Presequence and Mature Part of Preproteins Strongly Influence the Dependence of Mitochondrial Protein Import on Heat Shock Protein 70 in the Matrix

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Abstract. To test the hypothesis that 70-kD mitochondrial heat shock protein (mt-hsp70) has a dual role in membrane translocation of preproteins we screened preproteins in an attempt to find examples which required either only the unfoldase or only the translocase function of mt-hsp70. We found that a series of fusion proteins containing amino-terminal portions of the intermembrane space protein cytochrome b_2 (cyt. b_2) fused to dihydrofolate reductase (DHFR) were differentially imported into mitochondria containing mutant hsp70s. A fusion protein between the amino-terminal 167 residues of the precursor of cyt. b_2 and DHFR was efficiently transported into mitochondria independently of both hsp70 functions. When the length of the cyt. b_2 portion was increased and included the heme binding domain, the fusion protein became dependent on the unfoldase function of mt-hsp70, presumably caused by a conformational restriction of the heme-bound preprotein. In the absence of heme the noncovalent heme binding domain in the longer fusion proteins no longer conferred a dependence on the unfoldase function. When the cyt. b_2 portion of the fusion protein was less than 167 residues, its import was still independent of mt-hsp70 function; however, deletion of the intermembrane space sorting signal resulted in preproteins that ended up in the matrix of wild-type mitochondria and whose translocation was strictly dependent on the translocase function of mt-hsp70. These findings provide strong evidence for a dual role of mt-hsp70 in membrane translocation and indicate that preproteins with an intermembrane space sorting signal can be correctly imported even in mutants with severely impaired hsp70 function.

THE import of proteins into mitochondria is a complex, multi-step process. In recent years, a number of components of the mitochondrial import machinery have been identified. Among them are six gene products which are essential for the growth of *Saccharomyces cerevisiae*: the outer membrane protein ISP42/MOM38 that forms part of the general insertion pore (Baker et al., 1990; Kiebler et al., 1990); the inner membrane protein Mpilp that seems to represent part of the inner membrane translocation machinery (Maarse et al., 1992); the 70-kD heat shock protein (hsp70)[1] in the matrix (termed Ssclp in yeast) that is involved in translocation and folding of proteins (Craig et al., 1987, 1989; Kang et al., 1990; Scherer et al., 1990); hsp60 in the matrix, the central component of the (re)folding machinery (Cheng et al., 1989; Ostermann et al., 1989; Horwich, 1990); and the two subunits of the mitochondrial processing peptidase (Hawlitschek et al., 1988; Pollock et al., 1988; Witte et al., 1988; Yang et al., 1988).

Of all soluble essential components, mitochondrial hsp70 (mt-hsp70) is the only one that seems to have a direct role in membrane translocation of preproteins. mt-hsp70 binds to the polypeptide chain in transit across mitochondrial outer and inner membranes (Kang et al., 1990; Ostermann et al., 1990; Scherer et al., 1990; Manning-Krieg et al., 1991). By using two temperature-sensitive yeast mutants of mt-hsp70 (*sscl-2* and *sscl-3*) we provided evidence that mt-hsp70 has a dual role in membrane translocation of preproteins (Gambill et al., 1993). (*a*) It facilitates unfolding of the polypeptide chain. Mutant mitochondria that are only defective in the "unfoldase function" (*sscl-2*) are able to completely import an unfolded preprotein. (*b*) It is a genuine component of the inner membrane translocation machinery and thereby is essential for the completion of preprotein translocation into the matrix, independently of the folding state of the polypeptide chain ("translocase function"). *Sscl-3* mitochondria were found to be affected in both, the unfoldase and the translocase functions of mt-hsp70 (Gambill et al., 1993).

We have attempted a detailed test of this hypothesis. We tried to find preproteins which depended only on the unfol-

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

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dase function or the translocase function of mt-hsp70 to ask which properties of the preproteins influenced the hsp70 dependence. This was possible with a series of preproteins derived from the precursor of cytochrome b_2 that is targeted to the intermembrane space by a bipartite presequence of 80 residues, consisting of a matrix-targeting signal and an intramitochondrial sorting signal. The distinct requirements for hsp70 could be correlated with the presence of intramitochondrial sorting signals and conformational restrictions of the preproteins that were fully consistent with the proposed hypothesis. Surprisingly, some of the preproteins did not require a functional mt-hsp70 at all. The implications of these findings on the role of mt-hsp70 in preprotein sorting to the intermembrane space are discussed.

Materials and Methods

Published procedures were used for the following: growth of *Saccharomyces cerevisiae* wild-type and *sscl-2* and *sscl-3* mutant strains, and isolation of mitochondria (Daum et al., 1982; Hartl et al., 1987; Kang et al., 1990, Gambill et al., 1993); synthesis of cytochrome b_2 and b_2-dihydrofolate reductase (DHFR) fusion proteins in rabbit reticulocyte lysates in the presence of [^{35}S]methionine (Rassow et al., 1989, 1990; Pfanner et al., 1990; Koll et al., 1992); incubation of energized mitochondria (50 μg protein; preincubated for 15 min at 37°C) with reticulocyte lysate at 25°C in the presence of BSA-buffer (with 3% [wt/vol] BSA) (Kang et al., 1990; Söllner et al., 1991); treatment with proteinase K (40–75 μg/ml) (Pfanner and Neupert, 1987); analysis by SDS-PAGE, fluorography, laser densitometry and Western blotting (Kang et al., 1990; Söllner et al., 1991).

Results

Import of the Fusion Protein b_2(167)-DHFR Does Not Require a Functional mt-hsp70

To find a preprotein that showed a low dependence on mt-hsp70, we tested the import of a number of authentic and artificial preproteins into isolated mitochondria from wild-type yeast, *sscl-2* and *sscl-3* mutants. Surprisingly, we found a preprotein that was imported into both types of mutant mitochondria with the same efficiency as into wild-type mitochondria. The precursor, called b_2(167)-DHFR, consisted of the 167 amino-terminal amino acid residues (the 80-residue presequence and 87 residues of the mature protein part) of the precursor of yeast cytochrome b_2 and the entire mouse DHFR (Rassow et al., 1989). In our experiments, this fusion protein was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated at 25°C with isolated energized mitochondria, that has been preincubated for 15 min at 37°C, leading to induction of the mt-hsp70 deficiency in the mutants (Kang et al., 1990; Gambill et al., 1993). Fig. 1, *A* and *B* shows that the rate of import of b_2(167)-DHFR was indistinguishable in the three kinds of mitochondria. It was processed in two steps, first to an intermediate-sized form by the matrix-localized processing peptidase (Hawlitschek et al., 1988; Yang et al., 1988) and then to the mature form by the inner membrane protease I whose catalytic activity resides on the intermembrane space side (Schneider et al., 1991). The imported mature-sized protein was protected against digestion by protease added to the mitochondria. However, it was accessible to added protease after opening of the intermembrane space by a mild swelling (Fig. 1 *C*), indicating that it was correctly located in the intermembrane space.

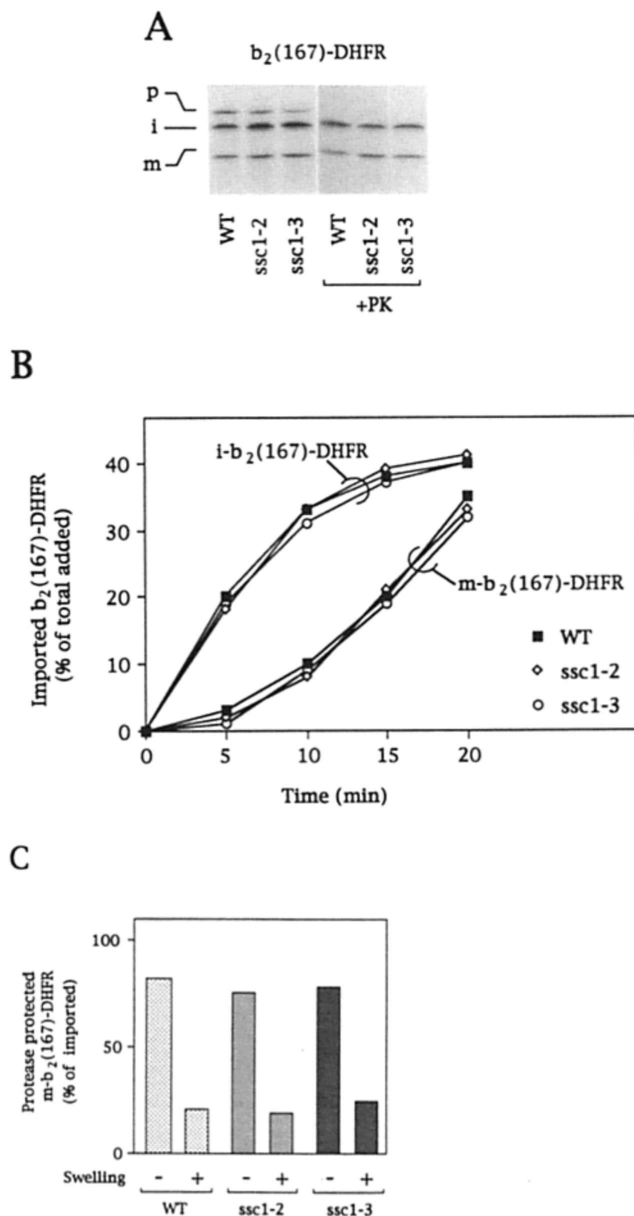

Figure 1. b_2(167)-DHFR is imported into isolated *sscl-2* and *sscl-3* mutant mitochondria. (*A* and *B*) b_2(167)-DHFR was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with isolated energized mitochondria from wild-type (*WT*) yeast or the *sscl-2* and *sscl-3* mutant strains for 15 min (*A*) or the indicated times (*B*) at 25°C. The mitochondria had been preincubated for 15 min at 37°C. After the import reaction, treatment with proteinase K (*B*, and *A* where indicated) was performed (see Materials and Methods). The mitochondria were reisolated and analyzed by SDS-PAGE, fluorography, and laser densitometry. In *B* the total amount of precursor added to the import reaction was set to 100%. (*C*) Imported b_2(167)-DHFR is located in the intermembrane space. b_2(167)-DHFR was imported as described above. The mitochondria were reisolated and resuspended in SEM-buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2). Half of the samples was diluted 10-fold in 25 mM Hepes/KOH, pH 7.4 (*swelling*), the other half in 25 mM Hepes/KOH, pH 7.4, 0.6 M sorbitol. Then treatment with proteinase K was performed for 15 min at 0°C. The organelles were reisolated and analyzed as described above. The amount of m-b_2(167)-DHFR obtained in parallel samples without protease treatment was set to 100%. *p*, precursor; *i*, intermediate; *m*, mature.

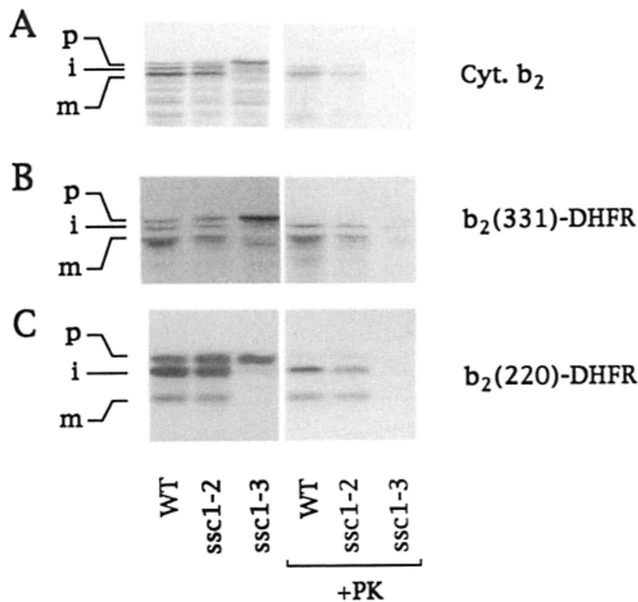

Figure 2. Import of authentic cytochrome b_2 and long b_2-DHFR fusion proteins into *sscl-2* and *sscl-3* mitochondria is inhibited. The precursors of cytochrome b_2 (*A*), b_2(331)-DHFR (*B*) or b_2(220)-DHFR (*C*) (Pfanner et al., 1990) were imported into isolated mitochondria for 10-15 min at 25°C and analyzed as described in the legend to Fig. 1. Quantitation of the amount of protein (p + i + m) imported into *sscl-2* mitochondria (+ proteinase K) revealed 28% for cytochrome b_2, 32% for b_2(331)-DHFR, and 44% for b_2(220)-DHFR (WT set to 100%).

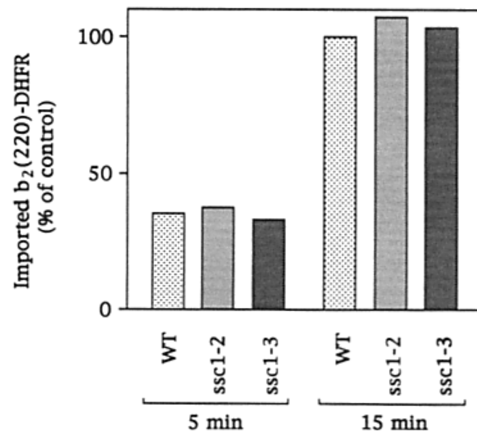

Figure 3. Urea-denatured b_2(220)-DHFR is imported with similar efficiency into wild-type, *sscl-2* and *sscl-3* mitochondria. Reticulocyte lysate containing b_2(220)-DHFR was precipitated with ammonium sulphate (66% saturation) and dissolved in 8 M urea, 10 mM DTT, 30 mM MOPS, pH 7.4, and diluted 40-fold into the import assay. Import into isolated mitochondria was performed for 5 or 15 min at 25°C as described in the legend of Fig. 1.

The import of b_2(167)-DHFR, as well as precursors shown to require mt-hsp70 function (Kang et al., 1990; Gambill et al., 1993), strictly depends on a membrane potential (Rassow et al., 1989; Martin et al., 1991). Therefore, the import defects seen in *sscl-2* and *sscl-3* mitochondria with preproteins other than b_2(167)-DHFR are not caused by a dissipation of $\Delta\Psi$. In subsequent experiments we attempted to unravel the properties of b_2(167)-DHFR that rendered its import independent of mt-hsp70.

Fusion Proteins Containing a Long Cytochrome b_2 Portion Require the Unfoldase Function of mt-hsp70

The processing of the authentic cytochrome b_2 precursor and its transport to a protease-protected location was partially inhibited in *sscl-2* mitochondria (Fig. 2 *A*; Kang et al., 1990) and almost completely inhibited in *sscl-3* mitochondria (Fig. 2 *A*). These results exclude the possibility that translocation of all preproteins with the targeting and sorting signals of cytochrome b_2 is independent of functional mt-hsp70.

It was thus possible that the presence of the DHFR-moiety conferred the independence of mt-hsp70. We used the fusion protein b_2(331)-DHFR in which the carboxyl-terminal portion of the authentic cytochrome b_2 precursor beyond residue 331 has been replaced by DHFR (Pfanner et al., 1990) to test this possibility. The import of b_2(331)-DHFR was inhibited in both *sscl-2* and *sscl-3* mitochondria (Fig. 2 *B*), suggesting that sequences between 167 and 331 of cytochrome b_2 were involved in conferring mt-hsp70 dependence of translocation. To further delimit the region, the translocation of a fusion protein containing 220 amino acid residues of cytochrome b_2 (b_2[220]-DHFR) was analyzed (Fig. 2 *C*). Import of b_2(220)-DHFR was inhibited, indicating that sequences between residues 167 and 220 of the preprotein were required for the mt-hsp70 dependence.

We then asked which of the functions of mt-hsp70 was needed by the longer preproteins. A requirement for the unfoldase function, but not for the translocase function, can be circumvented by artificially unfolding the preprotein by preincubating in 8 M urea (Gambill et al., 1993). Fig. 3 shows that the import of denatured b_2(220)-DHFR into *sscl-2* and *sscl-3* mitochondria occurred with the same efficiency as the import into wild-type mitochondria. The import of b_2(220)-DHFR thus mainly depended on the unfoldase function of mt-hsp70.

What is the reason for the conformational restriction of b_2(220)-DHFR? The domain of cytochrome b_2 that binds one heme noncovalently is located within the first 99 amino acids of the mature protein (Zia and Mathews, 1990). The b_2(167)-DHFR fusion protein contains only 87 residues of the mature protein and thus does not contain a functional heme binding domain. However, b_2(220)-DHFR and the larger fusions contain complete heme binding domains. As hemin is present in the reticulocyte lysate system, its binding to the longer preproteins may stabilize the conformation of the preprotein and therefore could explain a strong requirement for unfolding during membrane translocation. To test this directly, b_2(220)-DHFR was synthesized in a rabbit reticulocyte lysate that was prepared in the presence of cAMP instead of hemin (Ernst et al., 1976; Nicholson et al., 1987). Fusion protein synthesized in hemin-depleted lysate was imported into *sscl-2* and *sscl-3* mitochondria (Fig. 4). Readdition of hemin inhibited its import (Fig. 4). The noncovalent binding of heme to the preprotein thus seems to lead to a conformational restriction of the polypeptide which renders its import dependent on the unfoldase function of fully active hsp70. It is interesting to note that the mitochondrial import of δ-aminolevulinate synthase was also found to be inhibited by addition of hemin. In this case, the inhibition

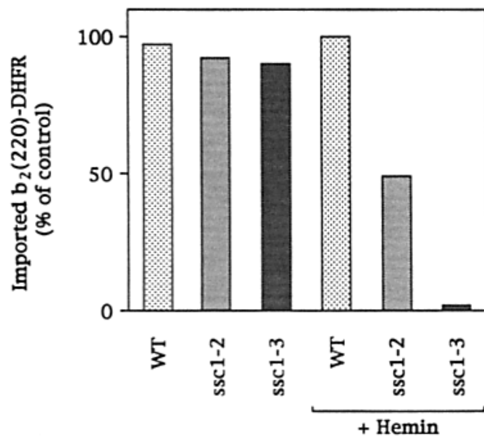

Figure 4. Import of B_2(220)-DHFR into *sscl-2* and *sscl-3* mitochondria is inhibited in the presence of hemin. Rabbit reticulocyte lysates were prepared in the presence of 5 mM cAMP instead of hemin (Ernst et al., 1976; Nicholson et al., 1987). The b_2(220)-DHFR was synthesized and imported as described in the legend to Fig. 1. Where indicated hemin (10 μM final concentration) was added back after synthesis, before the import reaction.

was mediated by a region in the presequence of the protein (Lathrop and Timko, 1993); the mode of interaction of this so-called heme regulatory motif with heme and the role of hsp70 in the translocation of δ-aminolevulinate synthase are unknown.

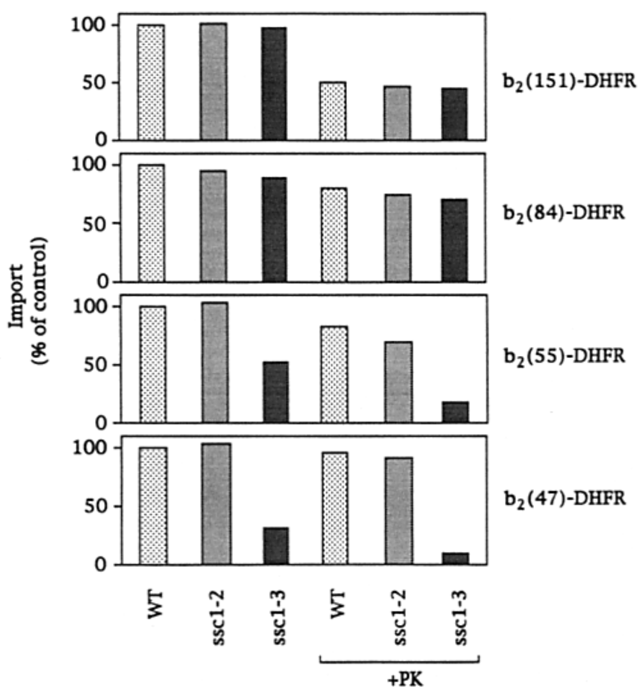

Figure 5. Short b_2-DHFR fusion proteins with an intact sorting signal are not inhibited in import into *sscl-2* and *sscl-3* mitochondria. The fusion proteins b_2(151)-DHFR, b_2(84)-DHFR, b_2(55)-DHFR, and b_2(47)-DHFR were synthesized in rabbit reticulocyte lysates and imported for 8 min at 25°C as described in the legend to Fig. 1. The amount of protein processed in wild-type mitochondria (no protease treatment) was set to 100% (control).

Fusion Proteins That Lack the Intramitochondrial Sorting Signal Require the Translocase Function of mt-hsp70

The experiments described above indicate that fusion proteins containing 167 or more amino-terminal amino acid residues of cytochrome b_2 do not require the translocase function of mt-hsp70, as indicated by their ability to be correctly imported into *sscl-3* mitochondria. We asked if certain sequences in the amino-terminal portion of cytochrome b_2 conferred this independence of the translocase function. b_2-DHFR fusion proteins with amino-terminal segments shorter than 167 residues were analyzed for import into the mutant mitochondria (Fig. 5). The preproteins b_2(151)-DHFR and b_2(84)-DHFR were imported efficiently into *sscl-2* and *sscl-3* mitochondria. The shorter preprotein b_2(55)-DHFR, however, was partially inhibited in import into *sscl-3* mitochondria. Import of the preprotein b_2(47)-DHFR was strongly inhibited in *sscl-3* mitochondria, while the import into *sscl-2* mitochondria was not affected (Fig. 5).

The dependence of the import of short preproteins on mt-hsp70 might be due to their reduced length or, more interestingly, caused by the lack of certain signals that are present in the longer preproteins. The intermembrane space sorting signal of cytochrome b_2 is located in the second half of the presequence. Similar to prokaryotic leader sequences (Wickner et al., 1991), the sorting signal consists of a hydrophobic segment that is preceded by positively charged residues (Guiard, 1985; van Loon et al., 1986; Hartl et al., 1987; Glick et al., 1992*a*,*b*; Koll et al., 1992). The positively charged residues, amino acids 47–49, are followed by a mainly hydrophobic segment of >20 residues. The deletion of a 19-residue fragment (amino acids 47 to 65) in b_2(167)-DHFR has been shown to fully disrupt the sorting signal and generate a preprotein that ended up in the mitochondrial matrix (Koll et al., 1992). Therefore, we asked if $b_2(167)_{\Delta 19}$-DHFR import into isolated mitochondria depended on mt-hsp70. In fact, processing and transport to a protease-protected location of $b_2(167)_{\Delta 19}$-DHFR were strongly inhibited in *sscl-3* mitochondria (Fig. 6). This inhibition of processing means that $b_2(167)_{\Delta 19}$-DHFR did not even go far enough into *sscl-3* mitochondria to be cleaved by the processing peptidase. We conclude that the intact intramitochondrial sorting signal makes the import of b_2(167)-DHFR independent of functional mt-hsp70. In *sscl-2* mitochondria, the import of $b_2(167)_{\Delta 19}$-DHFR was only slightly inhibited (Fig. 6), suggesting that $b_2(167)_{\Delta 19}$-DHFR predominantly depended on the translocase function of mt-hsp70. Consistently, unfolding of $b_2(167)_{\Delta 19}$-DHFR did not allow transport of the preprotein into *sscl-3* mitochondria (Fig. 7).

$b_2(167)_{\Delta 19}$-DHFR, but Not b_2(167)-DHFR, Is Found in a Complex with mt-hsp70

$b_2(167)_{\Delta 19}$-DHFR was accumulated in *sscl-2* mitochondria and b_2(167)-DHFR imported into *sscl-2* or *sscl-3* mitochondria. The mitochondria were then lysed with detergent and co-immunoprecipitations with antibodies directed against mt-hsp70 were performed. $b_2(167)_{\Delta 19}$-DHFR was efficiently co-immunoprecipitated out of *sscl-2* mitochondria (Fig. 8). This co-immunoprecipitation indicated that the preprotein was accumulated in a complex with Sscl-2p which has a

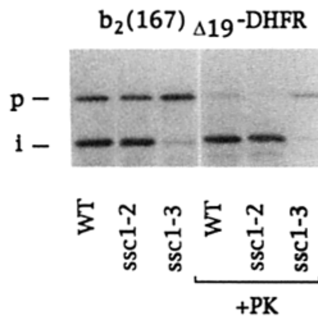

Figure 6. $b_2(167)_{\Delta 19}$-DHFR is inhibited in import into *sscl-3*, but not *sscl-2* mitochondria. The precursor of $b_2(167)_{\Delta 19}$-DHFR was imported into isolated mitochondria for 15 min at 25°C as described in the legend to Fig. 1.

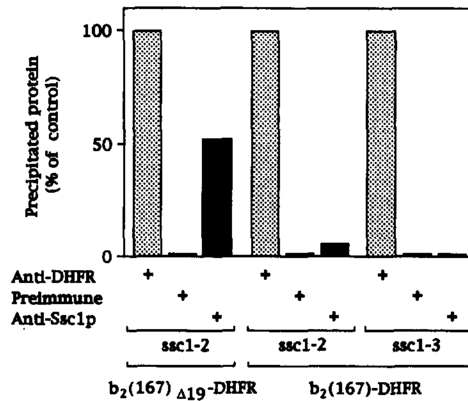

Figure 8. Co-immunoprecipitation of $b_2(167)_{\Delta 19}$-DHFR, but not $b_2(167)$-DHFR, with mt-hsp70. Urea denatured $b_2(167)_{\Delta 19}$-DHFR and $b_2(167)$-DHFR were imported as described in the legends to Figs. 1 and 7 for 10 min at 25°C. The reisolated mitochondria were lysed in 0.1% Triton X-100, 100 mM NaCl, 10 mM Tris/HCl, pH 7.5, 5 mM EDTA and immunoprecipitation with antiserum against DHFR, preimmune serum and antiserum against mt-hsp70 (*Ssclp*) were performed (Gambill et al., 1993). The amount of protein precipitated with anti-DHFR serum was set to 100% (control).

strong binding activity for preproteins (Kang et al., 1990; Gambill et al., 1993). $b_2(167)$-DHFR was not co-precipitated with mt-hsp70s (Fig. 8). This result was expected as the import of $b_2(167)$-DHFR was not inhibited in the mutant mitochondria and thus the imported protein was in the intermembrane space (see Fig. 1), while mt-hsp70 resides in the matrix.

We therefore accumulated $b_2(167)_{\Delta 19}$-DHFR and $b_2(167)$-DHFR at the same stage of import, in a two-membrane spanning fashion in translocation contact sites (Fig. 9, *A* and *B*). To accomplish this the preproteins were preincubated with the specific DHFR-inhibitor methotrexate that stabilizes the tertiary structure of the DHFR-moiety (Eilers and Schatz, 1986; Rassow et al., 1989, 1990; Koll et al., 1992). The amino-terminal b_2 portion was inserted into the membranes of wild-type, *sscl-2*, and, in the case of $b_2(167)$-DHFR, also *sscl-3* mitochondria and processed to the intermediate-sized form, while the folded DHFR remained on the outside of the outer membrane and was accessible to added protease. In co-immunoprecipitations with anti-mt-hsp70 antibodies, $b_2(167)_{\Delta 19}$-DHFR was found associated with mt-hsp70 of wild-type and *sscl-2* mitochondria (Fig. 9 *A*). $b_2(167)$-DHFR, however, could not be co-immunoprecipitated in wild-type, *sscl-2* and *sscl-3* mitochondria (Fig. 9 *B*), although it was accumulated in translocation contact sites and thus would have been in a stage where it should have access to mt-hsp70. We conclude that $b_2(167)_{\Delta 19}$-DHFR has to interact with mt-hsp70 to be imported into the mitochondrial matrix, whereas for the import of $b_2(167)$-DHFR into the intermembrane space functional mt-hsp70 is not required and an interaction between the preprotein and mt-hsp70 cannot be detected.

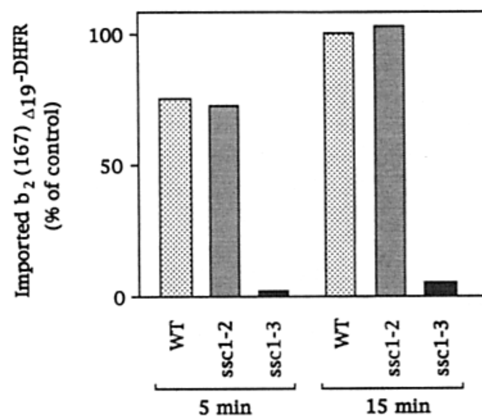

Figure 7. Unfolding of $b_2(167)_{\Delta 19}$-DHFR does not allow import into *sscl-3* mitochondria. $b_2(167)_{\Delta 19}$-DHFR was denatured in urea as described in the legend of Fig. 3 and imported for the times indicated as described in the legends to Figs. 1 and 3. The amount of protein protease-protected in wild-type mitochondria after 15 min incubation was set to 100% (control).

Discussion

The experiments reported here show that the properties of mitochondrial preproteins strongly influence their dependence on mt-hsp70 for membrane translocation. The results obtained with the precursor of cytochrome b_2 and fusion proteins between amino-terminal portions of b_2 and DHFR are best discussed by considering a dual role for mt-hsp70 in membrane translocation (unfolding of the preprotein and actual translocation; summarized in Fig. 10).

DHFR fusion proteins with a relatively short cytochrome b_2 part are nearly independent of the unfoldase function of mt-hsp70 (Fig. 10, *d–i*). This group of precursors can be subdivided into those which require no mt-hsp70 unfolding or translocation functions (Fig. 10, *d*, *f*, and *g*) or require only the translocase function (Fig. 10, *e*, *h*, and *i*). This distinction depends on the presence of the intermembrane space sorting signal. Short preproteins which contain the intermembrane space sorting signal are independent of mt-hsp70 and can thus be imported efficiently into *sscl-2* and *sscl-3* mitochondria where no association with the hsp70 can be observed. Preproteins which lack the intermembrane space sorting signal are imported completely into the matrix of wild-type mitochondria and depend strictly on binding to mt-hsp70 to reach the matrix. Unfolding of the preproteins does not promote their import into the matrix of *sscl-3* mitochondria where mt-hsp70 appears to be defective in binding to preproteins. Transport into *sscl-2* mitochondria is possible, however, as the mutant Sscl-2p shows a strong binding activity for the preproteins.

Long cytochrome b_2 preproteins that require unfoldase function are completly imported upon artificial unfolding of the preprotein. Therefore, an interaction with mt-hsp70 does

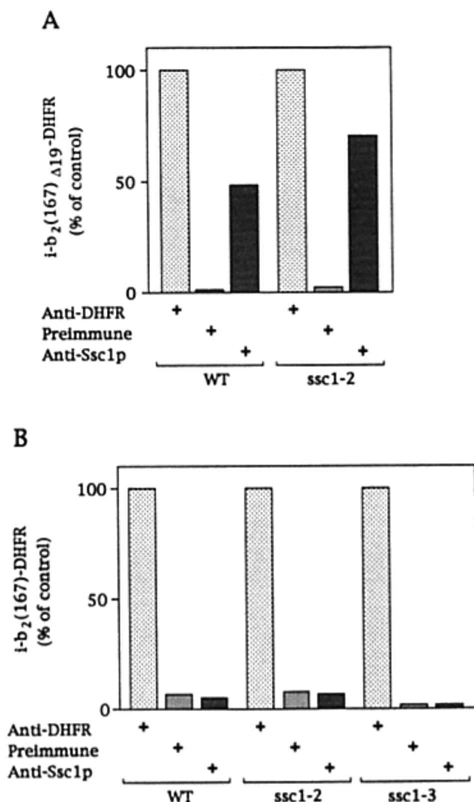

Figure 9. Differential co-immunoprecipitation of contact site-intermediates of b_2–DHFR fusion proteins with mt-hsp70. $b_2(167)_{\Delta 19}$–DHFR (*A*) and $b_2(167)$–DHFR (*B*) were preincubated with 4 μM methotrexate for 10 min at 0°C (Eilers and Schatz, 1986; Rassow et al., 1989, 1990) and incubated with the energized mitochondria for 20 min at 25°C. The mitochondria were reisolated, washed, and subjected to immunoprecipitation as described in the legend of Fig. 8. In parallel samples the mitochondria were treated with protease, leading to degradation of the fusion proteins, confirming that the fusion proteins were accumulated in mitochondrial contact sites as described (Rassow et al., 1989, 1990).

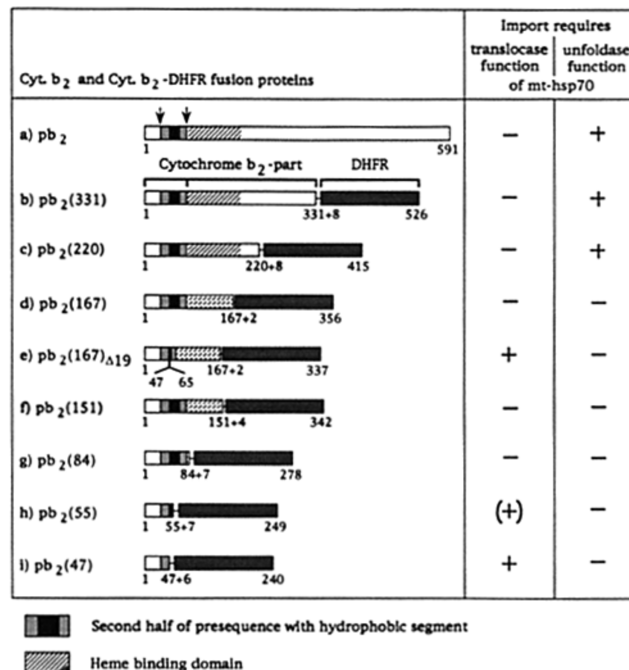

Figure 10. Cytochrome b_2 and b_2–DHFR fusion proteins and their requirement for the translocase function or unfoldase function of mitochondrial hsp70. The numbers of amino acid residues of the preproteins used in this study are given (starting with the first amino-terminal residue of the precursor of cytochrome b_2[pb_2]). The arrows indicate the processing sites.

not seem to be essential for correct import and sorting of preproteins with an intermembrane space-sorting signal. Since even the first processing step of preproteins without the sorting signal is blocked in *ssc1-3* mutant mitochondria, it appears that precursors with a sorting signal can use an alternative mechanism that promotes their insertion into the inner membrane followed by correct localization to the intermembrane space. A yet to be identified sorting component may thus be able to substitute for the translocase function of mt-hsp70 when preproteins possess the intermembrane space-sorting signal.

The sorting pathway of the precursor of cytochrome b_2 is currently the subject of a controversial debate. According to the "stop transfer" hypothesis, the sorting signal in the second portion of the presequence is arrested in the inner membrane and the mature portion of the protein is prevented from entering the matrix (van Loon et al., 1986; Glick et al., 1992*a*,*b*). The lack of a mt-hsp70 requirement for cytochrome b_2 import would be in agreement with the predictions of the stop transfer hypothesis, however, our findings do not imply that precursors with the b_2-sorting signal do not interact with mt-hsp70 at all; rather, longer b_2–DHFR fusion proteins as well as the authentic cytochrome b_2 preprotein require mt-hsp70 for unfolding. According to the stop transfer model, mt-hsp70 would only be able to bind to the presequence of cytochrome b_2 to facilitate unfolding of the mature part of the protein. While this possibility cannot be excluded, one could perhaps more easily imagine that interaction of mt-hsp70 with the mature part of the protein is involved in facilitating the unfolding of large proteins.

According to the original "conservative sorting" hypothesis, the preprotein is first imported completely into the matrix and then exported to the intermembrane space (Hartl et al., 1987). Recently, Koll et al. (1992) suggested that the import and export steps are coupled. A sorting component may recognize the sorting signal of the precursor during translocation and redirect translocation back across the inner membrane. In this modified view of conservative sorting, one might not expect to find the entire translocation intermediate in the matrix. Since interaction with mt-hsp70 is not essential for the cytochrome b_2 sorting pathway, cytochrome b_2 is apparently not imported like a matrix-targeted precursor as would be predicted in the original conservative sorting hypothesis. However, the independence of import of some b_2 fusion proteins from mt-hsp70 is compatible with the modified version of the model, with mt-hsp70 required for unfolding of certain fusion proteins. Therefore, although at a first glance, our findings may appear to favor the stop transfer model, they do not allow a definitive conclusion as to which model is correct.

The ongoing debate focuses on the issue of whether or not the sorting signal and the mature part of cytochrome b_2 pass through the matrix during the import process. The identification and localization of a sorting component that recognizes the intermembrane space-sorting signal will be

crucial for the determination of the mechanism of sorting to the intermembrane space. The localization of the sorting component to the matrix or the matrix face of the inner membrane would strongly favor the conservative sorting model, while localization to the intermembrane space or the outer face or core of the inner membrane would favor the stop transfer model. Surprisingly, the studies reported here indicate that the hypothetical sorting component has to operate in a very early step of translocation since preproteins lacking the sorting signal do not undergo even the first processing step in *sscl-3* mitochondria.

Independence from the unfoldase function of mt-hsp70 seems to depend on the structure of the protein to be imported. Apparently the amino-terminal portions of cytochrome b_2 do not have a tendency to fold into stable tertiary structures. The low energy required for unfolding of DHFR (6 kcal/mol for authentic DHFR) (Pace et al., 1990) may be further lowered due to its fusion with segments of cytochrome b_2. Interestingly, the (short) Su9–DHFR fusion protein (presequence of F_o-ATPase subunit 9 fused to DHFR) shows a dependence on mt-hsp70 to promote unfolding of the DHFR-moiety (Kang et al., 1990; Gambill et al., 1993), indicting that indeed the context of DHFR in a fusion protein modulates the requirements for unfolding.

Preproteins with longer segments of cytochrome b_2, particularly the heme-binding cytochrome domain (Fig. 10, *a–c*), have conformational restrictions that require unfolding by mt-hsp70. Strikingly, a preprotein containing the cytochrome domain could be imported independently of functional mt-hsp70 when the reticulocyte lysate was prepared in the absence of hemin, but its import was inhibited by readdition of hemin. This suggests that binding of heme to the preprotein in the cytosol promotes folding of the polypeptide chain. The translocation of those preproteins then apparently needs the full unfolding activity of mt-hsp70 as preproteins seem to be translocated across mitochondrial membranes in an extended conformation (Rassow et al., 1990).

It is likely that mt-hsp70 is not the only component of the import machinery that is involved in unfolding of preproteins. These yet to be characterized components seem to be sufficient for preproteins that require only low energy inputs for unfolding. It is obvious that the translocation of an initial segment of a preprotein across the inner membrane includes the unfolding of this segment and thus facilitates a further unfolding of the preprotein (Neupert et al., 1990). Cytochrome b_2-preproteins, that depend strictly on the translocase function of mt-hsp70, are apparently not inserted far enough into the inner membrane of *sscl-3* mitochondria to expose the processing site to the matrix processing peptidase and thus lack the unfolding and import promoting force provided by a translocation of the presequence across the inner membrane. On the other hand, the membrane potential is sufficient to drive the translocation of the more positively charged matrix-targeting sequence of Su9–DHFR across the *sscl-3* inner membrane (Gambill et al., 1993; Martin et al., 1991). Consistently these cytochrome b_2 preproteins are not even transported across the outer membrane in *sscl-3* mitochondria. Interaction of a polypeptide chain with the sorting component postulated above may provide a low unfolding force sufficient for short b_2–DHFR proteins carrying the sorting signal. With the longer fusion proteins, the unfoldase function of mt-hsp70 is needed in addition to remove stronger conformation restrictions. The requirement for mt-hsp70 correlates with the ATP dependence of import, as the short fusion proteins (containing the sorting signal) were found to be efficiently imported into ATP-depleted mitochondria, while import of the longer fusion proteins required the presence of ATP (Pfanner et al., 1990).

In summary, the differential requirements of the various b_2–DHFR fusion proteins for mt-hsp70 provide strong support for our hypothesis of a dual role of mt-hsp70 in membrane translocation of preproteins. We do not intend to imply that in the normal translocation process mt-hsp70 acts independently in the unfolding and the translocation processes. Rather, we envision that, while all proteins require binding for translocation into the matrix, preproteins with a greater degree of structure would reasonably be expected to require binding of more molecules of mt-hsp70 or mt-hsp70 with a higher affinity than is required for preproteins that are only loosely folded. Moreover, our results show that the mechanism of insertion into the inner membrane is not identical for matrix-targeted preproteins and preproteins with an intermembrane space-sorting signal. Our findings also emphasize that an analysis of sorting routes of preproteins should not only include the question of involvement of a certain component such as mt-hsp70, but also take into account that a single component can have multiple functions.

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