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The Function of Chaperones During Intracellular Protein Sorting, Folding and Assembly

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Introduction

Little is known about the fate of a polypeptide chain between its emergence from the ribosome and its assembly into the final three-dimensional structure. Proteins destined for cellular organelles or proteins that are exported from the cell have to be transported across membranes. The sorting to the correct subcompartment within the organelles involves, in many cases, further membrane translocation steps. In addition to membrane translocation, many proteins must be assembled into protein complexes. All these steps depend critically on the correct conformation of the protein. Premature folding of the newly synthesized proteins may result in a structure that is incompetent for further sorting and assembly steps. Nascent polypeptide chains or incompletely folded proteins might form aggregates that have to be resolved in order to allow sorting and assembly of the polypeptides. Studies in recent years revealed that the cell needs, in each compartment, a set of proteins that

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Abbreviations: AMP-PCP, adenosine 5′-(β, γ-methylene)-triphosphate; AMP-PNP, adenosine 5′-(β, γ-imido)-triphosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; BiP, immunoglobulin heavy-chain-binding protein; Bla, β-lactamase; DHFR, dihydrofolate reductase; DTT, dithiothreitol; ER, endoplasmic reticulum; GIP, general insertion protein; grp, glucose-regulated protein; grp78, 78 kDa glucose-regulated protein; HA0, influenza haemagglutinin; hsc, protein homologous to heat-shock protein; hsp, heat-shock protein; hsp60, 60 kDa heat-shock protein; hsp70, 70 kDa heat-shock protein; MBP, maltose-binding protein; mif, mitochondrial import function; OmpA, outer membrane protein A; PCB, polypeptide chain binding proteins; preBla, precursor of β-lactamase; preMBP, precursor of the maltose-binding protein; proOmpA, pro-outer membrane protein A; RBP, ribulose bisphosphate carboxylase–oxygenase (Rubisco) subunit-binding protein; SDS, sodium dodecylsulphate; SRP, signal recognition particle.

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keeps newly synthesized proteins in a conformation competent for the events of sorting, folding and assembly. These proteins are believed to prevent spontaneous but premature folding immediately after synthesis and to suppress the formation of aggregates of newly synthesized nascent chains.

The term 'chaperone' was introduced to describe this class of proteins. Originally, chaperones were defined as proteins that are needed for the assembly of protein complexes without being part of the final structure (Ellis, 1987). The first chaperone to be identified was nucleoplasmin, which assists the assembly of nucleosomes (Laskey *et al.*, 1978). The function of chaperones seems to be more general and not restricted to assembly alone. As the common mechanism of chaperone action involves binding to polypeptide chains, the term 'polypeptide chain binding proteins' (PCB) was proposed for the whole class of proteins involved in sorting, folding and assembly of polypeptides (Rothman, 1989). Many chaperones were identified as members of heat-shock protein (hsp) families. Heat-shock proteins were originally identified by their selective induction in cells that are incubated at a higher temperature or, more generally, subjected to stress conditions (reviewed by Lindquist and Craig, 1988). But not only stress-induced proteins belong to this class, some of them are expressed constitutively. These proteins are highly conserved during evolution, indicating important functions for the cell.

Two different functions of chaperones should be distinguished: first, maintenance of a precursor protein conformation that is competent for translocation across membranes, folding or assembly; and secondly, assistance in the process of folding or assembly. The first function can be accomplished by a chaperone that solely stabilizes a bound protein in a loosely folded conformation to prevent misfolding or the formation of aggregates. For this purpose a relatively low binding specificity would be needed. Due to the highly co-operative interactions in the protein structure and the low energy difference between the folded and the unfolded state, binding to any part of an unfolded protein that is not exposed in a native protein would be sufficient to destabilize the whole protein and prevent folding. A chaperone of the second class should be attached to the protein during its folding and assembly process. These chaperones could have a guiding function to suppress unwanted folding pathways that lead to unspecific protein aggregation. Their mode of action is completely unknown, but one could imagine that they act by a stepwise release of parts of the bound protein.

In this chapter we summarize what is known about the functions and mechanisms of the various chaperones for protein sorting, folding and assembly.

General features of protein translocation across membranes

TRANSLOCATION COMPETENCE AND UNFOLDING

Proteins are transported across membranes in an unfolded conformation. The first evidence for this was found by studying the export of proteins from the

bacterial cytosol (Randall and Hardy, 1986). When the export machinery was inhibited by dissipation of the proton motive force, a newly synthesized precursor of the maltose-binding protein (preMBP) remained in a conformation which was sensitive to proteolysis. In contrast, a mutant form of preMBP, which was export incompetent due to a mutated presequence, was protease resistant. In pulse-chase experiments the amount of export-competent preMBP correlated with protease sensitivity of the precursor protein. It was concluded that the precursor protein was originally synthesized in a loosely folded conformation and, if subsequent tight folding should occur, membrane translocation would be inhibited. When the import apparatus was blocked by saturating amounts of an overexpressed exported protein, preMBP accumulated in a protease-resistant rather than a protease-sensitive form. These results indicate that a component is needed to maintain the unfolded conformation.

A correlation between folding and translocation competence was also found for mitochondrial precursor proteins. The precursors are synthesized in the cytosol with an amino-terminal extension (termed presequence) and are post-translationally imported into the organelle (reviewed by Attardi and Schatz, 1988; Hartl *et al.*, 1989; Pfanner and Neupert, 1990). After binding to specific receptors at the mitochondrial surface (Söllner *et al.*, 1989, 1990) the precursor proteins are transported through contact sites between the outer and inner mitochondrial membranes (Schleyer and Neupert, 1985; Rassow *et al.*, 1989). When the unfolding of the mature part of the protein was inhibited, e.g. at low temperature or by binding of an antibody to the protein, only partial translocation of the precursor was possible. While the presequence had reached the mitochondrial matrix and was processed by the matrix processing peptidase, the mature part remained outside and was still accessible to externally added protease (Schleyer and Neupert, 1985). A different approach has been made with a fusion protein consisting of a mitochondrial presequence and the cytosolic protein dihydrofolate reductase (DHFR). The resulting protein was imported into mitochondria like an authentic mitochondrial protein. When the specific ligand methotrexate was bound to the DHFR moiety and thereby stabilized its tertiary structure, import of the fusion protein was blocked (Eilers and Schatz, 1986). Import of authentic mitochondrial precursor proteins was not inhibited, excluding a general inhibition of the import apparatus by methotrexate. Thus, binding of methotrexate to DHFR prevented the unfolding of the precursor protein and thereby, inhibited the import.

ATP-DEPENDENT MEMBRANE TRANSLOCATION

The unfolding reaction prior to membrane translocation of proteins is expected to be an energy-consuming process. As almost all membrane translocation steps examined so far require ATP, the unfolding reaction was thought to be linked to this ATP dependence. It has not been shown, however, that the unfolding reaction is directly ATP-dependent, ATP is most likely needed at several stages during protein transport across membranes.

Membrane translocation of bacterial and mitochondrial proteins depends additionally on a membrane potential.

ATP-dependent membrane translocation was first shown for bacterial proteins. Post-translational import of bacterial proteins into inverted vesicles of the bacterial plasma membrane was only possible when ATP was present. Non-hydrolysable ATP analogues inhibited the translocation (Chen and Tai, 1985). In a bacterial strain with a defect in the F_1-ATPase (EC 3.6.1.34) it was possible to analyse separately the effects of the membrane potential and ATP. Both were needed together for efficient protein translocation (Geller, Movva and Wickner, 1986). Not only the post-translational uptake is ATP-dependent. Co-translational transport was found to be inhibited by levels of an ATP analogue that do not inhibit synthesis of the precursor on the ribosomes (Chen and Tai, 1987).

With mitochondria, the different requirements for ATP and the membrane potential during the various steps of protein import could be clearly separated. The membrane potential across the inner mitochondrial membrane is needed for the initial translocation of the presequence. Once the presequence is translocated the import of the residual protein is independent of the membrane potential (Schleyer and Neupert, 1985). When the cell-free import system (isolated mitochondria and rabbit reticulocyte lysate, in which the precursor proteins were synthesized) was depleted of ATP, protein import was blocked (Pfanner and Neupert, 1986; Eilers, Oppliger and Schatz, 1987; Chen and Douglas, 1987; Pfanner, Tropschug and Neupert, 1987). At least for some precursor proteins the lack of import competence correlated with an increased protease resistance (Pfanner, Tropschug and Neupert, 1987). This indicated that ATP depletion resulted in a more tightly folded conformation of the pre-protein and that the presence of ATP was needed to maintain a translocation-competent conformation.

The mitochondrial outer membrane protein, porin, which is synthesized without a cleavable presequence, can be imported both in an ATP-dependent and an ATP-independent reaction. The comparison of the two import modes allowed the identification of the ATP-dependent step in the import of this protein (Kleene *et al.*, 1987; Pfanner *et al.*, 1988). When porin was synthesized in reticulocyte lysate *in vitro* its import depended on the presence of ATP. In contrast, isolated porin that was converted into a soluble form by unfolding was competent for insertion into the outer membrane even in the absence of ATP. Also, the *in vitro* synthesized precursor could be converted to a loosely folded form that was imported independently of ATP. With this example the ATP-dependent import step can be circumvented by artificial unfolding of the precursor protein.

Efficient post-translational uptake of pre-pro-α-factor by yeast microsomes is only possible in the presence of ATP (Hansen, Garcia and Walter, 1986; Mückler and Lodish, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). ATP is not needed for the interaction between ribosome and ribosome receptor or signal recognition particle (SRP) and docking protein (SRP receptor). Proteins that do not need the SRP/docking protein system or the ribosome receptor are imported in an ATP-dependent reaction (Schlenstedt

and Zimmermann, 1987; Wiech *et al.*, 1987). In these studies, ATP seemed to be needed to maintain a conformation of the precursor which is competent for post-translational translocation into microsomes.

The hypothesis that ATP is needed for the unfolding of precursor proteins during translocation has been weakened by recent results. Eilers, Hwang and Schatz (1988) describe an import intermediate of a mitochondrial precursor that accumulates in the presence of a membrane potential but without ATP. This 'ATP depletion intermediate' is loosely folded and was localized bound at the outer membrane. The ATP-dependent step would therefore be the translocation itself and not the unfolding. On the other hand, it was shown that an artificially unfolded precursor protein could be imported even at very low levels of ATP (Ostermann *et al.*, 1989). In the absence of ATP, this import intermediate was in an unfolded conformation, associated with the mitochondrial hsp60 in the matrix and was (partially) processed by the matrix processing peptidase. In these experiments, ATP was needed for the refolding reaction and release from hsp60 and not for the membrane translocation.

A more detailed analysis of the unfolding step was made by studying the import pathway of a fusion protein consisting of an amino-terminal portion of the cytochrome b_2 precursor and the entire DHFR. When unfolding of DHFR was inhibited (e.g. in the presence of methotrexate or at low temperature), complete import was blocked but the presequence was translocated into the mitochondria and was processed in the matrix (Rassow *et al.*, 1989). The DHFR part of the fusion protein remained outside, such that the import intermediate spanned both mitochondrial membranes. When the block of unfolding was released (by removal of the methotrexate or by increasing the temperature), import of the fusion protein was completed. This reaction was independent of ATP, even though it involved unfolding of the DHFR moiety (Pfanner *et al.*, 1990).

When both unfolding and membrane translocation are possible in the absence of ATP, what is then the ATP-dependent step found in many import systems? An alternative explanation for the ATP-dependence of the import of precursor proteins that were synthesized in cell-free translation systems is that cytosolic factors bind to and stabilize the nascent chain after its emergence from the ribosome. These putative factors would have to be released prior to membrane translocation. Several of these factors are members of the heat-shock protein family, and release of these proteins is ATP-dependent.

hsp70: Maintenance of competence for protein transport, folding and assembly

THE ROLE OF CYTOSOLIC hsp70 IN MAINTAINING TRANSLOCATION COMPETENCE OF PRECURSOR PROTEINS

The various members of the hsp70 family are among the most conserved proteins present in the cell. In addition to proteins that are induced by heat, the hsp70 family also contains proteins that are expressed constitutively. These proteins, which are highly homologous to the originally identified

hsp70, are called hsc70. A glucose-regulated protein, grp78, is also a member of this class (Munro and Pelham, 1986). The bacterial hsp70 cognate, namely the DnaK protein, was originally identified as a factor involved in λ-DNA replication (Georgopoulos, 1977; Sunshine *et al.*, 1977; Bardwell and Craig, 1984). All these proteins seem to fulfil related functions, as one should expect from their high degree of sequence identity. The differences in the regulation of their expression has been reviewed extensively (Lindquist and Craig, 1988). For the purpose of this review these proteins are collectively called hsp70 proteins.

The involvement of hsp70 in the process of membrane transport of precursor proteins has been demonstrated by a combination of biochemical and genetic methods. A yeast mutant was generated in which a subset of cytosolic *hsp70* genes was deleted (SSA1, SSA2 and SSA4). The cells were rescued by transformation with a plasmid containing the *hsp70* gene under control of a galactose-inducible promotor (Deshaies *et al.*, 1988). When cells were grown in the presence of glucose the amount of cytosolic hsp70 was drastically reduced and the cells were non-viable. After transfer to glucose medium and pulse-chase labelling, precursor proteins both for mitochondria and the endoplasmic reticulum (ER) accumulated in the cytosol, demonstrating that lack of hsp70 resulted in an inhibition of protein transport from the cytosol into organelles. The requirement for hsp70 during membrane translocation of proteins was also shown in an *in vitro* assay. Precursor proteins synthesized in a wheat-germ extract were incompetent for post-translational import into the ER or mitochondria, unless additional factors present in reticulocyte lysate were added to the import reaction (Chirico, Waters and Blobel, 1988; Murakami, Pain and Blobel, 1988; Zimmermann *et al.*, 1988). Fractionation of the reticulocyte lysate identified hsp70 as one factor. At least one other, so far unidentified, factor was needed, which was sensitive to the alkylating reagent *N*-ethyl maleimide. Similarly, a purified chloroplast precursor protein was only imported in the presence of hsp70 plus an unknown second cytosolic component (Waegemann, Paulsen and Soll, 1990). In a very recent study, the role of the bacterial DnaK protein in the export of proteins from the cytosol was studied (Phillips and Silhavy, 1990). Overexpression of DnaK markedly stimulated the export of a fusion protein that was only inefficiently exported at low levels of DnaK. Similar effects were also found for another bacterial heat-shock protein, the GroEL (*see* below).

All hsp70 proteins have a binding site for ATP and a low ATPase activity. It was therefore speculated that hsp70 was required for the ATP-dependent unfolding of precursor proteins (Pelham, 1986, 1988; Rothman and Kornberg, 1986). So far, however, a direct interaction between hsp70 and precursor proteins has not been demonstrated. In one study a precursor protein was incubated with hsp70 both in the presence and absence of ATP. In the absence of ATP the precursor protein was more resistant to proteolysis than in the presence of ATP (Zimmermann *et al.*, 1988). This supports the idea that hsp70, in the presence of ATP, stabilizes a loosely folded conformation and thereby promotes membrane translocation of proteins.

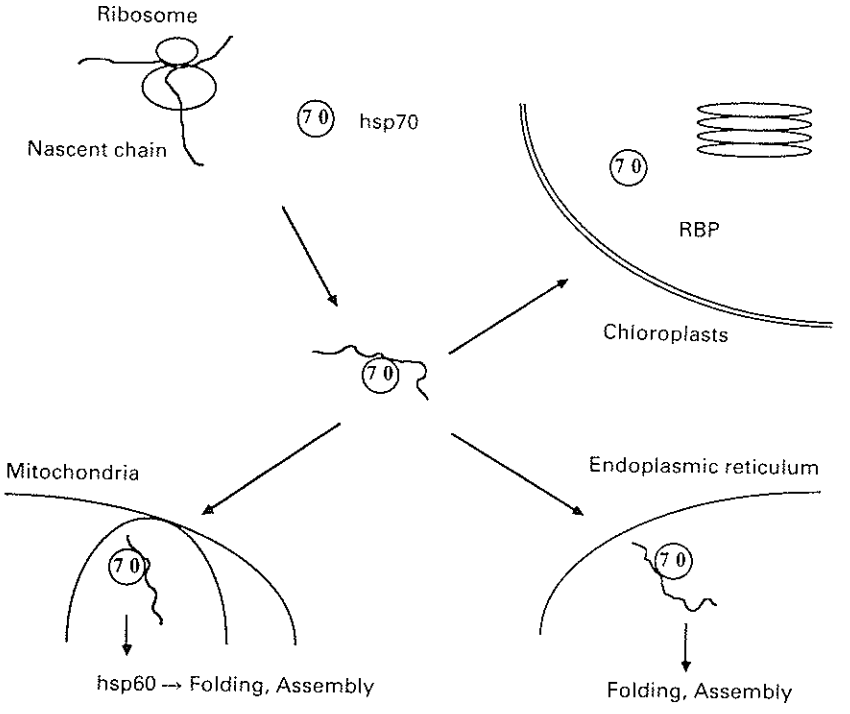

Figure 1. A model for the action of hsp70. During or immediately after synthesis on cytosolic ribosomes precursor proteins might bind to hsp70 in the cytosol. This binding would keep the pre-protein in a loosely folded conformation that is competent for membrane translocation of the pre-protein into the target organelle. After membrane translocation, binding to hsp70 on the lumenal side of the membrane possibly supports translocation, prevents unspecific aggregation and maintains the competence for the following sorting, folding and assembly steps. (Chirico, Waters and Blobel, 1988; Deshaies, Koch and Schekman, 1988; Pelham, 1988; Rothman, 1989; Vogel, Misra and Rose, 1990.)

INTERACTION OF PRECURSOR PROTEINS WITH ORGANELLAR hsp70

Many subcellular compartments contain at least one member of the hsp70 family. For example, yeast mitochondria contain Ssc1p, a member of the yeast hsp70 family that is essential for growth (Craig, Kramer and Kosic-Smithers, 1987; Craig *et al.*, 1989). Hsp70 was also found in the mitochondria of other organisms (Engman, Kirchhoff and Donelson, 1989; Leustek *et al.*, 1989; Mizzen *et al.*, 1989; Amir-Shapira *et al.*, 1990). Ssc1p is a soluble protein in the mitochondrial matrix. Chloroplasts contain three different hsp70-related proteins. One of them is bound to the inner surface of the outer membrane, the other two are localized in the stroma (Marshall *et al.*, 1990).

An hsp70-related protein in the ER was identified by its association with immunoglobulin heavy chains that were synthesized in excess over light chains and were not assembled into functional antibody molecules (Haas and Wabl, 1983). This immunoglobulin heavy-chain-binding protein (BiP) was found to be identical to grp78, a protein that is induced under stress conditions such as glucose deprivation or (in yeast) heat shock. Sequencing of the gene has revealed that BiP/grp78 is a member of the hsp70 family (Munro

and Pelham, 1986). Temperature-sensitive yeast mutants with a defect in the gene coding for BiP indicated an essential function of BiP (Normington *et al.*, 1989; Rose, Misra and Vogel, 1989). These *Kar2* mutants were originally identified as mutants with a defect in karyogamy. How the observed phenotype is related to the function of BiP in protein assembly remains unclear.

Association of newly secreted proteins with BiP has been found under various conditions. Immunoglobulin heavy chains interact transiently with BiP before they assemble with light chains into functional antibody molecules and are exported from the ER (Bole, Hendershot and Kearney, 1986). Inhibition of glycosylation of secretory proteins also leads to an accumulation of underglycosylated, misfolded proteins bound to BiP (Gething, McCommon and Sambrook, 1986; Dorner, Bole and Kaufman, 1987; Hurtley *et al.*, 1989). While the wild-type influenza virus haemagglutinin (HA0) interacts only transiently with BiP, a mutated form that cannot fold properly remains bound to BiP (Gething, McCommon and Sambrook, 1986). Accumulation of unfolded protein in the ER leads to an induction of BiP and another stress protein in the ER, the grp94 (Kozutsumi *et al.*, 1988). Release from BiP occurs only in the presence of ATP. Proteins passing through the ER must be folded correctly and assembled into oligomers before they leave the ER and are transported to the Golgi apparatus (reviewed by Rose and Doms, 1988; Hurtley and Helenius, 1989). Thus, the function of BiP could be to retain misfolded or aggregated proteins in the ER, which otherwise would be transported to the Golgi via the bulk flow. Binding to BiP keeps the misfolded proteins in a conformation which is more sensitive to proteolysis and thereby facilitates degradation. On the other hand, BiP could also have a function in the assembly of proteins in the ER. Binding of BiP to surfaces that are not exposed in the assembled structure could stabilize the newly secreted subunits.

The association with BiP could also be found in an *in vitro* translocation system (Kassenbrock *et al.*, 1988). Only completely translocated but incorrectly folded proteins were found associated with BiP. Binding of nascent chains to BiP could not be demonstrated. On the other hand, recent genetic evidence might suggest a role of BiP during membrane translocation (Vogel, Misra and Rose, 1990). In a detailed analysis of the phenotype of the temperature-sensitive *Kar2* mutant (where BiP is mutated), it was shown that translocation into the ER was defective. Instead, precursor proteins accumulated in the cytosol. Thus, BiP may have a more general function than only binding to misfolded proteins in the ER. One function of BiP could be to bind transiently to newly incoming proteins. It could stabilize newly translocated proteins and, by preventing premature folding, facilitate the process of productive folding and assembly. On the other hand, Nicchitta and Blobel (1990) in a reconstituted system observed translocation into microsomes even in the absence of BiP. However, it is not clear whether this *in vitro* assay represents all steps that are necessary for efficient translocation *in vivo*. Furthermore, small amounts of BiP that were not detectable might still be present.

Recently we have studied the role of the mitochondrial hsp70 in the import of mitochondrial precursor proteins (Kang *et al.*, 1990). A yeast mutant was used that was defective in the *SSC1* gene in a temperature-sensitive manner. Similar to the results with the *Kar2* mutant, import into *SSC1* mutant mitochondria was reduced. The first step of the translocation of precursor proteins into mitochondria was unaffected, i.e. the formation of a contact side intermediate was possible in mutant mitochondria at a non-permissive temperature with the same ratio as in wild-type mitochondria. However, the translocation of the mature part of the precursor protein was strongly retarded. In addition to the defect in membrane translocation, the refolding of imported proteins was slowed down. This could be analysed when the translocation defect was bypassed by artificial unfolding of the precursor protein. Thus, imported proteins appear to interact with hsp70 during membrane translocation. Folding of mitochondrial proteins occurs in association with hsp60 (see below). A role of hsp70 may therefore be in binding the newly incoming proteins at the import sites and delivery to hsp60 for folding. The precise mechanism of membrane translocation remains elusive but one might speculate that the free energy of binding of hsp70 to proteins on the *trans*-side of the membrane provides the driving force for translocation.

A MODEL FOR THE ACTION OF hsp70

Among the known functions of hsp70 is the disruption of protein complexes, e.g. in the process of uncoating clathrin-coated vesicles (Ungewickell, 1985; Chappell *et al.*, 1986; Rothman and Schmid, 1986). A further function is maintenance or even generation of a loosely folded conformation of proteins, thereby conferring competence for membrane translocation or for assembly. How are these functions achieved? A model suggested by Pelham started with the observation that, in the presence of ATP, hsp70 bound to denatured nucleoli in heat-shocked cells (Lewis and Pelham, 1985). Release of hsp70 required hydrolysis of ATP. It was therefore suggested that hsp70 recognized unfolded proteins and bound to hydrophobic surfaces that are not exposed in native proteins. The energy of ATP-hydrolysis might be used in a way that somehow changes the conformation of the bound proteins. Cycles of ATP-dependent binding and release of hsp70 could therefore dissolve the protein aggregates (Pelham, 1986). In a similar way, hsp70 might act in the process of uncoating clathrin-coated vesicles (Ungewickell, 1985; Chappell *et al.*, 1986; Rothman and Schmid, 1986). Here hsp70 binds to the clathrin coat in the presence of ATP. ATP-hydrolysis is needed to displace one of the legs of a clathrin triskelion monomer from the clathrin network.

A first clue to the specificity of hsp70 binding and the mechanism of its action came from a study of binding of peptides to hsp70 or BiP (Flynn, Chappell and Rothman, 1989). Randomly chosen peptides could be bound to hsp70 and released in the presence of ATP. The presence of peptides stimulated the ATPase activity of hsp70. All peptides studied bound to hsp70, but the measured affinities for the various sequences varied over several orders of magnitude. As the assay only allows the study of water-soluble

Table 1. The hsp70 family

Localization	Organism	Protein	Gene	Expression	Putative functions	Ref.*
Prokaryotes						
Cytosol	*E. coli*	DnaK	*dnaK*	Heat induced	DNA replication, protein export	1
Eukaryotes						
Cytosol	Yeast	Ssa1-4p	*SSA1-4*	Heat induced (*SSA1,3,4*) Constitutive (*SSA2*)	Protein translocation into organelles	2
	Mammals	hsp70	*hsp70*	Heat induced	Protein translocation into organelles	3
	Drosophila	hsp70	*hsp70*	Heat induced		4
	Yeast	Ssb1,2p	*SSB1,2*	Heat repressed	?	5
	Mammals	hsc70	*hsc70*	Constitutive	Uncoating of clathrin-coated vesicles	6
Endoplasmic reticulum	Yeast	Kar2	*KAR2*	Heat induced	Prevention of protein aggregation, assistance of productive folding and assembly, protein translocation	7
	Mammals	BiP/Grp78	*BiP/Grp78*	Glucose regulated		8
Mitochondrial matrix	Yeast	Ssc1p	*SSC1*	Heat induced	Protein import into mitochondria, refolding of newly imported proteins	9
	Mammals	P71/Grp75	*P71/Grp75*	Glucose regulated		10
?	Yeast	Ssd1p	*SSD1*	Constitutive	?	11

* (1) Georgopoulos, 1977; Sunshine *et al.*, 1977; Bardwell and Craig, 1984; Phillips and Silhavy, 1990; (2) Deshaies, Koch and Schekman, 1988; Deschaies *et al*, 1988; Lindquist and Craig, 1988; (3) Chirico, Waters and Blobel, 1988; (4,5, 11) Deshaies, Koch and Shekman, 1988; Lindquist and Craig, 1988; (6) Ungewickell, 1985; Chappell *et al.*, 1986; (7) Normington *et al.*, 1989; Rose, Misra and Vogel, 1989; Vogel, Misra and Rose, 1990; (8) Haas and Wabl, 1983; Munro and Pelham, 1986; Rose and Doms, 1988; Hurtley and Helenius, 1989; (9) Craig, Kramer and Kosic-Smithers, 1987; Craig *et al.*, 1989; (10) Engmann, Kirchhoff and Donelson, 1989; Leustek *et al.*, 1989; Mizzen *et al.*, 1989; Amir-Shapira *et al.*, 1990.

peptides, the question whether hsp70 binds to hydrophobic sequences with higher affinities could not be addressed directly. However, there was no clear correlation between the overall charge or hydrophobicity of a peptide with its binding affinity.

These observations led to a different model of hsp70 action. As protein folding is a highly co-operative process, binding of hsp70 to a small region in a protein that is only transiently accessible during thermal oscillations might provide enough energy to unfold the whole structure. Similarly, binding of BiP to a small sequence in a newly secreted protein would prevent premature folding. This binding would reduce the number of possible conformations of the bound protein. Thereby a certain conformation could be selected as the starting point for protein folding. A certain part of the bound protein would be allowed to fold whereas another part remained bound to BiP and therefore remained unfolded (Flynn, Chappell and Rothman, 1989).

The chaperonin family: assistance of folding and assembly

It is assumed that hsp70 binds proteins to stabilize a loosely folded conformation that is competent for later steps of sorting or assembly. So far, however, there is no clear evidence that hsp70 directly assists the process of folding or assembly. Another class of proteins, the chaperonins, seems to fulfil these functions.

COMMON FEATURES OF CHAPERONINS

Chaperonins were defined as a subclass of chaperones (Hemmingsen *et al.*, 1988). They are highly conserved during evolution and are found in bacteria, mitochondria and chloroplasts. The bacterial chaperonin, GroEL, was identified as a protein involved in λ-phage assembly (Georgopoulos *et al.*, 1972; Takaus and Kakefuda, 1972). Temperature-sensitive lethal mutations in the *groEL* gene were isolated, indicating an essential function for normal growth of bacteria. GroEL is closely related to the chloroplast ribulose bisphosphate carboxylase–oxygenase (Rubisco) subunit-binding protein (RBP) (Hemmingsen *et al.*, 1988). This protein was identified by its ability to bind newly synthesized large subunits of Rubisco prior to their assembly (Barraclough and Ellis, 1980). The mitochondrial counterpart, hsp60, was identified as a major mitochondrial heat-shock protein of approximately 60 kDa (McMullin and Hallberg, 1987, 1988). It is involved in several steps of mitochondrial protein sorting, folding and assembly (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). The amino-acid sequence identity between GroEL and RBP (α-subunit) is 46%, between GroEL and hsp60 54% and between hsp60 and RBP 43%. A chaperonin in the eukaryotic cytosol has not been found so far.

GroEL, RBP and hsp60 form a tetradecameric structure of identical subunits (Hendrix, 1979; Hohn *et al.*, 1979; Pushkin *et al.*, 1982; Hutchinson *et al.*, 1989). The molecular mass of the monomers is approximately 60 kDa, the apparent molecular mass of the native structure is between 700 and 800 kDa. Electron microscopic studies showed that the 14 subunits form a doughnut-like structure of two heptamer rings lying on top of each other. The

GroEL particle is 13 nm in diameter and 10 nm in height, leaving a central hole in the middle of the ring. GroEL and hsp60 are heat-shock proteins. GroEL is induced tenfold upon heat shock whereas the level of hsp60 is only moderately increased.

GroEL, A CHAPERONIN INVOLVED IN BACTERIAL PROTEIN ASSEMBLY AND EXPORT

The GroEL protein is an essential host protein for the biogenesis of several bacterial phages. Mutations in the *groEL* gene of *E. coli* results in inability to correctly assemble phage particles (reviewed by Georgopoulos *et al.*, 1990). In the case of λ- or T4-phage the head assembly is defective. Instead of functional head particles they form insoluble aggregates. Interestingly, during T5-phage assembly the formation of the tail and not the head is defective. These mutations also result in a growth defect of the host cell, indicating that the protein has an essential function for the host cell itself. Cloning of the *groEL* gene revealed that it is encoded as an operon together with a second gene, the *groES*. Both genes are needed for phage assembly or normal growth (Tilly, Murialdo and Georgopoulos, 1981). The genes were sequenced: the calculated molecular weights of GroEL and GroES are 57 259 and 10 368, respectively (Hemmingsen *et al.*, 1988).

The GroES also forms an oligomer with a molecular mass of approximately 70–80 kDa (Chandrasekhar *et al.*, 1986). The precise number of subunits is as yet unknown, but it is anticipated that it forms a heptamer similar to GroEL. The subunits form a single ring with a diameter of 8 nm and a central hole of 2 nm. Both genes are transcribed as a single mRNA and the amount of the mRNA is induced four- to fivefold after heat shock (Hemmingsen *et al.*, 1988).

Mutations in both GroEL or GroES were identified that render the bacteria growth sensitive to high temperature. Moreover, mutations were found that result in a cold-sensitive phenotype, indicating that the function of GroEL and GroES is needed at all temperatures, not only high ones (Fayet, Ziegelhoffer and Georgeopoulos, 1989). GroEL has an ATPase activity, which is inhibited in the presence of equimolar amounts of GroES. In the presence of MgATP a complex between GroEL and GroES is formed (Chandrasekhar *et al.*, 1986). ATP hydrolysis is necessary for this interaction, a non-hydrolysable ATP analogue like adenosine 5′-(β,γ-methylene)-triphosphate (AMP-PCP) cannot substitute for ATP.

GroEL BINDS NEWLY SYNTHESIZED BACTERIAL PROTEINS

A first hint of the function of GroEL came from the study of its interaction with newly synthesized proteins (Bochkareva, Lissin and Girshovich, 1988). The precursor of the secretory protein β-lactamase (preBla) was synthesized in a cell-free bacterial translation system with a photo-activatable cross-linker attached to the initiator methionine. After *in vitro* translation and illumination, a cross-linked product was found with a molecular mass on SDS gels of 120 kDa containing the radiolabelled preBla. On sucrose gradients it sedi-

mented with a sedimentation coefficient of 20S and co-fractionated with the GroEL present in the translation extract. The cross-linked product could be co-precipitated with an antibody directed against GroEL. When the translation system was depleted of GroEL no cross-linked protein was found. From these results the authors concluded that newly synthesized preBla binds to GroEL immediately after synthesis. Thus a secretory protein was found in a complex with GroEL after its synthesis, but in addition, a non-secretory protein, the chloramphenicol acetyl transferase, was found associated with GroEL when synthesized in the *in vitro* translation system. Only these two proteins were studied but probably many more, if not most, bacterial proteins interact with GroEL during or immediately after synthesis (Bochkareva, Lissin and Girshovich, 1988).

When ATP (but not non-hydrolysable ATP analogues) was added to the complex, the newly synthesized proteins were released from GroEL. PreBla bound to GroEL was in a translocation-competent conformation which was preserved even after prolonged incubation. Thus, one function of GroEL seems to be maintaining secretory proteins in an unfolded state as a prerequisite for membrane translocation. The observation that incubation with dithiothreitol (DTT) increased the amount of cross-linked protein further substantiated the idea that GroEL binds newly synthesized proteins in an unfolded conformation. DTT interferes with the formation of disulphide bridges and can therefore promote destabilization of newly synthesized proteins. Addition of unfolded protein competed for the binding of preBla to GroEL (Bochkareva, Lissin and Girshovich, 1988). The complex between GroEL and an exported protein could be reconstituted *in vitro* by adding denatured proOmpA to GroEL. Each GroEL tetradecamer bound a single proOmpA (Lecker *et al.*, 1989).

GroEL is also needed for export of preBla *in vivo*. Two temperature-sensitive mutants, one with a defect in the *groEL* and the other in the *groES* gene, accumulated precursor of preBla at the non-permissive temperature (Kusukawa *et al.*, 1989). However, not all secretory proteins were affected. Export of the maltose-binding protein or proOmpA was unchanged as compared to wild type. Further evidence for the involvement of GroEL in bacterial protein export came from the observation that overexpression of the GroE proteins stimulated the export of an otherwise inefficiently exported fusion protein (Phillips and Silhavy, 1990).

The involvement of GroEL in the assembly of a large number of different proteins under *in vivo* conditions is documented by the observation that a number of temperature-sensitive mutations can be suppressed by GroEL. For example, overexpression of the *groE* operon rescued a *dnaA* mutant which had a defect in DNA replication (Fayet, Louran and Georgopoulos, 1986; Jenkins *et al.*, 1986). A temperature-sensitive mutation in the single-strand DNA binding protein, Ssb1, was suppressed by an allele of *groEL* (Ruben *et al.*, 1988). Not only mutations in genes involved in DNA replication were suppressed. A number of mutations in the *his* operon or mutations in genes needed for the secretory pathway could be suppressed by overexpression of GroEL together with GroES (van Dyk, Gatenby and LaRossa, 1989). Thus,

Table 2. The chaperonin family

Localization	Organism	Protein	Gene	Expression	Putative functions	Ref.*
Bacteria						
Cytosol	*E. coli*	GroEL	*groEL*	Heat induced	Phage assembly, protein translocation, assistance of protein folding and assembly	1
Eukaryotes						
Mitochondrial matrix	Fungi	hsp60	*MIF4, hsp60*	Heat induced	Refolding and assembly of newly imported proteins, intramitochondrial protein sorting	2
	Mammals	P1, hsp58	*P1, hsp58*			3
	Human	HuCha60				4
Chloroplast stroma	Plants	Rubisco subunit-binding protein	*RBP*	?	Rubisco assembly	5

* (1) Hemmingsen *et al.*, 1988; Georgopoulos *et al.*, 1990; (2) McMullin and Halberg, 1987, 1988; Cheng *et al.*, 1989; Ostermann *et al.*, 1989; (3) Jindal *et al.*, 1989; Mizzen *et al.*, 1989; (4) Waldinger *et al.*, 1988; Waldinger, Subramanian and Cleve, 1989; (5) Hemmingsen *et al.*, 1988; Gatenby and Ellis, 1990.

the GroE protein appear to somehow stabilize a very large class of other proteins. They could do this by some kind of repair mechanism for unfolded proteins or their overexpression might facilitate the assembly of newly synthesized labile proteins.

THE RUBISCO SUBUNIT-BINDING PROTEIN (RBP)

Ribulose bisphosphate carboxylase–oxygenase (Rubisco) is the major protein of the chloroplast stroma and of photosynthetic bacteria. It catalyses the crucial step of photosynthesis, the fixation of carbon dioxide, and it determines the relative rates of photosynthesis and photorespiration. As the productivity of a plant is essentially limited by this ratio, improving the enzyme by genetic methods is believed to be of considerable biotechnical interest. In chloroplasts, as well as in most photosynthetic bacteria, Rubisco is a multimer of eight catalytic large and eight regulatory small subunits. In *Rhodospirillum rubrum* a simpler form of Rubisco exists which is a dimer of large subunits only (Schneider *et al.*, 1986). The dimer motif seems to be conserved in the hexadecameric form of Rubisco, which has been described as a tetramer of large subunit dimers with eight small subunits attached to it (Chapman *et al.*, 1987; Andersson *et al.*, 1989).

While the large subunit of plant Rubisco is encoded in the chloroplast genome, the small subunit is encoded in the nucleus. The small subunit is synthesized with an amino-terminal presequence in the cytosol, is post-translationally imported into the chloroplast and is processed to the mature-sized form in the chloroplast stroma (Smeekens, Weisbeck and Robinson, 1990). Here, large and small subunits assemble to form functional Rubisco (reviewed by Gatenby and Ellis, 1990). In order to study the enzyme mechanism of Rubisco it is important to analyse the effect of specific mutations in the protein. This could best be done by expressing large and small subunits together in *E. coli*. This approach was successful for a number of prokaryotic Rubisco enzymes (Somerville and Sommerville, 1984; Gatenby, van der Viess and Bradley, 1985; Gurevitz, Somerville and McIntosh, 1985; Viale *et al.*, 1985; Gibson and Tabita, 1986), but not for the Rubisco from crop plants. Expression of the plant subunits alone did not result in functional Rubisco. Obviously specific factors in the chloroplast are needed for the assembly. Therefore, the assembly of Rubisco in isolated chloroplasts has been studied extensively.

When isolated chloroplasts were incubated under appropriate conditions in the presence of [^{35}S]methionine, they continued to synthesize proteins. The major labelled protein was the large subunit of Rubisco (Blair and Ellis, 1973). Upon analysis by SDS gel electrophoresis the large subunit migrated with a molecular mass of 52 kDa. Analysis of the chloroplast proteins by non-denaturing gel electrophoresis yielded an unexpected result. The endogenous functional Rubisco migrated with a molecular mass of approximately 500 kDa, which is the molecular mass of the hexadecameric complex. On the other hand, the newly synthesized large subunits were found in a complex of 720 kDa. Only after prolonged incubation of the chloroplasts in the presence

of light as the energy source were newly synthesized large subunits found in assembled Rubisco (Barraclough and Ellis, 1980). The band corresponding to the 720 kDa complex was also identified by protein staining, indicating that the newly synthesized large subunits were bound to an endogenous chloroplast protein. When the 720 kDa band was analysed by SDS gel electrophoresis the stained protein migrated with a molecular mass of 60 kDa and the radiolabelled large subunit with a molecular mass of 52 kDa. Thus, the 720 kDa form of the large subunit is not an aggregated form of large subunit alone but large subunit bound to a different chloroplast protein (Barraclough and Ellis, 1980). As this protein apparently forms a complex with the newly synthesized large subunit, it was termed Rubisco large-subunit-binding protein (RBP). The best direct evidence for the importance of the RBP for the assembly of Rubisco was obtained by studying the assembly of Rubisco in chloroplast extracts. When the chloroplasts were lysed after synthesis of labelled large subunit and extracts were prepared, the large subunit could be chased from the 720 kDa form in the assembled Rubisco. This reaction could be inhibited by the addition of antibodies against the binding protein (Cannon, Wang and Roy, 1986).

RBP does not only bind to the large subunit synthesized within the chloroplast (Ellis and van der Vies, 1988; Gatenby *et al.*, 1988). When the target sequence of the small subunit was attached to the large subunit and the protein expressed in the cytosol, the large subunit was imported into chloroplasts. Like the chloroplast-encoded large subunit, it bound to RBP prior to its assembly into Rubisco. Furthermore, small subunits interacted with RBP, hence the name Rubisco large-subunit-binding protein was changed to Rubisco subunit-binding protein. It is not known whether other chloroplast proteins also use the same machinery for their assembly.

The RBP was identified by electron microscopy as a particle structurally related to GroEL (Pushkin *et al.*, 1982). It was originally proposed to form a dodecamer (Musgrove, Johnson and Ellis, 1987), but in the light of the extensive similarity in both primary sequence and function it was assumed that RBP forms a tetradecamer like GroEL (Hemmingsen *et al.*, 1988). A GroES homologue has not been identified in chloroplasts so far.

In contrast to GroEL, RBP is not a homo-oligomer but consists of two different subunits, the α- and β-subunits (Musgrove, Johnson and Ellis, 1987). Both subunits are present in equimolar amounts, so the composition of the RBP is probably $\alpha_7\beta_7$. However, the presence of two isoenzymes of RBP, an α_{14} and a β_{14} RBP, is a possibility that has not been ruled out. The molecular masses of the two subunits are similar (60 kDa for the α-subunit and 61 kDa for the β-subunit). Incubation of RBP with MgATP leads to a dissociation of the 14mer into monomers. Only ATP (but no other nucleoside triphosphates or non-hydrolysable ATP analogues) was effective. The role of this dissociation reaction for the assembly of Rubisco is unclear. When chloroplast extracts containing newly synthesized large subunits were analysed by sucrose gradient centrifugation, part of the newly synthesized large subunits were found in a 7S complex (Roy *et al.*, 1982). This low molecular mass form was not detected by native gel electrophoresis; the monomeric

RBP formed a distinct band in non-denaturing gels, whereas the 7S intermediate was only found as a smear in this gel system. This 7S form could correspond to a large subunit bound to monomeric RBP, or it could be a dimer of large subunits. As this dimer is a structural motif present in the hexadecameric Rubisco, such a dimer could be expected as an assembly intermediate. The 7S intermediate is competent for assembly in the hexadecameric Rubisco *in vitro*, but it is not clear whether it is an obligatory intermediate on the assembly pathway.

EXPRESSION OF RUBISCO IN BACTERIA AND RECONSTITUTION OF DENATURED RUBISCO

Attempts to express Rubisco in *E. coli* were successful with a number of prokaryotic forms. In contrast, expression of plant Rubisco was not possible despite many attempts. The observation that the plant chaperone RBP is highly homologous to the bacterial chaperone GroEL led to the idea that GroEL might substitute for RBP in bacteria.

To test this idea both subunits of (hexadecameric) Rubisco of *Anacystis nidulans* were transformed into *E. coli* (Goloubinoff, Gatenby and Lorimer, 1989). When synthesis of the subunits was induced, only a small amount of the synthesized material assembled into functional enzyme. When Rubisco

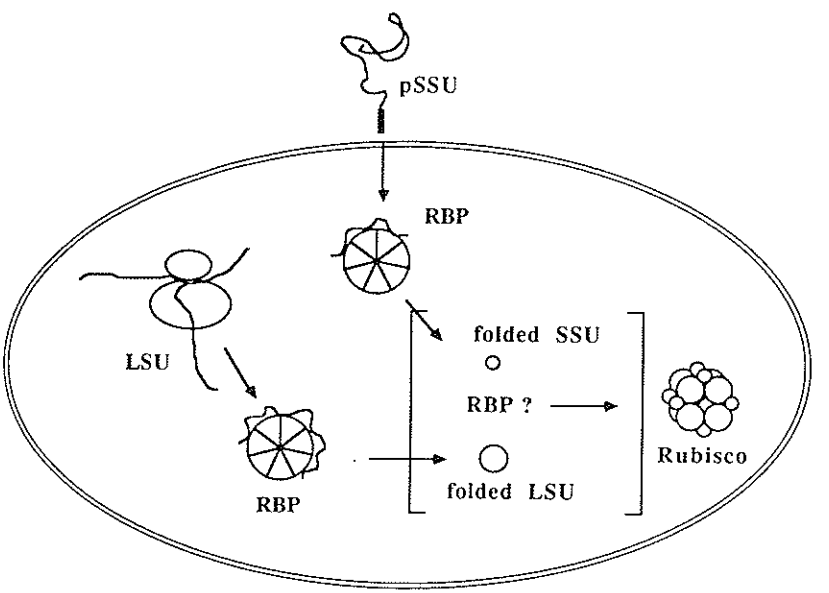

Figure 2. The Rubisco subunit-binding protein (RBP). Large subunits (LSU) of ribulose bisphosphate carboxylase–oxygenase (Rubisco) bind after their synthesis in the chloroplast stroma to the Rubisco subunit-binding protein (RBP). Small Rubisco subunits (SSU) are synthesized in the cytosol and bind to RBP after post-translational import into the chloroplast stroma (Ellis and van der Vies, 1988). The detailed steps of the subsequent folding and assembly steps are unknown. It is possible that the monomeric, folded subunits are released from RBP and assemble (spontaneously or assisted by RBP) into functional, hexadecameric Rubisco (Goloubinaoff *et al.*, 1989). Alternatively, assembly could occur while the subunits are still bound to RBP.

was expressed together with high levels of GroEL and GroES the amount of functional Rubisco was greatly increased. Both GroE proteins together appeared to be needed in this system. The GroE proteins probably assist assembly at an early step, the formation of a dimer of large subunits or earlier. However, possibly, later steps of assembly depend on GroEL and GroES, such as formation of the octameric core of large subunits and addition of the small subunits.

In a recent study, the assembly of active dimeric Rubisco could be reconstituted with purified components in an *in vitro* system (Goloubinoff *et al.*, 1989). Large subunits from *R. rubrum* were isolated and denatured by urea, guadinium chloride or acid treatment. Refolding of the large subunits and formation of enzymatically active dimers after dilution of the denaturant were only observed in the presence of both GroE proteins and MgATP. The GroE proteins were needed in large molar excess over the Rubisco subunits. Probably the unfolded large subunits must bind immediately to GroEL after dilution of the denatured subunits into the refolding assay to prevent the formation of unspecific aggregates. GroEL could be substituted for by hsp60 or RBP, but the addition of GroES is still necessary. This indicates that not only GroEL but also hsp60 and RBP must interact with GroES for proper function, suggesting that mitochondria and chloroplasts contain a protein related to GroES.

THE MITOCHONDRIAL CHAPERONIN hsp60

The mitochondrial chaperonin hsp60 was discovered by studying the effect of heat shock on *Tetrahymena thermophila* (Sinibaldi and Turpen, 1985; McMullin and Hallberg, 1987, 1988) and on cells of a number of other organisms (Hutchinson *et al.*, 1989; Picketts, Mayanil and Gupta, 1989; Reading, Hallberg and Myers, 1989) including human (Jindal *et al.*, 1989; Mizzen *et al.*, 1989; Waldinger *et al.*, 1988; Waldinger, Subramanian and Cleve, 1989). Hsp60 is a major protein even in unstressed cells. In yeast it comprises about 1% of total mitochondrial protein. The effect of heat shock on its expression is not very strong, as it is induced only about threefold. In contrast to RBP, all subunits of hsp60 are identical. A protein similar to GroES has not been found up to now.

Hsp60 is synthesized in the cytosol with an amino-terminal presequence which is cleaved off after import into the mitochondrial matrix. Cells without a functional *hsp60* gene are not viable at all temperatures (Cheng *et al.*, 1989). Hsp60 apparently fulfils an essential cellular function as well as its role after heat shock or other kinds of stress.

Information about the function of hsp60 was obtained by analysing the mitochondrial protein import. Most mitochondrial proteins are synthesized in the cytosol, become post-translationally imported into the organelle and are subsequently sorted to the pertinent submitochondrial compartment, i.e. outer membrane, intermembrane space, inner membrane or matrix. After reaching their respective subcompartments the proteins have to refold and the majority of them assemble into supramolecular structures.

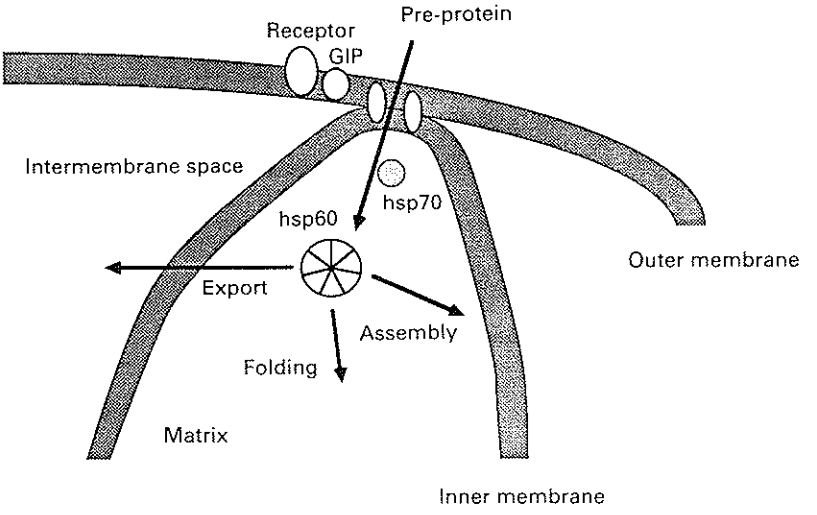

Figure 3. The role of hsp60 in intramitochondrial protein sorting. After synthesis on cytosolic ribosomes, mitochondrial precursor proteins are recognized by proteinaceous receptors on the surface of the outer membrane, are inserted into the outer membrane via the general insertion protein (GIP) and are translocated into the mitochondrial matrix at contact sites between outer and inner membrane. Membrane translocation is assisted by binding to the mitochondrial hsp70 in the matrix. Pre-proteins that are destined for the intermembrane space are kept in an unfolded conformation by binding to hsp60 and are subsequently exported across the inner membrane. Proteins that remain in the matrix fold in association with hsp60, are released as folded monomers or assemble into protein complexes. (Cheng *et al.*, 1989; Ostertmann *et al.*, 1989; Hartl and Neupert, 1990.)

The sorting pathways of a number of proteins in the intermembrane space are particularly complex and involve an additional membrane translocation step (Hartl *et al.*, 1986, 1987). These proteins are first completely translocated at contact sites between both mitochondrial membranes into the matrix. Proteins destined for the matrix remain in this compartment, but proteins destined for the intermembrane space, like cytochrome b_2, or the intermembrane space side of the inner membrane, like cytochrome c_1, are subsequently retranslocated across the inner membrane. These two cytochromes contain a bipartite presequence: at the amino-terminal end they contain a typical mitochondrial targetting sequence that directs the proteins into the matrix. This is followed by a second sequence which comprises a stretch of hydrophobic amino acids resembling a bacterial export sequence. This second part contains the information for the export from the matrix into the intermembrane space, a step probably related to the bacterial protein export from the cytosol. According to the endosymbiont hypothesis, mitochondria are derived from prokaryotes. Mitochondrial genes were transferred to the nucleus of the proto-eukaryotic cell and targetting sequences were attached. These sequences direct the proteins to the mitochondrial matrix where they enter the 'conservative sorting pathway' (Hartl and Neupert, 1990). There-

fore it was to be expected that components involved in mitochondrial and bacterial protein transport would be homologous in function and structure.

By screening a library of yeast mutants that were temperature sensitive for growth, a number of mutants were identified defective in the import of mitochondrial proteins (Pollock *et al.*, 1988; Cheng *et al.*, 1989). These mutants were termed *mif* (mitochondrial import function) mutants. One of these mutants, the *mif4* mutant, had a defect in the *hsp60* gene. At the non-permissive temperature the mutated hsp60 formed insoluble aggregates.

A detailed analysis of the phenotype of the *mif4* mutant provided information about the functions of hsp60 in mitochondrial biogenesis. The import of cytochrome b_2 was studied as an example of a protein destined for the intermembrane space. At the permissive temperature, or in wild-type mitochondria, this protein is first processed in the matrix to an intermediate-sized form, then exported to the intermembrane space and there becomes processed to the mature-sized protein. The precursor protein could still be imported into the mitochondrial matrix when *mif4* mitochondria were preincubated at non-permissive temperatures. However, formation of the mature-sized protein was inhibited, indicating that the export to the intermembrane space was defective. When *mif4* mitochondria were pulse-labelled at the non-permissive temperature *in vivo*, intermediate-sized forms of cytochrome b_2 accumulated. Moreover, $F_1\beta$ imported into *mif4* mitochondria did not assemble into the F_1-ATPase.

hsp60 BINDS NEWLY IMPORTED PROTEINS AND ASSISTS THEIR FOLDING

In a biochemical approach, the function of hsp60 in wild-type mitochondria of *Neurospora crassa* was studied (Ostermann *et al.*, 1989). A stable interaction between newly imported proteins with hsp60 was observed when the import reaction was performed in the absence of ATP. In order to find conditions at which import was independent of ATP, the precursor proteins were treated with 8M urea ('unfolded') and subsequently diluted into the import reaction. To deplete mitochondria of ATP, the mitochondria were preincubated with the ATP and ADP hydrolysing enzyme, apyrase.

One of the proteins studied was a hybrid protein that was constructed by attaching the presequence of F_0-ATPase subunit 9 (Su9) to the cytosolic protein dihydrofolate reductase (DHFR). This fusion protein can be imported into mitochondria like an authentic mitochondrial precursor protein. The Su9-presequence contains solely information for the translocation into the mitochondrial matrix and no further sorting information. The folding state of the DHFR can be easily monitored by the protease sensitivity of the protein. The folded DHFR is highly resistant to proteolysis whereas the unfolded form is very sensitive.

The fusion protein was imported into mitochondria and the protease resistance of the imported protein was measured after lysis of the mitochondrial membranes by detergent. Protease-resistant DHFR was found only when mitochondria contained ATP, indicating that folding is an ATP-requiring step. Non-hydrolysable ATP analogues like adenosine 5'-(β,γ-

imido)-triphosphate (AMP-PNP) or AMP-PCP did not support the folding. The unfolded protein formed a stable complex with hsp60. Hsp60 and the unfolded DHFR co-migrated upon gel filtration and upon non-denaturing gel electrophoresis. An antibody against hsp60 could precipitate this import intermediate. Not only DHFR but also authentic mitochondrial proteins were associated with hsp60 when they were imported in the absence of ATP.

Why are unfolded proteins associated with hsp60? A first clue to the answer of this problem was found by studying the effect of preincubating the mitochondria with *N*-ethyl maleimide. Under this condition the folding was blocked even in the presence of ATP. When, in addition, mitochondria were depleted of ATP, the hsp60-bound intermediate could still be accumulated. Upon incubation with ATP the protein was released from hsp60 and aggregated in insoluble form. Thus, release of the unfolded form from hsp60 alone is not sufficient for folding. The released protein could not spontaneously fold into its native structure in the mitochondrial matrix. Rather, a partial folding of the protein does occur in association with hsp60. This folding reaction could be demonstrated by incubating partially purified hsp60 with bound DHFR in the presence of ATP. The release reaction seemed to be dependent on another factor present in the mitochondrial matrix that was lost in the purification procedure. This factor could be the mitochondrial equivalent of the bacterial GroES. Folding of newly imported mitochondrial proteins therefore occurs in association with hsp60.

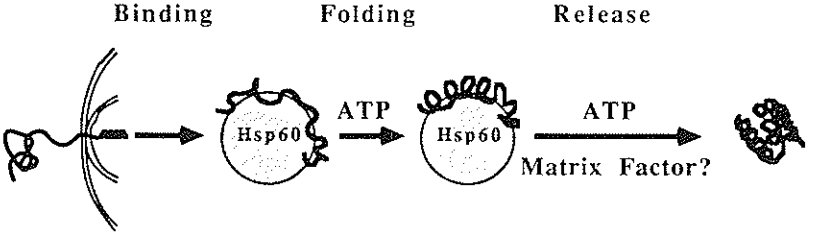

Figure 4. Folding of hsp60-bound proteins. After import into the mitochondrial matrix, the pre-proteins bind to hsp60 in an unfolded conformation. ATP-hydrolysis is needed for folding while the protein is still bound to hsp60. In a second reaction, ATP and an additional factor present in the matrix are needed for release of the partially folded protein, followed by completion of the folding into the final tertiary structure (Ostermann *et al.*, 1989).

Similarly, mitochondrial proteins that are exported from the matrix to the intermembrane space bind to hsp60 after their translocation into the matrix (Ostermann, 1989; Koll, Ostermann and Neupert, in preparation). In contrast to matrix proteins, they do not fold but are delivered to the export apparatus. Here, the function of hsp60 is probably to keep these proteins in a conformation competent for export.

Further chaperones of the bacterial export apparatus: SecB and trigger factor

Bacteria appear to contain several proteins that function in maintaining an export-competent conformation. Two of them, the chaperonin GroEL and the hsp70-related protein DnaK, were discussed above. The other two identified so far, SecB and trigger factor, were found by genetic and biochemical studies, respectively, of the export pathway.

SecB has an apparent molecular mass of 64 kDa and is a 4- to 6mer of identical 16 kDa subunits (Weiss, Ray and Bassford, 1988; Watanabe and Blobel, 1989). It was identified by analysis of a bacterial mutant with a defect in protein export (Emr, Hanley-Way and Silhavy, 1981; Oliver and Beckwith, 1981). The antifolding activity of SecB was discovered by studying the requirement of different mutated forms of the pre-maltose-binding protein (preMBP) for SecB (Collier *et al.*, 1988; Kumamoto and Gannon, 1988). An export-defective preMBP with a mutated leader sequence was overexpressed in bacteria and accumulated in the cytosol. This accumulation led to an inhibition of export of several (but not all) precursor proteins. The export block could be relieved by overexpression of SecB. SecB was assumed to be saturated by the mutated preMBP. What is the function of SecB? The requirement for SecB was studied by analysing the export of a number of preMBP mutants in a SecB-defective strain. Whereas wild-type preMBP accumulated in the cytosol, a mutated protein where the mature part was changed was still export competent. The export competence of precursors correlated with their sensitivity towards proteolytic attack after lysis of the cells. In the SecB-deficient strain, export competence of preMBP was rapidly lost, but in a SecB overproducer, preMBP remained protease sensitive. Isolated SecB could stabilize unfolded, export-competent preMBP in a similar fashion. Thus, the function of SecB is to stabilize preMBP in an export-competent, loosely folded conformation. Only mutated preMBP that cannot acquire a stably folded conformation can bypass SecB (Weiss, Ray and Bassford, 1988).

Upon artificial denaturation, the bacterial outer membrane protein, proOmpA, bound to SecB *in vitro* with a stoichiometry of one proOmpA bound to one SecB multimer (Lecker *et al.*, 1989). Mature OmpA is largely insoluble in water, but when it was denatured and diluted into a solution containing SecB the small soluble part of OmpA is bound to SecB. In these studies, binding of SecB to proteins did not depend on the presence of the presequence but on some structural motifs present in denatured but not in native proteins. Similarly, SecB bound to export-deficient preMBP with an almost completely deleted presequence with similar efficiency as native preMBP (Collier *et al.*, 1988). In contrast to these findings, Blobel and co-workers reported that mature MBP did not interact with SecB, and concluded that SecB binds to the presequence, similar to the eukaryotic signal-recognition particle (Watanabe and Blobel, 1989). Probably the interaction of SecB with denatured proteins is a kinetic competition between folding into the native conformation which does not bind to SecB and binding of the unfolded protein to SecB. The presence or absence of the presequence

will critically influence the speed of the refolding reaction and therefore facilitate the binding to chaperones (Randall and Hardy, 1989).

Another bacterial chaperone, termed trigger factor, with an apparent molecular mass of 63 kDa was reported to be involved in the export of proOmpA (Crooke and Wickner, 1987; Crooke *et al.*, 1988a). Trigger factor is attached to ribosomes and probably binds to proOmpA during its synthesis (Lill *et al.*, 1988). Isolated, urea-denatured proOmpA rapidly lost its export competence when it was diluted into buffer without trigger factor. When trigger factor was added, export competence was retained (Crooke *et al.*, 1988b). Other exported proteins, like preMBP or prePhoE, did not form complexes with trigger factor. When proOmpA was prebound to trigger factor and incubated in the presence of SecB or GroEL, it could exchange between different chaperones. Most of the proOmpA was then found attached to SecB or GroEL (Lecker *et al.*, 1989).

Perspectives

More and more proteins are now being identified that protect other proteins during intracellular sorting or assembly. Various assay systems have been established to study their function which allow us to address quite a number of intriguing questions.

The nature of the binding between chaperones and unfolded proteins is still unknown. Which structural element is recognized that is only present in unfolded but not in native proteins? Do the chaperones bind only to a small part of the polypeptide chain, or is the complete protein bound at the surface of the chaperone? The first alternative is more likely for the relatively small hsp70, but the tetradecameric chaperonins hsp60, GroEL and RBP could provide enough sites to bind more than a small part of the protein.

For the chaperonins at least, it has been shown that they are directly involved in protein folding and assembly, but the mechanism of their action is still mysterious. One possible mechanism could be that the bound protein is released in a stepwise fashion, allowing certain parts of the protein to fold earlier than others. Another possible mechanism could be that the bound protein is released from the chaperone for a short time. Then partial folding could occur before the protein binds again to the chaperone. The first crystal structure for a chaperone, the PapD (a protein involved in the formation of pili in *E. coli*) has been obtained recently (Halmgren and Bränden, 1989). Increasing knowledge of the chaperone structure will certainly help to elucidate their mechanism.

Do different chaperones act in a concerted fashion? In all compartments where hsp60-related proteins were identified, hsp70 is also present. They could act independently from each other, but their closely related functions suggest that they might co-operate. Is there a transfer of unfolded proteins between different chaperones? For example, proteins that were translocated across membranes might be transferred via a chaperone like hsp70 from the translocation machinery to a different chaperone like hsp60 where folding can occur.

Several biotechnological procedures critically depend on the correct localization and conformation of newly expressed proteins. Our understanding of these processes is a prerequisite to improve this technology. Thus, the problems of chaperone-assisted membrane translocation, folding and assembly of proteins are a rapidly developing area of both basic and applied research that will be of increasing interest for biotechnological procedures.

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