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 b2 (cyt. b2) 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. b2 and DHFR was efficiently transported into mitochondria independently of both hsp70 functions. When the length of the cyt. b2 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. b2 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 Mipilp that seems to represent part of the inner membrane translocation machinery (Maarse et al., 1992); the inner membrane protein Mipilp that seems to represent part of the inner membrane translocation machinery (Maarse et al., 1992); the 70-kD heat shock protein (hsp70) 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; 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. 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" 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 unfold-
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 [35S]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 [35S]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 (Hawlischek 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 2. Import of authentic cytochrome b2 and long b2-DHFR fusion proteins into sscl-2 and sscl-3 mitochondria is inhibited. The precursors of cytochrome b2 (A), b2(331)-DHFR (B) or b2(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 b2, 32% for b2(331)-DHFR, and 44% for b2(220)-DHFR (WT set to 100%).

The import of b2(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 b2(167)-DHFR are not caused by a dissipation of ΔΨ. In subsequent experiments we attempted to unravel the properties of b2(167)-DHFR that rendered its import independent of mt-hsp70.

**Fusion Proteins Containing a Long Cytochrome b2 Portion Require the Unfoldase Function of mt-hsp70**

The processing of the authentic cytochrome b2 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 b2 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 b2(331)-DHFR in which the carboxyl-terminal portion of the authentic cytochrome b2 precursor beyond residue 331 has been replaced by DHFR (Pfanner et al., 1990) to test this possibility. The import of b2(331)-DHFR was inhibited in both sscl-2 and sscl-3 mitochondria (Fig. 2 A), suggesting that sequences between 167 and 331 of cytochrome b2 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 b2 (b2(220)-DHFR) was analyzed (Fig. 2 C). Import of b2(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 b2(220)-DHFR into sscl-2 and sscl-3 mitochondria occurred with the same efficiency as the import into wild-type mitochondria. The import of b2(220)-DHFR thus mainly depended on the unfoldase function of mt-hsp70.

What is the reason for the conformational restriction of b2(220)-DHFR? The domain of cytochrome b2 that binds one heme noncovalently is located within the first 99 amino acids of the mature protein (Zia and Mathews, 1990). The b2(167)-DHFR fusion protein contains only 87 residues of the mature protein and thus does not contain a functional heme binding domain. However, b2(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, b2(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
Import of B₂(220)-DHFR into sscl-2 and sscl-3 mitochondria is inhibited in the presence of heroin. Rabbit reticulocyte lysates were prepared in the presence of 5 mM cAMP instead of heroin (Ernst et al., 1976; Nicholson et al., 1987). The b₂(220)-DHFR was synthesized and imported as described in the legend to Fig. 1. Where indicated heroin (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.

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 b₂ 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 b₂ conferred this independence of the translocase function. b₁-DHFR fusion proteins with amino-terminal segments shorter than 167 residues were analyzed for import into the mutant mitochondria (Fig. 5). The preproteins b₁(151)-DHFR and b₁(84)-DHFR were imported efficiently into sscl-2 and sscl-3 mitochondria. The shorter preprotein b₁(55)-DHFR, however, was partially inhibited in import into sscl-3 mitochondria. Import of the preprotein b₁(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 b₂ 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 (Giardi, 1985; van Loon et al., 1986; Hartl et al., 1987; Glick et al., 1992a,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 b₁(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 b₁(167)ₜₐ₋ₐₐ-DHFR import into isolated mitochondria depended on mt-hsp70. In fact, processing and transport to a protease-protected location of b₁(167)ₜₐ₋ₐₐ-DHFR were strongly inhibited in sscl-3 mitochondria (Fig. 6). This inhibition of processing means that b₁(167)ₜₐ₋ₐₐ-DHFR did not even go far enough into sscl-3 mitochondria to be cleaved by the processing peptidase. We conclude that the intact intermembrane sorting signal makes the import of b₁(167)-DHFR independent of functional mt-hsp70. In sscl-2 mitochondria, the import of b₁(167)ₜₐ₋ₐₐ-DHFR was only slightly inhibited (Fig. 6), suggesting that b₁(167)ₜₐ₋ₐₐ-DHFR predominantly depended on the translocase function of mt-hsp70. Consistently, unfolding of b₁(167)ₜₐ₋ₐₐ-DHFR did not allow transport of the preprotein into sscl-3 mitochondria (Fig. 7).

b₁(167)ₜₐ₋ₐₐ-DHFR, but Not b₁(167)-DHFR, Is Found in a Complex with mt-hsp70

b₁(167)ₜₐ₋ₐₐ-DHFR was accumulated in sscl-2 mitochondria and b₁(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. b₁(167)ₜₐ₋ₐₐ-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 Ssc1-2p which has a
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 $b_2(167)_{\text{A19}}$-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, $b_2(167)_{\text{A19}}$-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 $b_2(167)_{\text{A19}}$-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.

**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 completely 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. \( b_2(167)_{19} \)-DHFR (A) and \( b_2(167)-\)DHFR (B) were preincubated with 4 \( \mu \)M 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).

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 \( rsc1-3 \) 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., 1992a,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 translation 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
compont to the matrix or the matrix face of the inner mem-
brane 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 indi-
cate 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 im-
ported. Apparently the amino-terminal portions of cyto-
chrome b₂ do not have a tendency to fold into stable ter-
tary 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 b₂. Interestingly, the (short) Su₉-DHFR fusion
protein (presequence of F₉-ATPase subunit 9 fused to
DHFR) shows a dependence on mt-hsp70 to promote unfold-
ing of the DHFR moiety (Kang et al., 1990; Gambill et al.,
1993), indicating that indeed the context of DHFR in a fusion
protein modulates the requirements for unfolding.

Preproteins with longer segments of cytochrome b₂, par-
ticularly the heme-binding cytochrome domain (Fig. 10,
a–c), have conformational restrictions that require unfolding
by mt-hsp70. Strikingly, a preprotein containing the cyto-
chrome domain could be imported independently of func-
tional mt-hsp70 when the reticulocyte lysate was prepared in
the absence of hemin, but its import was inhibited by readdi-
tion 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 prepro-
teins. 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). Cyto-
chrome b₂-preproteins, that depend strictly on the translo-
cation 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 pro-
vided 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 b₂ preproteins are not
even transported across the outer membrane in sscl-3 mito-
chondria. Interaction of a polypeptide chain with the sort-
ing component postulated above may provide a low unfold-
ing force sufficient for short b₂-DHFR proteins carrying the
sorting signal. With the longer fusion proteins, the unfoldase
function of mt-hsp70 is needed in addition to remove stronger
conformational 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 pres-
ence of ATP (Pfanner et al., 1990).

In summary, the differential requirements of the various
b₂–DHFR fusion proteins for mt-hsp70 provide strong sup-
port for our hypothesis of a dual role of mt-hsp70 in mem-
brane translocation of preproteins. We do not intend to imply
that in the normal translocation process mt-hsp70 acts inde-
pendently 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 mecha-


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