69)DHFR and pSu9 (1–69)HBD consist of the presequence of the subunit 9 of the ATP synthase of *Neurospora crassa* (residues 1–69) fused either to DHFR or to the HBD (Xia and Mathews, 1990). To characterize the stability of the folded protein moieties, radiolabeled preproteins were synthesized *in vitro* in reticulocyte lysate and then treated with proteinase K (PK) at 4 and 37°C (Figure 1A). The presequence of both chimeric preproteins was degraded readily by low concentrations of PK and the folded protein domains remained. The sensitivity towards PK treatment of these folded domains differed significantly. The DHFR moiety was degraded by 1 mg/ml PK at 4°C and by 5 μg/ml PK at 37°C. The HBD was clipped by 100 μg/ml PK at 4°C or by 5 μg/ml PK at 37°C, and a folded core fragment was generated which was resistant to 1 mg/ml PK at 37°C. As determined by sequencing, the N-terminus of this core fragment corresponds to Gln9 of cytochrome b_2. The C-terminus of the core fragment corresponds approximately to residue 90 (unpublished observation). This core fragment was degraded by PK (1 mg/ml) only in the presence of SDS or 4 M urea (data not shown).

We measured import into mitochondria at different temperatures of the two chimeric preproteins and, as a control, of pSu9(1–69)DHFRmut which contains a folding-incompetent DHFR moiety (Vestweber and Schatz, 1988; Teichmann *et al*., 1996) (Figure 1B). The three preproteins were imported efficiently at 25°C. At 4°C, pSu9(1–69)DHFRmut was still imported efficiently, while import of pSu9(1–69)DHFR was not observed; import of pSu9(1–69)HBD was almost as efficient as import of pSu9(1–69)DHFRmut at this low temperature.

The ATP requirement for the import of pSu9(1–69)DHFRmut, pSu9(1–69)DHFR and pSu9(1–69)HBD was investigated. Mitochondria were pre-incubated to manipulate the levels of ATP in the matrix (Stuart *et al*., 1994; Wachter *et al*., 1994). Then radiolabeled preproteins were added to the mitochondria and import at 25°C was determined (Figure 1C). When levels of ATP in the matrix were high, i.e. ~1 mM (Stuart *et al*., 1994), all three precursors were imported efficiently and processed to their mature forms. Upon reduction of ATP levels to a concentration in the low micromolar range (Stuart *et al*., 1994), pSu9(1–69)DHFRmut was still imported efficiently, while import of pSu9(1–69)DHFR was reduced. Under these conditions, the intermediate form, iSu9(1–69)DHFR, accumulated. It was protected from digestion by PK in mitochondria but was sensitive to PK in mitoplast

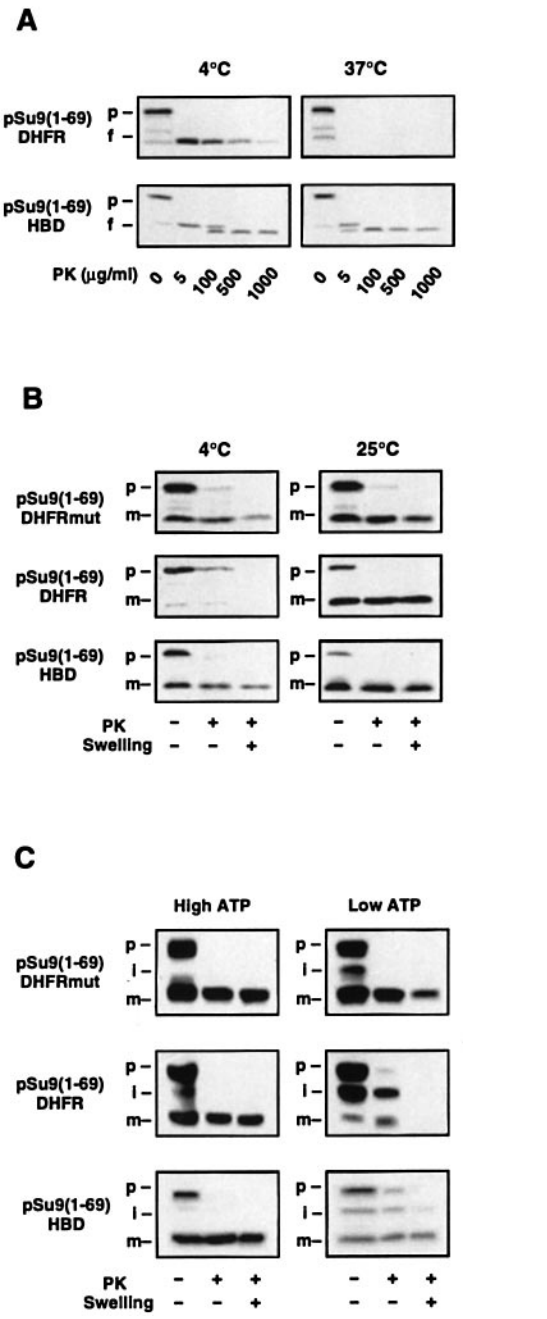

Fig. 1. Folded state and import efficiency of preproteins. (**A**) Resistance towards proteolytic degradation of chimeric preproteins. Radiolabeled pSu9(1–69)DHFR and pSu9(1–69)HBD were incubated at 4 and 37°C with increasing concentrations of PK. The samples were analyzed by SDS–PAGE and fluorography. Precursor (p) and the PK-resistant fragments (f) are indicated. (**B**) Import of the chimeric preproteins into mitochondria. Radiolabeled pSu9(1–69)DHFRmut, pSu9(1–69)DHFR and pSu9(1–69)HBD were incubated with energized mitochondria for 10 min at 4 or 25°C. When indicated, samples were treated with PK (200 μg/ml), or mitoplasts were generated by 'swelling' of mitochondria and treated with PK. Samples were analyzed by SDS–PAGE and fluorography. Precursor (p) and mature form (m) of the fusion proteins are indicated. (**C**) Requirement of matrix ATP for import of preproteins. Mitochondria were pre-incubated to adjust high and low levels of matrix ATP (see Materials and methods, levels I and IV). Radiolabeled preproteins were imported into the pre-treated mitochondria for 10 min at 25°C. i: intermediate form.

preparations, demonstrating that iSu9(1–69)DHFR accumulated in the intermembrane space. Similarly, most of the mature form accumulated in the intermembrane space. The DHFR moiety was folded (not shown), indicating that it refolded after translocation across the outer membrane. Obviously, at low levels of matrix ATP the preprotein was not translocated across the inner membrane. However, the DHFR domain was unfolded and translocated across the outer membrane; it refolded in the intermembrane space and impaired further import into the matrix. This indicates that unfolding and translocation of DHFR across the outer membrane do not require high ATP levels in the matrix. High ATP levels are required for unfolding and translocation across the inner membrane of DHFR but not for translocation of DHFRmut. Thus, the levels of ATP required for import into the matrix are different for different precursors of the same length. ATP consumption appears not to be tightly coupled to translocation of segments of defined length of a preprotein.

pSu9(1–69)HBD, in contrast to pSu9(1–69)DHFR, was imported at low levels of ATP in the matrix (Figure 1C). The intermediate form did not accumulate, suggesting that the HBD did not refold in the intermembrane space. These observations indicate that the levels of ATP required for unfolding are different for different folded protein domains.

In summary, the HBD was unfolded readily by the mitochondrial import machinery despite its resistance towards proteolytic degradation, and it was imported at low levels of ATP. On the other hand, the import machinery of the inner membrane could not unfold and import the DHFR domain efficiently at low temperature or at low levels of matrix ATP, although the DHFR is degraded readily by proteases. Thus, the overall stability of protein domains, as monitored by protease resistance, does not correlate with the ability of the mitochondrial import machinery to unfold protein domains during import. The rates of unfolding and/or refolding of protein domains rather than their thermodynamic stability may determine the efficiency of their import into mitochondria

Thermal unfolding of DHFR

In order to estimate rates of transient unfolding of DHFR and of the HBD, we measured the kinetics of chemical modification of amino acid side-groups that become exposed to solvent only after unfolding due to thermal breathing.

Mouse DHFR contains a unique cysteine residue in position 7 (Cys7) which is buried inside the protein (Stammers *et al*., 1987; Oefner *et al*., 1988). We monitored the accessibility of Cys7 to the water-soluble alkylating reagent *N*-ethylmaleimide (NEM) in order to assess transient unfolding of DHFR due to thermal motion. Modification of Cys7 by NEM was followed by subsequent detection of free sulfhydryl groups with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Habeeb, 1972). Cys7 of native DHFR was not modified by NEM at 4°C (Figure 2A), whereas the reaction of NEM with dithiothreitol (DTT) at this temperature was complete in ~2 min (not shown). This demonstrates that NEM had no access to the buried sulfhydryl group of Cys7 at 4°C. At 20°C, however, Cys7 was alkylated by NEM, indicating that it became exposed to solvent due to unfolding of DHFR driven by thermal

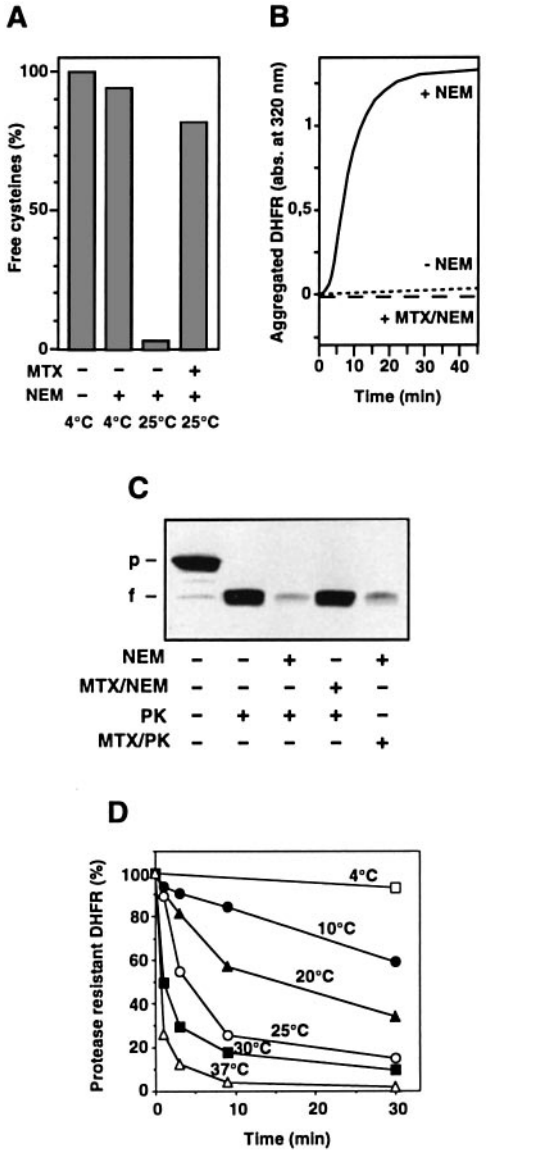

Fig. 2. Thermal unfolding of DHFR. (**A**) Modification of Cys7 of DHFR with NEM. Recombinant DHFR (50 μM) was incubated with 3 mM NEM for 60 min at 4 or 25°C in the absence or presence of 100 μM MTX. Subsequently, 5 mM DTT was added and the incubation was continued for 30 min. Free sulfhydryl groups were then determined by reaction with DTNB (Habeeb, 1972). Absorbance at 412 nm was monitored. (**B**) NEM modification causes aggregation of DHFR. Purified DHFR (50 μM) was incubated at 20°C and the time course of aggregation of DHFR was monitored by light scattering at 320 nm (Langer *et al*., 1992). The samples contained: 3 mM NEM (+NEM, solid line); no additions (–NEM, dotted line); 3 mM NEM and 75 μM MTX (+MTX/NEM, dashed line). (**C**) NEM modification of pSu9(1–69)DHFR. Reticulocyte lysate containing *in vitro* synthesized radiolabeled pSu9(1–69)DHFR was diluted 10-fold in import buffer and incubated at 25°C with 3 mM NEM either directly (NEM) or after pre-incubation with 1 μM MTX (MTX/NEM). The diluted lysate contained 0.1 mM DTT. After 30 min, free NEM was quenched with a 15-fold molar excess of DTT. Control reactions received NEM which previously was quenched with DTT. Samples were then incubated at 4°C with 50 μg/ml PK. One sample received MTX prior to the addition of PK (MTX/PK). Samples were analyzed by SDS–PAGE and fluorography. pSu9(1–69)DHFR (p) and the protease-resistant DHFR fragment (f) are indicated. (**D**) Kinetics of modification of Cys7 at different temperatures. Radiolabeled pSu9(1–69)DHFR was incubated at the different temperatures with NEM. After the indicated times, samples were quenched with DTT, treated with PK, analyzed by SDS–PAGE and quantified with a phosphoimaging system.

motion. Alkylation of Cys7 was completely abolished in the presence of methotrexate (MTX) although Cys7 is not involved in MTX binding (Stammers *et al.*, 1987; Oefner *et al.*, 1988). NEM treatment caused aggregation of DHFR (Figure 2B). The time course of aggregation was sigmoidal, and aggregation was half-maximal after 8 min. This indicates that aggregation is concentration-dependent and that thermal unfolding of DHFR is fast at 20°C. In the absence of NEM, DHFR did not aggregate. NEM-induced aggregation of DHFR was completely abolished in the presence of MTX. In conclusion, unfolding of DHFR due to thermal motion does occur at physiological temperature and can be monitored by modification of Cys7 with NEM. Alkylation of Cys7 interferes with refolding and destabilizes the structure of DHFR, which results in aggregation. In the absence of NEM, DHFR refolds efficiently and no net unfolding of the protein is observed.

Modification of Cys7 increased the protease sensitivity of DHFR. This was used to follow unfolding of radiochemical amounts of pSu9(1–69)DHFR. When the preprotein was treated with PK, it was degraded and the folded protease-resistant DHFR domain was recovered (Figure 2C). The preprotein was degraded completely by PK when it was pre-incubated for 30 min at 20°C with NEM. When the preprotein was incubated with NEM in the presence of MTX, the DHFR domain remained protease resistant. pSu9(1–69)DHFR which was alkylated with NEM was not protected by MTX against proteolytic degradation, indicating that it could not bind the antagonist. Thus, unfolding of the DHFR domain in the preprotein can be analyzed through its protease sensitivity after NEM treatment.

Using this assay, we measured the kinetics of unfolding of pSu9(1–69)DHFR due to thermal breathing (Figure 2D). The half-time for alkylation of pSu9(1–69)DHFR was ~30 min at 10°C and ~3 min at 25°C. At 37°C, the half-time for alkylation was faster than 1 min. This indicates that the DHFR domain unfolds spontaneously in a temperature-dependent manner.

Unfolding of the HBD

The HBD of yeast cytochrome b_2 consists of 99 amino acid residues (Xia and Mathews, 1990). It contains two cysteine residues, Cys25 and Cys94, and three histidine residues, His19, His43 and His66. Cys25 is buried inside the folded protein while Cys94 is solvent exposed. Cys94 is not part of the protease-resistant folded core of the HBD, which ends at approximately residue 90 (unpublished observation). His19 is solvent exposed; His43 and His66 are located in the heme-binding pocket and they are both involved in heme binding (Xia and Mathews, 1990). We expressed a His-tagged version of the HBD and purified the protein in a heme-free form, HBD(–H), and in a form where ~30% of the molecules had hemin bound, HBD(+H) (see Materials and methods). Both preparations were treated with PK to assess the folded state of the HBD (Figure 3A). HBD(–H) was degraded by PK at 4 and 37°C. In contrast, HBD(+H) was resistant to proteolytic degradation at both temperatures. This indicates that hemin stabilizes the structure of the HBD and that the protein is not tightly folded in the absence of the ligand. When the HBD(+H) was pre-incubated with NEM at 37°C and subsequently incubated with PK, it was degraded readily,

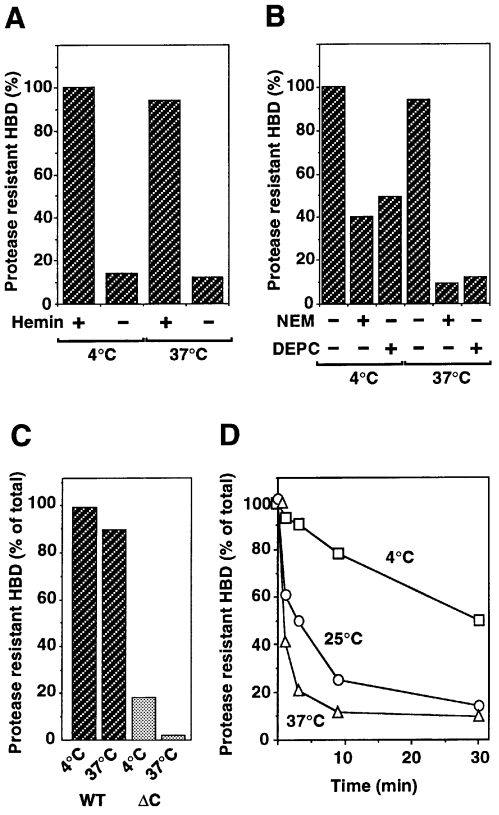

Fig. 3. Thermal unfolding of the heme-binding domain of cytochrome b_2. (**A**) Hemin contributes to the overall stability of the HBD. PK resistance of the HBD. Recombinant HBD (100 μM) containing hemin when indicated was incubated for 30 min with 1 mg/ml PK at either 4 or 37°C. The samples were precipitated with TCA, followed by SDS–PAGE and quantification with a phosphoimager. (**B**) Protease sensitivity of recombinant HBD induced by chemical modification with NEM or DEPC. When indicated, the HBD containing hemin was incubated for 30 min at the indicated temperature with 3 mM NEM and/or 7 mM DEPC. Subsequently, NEM was quenched with DTT and/or DEPC with imidazole. Samples were then treated with 1 mg/ml PK and analyzed as described in (A). (**C**) Protease sensitivity of the HBD synthesized in reticulocyte lysate. pSu9(1–69)HBD, which contains a full size HBD (WT), and pb_2(1–167)DHFR, which contains a truncated HBD that lacks residues 85–99 (ΔC), were synthesized in reticulocyte lysate containing 20 μM hemin. The radiolabeled precursors were diluted 20-fold in import buffer and incubated for 30 min with 1 mg/ml PK. PK was inactivated with PMSF and TCA precipitation. Samples were analyzed by SDS–PAGE and quantified with a phosphorimager. Values were corrected for the loss of ^{35}S-labeled methionines due to protease treatment. (**D**) Kinetics of unfolding of pSu9(1–69)HBD. Radiolabeled pSu9(1–69)HBD was diluted 50-fold in 100 mM KH_2PO_4, pH 7.4. Samples were pre-incubated for 5 min at 4, 25 and 37°C, and subsequently treated with DEPC (7 mM) for the indicated time periods and analyzed as described above.

indicating that modification of sulfhydryls destabilizes the protein (Figure 3B). In addition, treatment of the HBD(+H) with diethylpyrocarbonate (DEPC), which modifies the imidazole of histidine residues, rendered the protein sensitive to degradation by PK.

We synthesized the radiolabeled precursors pSu9(1–69)HBD and pb_2(1–167)DHFR in reticulocyte lysate, which contains 20 μM hemin. pSu9(1–69)HBD contains the full size HBD corresponding to residues 1–99 of mature cytochrome b_2, while pb_2(1–167)DHFR contains a C-terminally truncated HBD (residues 1–84) fused to DHFR. Both precursors were diluted in import buffer and

incubated with 1 mg/ml PK at either 4 or 37°C (Figure 3C). The full size HBD was completely resistant to PK treatment at both temperatures, while the C-terminally truncated HBD was degraded. This indicates that pSu9(1–69)HBD contained a tightly bound hemin that stabilized the HBD.

We measured modification by DEPC of the pSu9(1–69)HBD at different temperatures to assess the kinetics of thermal breathing (Figure 3D). The half-time of modification of HBD with DEPC was ~80 s at 37°C, 2 min at 25°C and 30 min at 4°C. Similar results were obtained when NEM was used for modification of the protein. This indicates that spontaneous transient unfolding of the HBD due to thermal breathing is faster than breathing of the DHFR domain at all temperatures.

Temperature dependence of import rates

pSu9(1–69)DHFRmut, pSu9(1–69)DHFR and pSu9(1–69)HBD were imported into mitochondria at various temperatures and the kinetics of import were measured (Figure 4A). pSu9(1–69)DHFRmut, which is folding incompetent, was taken up rapidly into the mitochondria at all temperatures, while the kinetics of import of pSu9(1–69)DHFR decreased strongly with decreasing temperature. The import kinetics of pSu9(1–69)HBD were fast and significantly less temperature dependent than those of the pSu9(1–69)DHFR.

To estimate the overall rate of protein import, the kinetic data were fitted to a simple first order equation (Figure 4B). The rates of import of pSu9(1–69)DHFRmut were high at all temperatures and they decreased <2-fold between 37 and 10°C (Figure 4B, left panel). Thus, translocation of an unfolded polypeptide chain is only slightly temperature dependent. Rates of import of pSu9(1–69)DHFR were slower than those of pSu9(1–69)DHFRmut at all temperatures (Figure 4B, central panel). In addition, they were strongly temperature dependent, decreasing 35-fold between 37 and 10°C which corresponds to a Q_{10} >3. This indicates that rate-limiting steps of import are different for the folding-competent DHFR and the folding-incompetent DHFRmut, in particular at lower temperatures.

We compared the rates of preprotein import with those of unfolding of the folded DHFR domain (Figure 4B, central panel). The rates of unfolding of the DHFR were significantly higher than those of import at all temperatures, indicating the existence of a rate-limiting step other than unfolding of the DHFR domain. As the folding-incompetent DHFRmut was imported readily, it appears that the refolding reaction of the breathing DHFR domain competes with the translocation of unfolded segments of the polypeptide chain.

The rates of import of pSu9(1–69)HBD were high at all temperatures. They decreased only ~3-fold between 37 and 4°C, corresponding to a Q_{10} <1.4 (Figure 4B, right panel). They were lower than those of import of Su9(1–69)DHFRmut at all temperatures. The rates of spontaneous unfolding of the HBD were virtually indistinguishable from those of preprotein import. This indicates that unfolding of the HBD was rate limiting. Thus, following unfolding, the protein was imported readily and tight refolding of the HBD did not occur on the time scale of translocation.

In summary, the rates of import of pSu9(1–69)HBD and their dependence on the temperature correlate

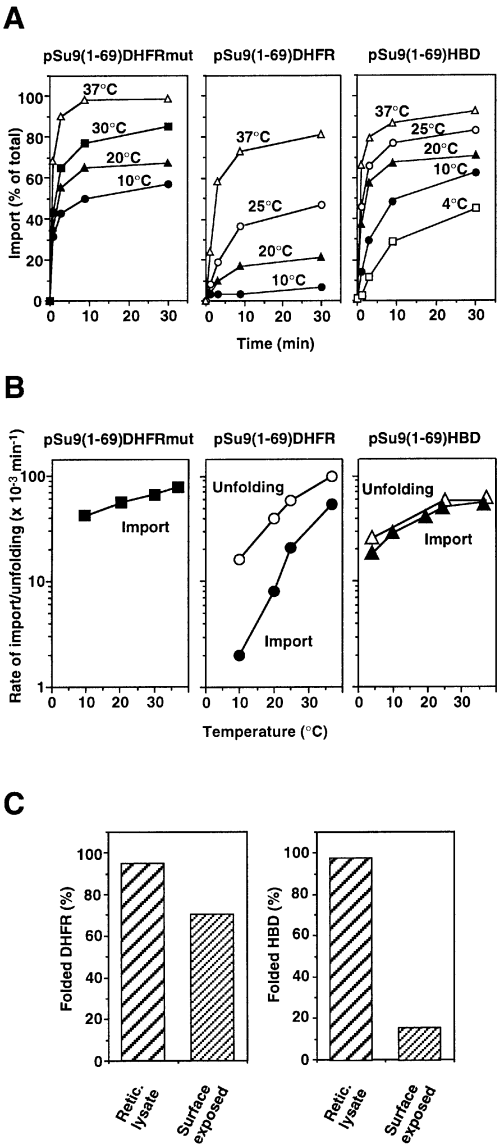

Fig. 4. Import and unfolding of preproteins. (**A**) Kinetics of import of preproteins of pSu9(1–69)DHFRmut, pSu9(1–69)DHFR and pSu9(1–69)HBD. Radiolabeled preproteins were imported into energized mitochondria at different temperatures. At the indicated time points, samples were removed and import was stopped with valinomycin. Samples were treated with PK, analyzed by SDS–PAGE and quantified with a phosphorimaging system. (**B**) Comparison of rates of import and unfolding. Kinetic data of unfolding and import shown in Figures 2D, 3D and panel (A) were fitted to first order equations and the corresponding rates were plotted versus temperature. (**C**) Folded state of passenger domains on the surface of mitochondria. pSu9(1–69)DHFR and pSu9(1–69)HBD were incubated for 10 min at 4°C with energized mitochondria and samples were divided into two aliquots. With one aliquot, mitochondria (M1) and soluble fraction (S1) were separated by centrifugation. S1 was then treated with 50 μg/ml PK. The second aliquot was first treated with PK, and mitochondria (M2) and soluble fraction (S2) were separated. Samples were analyzed by SDS–PAGE, and precursors associated with the mitochondria (M1 and M2) and folded passengers in the supernatant (S1 and S2) were quantified with a phosphorimager. S1 and S2 values were corrected for the loss of ^{35}S due to removal of the presequence upon PK treatment. The fractions of DHFR and the HBD released from the mitochondria as folded species were calculated: F = (S2–S1)/(P1–P2). For control, the PK resistance of DHFR and the HBD in the two preproteins was determined (reticulocyte lysate).

well with the kinetics of unfolding of the HBD. For pSu9(1–69)DHFR, the import rates as well as the rates of unfolding of DHFR were strongly temperature dependent. Unfolding of DHFR was significantly faster than import at all temperatures, suggesting that several rounds of unfolding and refolding of the DHFR domain precede its import.

To assess the folded state of passengers on the surface of the outer membrane, pSu9(1–69)DHFR and pSu9(1–69)HBD were incubated for 10 min at 4°C with energized mitochondria. Under these conditions, DHFR was not imported, and ~20% of the HBDs were imported. The majority of both preproteins, however, were found associated with the mitochondria. The mitochondria were then treated with 50 μg/ml PK, reisolated by centrifugation and the supernatants and mitochondrial pellets were analyzed. PK treatment removed most of the associated precursors from the mitochondria. A total of 60% of the DHFRs that were removed were recovered in the supernatant in a folded form where only the presequence had been clipped (Figure 4C). When reticulocyte lysate containing pSu9(1–69)DHFR was treated with PK, ~95% was protease resistant. This indicates that DHFR on the mitochondrial surface is folded to a similar extent as in solution. In contrast, 98% of the HBDs were folded in reticulocyte lysate but only 16% of the HBDs that were released from the mitochondria were recovered in a folded form. Apparently, the interaction of the precursors with energized mitochondria destabilizes the HBD but not DHFR.

Interaction of precursors with mt-Hsp70

Unfolding of folded preproteins on the surface of the mitochondria requires their interaction with mt-Hsp70 in the matrix. To determine what length of a segment in front of a folded passenger is required to reach mt-Hsp70, we used a number of chimeric preproteins which differ in the length of their presequence in front of the DHFR moiety (Figure 5A). The shortest precursor, pCoxIV-DHFR, had a presequence of 28 amino acid residues. The other precursors carried presequences which were derived from the precursor of subunit 9 of the ATPase. They were 52, 72, 83, 90 and 98 amino acid residues in length. These preproteins were incubated with energized mitochondria in the presence of MTX to stabilize the folded DHFR domain (Rassow *et al*., 1989). As the MTX-stabilized DHFR cannot be unfolded by the import machinery, the preproteins accumulate in a membrane-spanning fashion,

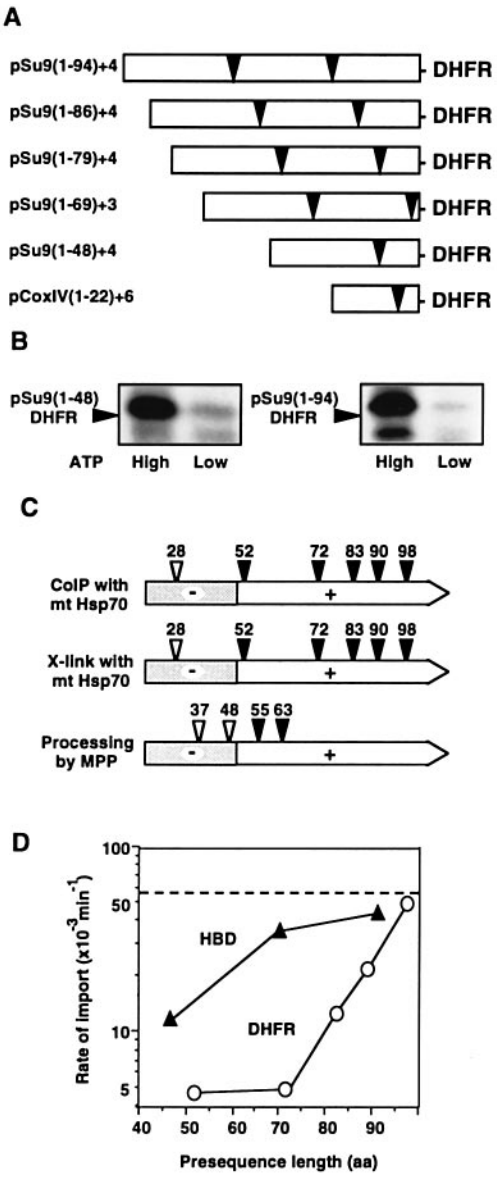

Fig. 5. Binding of mt-Hsp70 to presequences and kinetics of import. (**A**) Schematic outline of precursors with presequences of different length in front of DHFR. Presequences were derived from the precursor of subunit 9 of the ATPase of *N.crassa*, pSu9, or from the precursor of subunit 4 of the cytochrome oxidase of *S.cerevisiae*, pCoxIV. Numbers in parentheses refer to authentic amino acid residues derived from the precursors, numbers after the parentheses indicate amino acid residues that were inserted between the presequences and the start methionine of DHFR during subcloning. MPP processing sites after residues 35 and 66 in pSu9 and after residue 17 in pCoxIV are indicated by arrowheads. (**B**) Interaction of mt-Hsp70 with arrested precursors requires high levels of matrix ATP. Radiolabeled pSu9(1–48)DHFR and pSu9(1–94)DHFR were incubated for 10 min at 25°C with mitochondria in the presence of MTX and the samples were then halved. One half was incubated to maintain high matrix ATP levels while the second half was incubated for 10 min at 25°C with 10 U/ml apyrase and 5 μg/ml oligomycin to reduce matrix ATP. Mitochondria were reisolated, solubilized with Triton X-100 and subjected to immunoprecipitation with antibodies against mt-Hsp70. Samples were analyzed by SDS–PAGE and fluorography. (**C**) Presequence length in front of folded DHFR required to interact with mt-Hsp70 and MPP in the matrix. pCoxIV-DHFR, and the pSu9-DHFR fusion proteins with presequences of 52, 72, 83, 90 and 98 amino acid residues were pre-incubated with MTX and imported into mitochondria. Translocation arrest of the DHFR fusion proteins was followed by co-immuno-precipitation with antibodies against mt-Hsp70 at high levels of matrix ATP (upper panel), cross-linking with DSS of the arrested proteins to mt-Hsp70 (central panel) and analysis of processing of the presequence by the matrix-processing peptidase, MPP (lower panel). Interaction with mt-Hsp70 and cleavage by MPP is indicated by filled arrowheads, open arrowheads indicate a negative result. Numbers refer to the length of the sequence preceding the start methionine of DHFR (upper and central panel) and to the distance of the MPP processing site from the DHFR (lower panel). (**D**) Dependence of the import rates on the length of the sequence preceding DHFR and HBD. pSu9-DHFR precursors with presequences of 52, 72, 83, 90 and 98 amino acid residues and pSu9-HBD precursors with presequences of 47, 71 and 92 amino acid residues were imported into energized mitochondria at 20°C and the import rate was determined as in Figure 4. The dashed line indicates the import rate of the folding-incompetent DHFRmut precursor.

with their presequence inserted into the mitochondria and the DHFR domain apposed to the surface of the mitochondria (Ungermann *et al*., 1994). Following the accumulation of the precursors, the levels of matrix ATP were manipulated (Stuart *et al*., 1994; Wachter *et al*., 1994). The mitochondria were then solubilized with Triton X-100 and immunoprecipitations with antibodies against mt-Hsp70 were performed. When the levels of matrix ATP were kept high, pSu9(1–48)DHFR and pSu9(1–94)DHFR were co-precipitated with antibodies against mt-Hsp70 (Figure 5B). Similarly, the precursors with presequences longer than 52 amino acid residues were precipitated with anti-mt-Hsp70 IgG while pCoxIV-DHFR was not. (Figure 5C, upper panel). This indicates that 52 amino acid residues in front of the folded DHFR domain are sufficient to span both mitochondrial membranes and to allow tight binding of mt-Hsp70 to the segment exposed into the matrix. At lowered levels of matrix ATP, co-precipitation of pSu9(1–48)DHFR and pSu9(1–94)DHFR with mt-Hsp70 was drastically reduced (Figure 5B), in agreement with observations by Matouschek *et al*. (1997). Thus, the association of mt-Hsp70 with an incoming precursor is promoted by matrix ATP. Tim44 has been shown to recruit mt-Hsp70 to the import sites in the presence of matrix ATP. When matrix ATP levels are reduced, the mt-Hsp70–Tim44 reaction cycle is interrupted (Schneider *et al*., 1996). As a consequence, mt-Hsp70 cannot rebind to the incoming precursor and the equilibrium ultimately is shifted towards dissociation of mt-Hsp70 from the precursor.

The interaction of mt-Hsp70 with the arrested preproteins of different length was also analyzed by cross-linking with disuccinimidyl suberate (DSS). As summarized in Figure 5C (central panel), cross-linking confirmed the co-immunoprecipitations. Thus, interaction with mt-Hsp70 was observed with arrested pSu9(1–48) DHFR and with longer presequences, but not with pCoxIV-DHFR.

The matrix-processing peptidase, MPP, cleaves the Su9 presequence after residues 35 and 66, generating intermediate and mature forms, respectively. When arrested in a membrane-spanning fashion, pSu9(1–86)DHFR and pSu9(1–94)DHFR were processed to their intermediate forms while arrested pSu9(1–79)DHFR was not. Thus, 55 residues in front of the folded DHFR are sufficient to allow cleavage of the presequence by MPP in the matrix but 48 amino acid residues are not (Figure 5C, lower panel).

In summary, these data demonstrate that ~52 amino acid residues in front of a folded protein domain are sufficient to span both mitochondrial membranes and allow the interaction with proteins in the matrix.

We asked how the overall rate of import of a folded passenger depends on the length of the presequence. The DHFR precursors were incubated with energized mitochondria at 20°C and the rates of import were determined (Figure 5D). pSu9(1–48)DHFR and pSu9(1–69)DHFR were imported slowly, with import rates of 4.7 and 5×10^{-3}/min. Import of pSu9(1–79)DHFR and pSu9(1–86)DHFR was ~3 times and 5 times faster than import of pSu9(1–48)DHFR. The rate of import of pSu9(1–94)DHFR was 5×10^{-2}/min, which was close to the rate of import of the unfolded precursor pSu9(1–69)DHFRmut (5.7×10^{-2}/min). Thus, at 20°C, the overall rate for unfolding and import of the folded DHFR increased more than one order of magnitude when the length of the presequence in front of the folded domain was increased from 52 to 98 amino acid residues. As shown above, a presequence of 52 amino acid residues spans both mitochondrial membranes and allows binding of a mt-Hsp70. However, this is not sufficient to achieve maximal rates of protein import. Apparently, more than a single binding site for mt-Hsp70 has to be exposed to the matrix in order to allow maximal import rates of DHFR.

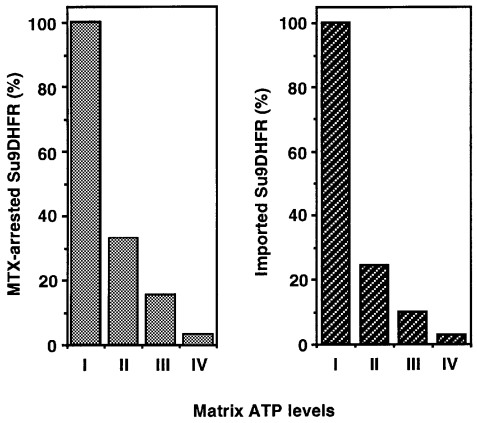

Fig. 6. ATP requirement of the import machinery is not strictly coupled to preprotein movement. Mitochondria were pre-incubated to adjust different levels of matrix ATP (see Materials and methods, levels I–IV). pSu9(1–69)DHFR was incubated for 5 min at 25°C with these mitochondria in the presence and absence of MTX. Samples were chilled on ice and incubated for 20 min with 50 μg/ml PK and analyzed by SDS–PAGE. PK-resistant pSu9(1–69)DHFR which was arrested in the presence of MTX (left panel) and mSu9(1–69)DHFR which was imported in the absence of MTX (right panel) were quantified with a phosphorimaging system. The amount of protein at high levels of matrix ATP (I) was set to 100%.

The import rates of the HBD fused with presequences of 47, 71 and 92 amino acid residues were 1.1, 3.6 and 4.4×10^{-2}/min (Figure 5D). Thus, in contrast to the loosely folded DHFR, the rates of import of the tightly folded HBD did not depend strongly on the length of the presequence.

ATP consumption and forward movement of preproteins

To assess how the requirement of the import machinery for ATP is linked to forward movement of the preprotein chain in the import channel, mitochondria were pre-incubated to reduce the levels of matrix ATP in a stepwise manner. pSu9(1–69)DHFR was then incubated with the pre-treated mitochondria in the presence of MTX to arrest the protein in a membrane-spanning fashion (Rassow *et al*., 1989) (Figure 6, left panel). The samples were then treated with PK. At high levels of matrix ATP, pSu9(1–69)DHFR associated with the mitochondria was resistant to PK treatment, indicating that the preprotein was arrested in a membrane-spanning fashion and that the DHFR domain was closely apposed to the outer membrane (Ungermann *et al*., 1994). When the ATP concentration was lowered, the arrested preprotein became accessible to PK, indicating that matrix ATP was required to maintain the DHFR domain closely apposed to the outer membrane. The level of ATP in the matrix which was required to hold the arrested preprotein firmly was similar to the ATP

level required for complete import of the preprotein in the absence of MTX (Figure 6, right panel). This indicates that ATP is required to hold a preprotein in the translocation channel and prevent retrograde movement of the polypeptide chain. When a folded passenger impairs further import of a preprotein, the ATP requirement of the import machinery is not strictly coupled to forward movement of the polypeptide chain of a preprotein in the import channel.

Discussion

Unfolding of proteins in the living cell has attracted little attention so far since the physiological relevance of unfolding has not been clear. In the past years, however, it has become obvious that energy-dependent unfolding of proteins is important in processes such as protein degradation by the proteasome and protein translocation into the endoplasmic reticulum and mitochondria (Hochstrasser, 1995; Pfanner and Meijer, 1995; Rubin and Finley, 1995). The molecular mechanisms underlying facilitated unfolding, therefore, is an important aspect in current cell biology.

Stability of folded passengers and import into mitochondria

The mitochondrial protein import apparatus is capable of unfolding folded protein domains which are about to be translocated across the mitochondrial membranes. We have analyzed unfolding and import of two types of chimeric precursors, one containing DHFR and the other containing the HBD of cytochrome b_2 as folded passenger proteins. The stabilities of the folded passengers, measured by resistance towards degradation by an unspecific PK, differed significantly. DHFR, according to this criterion, is not very stable, while the HBD appears to be tightly folded. Resistance to PK correlates well with other parameters characterizing the folding state of proteins such as the free-energy of folding of DHFR (Endo and Schatz, 1988) or the melting temperature of HBD (Glick *et al*., 1993).

The overall stabilities of the two folded protein domains do not, however, correlate with the ability of the translocation machinery to import these proteins when fused to a mitochondrial targeting signal. The HBD as a passenger was imported efficiently at all temperatures, while import of DHFR was strongly temperature-dependent and very inefficient at low temperature.

The kinetics of import of DHFR and the HBD were slower at all temperatures than those of the folding-incompetent DHFRmut. The folded passengers obviously slowed down the import process. A comparison of the rates of unfolding and import revealed that DHFR was imported efficiently at temperatures where it unfolded due to thermal breathing but not at low temperature where extensive breathing was not observed. At all temperatures, the rates of thermal unfolding of DHFR were faster than the rates of its import. Apparently, the DHFR domain unfolds, and then refolding of DHFR on the outside of the mitochondria and forward movement of unfolded segments of the precursor in the TOM and TIM translocases are competing reactions (Figure 7). As a consequence, a single unfolding step of DHFR may not be sufficient for import, and several rounds of unfolding and refolding precede its translocation into the matrix.

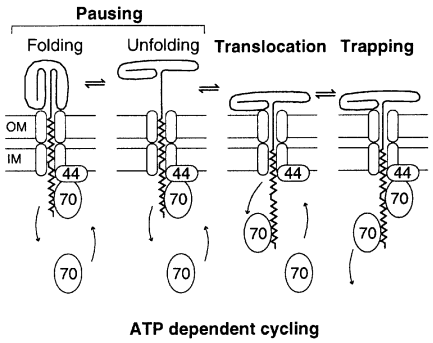

Fig. 7. Model for preprotein translocation and unfolding at the import site. Translocation of precursors relies on unfolding of passengers on the outside of the mitochondria. After spontaneous unfolding of a passenger domain, refolding competes with membrane translocation of unfolded segments of the polypeptide chain. Several rounds of unfolding and refolding precede its import. In the matrix, cycling of mt-Hsp70 onto Tim44 and its transfer to the preprotein occurs in an ATP-dependent manner. Hydrolysis of ATP by mt-Hsp70 in complex with Tim44 is not necessarily coupled to movement of the polypeptide chain in the channel. mt-Hsp70 bound to the preprotein is eventually released by uptake of ATP. Another mt-Hsp70 binds to Tim44 and initiates a new cycle. High ATP levels ensure saturation of Tim44 with mt-Hsp70 at the import site where the unfolded polypeptide chain can be trapped efficiently. This prevents retrograde movement of the preprotein and refolding on the surface of the mitochondria.

Why is import of the tightly folded HBD only slightly affected by lowering the temperature or by reduction of matrix ATP levels? The kinetics of thermal unfolding of the HBD at all temperatures were virtually indistinguishable from the kinetics of its import. This indicates that unfolding determines the rate of the overall import reaction. The fold of the HBD is stabilized by heme, which becomes released upon unfolding. The heme-free form of the HBD was degraded readily by PK, indicating that it was loosely folded. Heme, once released, becomes diluted and may not rebind efficiently to the refolding HBD under import conditions. Thus, in contrast to DHFR, a single round of unfolding of the HBD may be sufficient to shift the equilibrium from a tightly folded to a loosely folded state. Import of the loosely folded heme-free HBD will then be efficient at low temperature and at low levels of matrix ATP, similar to import of the folding-incompetent DHFRmut.

Interaction of mt-Hsp70 with the incoming precursor

Mt-Hsp70 cooperates with Tim44 and binds segments of the incoming preprotein as they emerge from the import channel (Schneider *et al*., 1994, 1996; Ungermann *et al*., 1994). Approximately 52 amino acid residues in front of the folded DHFR were sufficient to span both mitochondrial membranes and allow binding of mt-Hsp70. Efficient binding of mt-Hsp70 to short, matrix-exposed segments of an arrested precursor required high levels of matrix ATP, in agreement with the notion that ATP is required to recruit mt-Hsp70 to the outlet of the import channel (Schneider *et al*., 1996). This reaction cycle is interrupted at low matrix ATP levels and, as a consequence, the Tim44-mediated association of mt-Hsp70 with the translocation intermediate is drastically reduced.

Import of the loosely folded DHFR required rather long presequences (98 residues); fusion proteins with shorter presquences (52–72 residues) were translocated with low efficiency. These observations are in agreement with those of Matouscheck *et al*. (1997). On the other hand, when short presequences were fused to the tightly folded HBD, the precursors were imported at rates similar to those with long presequences. Thus, the interaction of mt-Hsp70 with a short matrix-exposed segment of a precursor is sufficient to unfold even a tightly folded passenger such as the HBD. However, mt-Hsp70 apparently cannot maintain the precursor efficiently in an unfolded state if only a short segment of the precursor is exposed in the matrix. When mt-Hsp70 is released from a spanning polypeptide chain, the precursor slides backwards out of the import channel (Ungermann *et al*., 1994, 1996). Passengers may then refold on the surface of the mitochondria or in the intermembrane space. With longer presequences, the probability increases of trapping a precursor at the import channel before it slides back and refolds. After refolding, the DHFR has the same stability as before, while the HBD would not refold tightly, with heme being released during the initial round of unfolding. A reduction of backsliding would, therefore, specifically accelerate the import of DHFR. Accordingly, the import rates of DHFR should strongly depend on the presequence length.

The dissociation of mt-Hsp70 from the translocating precursor in the course of a reaction cycle allows a relaxation of the extended polypeptide chain. In this way, secondary structures such as sorting signals and transmembrane helices may be formed, a process that would not be favored by constant pulling. This may represent a crucial function of the import motor. Indeed, missorting into the matrix of intermembrane space proteins with impaired sorting signals was observed to be relieved when mt-Hsp70 function was compromised by mutation or by reduction of ATP levels (Schwarz *et al*., 1993; Gärtner *et al*., 1995; Gruhler *et al*., 1997).

ATP and translocation of preproteins

Two observations suggest how matrix ATP may be utilized by the mitochondrial import motor. First, translocation of folding-incompetent DHFR was fast and required lower ATP levels than import of folded DHFR. Secondly, an MTX-stabilized DHFR domain cannot be unfolded by the import motor; yet high ATP levels were required to hold the arrested translocation intermediate in the import channel. When a folded DHFR domain impairs further inward movement of the preprotein, ATP consumption at the import site is obviously not coupled to movement of the polypeptide chain in the translocation channel. What force is generated by the import motor and how does it promote unfolding of folded passengers? The import motor is operating in an ATP-dependent manner at all temperatures and promotes import of DHFRmut and of the tightly folded HBD. The force generated by this machinery is, however, not sufficient to unfold DHFR at low temperature or to unfold it at high temperature in the presence of MTX.

Conclusions

Methods are not available to discriminate directly whether unfolding of folded passengers and forward movement in the import channel occur spontaneously or are facilitated by active pulling of mt-Hsp70 in the matrix since both processes are governed by similar forces. Theoretical modeling of protein translocation is compatible with both mechanisms, the results depending mainly on the parameters chosen for the oscillation of precursors in the import channel (Chauwin *et al*., 1998). Data presented here are compatible with various modes of mt-Hsp70-mediated unfolding. However, as shown here, mt-Hsp70 does not keep the unfolded polypeptide chain under constant tension but rather appears to cycle off and on the precursor. This allows a relaxation of the extended polypeptide chain which then slides back in the import channel. A previously unfolded passenger, such as DHFR, may then refold on the outside and, thus, several rounds of unfolding and refolding may occur before a sizeable segment becomes translocated. To drive unfolding of passengers such as DHFR and the HBD on the surface of the mitochondria thermodynamically, mt-Hsp70 must reduce retrograde movements of the translocating polypeptide chain. Thus, efficient trapping by mt-Hsp70 of segments of a precursor as they emerge from the import channel is critical for import under conditions where retrograde movement allows refolding of a passenger on the outside but not for import of folding-incompetent precursors. Long presequences, high matrix ATP levels and elevated temperature, which favor binding of mt-Hsp70 at the outlet of the import channel, are then required to promote unfolding and import of the folded passenger at a high rate.

Materials and methods

Plasmid constructions

For construction of pSu9(1–69)HBD, a DNA fragment encoding the N-terminal 99 amino acids of the mature cytochrome b_2 (Xia and Matthews, 1990) was amplified by PCR using the upstream primer, oligo 1 (5′-CCCCAGATCTGAGCCGAAACTGGAT), and the downstream primer, oligo 2 (5′-CCCCAGATCTAGGAGCATAAGGAGG). The PCR fragment was digested with *Bgl*II and cloned into the *Bam*HI site of a pGem4. A second PCR was performed on this plasmid with oligo 1 and with the T7 primer. The resulting PCR fragment was digested with *Bgl*II–*Hin*dIII and subcloned into the *Bam*HI–*Hin*dIII sites of the pGem4-Su9(1–69)DHFR, Su9(1–45)DHFR and $Su9(1–45)_2$DHFR (Ungermann *et al*., 1994). For construction of His-tagged HBD, the PCR fragment obtained with oligo 1 and oligo 2 was digested with *Bgl*II and cloned into the *Bam*HI site of pQE9 (Qiagen).

Protein translocation into isolated mitochondria

Mitochondria were isolated from yeast strain D27310-B (Daum *et al*., 1982). Mitochondria (1 mg/ml protein) were incubated in import buffer [500 mM sorbitol, 50 mM HEPES–KOH, pH 7.2, 10 mM $MgCl_2$, 80 mM KCl, 2 mM potassium phosphate and 0.01% fatty acid-free bovine serum albumin (BSA)] containing 2.5 mM ATP, 5 mM NADH, 10 mM phosphocreatine and 0.1 mg/ml creatine kinase. Import reactions were started by addition of ^{35}S-labeled precursor protein synthesized by *in vitro* transcription and translation in rabbit reticulocyte lysate (Pelham and Jackson, 1976). After incubation as indicated, the samples were split into three aliquots. One aliquot (–PK) was diluted 2-fold in cold SEM buffer (10 mM MOPS–KOH pH 7.2, 1 mM EDTA, 250 mM sucrose) containing 1 μM valinomycin. The second aliquot was diluted in SEM buffer containing 200 μg/ml PK (+ PK). The third aliquot was diluted 5-fold in ice-cold hypotonic buffer (20 mM HEPES–KOH pH 7.2) containing 200 μg/ml PK (swelling + PK). The samples were incubated for 25 min on ice. Then 1 mM phenylmethylsulfonyl fluoride (PMSF) was added and, after 5 min, mitochondria or mitoplasts were reisolated by centrifugation. The pellet was resuspended in sample buffer. Translocation arrest of DHFR fusion proteins with MTX was performed as described (Ungermann *et al*., 1994, 1996).

Immunoprecipitations and cross-linking of proteins

Mitochondria with MTX-arrested DHFR fusion proteins were reisolated by centrifugation and resuspended at a protein concentration of 1 mg/ml in solubilization buffer (0.4% Triton X-100, 20 mM Tris–HCl pH 7.4, 150 mM NaAc, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 1 mg/ml BSA, 0.1 mg/ml α_2-macroglobulin). After 15 min on ice, the samples were subjected to a clarifying spin and the supernatants were incubated at 4°C for 1 h with anti-mtHsp70 IgG covalently coupled to protein A–Sepharose beads. The beads were washed three times with solubilization buffer. Samples were analyzed by SDS–PAGE and fluorography. For ATP depletion, samples were incubated further for 10 min at 25°C with MTX, 20 µM oligomycin and 10 U/ml apyrase. Mitochondria were then reisolated and subjected to immunoprecipitation as described. For the cross-linking experiments, 200 µM DSS cross-linker was added just after the import of MTX-arrested precursors, and samples were incubated for 30 min on ice. The cross-linker was quenched with 100 mM glycine, pH 7.2. The mitochondria were subjected to immunoprecipitation as described above.

Kinetics of import and determination of import rates

Mitochondria were pre-incubated for 3 min at the import temperature in import buffer and reactions were started by addition of ^{35}S-labeled precursors. Aliquots (50 µl) were removed after 0, 1, 3, 9, 30 and 90 min and treated with PK. Samples were then processed as described above and subjected to SDS–PAGE. For each time point, the imported protein was quantified with a phosphoimaging system. The imported protein (expressed as a fraction of the total preprotein added) was plotted versus the time, and the import rate (fraction of imported protein/min) was determined by fitting the data to a first order equation.

Kinetics of unfolding

^{35}S-Labeled precursors were diluted 10-fold with import buffer for NEM treatment or 50-fold with KH_2PO_4 pH 7.4 buffer for DEPC treatment and pre-incubated at the indicated temperatures. Reactions were started by addition of NEM or DEPC, aliquots were removed after 0, 1, 3, 9 and 30 min and the reactions were quenched with either DTT or imidazole. Samples were treated with PK and subjected to SDS–PAGE. For each time point, the PK-resistant fragment was quantified with a phosphorimaging system. Untreated material was set to 100%. The percentage of protease-resistant domain was plotted versus the time, and the unfolding rate (percent degraded protein/min) was determined by fitting the data to a first order equation.

Manipulation of ATP levels in the matrix

To manipulate matrix ATP levels, mitochondria were pre-treated as described previously (Stuart *et al*., 1994). I: no pre-incubation. II and III: 1 mg/ml mitochondria were pre-incubated in import buffer without ATP and without NADH for 15 min at 25°C. Then 50 µM oligomycin and 5 mM NADH were added together with either 1 mM ATP (II) or 0.1 mM ATP (III) and mitochondria were incubated for 15 min at 4°C. IV: 1 mg/ml mitochondria were pre-incubated for 30 min at 25°C with 40 U/ml apyrase and 50 µM oligomycin. Carboxyatractyloside (5 µM) was added and mitochondria were incubated for 5 min at 4°C and then NADH was added. Untreated and pre-treated mitochondria were warmed for 3 min at 25°C and then radiolabeled preprotein was added.

Purification and analysis of recombinant DHFR and HBD

His-tagged DHFR was expressed in *E.coli* XL-blue transformed with pQE16. The protein was purified on Ni-NTA–agarose (Stüber *et al*., 1990) and dialyzed against 30 mM Tris–HCl, pH 7.2, 100 mM KCl. To determine free sulfhydryl groups in DHFR, the protein was precipitated with trichloroacetic acid (TCA), the pellet was washed four times with 1 ml of 30 mM Tris–HCl, pH 7.2, 100 mM KCl and dissolved at a concentration of 4 mg/ml in 8 M urea, 30 mM Tris–HCl, pH 8. DTNB (0.2 mM) was added and the samples were incubated for 15 min at 25°C. Thionitrobenzoate produced upon reaction of DTNB with free sulfhydryls was monitored by its absorbance at 412 nm (Habeeb, 1972). His-tagged HBD was expressed in *E.coli* XL-blue transformed with pQE9. The protein was purified on Ni-NTA–agarose (Stüber *et al*., 1990). The concentration of heme bound to the HBD was determined by spectrophotometry. Bound heme specifically absorbs at 413 nm with e_{ox} = 129 500/M/cm. To load the purified HBD with hemin, it was incubated with twice the amount of hemin for 1 h at 37°C. The protein was then recovered on Ni-NTA–agarose.

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