# The Biology of HEAT SHOCK PROTEINS and MOLECULAR CHAPERONES

Edited by

**Richard I. Morimoto**
Northwestern University

**Alfred Tissières**
University of Geneva

**Costa Georgopoulos**
University of Geneva


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# Chaperoning Mitochondrial Biogenesis

**Thomas Langer and Walter Neupert**
Institut für Physiologische Chemie
München, Germany



## I. INTRODUCTION

The parallel development of powerful in vitro systems and of genetic approaches has allowed considerable progress in understanding the mechanisms of protein transport into various cellular compartments. It is becoming ever more evident that transport processes across different cellular membranes are based on similar principles. Polypeptide chains appear to traverse lipid bilayers through proteinaceous pores. Translocation requires a "translocation-competent," rather unfolded conformation. As a consequence, proteins must be partially unfolded or kept in an unfolded conformation prior to the translocation event and must refold after crossing the lipid membrane bilayer. In recent years, increasing evidence was obtained that both represent assisted processes. Molecular chaperones, in many cases originally identified as heat shock proteins, modulate the folding state of polypeptide chains in different cellular compartments.

*The Biology of Heat Shock Proteins and Molecular Chaperones*

Mitochondria, which contain heat shock proteins of the hsp70 and hsp60 family, proved to be a useful model system to study the function of chaperone proteins in protein translocation and folding. Although mitochondria contain their own DNA and independent systems for replication and protein synthesis, only a few subunits of the oxidative phosphorylation system and, in some organisms, components mediating splicing and translation of mitochondrial mRNA are encoded by the mitochondrial genome (Grivell 1989). About 95% of the total mass of mitochondrial proteins are encoded in the nucleus. They are synthesized on cytosolic polyribosomes, many of them as precursor molecules with amino-terminal presequences containing the targeting information. Import can occur posttranslationally followed by sorting to the various subcompartments of mitochondria, the outer and inner membranes, the intermembrane, and the matrix space.

In recent years, an increasing number of components have been identified that are involved in the import and sorting of mitochondrial proteins (for reviews, see Glick and Schatz 1991; Segui-Real et al. 1992; Hannavy et al. 1993; Kiebler et al. 1993). In this chapter, we focus on the function of molecular chaperones in import and folding of mitochondrial proteins. In particular, we discuss their roles in maintaining a translocation-competent conformation of mitochondrial precursor molecules in the cytosol, in mediating the translocation process across mitochondrial membranes, and in the folding of matrix-localized proteins.

## II. MAINTENANCE OF TRANSLOCATION COMPETENCE IN THE CYTOSOL

### A. Conformation of Mitochondrial Proteins during Membrane Translocation

It is now generally agreed that proteins must attain a loosely folded conformation to traverse biological membranes, although with some organelles, in particular peroxisomes and glyoxysomes, the need for unfolding has not been proven. Studies of mitochondrial protein import provided direct experimental evidence for the requirement of a "translocation-competent" conformation of precursor proteins during the translocation process, which differs from the completely folded, native state: (1) Tight folding into a stable tertiary structure, e.g., induced by the presence of substrate analogs or cofactors, was found to prevent the import of precursor proteins into mitochondria (Eilers and Schatz 1986; Chen and Douglas 1987; Rassow et al. 1989; Wienhues et al. 1991). Removal of the ligand restored import competence of the precursor protein. Conversely, destabilization of the native conformation by point

mutations results in a more efficient import into mitochondria (Chen and Douglas 1988; Vestweber and Schatz 1988). (2) A nonnative conformation of precursor proteins during the translocation process is suggested by the identification of translocation intermediates spanning the inner and outer membranes (Schleyer and Neupert 1985). The two mitochondrial membranes form a barrier of about 10–12 nm as measured by electron microscopy. Using a set of fusion proteins consisting of amino-terminal parts of cytochrome $b_2$ of various lengths and dihydrofolate reductase (DHFR), Rassow et al. (1990) showed that about 50 amino acid residues are sufficient to span both mitochondrial membranes. This excludes that precursor proteins traverse membranes in their native conformation and suggests an extended or β-sheet structure of the spanning portion of a polypeptide chain, rather than an α-helical structure.

Up to now, physicochemical data have not been available that describe directly the conformation of translocation competent, mitochondrial precursor proteins. However, in view of the rapid collapse of proteins into a compact conformation after dilution from denaturant in vitro (Kim and Baldwin 1990), a completely unfolded conformation of mitochondrial precursor proteins prior to membrane translocation seems to be very unlikely. Proteins were proposed to traverse membranes in a "molten-globule"-like conformation characterized by the presence of secondary structural elements and a flexible, disordered tertiary structure (Bychkova et al. 1988). At this point, it should be noted that molecular chaperones, whose function in maintaining translocation competence is discussed in the following section, were found to stabilize unfolded proteins in a compact conformation without ordered tertiary structure (Martin et al. 1991b; Langer et al. 1992b).

### B. Function of Cytosolic Chaperone Proteins

A nonnative conformation of precursor proteins during membrane translocation implies that their folding must be modulated in the cytosol (Fig. 1). Precursor proteins to be transported across membranes could fold to the native state and become unfolded during membrane translocation (Pfanner et al. 1990; Skerjanc et al. 1990) or their folding is prevented in the cytosol. One obvious possibility would be that the amino-terminal presequence modulates the folding state of precursor molecules. After translocation, specific proteases within mitochondria cleave off the presequence, which would then allow folding to the native structure. However, the presequence is not sufficient to confer prolonged translocation competence. After dilution of mitochondrial precursor proteins from denaturant into in vitro import assays, translocation competence is usual-

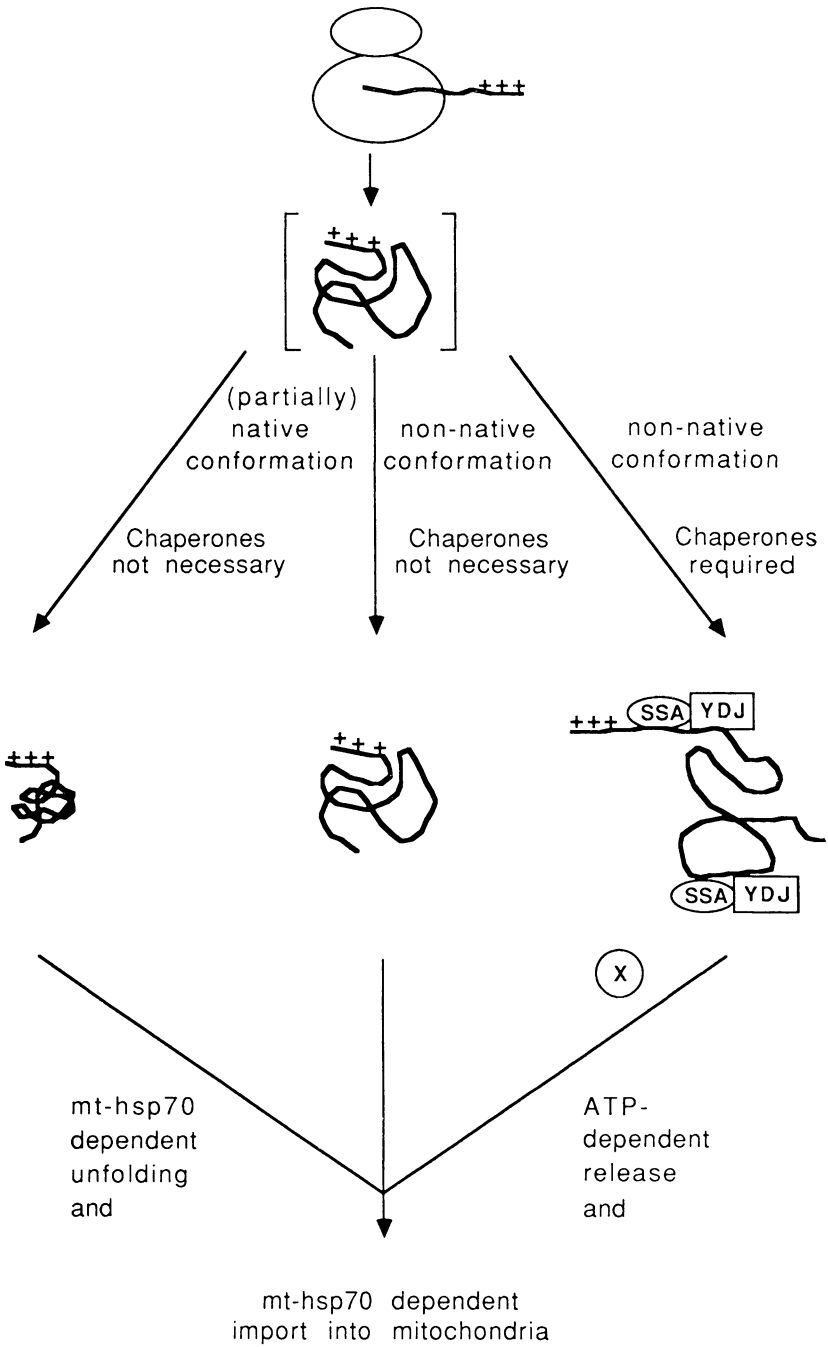


*Figure 1* Possible mechanisms of maintenance of translocation competence of precursor proteins in the cytosol. (SSA) Ssa1p/Ssa2p; (YDJ) Ydj1p; (X) NEM-sensitive factor; (mt-hsp70) mitochondrial hsp70.

ly rapidly lost. In contrast, it was shown that precursor proteins are kept transport-competent for long periods in the cytosol, in many cases depending on the presence of ATP (Pfanner et al. 1987, 1990). Indeed, in recent years, several ATP-dependent cytosolic factors have been identified that stabilize various precursor proteins in the cytosol, preventing their folding or aggregation. As discussed in a later section, however, (partial) folding of precursor proteins in the absence of these factors does not necessarily abolish translocation competence. Rather, unfolding in some cases can be promoted by the mitochondrial import machinery, in particular hsp70 (mt-hsp70), in the matrix (Fig. 1; see Section III.D).

Studies in yeast revealed that molecular chaperones of the hsp70 family help to maintain a translocation-competent conformation of mitochondrial precursor proteins as well as proteins targeted to the endoplasmic reticulum, chloroplasts, and the nucleus (Chirico et al. 1988; Deshaies et al. 1988; Murakami et al. 1988; Waegemann et al. 1990; Dingwall and Laskey 1992). It is well established that hsp70 proteins interact with unfolded polypeptide chains in an ATP-dependent manner. Among the six cytosolic hsp70 proteins identified in yeast, evidence for a role in maintaining transport competence exists for Ssa1p and Ssa2p. A yeast strain, in which the *SSA1*, *SSA2*, and *SSA4* genes are deleted, could be rescued by expression of *SSA1* from a galactose-regulated promoter (Deshaies et al. 1988). Genetic depletion of Ssa1p resulted in the accumulation of precursor forms of the mitochondrial inner membrane protein $F_1\beta$ and of α-factor in the cytosol, suggesting a common step in posttranslational protein transport across different membranes. The stimulating effect of Ssa1p/Ssa2p on import of prepro-α-factor into microsomes was also demonstrated biochemically in in vitro transport systems (Chirico et al. 1988). The identification of the temperature-sensitive yeast mutant *mas3*, which maps to the yeast heat shock factor (HSF), provides further evidence for a function of heat shock proteins in protein transport (Smith and Yaffe 1991). At the nonpermissive temperature, in the absence of an induction of *SSA1*, the rate of posttranslationally imported mitochondrial precursor proteins was decreased drastically. Interestingly, overexpression of Ssa1p alone did not relieve this phenotype, indicating that additional heat shock proteins are functioning during early steps of mitochondrial protein import (Smith and Yaffe 1991).

Although a direct physical interaction of Ssa1p/Ssa2p with prepro-α-factor was recently demonstrated by coimmunoprecipitation (Chirico 1992), so far, no stable binary complexes were isolated between cytosolic hsp70 and mitochondrial precursor proteins. Therefore, a detailed description of the mode of action of hsp70 in the cytosol is still not available. Precursor proteins were described to be part of a 200–250-kD

protein complex, which contains cytosolic hsp70 (Sheffield et al. 1990). ATP-dependent dissociation of the complex could be prevented by *N*-ethylmaleimide (NEM) treatment of the cytosol. The NEM insensitivity of hsp70 proteins suggests the presence of an additional, NEM-sensitive subunit of the complex. Although this component has not yet been identified, eukaryotic homologs of the *Escherichia coli* heat shock proteins DnaJ and GrpE are attractive candidates. DnaJ and GrpE interact functionally with the *E. coli* hsp70 homolog DnaK (Liberek et al. 1991) and modulate its ATP-dependent interaction with unfolded polypeptide chains (Zylicz et al. 1989; Liberek et al. 1991; Langer et al. 1992b). Indeed, a number of homologs of DnaJ were recently identified localized in various compartments of a eukaryotic cell (Kurihara and Silver 1992; Caplan et al. 1993).

The *YDJ1* gene (also called *MAS5*) was identified by screening a yeast expression library with a polyclonal antiserum raised against a partially purified nuclear fraction (Caplan and Douglas 1991) and independently by screening for yeast mutants displaying a defect in mitochondrial protein import (Atencio and Yaffe 1992). Subsequent biochemical analysis clearly demonstrated that Ydj1p is required for efficient post-translational protein import into mitochondria (Caplan et al. 1992a). In temperature-sensitive yeast mutant strains at the nonpermissive temperature, precursor proteins of the α, β, and γ subunits of the $F_1$-ATPase and of citrate synthase accumulate in the cytosol. The dependence of import on intact Ydj1p is obviously more strict at higher temperature. Only minor import defects were observed in a *mas5* deletion mutant at 23°C, whereas cells were not viable at 37°C. Interestingly, Ydj1p is farnesylated in vivo, which is essential for the function of the protein at high temperatures (Caplan et al. 1992b). Upon shift of the temperature to 37°C, the protein was partially relocalized to the membrane fraction dependent on the presence of the farnesyl lipid moiety. However, although enriched at the cytosolic side of the endoplasmic reticulum membrane, Ydj1p was not found in the outer membrane of mitochondria. A specific targeting function of the farnesyl group of Ydj1p during protein transport is therefore still speculative.

How does Ydj1p affect mitochondrial precursor proteins in the cytosol? The prokaryotic homolog DnaJ slightly stimulates the ATPase activity of DnaK (Liberek et al. 1991). This effect is far more pronounced in the presence of another heat shock protein, GrpE. Therefore, it is likely that Ydj1p exerts its effects in collaboration with hsp70 proteins in the cytosol. Indeed, purified Ydj1p functionally interacts with Ssa1p, as it stimulates the ATPase activity of Ssa1p up to ninefold (Cyr et al. 1992). Under these conditions, a permanently unfolded polypeptide

chain, carboxymethylated α-lactalbumin, was released from Ssa1p/Ssa2p in vitro. It remains to be determined whether Ydj1p is indeed part of the described cytosolic complex of about 200–250 kD containing Ssa1p/Ssa2p and mitochondrial precursor proteins (Sheffield et al. 1990). Because Ydj1p, as DnaJ, is found to be insensitive to NEM (D. Cyr, pers. comm.; T. Langer, unpubl.), the complex should contain additonal component(s). Although not identified in the cytosol of eukaryotic cells up to now, a protein homologous to the *E. coli* GrpE protein is a likely candidate.

### C. Cytosolic Factors with Targeting Function for Mitochondria

In view of the involvement of Ssa1p/Ssa2p and Ydj1p in the import of proteins into both mitochondria and endoplasmic reticulum, interaction of these molecular chaperones with the presequences, if it exists, apparently does not contribute to the specificity of targeting. However, chaperone proteins may stabilize precursor proteins in a conformation that allows interaction of the presequence with specific receptor proteins at the outer surface of mitochondria. Indeed, cytosolic targeting factors seem not to be absolutely required, as efficient import of a chemically pure preprotein into isolated yeast mitochondria was described to occur (Becker et al. 1992). The specific recognition of transport-competent precursor proteins by receptor proteins in the outer mitochondrial membrane is apparently sufficient for correct targeting in vitro. Nevertheless, cytosolic factors that bind specifically to mitochondrial presequences appear to exist, and several such factors have been identified in mammalian cells.

A presequence-binding factor (PBF) was purified from rabbit reticulocyte lysate (Murakami and Mori 1990). Whereas no interaction was observed with mature ornithine transcarbamoyltransferase (OTC), the precursor form was efficiently bound by PBF (Murakami et al. 1992). PBF is a homo-oligomeric protein of 50-kD subunits, with an $s_{20,w}$ value of 5.5S. Depletion of rabbit reticulocyte lysate from PBF prevented import of OTC, aspartate aminotransferase, and malate dehydrogenase into mitochondria. Readdition of purified PBF fully restored import. In contrast, import of 3-oxoacyl-CoA thiolase, which lacks a cleavable presequence, did not depend on PBF. The mode of PBF action has not been understood up to now. Direct evidence for a chaperone-like role of PBF is so far lacking. It has been suggested that PBF might modulate the conformation of precursor proteins synergistically, with hsp70 proteins conferring additional mitochondrion-specific targeting information to the complex (Murakami et al. 1992).

Another cytosolic factor that stimulates mitochondrial protein import was isolated from rat liver (Ono and Tuboi 1988, 1990; Hachiya et al. 1993). This factor, termed mitochondrial-import-stimulating factor (MSF), is composed of two subunits of 30 and 32 kD. In contrast to PBF, MSF exhibits strong ATPase activity in the presence of a transport-incompetent precursor protein (Hachiya et al. 1993). ATP hydrolysis was reported to result in depolymerization of an in-vitro-synthesized mitochondrial precursor protein. Therefore, MSF may represent a novel chaperone protein specific for mitochondrial precursor proteins with a dual function: On the one hand, it may recognize presequences and confer translocation competence to precursor proteins; on the other hand, it may target precursor proteins to mitochondria. Interestingly, the two activities were affected differently by NEM treatment (Hachiya et al. 1993). Whereas no effect of the alkylating agent was observed on presequence binding and the ATPase activity of MSF, the stimulating effect of MSF on mitochondrial import was abolished, suggesting impairment of the release from MSF. NEM exhibited a similar effect on a cytosolic complex containing hsp70 and a mitochondrial precursor protein (Murakami et al. 1988; Sheffield et al. 1990). However, in contrast to PBF, stimulation of import by MSF did not depend on cytosolic hsp70.

The relative importance of MSF, PBF, or hsp70 for mitochondrial protein import in vivo remains to be determined. It might well be that a diverse set of factors interact with various parts of a precursor protein, resulting in stabilization of a transport-competent conformation and efficient targeting to mitochondria.

## III. PROTEIN TRANSLOCATION ACROSS MITOCHONDRIAL MEMBRANES

Translocation-competent precursor proteins are specifically recognized by receptor proteins at the outer surface of mitochondria, which are part of a protein complex in the outer membrane. This receptor complex consisting of at least six different proteins mediates binding and insertion into the translocation pore in the outer membrane of mitochondria (for review, see Kiebler et al. 1993). The targeting sequences are then thought to make contact with components of the inner membrane. Translocation of the presequence across the inner membrane into the matrix strictly depends on an energized inner membrane (Gasser et al. 1982; Schleyer and Neupert 1982). The electrical potential ($\Delta\psi$) may exert an electrophoretic effect on the positively charged presequence or influence the conformation of an inner membrane component in a manner such that translocation is triggered. This hypothesis is supported by the finding that differences in the positive charge of presequences are reflected in a

different sensitivity of import for the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Martin et al. 1991a). Further translocation into the matrix does not require an energized inner membrane, but it does require the hydrolysis of ATP. The role of ATP for mitochondrial protein import was a matter of debate for a long time, mainly because ATP depletion experiments with isolated mitochondria were performed under various conditions, resulting in different ATP levels both outside and inside mitochondria. Two ATP-dependent steps of mitochondrial protein import are now well characterized. (1) In the cytosol, ATP is required to maintain a transport-competent conformation of precursor proteins, as discussed above. (2) The translocation of polypeptide chains across the inner mitochondrial membrane is mediated by a matrix-localized hsp70 protein (mt-hsp70) in an ATP-dependent manner.

### A. mt-hsp70-dependent Membrane Translocation

First evidence for a function of mt-hsp70 in the translocation process was obtained upon characterization of the yeast mutant *ssc1-2*, which contains a temperature-sensitive allele of the mt-hsp70 gene *SSC1* (Kang et al. 1990; Ostermann et al. 1990). At the nonpermissive temperature, precursor proteins of $F_1\beta$, hsp60, and Ssc1p (mt-hsp70) itself accumulated in the cytosol in vivo. Consistently, import was impaired in in vitro systems. The mutation in the *SSC1* gene affected import of proteins of the inner membrane (e.g., the Rieske-Fe/S-protein and the ADP/ATP carrier), the intermembrane space (e.g., cytochrome $c_1$), and the matrix (e.g., the $\beta$ subunit of the $F_1$-ATPase). At nonpermissive temperature in the *ssc1-2* mutant, these proteins accumulated at the surface of mitochondria, as assessed by their accessibility to externally added protease. However, the amino-terminal presequences reached the matrix space and were cleaved off by the matrix-processing peptidase. Obviously, the accumulated translocation intermediates were spanning both mitochondrial membranes, indicating that mt-hsp70 acts already during membrane translocation. Indeed, a precursor protein partly translocated into the matrix could be cross-linked to mt-hsp70 (Scherer et al. 1990). In addition, electron microscopic studies revealed a localization of mt-hsp70 near the inner membrane (Carbajal et al. 1993).

On the basis of these results, a model for the translocation of proteins across mitochondrial membranes mediated by mt-hsp70 was proposed (Fig. 2) (Neupert et al. 1990; Neupert and Pfanner 1993). This model predicts cycles of binding of mt-hsp70 to an incoming precursor to provide the driving force for the translocation across the membrane. Spontaneous "breathing" of the polypeptide on the outside would be suf-

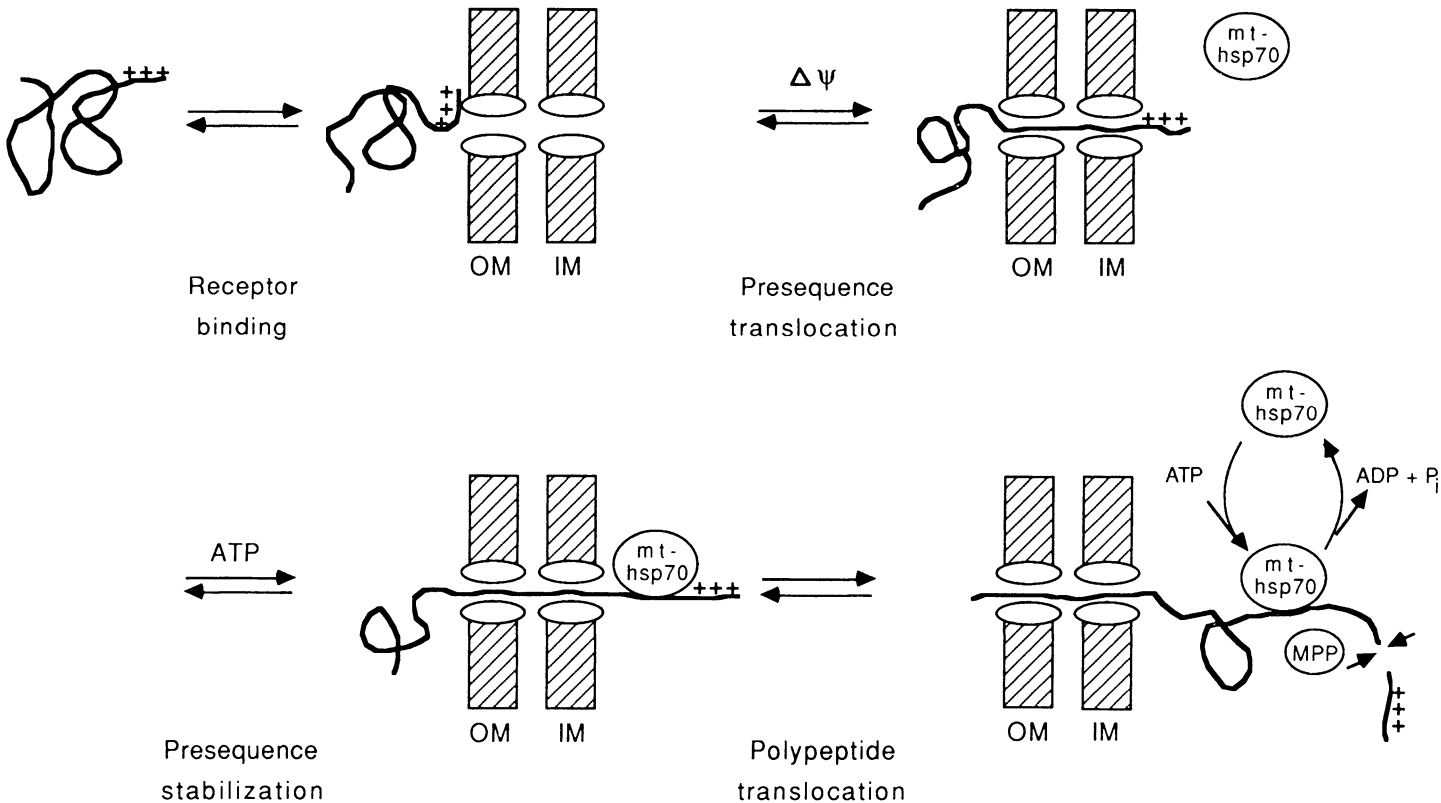


*Figure 2* Model of mt-hsp70-mediated membrane translocation of mitochondrial precursor proteins. A functional interaction of proteins homologous to *E. coli* DnaJ and GrpE is conceivable but remains to be demonstrated. (Mt-hsp70) Mitochondrial hsp70; (MPP) matrix processing peptidase; (OM) outer mitochondrial membrane; (IM) inner mitochondrial membrane.

ficient to allow the passage of limited segments of the precursor through the translocation pores in the outer and inner membranes. According to this view, binding of mt-hsp70 to incoming segments of the precursor protein shifts the equilibrium of folded and unfolded state on the outside by trapping the unfolded precursor in a stepwise fashion on the *trans* side of the two mitochondrial membranes. The model would also imply that breakdown of folded domains on the outside is a cooperative effect. After initial unfolding steps, only little energy input is necessary, since then free energy stabilizing the folded conformation is no longer existing as a force preventing complete unfolding. The free energies that further stabilize a folded conformation upon binding of a ligand are usually in the range of a few kcal/mole, thus relatively small. Still, they are sufficient to block import efficiently (Eilers and Schatz 1986; Chen and Douglas 1987; Rassow et al. 1989; Wienhues et al. 1991). This would support the view that advantage is taken of the spontaneous reversible unfolding on the outside by the mt-hsp70-binding system inside. Obviously, the hsp70-binding/ATP hydrolysis system cannot work when spontaneous unfolding outside is strongly impaired by binding of a ligand.

### B. Requirement for ATP in the Matrix

To test some predictions of this model, the energetics of membrane translocation was studied in more detail. mt-hsp70 mediates at least one important ATP-dependent step in the mitochondrial matrix during translocation. Therefore, the requirement of ATP in the matrix most likely reflects the function of mt-hsp70. ATP concentrations can be modulated in the matrix under various import conditions (Hwang and Schatz 1989; Stuart et al. 1994). In the absence of substrates for the respiratory chain and by inhibition of the ATP synthase and the ADP/ATP carrier, ATP levels in the matrix can be decreased drastically in vitro. Reduction of the ATP concentration from normal levels of about 1.4 mM to 280 μM did not impair the translocation of matrix-localized proteins or proteins finally localized in the intermembrane space (Stuart et al. 1994). However, at ATP concentrations of about 150 μM, import of matrix-localized proteins like the β subunit of the $F_1$-ATPase or the Su9(1-69)-DHFR fusion protein was affected. The ATP available to mt-hsp70 under these conditions is extremely low since a considerable amount of total ATP in the matrix is bound to mitochondrial proteins, in particular to $F_1$-ATPase with affinities in the nanomolar range (Cross and Nalin 1981). Although the binding constant for ATP of mt-hsp70 has not been determined so far, it is expected to be in the micromolar range. DnaK, the *E. coli* hsp70

homolog, has an ATP-binding constant of about 20 μM (Liberek et al. 1991). Therefore, most likely under conditions of extreme ATP depletion, mt-hsp70 in the matrix becomes inactive.

Interestingly, at these extremely low matrix ATP levels, processing of precursor proteins was very inefficient and import-competent proteins accumulated at the surface of the mitochondria (Cyr et al. 1993). The observation of inefficient processing of precursor proteins in the presence of $\Delta\psi$ indicates that the presequence can reach the matrix. However, the membrane potential is not sufficient to translocate presequences across the inner membrane in a stable manner. In addition to $\Delta\psi$, ATP is required in the matrix. Precursor proteins, accumulated outside the inner membrane of mitochondria in the presence of $\Delta\psi$, but absence of matrix ATP, could be chased into the matrix by adding ATP. Most likely, the ATP-dependent interaction of mt-hsp70 with the incoming polypeptide chain arrests the presequence on the matrix side of the inner membrane in a topology that allows cleavage by the matrix-processing peptidase (MPP) (Cyr et al. 1993). The observation of only inefficient processing at low ATP concentrations suggests that already the binding of amino-terminal segments of the precursor protein to mt-hsp70 requires the presence of ATP. Indeed, after import in ATP-depleted mitochondria, partly translocated Su9(1-69)-DHFR could only be coimmunoprecipitated with mt-hsp70 shortly after readdition of ATP (Manning-Krieg et al. 1991). Consistent results were obtained when two temperature-sensitive alleles of mt-hsp70, *ssc1-2* and *ssc1-3*, were analyzed (Kang et al. 1990; Gambill et al. 1993). In the temperature-sensitive mutant *ssc1-2*, carrying a mutation in the putative peptide-binding domain, precursor proteins are bound to mt-hsp70 and processed efficiently at normal ATP levels. However, the release of bound polypeptides is impaired. On the other hand, in the temperature-sensitive mutant *ssc1-3*, carrying a point mutation near the ATP-binding site, which may prevent binding of ATP to mt-hsp70, binding and efficient processing were not observed.

Taken together, these results indicate that ATP-dependent mt-hsp70 binding is sufficient to arrest the presequence in a stable manner on the matrix side of the inner membrane and allow efficient processing. Complete translocation of matrix-localized proteins into the matrix, however, requires several cycles of ATP-dependent binding and release from mt-hsp70. Even after unfolding of precursor proteins in vitro, import of matrix-targeted precursor proteins did not occur under conditions of extreme ATP depletion or at nonpermissive temperature in *ssc1-3* mutant mitochondria (Gambill et al. 1993; Stuart et al. 1994). Interestingly, under these conditions, efficient in vitro import of polypeptides into the matrix was observed in the *ssc1-2* mutant, in which binding of precursor

proteins to mt-hsp70 is still possible. This suggests that already the ATP-dependent binding of (several) mt-hsp70 by itself to newly imported amino-terminal segments of precursor proteins may be sufficient to drive the translocation, independent of the hydrolysis of ATP.

### C. Matrix ATP Requirement for Import of Intermembrane Space Proteins

Import of several proteins localized to the intermembrane space was also found to depend on mt-hsp70 and matrix ATP. Whereas cytochrome $b_2$ accumulates as a translocation intermediate spanning both mitochondrial membranes in the *ssc1-2* mutant under nonpermissive temperature, no processing was observed in the *ssc1-3* mutant or after ATP depletion of the matrix (Voos et al. 1993; Stuart et al. 1994). A stepwise reduction of ATP levels in the mitochondrial matrix during import revealed a less-stringent ATP requirement for sorting of proteins to the intermembrane space compared to matrix-localized proteins. The hydrophobic part of the bipartite presequences in intermembrane space proteins that contain the sorting information relieves the requirement for the import for matrix ATP/mt-hsp70 (Voos et al. 1993; Stuart et al. 1994). A fusion protein containing the complete presequence of cytochrome $b_2$ fused to mouse DHFR is transported to the intermembrane space even at very low matrix ATP levels or in the absence of functional mt-hsp70. In contrast, after deletion of the hydrophobic part of the presequence, which results in missorting of the otherwise identical precursor protein into the matrix, import is strictly dependent on the presence of ATP. Similar observations were made studying the import of cytochrome $c_1$ (Stuart et al. 1994). The efficient, mt-hsp70-independent processing of intermembrane space proteins in the matrix indicates that in this case, stable translocation of the presequence across the inner membrane is achieved by binding to another, not yet identified, protein that may interact with the hydrophobic part of the bipartite presequence.

The less-stringent dependence of intermembrane space proteins on mt-hsp70 does not allow a differentiation between the models presently proposed for the sorting of intermembrane space proteins, namely, the stop-transfer model and the conservative sorting model (Hartl and Neupert 1990; Glick et al. 1992). However, the efficient sorting at low matrix ATP levels of preproteins, loosely folded prior to import and destined to the intermembrane space, indicates that the precursor protein may be present in the matrix only with parts of the entire length at a given time. In frame of the conservative sorting model, this suggests that the polypeptide chain is exported to the intermembrane space in a co-

translocational manner as proposed earlier (Koll et al. 1992). This mechanism could provide the energy for the movement of the polypeptide chain from the matrix to the intermembrane space.

### D. mt-hsp70-mediated Unfolding of Precursor Proteins

ATP-dependent binding to mt-hsp70 drives the vectorial movement of a precursor protein across mitochondrial membranes into the matrix. Several lines of evidence indicate that binding of mt-hsp70 to newly imported segments of precursor proteins can indirectly promote the unfolding of domains at the outer surface of mitochondria, another key element of the model for its function in membrane translocation (Fig. 2): (1) The block of complete import of several precursors into *ssc1-2* mitochondria at nonpermissive temperature can be circumvented by urea denaturation of precursor proteins prior to import (Kang et al. 1990; Gambill et al. 1993). (2) In contrast to various fusion proteins containing the presequence of cytochrome $b_2$, import and sorting of cytochrome $b_2$ itself to the intermembrane space require matrix ATP and mt-hsp70 (Voos et al. 1993; Stuart et al. 1994). Cytochrome $b_2$, a lactate dehydrogenase, contains a tightly folded heme-binding domain (cytochrome-$b_5$-like) followed by a flavin-containing domain. Upon protease treatment of the precursor, the cytochrome $b_5$ domain is found to form a protease-resistant fragment prior to import (B. Glick; R. Stuart; both pers. comm.). A precursor protein, in which this domain was deleted, did not depend on matrix ATP and mt-hsp70 in its import (Stuart et al. 1994). Consistently, to reach the intermembrane space, fusion proteins containing amino-terminal parts of cytochrome $b_2$ of various lengths and DHFR only required matrix ATP if the cytochrome $b_5$ domain was intact. As shown in *ssc1-2* mitochondria, urea denaturation of the precursor protein prior to import circumvented the necessity of ATP in the matrix for the translocation process.

An unfolding reaction on the outside of the mitochondrion mediated by mt-hsp70 implies that folding of precursor proteins in the cytosol does not necessarily prevent efficient import (Fig. 1). Rather, import of folded (partially) proteins depends strictly on the action of mt-hsp70, which promotes unfolding outside. This is consistent with a mechanism by which unfolding at the mitochondrial surface occurs essentially in a spontaneous reaction. As a consequence, cytosolic chaperones may not even be required to maintain a translocation-competent conformation of certain precursor proteins, one such example being cytochrome $b_2$. However, after stabilization of the folded structure by adding substrate analogs or ligands, e.g., methotrexate for DHFR fusion proteins or heme

to a heme-binding domain, the energy provided by binding to mt-hsp70 (followed by ATP-dependent release) seems not to be sufficient to facilitate the unfolding. Under these conditions, translocation intermediates spanning across inner and outer membranes accumulate. From this scenario, it can be predicted that after removal of the ligand, e.g., methotrexate in the case of DHFR fusion proteins, the import of the translocation intermediates requires matrix ATP and mt-hsp70.

Taken together, several key predictions of the current view of the mt-hsp70-mediated membrane translocation process (Fig. 2) received experimental support. mt-hsp70 function could be studied either by ATP depletion of the matrix, preventing binding to mt-hsp70, or by characterizing temperature-sensitive mutants with a defect in binding (*ssc1-3*) or in release of polypeptides (*ssc1-2*). These approaches unraveled several functions of mt-hsp70: Presequences are stabilized in the matrix by ATP-dependent binding to mt-hsp70. The vectorial movement of the complete polypeptide chain across the two mitochondrial membranes requires several cycles of ATP-dependent binding and release from mt-hsp70. These interactions not only provide the energy for the translocation process itself, but can also promote unfolding of precursor proteins outside of mitochondria by shifting the equilibrium of folding to the unfolded state.

### E. mt-hsp70-independent Translocation Across the Outer Membrane of Mitochondria

Whereas the complete transport of polypeptide chains across the mitochondrial inner membrane requires mt-hsp70 and the membrane potential, several precursor proteins can be translocated across the outer mitochondrial membrane in a manner independent of mt-hsp70. Cytochrome *c*, a soluble protein of the intermembrane space, follows a quite exceptional import pathway (for review, see Stuart and Neupert 1990; Lill et al. 1992b). Efficient import does not require receptor proteins at the surface of mitochondria or the hydrolysis of ATP. Attachment of the heme group in the intermembrane space, catalyzed by cytochrome *c* heme lyase (CCHL), and subsequent folding are thought to drive membrane translocation (Nicholson et al. 1988). CCHL, on the other hand, is transported via the receptor complex in the outer membrane into the intermembrane space seemingly independent from an external energy source (Lill et al. 1992a). Neither ATP depletion nor destruction of the membrane potential across the inner membrane reduced the import efficiency. It is so far not clear how the energy is provided for the vectorial movement of CCHL across the lipid bilayer. Fold-

ing of CCHL or binding to a yet unidentified factor in the intermembrane space could drive the import reaction. Similarly, as in the matrix, a chaperone-like protein might be involved in these processes. However, the observed import of CCHL into isolated outer membrane vesicles argues against the requirement of a soluble factor in the intermembrane space (Mayer et al. 1993). Matrix-localized proteins cannot be imported into these vesicles, most likely because a driving force is missing that in intact mitochondria is provided by the simultaneous, mt-hsp70-dependent translocation across the inner membrane.

## IV. FOLDING AND ASSEMBLY OF MITOCHONDRIAL PROTEINS

After membrane translocation, newly imported polypeptides have to attain their native conformation at their site of function. In many cases, this seems to be an assisted process. An increasing number of genes are being characterized whose functions are required for the assembly of protein complexes in the inner membrane, e.g., the $F_1F_0$-ATPase, the ubiquinol–cytochrome *c* oxidoreductase, and cytochrome *c* oxidase (for review, see Grivell 1989; Ackermann and Tzagoloff 1990; Luis et al. 1990; Buchwald et al. 1991). In many cases, the function of the products of these genes seems to be restricted to assisting assembly of a particular protein complex, i.e., they may function as "private" chaperones. In contrast, the molecular chaperone hsp60, localized in the matrix, was shown to mediate folding and assembly of many mitochondrial proteins (Cheng et al. 1989; Martin et al. 1992; Hallberg et al. 1993).

hsp60 belongs to a family of highly conserved proteins, termed chaperonins (cpn60) (Hemmingsen et al. 1988), that occur in prokaryotes (Hendrix 1979; Hohn et al. 1979) and eukaryotes, where it is present in mitochondria (McMullin and Hallberg 1987, 1988; Jindal et al. 1989; Mizzen et al. 1989; Picketts et al. 1989) and in chloroplasts (Barraclough and Ellis 1980; Martel et al. 1990). hsp60 is encoded by an essential gene whose transcription is increased two- to threefold upon temperature shift to 39°C (Reading et al. 1989). Under these conditions, the protein represents about 0.3% of total cell protein. As other chaperonins, hsp60 is a homo-oligomeric protein composed of 14 subunits with a molecular mass of 60 kD. These subunits are arranged in two-stacked heptameric rings, thereby forming the characteristic barrel-like structure (Hutchinson et al. 1989). hsp60 exhibits an ATPase activity that is modulated by a cochaperonin (cpn10) homologous to GroES in *E. coli* (Goloubinoff et al. 1989a). Although so far only identified in mammalian and plant mitochondria (Lubben et al. 1990; Hartman et al. 1992a,b), the ubiquitous occurrence of GroES homologs is very likely. Mitochondrial cpn10

proteins consist of seven identical 10-kD subunits that form a ring-like structure.

### A. hsp60-dependent Assembly of Matrix-localized and Inner Membrane Proteins

The yeast *HSP60* gene was originally identified in the mutant *mif4* that lacked enzymatic activity of imported mitochondrial proteins (Cheng et al. 1989). At the same time, hsp60 was found in the yeast genome and its DNA sequence was determined (Johnson et al. 1989; Reading et al. 1989). Subsequent biochemical characterization of the temperature-sensitive mutant *mif4* provided direct evidence for the involvement of hsp60 in the assembly of mitochondrial proteins (Cheng et al. 1989). Import of a number of precursor proteins localized in the matrix or the inner membrane was analyzed and found not to be affected at nonpermissive temperature in vivo. However, assembly of the β subunit of the $F_1$-ATPase or of ornithine transcarbamoylase and the maturation of the Rieske-Fe/S-protein were impaired. Under these conditions, a large number of matrix proteins, including Mif4p (hsp60), were found as aggregates in the membrane pellet after extraction of mitochondria. This points to a general role of hsp60 in the assembly of mitochondrial matrix proteins. Recently, these observations were further confirmed by genetic depletion of hsp60 (Hallberg et al. 1993). Yeast strains with a disrupted *HSP60* gene were rescued by expression of the wild-type gene from a galactose-inducible promoter. Growth of cells on glucose-containing medium resulted in depletion of hsp60. As in *mif4* mitochondria, proteins were imported normally but remained insoluble. Interestingly, as with other matrix proteins, hsp60 is required for its own assembly (Cheng et al. 1990; Hallberg et al. 1993). In addition, hsp60 also seems to be required for the assembly of some mitochondrially encoded proteins. In plant mitochondria, the newly synthesized α subunit of the $F_1$-ATPase was found to be associated with hsp60 (Prasad et al. 1990). Taken together, these results demonstrate the requirement of the hsp60 complex for the biogenesis of mitochondrial matrix proteins. However, it was not possible on the basis of these studies to distinguish whether hsp60 affects folding or oligomerization of newly imported proteins.

### B. Role of hsp60 for Sorting Proteins to the Intermembrane Space

A function of hsp60 for sorting of proteins to the intermembrane space is currently a matter of debate. At nonpermissive temperature, accumula-

tion of the intermediate form of cytochrome $b_2$ was observed in the *mif4* strain in vivo (Cheng et al. 1989). Consistently, cytochrome $b_2$ or hybrid proteins containing various amino-terminal parts of cytochrome $b_2$ fused to DHFR were found in association with hsp60 in in vitro experiments (Koll et al. 1992). The association with hsp60 was taken as an additional evidence for the conservative sorting model which predicts that cytochrome $b_2$ traverses the matrix on its sorting pathway to the intermembrane space. As demonstrated in vitro using the purified *E. coli* hsp60 homolog GroEL, ATP hydrolysis resulted in efficient release of the bound protein only if the hydrophobic part of the bipartite presequence of cytochrome $b_2$ was deleted (Koll et al. 1992). This was interpretated to suggest an antifolding effect of hsp60 on intermembrane space proteins with a bipartite presequence. The hydrophobic part of the presequence may promote a prolonged association with hsp60 that keeps the import intermediate in a conformation competent for re-export.

Recently, however, these results were challenged. A different phenotype was described for import of cytochrome $b_2$ and cytochrome $c_1$ into the intermembrane space of mitochondria isolated from the *mif4* strain (Glick et al. 1992). In addition, both proteins were found to be imported with unchanged efficiency after genetic depletion of hsp60 (Hallberg et al. 1993). Although the latter result suggests that hsp60 may not be essential for correct sorting of cytochrome $b_2$ and cytochrome $c_1$ into the intermembrane space, a kinetic effect of hsp60 was not excluded. hsp60 may stabilize intermediates in an export-competent conformation especially under conditions that favor import into the matrix over re-export into the intermembrane space. This might explain the accumulation of the intermediate form of cytochrome $b_2$ that was synthesized and imported into mitochondria after almost complete depletion of hsp60 (Hallberg et al. 1993).

### C. hsp60-mediated Folding of Monomeric Proteins in the Matrix

The demonstration of an impaired assembly of several newly imported proteins in the *mif4* mutant strain at nonpermissive temperature raised the intriguing question of whether folding of monomeric proteins is mediated by molecular chaperones in vivo. An assisted folding reaction might be required to cope with the high protein concentration in the mitochondrial matrix, which may be as high as 500 mg/ml (Schwerzmann et al. 1986), and thus favor aggregation of newly imported or synthesized polypeptides. A hybrid protein containing DHFR fused to a mitochondrial targeting domain (amino acids 1–69 of subunit 9 of the ATP synthase;

Su9[1-69]-DHFR) was used to study folding of proteins within mitochondria (Ostermann et al. 1989). In the native conformation, DHFR exhibits an intrinsic protease resistance, allowing assessment of the folding state of the protein. In *Neurospora crassa*, folding of the DHFR was found to occur with a half-time of about 2 minutes (Ostermann et al. 1989). In contrast, spontaneous refolding of purified DHFR from denaturant in vitro takes place at a considerably faster rate (Touchette et al. 1986). Together with the observed ATP dependence of the folding reaction in mitochondria, these results pointed to a role of hsp60 in mediating the folding of DHFR after import. Indeed, a stable complex of newly imported, unfolded DHFR with hsp60 was isolated from a mitochondrial matrix extract at reduced ATP levels (Ostermann et al. 1989). Addition of ATP resulted in folding of the DHFR in a protease-resistant conformation. Besides hsp60, an additional factor in the matrix was required for efficient folding, most likely a protein homologous to *E. coli* GroES, in the meantime identified in mitochondria of various organisms (Lubben et al. 1990; Hartman et al. 1992a,b). Additional evidence for an hsp60 function in folding of DHFR was obtained by importing a fusion protein, pOTC-DHFR, into *mif4* mitochondria in vivo (Martin et al. 1992). DHFR could only be extracted in a soluble, enzymatically active conformation at 23°C, whereas at the nonpermissive temperature, most of the protein was recovered in the membrane pellet. Therefore, despite the ability of DHFR to refold in vitro spontaneously after dilution from denaturant, in vivo folding is mediated by hsp60. This is also suggested by the slower kinetics of DHFR folding observed in vivo.

These studies established the role of hsp60 in mediating the folding of newly imported, monomeric proteins. The general function of hsp60 is underlined by the observation that under stress conditions (e.g., high temperature), hsp60 prevents the denaturation of a large number of preexisting mitochondrial proteins as well (Martin et al. 1992). After import into mitochondria in vivo, DHFR, a thermolabile protein, was inactivated at 37°C in the absence of functional hsp60 but was stabilized in an enzymatically active conformation in the presence of hsp60. An ATP-dependent association with hsp60 was only detected at high temperature, conditions that result in denaturation of DHFR. This suggests that under stress conditions in vivo, proteins are stabilized by ATP-dependent association with hsp60. Interestingly, in vivo, mt-hsp70 is not able to compensate for hsp60 in maintaining DHFR enzymatically active at 37°C.

A detailed characterization of the folding activity of chaperonins, including mitochondrial hsp60, was performed by reconstitution of the folding reaction in vitro using purified components. These studies revealed principles of chaperonin action as reviewed elsewhere (Gething

and Sambrook 1992; Hendrick and Hartl 1993). Chaperonins of different origins, such as mitochondrial hsp60 and *E. coli* GroEL, can substitute for each other in in vitro folding assays, although with reduced efficiency (Goloubinoff et al. 1989b). This demonstrates the existence of a conserved, ATP-dependent mechanism of chaperonin action. The reduced efficiency of the folding reaction observed when using heterologous components may reflect the parallel evolution of the cochaperonin.

### D. Sequential Action of mt-hsp70 and hsp60 in the Mitochondrial Matrix

In addition to hsp60, folding of newly imported proteins in the mitochondrial matrix requires functional mt-hsp70 (Fig. 3). As discussed in an earlier section, mt-hsp70 promotes the translocation of polypeptide chains across mitochondrial membranes by cycles of ATP-dependent binding to the incoming protein. In the yeast mutant *ssc1-2* at the nonpermissive temperature, the block of import could be circumvented by urea denaturation of precursor proteins (Kang et al. 1990; Gambill et al. 1993). Under these conditions, a fusion protein containing DHFR (Su9[1-69]-DHFR) was imported completely into the matrix. However, DHFR remained bound to mt-hsp70 in an unfolded conformation as demonstrated by coimmunoprecipitation and by assessing its protease sensitivity after lysis of mitochondria. Obviously, protein folding requires the ATP-dependent release of newly imported proteins from mt-hsp70 and most likely transfer to hsp60. A sequential interaction of mt-hsp70 and hsp60 with newly imported matrix proteins was already suggested by the observation that in contrast to mt-hsp70, functional inactivation of hsp60 did not affect the import reaction (Cheng et al. 1989). Indeed, upon import in vitro, the precursor of β-MPP, a subunit of the dimeric matrix processing peptidase of yeast, could be coimmunoprecipitated with mt-hsp70 and hsp60 successively (Manning-Krieg et al. 1991). ATP hydrolysis promotes release from mt-hsp70 and binding to hsp60. In addition, newly imported hsp60 was found in a transient complex with mt-hsp70 prior to its assembly (Manning-Krieg et al. 1991). Interestingly, in hsp60-depleted mitochondria, newly imported hsp60 remained associated with mt-hsp70 even in the presence of ATP (Hallberg et al. 1993). Because of the lack of preexisting hsp60 oligomers, which are required for assembly (Cheng et al. 1990), hsp60 subunits remain bound to mt-hsp70.

The cooperation of mt-hsp70 and hsp60 in mediating folding of proteins localized in the mitochondrial matrix raises the intriguing question of how the ATP-dependent transfer of a polypeptide chain from mt-

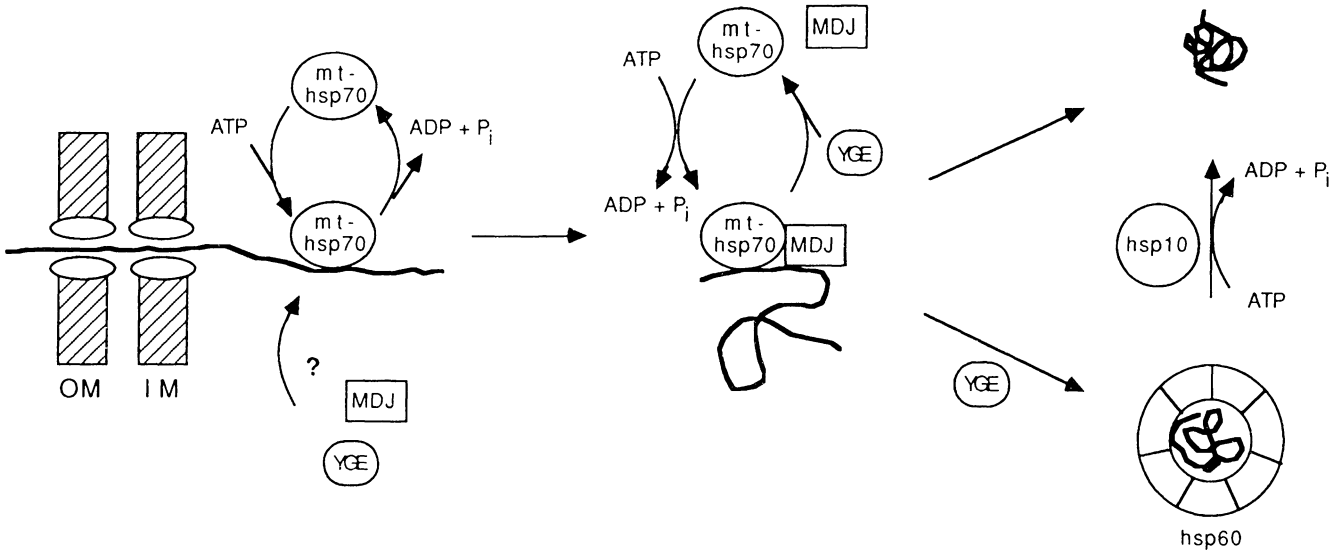


*Figure 3* Hypothetical model of the role of mitochondrial chaperone proteins in protein folding in the mitochondrial matrix. The direct functional interaction of Mdj1p and of Yge1p (see Section IV.D and E) with mt-hsp70 in this process remains to be demonstrated. (Mt-hsp70) Mitochondrial hsp70; (MDJ) mitochondrial DnaJ homolog Mdj1p; (YGE) mitochondrial GrpE-homolog Yge1p; (OM) outer mitochondrial membrane; (IM) inner mitochondrial membrane.

hsp70 to hsp60 is regulated. Reconstitution experiments with purified components allowed further insights into the mechanism of chaperone-mediated protein folding (Langer et al. 1992b). The homologous proteins from *E. coli*, DnaK and GroEL, were used in these studies that share 58% (DnaK and Ssc1) and 54% (GroEL and hsp60) sequence identity with their mitochondrial counterparts (Craig et al. 1989; Reading et al. 1989). The transfer of a protein from DnaK to GroEL was found to be tightly regulated. The ATP-dependent interaction of an unfolded polypeptide chain with DnaK is modulated by two other heat shock proteins of *E. coli*, DnaJ and GrpE (Liberek et al. 1991; Langer et al. 1992b). Binding of DnaJ, which can act as a molecular chaperone on its own, increases the affinity of DnaK for an unfolded protein (Zylicz et al. 1989; Wickner et al. 1991; Langer et al. 1992b). A complex between DnaK and DnaJ is formed that is stabilized by ATP hydrolysis by DnaK. GrpE mediates the ADP release from DnaK, resulting in a decreased substrate affinity of DnaK. Under these conditions, an efficient transfer of the protein to GroEL is observed. Subsequently, folding of the polypeptide chain occurs in association with GroEL in an ATP-dependent manner, most likely within the central cavity of the GroEL cylinder (Martin et al. 1991b; Langer et al. 1992a; Braig et al. 1993). The sequential interaction of the molecular chaperones, DnaK and GroEL, seems to be directed by their binding specificity (Langer et al. 1992b). Whereas DnaK, like various eukaryotic hsp70 proteins (Palleros et al. 1991), exhibits high substrate affinity for polypeptides that are in an unfolded conformation lacking secondary structures, a polypeptide chain in the process of folding to its native state is stabilized by GroEL in a collapsed state characterized by a disordered tertiary structure (Martin et al. 1991b).

The successive interaction of DnaK and GroEL with a polypeptide chain during its folding in vitro may mimic the situation prevailing in mitochondria. Participation in mitochondria of proteins with a function similar to that of *E. coli* DnaJ and GrpE is an attractive possibility. Indeed, a protein homologous to *E. coli* GrpE was recently identified (Yge1p; E. Craig, pers. comm.) that was localized to the mitochondrial matrix. In agreement with the predicted function, the protein is encoded by an essential gene (E. Craig, pers. comm.). On the other hand, a general importance of DnaK-DnaJ-like interactions in eukaryotes is suggested by the identification of proteins homologous to *E. coli* DnaJ in various compartments of a eukaryotic cell; part of them has already been shown to interact functionally with hsp70 proteins (Kurihara and Silver 1992; Caplan et al. 1993). In *Saccharomyces cerevisiae*, *SCJ1* (37% sequence identity to DnaJ) was identified as a gene whose overexpression results

in missorting of a nucleus-targeted cytochrome $c_1$ fusion protein to mitochondria (Blumberg and Silver 1991). However, a mitochondrial localization of Scj1p by cellular subfractionation has not been demonstrated.

### E. Identification of the Mitochondrial DnaJ Homolog Mdj1

Recently, during DNA sequencing of an *S. cerevisiae* λ clone library, another gene was identified that turned out to encode a mitochondrial DnaJ homolog and was therefore termed Mdj1p (mitochondrial DnaJ) (N. Rowley et al., in prep.). The *MDJ1* gene exhibits striking similarity with already known DnaJ homologs. The gene encodes a protein of 511 residues that is 33% identical to *E. coli* DnaJ over the entire length. Moreover, the characteristic sequence motifs found in DnaJ homologs are also present in the *MDJ1* gene. The "J region" of Mdj1p is 50% identical to that in *E. coli* DnaJ and 54% identical to that in Ydj1p, which is located in the cytosol of *S. cerevisiae*. In addition, *MDJ1* contains a glycine-rich region as well as a four times repeated cysteine-containing motif in the central part of Mdj1p, both motifs being characteristic for members of the DnaJ family. In contrast to other known DnaJ homologs, an amino-terminal extension is found in Mdj1p that is rich in basic amino acids, a characteristic feature of mitochondrial presequences. Indeed, Mdj1p is synthesized as a larger presursor protein and imported into isolated mitochondria followed by cleavage of the presequence. The protein was localized to the mitochondrial matrix, more precisely to the inner side of the inner membrane.

To analyze the function of Mdj1p within mitochondria, a gene disruption was carried out (Δ*mdj1*). As with other DnaJ homologs, Mdj1p is not essential for viability. Disruption of the *MDJ1* gene resulted in a *petite* phenotype in yeast. Whereas normal growth on fermentable carbon sources at 30°C was observed, cells were inviable at 37°C and unable to grow on nonfermentable carbon sources at any temperature. Growth at 37°C could be restored by transformation of the disruptant strain with the complete *MDJ1* gene.

Interestingly, *MDJ1* is required for the maintenance of mitochondrial DNA. No mitochondrial DNA was found in the disruptant strain. In view of the known functions of other DnaJ homologs, in particular their functional interaction with hsp70 proteins, an impaired protein import or folding may account for this effect. Alternatively, the observed $\rho^o$ phenotype may reflect a function of Mdj1p in mitochondrial DNA replication or in translation, which is required for maintaining mitochondrial DNA. To obtain further evidence for the role of Mdj1p in mitochondrial biogenesis, protein import and folding within mitochondria were studied.

In contrast to an inactivation of mt-hsp70, disruption of *MDJ1* did not affect protein import into different mitochondrial subcompartments. Matrix-localized proteins (e.g., β subunit of the $F_1$-ATPase), proteins located in the intermembrane space (e.g., cytochrome $b_2$), as well as proteins of the inner (e.g., ADP/ATP-carrier) and outer membranes (e.g., MOM38) were imported in the absence of Mdj1p with the same efficiencies and kinetics as those observed in wild type. In contrast, Mdj1p seems to participate in the folding of both newly imported and preexisting proteins within mitochondria. After import of DHFR fusion proteins into mitochondria, protease-resistant folded DHFR was formed even in the absence of Mdj1p, however, with reduced efficiency. In the Δ*mdj1* strain, insoluble DHFR was in the pellet fraction after low-speed centrifugation, most likely representing aggregated protein. This indicates a role of Mdj1p in folding of newly imported proteins and, in addition, in folding of preexisting proteins. Completely imported DHFR exhibited a decreased heat stability at 37°C in Δ*mdj1*, suggesting a role of Mdj1p in stabilization of preexisting mitochondrial proteins against heat denaturation.

Taken together, these results demonstrate the importance of Mdj1p for the formation of respiratory-competent mitochondria. Further experiments are required to demonstrate a functional interaction with Ssc1p in mediating import and folding of proteins. Such a cooperation would also point to a protein homologous to *E. coli* GrpE recently identified in yeast (as discussed above). It may seem surprising that deletion of *MDJ1* affects folding of mitochondrial proteins, but not membrane translocation, in view of the participation mt-hsp70 in both processes. Studies of the function of *E. coli* DnaK demonstrated that DnaK binds extended polypeptide chains with high affinity, whereas a stable complex with compact folding intermediates can only be detected in the presence of DnaJ (Langer et al. 1992b). Therefore, different conformational states of precursor proteins during membrane translocation and during subsequent folding may account for the different effects of *MDJ1* deletion. The observed requirement of Mdj1p for maintenance of mitochondrial DNA may suggest a role of Mdj1p in mitochondrial DNA replication or protein synthesis, comparable to DnaJ in the replication of viral DNA in *E. coli* (Georgopoulos et al. 1990) or Sis1p in initiation of translation in *S. cerevisiae* (Zhong and Arndt, 1993).

## V. PERSPECTIVES

Molecular chaperones are known to fulfill essential functions during biogenesis of mitochondria. Although general functions are recognized,

in many cases, a detailed analysis of the mode of action of the various chaperones is still awaited. This holds particularly true for their role in maintaining a translocation-competent conformation in the cytosol. Questions addressing the composition of cytosolic complexes that contain mitochondrial precursor proteins, the coordination of various chaperone proteins in the cytosol, or the interplay with targeting factors specific for mitochondrial presequences need to be answered.

Future studies should also allow further insights into the function of chaperone proteins within mitochondria. Although a sequential action of mt-hsp70 and hsp60 has been described, the general importance of this pathway is still a matter of debate. The analysis of Mdj1p mutants may provide further clues as to how the cooperation is regulated. Moreover, mitochondrial chaperone proteins may also participate in processes other than protein import and folding, such as DNA replication, DNA recombination, protein synthesis, and degradation. Again, mitochondria may turn out to represent a useful model system to discover novel functions of molecular chaperones.

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