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Minireview

Molecular Chaperones and Mitochondrial Protein Folding

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Precursor proteins destined for the mitochondrial matrix traverse inner and outer organelle membranes in an extended conformation. Translocation events are therefore integrally coupled to the processes of protein unfolding in the cytosol and protein refolding in the matrix. To successfully import proteins from the cytoplasm into mitochondria, cells have recruited a variety of molecular chaperone systems and folding catalysts. Within the organelles, mitochondrial Hsp70 (mt-Hsp70) is a major player in this process and exerts multiple functions. First, mt-Hsp70 binds together with cohort proteins to incoming polypeptide chains, thus conferring unidirectionality on the translocation process, and then assists in their refolding. A subset of imported proteins requires additional assistance by chaperonins of the Hsp60/Hsp10 family. Protein folding occurs within the cavity of these cylindrical complexes. A productive interaction of precursor proteins with molecular chaperones in the matrix is not only crucial for correct refolding and assembly, but also for processing of presequences, intramitochondrial sorting, and degradation of proteins. This review focuses on the role of mt-Hsp70 and Hsp60/Hsp10 in protein folding in the mitochondrial matrix and discusses recent findings on their molecular mechanism of action.

KEY WORDS: Chaperonins; heat-shock proteins; mitochondria; molecular chaperones; protein folding; protein import.

INTRODUCTION

The analysis of the folding of proteins imported into mitochondria from the cytosol has contributed considerably to our present understanding of chaperone-mediated protein folding. Molecular chaperones in the mitochondrial matrix are intimately involved in major steps of mitochondrial biogenesis, including translocation, refolding, and assembly of both imported and mitochondrially encoded proteins, protein sorting to the inner membrane and intermembrane space, removal of presequences, and protein degradation. Over the last few years, the molecular mechanisms of chaperone action have been established primarily with the help of reconstituted *in vitro* systems, using purified *Escherichia coli* chaperones. The biochemical findings resulting from these studies correlate well with the information obtained from cell biological and genetic studies with yeast mitochondria. In accordance with the endosymbiotic origin of these organelles, the mitochondrial and bacterial protein folding machineries appear to resemble each other closely. The following sections relate recent models for the action of the Hsp70 chaperone system and the reaction cycle of Hsp60/Hsp10 chaperonins to the various functions of these complexes inside the mitochondrial matrix.

MT-HSP70: DUAL ROLE IN TRANSLOCATION AND FOLDING

Mt-Hsp70 is one of the first matrix proteins to interact with an incoming polypeptide chain. This is reminiscent of the situation in bacteria where the prokaryotic Hsp70 homologue DnaK interacts already with nascent polypeptide chains while they are being synthesized at ribosomes (Gaitanaris *et al.*, 1994). How

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does DnaK assist in folding of these proteins? DnaK has the ability to bind and hydrolyze ATP, which modulates its affinity for (poly)peptide substrates. The ATP-bound state of DnaK has low substrate affinity, whereas the ADP state binds peptides strongly. Interconversion between the two states depends on the regulation of DnaK by the two cooperating proteins DnaJ, a chaperone itself, and GrpE (Langer *et al.*, 1992a; Szabo *et al.*, 1994). In a typical reaction cycle, DnaJ docks on to DnaK via its conserved J-domain and stimulates ATP hydrolysis by DnaK. The result is a stable ternary complex consisting of DnaK in its ADP state, DnaJ, and unfolded substrate protein. Binding of the 21-kDa GrpE protein to the ATPase domain of DnaK induces then dissociation of ADP and causes polypeptide release for folding or interaction with other chaperone components (see below). In mitochondria, mt-Hsp70 cooperates with at least two J-domain-containing proteins, Tim44 and Mdj1 (Fig. 1). Tim44 works together with mt-Hsp70 in completing transport of proteins across the inner membrane (Schneider *et al.*, 1994; Scherer *et al.*, 1992; Rassow *et al.*, 1994). Apart from the J-domain, the membrane-associated Tim44 has no homology to DnaJ. One of its functions is to provide a docking place for mt-Hsp70 at the translocation site at the inner membrane to bring the chaperone close to the incoming polypeptide chain. The mt-Hsp70/Tim44 complex binds first to the incoming presequence. The general motif recognized by Hsp70 proteins consists of 7–8 amino acid long extended peptide segments of primarily hydrophobic character (Blond-Elguindi *et al.*, 1993). Due to the presence of polar and positively charged amino acid residues in the presequence, the initial binding affinity is rather low. Mt-Hsp70 would be expected to have a higher affinity for the following mature parts of the incoming precursor protein. Binding of a second mt-Hsp70 via Tim44 leads indeed to a more stable complex that allows for net movement of the precursor protein into the matrix (Ungermann *et al.*, 1996). Dissociation of mt-Hsp70 from its sub-

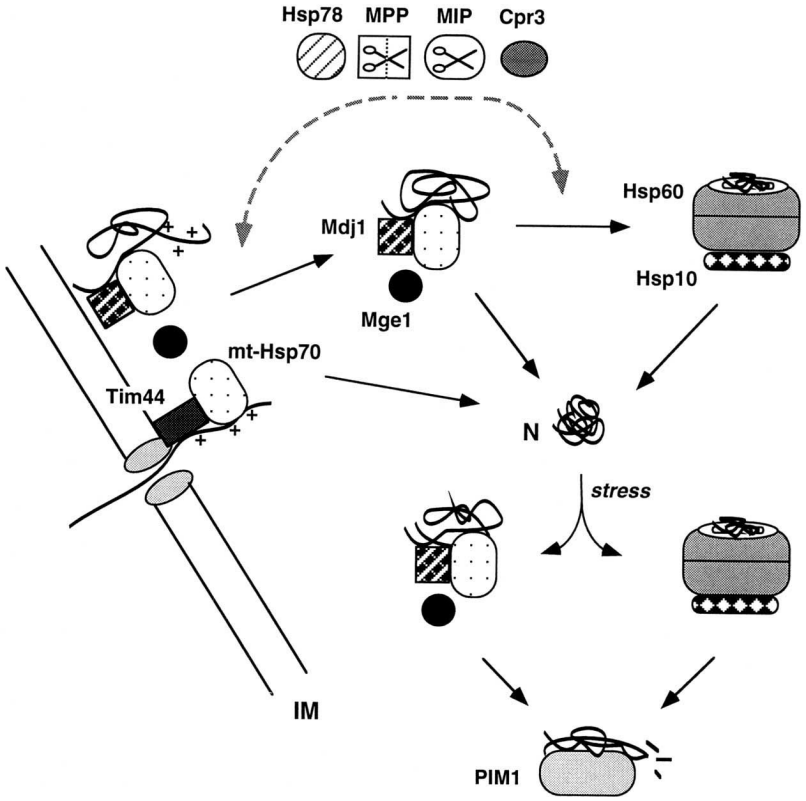

Fig. 1. Model for the interaction of matrix-destined precursor proteins with components of the translocation, folding, and degradation machinery in the mitochondrial matrix. Only the final stages of translocation across the inner membrane (IM) are shown. See text for details of the pathway.

Table I. Mitochondrial Matrix Components Involved in Translocation, Folding, and Degradation of Imported Precursor Proteins in Yeast

Component	Function	Essential	References
Ssc1/mt-Hsp70	Chaperone; partially membrane associated; translocation and folding	Yes	Kang *et al.*, 1990
Tim44	Peripheral membrane protein; cooperating with mt-Hsp70 in translocation	Yes	Maarse *et al.*, 1992; Scherer *et al.*, 1992
Mdj1	Chaperone; partially membrane associated; cooperating with mt-Hsp70 in folding	No	Rowley *et al.*, 1994
Mge1	Cooperating with mt-Hsp70 in translocation and folding	Yes	Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Laloraya *et al.*, 1994
Hsp78	Member of Clp family; folding?	No	Leonhardt *et al.*, 1993; Moczko *et al.*, 1995; Schmitt *et al.*, 1995
Hsp60	Chaperonin system; folding	Yes	Cheng *et al.*, 1989; Rospert *et al.*, 1993; Höhfeld and Hartl, 1994
Hsp10		Yes	
Cpr3	Peptidyl prolylisomerase; folding	No	Davis *et al.*, 1992; Matouschek *et al.*, 1995; Rassow *et al.*, 1995
MPP	Peptidases; processing of presequences	Yes	Pollock *et al.*, 1988, Yang *et al.*, 1988; Isaya *et al.*, 1992
MIP		?	
Lon/PIM1	Protease; degradation	No	Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994; Wagner *et al.*, 1994

strate is mediated by the GrpE homologue Mge1. Temperature-sensitive mutants of this essential nucleotide-exchange factor are defective in translocation and folding of newly imported proteins (Bolliger *et al.*, 1994; Laloraya *et al.*, 1994; Westermann *et al.*, 1995). Mge1 exerts its function probably on other types of mt-Hsp70 complexes in the matrix as well. In addition to its interaction with Tim44, mt-Hsp70 cooperates also with a bona fide DnaJ homologue, Mdj1 (Rowley *et al.*, 1994) (Fig. 1). Although some of the mitochondrial Mdj1 is associated with the inner face of the inner membrane, Δ*mdj1* mutants are fully functional in translocation. Refolding of proteins and stability against heat denaturation, however, are affected in the deletion mutant. It is possible that additional DnaJ homologues exist in mitochondria which partially compensate for loss of Mdj1 function. Multiple DnaJ proteins have been found in *E. coli* and in the eukaryotic cytosol (reviewed in Cyr *et al.*, 1994). Alternatively, the initial 1–2 rounds of interaction with mt-Hsp70/Tim44 are the most critical ones for translocation and subsequent folding of imported precursor proteins, and later rounds with Mdj1 increase merely the efficiency of the process. A precursor protein may also perform more reaction cycles with the mt-Hsp70/Tim44 complex in a Δmdj1 mutant. While a major reason for the tight membrane association of Tim 44 can be seen in coupling the inner membrane translocation machinery to the matrix-located mt-Hsp70, Mdj1 may primarily assist the mt-Hsp70 functions in folding, stress protection, and degradation, which appear to require a free form of the chaperone. It is unclear how important the intrinsic chaperone activity of Mdj1 is in this regard. As the substrate specificities of bacterial DnaJ and DnaK have been shown to be different (Langer *et al.*, 1992a), Mdj1 may considerably modulate the spectrum of substrate protein interactions with mt-Hsp70.

HSP60/HSP10: PROTEIN FOLDING IN ASSOCIATION WITH CHAPERONINS

What is the fate of precursor proteins that originate from mt-Hsp70 complexes after import into the matrix is completed? Smaller proteins with fast folding kinetics may fold after release from the chaperone complex to the native form without further assistance. A subset of the imported proteins requires, however, more substantial folding assistance and has to be transferred from mt-Hsp70 to Hsp60. Hsp60 chaperonins mediate the folding of proteins to their native state or into monomers that subsequently undergo oligomeric assembly. Hsp60 function requires ATP-hydrolysis and regulation by Hsp10 (Martin *et al.*, 1991, 1993; Höhfeld and Hartl, 1994). Like mt-Hsp70, the mitochondrial chaperonins have evolutionary related bacterial counterparts, named GroEL and GroES. The general architecture of *E. coli* GroEL and GroES has been

established by electron microscopy and X-ray crystallography (Langer *et al.*, 1992b; Braig *et al.*, 1994; Hunt *et al.*, 1996). Due to the high sequence conservation between the chaperonins, data obtained with the bacterial components can be directly applied to the mitochondrial Hsp60/Hsp10 system with some confidence. GroEL is a cylinder composed of two heptameric rings of identical 58-kDa subunits that are stacked back-to-back. The equatorial domain of each subunit contains an ATP-binding site, and the apical domains provide binding sites for unfolded polypeptides at the inner surface of the ring, facing the cavity (Fig. 2). Substrate proteins bind in the form of "molten-globule"-like folding intermediates that contain secondary structure but only loosely ordered tertiary structure (Martin *et al.*, 1991). Chaperonins recognize probably hydrophobic secondary structure elements that are exposed in these folding intermediates. The reaction cycle of chaperonin-mediated protein folding has been elucidated during the last couple of years (reviewed in Hartl and Martin, 1995). According to the model shown in Fig. 2, the chaperonin system exists in the cell as a GroEL/GroES complex (step 1). The heptameric single-ring complex GroES binds asymmetrically on top of GroEL in the presence of nucleotides and locks the interacting GroEL-ring in the ADP state. As one of the ring cavities is occluded now, unfolded polypeptide can initially only bind within the GroEL toroid that is in *trans* to GroES. Binding of substrate protein induces conformational changes in GroEL which affect nucleotide binding and facilitate dissociation of GroES (step 2) (Martin *et al.*, 1993). When the smaller chaperonin protein rebinds together with ATP to the GroEL toroid that is in *cis* to the bound substrate protein, the unfolded polypeptide is displaced into the cavity for folding to the native state (steps 3, 4) (Martin *et al.*, 1993; Mayhew *et al.*, 1996). Release into the cavity is probably caused by affinity changes in GroEL during ATP hydrolysis and direct competition of unfolded protein with GroES for the same binding sites at GroEL. After having folded in the cavity, the protein emerges from the inside of the cylinder after dissociation of GroES, which is triggered by ATP hydrolysis in the opposite GroEL toroid (step 5). If the protein exposes after one such reaction cycle still high-affinity binding sites for GroEL, it rebinds before it can leave the complex to undergo another cycle of GroEL/GroES mediated protein folding in the cavity of the chaperonin (step 6). The function of GroES in the cycle is to (a) coordinate and regulate nucleotide binding events in the larger chaperonin GroEL and (b) enclose the folding substrate protein in the cavity and prevent its premature release. It is presently not clear whether during these cycles *in vivo* GroES rebinds exclusively to the same side as the substrate or also in *trans*. In the latter case, unproductive release of non-native polypeptides could occur, as has been demonstrated *in vitro* (Weissman *et al.*, 1994). Under these experimental conditions, release of some non-native protein may reflect a certain leakiness of the chaperonin system which can be prevented by providing more physiological conditions for the folding machinery. Of particular importance here is the molecular crowding phenomenon, which takes into account the high intracellular protein concentrations of more than 300 mg/ml and the resulting excluded volume effects (Zimmerman and Trach, 1991). Physico-chemical properties of the complexes change when chaperonin-mediated protein folding is performed in highly concentrated protein extracts or in inert polymer solutions, where the chaperonin appears to function in a more tightly coupled manner without release of non-native folding intermediates (Martin and Hartl, unpublished).

Assuming that a very similar chaperonin reaction cycle operates in the mitochondrial matrix, we can define a folding pathway in which proteins that have been released from mt-Hsp70 bind to a preexisting Hsp60/Hsp10 complex, will be eventually encapsulated in the chaperonin cavity for folding, and will be released as native proteins or advanced folding intermediates (Fig. 1). The importance of Hsp60/Hsp10 for the biogenesis of mitochondrial proteins has been established early on using temperature-sensitive yeast mutants. The defect of Hsp60 in the *mif4* mutant strain results in misfolding of a variety of imported proteins such as the β-subunit of the F1-ATPase, trimeric ornithine transcarbamoylase (OTC), and the Rieske Fe/S-protein (Cheng *et al.*, 1989). These proteins accumulate as aggregates in the matrix. In line with these results is a recent study with human mitochondria that suggests a deficiency of Hsp60 as the underlying cause for a form of mitochondrial encephalomyopathy (Huckriede and Agsteribbe, 1994). The affected organelles show defects in various matrix enzyme activities and display strongly reduced Hsp60 levels.

Hsp60 is not involved in the actual import and translocation process, either because Tim44 channels incoming precursors directly to mt-Hsp70 and Hsp60 has no access to them, or because the affinity of early import intermediates for the chaperonin is not high enough. Similarly, in bacterial translation lysates GroEL is not found associated with nascent polypeptide chains at ribosomes (Gaitanaris *et al.*, 1994). It is

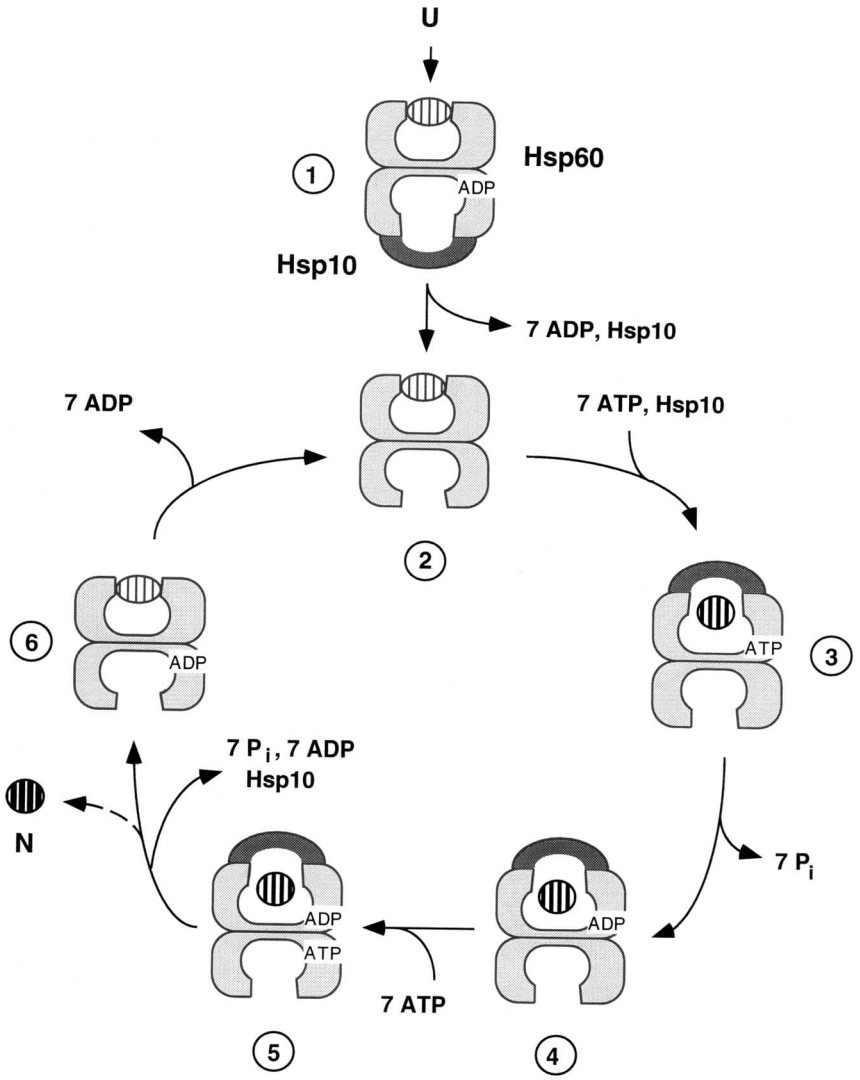

Fig. 2. Model for the reaction cycle of chaperonin-mediated protein folding in the mitochondrial matrix. Most of our knowledge about the mechanism of chaperonin action is derived from *in vitro* studies with the *Escherichia coli* homologues of Hsp60 (GroEL) and Hsp10 (GroES). Structural and functional studies suggest that these evolutionary related and conserved high molecular weight complexes function in a very similar fashion in bacteria and mitochondria. The individual steps of the reaction cycle, leading from unfolded (U) to native (N) protein, are described in the text.

evident from the chaperonin reaction cycle outlined above that productive folding in the chaperonin cavity requires also the participation of Hsp10. Not surprisingly, Hsp10 is essential for the viability of yeast cells at normal growth conditions (Rospert *et al.*, 1993b; Höhfeld and Hartl, 1994). Conditional mutants that are defective in Hsp10 function at higher temperature fail to correctly fold and assemble a number of imported proteins in the matrix, including OTC, the Rieske Fe/S-protein, and the α-subunit of the mitochondrial matrix-processing peptidase (αMPP) (Höhfeld and Hartl, 1994).

PATHWAYS OF PROTEIN FOLDING IN THE MITOCHONDRIAL MATRIX

Do all imported matrix proteins take the same chaperone route to the native state after reaching the matrix and do all precursor proteins interact with the

full spectrum of folding assistants? Probably not. It is likely that all incoming precursors go through at least one or two rounds of interaction with Tim44/mt-Hsp70. These binding and release cycles are accompanied or followed by cleavage of the N-terminal presequence and in several cases also interaction with peptidyl prolylisomerases (see below). Because of the transient nature of interaction with mt-Hsp70 it can be difficult to accumulate certain precursor proteins at the chaperone (Rospert *et al.*, 1996). After completion of translocation, folding routes may begin to differ for individual proteins. Some proteins may bypass free mt-Hsp70 and fold directly after release from the membrane-associated form of the mt-Hsp70 chaperone, or they may be transferred from there directly to Hsp60. For rhodanese, a matrix protein that folds strictly chaperonin-dependent, a pathway of chaperone action has been established *in vitro* with purified proteins and in bacterial cell-free translation systems (Langer *et al.*, 1992a; Kudlicki *et al.*, 1994). After interaction with DnaK/DnaJ, folding intermediates are released in an unfolded form and bind to GroEL/GroES where folding to the native state proceeds. Similar pathways of sequential mt-Hsp70 and Hsp60 action have been demonstrated for mitochondrial import reactions (Manning-Krieg *et al.*, 1991). Between the two extremes of proteins that either bypass Hsp60 or are strictly dependent on interaction with the chaperonin, there are substrates with a modest affinity for Hsp60 which may use the chaperonin system but may not depend on it as an essential requirement for folding. A well-examined example is dihydrofolate reductase (DHFR), a 21-kDa model substrate for import and folding studies. DHFR binds *in vitro* efficiently to GroEL and folds in the cavity of the chaperonin when GroES is present (Martin *et al.*, 1991; Mayhew *et al.*, 1996). In the absence of GroES, ATP hydrolysis leads to release of non-native protein into solution. While this does not represent the physiological situation, DHFR is able to reach the native state nonetheless, as it can fold under many conditions spontaneously. In agreement with these results is the finding that folding of DHFR in mitochondria is not affected by a temperature-sensitive mutant defective in Hsp10 activity (Höhfeld and Hartl, 1994). The intimate coupling between Hsp60 and Hsp10 function and the nature of the reaction cycle suggests, however, that also for those proteins that could manage to fold without Hsp10, folding occurs within the chaperonin cavity with Hsp10 as an integral part of the reaction cycle. It should be noted here that under conditions that make spontaneous folding in solution more difficult, such as heat stress, folding of DHFR becomes GroES-dependent (Martin *et al.*, 1992). DHFR can also be used to demonstrate the subtle differences that exist between chaperonin proteins in the affinity towards their substrates. Unlike with GroEL, DHFR binds at lower temperatures only weakly to yeast Hsp60 and has been proposed to bypass this chaperonin system after import into the matrix (Rospert *et al.*, 1996). Yeast Hsp60 may have indeed a particular low affinity for DHFR, as indicated by the much more stable binding of this protein to the bacterial chaperonin GroEL and the Hsp60s from *Neurospora crassa* and porcine mitochondria (Ostermann *et al.*, 1989; Martin *et al.*, 1991; Rassow *et al.*, 1995; Itoh *et al.*, 1995). Whether a certain protein uses a chaperonin system or folds without it may thus depend not only on the intrinsic folding capabilities of the protein, but also on the particular binding specificities of the chaperonins that are present. Furthermore, the internal diameter of the Hsp60 cylinder sets limits to the size of proteins that fit into the Hsp60 cavity. Proteins that are bigger than 60 kDa are not likely to fold with the complete Hsp60/Hsp10 system.

PROCESSING PEPTIDASES AND PEPTIDYL PROLYLISOMERASES AS MODULATORS OF PROTEIN FOLDING IN THE MATRIX

In addition to molecular chaperones, there are several other matrix proteins involved in protein maturation and folding. The matrix processing peptidases MPP and MIP remove amino-terminal presequences from the imported precursor proteins. They may become already engaged in the maturation process before completion of translocation, when Tim44/mt-Hsp70 interact with the incoming polypeptide chains. Supportive evidence for such an early action comes from studies with plant mitochondria, where the processing enzymes are found tightly associated with the inner membrane (Braun *et al.*, 1992). It appears that the peptidases have no access to the precursors while they are bound to the chaperones and rather bind their substrates after they become released from mt-Hsp70 (Rospert *et al.*, 1996; Ungermann *et al.*, 1996). As has been shown most clearly for bacterial precursor proteins, the presence of presequences can retard folding and may also impair assembly of proteins into oligomeric complexes. The effect of presequences on slowing down folding rates would explain why almost exclusively precursor forms but not mature proteins

are found associated with the mt-Hsp70 chaperone in cofractionation or co-immunoprecipitation experiments (see, for example, Rospert *et al.*, 1996). After their presequences have been cleaved off, many proteins may be able to fold more rapidly. Efficient internalization of hydrophobic structure elements would then make mt-Hsp70 or Hsp60 obsolete for these proteins. Under normal folding conditions MPP and MIP will have completed their processing job before proteins are transferred to Hsp60/Hsp10. A later interaction with their substrate proteins would prove indeed difficult, given that folding to the native state proceeds in the cavity of the chaperonin which is capped by Hsp10.

Whereas the presence of a presequence as an impediment for efficient protein folding is restriced to precursor proteins, a more general factor influencing folding rates is the *cis–trans* isomerization of peptide bonds containing prolyl residues. A ubiquitous family of peptidyl prolylisomerases (PPIase) is supposed to speed up this reaction. The best characterized mitochondrial member of this class of enzymes is the 20-kDa cyclophilin Cpr3 (CyP20) (Davis *et al.*, 1992; Matouschek *et al.*, 1995; Rassow *et al.*, 1995). Like MPP and MIP, this folding catalyst appears to exert its function at an early stage on a relatively unfolded substrate protein (Rassow *et al.*, 1995; Rospert *et al.*, 1996). An early interaction seems advantageous not only because Cpr3 would have difficulty interacting with its substrate in the Hsp60 chaperonin cavity. Cpr3 should be most effective by correcting non-native peptide bonds at an already early folding stage when the recognized peptide regions are most accessible for the enzyme. In this respect it will be interesting to compare the function of Cpr3 with that of the recently discovered ribosome-associated PPIase in *E. coli* (Stoller *et al.*, 1995). Interestingly, in a Δ*cpr3* mutant mature DHFR is found in association with Hsp60 and complexed to mt-Hsp70, indicating that even in the absence of a presequence slow folding kinetics can result in prolonged chaperone interactions (Rassow *et al.*, 1995).

MISFOLDING AND DEGRADATION

If cells are exposed to unfavorable conditions like heat shock, proteins face an increasing risk of becoming unfolded and denatured. Consequently, molecular chaperones are in higher demand and the expression of most of the above described components is increased after heat stress. Mt-Hsp70, Mdj1, Mge1, Hsp60, and Hsp10 are all heat-shock proteins and participate in the protection of cells and mitochondria under adverse conditions. In addition to their function in *de novo* protein folding, chaperones bind to preexistent proteins that have become unfolded under stress and maintain them in a soluble state till more favorable conditions have been reestablished. Proteins like DHFR, which under optimal folding conditions bind to Hsp60 with only moderate affinity, have been shown to depend strongly on the chaperonin at elevated temperatures both in mitochondria *in vivo* and in reconstituted folding reactions *in vitro* (Martin *et al.*, 1992). It is likely that the time a substrate protein spends in a complex with chaperones and the number of reaction cycles that a protein undergoes with the matrix folding machinery vary strongly with the external conditions for folding. In extreme cases, even a permanent association with the chaperones could result. Such a situation is also likely to occur when a protein is intrinsically unable to fold correctly because of a chemical modification, lack of assembly partners, or because of a mutation. An example is provided by hereditary medium-chain acyl-CoA dehydrogenase (MCAD) deficiency where a mutation in the MCAD gene results in impaired folding and assembly rates. The monomer form of the mutant protein forms a much more stable complex with Hsp60 than its wild-type counterpart (Saijo *et al.*, 1994).

A likely fate for such permanently misfolded proteins is degradation. In order to effectively attack their proteolytic substrates, proteases need access to their targets. Recent results indicate that chaperones enable proteases to take over proteins that are destined for degradation by keeping these proteins soluble and preventing them from aggregation. Mt-Hsp70 and Hsp60 form complexes with short-lived and permanently misfolded proteins (Sherman and Goldberg, 1991; Wagner *et al.*, 1994) and the association with the chaperonin was shown to be a rate-limiting step in protein degradation (Kandror *et al.*, 1994). A detailed study by Wagner *et al.* (1994) has shown that ATP-dependent degradation of misfolded proteins in the mitochondrial matrix by the PIM1 protease is also dependent on binding of unfolded polypeptides to mt-Hsp70 and Mdj1 (Fig. 1). In the absence of their correct chaperone function, these proteins aggregate and cannot be degraded. PIM1 protease is the mitochondrial homologue of the *E. coli* protease Lon. A second major type of *E. coli* proteases is represented by the multimeric Clp-like proteins. Only one member of this family, Hsp78, has been

identified so far in mitochondria (Leonhardt *et al.*, 1993). Although deletion of the *HSP78* gene has no discernible effects, in a mutant mt-Hsp70 background loss of mitochondrial DNA and reduced membrane potential were observed. It was concluded that some sort of cooperation between Hsp78 and mt-Hsp70 exists, perhaps with Hsp78 at the center of a salvage pathway under limiting mt-Hsp70 conditions (Moczko *et al.*, 1995; Schmitt *et al.*, 1995) (Fig. 1). It remains to be seen whether Hsp78 activity is restricted to assistance in protein folding or whether its function extends to a role in proteolysis as well.

OUTLOOK

Although the principles of molecular chaperone action in the mitochondrial matrix are established by now, numerous cell biological aspects deserve further investigation. What is the percentage of precursor proteins that actually require and use chaperonins or soluble mt-Hsp70 for refolding? Finding out what precisely determines whether a precursor protein interacts with these chaperones might also provide information about their role in protein sorting to the inner membrane and the intermembrane space, the latter being still a contentious issue. Other open questions concern the function of Hsp78 and the extent of cooperation between folding and degradation machineries. The next few years may also see the discovery of additional folding factors, such as peptidyl prolylisomerases or new members of the Mdj1 family. Finally, a better knowledge of protein folding pathways in the mitochondrial matrix should help us to understand the nature of mitochondrial diseases which are related to protein misfolding and defects in chaperone function. Undoubtedly, mitochondria will continue to be a model system for cellular protein folding in the future.

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