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Differential requirement for the mitochondrial Hsp70–Tim44 complex in unfolding and translocation of preproteins

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The mitochondrial heat shock protein Hsp70 is essential for import of nuclear-encoded proteins, involved in both unfolding and membrane translocation of preproteins. mtHsp70 interacts reversibly with Tim44 of the mitochondrial inner membrane, yet the role of this interaction is unknown. We analysed this role by using two yeast mutants of mtHsp70 that differentially influenced its interaction with Tim44. One mutant mtHsp70 (Ssc1-2p) efficiently bound preproteins, but did not show a detectable complex formation with Tim44; the mitochondria imported loosely folded preproteins with wild-type kinetics, yet were impaired in unfolding of preproteins. The other mutant Hsp70 (Ssc1-3p′) bound both Tim44 and preproteins, but the mitochondria did not import folded polypeptides and were impaired in import of unfolded preproteins; Ssc1-3p′ was defective in its ATPase domain and did not undergo a nucleotide-dependent conformational change, resulting in permanent binding to Tim44. The following conclusions are suggested. (i) The import of loosely folded polypeptides (translocase function of mtHsp70) does not depend on formation of a detectable Hsp70–Tim44 complex. Two explanations are possible: a trapping mechanism by soluble mtHsp70, or a weak/very transient interaction of Ssc1-2p with Tim44 that leads to a weak force generation sufficient for import of loosely folded, but not folded, polypeptides. (ii) Import of folded preproteins (unfoldase function of mtHsp70) involves a reversible nucleotide-dependent interaction of mtHsp70 with Tim44, including a conformational change in mtHsp70. This is consistent with a model that the dynamic interaction of mtHsp70 with Tim44 generates a pulling force on preproteins which supports unfolding during translocation.

Keywords: heat shock protein/Hsp70–Tim44 complex/translocase/unfoldase action

Introduction

The biogenesis of mitochondria requires the translocation of a large number of precursor polypeptides from the cytosol into the organelle (Baker and Schatz, 1991; Stuart *et al.*, 1994a; Kübrich *et al.*, 1995). Mitochondrial preproteins are typically synthesized with positively charged amino-terminal signal sequences (presequences) (Hurt *et al.*, 1984; Horwich *et al.*, 1985). Translocation of the first portion of a presequence across the mitochondrial inner membrane is driven by the membrane potential $\Delta\psi$ (negative on the inside) (Schleyer and Neupert, 1985; Pfanner *et al.*, 1987; Martin *et al.*, 1991). Translocation of the remainder of the presequence and of the mature part of the preprotein requires interaction with a molecular chaperone (Ellis and Hemmingsen, 1989; Rothman, 1989), the heat shock protein of 70 kDa in the mitochondrial matrix (mtHsp70, also termed Ssc1p) (Kang *et al.*, 1990; Scherer *et al.*, 1990, 1992; Cyr *et al.*, 1993, Gambill *et al.*, 1993; Ungermann *et al.*, 1994). Two roles have been ascribed to mtHsp70 in membrane translocation of preproteins: facilitation of unfolding of precursor polypeptides (unfoldase function) and driving the complete import into the matrix of polypeptides irrespective of their folding state (translocase function) (Gambill *et al.*, 1993; Voos *et al.*, 1993; Rassow *et al.*, 1995). The unfoldase function of mtHsp70 is needed for preproteins with (tightly) folded domains and can be circumvented by artificial unfolding of preproteins. The translocase function is required for every preprotein which has to be completely imported into the matrix; it is not required for some preproteins with an inner membrane/intermembrane space sorting signal which promotes their divergence from the general import pathway (Voos *et al.*, 1993; Glick *et al.*, 1993; Stuart *et al.*, 1994b; Gärtner *et al.*, 1995a). The biochemical mechanisms underlying the two postulated functions of mtHsp70 are unknown.

Recently it was observed by biochemical and genetic means that mtHsp70 interacts with a component of the preprotein translocase of the inner membrane, the protein Tim44 [new uniform nomenclature (Pfanner *et al.*, 1996); Tim 44 was previously termed Mpi1, Isp45 or Mim44] (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). The complex between these two proteins, each of which is essential for the viability of the yeast *Saccharomyces cerevisiae* (Craig *et al.*, 1987; Maarse *et al.*, 1992), is dissociated by Mg-ATP. These findings raised several speculations about a function of the mtHsp70–Tim44 interaction in promoting protein import, including possible roles of mtHsp70 in pulling or trapping preproteins (Kronidou *et al.*, 1994; Pfanner *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994; Stuart *et al.*, 1994b; Ungermann *et al.*, 1994; Berthold *et al.*, 1995; Glick, 1995; Pfanner and Meijer, 1995). However, the role of the Hsp70–Tim44 interaction for preprotein import has not been characterized by experimental studies.

For this study, we asked in which way the interaction of mtHsp70 with Tim44 is related to the different functions of mtHsp70 (unfoldase, translocase) in the import of

preproteins. We characterized conditions of an altered interaction between mtHsp70 and Tim44 and asked for the consequences on membrane translocation of preproteins. A *S.cerevisiae* mutant of mtHsp70 (*ssc1-2*) which lacked a detectable complex formation between mtHsp70 and Tim44 was still able to promote translocation of loosely folded polypeptides (translocase function), but was impaired in the unfoldase function of mtHsp70. Binding of mtHsp70 to Tim44, however, was not sufficient to drive unfolding of preproteins since a different *S.cerevisiae* mutant of mtHsp70 (*ssc1-3'*) which showed a non-dissociable binding of mtHsp70 to Tim44 was defective in protein import. These results suggest that the translocase function of mtHsp70 does not depend on a detectable complex formation with Tim44. The unfoldase function of mtHsp70 requires a dynamic interaction with Tim44, including a nucleotide-dependent conformational change of mtHsp70, supporting a mechanism of active pulling.

Results

A mutant mtHsp70 (Ssc1-2p) unable to bind to Tim44 is defective in the unfoldase function, but not in the translocase function

To differentiate between the unfoldase and translocase function of mtHsp70 in preprotein translocation across the mitochondrial membranes, we used two closely related preproteins that are targeted into the matrix space. At the N-terminus, both contain the presequence of cytochrome b_2 with a 19-residue deletion that inactivates the intermembrane space targeting signal, and at the C-terminus, both carry the entire mouse dihydrofolate reductase (DHFR) (Figure 1A). The fusion proteins carry segments of different length of the mature part of cytochrome b_2 in their middle portion. $b_2(167)_{\Delta 19}$-DHFR contains the first 87 residues, whereas $b_2(220)_{\Delta 19}$-DHFR contains the first 140 residues of mature cytochrome b_2 (Figure 1A). The first ~100 residues of mature cytochrome b_2 form a tightly folded non-covalent haem-binding domain (Xia and Mathews, 1990) which must be unfolded to allow translocation across the mitochondrial membranes (Glick *et al.*, 1993; Voos *et al.*, 1993). The DHFR moiety of the b_2-fusion proteins requires only very low energy input for unfolding (Pace, 1990) and therefore does not require the unfoldase function of mtHsp70 for membrane translocation (Voos *et al.*, 1993).

Cells of the temperature-sensitive mtHsp70 mutant *ssc1-2* (Pro419 substituted by serine; Gambill *et al.*, 1993) were grown at the permissive temperature of 25°C, and mitochondria were isolated. The mitochondria were preincubated at 37°C to induce the mutant phenotype and incubated at 25°C with rabbit reticulocyte lysate containing the ^{35}S-labelled preproteins $b_2(167)_{\Delta 19}$-DHFR and $b_2(220)_{\Delta 19}$-DHFR. $b_2(167)_{\Delta 19}$-DHFR was processed to the intermediate-sized form and transported to a protease-protected location by *ssc1-2* mitochondria with the same efficiency and kinetics as by wild-type mitochondria (Figure 1B). The processing and import of $b_2(220)_{\Delta 19}$-DHFR, however, were significantly inhibited in *ssc1-2* mitochondria compared with wild-type mitochondria (Figure 1C).

To assess the defect of *ssc1-2* mitochondria, we compared the interaction of mutant mtHsp70 (Ssc1-2p) and wild-type mtHsp70 with preproteins and Tim44 by co-immunoprecipitations (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). Mitochondria which accumulated $b_2(167)_{\Delta 19}$-DHFR or $b_2(220)_{\Delta 19}$-DHFR in the presence of a membrane potential (Figure 2A and B, columns 1 and 3) were lysed in non-ionic detergent. The co-precipitation of either preprotein with Ssc1-2p (Figure 2A and B, columns 3) was even more efficient than the one with wild-type mtHsp70 (Figure 2A and B, columns 1) (Voos *et al.*, 1994; von Ahsen *et al.*, 1995). The efficient interaction with the two preproteins was in strict contrast, however, to the effect of the *ssc1-2* mutation on the interaction between Ssc1-2p and Tim44. While the wild-type complex Hsp70–Tim44 was fully stable also after four or five washing cycles (in the presence of EDTA) (Figure 2C, lane 1), no binding of Ssc1-2p to Tim44 was detectable (Figure 2C, lane 3). Ssc1-2p was not impaired in binding of nucleotides: it bound to ATP-agarose and ADP-agarose as efficiently as wild-type mtHsp70 (von Ahsen *et al.*, 1995); a titration showed that the ATP-induced release of the co-chaperone Mge1p (Voos *et al.*, 1994) from mtHsp70 was indistinguishable between wild-type and mutant (Figure 2 legend); and the amino acid alteration in Ssc1-2p that is outside of the ATPase domain (Gambill *et al.*, 1993) did not disturb the domain folding of the ATPase domain (von Ahsen *et al.*, 1995).

In summary, *ssc1-2* mitochondria are fully competent in the translocase function (evidenced by the undisturbed import of $b_2(167)_{\Delta 19}$-DHFR), yet are impaired in the unfoldase function of matrix Hsp70. Since *ssc1-2* mitochondria do not show any detectable binding of the mutant mtHsp70 to Tim44, the translocase function does not seem to depend on the complex formation Hsp70–Tim44.

mtHsp70 of ssc1-3' mitochondria binds to Tim44, but the mitochondria are impaired in protein import

We asked if binding of mtHsp70 to Tim44 is sufficient to drive import of folded preproteins. We used a different mtHsp70 mutant; the *ssc1-3* mutation causes the exchange of Gly56 in the ATPase domain by serine (Gambill *et al.*, 1993). *ssc1-3* cells are unable to grow at 37°C on every carbon source, yet are already impaired in growth at 25°C ('permissive conditions') (Gambill *et al.*, 1993; Schmitt *et al.*, 1995). Under non-permissive conditions (37°C), the mutant mtHsp70 (Ssc1-3p) is unable to bind to Tim44 and does not interact with preproteins, leading to a full block of protein import (Gambill *et al.*, 1993; Rassow *et al.*, 1994; Schneider *et al.*, 1994). At lower temperature, the mutant Hsp70 showed a considerable binding activity to Tim44 when compared with wild-type mtHsp70, as demonstrated in the co-precipitation with anti-Tim44 antibodies (Figure 3A, lane 3 versus lane 1) [the total amount of mtHsp70 is comparable between wild-type and mutant mitochondria (see below, Figure 4C)]. We use a prime (') *ssc1-3'* for mitochondria kept at 'permissive conditions' to distinguish them from *ssc1-3* mitochondria which were treated at 37°C.

To test for the import activity of *ssc1-3'* mitochondria, they were incubated with rabbit reticulocyte lysates containing ^{35}S-labelled $b_2(167)_{\Delta 19}$-DHFR or $b_2(220)_{\Delta 19}$-DHFR in the presence of energy substrates. Both processing (Figure 3B, lanes 2 and 4) and transport to a protease-

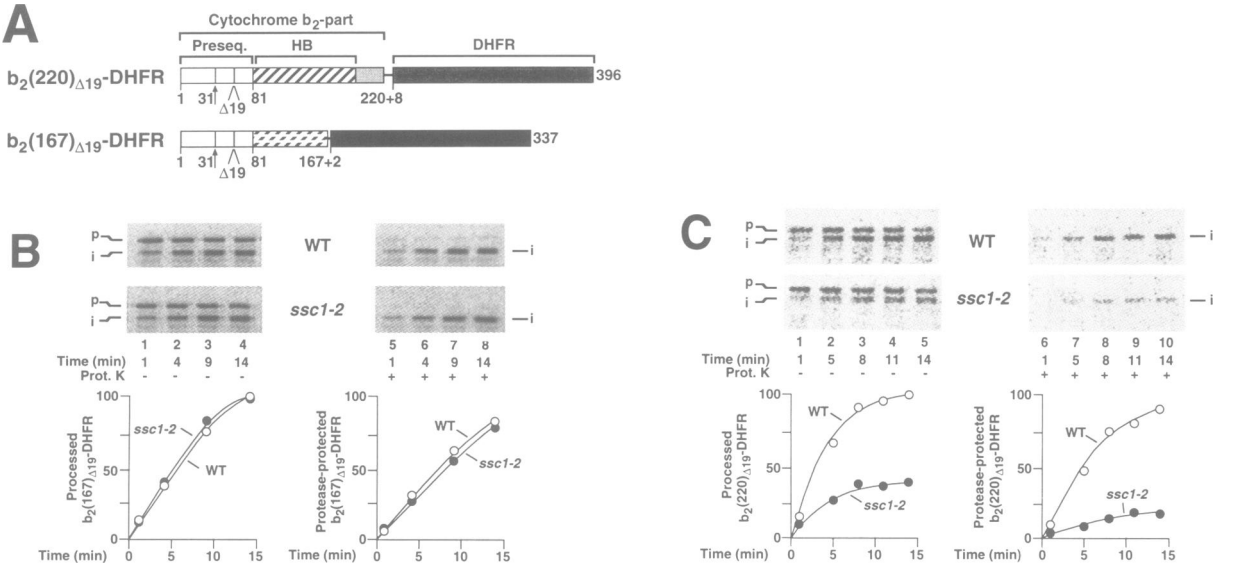

Fig. 1. *ssc1-2* mitochondria are impaired in import of $b_2(220)_{\Delta 19}$-DHFR, but not $b_2(167)_{\Delta 19}$-DHFR. (**A**) Preproteins used. Both contain at the N-terminus the presequence of *S.cerevisiae* cytochrome b_2 with a 19-residue deletion (amino acids 47–65) that inactivates the intermembrane space sorting signal and leads to complete transport of the preproteins into the matrix, and at the C-terminus the entire mouse dihydrofolate reductase (DHFR; 187 residues) connected by an eight-residue linker or two-residue linker, respectively. $b_2(220)_{\Delta 19}$-DHFR contains the first 140 amino acid residues of mature cytochrome b_2, including the non-covalent haem-binding (HB) domain within the first 99 residues of the mature part. $b_2(167)_{\Delta 19}$-DHFR does not contain the complete haem-binding domain and thus cannot form the tightly folded HB-domain (Glick *et al.*, 1993; Voos *et al.*, 1993). The hybrid proteins are cleaved to the intermediate-sized form by the matrix-localized processing peptidase (MPP) between residues 31 and 32; an additional proteolytic processing can occur in the matrix, probably performed by the mitochondrial intermediate peptidase, yielding a slightly smaller second intermediate form, visible in Figures 1B and 3B (Schwarz *et al.*, 1993; Voos *et al.*, 1994; Wagner *et al.*, 1994; Laloraya *et al.*, 1995). Due to inactivation of the intermembrane space sorting signal, the preproteins are not processed to the mature-sized form. The preproteins $b_2(167)_{\Delta 19}$-DHFR (**B**) and $b_2(220)_{\Delta 19}$-DHFR (**C**) were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine and incubated with isolated *S.cerevisiae* mitochondria (40 μg of protein in a total volume of 100 μl) at 25°C in the presence of 10 μM haemin for the times indicated. The mitochondria were preincubated at 37°C for 15 min to induce the mutant phenotype (the *ssc1-2* phenotype is stable at 25°C or 0°C for at least 1 h). The import reactions were stopped by cooling the samples on ice and addition of 1 μM valinomycin to dissipate the membrane potential. The samples were divided, and one half was treated with proteinase K (Prot. K; 40 μg/ml final concentration). After reisolation and separation by SDS–PAGE, the imported proteins were analysed by a storage phosphor imaging system. p, i, precursor protein and intermediate-sized protein after cleavage of the first part of the presequence. WT, wild-type mitochondria (○); *ssc1–2*, mitochondria from the mtHsp70 mutant *ssc1-2* (●).

protected location (Figure 3B, lanes 6 and 8) of $b_2(167)_{\Delta 19}$-DHFR were significantly reduced with *ssc1-3′* mitochondria compared with wild-type mitochondria (Figure 3B, lanes 1, 3, 5 and 7). Processing and import of $b_2(220)_{\Delta 19}$-DHFR were nearly completely blocked (Figure 3C, lanes 2, 4, 6 and 8). We conclude that the *ssc1-3′* mutation caused a reduction of the translocase function and a block of the unfoldase function of mtHsp70.

To exclude that the import defect was indirectly caused by a reduction of the membrane potential $\Delta\psi$ of *ssc1-3′* mitochondria, we assessed the membrane potential by use of the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] (Sims *et al.*, 1974; Eilers *et al.*, 1987; Gärtner *et al.*, 1995b). Both wild-type and *ssc1-3′* mitochondria led to a comparable fluorescence decrease and subsequent increase after adding the potassium ionophore valinomycin and blocking the respiratory chain by potassium cyanide (Figure 3D), indicating that *ssc1-3′* mitochondria were not impaired in generation of a $\Delta\psi$.

Ssc1-3p′ binds substrate proteins, but does not bind ATP

To further characterize the properties of *ssc1-3′* mitochondria, we investigated the interaction of Ssc1-3p′ with additional binding partners besides Tim44: substrate proteins and nucleotides.

Ssc1-3p′ was able to bind both preproteins $b_2(167)_{\Delta 19}$-DHFR and $b_2(220)_{\Delta 19}$-DHFR when they were accumulated at the mitochondria in the presence of a membrane potential, followed by lysis of the mitochondria with non-ionic detergent and co-immunoprecipitation with antibodies directed against mtHsp70 (Figure 4A, column 3; Figure 4B, column 3). The calculation of the binding efficiency of Ssc1-3p′ in comparison with wild-type mtHsp70 shown in Figure 4A and B had to take into account the considerably lower amount of preprotein accumulated at *ssc1-3′* mitochondria (Figure 3B and C). Therefore we investigated in addition whether solubilized Ssc1-3p′ was able to bind to substrate proteins. A permanently unfolded artificial substrate, reduced carboxymethylated α-lactalbumin (RCMLA), was covalently coupled to CNBr-activated Sepharose and incubated with lysed mitochondria. Ssc1-3p′ was able to bind to the RCMLA–Sepharose with an efficiency of ~75% compared with wild-type mtHsp70 (Figure 4C, lanes 3 and 4, and figure legend). The selectivity of binding was demonstrated by the lack of binding of Ssc1-3p′ to the folded protein lactalbumin (Figure 4C, lane 6).

Since the *ssc1-3* mutation caused an amino acid exchange in the ATPase domain of Hsp70, we wondered whether the mutant protein was still able to bind to ATP or ADP. Mitochondria were lysed with non-ionic detergent and incubated with ATP-, ADP- or AMP-agarose. Ssc1-3p′ did not bind to any of the nucleotides (Figure 5A,

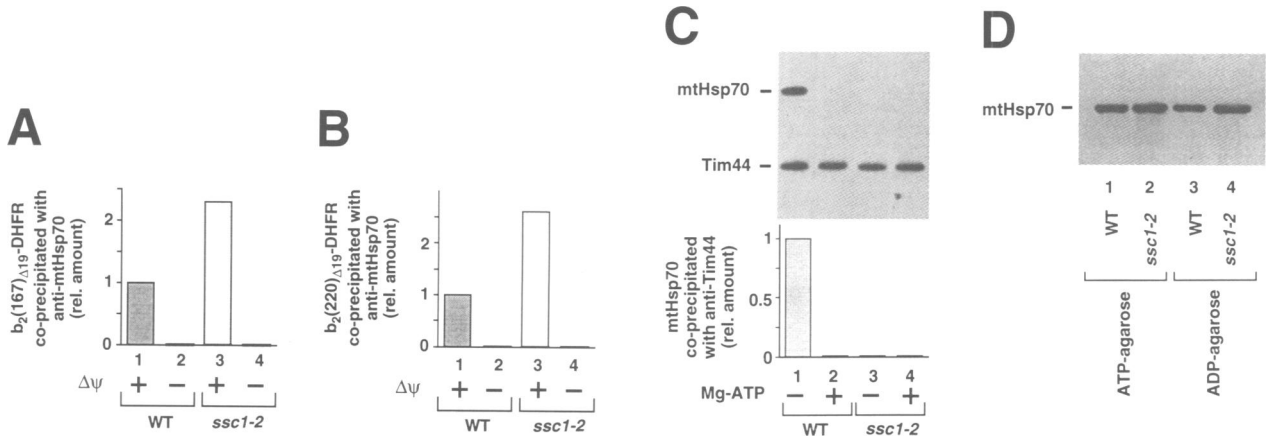

Fig. 2. Ssc1-2p efficiently binds $b_2(167)_{\Delta 19}$-DHFR and $b_2(220)_{\Delta 19}$-DHFR, but not Tim44. (**A** and **B**) Co-immunoprecipitation of preproteins by antibodies against mtHsp70. Radiolabelled precursor proteins $b_2(167)_{\Delta 19}$-DHFR and $b_2(220)_{\Delta 19}$-DHFR were synthesized in reticulocyte lysate and imported into mitochondria isolated from the wild-type strain (WT) and from the mutant strain *ssc1-2* as described in the legend to Figure 1. Where indicated, the samples received 1 μM valinomycin to dissipate the membrane potential Δψ and block protein import. The mitochondria were reisolated and subsequently lysed in 0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5. The imported proteins were co-immunoprecipitated in the absence of ATP by antibodies against mtHsp70, prebound to protein A–Sepharose, and analysed by SDS–PAGE and digital autoradiography. The amount of preprotein co-precipitated from wild-type mitochondria was set to 1. The amount of preprotein co-precipitated from *ssc1-2* mitochondria was corrected for the different amounts of preprotein accumulated with wild-type and *ssc1-2* mitochondria in the presence of a Δψ (as shown in Figure 1B and C). (**C**) Co-immunoprecipitation of mtHsp70 by antibodies against Tim44. Isolated mitochondria from the wild-type and from *the ssc1-2* strain were preincubated at 37°C and lysed in the same buffer as described in (A). mtHsp70 was co-immunoprecipitated by affinity-purified antibodies from a polyclonal antiserum raised against Tim44. The antibodies were prebound to protein A–Sepharose and the precipitation was performed in the presence of 1 mM ATP/5 mM Mg-acetate (+ATP) or 5 mM EDTA (–ATP) at 4°C. Precipitates were analysed by SDS–PAGE and immunoblotting. The ATP-binding properties of wild-type mtHsp70 and Ssc1-2p were comparable (von Ahsen *et al.*, 1995); the curves of ATP-induced release of the co-chaperone Mge1p from mtHsp70 (5 min at 4°C) were indistinguishable between wild-type mtHsp70 and Ssc1-2p: the half-maximal release occurred at 0.1 μM ATP in both cases.

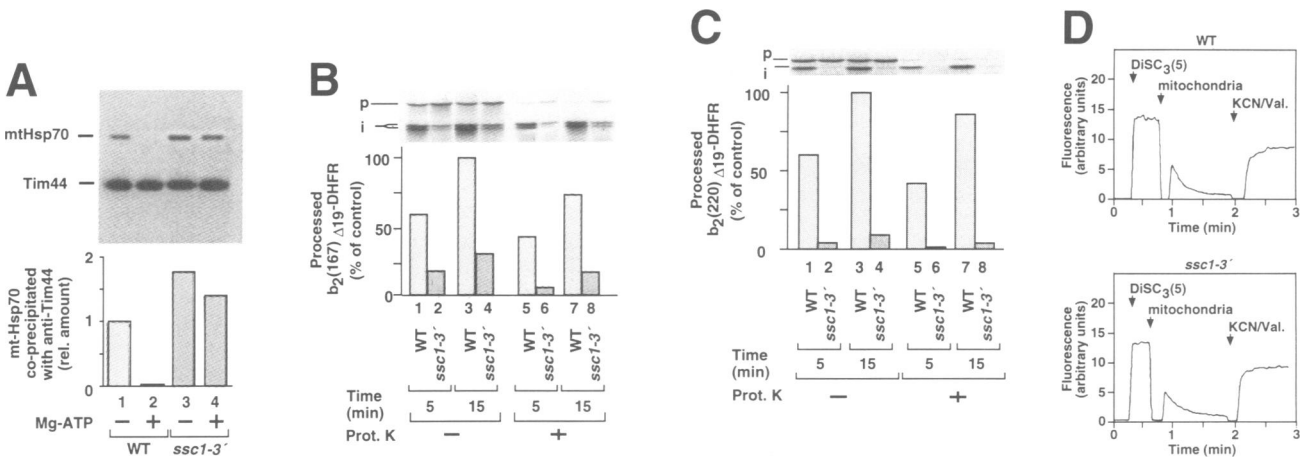

Fig. 3. Ssc1-3p′ binds to Tim44, but *ssc1-3′* mitochondria are defective in import of preproteins. (**A**) Co-immunoprecipitation of mtHsp70 by antibodies directed against Tim44. Isolated wild-type or *ssc1-3′* mitochondria were lysed in 0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5. mtHsp70 was co-immunoprecipitated by affinity-purified antibodies from a polyclonal antiserum raised against Tim44. The antibodies were prebound to protein A–Sepharose and the precipitation was performed in the presence of 1 mM ATP/5 mM Mg-acetate (+ATP) or 5 mM EDTA (–ATP). Precipitates were analysed by SDS–PAGE and immunoblotting. (**B**) Import of $b_2(167)_{\Delta 19}$-DHFR into isolated mitochondria. Radiolabelled $b_2(167)_{\Delta 19}$-DHFR was synthesized in reticulocyte lysate and imported into wild-type (WT) and *ssc1-3′* mitochondria (*ssc1-3′*) at 25°C for the times indicated. The import reaction was stopped by addition of 1 μM valinomycin, the samples were divided into halves, and one half was treated with 150 μg/ml proteinase K (Prot. K). Mitochondria were reisolated and analysed by SDS–PAGE and autoradiography. The amount of processed protein in wild-type mitochondria after 15 min was set to 100% (control). (**C**) Import of $b_2(220)_{\Delta 19}$-DHFR. The import assay was performed as described for (B). (**D**) *ssc1-3′* mitochondria can generate a strong membrane potential. The membrane potential of isolated mitochondria from the wild-type strain and from the strain *ssc1-3′* was assessed at 25°C by use of the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] as described in Gärtner *et al.* (1995b). The membrane potential is indicated by the difference in fluorescence before and after addition of potassium cyanide/valinomycin (KCN/Val.).

lanes 4–6). In order to study the nucleotide binding properties in an independent approach, mitochondria were loaded with [α-^{32}P]ATP. Both wild-type and *ssc1-3′* mitochondria accumulated comparable amounts of labelled nucleotides (Figure 5B, lanes 1 and 2). After lysis of the mitochondria with detergent, the interaction of labelled nucleotides with mtHsp70 was analysed by immunoprecipitation with anti-mtHsp70 antibodies. Ssc1-3p′ did

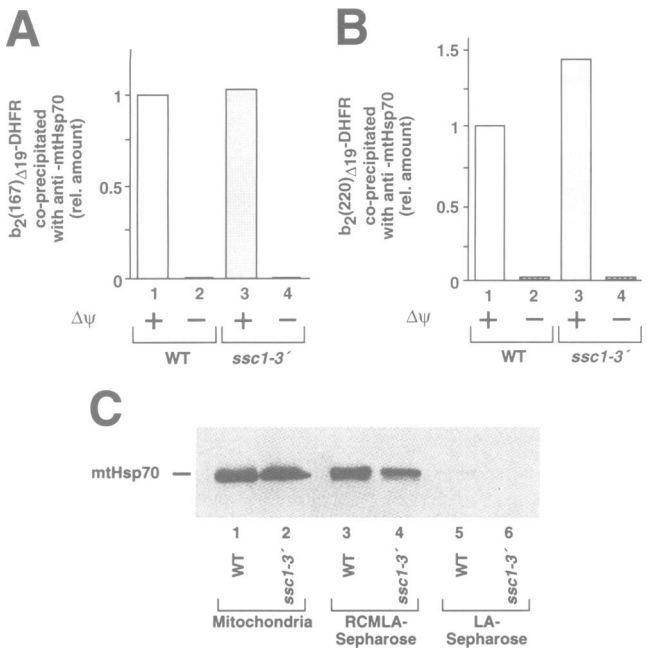

Fig. 4. Ssc1-3p′ can bind polypeptides. (**A**) Binding of Ssc1-3p′ to $b_2(167)_{\Delta 19}$-DHFR. Radiolabelled $b_2(167)_{\Delta 19}$-DHFR was imported into isolated wild-type and *ssc1-3′* mitochondria. The import reaction was stopped by addition of valinomycin, the mitochondria were reisolated and lysed in 0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl pH 7.5. Imported $b_2(167)_{\Delta 19}$-DHFR was co-immunoprecipitated by antibodies directed against mtHsp70. After separation by SDS–PAGE, precipitated $b_2(167)_{\Delta 19}$-DHFR was quantified by digital autoradiography as described in the legend to Figure 2A and B (co-precipitated amount from wild-type set to 1; amount from *ssc1-3′* corrected for the different amounts of preprotein accumulated at wild-type and *ssc1-3′* mitochondria, compare with Figure 3B and C). (**B**) Binding of Ssc1-3p′ to $b_2(220)_{\Delta 19}$-DHFR. The experiment was performed as described in (A). (**C**) Binding of Ssc1-3p′ to carboxy-methylated α-lactalbumin (RCMLA). Mitochondria were lysed in the same lysis buffer as described in (A) with the addition of 1% BSA and shaken for 1 h at 37°C with RCMLA or native lactalbumin (LA) covalently coupled to CNBr-activated Sepharose. The Sepharose was washed three times with lysis buffer, and bound proteins were eluted with sample buffer and applied to SDS–PAGE. As a standard, 20% of the mitochondrial protein applied to the Sepharose was loaded on the gel (mitochondria). Quantitation revealed that the amount of mtHsp70 from *ssc1-3′* mitochondria bound to RCMLA–Sepharose represented ~75% of the amount of wild-type mtHsp70 bound to RCMLA–Sepharose.

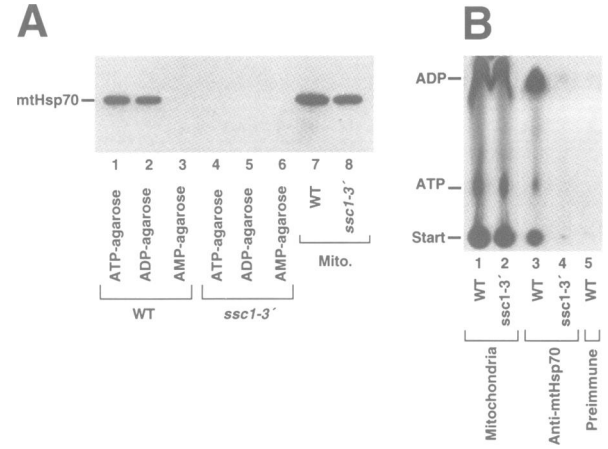

Fig. 5. Ssc1-3p′ does not bind nucleotides. (**A**) Binding of mtHsp70 to agarose-bound nucleotides. Isolated wild-type or *ssc1-3′* mitochondria were ATP-depleted by preincubation with apyrase and oligomycin. The mitochondria were then lysed in 0.3% Triton X-100, 1% BSA, 300 mM KCl, 5 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.5, and gently shaken with ATP-, ADP- or AMP-agarose for 30 min at 4°C. After three washing cycles with lysis buffer, bound mtHsp70 was eluted by sample buffer and subjected to SDS–PAGE and Western blotting. 50% of the amount of mitochondria added to agarose was directly analysed by SDS–PAGE (Mito., samples 7 and 8). (**B**) Thin-layer chromatography of ^{32}P-labelled nucleotides bound to mtHsp70. Isolated mitochondria were incubated with [α-^{32}P]ATP and subsequently lysed in 0.3% Triton X-100, 5 mM glycerol, 150 mM KCl, 5 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.4. Immunoprecipitations were performed with antibodies from preimmune serum and from a serum directed against mtHsp70. Co-precipitated nucleotides were analysed by thin-layer chromatography. 10% of the lysed mitochondria were subjected directly to chromatography (samples 1 and 2). Labelled nucleotides were visualized by autoradiography.

not carry labelled nucleotides (Figure 5B, lane 4) in contrast to wild-type mtHsp70 (Figure 5B, lane 3). The specificity of the immunoprecipitation was confirmed by the lack of precipitation of labelled nucleotides from wild-type mitochondria by preimmune antibodies (Figure 5B, lane 5). We conclude that Ssc1-3p′ is unable to bind nucleotides.

The defect in binding of nucleotides explains why the interaction of Ssc1-3p′ with Tim44 was not dissociated by addition of Mg-ATP (Figure 3A, lane 4), whereas the interaction between wild-type mtHsp70 and Tim44 was completely dissociated by Mg-ATP (Figure 3A, lane 2) (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). We conclude that Ssc1-3p′ can bind substrate proteins and Tim44, but is defective in nucleotide binding, leading to a permanent binding of the mutant Hsp70 to Tim44.

Ssc1-3p′ lacks protease-resistant domains, indicating a conformational alteration

Does the defect in nucleotide binding lead to structural changes in the mutant mtHsp70? Wild-type and *ssc1-3′* mutant mitochondria were lysed in non-ionic detergent, and a time-course of digestion with trypsin was performed. In the presence of Mg-ATP, wild-type mtHsp70 was digested to a 56 kDa fragment (f_1) and a 45 kDa fragment (f_2) that were detectable by immunodecoration with antibodies directed against Hsp70 (Figure 6, lanes 3–5). The 45 kDa fragment was recognized by an antibody directed against the N-terminus of Hsp70, demonstrating that it represented the N-terminal ATPase domain (von Ahsen *et al.*, 1995) [similar to the ATPase domain of the homologous Hsp70s from the cytosol, endoplasmic reticulum and *Escherichia coli* (Chappell *et al.*, 1987; Kassenbrock and Kelly, 1989; Liberek *et al.*, 1991; Buchberger *et al.*, 1995; Freeman *et al.*, 1995)]. The size of the 56 kDa fragment indicates that it comprises parts of both the ATPase domain and the C-terminal portion of mtHsp70.

In the presence of Mg-ADP the full-length mtHsp70 revealed a significantly higher stability than in the presence of Mg-ATP (Figure 6, compare the quantifications of samples 8–10 with that of samples 3–5). Moreover, in the presence of Mg-ADP the 56 kDa fragment (f_1) was not observed (Figure 6, lanes 8–10). The quantification of the time-course of tryptic digest indicates that the absence of the 56 kDa fragment in the ADP-containing samples is not simply caused by the slower degradation of full-length

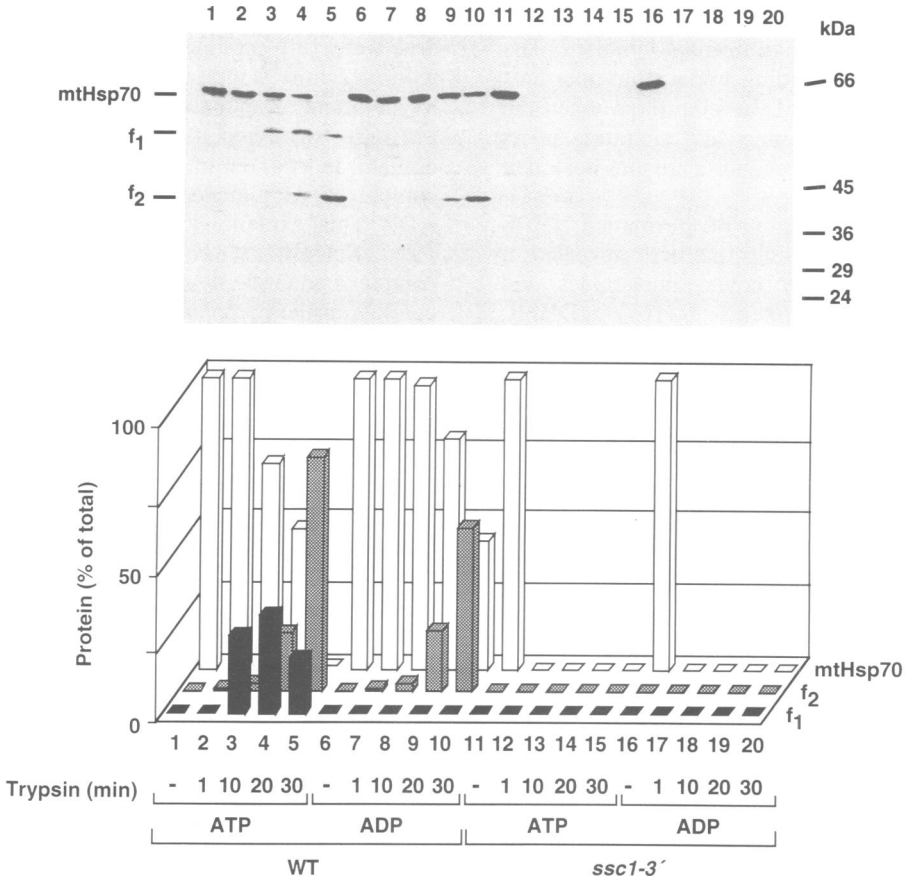

Fig. 6. Ssc1-3p′ is in a loosely folded conformation as assessed by proteolytic fragmentation. The pattern of tryptic fragments (f_1 and f_2) of mtHsp70 was compared for *ssc1-3′* mitochondria and wild-type mitochondria in the presence of ATP and ADP. Isolated mitochondria were pretreated with apyrase and oligomycin to deplete endogenous ATP. The mitochondria were lysed in 0.1% Triton X-100, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS–KOH, pH 7.2, with addition of 5 mM ATP or 5 mM ADP. The lysates were treated with trypsin (50 μg/ml) at room temperature for the times indicated. After addition of PMSF and TCA precipitation, mtHsp70 and the degradation products were analysed by SDS–PAGE and immunoblotting with antibodies directed against mtHsp70.

mtHsp70; the degree of digestion of mtHsp70 after 10–20 min in the presence of ATP is in a similar range as that after 20–30 min in the presence of ADP (Figure 6, samples 3 and 4 compared with samples 9 and 10), and the 56 kDa fragment is clearly detectable in the ATP-containing samples after 10–20 min (Figure 6, samples 3 and 4). The differential stability and fragmentation of wild-type mtHsp70 in the presence of ATP and ADP indicates a nucleotide-dependent conformational change of mtHsp70.

In strong contrast, Ssc1-3p′ was rapidly digested by trypsin without formation of detectable fragments (Figure 6, right half). The high protease susceptibility was found independently if ATP or ADP were present (Figure 6, lanes 12–15 versus 17–20). These results indicate that the mutant mtHsp70 has a profound structural alteration.

Discussion

We have analysed the relevance of the mtHsp70–Tim44 interaction for translocation of preproteins across the mitochondrial membranes. This provides the biochemical basis to explain the different functions of mtHsp70 (unfoldase and translocase) in membrane translocation of preproteins and allows assessment of the various hypotheses that were made on the molecular mechanism of mtHsp70 action.

Mitochondria with a defective complex formation Hsp70–Tim44 are fully competent in the translocase function, but impaired in the unfoldase function of mtHsp70

To distinguish between the translocase and unfoldase function of mtHsp70, we employed two preproteins which differed only in the middle portion, but contained the identical N-terminal matrix targeting signal and the identical C-terminal domain. $b_2(220)_{\Delta 19}$-DHFR contained a longer portion of mature cytochrome b_2 than $b_2(167)_{\Delta 19}$-DHFR and was thus able to form a tightly folded domain, the non-covalent haem-binding domain (Xia and Mathews, 1990; Glick *et al.*, 1993) in its middle portion (amino acids 81–179 of cytochrome b_2). Previous work, using urea-denatured preproteins and preproteins targeted to the intermembrane space, had suggested that *ssc1-2* mitochondria were not impaired in the translocase function of mtHsp70 (Gambill *et al.*, 1993; Voos *et al.*, 1993). Here we show that $b_2(167)_{\Delta 19}$-DHFR was imported into *ssc1-2* mitochondria with the identical kinetics and efficiency as into wild-type mitochondria, directly demonstrating that *ssc1-2* mitochondria are fully competent in the translocase

function. The import of $b_2(220)_{\Delta 19}$-DHFR into *ssc1-2* mitochondria, however, was significantly inhibited. The presence of the folded haem-binding domain thus interferes with membrane translocation of the preprotein when the mtHsp70 carries the *ssc1-2* amino acid alteration in its C-terminal domain. *ssc1-2* mitochondria are thus defective in the unfoldase function.

A comparison of the interaction of the mutant Hsp70 (Ssc1-2p) with different binding partners revealed a remarkable situation. Ssc1-2p bound nucleotides and substrate proteins, the preproteins $b_2(167)_{\Delta 19}$-DHFR, $b_2(220)_{\Delta 19}$-DHFR and other polypeptides (Voos *et al.*, 1994; von Ahsen *et al.*, 1995), at least as efficiently as wild-type mtHsp70. The binding of Ssc1-2p to polypeptides was even of higher efficiency and longer than that of wild-type mtHsp70. Binding of Ssc1-2p to Tim44, however, could not be observed. It cannot be fully excluded that Ssc1-2p binds to Tim44 very transiently, yet this binding would have to occur with very low affinity since no complex between Ssc1-2p and Tim44 was detectable after immunoprecipitation, whereas the binding of wild-type mtHsp70 to Tim44 was stable even for five and more washes of the immunoprecipitates (in the absence of nucleotides). In view of the sensitivity of our binding assay, the yield of complex formation between Ssc1-2p and Tim44 is at least 500-fold lower than the yield of complex formation between wild-type mtHsp70 and Tim44. We conclude that a detectable complex formation between mtHsp70 and Tim44 is not required for the translocase function of mtHsp70, i.e. the import of loosely folded precursor polypeptides, including domains such as DHFR which need only low energy input for unfolding (Pace, 1990; Voos *et al.*, 1993).

Since Ssc1-2p is not defective in binding to precursor polypeptides or nucleotides, the impaired binding to Tim44 seems to be of critical importance to explain the defect of *ssc1-2* mitochondria in promoting unfolding of precursor polypeptides.

Mitochondria with permanently stable, nucleotide-resistant mtHsp70–Tim44 complexes are impaired in the translocase function and blocked in the unfoldase function

The results with *ssc1-2* mitochondria suggest that binding of mtHsp70 to Tim44 is required to promote unfolding of preproteins during import. We asked if such binding was sufficient to drive protein import and found that the mutant mtHsp70 Ssc1-3p′ showed interesting differences to Ssc1-2p. Ssc1-3p′ efficiently bound Tim44, and it efficiently bound various substrate proteins (the b_2-fusion proteins and artificial substrate), suggesting that it should be a competent mediator of protein import. However, we found that *ssc1-3′* mitochondria showed considerable import defects. The import of $b_2(167)_{\Delta 19}$-DHFR into *ssc1-3′* mitochondria was strongly reduced, indicating an impairment of the translocase function. The import of $b_2(220)_{\Delta 19}$-DHFR was almost completely inhibited; that is, the unfoldase function was blocked.

How can this phenotype of the *ssc1-3′* mutation be explained? A trivial reason would be an indirect effect of the *ssc1-3′* mutation on the ability of mitochondria to generate a membrane potential $\Delta\psi$ across the inner membrane. This possibility could be excluded as measurements with a fluorescent dye demonstrated that *ssc1-3′* mitochondria were fully competent for generation of a $\Delta\psi$-like wild-type mitochondria. Since the *ssc1-3* mutation leads to an amino acid exchange in the ATPase domain of mtHsp70, we asked if Ssc1-3p′ had a defective ATPase domain. Indeed, the mutant mtHsp70 was unable to form complexes with nucleotides, it neither bound ATP nor ADP. This explains the observation (Schneider *et al.*, 1994; Schmitt *et al.*, 1995) that the Ssc1-3p′–Tim44 complex cannot be dissociated by addition of Mg-ATP in contrast to the complex between wild-type mtHsp70 and Tim44 (Figure 3).

The defect of the ATPase domain is also evidenced by lack of formation of a protease-resistant 45 kDa domain of Ssc1-3p′. Wild-type mtHsp70 undergoes a nucleotide-dependent conformational change, analysed by the differential proteolytic fragmentation in the presence of ATP and ADP. Ssc1-3p′, however, is completely degraded by added protease, demonstrating a profound influence of the mutation on the domain folding of mtHsp70. The exchange of Gly56 by a serine (Gambill *et al.*, 1993) thus leads to a defective ATPase domain, the inability to bind nucleotides and lack of a nucleotide-dependent conformational change of Ssc1-3p′, causing a permanent binding of Ssc1-3p′ molecules to Tim44.

The strong import defect of *ssc1-3′* mitochondria and the amount of Ssc1-3p′ found in complexes with Tim44 suggest that a considerable fraction of inner membrane import sites are blocked by irreversibly bound mtHsp70. The residual import of loosely folded preproteins (residual translocase function) probably occurs via import sites that are not fully blocked by Ssc1-3p′. Since the results with *ssc1-2* mitochondria raised the possibility that soluble mtHsp70 may be sufficient to promote import of loosely folded polypeptides as long as it retains a binding activity for preproteins, soluble Ssc1-3p′ may promote import of some precursor polypeptides [wild-type and mutant mtHsp70s are ~5-fold more abundant than Tim44, and the complexes of mtHsp70–Tim44 are of a molar ratio of 1:1 (Gambill *et al.*, 1993; Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994)]. *ssc1-3′* mitochondria are virtually blocked in the unfoldase function; the few percent of imported $b_2(220)_{\Delta 19}$-DHFR are explained best by a small fraction of not completely folded haem-binding domains (Glick *et al.*, 1993; Voos *et al.*, 1993).

This suggests that neither soluble mtHsp70 nor permanently Tim44-bound mtHsp70 are able to promote unfolding of precursor polypeptides during membrane translocation. The unfoldase function apparently requires a dynamic interaction of mtHsp70 with Tim44.

Conclusions and hypotheses of mtHsp70 action in protein import

Conclusions. (i) Formation of a complex between mtHsp70 and Tim44 is not required for import of loosely folded preproteins (translocase function), but needed to promote unfolding of polypeptides by mtHsp70 (unfoldase function). (ii) The complex formation mtHsp70–Tim44 is not sufficient for the unfoldase function of mtHsp70, but the dynamics of the interaction is of critical importance. A nucleotide-induced conformational change of mtHsp70 and release from Tim44 are involved in the unfoldase function.

Various hypotheses have been proposed of how mtHsp70 acts to drive the import of precursor polypeptides across the inner membrane, ranging from a more passive role (trapping, Brownian ratchet) to a more active role (pulling, generation of force), yet experimental evidence has remained scarce (summarized in Wickner, 1994). The *in organello* and *in vitro* analysis of yeast mutants of mtHsp70 that alter the interaction with Tim44, but do not destroy the binding of preproteins, now permit us to relate hypotheses to experiments and lead to a unifying conclusion.

Brownian ratchet (trapping) (Simon et al., 1992). The precursor polypeptides slide back and forth in the import channel (Ungermann *et al.*, 1994). mtHsp70 molecules bind to unfolded segments of the polypeptide emerging on the matrix side and thereby trap it in the matrix. In this model, the bidirectional movement of the polypeptide driven by Brownian motion is rendered unidirectional by binding to mtHsp70 (Schneider *et al.*, 1994; Stuart *et al.*, 1994a). The energy of ATP is then needed to release the polypeptide from mtHsp70s in the matrix. An import of loosely folded polypeptides driven by soluble mtHsp70 (not bound to Tim44; Ssc1-2p and soluble Ssc1-3p′) is consistent with this model. It is important to note that the unfoldase function, i.e. action of mtHsp70 (bound to the precursor polypeptide) against conformational restrictions, is difficult to reconcile with the trapping model, and as demonstrated in this report cannot be efficiently performed by soluble mtHsp70.

Pulling by mtHsp70. mtHsp70 generates an inward-directed force on the precursor polypeptide that supports its translocation and unfolding (Kang *et al.*, 1990; Scherer *et al.*, 1990; Pfanner *et al.*, 1994; Glick, 1995; Pfanner and Meijer, 1995). An essential prerequisite is that the generated force has a direction, i.e. this cannot be accomplished by soluble mtHsp70. Horst *et al.* (1996) showed that mtHsp70 can bind simultaneously to Tim44 and a peptide substrate. Together with our observation of an opposite effect of the *ssc1-2* mutation on binding of Tim44 and various substrates, this demonstrates that the binding sites of mtHsp70 for Tim44 and substrates are different. While bound to Tim44, mtHsp70 is fixed, and a force generated onto a polypeptide by conformational changes of mtHsp70 will be directional. mtHsp70 with the bound preprotein is then released from Tim44 and a new mtHsp70 can bind to Tim44, bind another segment of the preprotein and perform a pulling action by a conformational change. ATP is needed to promote the conformational change and release of mtHsp70. We demonstrate here that interaction of mtHsp70 with Tim44 is required for, but not sufficient for the unfoldase function. mtHsp70 has to undergo a nucleotide-dependent conformational change and release from Tim44 to promote unfolding of polypeptides.

We propose that the translocase function of mtHsp70 can occur by a trapping-like mechanism (soluble mtHsp70), whereas the unfoldase function requires a dynamic interaction of mtHsp70 with Tim44 (pulling mechanism). Thus, both mechanisms, trapping and pulling by mtHsp70, can operate in the import of preproteins and are, in contrast to previous assumptions, not controversial or mutually exclusive.

An alternative proposal is conceivable under the assumption that the mutant mtHsp70 Ssc1-2p very transiently interacts with Tim44 in intact mitochondria such that the interaction is too weak to be detected by co-immunoprecipitation in detergent-containing buffer. Such an interaction between mtHsp70 and Tim44 could lead to the generation of a weak force that is just sufficient to promote the import of loosely folded preproteins, but not of preproteins with stably folded domains. The translocase function could then be driven by a weak pulling force, whereas the unfoldase function requires a strong pulling force. Moreover, in wild-type mitochondria, mtHsp70 seems to be bound to Tim44 and a membrane-spanning preprotein at the same time. Under these conditions, a trapping mechanism can hardly drive translocation, suggesting that generation of a pulling force is the initial and probably major driving force for membrane translocation of preproteins.

Interestingly, trapping or pulling mechanisms are also discussed for the role of the endoplasmic reticulum Hsp70 (BiP, Kar2p) in protein translocation; BiP transiently interacts with Sec63p of the endoplasmic reticulum membrane (Simon *et al.*, 1992; Brodsky and Schekman, 1993; Nicchitta and Blobel, 1993; Brodsky *et al.*, 1995; Panzner *et al.*, 1995). The characterization of a dynamic membrane interaction of Hsp70s and its effect on import of polypeptides with different folding states may thus be of general importance to differentiate distinct functions of Hsp70s in membrane translocation of preproteins.

Materials and methods

Construction of cytochrome b_2 hybrid proteins

$b_2(167)_{\Delta 19}$-DHFR was described by Koll *et al.* (1992). For construction of a plasmid encoding $b2(220)_{\Delta 19}$-DHFR, a plasmid encoding $b_2(220)$-DHFR (Koll *et al.*, 1992) was used; the *Cla*I–*Hin*dIII fragment of this construct, carrying the coding region for cytochrome b_2 residues 151–220 and entire mouse DHFR, was used to replace the *Cla*I–*Hin*dIII fragment of $b_2(167)_{\Delta 19}$-DHFR, yielding $b_2(220)_{\Delta 19}$-DHFR.

Import of preproteins into isolated mitochondria

Mitochondria were isolated from three *S.cerevisiae* strains: the wild-type strain PK82 (MATα, *his4-713*, *lys2*, *ura3-52*, *leu2-3,112*, *Δtrp1*), the *ssc1-2* strain PK81 [MATα, *ade2-101*, *lys2*, *ura3-52*, *leu2-3,112*, *Δtrp1*, *ssc1-2(LEU2)*], and the *ssc1-3* strain PK83 [MATα, *ade2-101*, *lys2 ura3-52*, *leu2-3,112*, *Δtrp1*, *ssc1-3(LEU2)*] (Gambill *et al.*, 1993). The cells were grown in YPG medium (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol), and mitochondria were prepared according to published procedures (Daum *et al.*, 1982; Gambill *et al.*, 1993). Preproteins were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine after *in vitro* transcription by SP6 RNA polymerase (Stratagene, Amersham). Import reactions were performed by incubation of 2 μl reticulocyte lysate (containing the preprotein) with isolated mitochondria (40 μg protein) in import buffer [3% (w/v) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.2] at 25°C. Mitochondria from the mutant *ssc1-2* were preincubated in import buffer for 15 min at 37°C. The phenotype was stable in subsequent import reactions or co-immunoprecipitation reactions at lower temperature (25°C, 4°C). Proteinase K treatment, reisolation of mitochondria by centrifugation, and separation of imported proteins by SDS–PAGE were performed as published previously (Söllner *et al.*, 1991). Autoradiographs were obtained and quantified using a storage phosphorimaging system (Molecular Dynamics, Inc.). The membrane potential of isolated mitochondria was determined using the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] as described by Gärtner *et al.* (1995b). Quantifications of import experiments or co-immunoprecipitation experiments were done by digital autoradiography or densitometry (typically from 3–5 independent experiments with standard errors of the means below 8% of the reported values).

Binding of mtHsp70 to ATP-, ADP- and AMP-agarose

0.5 mg ADP- or ATP-agarose (Sigma) and 50 μg mitochondrial protein were used per sample. The mitochondria were preincubated in import buffer (3% BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS–KOH, pH 7.2) together with 20 μM oligomycin and 5 U/ml apyrase for 15 min at 0°C. After reisolation and washing in import buffer, the mitochondria were lysed in 0.3% Triton X-100, 300 mM KCl, 5 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.5 and shaken for 15 min at 4°C. After a clarifying spin for 10 min at 16 000 *g*, the supernatant was incubated with ATP-, ADP- or AMP-agarose by end-over-end-rotation for 30 min at 4°C. After three washing cycles in lysis buffer, the agarose pellets were boiled in sample buffer and applied to SDS–PAGE.

Co-immunoprecipitation of Tim44–mtHsp70 complexes

A polyclonal rabbit antiserum was raised against a fragment of Tim44 (residues 68–345; Blom *et al.*, 1993). Antibodies were affinity purified on a Tim44–Sepharose column and covalently coupled to protein A–Sepharose. Affinity-purified antibodies from 1 ml serum were bound to 0.5 ml (wet volume) protein A–Sepharose in 3.3 ml 100 mM potassium phosphate buffer, pH 7.5, by shaking for 1 h at room temperature. After two washing-steps with 0.1 M sodium borate buffer, pH 9, antibodies and protein A–Sepharose were cross-linked by addition of solid dimethylpimelimidate (DMP) at a final concentration of 5 mM in borate buffer and incubation for 30 min. Cross-linking was stopped by washing and incubation in 1 M Tris–HCl, pH 7.5, for 2 h. Both reactions were performed at room temperature. The Sepharose matrix with coupled antibodies was stored in 10 mM Tris–HCl, pH 7.5, 0.9% (w/v) NaCl at 4°C. For co-immunoprecipitations, mitochondria were preincubated in import buffer together with 20 μM oligomycin and 5 U/ml apyrase for 15 min at 0°C. After reisolation and washing, the mitochondria were resuspended in lysis buffer (0.1% Triton X-100, 250 mM sucrose, 80 mM KCl, 5 mM EDTA, 20 mM MOPS–KOH, pH 7.2) shaken for 5 min at 4°C, then subjected to a clarifying spin of 10 min at 16000 *g* and applied to anti-Tim44 protein A–Sepharose. The samples were incubated at 4°C by end-over-end rotation for 1 h, washed, and analysed by SDS–PAGE and Western-blotting. Binding of imported proteins to mtHsp70 was assayed in 0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.5. After a clarifying spin, the extract was incubated for 1 h at 4°C by end-over-end shaking with antibodies prebound to protein A–Sepharose. The samples were washed three times with lysis buffer and finally with 10 mM Tris–HCl, pH 7.0.

Binding of mtHsp70 to RCMLA–Sepharose

For coupling of reduced carboxy-methylated α-lactalbumin (RCMLA; Sigma) to Sepharose, 0.25 g CNBr-activated Sepharose 4B (Pharmacia) was first washed in 1 mM HCl and subsequently in 4 ml 500 mM NaCl, 100 mM $NaHCO_3$, pH 8.3. 10 mg RCMLA were added and coupled for 1 h at 25°C. The Sepharose was washed and blocked by incubation in 100 mM Tris–HCl, pH 8.0, for 2 h. The preparation was washed six times with 500 mM NaCl, 0.1 M sodium acetate, pH 4.0, or 500 mM NaCl, 0.1 M Tris–HCl, pH 8.0, in alternating cycles. The preparation was stored in 100 mM Tris–HCl, pH 8.0.

For binding of mtHsp70 to RCMLA, 50 μg mitochondrial protein and 50 μg RCMLA (Sepharose-coupled) were used per lane. Mitochondria were preincubated with 20 μM oligomycin and 5 U/ml apyrase for 15 min at 0°C. After washing, the mitochondria were lysed in 0.3% Triton-X-100, 1% BSA, 300 mM KCl, 5 mM $MgCl_2$, 50 mM sodium phosphate, pH 7.5. After a clarifying spin, RCMLA–Sepharose and mitochondrial lysate were mixed for 1 h at 37°C. After three washing steps, bound proteins were eluted from the RCMLA–Sepharose by sample buffer and analysed by SDS–PAGE.

Thin-layer chromatography of nucleotides

Mitochondria were incubated with 250 μCi/ml [α-^{32}P]ATP for 30 min at 25°C in the presence of 8 μM antimycin A, 20 μM oligomycin and 0.5 μM valinomycin. After washing in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2, the mitochondria were lysed in 0.3% Triton X-100, 150 mM KCl, 5 mM glycerol, 5 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.4. After a clarifying spin, the supernatants were used for immunoprecipitations by antibodies against mtHsp70, prebound to protein A–Sepharose. Bound proteins were eluted from the Sepharose by 2.5 M perchloric acid and finally neutralized by addition of 2.5 M KOH. Nucleotides were separated by thin-layer chromotography using polyethyleneimine plates 5579 (E.Merck). 0.5 M potassium phosphate, pH 4.2, was used as liquid phase.

Tryptic digest of mtHsp70

After a pretreatment with oligomycin (20 μM) and apyrase (10 U/ml) in order to deplete endogenous ATP, mitochondria were lysed in 0.1% Triton X-100, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS–KOH, pH 7.2, in the presence of different nucleotides (15 min at 4°C). After a clarifying spin of 10 min at 16 000 *g*, trypsin was added to a final concentration of 50 μg per ml. The samples were shaken at room temperature. The reaction was stopped by addition of 1 mM PMSF and the samples were precipitated by TCA. mtHsp70 and its degradation products were analysed by SDS–PAGE and Western-blotting.

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