

# THE ROLE OF MOLECULAR CHAPERONES IN TRANSPORT OF PROTEINS ACROSS MEMBRANES

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## I. INTRODUCTION

### A. hsp70 Structure and Biochemical Properties

The process of transporting proteins from their site of synthesis in the cytosol into organelles such as the mitochondrion or endoplasmic reticulum is facilitated by a group of proteins known as the molecular chaperones. Among the best characterized molecular chaperones are the hsp70 proteins. The hsp70s are highly conserved polypeptides found in all species, with identity ranging from 50% between procaryotic and eucaryotic examples to as high as 99% between members of a HSP70 gene family in yeast (Boorstein et al., 1994). The hsp70s possess two biochemical activities: a weak ATPase activity and peptide binding activity (reviewed in Gething and Sambrook, 1992). The ATPase activity can be stimulated severalfold by binding the hsp70 to peptides or polypeptides. Biochemical and structural studies of mammalian cytosolic hsp70 have shown that an N-terminal 44-kDa proteolytic fragment has ATPase activity that cannot be stimulated by peptide binding. The structure of the N-terminal proteolytic fragment of mammalian HSP70 has been solved by X-ray crystallography (Flaherty et al., 1990). The roughly U-shaped structure consists of two lobes separated by a deep cleft in which ATP binds. The most highly conserved amino acids of the amino-terminal domain are found lining this cleft.

The peptide binding activity resides in the carboxyl-terminal portion of the protein. It should be emphasized that there is no "consensus" binding site of hsp70s, inasmuch as they are able to interact with a wide variety of peptides. However, studies of a mammalian hsp70 indicated a preference for binding of peptides rich in aliphatic and hydrophobic amino acids that were at least seven residues in length (Flynn et al., 1991). The cycles of binding and release of peptide require the hydrolysis

of ATP, implying an interaction between the C-terminal peptide binding domain and the N-terminal ATPase domain. The structure of the C-terminal domain of hsp70 has not been solved; however, two groups have proposed a structure similar to that of the well-characterized major histocompatibility complex class I antigen presenting (HLA) molecules, based on slight similarities in primary sequence and secondary-structure predictions (Rippmann et al., 1991; Flajnik et al., 1991). The HLA structure and the proposed hsp70 peptide binding region consist primarily of  $\beta$  sheets with peptides binding in an extended conformation in the groove bounded by  $\alpha$  helices (Fremont et al., 1992).

The universal ability of hsp70s to undergo cycles of binding and release with short regions of peptides determines their role in a great variety of intracellular functions. In this chapter we will discuss how, based on analysis of mutants of the yeast *Saccharomyces cerevisiae*, hsp70s function in the translocation of proteins from the cytosol into mitochondria and the endoplasmic reticulum has begun to be elucidated.

## B. The hsp70s of *Saccharomyces cerevisiae*

Like other eucaryotes, the budding yeast *Saccharomyces cerevisiae* has multiple hsp70 proteins. This large gene family is divided into subfamilies that correlate with the subcellular localization of the gene products (reviewed in Craig et al., 1993). The *SSA* and *SSB* subfamilies of proteins are found in the cytosol. The *SSB* subfamily has two members that are not absolutely essential for growth. This subfamily of hsp70s is found in association with translating ribosomes, perhaps bound to the nascent polypeptide chain. The four *SSA* gene products are found dissolved in the cytosol. The *SSA* subfamily is essential; cells require at least one of the *SSA* proteins in substantial amounts for growth at any temperature. *SSA* proteins function to regulate the transcription of heat shock responsive genes (Stone and Craig, 1990) and as discussed below are important for efficient translocation of at least some proteins from the cytosol into the ER and mitochondria. The other two yeast hsp70s are organellar. Kar2p is an essential protein found in the lumen of the endoplasmic reticulum (Rose et al., 1989; Normington et al., 1989). The mitochondrial hsp70, Ssc1p, is a soluble protein of the matrix and is essential for growth (Craig et al., 1987, 1989). As discussed below both organellar hsp70s are required for transport of proteins into their respective organelles. Here, we focus on the role of mitochondrial hsp70 in translocation since it is the subject of work from our laboratories.

### C. The Basics of Import into Mitochondria

The biogenesis of mitochondrial proteins is a multistep process (reviewed in Pfanner et al., 1991; Glick and Schatz, 1991). Precursor proteins synthesized in the cytosol with an N-terminal presequence and probably bound to cytosolic hsp70s associate with receptors on the mitochondrial surface. The presequence is then inserted into the outer membrane and then across the inner membrane triggered by the membrane potential,  $\Delta\psi$ , across the inner membrane. The presequence is proteolytically removed by the processing protease of the mitochondrial matrix. Then the bulk of the polypeptide moves vectorially across the outer and inner membranes. Experimentally the translocation of precursor proteins across the inner membrane can be broken down into two steps: (1) the insertion of the presequence and (2) the translocation of the remainder of the polypeptide. The first step is dependent on  $\Delta\psi$ ; the second is not. As described below Ssc1p mainly functions in the second step, the movement of the bulk of the polypeptide into the matrix.

A genetic approach has proved to be a very useful companion to biochemical analysis in understanding a number of physiological processes in both eucaryotes and procaryotes. The translocation of proteins from the cytosol into mitochondria has been no exception. Temperature-sensitive mutants have allowed the assessment of the effect of the absence of a single functional protein *in vivo*. When cells are grown under permissive conditions, the protein of interest is functional. Defects that are detected within a very short time after a shift to the nonpermissive temperature are likely a direct result of the inactivation of the protein. In this chapter we discuss the results of our combined genetic and biochemical analysis of the role of mitochondrial hsp70 in the translocation of proteins into mitochondria using temperature-sensitive hsp70 mutants.

## II. A ROLE FOR CYTOSOLIC hsp70 IN PROTEIN TRANSLOCATION INTO ORGANELLES

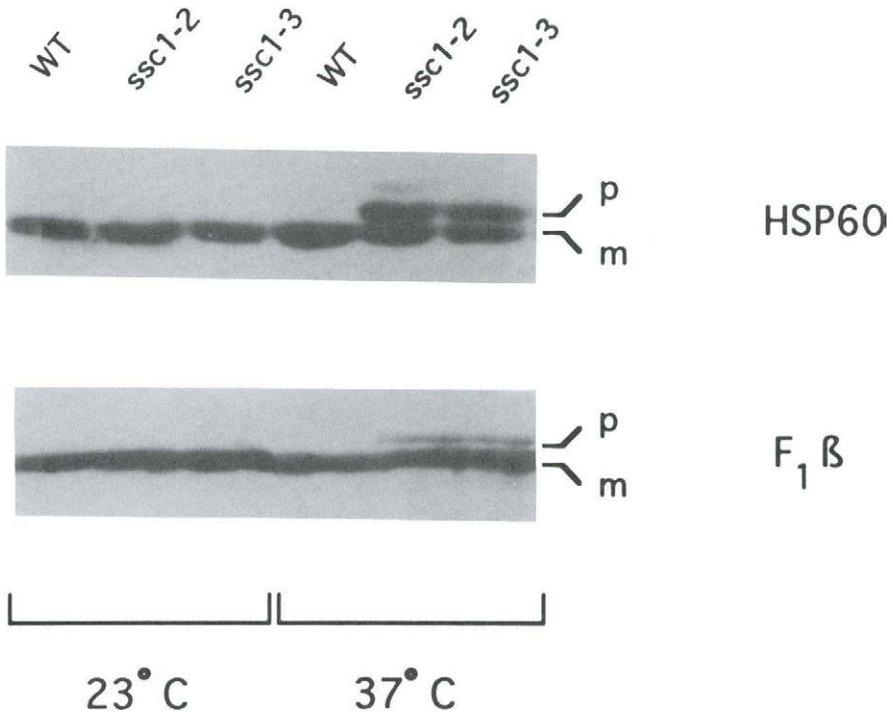
Several years ago it was suggested that cytosolic hsp70s of *S. cerevisiae* were important for efficient translocation of at least some proteins from the cytosol to the lumen of the ER or matrix of the mitochondria. *In vivo* evidence came from the observation that strains depleted of the SSA proteins accumulated precursors of two proteins, the  $\beta$  subunit of the  $F_1F_0$  ATPase, a mitochondrial protein, and  $\alpha$  factor, a secreted protein, as the amount of SSA proteins dropped below the level found in wild-type

(wt) cells (Deshaies et al., 1988). This initial experiment suffered from the fact that several hours elapsed after new expression of SSA proteins was stopped before a decrease in protein translocation was observed. Therefore it was possible that the effect of SSA depletion on translocation was an indirect one. However, recent experiments with temperature-sensitive SSA mutants have shown a defect in translocation of the same proteins within 10 minutes after a shift to the nonpermissive temperature, suggesting a direct effect (Becker and Craig, unpublished results). *In vitro* experiments supported the notion that SSA proteins are important for translocation. Translocation of radiolabeled proteins into microsomal vesicles and mitochondria was facilitated by the addition of SSA proteins (Chirico et al., 1988). Incubation of the precursor with urea substituted for the addition of SSA proteins in facilitating translocation, suggesting that the SSA proteins may aid in maintaining the precursor in a partially unfolded, translocation-competent conformation. This is an appealing idea because of the known peptide-binding activity of hsp70s and because of their propensity to bind to regions rich in hydrophobic residues as would be exposed in unfolded proteins. However, at this point there is no demonstration *in vivo* of a direct interaction of SSA proteins with precursors, although such interactions have been observed in reticulocyte lysates (Chirico, 1992).

### III. ROLE OF MITOCHONDRIAL hsp70 IN PROTEIN TRANSLOCATION

#### A. *In vivo* Analysis of a Mitochondrial hsp70 Mutant

The first indication that mitochondrial hsp70 is required for the translocation of proteins from the cytosol into the matrix of mitochondria came from analysis of a temperature-sensitive mutant of *S. cerevisiae* (Kang et al., 1990). This mutant, called *ssc1-2*, was obtained by directly mutagenizing *SSC1* DNA *in vitro*. Because of the suggestion that the cytosolic hsp70s, the SSA proteins, are involved in the translocation of at least some proteins from the cytosol into the endoplasmic reticulum and mitochondria, the *ssc1-2* mutant was tested for its ability to carry out translocation at the nonpermissive temperature of 37°C. Specifically, the effect of a shift to 37°C on the conversion of the precursor form of mitochondrial proteins to the mature form was tested. The proteins in extracts made from cells 30 minutes after a shift from 23°C to 37°C were separated by electrophoresis and reacted with antibodies specific for



**Figure 1.** Accumulation of precursor proteins in *SSC1* mutants *in vivo*. Cultures of WT, *ssc1-2* and *ssc1-3* cells growing at 23°C were divided, and half of each culture was shifted to 37°C for 30 minutes prior to harvest. Protein extracts were fractionated by SDS-PAGE, electrotransferred to nitrocellulose filters, and probed with hsp60- and F<sub>1</sub>β-specific antiserum. p, precursor, m, mature.

mitochondrial proteins. Accumulation of the precursor form of hsp60 (Fig. 1) as well as a number of other proteins was observed. Since the protease that catalyzes the cleavage of matrix-destined mitochondrial proteins is located in the matrix, the lack of efficient cleavage in the *ssc1-2* mutant suggested a defect in the translocation process. Since initially cells were shifted to the nonpermissive temperature for 30 minutes prior to harvest of the cells, the effect on processing could easily have been due to a secondary rather than a direct effect of a mutation. To determine how rapidly the processing defect occurred after temperature shift, cells were labeled between 5 and 10 minutes after the shift and the

$\beta$  subunit of the  $F_1F_0$  ATPase ( $F_1\beta$ ) was precipitated using specific antibodies. Whereas in wild-type (wt) cells all of the  $\beta$  subunit synthesized in this period was converted to the mature form, all of the detectable  $\beta$  subunit in the mutant was found in the precursor form, indicating a rapid decrease in precursor processing after the shift to the nonpermissive temperature.

To ascertain the location of the precursor in the *ssc1-2* cells, pulse-labeled cells were fractionated prior to immunoprecipitation with  $F_1\beta$ -specific antibody (Kang et al., 1990). After a 5-minute pulse, nearly all of the labeled subunit was associated with the mitochondria. However, it remained sensitive to added protease compared to matrix-localized proteins, indicating that it was associated with the mitochondrial surface.

### **B. Mitochondrial hsp70 Is Required for Translocation into the Matrix**

#### ***Functional Ssc1p Is Required for Translocation into Isolated Mitochondria***

*In vivo* experiments are limited in allowing the determination of a specific defect in the translocation process; therefore we attempted to analyze translocation in *ssc1-2* mitochondria in a cell-free system (Kang et al., 1990). Mitochondria were isolated from wt and *ssc1-2* cells grown at 23°C. Prior to use in *in vitro* translocation assays the mitochondria were incubated at 37°C for 15 minutes to inactivate *SSC1* protein. Radiolabeled precursor proteins synthesized in the presence of [<sup>35</sup>S] methionine in reticulocyte lysates were added to these isolated, energized mitochondria. Although efficient processing of the precursor proteins occurred, the proteins remained sensitive to exogenously added protease, indicating that the bulk of the proteins was not imported. In initial experiments similar results were obtained with precursors of Fe/S protein, the  $\beta$  subunit of the  $F_1F_0$  ATPase, and cytochrome *b*<sub>2</sub>, suggesting that a decrease in *SSC1* function causes a general defect in translocation.

The *in vitro* and *in vivo* results are in apparent contradiction. The *in vivo* experiments suggested a defect in translocation resulting in failure of insertion of the N-terminal targeting sequence across the inner membrane. However, the *in vitro* experiments suggested that the membrane potential-dependent insertion of the targeting sequence is not inhibited, whereas translocation of the remainder of the protein is defective. We think that the *in vitro* experiments provide a better indication of the role

of mitochondrial hsp70 in translocation. An explanation of the apparent discrepancy may be the differences in the conditions. In an *in vitro* assay, radiochemical amounts of precursor are being imported; sites of import are in vast excess over the number of precursor proteins. However, in the *in vivo* experiment cells are labeled over a period of 5 minutes, 5 minutes after the shift to the nonpermissive temperature. The amount of precursor synthesized during the labeling time exceeds the number of import sites available. Therefore if translocation were blocked as indicated by the *in vitro* experiments with precursors "stuck" in an import site, the precursors synthesized after this point would be expected to accumulate in the unprocessed form. The observed association of precursors with mitochondria is probably due to interaction with receptors or other interactions with the outer surface of the outer membrane. Precursors labeled at later times after the temperature shift were found in the cytosol, suggesting that binding sites on the mitochondrial surface were saturated.

### ***Ssc1p Interacts Directly with Precursor Proteins***

Since hsp70s were known to bind to unfolded proteins it was not unreasonable to propose that Ssc1p's role in translocation might be due to its direct association with the precursor as it enters the mitochondria. An indication of a direct association came from co-immunoprecipitation experiments (Ostermann et al., 1990). Labeled proteins that were trapped during translocation into *ssc1-2* mitochondria at the nonpermissive temperature were precipitated by Ssc1p-specific antibody. In agreement with these findings, cross-linking experiments identified Ssc1p as a protein in extremely close proximity to translocating polypeptides in wild-type mitochondria (Scherer et al., 1990).

To test the effect of the conformation of the precursor on its ability to be translocated into mutant mitochondria, the fusion protein Su9-DHFR was denatured by incubation in 8 M urea. The denatured preprotein was diluted directly into import reactions. Unlike partially folded precursor added directly from reticulocyte lysates, the import rates for the unfolded precursors into wt and *ssc1-2* mitochondria were very similar. These results suggested that Ssc1p is involved in the unfolding of preproteins during translocation into mitochondria.

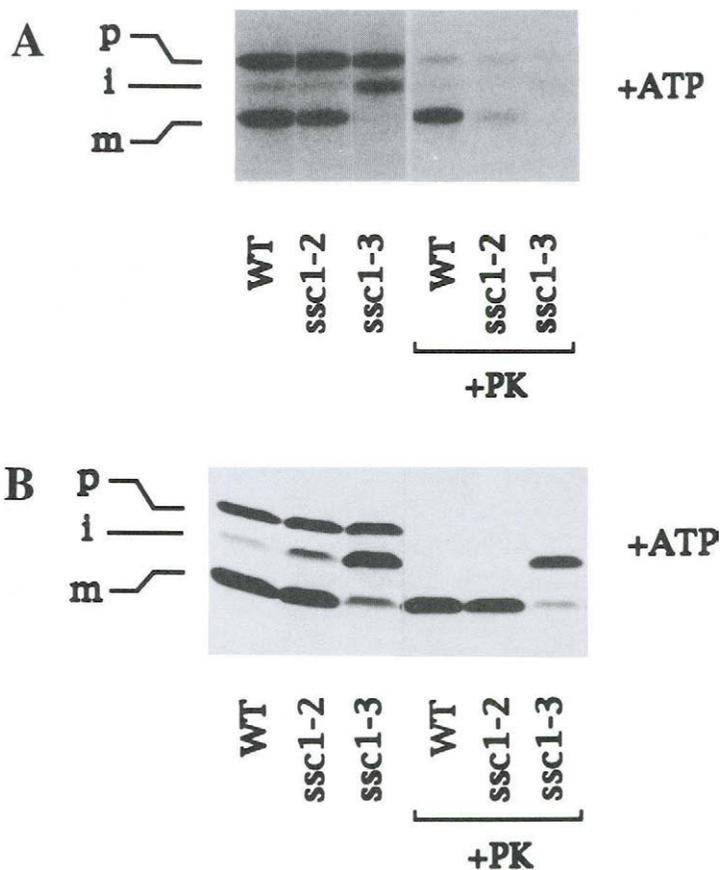
In summary: initial studies indicated that mitochondrial hsp70 was required for translocation of a number of proteins into mitochondria. Initial insertion of the targeting sequence across the outer and inner membrane is not dependent upon hsp70 function. However, the translo-

cation of the bulk of the protein across the membranes requires functional hsp70 and involves a direct interaction between the precursor protein and mitochondrial hsp70.

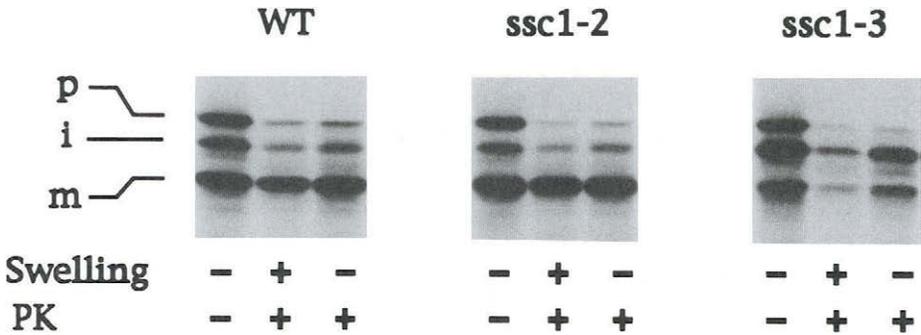
### ***Isolation and Analysis of a Second SSC1 Allele, ssc1-3***

In order to more fully understand the role of mitochondrial hsp70 in translocation, additional temperature-sensitive *SSC1* mutants were sought. One additional mutant, called *ssc1-3*, was isolated by the same procedure used in the isolation of *ssc1-2* (Gambill et al., 1993) and was found to be defective in processing precursor proteins at the nonpermissive temperature of 37°C (Fig. 1). Surprisingly, *ssc1-2* and *ssc1-3* mitochondria had different properties with regard to the translocation of the fusion protein Su9-DHFR (dihydrofolate reductase) (Fig. 2). Su9-DHFR contains the presequence plus three amino acids of the mature portion of the *Neurospora crassa* F<sub>o</sub>-ATPase subunit 9 and the entire protein coding region of murine DHFR (Pfanner et al., 1987). Unlike most presequences the Su9 presequence contains two cleavage sites for the matrix-localized protease, after amino acids 35 and 66. Wild-type mitochondria efficiently cleaved Su9-DHFR to the mature form and translocated it to a protease-resistant location. Consistent with the initial results described above, addition of Su9-DHFR to heat-treated *ssc1-2* mitochondria resulted in cleavage to the mature form, but it did not result in translocation to a protease-resistant location. However, most of the Su9-DHFR added to *ssc1-3* mitochondria was only cleaved at the first cleavage site at amino acid 35 and not translocated to a protease-resistant location. This difference in precursor translocation into mitochondria suggested that the *ssc1-3* mitochondria might be more defective in translocation than *ssc1-2* mitochondria.

To further address this difference, we compared the ability of the two types of mitochondria to import Su9-DHFR that was artificially denatured by incubation in 8 M urea (Gambill et al., 1993). Both *ssc1-2* and *ssc1-3* mitochondria efficiently translocated denatured precursor to a protease-protected location (Fig. 2). However, while the precursor was translocated across both the inner and outer membranes in *ssc1-2* mitochondria, it was translocated across only the outer membrane of the *ssc1-3* mitochondria. This was shown by subjecting the mitochondria to a hypotonic solution after import, causing rupture of the outer, but not the inner membrane (Fig. 3). After swelling, most of the protein imported into *ssc1-2* mitochondria was still resistant to exogenously added pro-



**Figure 2.** Undenatured Su9-DHFR precursor accumulates in *ssc1-2* and *ssc1-3* mitochondria in a protease-sensitive form, but after denaturation is translocated to a protease-resistant location. <sup>35</sup>S-labeled precursor of Su9-DHFR synthesized in reticulocyte lysate was incubated with isolated mitochondria (25 µg/lane) from WT, *ssc1-2*, or *ssc1-3* that had been preincubated for 15 minutes at 37°C to inactivate mutant Ssc1p. Where indicated the mitochondria were treated with proteinase K (PK) after the import reaction. The reisolated mitochondria were analyzed by SDS-PAGE and fluorography. **(A)** The lysate was added directly to the mitochondria. **(B)** The reticulocyte lysate containing the labeled precursor was precipitated with ammonium sulfate and dissolved in 8 M urea to denature it (Kang et al., 1990). The precursor was then added directly to mitochondria; the final import mix had a concentration of 200 mM urea. Reprinted (Gambill et al., 1993) with permission.



**Figure 3.** Urea-denatured Su9-DHFR is transported into the matrix of *ssc1-2* mitochondria but remains in the intermembrane space in *ssc1-3* mitochondria. Import was performed as described in Figure 2B. After import mitochondria were reisolated, a portion was diluted into a hypotonic solution (+ swelling), and a portion was diluted into an isotonic buffer containing 0.6 M sorbitol (– swelling). Where indicated (+ PK), proteinase K was added for 20 minutes after the dilution. The mitochondria were reisolated and analyzed by SDS-PAGE and fluorography. The treatment in a hypotonic solution results in the rupture of the outer but not the inner membrane, as confirmed by the localization of the intermembrane space protein cytochrome  $b_2$ , the inner membrane protein ADP/ATP carrier, and the matrix protein Ssc1p. Taken from Gambill et al. (1993) with permission.

tease, indicating that the protein had been completely translocated into the matrix. But a large portion of the protein imported into *ssc1-3* mitochondria became susceptible. Therefore in *ssc1-3* mitochondria most of the Su9-DHFR protein accumulated as an intermediate that was cleaved once and spanned from the matrix, across the inner membrane into the intermembrane space. This type of intermediate has been termed the “intermembrane space intermediate” (Hwang et al., 1989; Rassow and Pfanner, 1991; Jascur et al., 1992; Pfanner et al., 1992). Even the small amount of mature form accumulated was exposed to the intermembrane space.

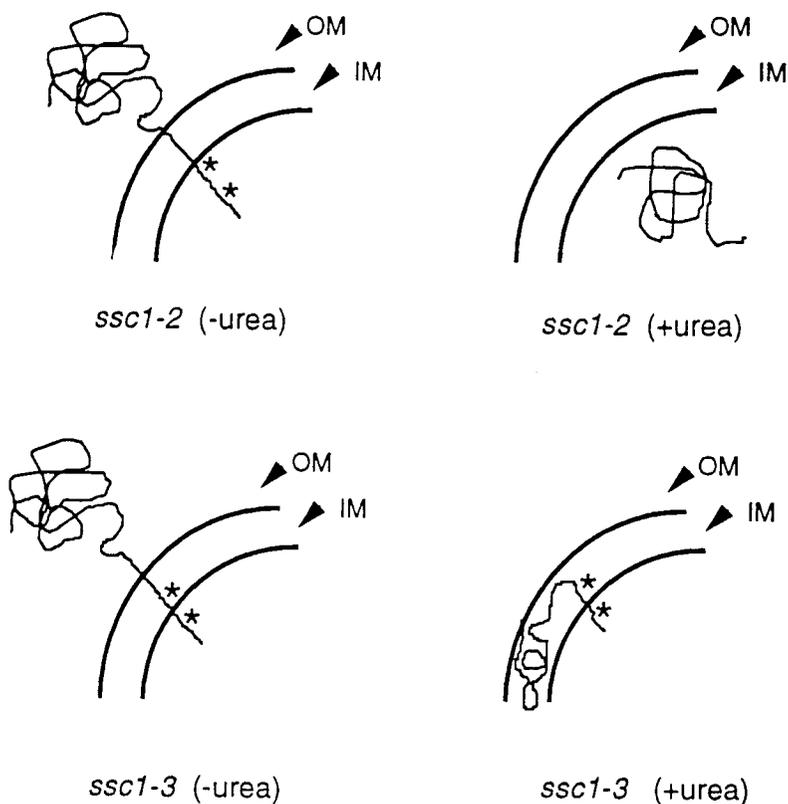
It is possible that the failure of the *ssc1-3* mitochondria to translocate Su9-DHFR across the inner membrane in these experiments was due to the refolding of the precursor prior to crossing the inner membrane into a conformation that prevented its passage. To test this possibility translocation was carried out with mitochondria whose outer membranes had been disrupted (Gambill et al., 1993). Previously Schatz and co-workers

(Hwang et al., 1989) had shown that such mitochondria, referred to as mitoplasts, were capable of translocating some preproteins. While denatured Su9-DHFR could be transported across the inner membrane of *ssc1-2* mitoplasts, almost no translocation into *ssc1-3* mitoplasts occurred. We concluded that unfolding of the polypeptide chain is not sufficient to allow translocation across the inner membrane of *ssc1-3* mitochondria. Since hsp70 function is required for translocation of proteins even when denatured, we concluded that mitochondrial hsp70 is a bonafide component of the mitochondrial translocation machinery.

### ***Comparison of the ssc1-2 and ssc1-3 Alleles***

In the *in vitro* translocation assays described above the *ssc1-3* mutant displayed a much stronger phenotype than the *ssc1-2* mutant (Fig. 4). In the *ssc1-3* mutant only the first site of cleavage of Su9-DHFR was cleaved, whereas in the *ssc1-2* mutant both were cleaved. In addition, after denaturation Su9-DHFR only traversed the outer membrane, whereas complete translocation into the matrix was observed in the *ssc1-2* mutant. These *in vitro* results were somewhat surprising to us since our initial observations of the growth phenotypes of the two strains suggested that *ssc1-3* was a “weaker” allele than *ssc1-2*. Upon the shift to 37°C *ssc1-3* is able to form small, barely visible colonies before growth ceases, whereas *ssc1-2* forms no visible colonies. *ssc1-3* cells can resume growth and form colonies at 23°C after being at 37°C for several days. While less than 1 day at 37°C prevents colony formation of *ssc1-2* at any temperature. Therefore the *ssc1-3* mutation is merely cytostatic at the nonpermissive temperature, whereas the *ssc1-2* mutation is cytotoxic.

This phenotypic difference is likely due to differences in the effect of the two mutations on the function of Ssc1p. We tested the ability of the mutant hsp70s to interact with translocating polypeptides. Urea-denatured and labeled Su9-DHFR was incubated with wt, *ssc1-2*, and *ssc1-3* mitochondria. The capacity of the mature protein to be co-immunoprecipitated by Ssc1p antibody was tested. At short incubation times, Su9-DHFR was co-immunoprecipitated in both wt and *ssc1-2* mitochondria. The immunoprecipitation was about 70% efficient as compared to precipitation with DHFR antibody in wt mitochondria and about 80% as efficient in *ssc1-2* mitochondria. Interestingly, after longer times of incubation the DHFR could no longer be precipitated by Ssc1p antibody in wt mitochondria, presumably because the DHFR protein had become properly folded. However, efficient precipitation was still observed in



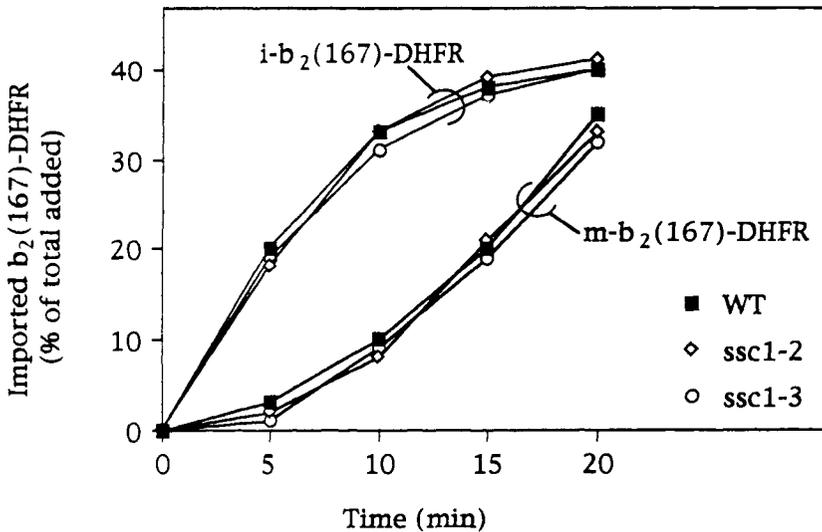
**Figure 4.** Depiction of the location of the Su9-DHFR in *ssc1-2* (A, B) and *ssc1-3* (C, D) mitochondria. As described in the text and shown in Figure 3. (A) When the precursor is not denatured by incubation in 8 M urea, both N-terminal cleavage sites of Su9-DHFR enter the matrix, but the bulk of the protein remains outside the outer membrane of *ssc1-2* mitochondria. (B) After denaturation with urea the entire protein is imported into the matrix of *ssc1-2* mitochondria. (C) In *ssc1-3* mitochondria the N-terminus of Su9-DHFR is imported to a point that allows cleavage only at the first site. (D) Upon denaturation with urea, the protein enters the intermembrane space, but no further import into the matrix is detected. Asterisk (\*) indicates the sites of cleavage of the matrix-localized processing protease. For diagrammatic purposes, cleavage is not shown.

*ssc1-2* mitochondria, suggesting that the Ssc1-2p remained bound to imported protein for a much longer time. Analysis of the interaction of Ssc1-3p with precursor was more difficult to evaluate. Only a small amount of mature protein was formed in the *ssc1-3* mitochondria and only about 20% of that imported was able to be co-immunoprecipitated with Ssc1p antibody. Thus it appears that Ssc1-3p is strongly impaired in binding Su9-DHFR, whereas Ssc1-2p fails to release from Su9-DHFR.

Although the exact effect of the two mutations on substrate protein binding and release will not be determined until purified protein is analyzed, the results from the analysis of the interaction of Su9-DHFR with the different *SSC1* proteins in isolated mitochondria suggests an explanation for the seemingly contradictory effects of the mutant proteins on translocation in the *in vitro* system and the effects on cell growth. The translocation assays suggest that *ssc1-3* mutant protein is less effective than *ssc1-2* in protein translocation into mitochondria. Our working model proposes that Ssc1-2p still has substantial affinity for substrate proteins but is also defective in releasing the substrates that are bound. This residual binding activity would be sufficient for translocation under certain conditions, such as when the precursor protein is denatured. However, the defect in release indicated by prolonged association with imported proteins could be problematic *in vivo*. After a shift to the nonpermissive temperature, failure to release from proteins after binding might well interfere with mitochondrial processes more than a simple failure to bind, causing a lethal effect. Because it could not be efficiently immunoprecipitated with precursor proteins, Ssc1-3p likely has a greatly decreased affinity for substrate proteins.

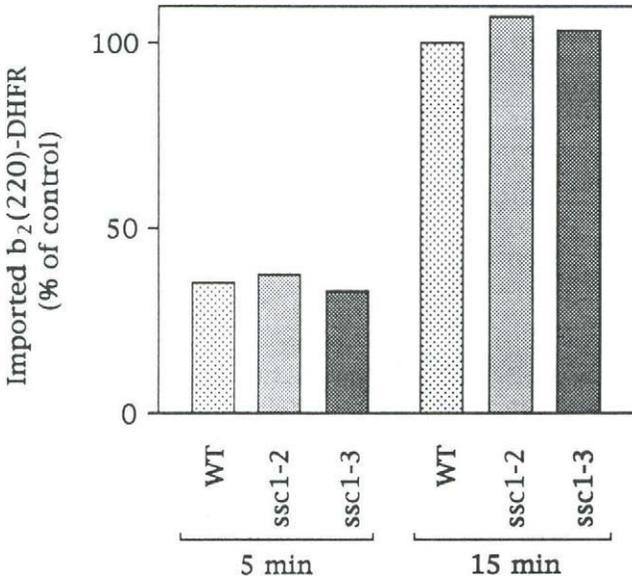
### **C. Degree of Requirement of Ssc1p Activity in Transport Is Correlated with the Conformation of the Precursor**

The results described above, concerning the failure of Su9-DHFR precursor to be imported across the inner membrane into *ssc1-3* mitochondria even when denatured by treatment with urea, indicate that Ssc1p is necessary for translocation across the inner membrane. However, the ability of urea-denatured Su9-DHFR to cross the inner membrane of *ssc1-2* mitochondria implies an effect of the structure of the precursor on its ability to be translocated. In order to better understand the role of Ssc1p on protein translocation we tested the ability of a variety of different precursors to be imported into isolated wt, *ssc1-2*, and *ssc1-3*



**Figure 5.**  $b_2(167)$ -DHFR precursor is imported into *ssc1-2* and *ssc1-3* mitochondria as well as into wt mitochondria. Import experiments using  $b_2(167)$ -DHFR precursor were carried out as described in Figure 2. After the import reaction was carried out for various times, the mitochondria were treated with proteinase K to degrade any protein on the mitochondrial surface and were reisolated and analyzed by SDS-PAGE and fluorography. Taken with permission from Voos et al. (1993).

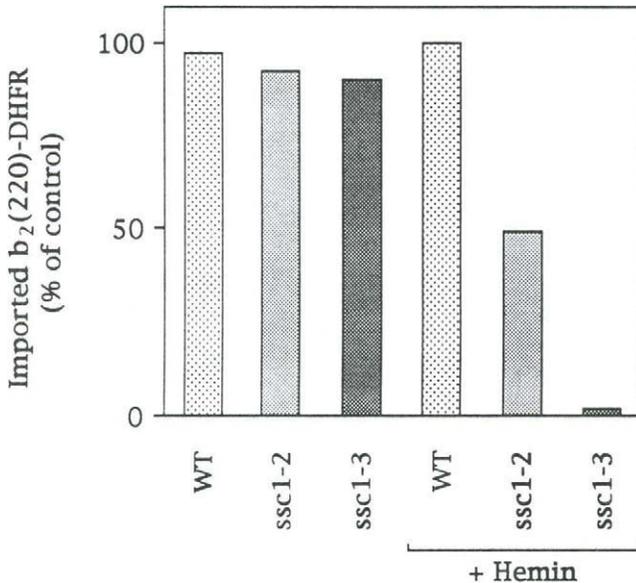
mitochondria (Voos et al., 1993). Surprisingly, we found a precursor that was imported into the three types of mitochondria with nearly identical efficiency (Fig. 5). The precursor,  $b_2(167)$ -DHFR, consisted of 167 N-terminal residues of the yeast cytochrome  $b_2$  protein and the entirety of murine DHFR. The 167 residues of the cytochrome  $b_2$  protein included 80 residues of the presequence followed by 87 residues of the mature protein. Cytochrome  $b_2$  is located in the intermembrane space; the 80 residues of the presequence contain the information for targeting to this compartment. In the process of import the precursor is cleaved once by the matrix processing protease to give an intermediate-sized form and then a second time on the outer side of the inner membrane by inner membrane protease I to yield the mature form. The processing of  $b_2(167)$ -DHFR into the intermediate and mature forms occurred at the same rate in wt, *ssc1-2*, and *ssc1-3* mitochondria that had been incubated at 37°C for 15 minutes prior to import to inactivate the mutant hsp70s.



**Figure 6.** Urea-denatured  $b_2(220)$ -DHFR is imported with similar efficiency into WT, *ssc1-2* and *ssc1-3* mitochondria. Reticulocyte lysate containing  $^{35}\text{S}$ -labeled  $b_2(220)$ -DHFR was precipitated with ammonium sulfate and dissolved in 8 M urea. The denatured precursor was diluted 40-fold into the import reaction containing mitochondria. Import into mitochondria was performed for 5 or 15 minutes, as described in Figure 2. Taken from Voos et al. (1993) with permission.

In all cases the mature form was protected against digestion with exogenous protease; however, it was susceptible to digestion after incubation with hypotonic buffer, indicating that the imported protein was appropriately localized to the intermembrane space.

We had previously observed that the import of authentic cytochrome  $b_2$  into *ssc1-2* mitochondria was partially inhibited (Kang et al., 1990). We repeated this result and determined that import of the full-length cytochrome  $b_2$  protein into *ssc1-3* mitochondria was almost completely blocked. To track down the reason for the difference in the translocation of authentic cytochrome  $b_2$  and the  $b_2$  167-DHFR fusion, we tested other fusions that contained larger portions of cytochrome  $b_2$ . It was possible that the DHFR moiety conferred the *SSC1* independence. However, when we tested two other fusions that contained larger portions (220 and 330 amino acids) of cytochrome  $b_2$  linked to DHFR we found that their



**Figure 7.** Import of  $b_2(220)$ -DHFR into *ssc1-2* and *ssc1-3* mitochondria is inhibited in the presence but not the absence of hemin. Rabbit reticulocyte lysates were prepared in the presence of 3'5' cyclic AMP rather than hemin. Imports were carried out as described in Figure 1. Where indicated (+ hemin), hemin was added to a final concentration of 10  $\mu$ M. Taken from Voos et al. (1993) with permission.

import into *ssc1-2* and *ssc1-3* mitochondria was inhibited (Voos et al., 1993). These results suggested that sequences between 167 and 220 conferred dependence on Ssc1p.

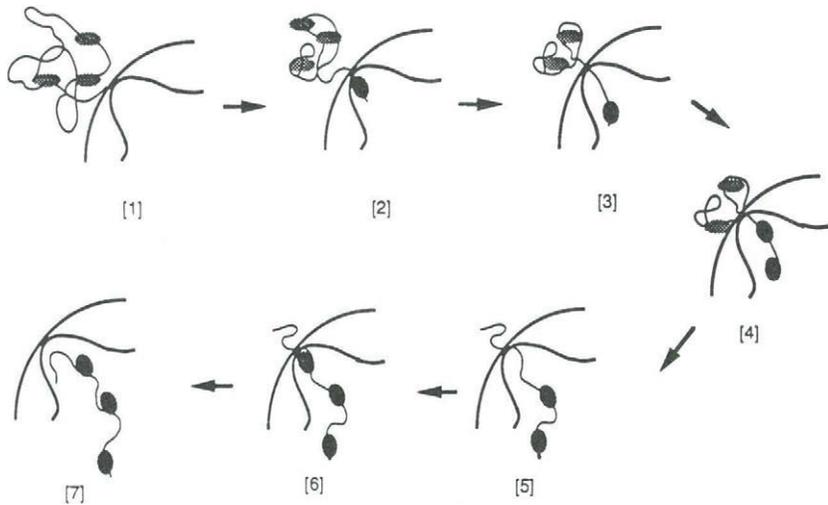
To test if the conformation of the preprotein was causing the hsp70-dependence, the  $b_2(220)$ -DHFR precursor was denatured in urea prior to addition to mitochondria. The denatured precursor was translocated into the three types of mitochondria at nearly identical rates, indicating that the conformational state of the longer fusion and the authentic cytochrome  $b_2$  prevented translocation (Fig. 6). Cytochrome  $b_2$  binds heme noncovalently; the heme-binding domain is located within the first 99 amino acids of the mature form of the protein. The  $b_2(167)$ -DHFR fusion, that is translocated into mutant mitochondria contains only 87 amino acids of the mature protein, whereas  $b_2(220)$ -DHFR includes the entire heme-binding domain. Since reticulocyte lysates contain hemin we thought that the heme might bind to cytochrome  $b_2$  and stabilize the

conformation of the heme-binding domain such that it would require unfolding in order to be translocated into the mitochondria. To test this idea, the  $b_2(220)$ -DHFR fusion was synthesized in a reticulocyte lysate that contained 3'5' cyclic AMP rather than hemin (Ernst et al., 1976; Nicholson et al., 1987). Indeed, the fusion was imported into *ssc1-2* and *ssc1-3* mitochondria as efficiently as into wt mitochondria (Fig. 7). Furthermore, addition of hemin to lysates that lacked hemin inhibited the translocation into the mutant mitochondria. Therefore we concluded that the dependence of cytochrome  $b_2$  and the fusions with DHFR are dependent upon mitochondrial hsp70 in the *in vitro* translocation assays because of the conformation of the precursor protein formed in the reticulocyte lysates.

#### D. Model of hsp70 Action in Translocation Across Mitochondrial Membranes

Putting together the data collected from analysis of the *ssc1-2* and *ssc1-3* mitochondria, we have concluded that mitochondrial hsp70 is required for the translocation of precursor proteins into the mitochondrial matrix. However, the degree of dependence, at least *in vitro*, varies among different precursors. Some precursors require a low level of hsp70 action, as evidenced by their ability to be imported into *ssc1-2* mitochondria. This is particularly evident in the complete translocation of denatured precursors into *ssc1-2* but not into the more severely affected *ssc1-3* mitochondria. This lack of translocation of denatured precursors into *ssc1-3* mitochondria indicates that mitochondrial hsp70 is a critical component of the mitochondrial translocation machinery. However, the greater dependence of undenatured precursors on mt-hsp70 indicates that it also plays a role in the unfolding of the precursor on the cytosolic side of the membranes.

How might mt-hsp70 act in the translocation and unfolding of a precursor protein? We propose a simple model (Fig. 8) that attributes action of mt-hsp70 in translocation and unfolding to its well-established peptide-binding activity. After translocation of the leader sequence across the outer and inner membranes, a process dependent upon a membrane potential and the targeting signal, mt-hsp70 binds to the precursor protein. In the simplest form of the model the movement of the protein across the membranes may simply be the result of Brownian motion. However, the binding of mt-hsp70 would prevent the movement of the translocating polypeptide back toward the cytosol by steric hin-



**Figure 8.** Model for mt-hsp70 action during translocation of precursor proteins into the matrix of mitochondria. (1) The precursor protein binds to import receptors on the outer surface of the mitochondria. c-hsp70 may be bound to the partially folded precursor. (2) The membrane potential-dependent step of import, the translocation of the N-terminus of the protein, occurs. m-hsp70 binds to the precursor and prevents any movement back toward the cytosol. (3) By Brownian motion (and possibly because of the action of some proteins) the precursor protein moves further into the matrix. (4) Another m-hsp70 binds to a site more toward the C-terminus of the protein and prevents movement back toward the cytosol. (5) More of the protein moves into the matrix as described in (3). (6) Another m-hsp70 binds as described in (4). (7) The entire protein moves into the matrix and translocation is complete. See text for discussion of the action of c-hsp70 versus m-hsp70.

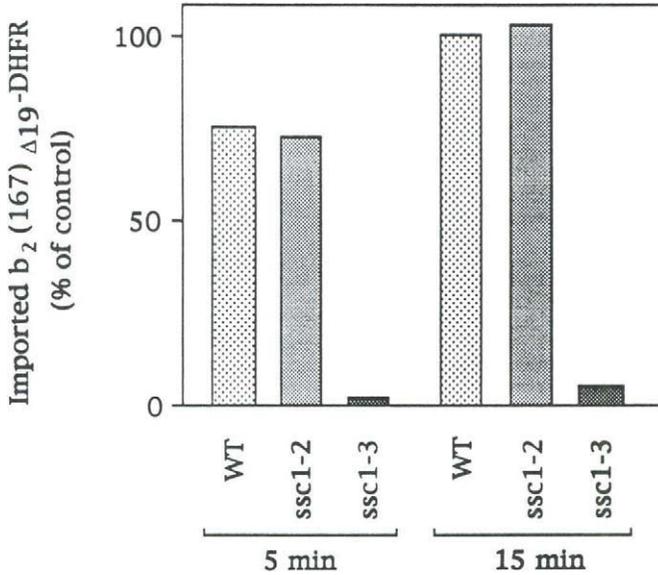
drance. As more of the protein is exposed in the matrix, additional high-affinity binding sites for mt-hsp70 are exposed, and binding to these sites prevents movement back toward the cytosol. In a more complex model components of the translocation machinery in the membrane, such as MIM44, MIM23, and MIM17, could also, in part, provide the driving force of translocation (Dekker et al., 1993; Blom et al., 1993; Emtage and Jensen, 1993).

Although this notion of binding of mt-hsp70 providing the driving force of translocation is fairly straightforward, how binding of an hsp70 inside the mitochondria could affect the “unfolding” of a protein on the

cytosolic side is more difficult to understand. Translocation may be a dynamic process, a competition between unfolding on the cytosolic side and translocation into the matrix. The secondary and tertiary structures of the precursor could sterically inhibit the translocation process. A partially folded protein in the act of translocation, perhaps bound to cytosolic chaperones, likely undergoes slight changes in conformation that expose unfolded regions. The higher the degree of structure on the cytosolic side, perhaps the greater the need for binding of mt-hsp70. If a protein is denatured, a partially active mt-hsp70 with a lower affinity may be sufficient. Such a model predicts a competition between cytosolic and mitochondrial hsp70s since translocation into the matrix occurs, binding to the mitochondrial form is favored over binding to the cytosolic form. This could be accomplished in several ways. For example, there may be an inherent difference in binding affinities between the hsp70s or the availability of binding sites may be different on opposite sides of the membranes. If the preprotein is in a partially folded form on the cytosolic side but in an unfolded form as it enters the matrix, there would be inherent differences in sites exposed for chaperone binding. Since hsp70s are thought to have a higher affinity for sequences rich in hydrophobic residues and since these are more exposed in unfolded proteins and buried in folded ones, mt-hsp70 may very well have available higher-affinity binding sites than cytosolic hsp70. According to this model, the role of mt-hsp70 in translocation and unfolding would mechanistically be the same, but an hsp70 with a lower binding activity would be sufficient to drive the translocation of a denatured protein, but not of a protein with substantial tertiary structure.

### **E. Requirements for Mitochondrial hsp70 in the Translocation of Proteins into the Matrix Compared to the Intermembrane Space**

In our analysis of the dependence of precursor proteins on mt-hsp70 we tested the import of shorter cytochrome  $b_2$ -DHFR fusions (Voos et al., 1993). While fusions containing 151 and 84 amino acids of the cytochrome  $b_2$  precursor were imported as efficiently into mutant mitochondria as wt mitochondria, fusions containing 55 or 47 amino acids were imported efficiently into wt and *ssc1-2* mitochondria but not *ssc1-3* mitochondria (Fig. 9). The sorting signal for the intermembrane space, a hydrophobic region preceded by a positively charged region, is located in the second half of the presequence, prior to amino acid 80 (van Loon



**Figure 9.**  $b_2(167)_{\Delta 19}$ -DHFR is inhibited in import into *ssc1-3*, but not *ssc1-2* mitochondria. The precursor of  $b_2(167)_{\Delta 19}$ -DHFR was imported into isolated mitochondria as described in Figure 2.

et al., 1986; Hartl et al., 1987; Koll et al., 1992; Glick et al., 1992). It seemed likely that the reason for the lack of import of the short fusions into *ssc1-3* mitochondria was due to the lack of a functional intermembrane space sorting signal. This idea was supported by the lack of import of a  $b_2(167)$ -DHFR fusion having a deletion between amino acids 47 and 65, which is known to prevent sorting of the inner membrane space (Koll et al., 1992) and which also was not imported into *ssc1-3* mitochondria. In fact, in *ssc1-3* mitochondria the first cleavage of this fusion did not occur even if the precursor was first treated with urea, whereas cleavage and translocation to a protease-resistant location occurred in wt and *ssc1-2* mitochondria.

$b_2(55)$ -DHFR,  $b_2(47)$ -DHFR, and  $b_2(167)_{\Delta 19}$  DHFR are known to be translocated into the matrix of the mitochondria because they lack a functional intermembrane space sorting signal. Therefore from the work discussed above, it is not surprising that their translocation into the matrix is dependent on Ssc1p. However, the dependence of authentic cytochrome  $b_2$  and the longer DHFR fusions on Ssc1p is more provocative since the sorting pathway of cytochrome  $b_2$  is currently the subject

of controversy. According to the “stop-transfer” hypothesis, targeting to the inner membrane space is caused by the arrest of the translocating polypeptide in the inner membrane. In other words, the sorting signal prevents the protein from entering the matrix. If this model is correct in its simplest form it would not be at all surprising if cytochrome  $b_2$  import was independent of hsp70 and if deletion of the sorting signal rendered import hsp70-dependent, since the protein would be imported into the matrix. However, the longer fusions and authentic cytochrome  $b_2$  are dependent on hsp70, as described above because of the conformation of the heme-binding domain. According to the “stop-transfer” model (Glick et al., 1992a,b), Ssc1p could only interact with the extreme N-terminus of the preprotein, but by this interaction it would be able to facilitate the unfolding of the protein on the other side of the membrane. Although this possibility cannot be excluded, it is easier for us to imagine this being accomplished by interaction of the protein with hsp70 along its entire length.

Along these lines, the original “conservative sorting” model proposed (Hartl et al., 1987) that cytochrome  $b_2$  was imported entirely into the matrix and then reexported to the inner membrane space. A revision of the model proposed recently (Koll et al., 1992) suggests that the import and export steps are coupled and that a sorting component may recognize the sorting signal and redirect translocation back across the inner membrane. Perhaps if a precursor protein has little tertiary structure then the import does not require hsp70; that is, interaction with the intermembrane space sorting machinery may be sufficient for import. However, if the conformation of the preprotein is more of a detriment to import, the additional action of hsp70 may be required, as it is for matrix-bound proteins.

#### **F. Components Other Than Mitochondrial hsp70 Required for Protein Movement across the Outer and Inner Membranes**

From the results described above it appears that mt-hsp70 is required for translocation of preproteins across the inner membrane regardless of the conformation of the precursor protein. However, in the mutant with very low mt-hsp70 activity proteins could be translocated across the outer membrane into the intermembrane space if they were denatured prior to addition to mitochondria. This dependence on denaturation to cross the outer membrane suggests that there is a component of the translocation machinery in addition to the hsp70 in the matrix that is

required for translocation across the outer membrane. This component may well reside in the intermembrane space and act in conjunction with mt-hsp70. This same component or another may also be involved in the translocation of proteins destined for the intermembrane space as well as those translocated into the matrix. This may explain the independence of certain precursors, such as  $b_2(167)$ -DHFR, on Ssc1p function. In cases where there is little conformational restriction the second component may be sufficient for translocation, whereas other precursors with more tertiary structure may require both components.

#### IV. ROLE OF ATP IN PROTEIN TRANSLOCATION

It has been appreciated for some time that ATP is required for the translocation of proteins into the mitochondrial matrix. Although some proteins require ATP on the outside of the outer membrane, all precursors tested require ATP inside mitochondria (Hwang and Schatz, 1989; Neupert et al., 1990; Manning-Krieg et al., 1991). Since hsp70s are ATP binding proteins and ATP hydrolysis is required for cycles of peptide binding and release, it is reasonable to propose that ATP is required because mt-hsp70 is required for translocation. Although there may be other requirements for ATP in the translocation and folding processes in the matrix, the close similarity between the effects of ATP depletion and lack of hsp70 function on translocation strongly supports the idea that ATP is required for hsp70 action *in vivo*, as described below. ATP levels were lowered by treating isolated mitochondria with apyrase and inhibition of the mitochondrial ATP-synthase with oligomycin (Voos et al., 1993). Upon ATP depletion both wt and *ssc1-2* mitochondria accumulated the intermediate form of Su9-DHFR in a protease-accessible form, just as did *ssc1-3* mitochondria in the presence of ATP. In addition, after ATP depletion the Su9-DHFR precursor could not be co-immunoprecipitated with Ssc1p antibody in wt or *ssc1-2* mitochondria, just as in *ssc1-3* mitochondria in the presence of ATP.

Although there is agreement in the literature that cycles of peptide binding and release require ATP hydrolysis, the mechanistic details of this cycle are not yet resolved. In some studies ADP-bound hsp70 has a higher affinity for peptide binding (Palleros et al., 1991), whereas in others ATP is required for the stabilization of the hsp70-protein complex when other accessory proteins such as DnaJ or GrpE are present (Langer et al., 1992; Georgopoulos, 1992). The failure of Ssc1p to bind the precursor protein in the absence of ATP (Manning-Krieg et al., 1991;

Gambill et al., 1993) indicates that in at least this *in vivo*-like situation, the ATP-bound form is required for efficient binding.

## V. ROLE OF hsp70 OF THE ENDOPLASMIC RETICULUM IN PROTEIN TRANSLOCATION

As with *SSC1*, temperature-sensitive alleles have been exploited to analyze the function of the hsp70 of the ER, Kar2p. When shifted to the nonpermissive temperature, *kar2-159* strains accumulate the precursor forms of several proteins normally translocated across the ER membrane, indicating a direct involvement in protein translocation (Vogel et al., 1990). An *in vitro* system was used to assess more directly the role of Kar2p in the process (Sanders et al., 1992). A translocation intermediate that becomes jammed during transit was used to freeze the translocation apparatus, allowing a study of its components. An association between the jammed precursor and Sec61p was detected, indicating that the precursor interacts directly with Sec61p. Sec61p, an integral membrane protein, is known to be critical for protein translocation and proposed to be a critical component of the translocation apparatus. The effect of three different *KAR2* alleles on the translocation of the precursor and its interaction with Sec61 was determined as a function of the temperature in the *in vitro* system. Microsomes were prepared from the three mutant strains grown at 23°C, and translocation assays were carried out over a range of temperatures. Although the alleles showed different levels of activity all showed decreased activity at higher temperatures, and microsomes from wt cells were unaffected. Two of the alleles, *kar2-113* and *kar2-159*, resulted in a severe reduction in complex formation. However, the third allele, *kar2-203*, did not significantly reduce the formation of the Sec61p-precursor complex. The authors suggested that this difference indicates that Kar2p acts at two points in the translocation process, in the formation of the Sec61p-precursor complex and in some undefined event important for translocation that occurs after the Sec61p-precursor interaction.

As is the case with Ssc1p, immunoprecipitation experiments indicate a direct interaction of Kar2p and the precursor protein. This interaction is consistent with results from mammalian cells showing a transient interaction between proteins entering the ER and the mammalian equivalent of Kar2p, BiP (reviewed in Gething and Sambrook, 1992).

From these experiments the roles of Ssc1p in the mitochondria and Kar2p in the ER appear to be similar. Both bind to proteins as they emerge

into the organelle. Both are needed for translocation for a variety of proteins and are thus involved in general protein translocation. Although the extent of the parallels between the roles of the two hsp70s remains to be seen, this initial work suggests that hsp70 function may be important for the biogenesis of proteins in other organelles and perhaps the cytosol as well. The *SSB* cytosolic hsp70s bind to translating ribosomes and are important for efficient translation. Evidence suggests that *SSB* proteins may interact with the nascent chain on the ribosome. Exit from the ribosome into the cytosol may be similar to entering organelles after traversing the mitochondrial or ER membranes, a process facilitated by hsp70s.

## VI. SUMMARY

In summary, mitochondrial hsp70 is a critical component of the apparatus required for translocation across the inner membrane of mitochondria. hsp70 function is required even for the translocation of denatured proteins. However, in the case of precursors with secondary and tertiary structure there is a greater requirement for hsp70 function, presumably because of the requirement for at least partial unfolding prior to translocation across the membranes. These studies concentrated on the role of hsp70 in the translocation process. However, clearly hsp70 does not act alone. In bacteria, hsp70 (DnaK) acts with two other proteins, DnaJ and GrpE. It will be of interest to analyze the function of homologous proteins in mitochondrial import. In addition, components of the inner membrane and the intermembrane space likely play critical roles. In the next few years an understanding of the interplay among these components of the translocation apparatus should be attained.

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