

The Protein Import Motor of Mitochondria: Unfolding and Trapping of Preproteins Are Distinct and Separable Functions of Matrix Hsp70

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Summary

Mitochondrial heat shock protein 70 (mtHsp70) functions in unfolding, translocation, and folding of imported proteins. Controversial models of mtHsp70 action have been discussed: (1) physical trapping of preproteins is sufficient to explain the various mtHsp70 functions, and (2) unfolding of preproteins requires an active motor function of mtHsp70 (“pulling”). Intragenic suppressors of a mutant mtHsp70 separate two functions: a nonlethal folding defect caused by enhanced trapping of preproteins, and a conditionally lethal unfolding defect caused by an impaired interaction of mtHsp70 with the membrane anchor Tim44. Even enhanced trapping in wild-type mitochondria does not generate a pulling force. The motor function of mtHsp70 cannot be explained by passive trapping alone but includes an essential ATP-dependent interaction with Tim44 to generate a pulling force and unfold preproteins.

Introduction

Most mitochondrial proteins are synthesized as preproteins on cytosolic ribosomes. The precursor polypeptides must be unfolded on the mitochondrial surface to allow translocation through the import channels of the outer and inner membranes. In the matrix, the N-terminal presequences are removed and the proteins are folded to the active form (Ryan and Jensen, 1995; Schatz and Dobberstein, 1996; Neupert, 1997; Pfanner et al., 1997). The essential heat shock protein 70 in the mitochondrial matrix (mtHsp70; also called Ssc1 in yeast) binds to the preproteins in transit. Inactivation of mtHsp70 by temperature-sensitive mutants inhibits unfolding, translocation, and the subsequent folding of preproteins (Kang et al., 1990; Gambill et al., 1993; Voos et al., 1993). The major ATP-consuming step in protein translocation is ascribed to the function of mtHsp70 (Glick et al., 1993; Stuart et al., 1994). While this central role of mtHsp70 in protein import is undisputed, a controversial debate is ongoing as to the molecular mechanism of mtHsp70 action.

On the one hand, a “Brownian ratchet or trapping-only model” has been proposed to explain the role of mtHsp70 in unfolding and membrane translocation of preproteins. According to this model, movement of the polypeptide chain in the translocation channels of the membranes would be caused solely by Brownian motion. Folded domains of preproteins that are located on the cytosolic side undergo a spontaneous partial unfolding (“breathing”). When the domains are in a loosely folded state, segments of the preproteins can traverse the membranes and bind to mtHsp70, thus preventing backsliding, arresting this state and thereby facilitating unfolding. Thus, unfolding and translocation are proposed to be spontaneous events that are made directional only by the binding of mtHsp70 to the preprotein in transit (Schneider et al., 1994, 1996; Ungermann et al., 1994, 1996; Gaume et al., 1998). The trapping-only model makes one clear and testable prediction, that is, that trapping of preproteins by mtHsp70 is sufficient for unfolding of preproteins.

On the other hand, it was proposed that mtHsp70 performs an active motor function in protein import, particularly in the unfolding of preproteins. According to this “pulling model,” membrane-bound mtHsp70 undergoes a conformational change that generates a “pulling force or power stroke” on a bound precursor polypeptide, driving the preprotein across the mitochondrial membranes. Thus, folded domains located in the cytosol are labilized and their unfolding facilitated (Kronidou et al., 1994; Rassow et al., 1994; Gärtner et al., 1995; Glick, 1995; Pfanner and Meijer, 1995; Horst et al., 1996; Voos et al., 1996; Matouschek et al., 1997). The observation that mtHsp70 binds to the membrane protein Tim44 of the inner membrane translocase in a 1:1 complex in an ATP-dependent manner (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994) provided an important prerequisite for a pulling model, the need for a membrane anchor for directed force generation (Glick, 1995; von Ahsen et al., 1995; Horst et al., 1996; Voos et al., 1996).

While numerous studies have been performed with various constructs and folding states of preproteins, little information is available on mtHsp70 itself at the molecular level. Since the *SSC1* gene is essential for cell viability, the inactivation of mtHsp70 *in vivo* and in organello could only be studied with conditional-lethal mutants. Two temperature-sensitive mutants of mtHsp70 have been described, *ssc1-2* and *ssc1-3*. Whereas the *ssc1-3* mutation affects the ATP binding/hydrolysis of mtHsp70 and thereby blocks protein transport into the matrix in general (Gambill et al., 1993), the *ssc1-2* mutation leads to a more complex pattern of effects.

While *ssc1-2* mitochondria are competent for the translocation of loosely folded preproteins, both unfolding of tightly folded preproteins and folding of imported preproteins are inhibited, and interaction of the mutant mtHsp70 (*Ssc1-2*) with Tim44 is impaired (Kang et al., 1990; Gambill et al., 1993; Voos et al., 1993, 1996; Schneider et al., 1994). We report that the trapping of

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preproteins by Ssc1-2 is severalfold enhanced and prolonged, questioning a coupling of trapping and unfolding of preproteins. We isolated suppressor mutations for the temperature-sensitive lethal effect of *ssc1-2* and found intragenic suppressors that alleviate a subset of the Ssc1-2 defects. Analysis of these suppressors, as well as wild-type mitochondria, excludes the trapping-only model as explanation of the role of mtHsp70 in the import of tightly folded preproteins.

Results

Prolonged Interaction of Preproteins with Ssc1-2: Two Working Hypotheses on mtHsp70 Function

To compare the interaction of wild-type and mutant (*Ssc1-2*) mtHsp70 with substrate polypeptides, a radiolabeled preprotein commonly used for translocation studies, Su9(70)-DHFR (DHFR, dihydrofolate reductase), was imported into isolated mitochondria and the time course of association with mtHsp70 analyzed. The mitochondria were preincubated at 37°C to induce the mutant phenotype in vitro (Kang et al., 1990). Denatured Su9(70)-DHFR (Figure 1A) was imported into wild-type and *ssc1-2* mitochondria with comparable efficiency (Figure 1B, lanes 17 and 18). Import was stopped by dissipation of the inner membrane potential ($\Delta\psi$). After distinct times of incubation, the mitochondria were lysed with nonionic detergent, and coimmunoprecipitations with antibodies directed against mtHsp70 were performed. The association of the preprotein with wild-type mtHsp70 rapidly decreased (Figure 1B, lanes 2–7). The yield of coprecipitation of 0.5%–2% of imported proteins with mtHsp70 (Figure 1C) was similarly observed in the studies favoring the trapping-only model (Ungermann et al., 1994, 1996). In contrast, the association of Su9(70)-DHFR with Ssc1-2 was enhanced by a factor of four to five (Figure 1B, lanes 10–15; Figure 1C). Even 30 min after import into the matrix, a significant fraction of the preprotein was still associated with Ssc1-2 (Figure 1B, lane 15; Figure 1C), demonstrating an increased trapping capacity of the mutant mtHsp70.

The fact that Ssc1-2 shows an enhanced trapping while having defects in unfolding of preproteins brings into question whether passive trapping of preproteins is sufficient for unfolding. We thus formulated two alternative hypotheses. (1) The single amino acid alteration of Ssc1-2 affects only one biochemical property, the release of bound substrate proteins. Retardation of the release (i.e., enhanced trapping) inhibits the reaction cycle of mtHsp70, leading to a general impairment of mtHsp70 function, including the defects in unfolding of preproteins, interaction with Tim44, and folding of proteins. This hypothesis is fully compatible with the trapping-only model, in that the roles of mtHsp70 can be explained by a single mechanism, the binding and release of protein substrates. (2) The *ssc1-2* mutation causes two separate biochemical defects. On the one hand, the enhanced trapping of preproteins by Ssc1-2 explains the folding defect, while on the other hand, the interaction of Ssc1-2 with Tim44 is blocked, causing the defect in unfolding of preproteins. This hypothesis implies that even an enhanced trapping of preproteins is not sufficient for promoting unfolding of preprotein

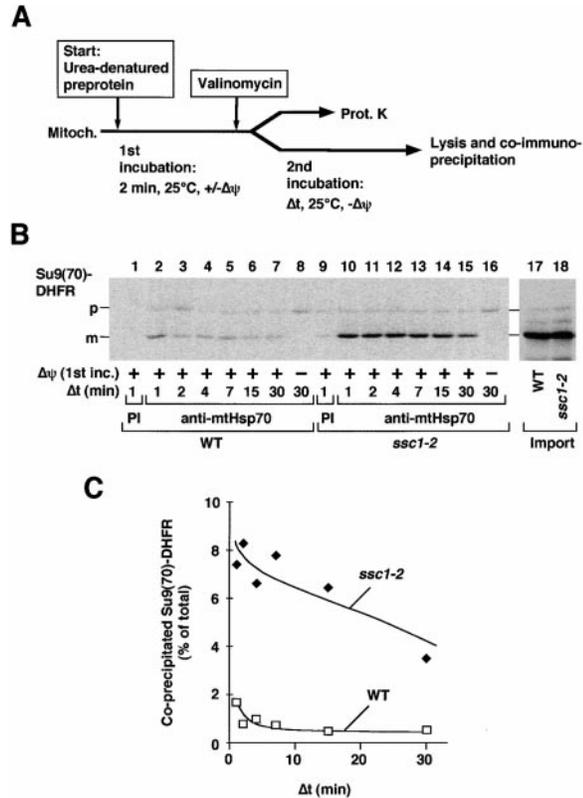


Figure 1. Enhanced and Prolonged Interaction of Preproteins with Ssc1-2

(A) Experimental approach.

(B) Urea-denatured radiolabeled preprotein Su9(70)-DHFR was imported into isolated wild-type (WT) or *ssc1-2* mutant mitochondria under nonpermissive conditions. After 2 min at 25°C, further import was stopped by dissipating $\Delta\psi$ in all samples (addition of valinomycin). Portions were directly assayed for total import efficiency by adding proteinase K (samples 17 and 18). The remaining mitochondria were kept at 25°C for the indicated times and then lysed, and imported proteins bound to mtHsp70 were coimmunoprecipitated (anti-mtHsp70). PI, preimmune serum. Su9(70)-DHFR was analyzed by SDS-PAGE and autoradiography.

(C) Quantitation of coimmunoprecipitation of imported precursor with anti-mtHsp70. The total amount of imported protein was set to 100%.

domains on the mitochondrial surface and thus would contradict the trapping-only model.

Suppressor Mutants of *ssc1-2* Have Amino Acid Substitutions in the Peptide-Binding Domain of mtHsp70

To discriminate between the two alternative hypotheses on the molecular mechanism of mtHsp70, we selected intragenic suppressor mutants of *ssc1-2* and tested distinct mtHsp70 properties. Six second-site suppressors that allow growth at the nonpermissive temperature of 34°C were identified that map to two codons, both in the region encoding the peptide-binding domain, the domain of the original *ssc1-2* mutation (Figure 2A). *ssc1-2* changes the proline at position 419, located at one end of a β strand that contacts the substrate at its opposite end, to a serine (Figure 2A). Four suppressors change the aspartic acid at position 496: *ssc1-201* to

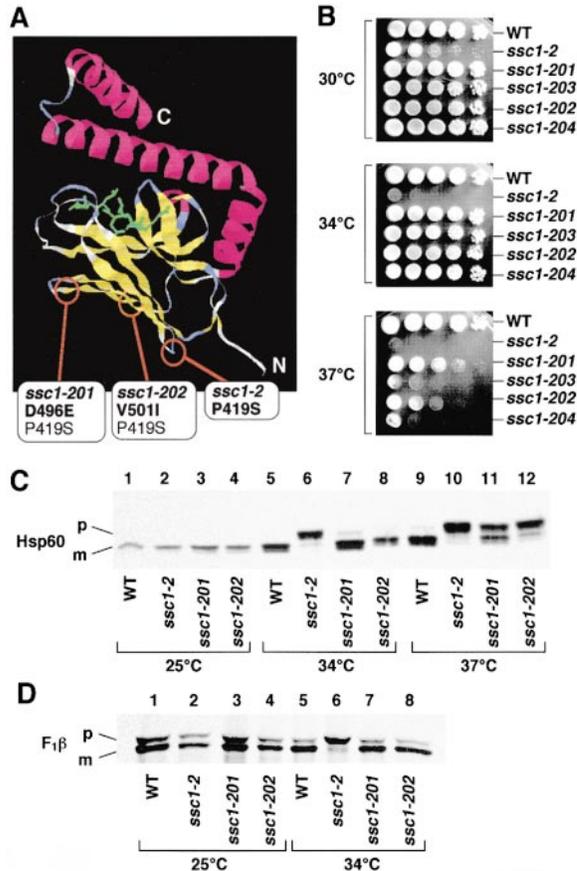


Figure 2. Intragenic Suppressor Mutants of *ssc1-2*

(A) Peptide-binding domain of Ssc1 modeled onto the known structure of DnaK (Zhu et al., 1996) by the program SWISS-MODEL (Peitsch, 1996). The Ssc1 backbone (amino acid residues 389–577) is represented as a ribbon structure, showing α helices in red, β sheet in yellow, turns in blue, and bound substrate peptide in green. Altered amino acid residues are indicated with orange circles.

(B) Temperature sensitivity of growth. Serial dilutions of yeast cells of wild-type, *ssc1-2*, *ssc1-201*, *ssc1-202*, *ssc1-203*, and *ssc1-204* strains were plated on fermentable medium and incubated for 3 days at the indicated temperatures.

(C) Restoration of protein import in vivo (steady state). Total cell extracts of wild-type, *ssc1-2*, *ssc1-201*, and *ssc1-202* strains were prepared after shifting the culture for 4 hr from 25°C to the indicated temperatures. Proteins were separated by SDS-PAGE and analyzed by immunodecoration using anti-Hsp60. p, precursor form; m, mature form.

(D) Restoration of protein import in vivo (pulse labeling). Total yeast proteins were radiolabeled in vivo. Cell extracts were prepared, and $F_1\beta$ was immunoprecipitated.

glutamic acid (two cases), *ssc1-203* to tyrosine, and *ssc1-204* to asparagine. Two suppressors change the valine at position 501 to isoleucine (*ssc1-202*). Both D496 and V501 are located on a single β strand that neighbors the strand carrying the *ssc1-2* point mutation.

The suppressor mutations restored growth of *ssc1-2* at 34°C but only partially restored growth at 37°C (Figure 2B). For further analysis, we focused on two suppressor mutations that mapped to two different amino acid codons and showed the best growth restoration: *ssc1-201* and *ssc1-202*. The *ssc1-2* mutation causes an arrest of mitochondrial protein import in vivo (Kang et al., 1990),

as evidenced by the accumulation of the precursor form of Hsp60 after shifting the cells from 25°C to 34°C or 37°C for 4 hr (Figure 2C, lanes 6 and 10). In *ssc1-201* and *ssc1-202* cells, the generation of mature Hsp60 was restored at 34°C (Figure 2C, lanes 7 and 8). At 37°C, the restoration was partial, yet again a stronger effect of *ssc1-201* could be observed (Figure 2C, lanes 11 and 12). Within the time frame of this experiment, the accumulation of precursor may result from a secondary effect of the *ssc1-2* mutation and not from the inactivation of Ssc1-2 itself. To address this possibility, cells were incubated for only 15 min at 25°C or 34°C prior to a labeling with [³⁵S]methionine for 2 min. The newly synthesized β subunit of the F_1 ATPase was immunoprecipitated from the cell lysates. After the brief preincubation, the precursor form of $F_1\beta$ accumulated in *ssc1-2* (Figure 2D, lane 6), while the suppressor mutations restored the level of mature protein to near wild-type levels (Figure 2D, lanes 7 and 8). Therefore, the suppressor mutations not only ameliorated the temperature-sensitive growth defect of *ssc1-2* but also reduced the level of precursor forms of mitochondrial proteins, suggesting a restoration of mitochondrial protein import in vivo.

The Suppressor Mutations Restore Preprotein Unfolding during Translocation

We used two preproteins that differ in the stability of their tertiary structure to analyze the effect of the suppressor mutations on the unfolding of preproteins during translocation across the mitochondrial membranes. $b_2(220)\Delta$ -DHFR carries an intact heme-binding domain that in the presence of heme is stably folded and has been one of the major substrates to assay unfolding activity during translocation into the matrix (Glick et al., 1993; Voos et al., 1993, 1996; Stuart et al., 1994). $b_2(167)\Delta$ -DHFR is identical to $b_2(220)\Delta$ -DHFR in its amino-terminal targeting sequence and the carboxy-terminal DHFR (Figure 3A). However, its heme-binding domain is not intact and thus not tightly folded. Therefore, this preprotein is not dependent on mtHsp70 activity to facilitate its unfolding (Voos et al., 1993, 1996). The preproteins were synthesized and radiolabeled in reticulocyte lysate in the presence of heme and incubated with isolated mitochondria from wild-type, *ssc1-2*, and the suppressor *ssc1-201* and *ssc1-202* strains. While $b_2(167)\Delta$ -DHFR was imported into both *ssc1-2* mitochondria and the suppressor mitochondria with comparable efficiency (Figure 3B), a difference was observed with $b_2(220)\Delta$ -DHFR. The import inhibition into *ssc1-2* mitochondria (Figure 3C, lanes 3 and 4) was fully restored by the *ssc1-201* mutation (Figure 3C, lanes 5 and 6) and partially restored by the *ssc1-202* mutation (Figure 3C, lanes 7 and 8). When heme was omitted from the in vitro import reaction, import of $b_2(220)\Delta$ -DHFR occurred at equal rates into wild-type and all mutant mitochondria (Figure 3D), demonstrating that the folding state of the heme-binding domain was the crucial factor in the differential import into *ssc1-2* and suppressor mitochondria. We conclude that the suppressor mutations restore the unfolding function of mtHsp70 for membrane translocation of preproteins.

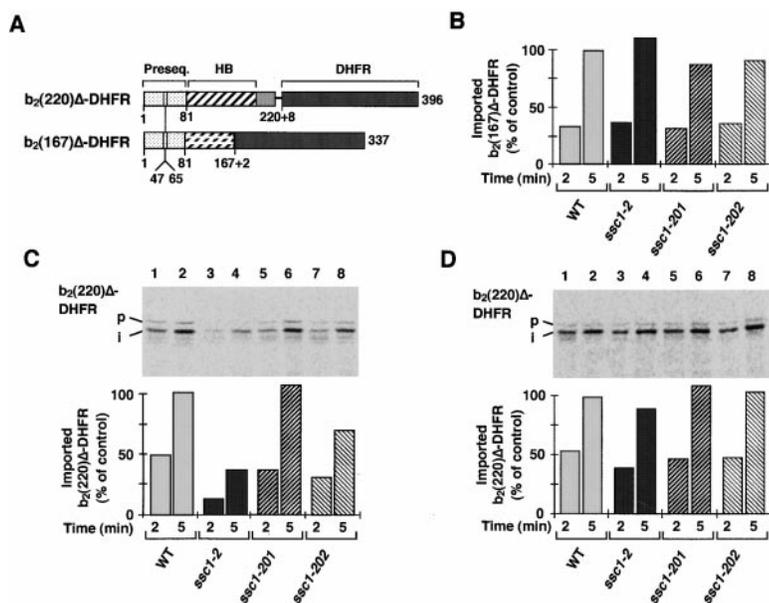


Figure 3. Restoration of Preprotein Unfolding by the Suppressor Mutations

(A) Matrix-targeted fusion proteins between cytochrome *b₂* and mouse DHFR used in this study, showing the location of the heme-binding domain (HB).

(B) Import of *b₂(167)Δ*-DHFR in the presence of heme. In vitro-synthesized, radiolabeled *b₂(167)Δ*-DHFR was imported into isolated mitochondria from the indicated strains. Import was assayed by treatment with proteinase K, analysis by SDS-PAGE, and digital autoradiography. The amount imported into wild-type mitochondria after 5 min was set to 100% (control).

(C) Import of *b₂(220)Δ*-DHFR in the presence of heme. p and i, precursor- and intermediate-sized forms of the preprotein, respectively. Because of the deletion in the second half of the presequence, processing to the mature form does not occur.

(D) Import of *b₂(220)Δ*-DHFR without conformational restrictions (no addition of heme).

The Suppressor Mutations Do Not Affect the Folding Defect and Enhanced Trapping in *ssc1-2* Mitochondria

ssc1-2 mitochondria are defective in the folding of proteins once they are imported into mitochondria. To assess folding of imported proteins, we again utilized the Su9(70)-DHFR precursor that is efficiently imported into *ssc1-2* mitochondria if denatured (Figure 1B and data not shown). However, in its folded native form, DHFR is protease resistant. Therefore, protease resistance of the imported protein can be used to monitor its folding state (Ostermann et al., 1989; Kang et al., 1990). Denatured Su9(70)-DHFR was imported into wild-type and mutant mitochondria; the membrane potential was dissipated to halt any further import. After further incubation, the mitochondria were lysed and immediately treated with proteinase K. In wild-type mitochondria, the imported DHFR was resistant to protease (Figure 4A, lanes 1–3). A strong folding defect of imported Su9(70)-DHFR was observed in *ssc1-2* (Figure 4A, lanes 4–6) as well as *ssc1-201* and *ssc1-202* mitochondria (Figure 4A, lanes 7–12), as indicated by greater susceptibility of DHFR to digestion. The relative intensity of the signals indicate that the folding efficiency is 5- to 7-fold lower in *ssc1-2* and suppressor mitochondria compared to that found in wild-type mitochondria. Therefore, the folding defect observed in *ssc1-2* mitochondria persists in the suppressor mitochondria.

We asked if the defects in folding and maturation of proteins were related to the enhanced association of preproteins with mtHsp70 observed in *ssc1-2*. Indeed, both suppressor mtHsp70s showed a strongly enhanced and prolonged association with imported Su9(70)-DHFR (Figure 4B, lanes 9–16; quantitation), demonstrating that the suppressor mutations did not relieve this *ssc1-2* defect. The prolonged association of imported proteins with mtHsp70 thus explains the retardation of folding in *ssc1-2* and suppressor mitochondria. However, the restoration of unfolding of preproteins is not coupled to these observations and points to a second independent effect of the *ssc1-2* mutation (see Discussion and Table 1).

The Suppressor Mutations Partially Reverse Conformational Alterations of Ssc1-2

To assess the conformational state of the mutant mtHsp70s, we assayed their sensitivity toward a treatment with trypsin. A striking difference was observed between wild-type mtHsp70 and Ssc1-2, particularly in the ATP-bound state (von Ahlsen et al., 1995). When the ATP-bound state was stabilized with the nonhydrolyzable ATP analog 5'-adenylyl-β,γ-imidodiphosphate (AMP-PNP), a portion of wild-type mtHsp70 remained full length, and a 56 kDa fragment (*f₁*) was formed by trypsin treatment (Figure 5, lanes 5–8). However, Ssc1-2 was rapidly degraded to a 45 kDa fragment (*f₂*) (Figure 5, lanes 9–12). Both the 45 kDa fragment and the 56 kDa fragment were recognized by an antiserum directed against an N-terminal peptide of mtHsp70, while an antiserum directed against the C terminus did not react with either fragment (not shown). The 45 kDa fragment thus represents the ATPase domain of mtHsp70, and the 56 kDa fragment approximately comprises the ATPase domain and the β sandwich portion of the peptide-binding domain. Ssc1-201 and Ssc1-202, which contain both the *ssc1-2* and suppressor alterations, yielded a split effect. While a fraction of the mtHsp70 was cleaved to the 45 kDa fragment, the full-length mtHsp70 was considerably more stable than Ssc1-2 (Figure 5, lane 13–20). The 56 kDa fragment was neither observed in Ssc1-2 nor in the suppressors Ssc1-201 and Ssc1-202. We conclude that the *ssc1-2* mutation causes two distinct conformational changes: formation of the 45 kDa fragment instead of the 56 kDa fragment and a high trypsin sensitivity of the full-length mtHsp70. The suppressor mutations affect only the second alteration.

The Suppressors Restore the Association of mtHsp70 with Tim44 and the Generation of an Inward-Directed Force on Membrane-Spanning Preproteins

As described above, preprotein unfolding during translocation is restored in the mitochondria from the suppressor strains. According to the pulling model of

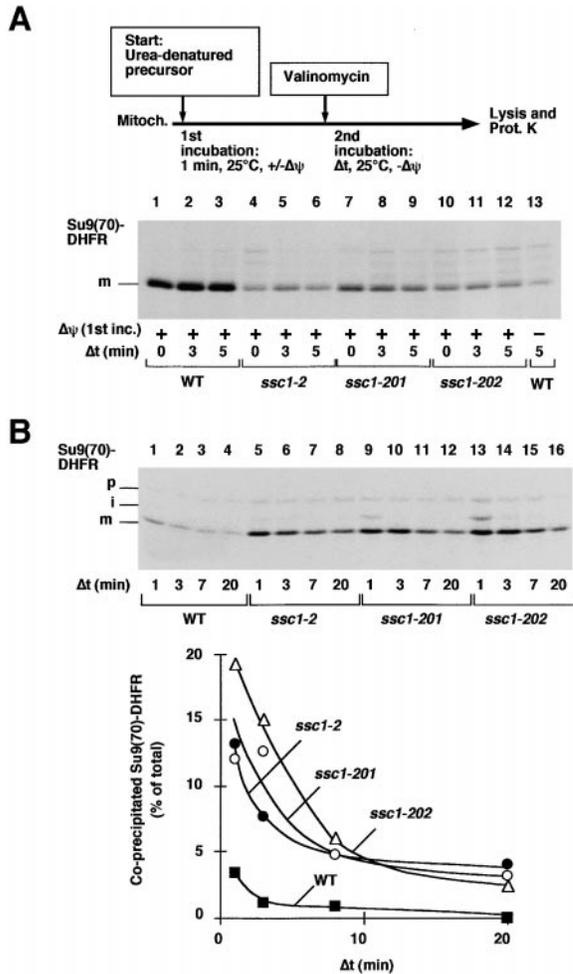


Figure 4. The Suppressor Mutants Do Not Restore Defects in Protein Folding and Maintain the Enhanced Association of Substrates with mtHsp70

(A) Folding of Su9(70)-DHFR. Urea-denatured Su9(70)-DHFR was imported into wild-type and *ssc1* mutant mitochondria as described in the legend of Figure 1. $\Delta\psi$ was dissipated, and the mitochondria were incubated for the indicated times and then lysed, and the folding state of Su9(70)-DHFR was determined by resistance of the mature protein (m) against treatment with proteinase K. As a control for import-independent folding of Su9(70)-DHFR (background), wild-type mitochondria with dissipated $\Delta\psi$ were used in the first incubation (sample 13).

(B) Interaction of mtHsp70 with the substrate protein Su9(70)-DHFR was assayed as described in the legend of Figure 1, using wild-type and mutant mitochondria. After completion of import, mitochondria were incubated at 25°C for the indicated times. Mitochondria were lysed, and material bound to mtHsp70 was analyzed by coimmunoprecipitation.

translocation, the unfolding process would require the interaction with the membrane anchor Tim44. Therefore, the complex between mtHsp70 and Tim44 was analyzed by lysing mitochondria with nonionic detergent followed by coprecipitation with antibodies directed against Tim44 (Figure 6A, lane 5). The disruption of this association in *ssc1-2* (Figure 6A, lane 6) was reversed by the suppressor mutations. *ssc1-201* and *ssc1-202* mitochondria contained a mtHsp70-Tim44 complex that was stable in the absence of ATP (Figure 6A, lanes 7 and 8).

The association of mtHsp70 with this inner membrane anchor was specific, as shown by the efficient dissociation in the presence of Mg-ATP (Figure 6A, lanes 9, 11, and 12).

The restoration of mtHsp70 interaction with Tim44 by the suppressors provides a possible explanation for the restoration of the unfolding activity during protein import. To ask directly if the suppressors restore an effect on a membrane-spanning preprotein that is defective in *ssc1-2* mitochondria, we used an established assay that monitors the tautness of the interaction of the preprotein against the outer membrane (Schwarz et al., 1993; Gärtner et al., 1995; Bömer et al., 1998). We utilized a fusion protein consisting of the 86 N-terminal amino acids of F_0 -ATPase subunit 9, a four-residue linker, and DHFR. In the presence of methotrexate, the DHFR domain is stabilized and prevented from entering the import channel (Eilers and Schatz, 1986). However, the subunit 9 portion is sufficiently long to be processed to the intermediate form (after residue 35) by the matrix-processing peptidase, since ~50 residues are sufficient to span both mitochondrial membranes (Rassow et al., 1990; Ungermann et al., 1994). In wild-type mitochondria, the folded DHFR is tightly "pulled" against the outer membrane, and added protease cannot access and cleave the polypeptide segment immediately in front of DHFR. The tightness of the interaction is generated by both the membrane potential $\Delta\psi$ and mtHsp70. When $\Delta\psi$ is dissipated, the polypeptide chain begins sliding back and forth in the import channel and thus becomes accessible to protease. Therefore, the effectiveness of the generation of an inward-directed force on the membrane-spanning preprotein by mtHsp70 can be monitored by the rate of cleavage of the intermediate form by externally added protease after dissipation of $\Delta\psi$.

We accumulated methotrexate-bound preprotein in mitochondrial import sites of wild-type and mutant mitochondria (Figure 6B). With increasing times of incubation in the absence of a $\Delta\psi$, a large fraction of the accumulated intermediate form of Su9(86)-DHFR was degraded in wild-type mitochondria (Figure 6B, lanes 1–4). In *ssc1-2* mitochondria, the intermediate form was even more rapidly degraded (Figure 6B, lanes 5–8 and quantitation), indicating an impairment of the mtHsp70 import driving system. In the suppressor mitochondria, the protease resistance was largely restored to levels found in wild-type mitochondria (Figure 6B, lanes 9–16 and quantitation). Since *Ssc1-2* as well as *Ssc1-201* and *Ssc1-202* possess an increased trapping activity, the ability of the mutant proteins to generate an inward-directed force (pulling) does not correlate with the trapping capacity (see below; Table 1).

Trapping of Preproteins Is Not Sufficient for Generating an Inward-Directed Force

The results described above make it highly unlikely that trapping of a preprotein by mtHsp70 is sufficient for driving the unfolding/pulling of a preprotein. However, since the unfolding function is related to the association of mtHsp70 with Tim44, according to a trapping-only model, it may be argued that the interaction of mtHsp70 with Tim44 is needed to increase the local concentration of the chaperone at the protein import site and thus enhance the trapping function (Schneider et al., 1994, 1996; Ungermann et al., 1996; Gaume et al., 1998).

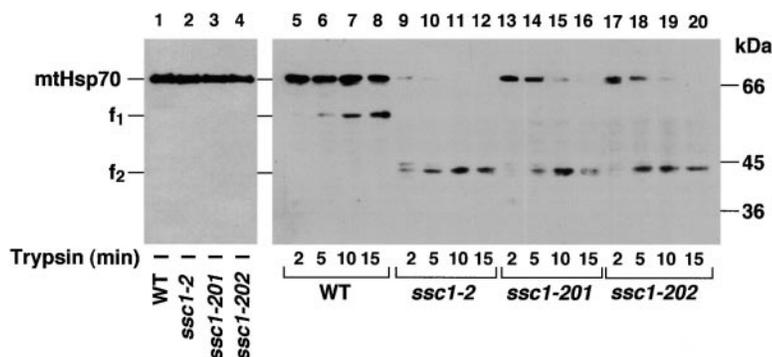


Figure 5. Two Distinct Conformational Alterations of Ssc1-2

Wild-type and mutant mitochondria were lysed in the presence of 5 mM AMP-PNP and treated with trypsin for the indicated times. Full-length mtHsp70 and partially protease-resistant fragments (f_1 and f_2) were detected by SDS-PAGE and immunodecoration using anti-mtHsp70 antiserum.

To obtain direct experimental evidence regarding this issue, we arrested membrane-spanning intermediates as described above with wild-type mitochondria using the preprotein $b_2(167)\Delta$ -DHFR. Previously, it was reported that at low levels of matrix ATP the preprotein slid back in the import channel, as evidenced by increased accessibility to added protease (Schwarz et al., 1993; Gärtner et al., 1995). We asked if this was correlated with a decreased binding of mtHsp70, as predicted by the trapping model. $b_2(167)\Delta$ -DHFR was preincubated with methotrexate and arrested in the import sites of wild-type mitochondria in the presence of a $\Delta\psi$ and ATP, that is, under the physiological conditions with a fully functional Tim44–mtHsp70 system. The mitochondria were reisolated and split into two samples, one of which was depleted of ATP (Figure 7A). Then the $\Delta\psi$ was dissipated, and each sample was analyzed over time by two parallel methods: determination of the protease accessibility of the accumulated preprotein to assay for generation of an inward-directed force (Figure 7B, lanes 1–8), and coimmunoprecipitation of the radiolabeled precursor to assay for trapping by mtHsp70 (Figure 7B, lanes 9–16). As expected, at a low level of ATP the inward-directed force generated on the preprotein was decreased (Figure 7B, lanes 5–8) compared to the samples that were kept at high ATP in the final incubation (Figure 7B, samples 1–4). However, the association of the membrane-spanning intermediate form with mtHsp70 (trapping) was increased under the low ATP conditions (Figure 7B, lanes 13–16; compare to lanes 9–12). Quantitation of the results (Figure 7C) demonstrates that trapping was enhanced by the subsequent ATP depletion, but the force generation on the membrane-spanning preprotein was decreased.

We also analyzed the fusion protein Su9(86)-DHFR, which has an N-terminal extension so short that, after methotrexate arrest and cleavage to the intermediate form, only approximately five (to maximally ten) residues extend into the matrix. This length is sufficient to bind one (maximally two) molecule(s) of mtHsp70. Also in this case, a lowering of the ATP level increased the association of the membrane-spanning preprotein with mtHsp70 (Figure 7D, right panel) but decreased the inward-directed force acting on the preprotein (Figure 7C, left panel). We conclude that trapping of a preprotein is not sufficient for generating an inward-directed force (pulling), even when mtHsp70 is available directly at the protein import site.

Discussion

We report that trapping of a preprotein by mtHsp70 is not sufficient to drive the unfolding of preprotein domains on the mitochondrial surface prior to translocation. Intensive discussion of the alternative models of the role of mtHsp70 in preprotein translocation, trapping, and pulling, has been based mainly on analysis using preproteins of different folding states (Ungermann et al., 1994, 1996; Glick, 1995; Schneider et al., 1996; Matouschek et al., 1997; Chauwin et al., 1998; Gaume et al., 1998). On the other hand, our conclusions are based on the analysis of the point mutant *ssc1-2* and intragenic suppressors that reverse some, but not all, of its defects. *ssc1-2* mitochondria are defective in both the unfolding of tightly folded preproteins during translocation and their subsequent folding after translocation. Moreover, the interaction of Ssc1-2 with Tim44 is defective. We report here that Ssc1-2 has a prolonged interaction with incoming precursor proteins, causing an enhanced trapping activity (Table 1). This enhanced trapping explains the folding defect due to a retarded release of proteins in the matrix yet implies that trapping of a preprotein is not sufficient to generate a pulling force, that is, to drive unfolding on the mitochondrial surface.

Analysis of intragenic suppressor mutations of *ssc1-2* revealed that these two biochemical defects, enhanced trapping of preproteins and defective interaction with Tim44, are separable. The suppressors reestablish the Tim44 interaction, but the prolonged interaction with protein substrates persists. The reestablishment of the Tim44 interaction correlated with the recovery of the ability of mitochondria to unfold preproteins (Table 1).

Table 1. Properties of Mutant mtHsp70s

mtHsp70	Ssc1 (WT)	Ssc1-2	Ssc1-201, Ssc1-202 (Suppressors)
Trapping of preproteins	+	+++	+++
Folding of imported proteins	+	–	–
Binding to Tim44	+	–	+
Import of preproteins with tightly folded domains (unfolding, “pulling”)	+	–	+
Import of loosely folded preproteins	+	+	+

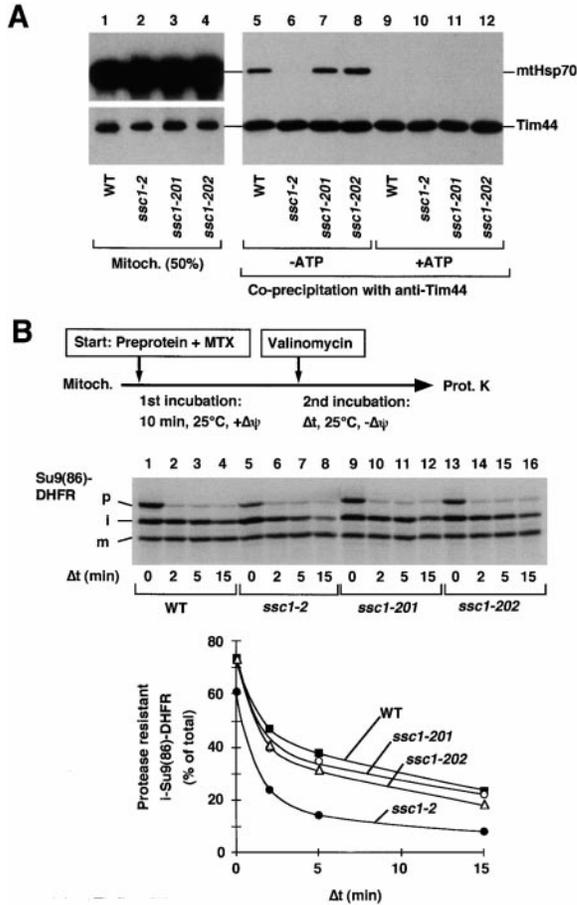


Figure 6. The Suppressors Restore the Association of mtHsp70 with Tim44 and the Generation of an Inward-Directed Force on a Membrane-Spanning Preprotein

(A) Interaction of Tim44 and mtHsp70. Complexes between Tim44 and mtHsp70 were purified from wild-type and mutant mitochondria by immunoprecipitation with antibodies against Tim44. Lysis was performed in the presence of 2 mM ATP/5 mM MgCl₂ (+ATP) or 5 mM EDTA (-ATP). In lanes 1-4, 50% of lysate was analyzed. Tim44 and mtHsp70 were detected by immunodecoration.

(B) Restoration of inward-directed force. Su9(86)-DHFR precursor proteins spanning both mitochondrial membranes were accumulated in wild-type and mutant mitochondria by pretreatment with methotrexate (MTX). After import, mitochondria were incubated in the absence of a membrane potential for the indicated times (Δt). The inward-directed force was assayed by the resistance of the accumulated preproteins (as processing intermediate form) to treatment with proteinase K. The total amount of accumulated intermediate (without protease treatment) was set to 100%. The standard errors of the means of three independent experiments were <5% for the 2 min and 5 min values and <2.5% for the 15 min values.

The folding defect was not corrected, in agreement with the assumption that this defect is due to the increased trapping of proteins. Therefore, it is highly unlikely that trapping is the causative mechanism for unfolding of preproteins.

These results could be compatible with the trapping-only model if the role of Tim44 was restricted to increase the local concentration of mtHsp70 at the protein import site. Due to the high concentration of mtHsp70 in the mitochondrial matrix and its excess over incoming preproteins, this possibility seems unlikely. In addition, we

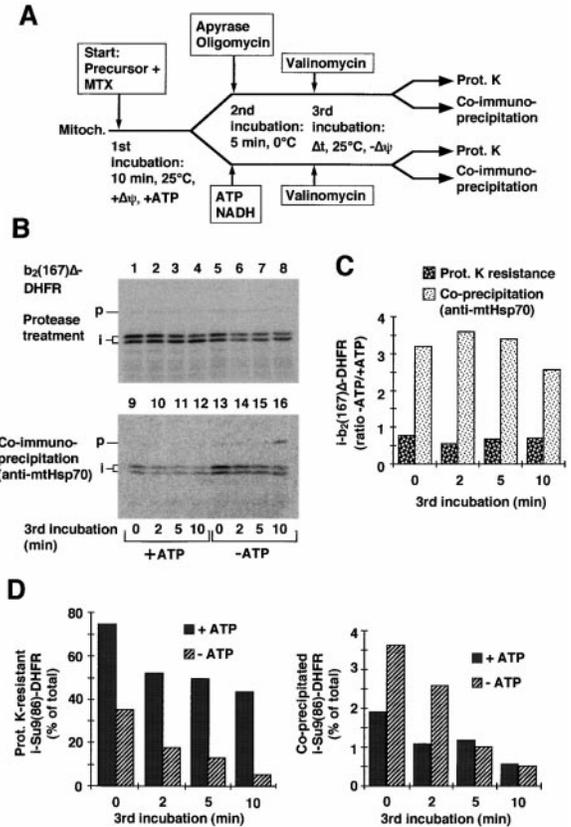


Figure 7. Enhanced Interaction of mtHsp70 with a Membrane-Spanning Preprotein Does Not Promote Generation of an Inward-Directed Force

(A) Experimental approach. Preproteins were accumulated as membrane-spanning intermediates in wild-type mitochondria in the presence of MTX. Samples were split. One-half was treated with oligomycin and apyrase to reduce the level of matrix ATP. After dissipation of $\Delta\psi$, samples were incubated for the indicated times at 25°C. One-third was treated with proteinase K to assess the inward-directed force. The other part was lysed and a coimmunoprecipitation with anti-mtHsp70 performed.

(B) Inward-directed force on b₂(167)Δ-DHFR. Both i forms represent membrane-spanning translocation intermediates (processing by matrix-processing peptidase and, in the case of the lower i form, additionally by the mitochondrial intermediate peptidase).

(C) Relation between inward-directed force and mtHsp70 substrate interaction. Proteinase K-resistant intermediate and coimmunoprecipitated b₂(167)Δ-DHFR intermediate forms were quantified. The ratio of the values obtained at low ATP versus high ATP is shown for each time point. The standard errors of the means of three independent pulling experiments were <0.09 for each ratio.

(D) Inward-directed force on Su9(86)-DHFR. Left panel: Su9-DHFR resistant to proteinase K; right panel: Su9-DHFR coimmunoprecipitated with anti-mtHsp70. The total amount of accumulated intermediate (without protease treatment) was set to 100%.

show here that even under conditions where the initial interaction of wild-type mtHsp70 with Tim44 is normal, leading to loading of the preprotein directly at the protein import site, the increased trapping activity found upon depletion of ATP is not correlated with a strong import driving force (pulling). Therefore, the trapping-only model can be excluded as an explanation for our results.

Previous results indicate that the diffusion rates of preproteins in the import channel are very low compared

to the free diffusion rates, suggesting an interaction of translocating preproteins with the walls of the import channel (summarized in Chauwin et al. [1998]). In addition, a translocase channel-preprotein complex could be isolated that was devoid of mtHsp70, indicating that the translocase was able to stably hold the preprotein in the absence of mtHsp70; a regulatory effect of mtHsp70 on the inner membrane channel was discussed (Bömer et al., 1997; Dekker et al., 1997). A calculation by Chauwin et al. (1998) provided theoretical evidence that additional driving forces besides trapping are required to overcome the diffusion constraints, particularly in the case of preproteins with folded domains. While these observations per se were not taken as sufficient evidence to exclude the trapping-only model (Gaume et al., 1998), they are in full agreement with the conclusions drawn here from analysis of the suppressor mutants, that is, that an additional import driving force besides trapping is generated by the mtHsp70 system.

Hsp70 (BiP/Kar2) is important in driving the translocation of preproteins into the endoplasmic reticulum from the *trans* side of the membrane. As in the case of mtHsp70, different mechanisms have been discussed for the molecular mode of BiP action (Simon et al., 1992; Panzner et al., 1995; Brodsky, 1996; Lyman and Schekman, 1997; Hamman et al., 1998). However, it remains unresolved whether the posttranslational translocation is driven by a trapping mechanism or whether BiP additionally functions as an active motor. For prokaryotic protein export, a mechanism evolved with a pushing force from the *cis* side of the membrane, generated by ATP-dependent conformational changes of SecA (Duong et al., 1997). Thus, the prokaryotic system includes an active transport motor on the *cis* side of the membrane, while mitochondria possess a transport motor on the *trans* side. A continuous supply of ATP is required to generate a pulling force on mitochondrial preproteins, supporting the view that an active ATP-dependent cycling of mtHsp70 via Tim44 concomitant with conformational changes of mtHsp70 is needed for pulling proteins in and promoting their unfolding. A basic import of loosely folded preproteins can occur in the absence of functional Tim44 (Bömer et al., 1998) and is ascribed to the trapping function of mtHsp70. We conclude that for tightly folded proteins, the function of the mitochondrial import motor involves two mechanisms, trapping and pulling.

Experimental Procedures

Yeast Strains and Plasmids

All yeast strains used in this study have the genotype *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2⁺ met2-Δ1 lys2-Δ2*. In *CMV1*, used for mutagenesis, the chromosomal copy of *SSC1* is replaced with the mutant allele *ssc1-2* and a *LEU2* auxotrophic marker gene 3' to the coding sequence of *ssc1-2* (Gambill et al., 1993). The other strains used in this study were constructed from *PJ53-52C*, which carries a chromosomal deletion of the 1.4 kb *Clal* fragment of *SSC1* (encoding amino acids 10 through 478) substituted with a *LEU2* auxotrophic marker gene. *PJ53-52C* also harbors a plasmid with *SSC1* under the control of the *GAL1* promoter (Gambill et al., 1993). This plasmid has been exchanged for a plasmid carrying the *ssc1-2* and wild-type *SSC1* alleles under the control of its own promoter in *CMV2* and *CMV3*, respectively. Strains *CMV201*, *CMV202*, *CMV203*, and *CMV204* carry the *ssc1-201*, *ssc1-202*, *ssc1-203*, and *ssc1-204* alleles, respectively.

The plasmid pS13 used as transcription template for Su9(86)-DHFR was constructed by cloning a PCR-generated Su9 fragment into a pGEM4 vector containing the mouse DHFR open reading frame (Bömer et al., 1998), resulting in a fusion protein consisting of the amino-terminal 86 amino acids of Su9 from *N. crassa*, a four amino acid linker (G, S, G, and I), and the entirety of DHFR.

Generation and Isolation of Suppressor Mutations

CMV1 cells were mutagenized with ultraviolet light to 50% viability; 7×10^8 cells were selected for growth at 37°C. Thirty-nine independent colonies grew at 37°C—twenty-seven as well as wild-type at all temperatures tested, twelve more slowly. Twenty-five candidates were tested for linkage of the mutation(s) to the *ssc1-2:LEU2* locus by mating to a wild-type strain and tetrad analysis. In all cases examined, all four segregants were viable at the nonpermissive temperature, suggesting that the suppression was within the coding sequence of *ssc1-2*. To determine whether the suppression resulted from a reversion of the point mutation within *ssc1-2* or an additional mutation within the coding sequence of *ssc1-2*, the sequence at the *ssc1-2:LEU2* locus was isolated using gap repair (Orr-Weaver et al., 1988). Gap repair was performed on one candidate exhibiting wild-type growth and six of the slower growing candidates using a centromeric plasmid carrying parts of the coding sequence of the *ssc1-2* allele. The circularized plasmid was rescued from the suppressor strains and retransformed into the parent strain to establish that suppression was dependent on the gene carried on the plasmid. The entire coding sequence was sequenced to determine the location of the mutations. The candidate showing wild-type growth had no mutations, indicating it was a true revertant. The remaining six candidates possessed two mutations, the original *ssc1-2* mutation and the one allowing the suppression; four of the strains with two mutations, representing alleles *ssc1-201*, *ssc1-202*, *ssc1-203*, and *ssc1-204*, were chosen for further analysis.

Analysis of Precursor Accumulation In Vivo

For steady-state analysis, yeast carrying either wild-type or mutant forms of *SSC1* were grown to early log phase. The cultures were divided into three: one was shifted to 34°C, the second to 37°C, and the third maintained at 25°C. Four hours after the temperature shift, total cell lysates were prepared by glass bead lysis and analyzed by SDS-PAGE and immunodecoration using Hsp60-specific antiserum.

For pulse labeling, cells were grown at 25°C in selective media lacking methionine to early log phase. After a 15 min preincubation at 25°C or 34°C, cells were labeled for 2 min at 25°C or 34°C. Immunoprecipitations were essentially carried out as previously described (Kang et al., 1990).

Import of Preproteins into Isolated Mitochondria

Mitochondria were isolated from wild-type and *ssc1* mutant (*ssc1-2*, *-201*, and *-202*) yeast strains under permissive conditions (Voos et al., 1996). In experiments using *ssc1* mutants, mitochondria were preincubated for 15 min at 37°C in import buffer (3% BSA, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS-KOH [pH 7.2]) immediately prior to import reactions to induce the *ssc1-2* phenotype that was maintained during subsequent incubation at lower temperature (Kang et al., 1990; Gambill et al., 1993). Where indicated, reticulocyte lysates were incubated with 10 μM heme for 5 min at 0°C after in vitro translation reactions but prior to import reactions. Import reactions were performed by incubation of radiolabeled preproteins with isolated mitochondria in import buffer at 25°C (Bömer et al., 1998). Proteinase K treatment, reisolation of mitochondria by centrifugation, and separation of imported proteins by SDS-PAGE were performed as published (Söllner et al., 1991).

Folding Assay

Import reactions were performed with urea-denatured preprotein (Ostermann et al., 1989; Kang et al., 1990). Reactions were stopped after 1 min by addition of 1 μM valinomycin and transfer to ice. Mitochondria were immediately lysed in lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 5 mM EDTA, and 0.3% Triton X-100) containing 180 μg/ml proteinase K. After incubation for 10 min at 0°C, digestion was stopped by addition of 1 mM PMSF. After a clarifying

spin, supernatants were precipitated with TCA and analyzed by SDS-PAGE and digital autoradiography.

Binding of Imported Proteins to mtHsp70 and Analysis of Tim44-Hsp70 Complexes by Coimmunoprecipitation

Urea-denatured preprotein was imported. After 2 min import at 25°C, mitochondria were washed with SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]) and lysed in buffer A (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.1% Triton X-100) at 125 µg mitochondrial protein/ml. After a clarifying spin, the extract was incubated for 1 hr at 4°C by end-over-end shaking with protein A-Sepharose-containing prebound antibodies against mtHsp70. After 1 hr incubation at 4°C, the samples were washed three times with buffer A and once with 10 mM Tris-HCl (pH 7.5) and analyzed by SDS-PAGE and autoradiography.

Immunoprecipitations assaying Tim44-mtHsp70 interactions were performed essentially as described (Voos et al., 1996). Mitochondria were lysed in buffer B (30 mM Tris-HCl [pH 7.4], 5% glycerol, 200 mM KCl, 0.5 mM PMSF, 0.1% Triton X-100) at 500 µg mitochondrial protein/ml, either including 5 mM EDTA (-ATP) or 5 mM MgCl₂/2 mM ATP (+ATP). Samples were applied to anti-Tim44-protein A-Sepharose and incubated at 4°C for 1 hr, washed three times in buffer B, and eluted by 5 min incubation in 100 mM glycine (pH 2.5). Eluted proteins were precipitated with TCA, separated by SDS-PAGE, and detected by immunodecoration.

Tryptic Digest of mtHsp70

After a pretreatment with oligomycin (20 µM) and apyrase (10 U/ml) to deplete endogenous ATP, mitochondria were lysed in 0.1% Triton X-100, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 20 mM MOPS-KOH (pH 7.2), 5 mM AMP-PNP for 15 min. After a clarifying spin, a treatment with trypsin (15 µg/ml) was performed at room temperature (von Ahsen et al., 1995). In control samples (10% of material), trypsin was omitted. After TCA precipitation, mtHsp70 and its degradation products were analyzed by SDS-PAGE and immunodecoration.

Assay for the Generation of an Inward-Directed Force

Radiolabeled preprotein-DHFR fusion proteins were arrested as translocation intermediates spanning both mitochondrial membranes by pretreating the precursors with 1 µM methotrexate (MTX) for 5 min at 0°C (Bömer et al., 1998). After import, mitochondria were reisolated and resuspended in SEM. In some experiments, matrix ATP levels were reduced by treating mitochondria with 20 µM oligomycin and 10 U/ml apyrase for 5 min at 0°C. The membrane potential was dissipated by addition of 1 µM valinomycin, and mitochondria were incubated up to several minutes at 25°C. Residual import force exerted on the translocation intermediate was assayed by resistance against treatment with 50 µg/ml proteinase K. Samples were either directly analyzed by SDS-PAGE, or mitochondria were lysed and mtHsp70-bound proteins were detected as described above.

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References

Bömer, U., Meijer, M., Maarse, A.C., Hönliger, A., Dekker, P.J.T., Pfanner, N., and Rassow, J. (1997). Multiple interactions of components mediating preprotein translocation across the inner mitochondrial membrane. *EMBO J.* **16**, 2205-2216.
Bömer, U., Maarse, A.C., Martin, F., Geissler, A., Merlin, A., Schönfisch, B., Meijer, M., Pfanner, N., and Rassow, J. (1998). Separation of

structural and dynamic functions of the mitochondrial translocase: Tim44 is crucial for the inner membrane import sites in translocation of tightly folded domains, but not of loosely folded preproteins. *EMBO J.* **17**, 4226-4237.

Brodsky, J.L. (1996). Post-translational protein translocation: not all hsc70s are created equal. *Trends Biochem. Sci.* **21**, 122-126.

Chauwin, J.F., Oster, G., and Glick, B.S. (1998). Strong precursor-pore interactions constrain models for mitochondrial protein import. *Biophys. J.* **74**, 1732-1743.

Dekker, P.J.T., Martin, F., Maarse, A.C., Bömer, U., Müller, H., Guiard, B., Meijer, M., Rassow, J., and Pfanner, N. (1997). The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70-Tim44. *EMBO J.* **16**, 5408-5419.

Duong, F., Eichler, J., Price, A., Leonard, M.R., and Wickner, W. (1997). Biogenesis of the gram-negative bacterial envelope. *Cell* **91**, 567-573.

Eilers, M., and Schatz, G. (1986). Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* **322**, 228-232.

Gambill, B.D., Voos, W., Kang, P.J., Miao, B., Langer, T., Craig, E.A., and Pfanner, N. (1993). A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J. Cell Biol.* **123**, 109-117.

Gärtner, F., Bömer, U., Guiard, B., and Pfanner, N. (1995). The sorting signal of cytochrome *b*₂ promotes early divergence from the general mitochondrial import pathway and restricts the unfoldase activity of matrix Hsp70. *EMBO J.* **14**, 6043-6057.

Gaume, B., Klaus, C., Ungermann, C., Guiard, B., Neupert, W., and Brunner, M. (1998). Unfolding of preproteins upon import into mitochondria. *EMBO J.* **17**, 6497-6507.

Glick, B.S. (1995). Can Hsp70 proteins act as force-generating motors? *Cell* **80**, 11-14.

Glick, B.S., Wachter, C., Reid, G.A., and Schatz, G. (1993). Import of cytochrome *b*₂ to the mitochondrial intermembrane space: the tightly folded heme-binding domain makes import dependent upon matrix ATP. *Protein Sci.* **2**, 1901-1917.

Hamman, B.D., Hendershot, L.M., and Johnson, A.E. (1998). BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation. *Cell* **92**, 747-758.

Horst, M., Oppliger, W., Feifel, B., Schatz, G., and Glick, B.S. (1996). The mitochondrial protein import motor: dissociation of mitochondrial hsp70 from its membrane anchor requires ATP binding rather than ATP hydrolysis. *Protein Sci.* **5**, 759-767.

Kang, P.J., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A., and Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* **348**, 137-143.

Kronidou, N.G., Oppliger, W., Bolliger, L., Hannavy, K., Glick, B.S., Schatz, G., and Horst, M. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. USA* **91**, 12818-12822.

Lyman, S.K., and Schekman, R. (1997). Binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP. *Cell* **88**, 85-96.

Matouschek, A., Azem, A., Ratliff, K., Glick, B.S., Schmid, K., and Schatz, G. (1997). Active unfolding of precursor proteins during mitochondrial protein import. *EMBO J.* **16**, 6727-6736.

Neupert, W. (1997). Protein import into mitochondria. *Annu. Rev. Biochem.* **66**, 863-917.

Orr-Weaver, T.L., Nicolas, A., and Szostak, J.W. (1988). Gene conversion adjacent to regions of double-strand break repair. *Mol. Cell. Biol.* **8**, 5292-5298.

Ostermann, J., Horwich, A.L., Neupert, W., and Hartl, F.U. (1989). Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature* **341**, 125-130.

Panzner, S., Dreier, L., Hartmann, E., Kostka, S., and Rapoport, T.A. (1995). Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell* **81**, 561-570.

- Peitsch, M.C. (1996). ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. *Biochem. Soc. Trans.* **24**, 274–279.
- Pfanner, N., and Meijer, M. (1995). Protein sorting: pulling in the proteins. *Curr. Biol.* **5**, 132–135.
- Pfanner, N., Craig, E.A., and Hönlinger, A. (1997). Mitochondrial pre-protein translocase. *Annu. Rev. Cell. Dev. Biol.* **13**, 25–51.
- Rassow, J., Hartl, F.U., Guiard, B., Pfanner, N., and Neupert, W. (1990). Polypeptides traverse the mitochondrial envelope in an extended state. *FEBS Lett.* **275**, 190–194.
- Rassow, J., Maarse, A.C., Krainer, E., Kübrich, M., Müller, H., Meijer, M., Craig, E.A., and Pfanner, N. (1994). Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J. Cell Biol.* **127**, 1547–1556.
- Ryan, K.R., and Jensen, R.E. (1995). Protein translocation across mitochondrial membranes: what a long, strange trip it is. *Cell* **83**, 517–519.
- Schatz, G., and Dobberstein, B. (1996). Common principles of protein translocation across membranes. *Science* **271**, 1519–1526.
- Schneider, H.-C., Berthold, J., Bauer, M.F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* **371**, 768–774.
- Schneider, H.-C., Westermann, B., Neupert, W., and Brunner, M. (1996). The nucleotide exchange factor MGE exerts a key function in the ATP-dependent cycle of mt-Hsp70-Tim44 interaction driving mitochondrial protein import. *EMBO J.* **15**, 5796–5803.
- Schwarz, E., Seytter, T., Guiard, B., and Neupert, W. (1993). Targeting of cytochrome *b*₂ into the mitochondrial intermembrane space: specific recognition of the sorting signal. *EMBO J.* **12**, 2295–2302.
- Simon, M.S., Peskin, C.S., and Oster, G.F. (1992). What drives the translocation of proteins? *Proc. Natl. Acad. Sci. USA* **89**, 3770–3774.
- Söllner, T., Rassow, J., and Pfanner, N. (1991). Analysis of mitochondrial protein import using translocation intermediates and specific antibodies. *Methods Cell Biol.* **34**, 345–358.
- Stuart, R.A., Gruhler, A., van der Klei, I., Guiard, B., Koll, H., and Neupert, W. (1994). The requirement of matrix ATP for the import of precursor proteins into the mitochondrial matrix and intermembrane space. *Eur. J. Biochem.* **220**, 9–18.
- Ungermann, C., Neupert, W., and Cyr, D.M. (1994). The role of hsp70 in conferring unidirectionality on protein translocation. *Science* **266**, 1250–1253.
- Ungermann, C., Guiard, B., Neupert, W., and Cyr, D.M. (1996). The $\Delta\psi$ - and Hsp70/MIM44-dependent reaction cycle driving early steps of protein import into mitochondria. *EMBO J.* **15**, 735–744.
- von Ahsen, O., Voos, W., Henninger, H., and Pfanner, N. (1995). The mitochondrial protein import machinery. Role of ATP in dissociation of the Hsp70-Mim44 complex. *J. Biol. Chem.* **270**, 29848–29853.
- Voos, W., Gambill, B.D., Guiard, B., Pfanner, N., and Craig, E.A. (1993). Presequence and mature part of preproteins strongly influence the dependence of mitochondrial protein import on heat shock protein 70 in the matrix. *J. Cell Biol.* **123**, 119–126.
- Voos, W., von Ahsen, O., Müller, H., Guiard, B., Rassow, J., and Pfanner, N. (1996). Differential requirement for the mitochondrial Hsp70-Tim44 complex in unfolding and translocation of preproteins. *EMBO J.* **15**, 2668–2677.
- Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E., and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606–1614.