A Dual Role for Mitochondrial Heat Shock Protein 70 in Membrane Translocation of Preproteins

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Abstract. The role of mitochondrial 70-kD heat shock protein (mt-hsp70) in protein translocation across both the outer and inner mitochondrial membranes was studied using two temperature-sensitive yeast mutants. The degree of polypeptide translocation into the matrix of mutant mitochondria was analyzed using a matrix-targeted preprotein that was cleaved twice by the processing peptidase. A short amino-terminal segment of the preprotein (40–60 amino acids) was driven into the matrix by the membrane potential, independent of hsp70 function, allowing a single cleavage of the presequence. Artificial unfolding of the preprotein allowed complete translocation into the matrix in the case where mutant mt-hsp70 had detectable binding activity. However, in the mutant mitochondria in which binding to mt-hsp70 could not be detected the mature part of the preprotein was only translocated to the intermembrane space. We propose that mt-hsp70 fulfills a dual role in membrane translocation of preproteins. (*a*) Mt-hsp70 facilitates unfolding of the polypeptide chain for translocation across the mitochondrial membranes. (*b*) Binding of mt-hsp70 to the polypeptide chain is essential for driving the completion of transport of a matrix-targeted preprotein across the inner membrane. This second role is independent of the folding state of the preprotein, thus identifying mt-hsp70 as a genuine component of the inner membrane translocation machinery. Furthermore we determined the sites of the mutations and show that both a functional ATPase domain and ATP are needed for mt-hsp70 to bind to the polypeptide chain and drive its translocation into the matrix.

HEAT shock proteins of 70 kD (hsp70s)[1] play important roles in intracellular protein transport and folding (reviewed in Lindquist and Craig, 1988; Ellis and Hemmingsen, 1989; Rothman, 1989; Gething and Sambrook, 1992; and references therein). The yeast *Saccharomyces cerevisiae* contains eight genes encoding hsp70s that are, according to their homologies, grouped into the four subfamilies: *SSA* (four genes, forming an essential subfamily), *SSB* (two genes required for normal growth), *SSC* (one essential gene), and *SSD* (one essential gene, *KAR2*) (Craig et al., 1987; Werner-Washburne et al., 1987; Rose et al., 1989). Members of the *SSB* subfamily are probably the first hsp's that are seen by a protein during biogenesis; the proteins Ssb1p and Ssb2p are associated with cytosolic ribosomes and interact with nascent polypeptide chains (Nelson et al., 1992). Members of the *SSA* subfamily seem to facilitate translocation of proteins from the cytosol to various cell organelles (Deshaies et al., 1988; Murakami et al., 1988; Zimmermann et al., 1988; Beckmann et al., 1990; Murakami and Mori, 1990; Waegemann et al., 1990; Dice, 1990; Dingwall and Laskey, 1992; Imamoto et al., 1992; Shi and Thomas, 1992) and are assumed to act by preventing misfolding or aggregation of preproteins. Ssc1p in the mitochondrial matrix (70-kD mitochondrial heat shock protein [mt-hsp70]; see references below) and Kar2p (Ssd1p, BiP) in the lumen of the endoplasmic reticulum are involved in import and folding of precursor proteins (Munro and Pelham, 1986; Hendershot et al., 1987; Kassenbrock et al., 1988; Kozutsumi, 1988; Vogel et al., 1990; Sanders et al., 1992; Brodsky et al., 1993; de Silva et al., 1993).

The analysis of mitochondrial protein import has proven to be an effective method for studying the functions of proteins involved in organellar protein translocation. The isolated mitochondria allow analysis of protein import independent of the energetics of protein synthesis; while at the same time, the matrix remains intact, closely resembling the in vivo situation. Functional analysis of the mt-hsp70 (Ssc1p) was initially carried out in such a system with a single temperature-sensitive yeast mutant (*ssc1-2*). In *ssc1-2* mitochondria, preproteins accumulated in the mitochondrial import sites after pretreatment at the nonpermissive temperature (Kang et al., 1990; Ostermann et al., 1990). The amino-terminal presequences of the preproteins were trans-

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1. *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; hsp70, 70-kD heat shock protein; mt-hsp70, mitochondrial hsp70.

located into the mitochondrial matrix and cleaved by the processing peptidase. However, at the same time, the preproteins were accessible to protease added to the isolated mitochondria, indicating that the polypeptide chains spanned both mitochondrial membranes at translocation contact sites. Artificial unfolding of a preprotein allowed a further import into mitochondria despite the partially defective hsp70 (Kang et al., 1990). Co-immunoprecipitation experiments demonstrated that the preproteins were associated with the mutant hsp70 (Sscl-2p). A transient interaction of preproteins and mt-hsp70 has also been demonstrated in wild-type mitochondria by co-immunoprecipitation and cross-linking experiments (Scherer et al., 1990; Manning-Krieg et al., 1991). We concluded that mt-hsp70 interacts with the polypeptide chain in transit across the mitochondrial membranes and thereby facilitates an unfolding of the preprotein on the cytosolic side of the mitochondrial outer membrane (Kang et al., 1990; Neupert et al., 1990).

To further unravel the role of mt-hsp70 in membrane translocation of preproteins, it is important to distinguish between two possibilities. Is the function of mt-hsp70 in membrane translocation fully explained by facilitating the unfolding of preproteins or could mt-hsp70 play an even more basic role in membrane translocation processes? To address this question, we tried to obtain additional temperature-sensitive mutants of mt-hsp70. A mutant allele that came out of the screen (*sscl-3*) encodes a mt-hsp70 that appears to be more severely affected than the *sscl-2* hsp70, in particular to have a strongly reduced binding activity for preproteins. Using a preprotein that is processed twice by the matrix processing peptidase and which can be presented to mitochondria in an unfolded conformation, we show that binding to mt-hsp70 is indeed required to drive the import of the preprotein after the initial triggering step by the membrane potential, $\Delta\Psi$. Unfolding of the preprotein allowed transport across the outer membrane of both *sscl-2* and *sscl-3* mitochondria; but, the preprotein was transported across the inner membrane only in *sscl-2* mitochondria. Binding of the polypeptide chain in transit by mt-hsp70 is thus not only required for the unfolding of the polypeptide chain, but represents an essential reaction for complete transport of a matrix protein across the inner membrane. Moreover, we found that the two mutations mapped in different domains of mt-hsp70. The *sscl-3* mutation was in the ATPase domain. Consistent with this, ATP depletion of wild-type and *sscl-2* mitochondria caused a phenotype comparable to that seen in *sscl-3* mitochondria, supporting the idea that ATP is needed for mt-hsp70 to bind to the polypeptide chain and promote its translocation across the inner membrane.

Materials and Methods

Generation and Sequencing of SSC1 Mutants

Isolation of *sscl-2* was described previously (Kang et al., 1990). *sscl-3* was isolated in the same manner. Briefly, plasmid DNA carrying *SSC1* (pJK808) was mutagenized in vitro with hydroxylamine and transformed into a strain carrying a wild-type *SSC1* gene under the control of the *GAL1* promoter on a plasmid and an *sscl* insertion mutation on the chromosome. *sscl-3* was isolated as a gene that allowed growth on glucose-based media at 23°C, but not 37°C. The auxotrophic marker *LEU2* was inserted into the BglII site immediately 3′ of *SSC1* for chromosomal integration into yeast strain T87 (*ade2-101/+ lys2/lys2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 Δtrp1/Δtrp1 SSC1/SSC1*). Haploids used in this study were obtained by sporulation and are described in Table I. Methods for yeast and *E. coli* cloning and transformation have been described (Sambrook et al., 1989; Rose et al., 1990).

Table I. Haploid Yeast Strains Used in This Study

Strain	Genotype
PK81	MATα *ade2-101 lys2 ura3-52 leu2-3,112 Δtrp1 sscl-2(LEU2)*
PK82	MATα *his4-713 lys2 ura3-52 Δtrp1 leu2-3,112*
PK83	MATα *ade2-101 lys2 ura3-52 leu2-3,112* Δtrp1 sscl-3(LEU2)

The *sscl-2* and *sscl-3* mutations were initially mapped within a 1.4-kb ClaI fragment between the codons encoding amino acids 10 and 478 of the mature protein, by replacing the 1.4-kb ClaI fragment of an unmutagenized plasmid containing the wild-type *SSC1* gene with the ClaI fragment from the mutant genes. Before DNA sequencing, the site of the *sscl-2* mutation was further mapped within a 484-bp SalI–ClaI fragment by the same approach. Only a single nucleotide difference compared to the wild-type sequence was present within the ClaI fragment of *sscl-3* and the SalI–ClaI fragment of *sscl-2*.

Western Analysis of Cell Lysates

Cells were grown to early log phase (OD_{600} 1.0) at 23°C in YPD medium (Rose et al., 1990). Whole cell lysates for Western analysis were prepared by the glass bead lysis method (Ausubel et al., 1989). Proteins were immunodetected using the ECL Western immunodetection system (Amersham International, Amersham, UK) as described by the manufacturer with the following modifications. Filters were washed in 50 mM Tris base, pH 10, 150 mM NaCl and blocked in wash buffer containing 0.5% Tween 20.

Import of Su9–dihydrofolate Reductase into Isolated Mitochondria

The following procedures were performed as published (Hartl et al., 1987; Pfanner and Neupert, 1987; Pfanner et al., 1987; Kang et al., 1990; Söllner et al. 1991): isolation of mitochondria from *Saccharomyces cerevisiae*, preincubation of the mitochondria for 15 min at 37°C; synthesis of Su9–dyhydrofolate reductase (DHFR) in rabbit reticulocyte lysate in the presence of [^{35}S]methionine; incubation of reticulocyte lysate (10 ml) with mitochondria (50 μg protein) in the presence of 2 mM ATP and 4 mM NADH for 5 min at 25°C in the presence of BSA buffer (with 3% [wt/vol] BSA) in a final volume of 100 ml ("import assay"); treatment with proteinase K (40–75 μg/ml); reisolation of mitochondria; analysis by SDS-PAGE, fluorography, Western blotting, and laser densitometry.

Results

A New Temperature-sensitive Mutant of mt-hsp70

A new *SSC1* temperature-sensitive (ts) allele was isolated using the strategy utilized in the isolation of *sscl-2* (Kang et al., 1990; see Materials and Methods). *sscl-3* is recessive as indicated by the ability of *SSC1/sscl-3* heterozygous diploids to grow at wild-type rates even at 37°C. When *sscl-3* cells were shifted to the nonpermissive temperature of 37°C, an accumulation of mitochondrial preproteins was observed in a Western analysis (Fig. 1), similarly to the situation found with *sscl-2* mutant cells (Kang et al., 1990).

The mutant hsp70s (Sscl-2p and Sscl-3p) were mainly found in the soluble fraction upon sonication of mitochondria (Fig. 2 *A*) or after lysis of mitochondria with detergent (Fig. 2 *A*). The mutant proteins were correctly localized to the matrix since they were resistant to digestion with protease after opening the intermembrane space (formation of "mitoplasts"), only becoming accessible to protease after

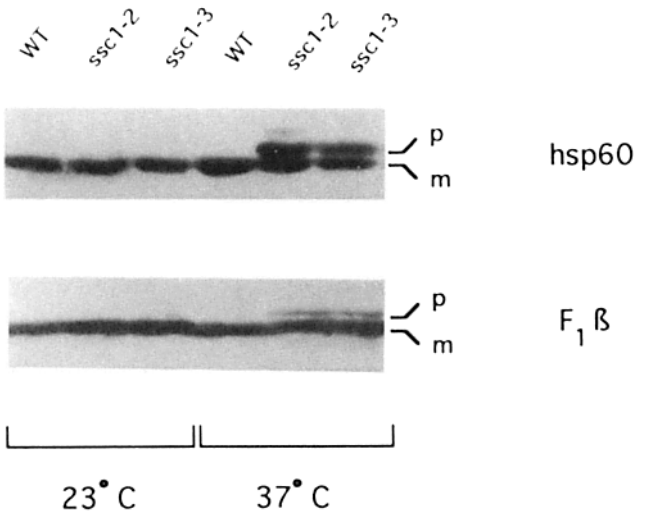

Figure 1. Accumulation of precursor proteins in *sscl-2* and *sscl-3* strains in vivo. Cultures of PK82 (*WT*), PK81 (*sscl-2*) and PK83 (*sscl-3*) were divided and half of each culture was shifted to 37°C for 30 min. Protein extracts (10 and 50 μg for hsp60 and $F_1\beta$ blots, respectively) were fractionated by SDS-PAGE, electrotransferred to nitrocellulose and probed with hsp60 or $F_1\beta$-specific antiserum. *p*, precursor; *m*, mature.

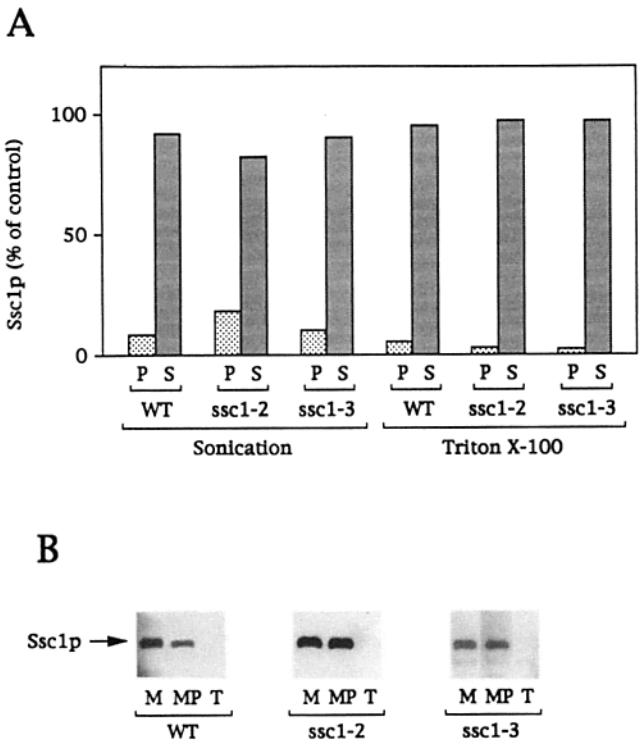

Figure 2. The mutant hsp70s (Sscl-2p and Sscl-3p) are located in the mitochondrial matrix. (*A*) Solubility of mt-hsp70s after sonication or after solubilization of mitochondria with Triton X-100. Isolated mitochondria in SEM-buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) (80 μg protein per sample) were preincubated for 15 min at 37°C and sonified for 3× 30 s (Branson sonifier setting 5; Branson Ultrasonics Corp., Danbury, CT) or extracted with 1% (wt/vol) Triton X-100, 300 mM NaCl, 10 mM Tris, pH 7.5. Supernatants and pellets were then separated by centrifugation for 60 min at 166,000 *g* or 15 min at 18,000 *g*. After precipitation with TCA, analysis was performed by Western blotting using antiserum directed against mt-hsp70. The total amount of mt-hsp70 in a sample (without sonication or treatment with Triton X-100) was set to 100% (control). At the sonication conditions used, membrane proteins remained in the pellet (Kang et al., 1990; Söllner et al., 1990). *P*, pellet; *S*, supernatant. (*B*) Mitochondria (*M*, 30 μg protein; in SEM 10-fold diluted in 25 mM Hepes/KOH, pH 7.4, 0.6 M sorbitol) or mitoplasts (*MP*, 30 μg protein; mitochondria diluted 10-fold in 25 mM Hepes/KOH, pH 7.4) or Triton-solubilized mitochondria (*T*, 30 μg protein; Triton X-100 buffer described above) were treated with proteinase K and analyzed by Western blotting with antiserum directed against mt-hsp70 (Ssclp). The formation of mitoplasts was controlled and confirmed by the release of cytochrome b_2 and the fragmentation of the ADP/ATP carrier as published (Hwang et al., 1991; Rassow and Pfanner, 1991; Glick et al., 1992).

disruption of the inner membrane (Fig. 2 *B*). Sscl-2p and Sscl-3p are thus soluble proteins of the mitochondrial matrix as is wild-type Ssclp (Fig. 2; Kang et al., 1990), indicating that the mutant phenotypes are caused by altered functions of mt-hsp70 and not by defects in its biogenesis or by aggregation of the mutant hsp70s.

The Single Mutations in Sscl-2p and Sscl-3p Map to Different Domains of hsp70

DNA sequence analysis of the *sscl-2* mutant revealed a single nucleotide difference compared to the wild-type sequence (see Materials and Methods), a C:G→T:A transition at the codon for amino acid 419 of the mature protein. The resulting proline → serine (CCA → TCA) change is in the putative peptide binding domain of hsp70s (Chappell et al., 1987). This proline residue is very highly conserved, present in all of the hsp70s whose DNA sequence has been determined so far (Fig. 3).

The *sscl-3* mutation also causes a single amino acid difference, a glycine → serine (GGT → AGT) change at position 56 of the mature protein, within the ATPase domain (Flaherty et al., 1990). The glycine at position 56 is highly conserved, present in nearly every hsp70 whose DNA sequence has been determined (Fig. 3). Actin has been shown to be structurally very similar to hsp70s even though there is little amino acid similarity (Flaherty et al., 1991). Glycine 56 is one the few amino acid identities between the two types of proteins.

Differential Accumulation of a Preprotein in sscl-2 and sscl-3 Mitochondria

We used a fusion protein containing the presequence plus three amino acid residues of the mature portion of *Neurospora crassa* F_o-ATPase subunit 9 and the entire mouse DHFR (Pfanner et al., 1987) to assess import into mutant mitochondria. As is the case with the authentic *N. crassa* preprotein, the Su9-DHFR presequence is cleaved twice upon import, after residues 35 and 66. This two step removal of the presequence is catalyzed by the matrix-localized processing peptidase (Schmidt et al., 1984; Hawlitschek et al., 1988). Mitochondria were isolated from wild-type, *sscl-2* and *sscl-3* yeast cells that were grown at the permissive temperature of 25°C. Before being energized and incubated with ^{35}S-labeled precursor protein at 25°C, mitochondria were pre-incubated at 37°C for 15 min.

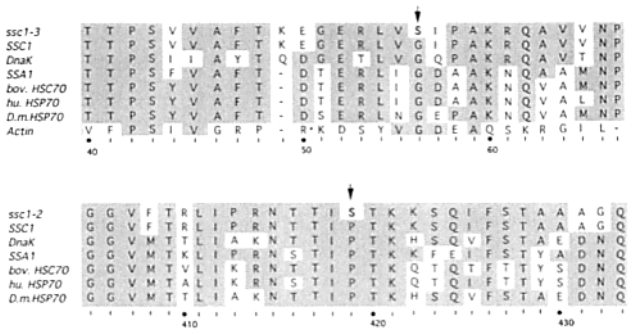

Figure 3. Alignment of *sscl-2* and *sscl-3* with representative *HSP70* genes and yeast actin (deduced amino acid sequences). Amino acid sequence identities are shaded; arrows denote *SSCI* mutations; "-" represents a gap in the sequence; "*" in the actin sequence represents 10 amino acids looped out to give optimal alignment. Residue numbers correspond to the sequence of mature Ssclp. GenBank accession codes are: *SSCI* - M27229; *DnaK* - K01298; *SSAI* - X12926; bovine *HSC70* - P19120 (Swiss-Prot); human *HSP70* - M11717; *Drosophila melanogaster HSP70* - J1104, J1105.

With wild-type mitochondria the Su9-DHFR was processed to the mature form and virtually all the mature form was resistant to exogenously added proteinase K (Fig. 4 *A*) as expected of protein completely imported into the matrix. Consistent with previous results (Kang et al., 1990), Su9-DHFR was processed to mature form in *sscl-2* mitochondria as in wild-type mitochondria, and only a small portion of this mature form was protected from protease (Fig. 4 *A*). With increased times of incubation the portion gaining protease resistance increased (data now shown), suggesting that import into *sscl-2* mitochondria is delayed but not completely blocked. With *sscl-3* mitochondria, Su9-DHFR was processed to the intermediate, but very little mature form was observed even at longer periods of incubation. This intermediate form was susceptible to digestion with proteinase K (Fig. 4 *A*), indicating that it was only partially translocated into the *sscl-3* mitochondria with a portion still present outside the outer membrane.

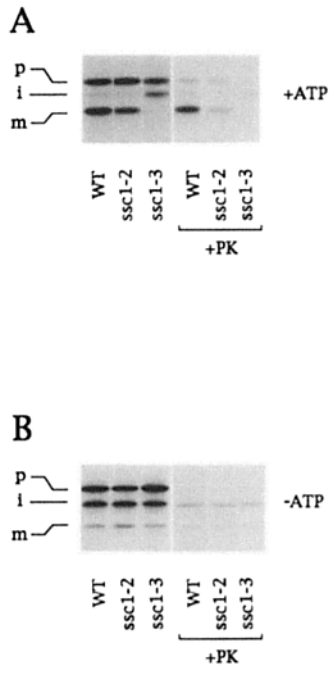

Figure 4. Accumulation of Su9-DHFR in *sscl-2* and *sscl-3* mitochondria. (*A*) Reticulocyte lysate containing ^{35}S-labeled precursor of Su9-DHFR was incubated with isolated mitochondria (25 μg protein per lane) from wild-type (*WT*), *sscl-2* or *sscl-3* mitochondria (that had been preincubated for 15 min at 37°C) as described in Materials and Methods. Where indicated the samples were treated with proteinase K (*PK*) after the import reaction. The reisolated mitochondria were analyzed by SDS-PAGE and fluorography. (*B*) The import was performed as described above except that the mitochondria were depleted of ATP by preincubation with apyrase (20 U/ml) (Pfanner and Neupert, 1986) in the presence of 20 μM oligomycin. Import was performed in the presence of oligomycin and carboxyatractyloside as described (Wachter et al., 1992). *p*, precursor; *i*, intermediate; *m*, mature Su9-DHFR.

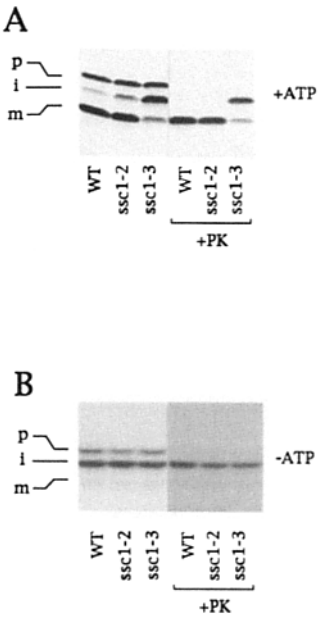

Figure 5. Urea-denatured Su9-DHFR is transported to a protease-protected location in *sscl-2* and *sscl-3* mitochondria. Reticulocyte lysate containing Su9-DHFR was precipitated with ammonium sulphate at 66% saturation and dissolved in 8 M urea, 30 mM MOPS, pH 7.4, 10 mM DTT (Ostermann et al., 1989; Kang et al., 1990). Import into isolated mitochondria was performed and analyzed as described in the legend to Fig. 4 (200 mM final concentration of urea in the import assay).

Since hsp70 action requires ATP and Sscl-3p is mutated in the ATPase domain, it was of interest to compare the effect of ATP depletion to the effect of mutations in *SSCI*. ATP levels were lowered by pretreatment of the mitochondria with apyrase and inhibition of the mitochondrial ATP-synthase with oligomycin. ATP-depleted wild-type and *sscl-2* mitochondria accumulated the protease-accessible intermediate-sized form as was found with *sscl-3* mitochondria in both the presence and absence of ATP (Fig. 4 *B*). This and results shown below thus indicate that depletion of ATP in wild-type or *sscl-2* mitochondria leads to the accumulation of the same intermediate form as in *sscl-3* mitochondria.

Mt-hsp70 Facilitates Unfolding of Preproteins for Translocation Across Mitochondrial Membranes

We previously showed that an artificial unfolding of Su9-DHFR by preincubation in 8 M urea allowed rapid translocation of the preprotein to a protease-protected location in *sscl-2* mitochondria (Kang et al., 1990). We presented *sscl-3* mitochondria with urea denatured Su9-DHFR to test whether denaturing the precursor allowed transport to a protease-protected location and complete processing of the precursor. Fig. 5 *A* shows that unfolded Su9-DHFR was indeed transported to a protease-protected location in *sscl-3* mitochondria, but only very little processing to the mature form was observed. At low levels of ATP, wild-type and *sscl-2* mitochondria also accumulated intermediate forms in a protease-protected location (Fig. 5 *B*). Unfolding of Su9-DHFR thus promoted the translocation of the entire fusion protein across the outer membrane in *sscl-3* mitochondria. However, the second processing site of most precursor molecules was not transported to a location where it was accessible to the pro-

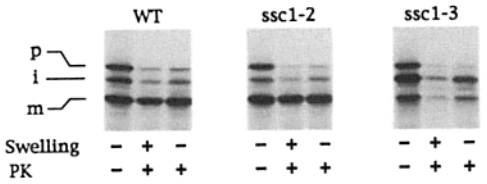

Figure 6. Urea-denatured Su9-DHFR is transported into the matrix of *sscl-2* mitochondria, but remains exposed to the intermembrane space in *sscl-3* mitochondria. Import was performed as described in the legend of Fig. 5 *A*. The mitochondria were reisolated, resuspended in SEM containing 3% (wt/vol) BSA and diluted 10-fold in either 25 mM Hepes/KOH, pH 7.4 (*swelling*) or 25 mM Hepes/KOH, pH 7.4, 0.6 M sorbitol (*no swelling*). Where indicated, proteinase K (*PK*) was added for 20 min at 0°C during the dilution. The mitochondria were reisolated and analyzed by SDS-PAGE and fluorography. The formation of mitoplasts by the swelling procedure (i.e. opening of the outer membrane, but not the inner membrane with the major fraction of the mitochondria) was controlled and confirmed by analyzing the marker proteins cytochrome b_2, ADP/ATP carrier and mt-hsp70 by Western blotting as described (Rassow and Pfanner, 1991; Hwang et al., 1991; Glick et al., 1992).

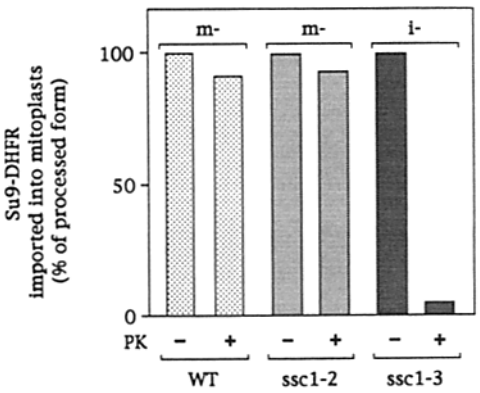

Figure 7. Denatured Su9-DHFR is directly translocated across the inner membrane of wild-type and *sscl-2* mitochondria, but gets stuck in the inner membrane of *sscl-3* mitochondria. Isolated mitochondria (preincubated for 15 min at 37°C) were pretreated with trypsin (40 μg/ml) to remove the surface receptors (Pfaller et al., 1988, 1989), swollen as described in the legend of Fig. 6 and reisolated. Denatured Su9-DHFR (see legend of Fig. 5) was then incubated with the resulting mitoplasts under import conditions (see Materials and Methods) for 10 min at 25°C. Where indicated the samples were treated with proteinase K. Quantitation was performed by laser densitometry of the fluorographs. To assess the amount of transport via the bypass route across the outer membrane of trypsinized mitochondria (Pfaller et al., 1989; Hwang et al., 1989), trypsinized mitochondria were incubated in SEM-buffer instead of the swelling buffer and used for the import of denatured Su9-DHFR; the amount of (bypass) import into these mitochondria was subtracted from the import value obtained with the mitoplasts.

cessing peptidase in the matrix either in *sscl-3* mitochondria or under conditions of low ATP in any of the mitochondria.

To determine the intramitochondrial location of Su9-DHFR transported into *sscl-2* and *sscl-3* mitochondria out of urea we fractionated mitochondria after the import reaction. In both *sscl-2* and *sscl-3* mitochondria the extreme amino terminus of the preprotein must have entered the matrix since processing occurred, at least at the first cleavage site. In the case of *sscl-3* mitochondria the preprotein was accessible to added protease after opening the intermembrane space (Fig. 6), demonstrating that Su9-DHFR accumulated as a so-called "intermembrane space intermediate" (Hwang et al., 1991; Rassow and Pfanner, 1991; Manning-Krieg et al., 1991; Jascur et al., 1992; Pfanner et al., 1992) that spans across the inner membrane, exposes the amino-terminal presequence to the matrix and a mature protein part to the intermembrane space. Both the intermediate form and the small amount of mature form that accumulated in *sscl-3* were found in this membrane-spanning form. In contrast, in *sscl-2* mitochondria the Su9-DHFR did not become accessible to protease until the matrix was opened by treatment with detergent. Thus, *sscl-2* mitochondria are able to transport the urea-denatured protein completely into the matrix, while in *sscl-3* mitochondria the preprotein is trapped as an intermediate in the intermembrane space with the amino-terminus spanning the inner membrane into the matrix.

Mt-hsp70 Is Required for Translocation of Unfolded Su9-DHFR Directly Across the Inner Membrane

There are two reasons why unfolded Su9-DHFR may not be completely transported across the inner membrane of *sscl-3* mitochondria. First, unfolded DHFR may partially refold during passage across the outer membrane and intermembrane space, thus preventing translocation across the inner membrane because of a conformational restriction. Second, unfolding, however, may not be the sole function of mt-hsp70 in membrane translocation and mt-hsp70 may be an essential component for translocation across the inner membrane. To differentiate between these two possibilities we made use of an observation of Schatz and co-workers (Ohba and Schatz, 1987; Hwang et al., 1989) that some preproteins could be directly transported across the inner membrane after disruption of the outer membrane. As expected, *sscl-2* mitochondria were able to translocate unfolded Su9-DHFR directly across the inner membrane (Fig. 7). When *sscl-3* mitochondria with disrupted outer membranes were used for import, Su9-DHFR accumulated in the inner membrane exposed to added protease (Fig. 7). We conclude that unfolding of the polypeptide chain is not sufficient to overcome the translocation defect across the inner membrane of *sscl-3* mitochondria.

In Contrast with sscl-2 Mitochondria, Most of Su9-DHFR Is Not Found in a Complex with mt-hsp70 of sscl-3 Mitochondria

Co-immunoprecipitation of several preproteins with anti-mt-hsp70 antibodies has been reported after lysis by detergent of *sscl-2* (Kang et al., 1990; Ostermann et al., 1990) and wild-type mitochondria (Scherer et al., 1990; Manning-Krieg et al., 1991). Fig. 8 shows the efficient co-immunoprecipitation of Su9-DHFR out of wild-type and *sscl-2* mitochondria. However, none of the intermediate-sized Su9-DHFR accumulated in *sscl-3* mitochondria was co-precipitated with anti-mt-hsp70 antibodies (Fig. 8, *A* and *B*), even though a wide variety of conditions including variations of import times, levels of ATP and mono- and divalent salts were tried. ~5–10% of the Su9-DHFR was processed to the mature-sized form in *sscl-3* mitochondria. Interestingly, a significant amount of this form was co-immunoprecipitated, though the efficiency of precipitation was far be-

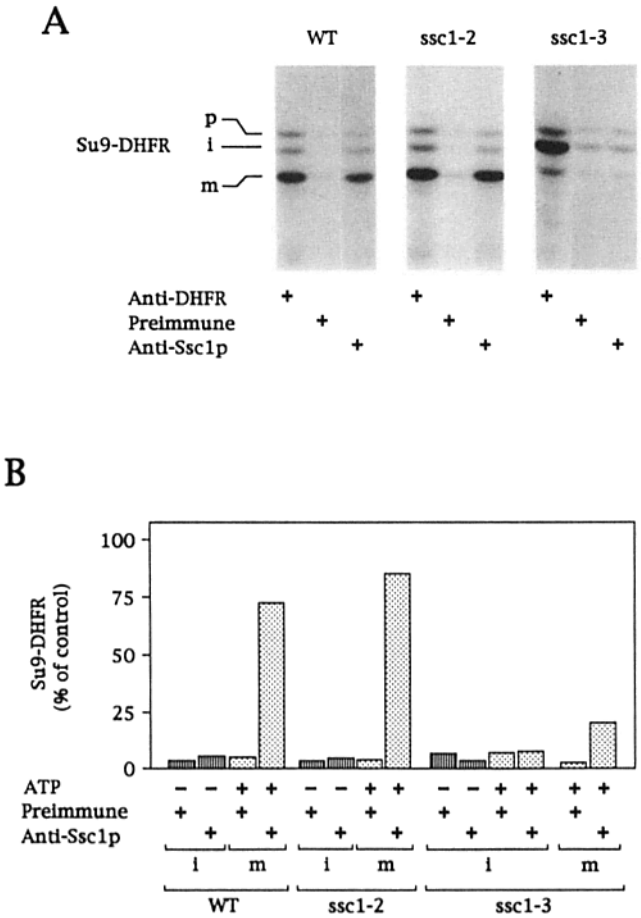

Figure 8. Co-immunoprecipitation of mature-sized Su9-DHFR with mt-hsp70. Import of Su9-DHFR was performed for 5 min at 25°C as described in the legend of Fig. 5 *A*; where indicated in *B* the mitochondria were depleted of ATP (−ATP) before the import reaction as described in the legend of Fig. 4 *B*. The mitochondria were lysed in 0.1% (wt/vol) Triton X-100, 100 mM NaCl, 10 mM Tris/HCl, pH 7.5, 5 mM EDTA and, after a clarifying spin (5 min, 27,000 *g*), incubated with antisera directed against DHFR, preimmune serum or antiserum directed against Ssclp. The immunoprecipitates were washed three times in the buffer described above and once in 10 mM Tris, pH 7.5, and analyzed by SDS-PAGE, fluorography (*A*) and laser densitometry (*B*). The amount of intermediate (*i*) or mature (*m*) Su9-DHFR that was precipitated with antiserum directed against DHFR was set to 100% (control), respectively.

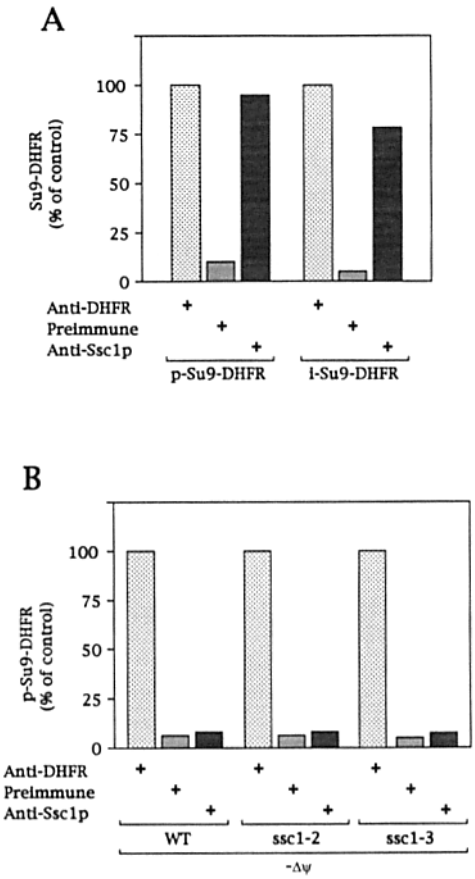

Figure 9. Co-immunoprecipitation of precursor and intermediate forms of Su9-DHFR with mt-hsp70. (*A*) Denatured Su9-DHFR was imported into *sscl-2* mitochondria (in the presence of ATP) and immunoprecipitations were performed as described in the legend of Fig. 8, except that the mitochondrial processing peptidase was partially inactivated by performing the import in the presence of 1 mM o-phenanthroline and 5 mM EDTA (Schmidt et al., 1984; Hawlitschek et al., 1988). (*B*) Su9-DHFR accumulated in the absence of a membrane potential is not co-immunoprecipitated with mt-hsp70. Denatured Su9-DHFR was incubated with the mitochondria as described in the legend of Fig. 8 (in the presence of ATP) except that NADH was omitted and the membrane potential was dissipated by addition of 0.5 μM valinomycin, 8 μM antimycin A and 20 μM oligomycin (Pfanner and Neupert, 1987). Immunoprecipitations were performed as described in the legend to Fig. 8.

low that observed with wild-type or *sscl-2* mitochondria (Fig. 8 *B*). We suspect that mutant Sscl-3p was able to bind a very small amount of the preprotein thus allowing further import. However, it should be noted that, in contrast to *sscl-2* mitochondria, the mature-sized protein in *sscl-3* mitochondria was not completely imported into the matrix, even after pretreatment with urea. Instead, it was arrested in its translocation across the inner membrane (Fig. 6). We then tested if a depletion of ATP in wild-type or *sscl-2* mitochondria led to a similar lack of co-immunoprecipitation as in *sscl-3* mitochondria. Fig. 8 *B* shows that this was indeed the case.

The fact that we observed co-immunoprecipitation of only the mature-sized form of Su9-DHFR in *sscl-3* mitochondria raises the question of whether the presence of the presequence prevents the binding of Su9-DHFR to mt-hsp70. Inhibition of binding by the presequence appears to be unlikely as Schmid et al. (1992) recently showed that the presence of a mitochondrial presequence strongly stimulated the interaction of DnaK, the bacterial homolog of hsp70, with a mitochondrial preprotein. For a direct experimental demonstration in our system we partially inhibited the mitochondrial processing peptidase, such that precursor- and intermediate-sized forms of Su9-DHFR were obtained during import. Both preprotein forms were efficiently co-immunoprecipitated with mt-hsp70 (Fig. 9 *A*).

The possibility that the association of Su9-DHFR with mt-hsp70 occurred after the lysis of mitochondria was of concern. This possibility is unlikely since Su9-DHFR which accumulated at the outer membrane in the absence of $\Delta\Psi$ could not be co-immunoprecipitated with mt-hsp70 (Ostermann et al., 1990). However, in this previously reported experiment

binding of the preprotein had been performed directly from a reticulocyte lysate, rather than after urea denaturation as was the case in the co-immunoprecipitation experiments reported in Fig. 8. Therefore as a more relevant control, Su9-DHFR was denatured in urea and bound to mitochondria in the absence of $\Delta\Psi$ (Fig. 9B). No co-immunoprecipitation with mt-hsp70 in wild-type, *sscl-2* or *sscl-3* mitochondria was observed. We conclude that the interaction between mt-hsp70 and preprotein shown in Fig. 8 occurred in the intact mitochondria.

Discussion

We compared the ability of mitochondria from two temperature-sensitive yeast mutants of mitochondrial hsp70 (*sscl-2* and *sscl-3*) to import preproteins. While the mutant protein Sscl-2p showed a strong binding activity for preproteins, only very small amounts of preproteins could be found associated with the Sscl-3p protein. The ability of the mutant hsp70s to interact with preproteins corresponded to the degree of polypeptide chain translocation into the mutant mitochondria. (*a*) In both mutants the first half of the presequence of F_o-ATPase subunit 9 was translocated into the mitochondrial matrix, a process mediated by the membrane potential $\Delta\Psi$. In *sscl-2* mitochondria the presequence could be translocated sufficiently far so that both cleavage sites were accessible to the matrix localized processing peptidase. However, in *sscl-3* mitochondria the preprotein accumulated predominantly in the intermediate-sized form. (*b*) The mature part of the preprotein spanned both membranes of the mutant mitochondria at translocation contact sites, exposed to the cytosolic side as evidenced by susceptibility to protease. With *sscl-2* mitochondria, the translocation of the mature part was slow; an increasing fraction of the preprotein was completely translocated across the outer membrane (and eventually across the inner membrane) at longer import times, while in *sscl-3* mitochondria the translocation block appeared to be quite stable. (*c*) When the preprotein was added to mitochondria in an unfolded conformation, it was completely translocated into the matrix of *sscl-2* mitochondria where it remained bound to the mutant hsp70. In *sscl-3* mitochondria, unfolding of the preprotein allowed complete translocation across the outer membrane, but not across the inner membrane, as the mature protein part of most precursor molecules accumulated in the intermembrane space; moreover, even the second portion of the presequence of most precursor molecules was not transported far enough to be cleaved by the processing peptidase in *sscl-3* mitochondria. Only a small amount of the preprotein was found associated with Sscl-3p; the proteins that were associated with Sscl-3p were processed twice. It appears that the very weak binding activity of Sscl-3p allowed binding of a minor amount of preproteins whose entire presequence was then moved into the matrix; apparently the binding activity was not sufficient to allow complete translocation of the mature protein part across the inner membrane even if it was unfolded before addition to the mitochondria.

As a class, hsp70s have an amino-terminal ATPase domain and a carboxyl-terminal peptide-binding region (Rothman, 1989; Gething and Sambrook, 1992). The structure of the 44-kD ATPase domain of bovine heat shock cognate protein, hsc70, has been determined (Flaherty et al., 1990) and shown to consist of two lobes with a deep cleft between them. Nucleotide binds at the base of the cleft. By comparing the sequences of bovine hsc70 and Ssc1p we were able to tentatively place the site of the amino acid change in Sscl-3p at the top of the cleft, at the surface of the so-called domain IB. Our inability to co-immunoprecipitate significant quantities of preprotein with anti-Ssc1p antibody suggests that Sscl-3p has a reduced binding affinity for proteins. A debate exists as to whether ATP-bound or ADP-bound hsp70 has a higher affinity for peptide. Since the depletion of ATP in wild-type or *sscl-2* mitochondria mimics the translocation defect of *sscl-3* mitochondria, in both the lack of co-immunoprecipitation of polypeptide with hsp70 and the extent of the translocation defect, it is tempting to speculate that the ATP-bound hsp70 is the active peptide-binding form of hsp70 in vivo. In support of this notion, preliminary experiments indicate that in crude lysates Sscl-3p has reduced binding to ATP-agarose compared to wild-type Ssc1p (unpublished data). Alternatively, the structure of domain IB may be important for interaction between the ATPase and peptide binding domains as has been proposed (Flaherty et al., 1990). Regardless of the mechanistic details behind the *sscl-3* phenotype, the fact, that reduction of ATP levels in the matrix led to an accumulation of the intermediate form of the preprotein in wild-type and *sscl-2* mitochondria as was found in *sscl-3* mitochondria independent of ATP, is in agreement with the view that ATP is needed for mt-hsp70 to drive protein import into the matrix (Neupert et al., 1990; Hwang et al., 1991; Manning-Krieg et al., 1991).

Although the structure of the peptide-binding domain has not been determined, structural models have been proposed based on slight similarities between hsp70s and the human type I major histocompatibility antigens (Rippmann et al., 1991; Flajnik et al., 1991). According to those models the amino acid change of *sscl-2* is located in a hinge region between two predicted β strands in the peptide binding region. An amino acid change in a hinge region might well be expected to alter both binding and release of peptide. An exact determination of the effect of the mutation on the interaction with a polypeptide awaits structural and functional analysis of purified wild-type and mutant protein.

It is important to note that the induction of the phenotypes did not require a temperature shift of the cells, but could be performed with the isolated mitochondria, thereby minimizing the chances of unspecific or indirect effects of the hsp70 mutations on the structure and function of the mitochondria. Moreover, it can be excluded that the import defect caused by the mutations is due to a dissipation of the membrane potential. $\Delta\Psi$ is needed for the translocation of the extreme amino terminus of preproteins (the positively charged matrix-targeting portion of the presequence), but not the mature protein part (Schleyer and Neupert, 1985; Martin et al., 1991), and thus $\Delta\Psi$ and mt-hsp70 are required for distinct steps in the translocation process. Moreover, in the accompanying manuscript (Voos et al., 1993) we show that a preprotein, which depends strictly on a membrane potential for import, is completely imported into mitochondria of both mutants.

We conclude that the role of mt-hsp70 for preprotein translocation across the outer membrane is explained by a facilitation of unfolding of the polypeptide chain. This conclusion fits with our previous proposal that a step-wise unfolding of

the polypeptide chain on the cytosolic side leads to a step-wise movement of preprotein segments into the matrix where they are trapped by mt-hsp70 (Kang et al., 1990; Neupert et al., 1990). The function of mt-hsp70 in preprotein translocation across the inner membrane is at least a dual one. First, it is similarly involved in facilitating the unfolding of the preproteins. Second, binding of the polypeptide chain to mt-hsp70 is essential to complete its translocation into the matrix, independent of its folding state. We propose that mt-hsp70 is a genuine component of the inner membrane translocation machinery. Binding of mt-hsp70 to the preprotein is required for its vectorial movement into the matrix and multiple cycles of hsp70 binding and ATP-dependent release will thus promote a step-wise movement of preproteins into the matrix. As it has been postulated that the interaction with the hsp70 in the endoplasmic reticulum (Kar2p, BiP) leads to trapping of a preprotein in the ER (Vogel et al., 1990; Ooi and Weiss, 1992; Sanders et al., 1992; Simon et al., 1992), it is tempting to speculate that Kar2p plays a dual role in protein translocation that is similar to that of mt-hsp70. Thus, the complexities of hsp70 function in protein translocation revealed in these studies of mitochondrial hsp70 may well be more generally applicable.

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