# Heat Shock Proteins and Immune Response

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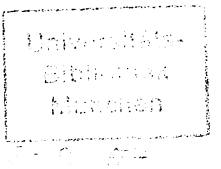

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# Basic Features of Heat Shock Proteins

# Heat Shock Proteins hsp60 and hsp70: Their Roles in Folding, Assembly and Membrane Translocation of Proteins

T. LANGER and W. NEUPERT



## 1 Introduction

Heat shock proteins were initially recognized by their increased expression after exposure of cells to elevated temperatures (RITOSSA 1962; LINDQUIST 1986; LINDQUIST and CRAIG 1988). Subsequently a number of exciting findings stimulated the interest in heat shock proteins. First, homologous proteins were identified in prokaryotic and eukaryotic organisms, suggesting an important general function. Second, it turned out that the various proteins can be grouped into a few distinct families with a high degree of structural conservation during evolution. Third, it was realized that besides elevated temperature other kinds of

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"stress" conditions lead to the induction of proteins which are identical or similar to those induced by heat. For instance, metabolic stress such as glucose starvation or the presence of amino acid analogues was observed to induce a subset of proteins which are immunologically related to heat shock proteins but can be differentiated on the basis of ionic charge (LEE 1987). Fourth, it was realized that some of these heat shock or stress proteins are expressed constitutively in the absence of any kind of stress in various cell types.

Two major lines of research were provoked by these observations. On the one hand, temperature induction proved to be a process well suited to study the molecular basis of regulation of gene expression in both bacteria and eukaryotes. On the other hand, the structure and function of heat shock or stress proteins attracted the attention of researchers in various fields of biology. The present review is not concerned with the complex patterns of regulation of heat shock gene expression (for a review see NEIDHART et al. 1984; LINDQUIST 1986; PELHAM 1989b). It should be pointed out, however, that despite the impressive achievements in defining regulatory elements involved in heat shock regulation, the complete chain of signal transmission from the heat effect to the turning on of specific genes remains elusive. Recent results indicate a role of denatured proteins as a common factor for induction of heat shock proteins (GOFF and GOLDBERG 1985; LEWIS and PELHAM 1985). It is reasonable to assume that the different kinds of stress promote denaturation of intracellular proteins which in turn signal the induction of heat shock proteins (ANANTHAN et al. 1986; KOZUTSUMI et al. 1988). The effect of denatured proteins may be indirect, however, for example by reducing the internal concentration of an unknown regulatory factor.

In the last few years molecular cloning, protein purification, and functional studies in vitro and in vivo have led to a better understanding of the role of heat shock proteins in the sorting, folding, and assembly of proteins, as will be discussed in the present review. It is generally accepted that heat shock proteins protect cells from the damaging effect of temperature or other kinds of metabolic stress. A wide variety of cells have higher thermotolerance after preincubation at an elevated temperature (reviewed in LINDQUIST and CRAIG 1988).

Strong evidence is accumulating that heat shock proteins are necessary for the acquisition of the native structure of monomeric and oligomeric proteins after their synthesis on ribosomes or after transfer across membranes. The transient exposure of hydrophobic or charged residues during these processes can result in misfolding or aggregation of proteins. Therefore, folding in vivo, at least in some cases, seems to require the presence of protein factors. The term "molecular chaperone" was proposed for such proteins (ELLIS 1987), which prevent incorrect interactions and assist assembly without being part of the final structure. It was first used by LASKEY et al. (1978) to illustrate the function of nucleoplasmin in the assembly of nucleosomes. It is now becoming apparent that heat shock proteins can act as chaperones in vitro and in vivo, as will be discussed in detail below. The strongest lines of evidence exist for the members of the hsp60 and hsp70 families. Therefore this review will focus on the role of

these proteins in the acquisition of the native structure of proteins in the cell and in the assembly of biological membranes.

## 2 The hsp70 Family

In each organism examined so far heat shock proteins with a molecular mass of about 70 kD have been found in abundance. They have been observed not only in yeast and mammalian cells, but also in organisms as diverse as plants, trypanosomes, and *Escherichia coli*. hsp70 proteins have a very high degree of evolutionary conservation. Between the bacterial members of the hsp70 family, DnaK, and eukaryotic members, the homology is higher than 45%.

Within the eukaryotic cell hsp70-related proteins were detected in the cytosol compartment, mitochondria (LEUSTEK et al. 1989; MIZZEN et al. 1989; ENGMAN et al. 1989; AMIR-SHAPIRA et al. 1990), chloroplasts (KRISHNASAMY et al. 1989; MARSHALL et al. 1990), and the endoplasmic reticulum (ER). They are members of a multigene family. Some are induced by various stress conditions, others are expressed constitutively. The most thoroughly studied system is that of yeast (as reviewed in detail by LINDQUIST and CRAIG 1988). Nine genes related to the *hsp 70* gene of higher eukaryotes were identified and cloned. Mutational analysis revealed five distinct groups. Ssa1-4, present in the cytosol, are essential for cell viability and may have interchangeable function. hsp70 proteins of the Ssb group (encoded in *ssb1* and *ssb2*), also present in the cytosol, and Ssd1 have not been further characterized. Ssc1 is a mitochondrial protein of 70627 daltons (CRAIG et al. 1989) with a characteristic amino terminal targeting sequence. Mutant strains in which the *ssc1* gene was deleted were not viable (CRAIG et al. 1987). Recently, *kar2* was identified as the yeast homologue of the mammalian *BiP/grp78* gene (ROSE et al. 1989; NORMINGTON et al. 1989), which is essential for cell viability and, in addition, is required for nuclear fusion. A cleavable amino terminal hydrophobic signal sequence targets the protein to its site of function, the lumen of the ER.

### 2.1 General Enzymatic and Structural Properties of hsp70 Proteins

Proteins of the hsp70 family tightly bind ATP, a property that can be used for purification by ATP–agarose chromatography (WELCH and FERAMISCO 1985; CHAPPELL et al. 1986). A weak ATPase activity appears to be a general feature of these proteins. Although several hsp70 proteins have been cloned and sequenced, no consensus sequence for an ATP binding site has been identified so far.

A first structural characterization of hsp70 proteins came from protease degradation studies and mutational analysis. The generation of a stable amino terminal fragment of roughly 44 kD after cleavage with proteases such as chymotrypsin and trypsin suggests a common domain structure of hsp70 proteins. CHAPPELL et al. (1987) purified and characterized this fragment of the uncoating ATPase. They proposed a two-domain model for the hsp70 proteins, which is supported by recent deletion studies with both a human hsp70 (MILARSKI and MORIMOTO 1989) and *E. coli* DnaK (CEGIELSKA and GEORGOPOULOS 1989). The amino terminal part of the molecules harbors the ATP-binding site and the ATPase activity. CHAPPELL et al. (1987) suggested the term "ATPase core" for this domain. Deletion of the N-terminal region in DnaK and human hsp70 results in the loss of both the ATPase activity and the ATP-binding ability. In the carboxy terminal domain the sequence homology between different hsp70 proteins is relatively low ($< 30\%$). According to the model, this part of the molecule contains activities specific for the different hsp70s, e.g., for the disassembly of coated vesicles in the case of uncoating ATPase. In keeping with the idea of the so-called substrate-binding domain of hsp70 proteins, truncated human hsp70 (lacking carboxy terminal amino acids) shows a lack of proper nucleolar localization after heat shock (MILARSKI and MORIMOTO 1989). A carboxy terminal deletion of DnaK results in the loss of its autophosphorylating activity (CEGIELSKA and GEORGOPOULOS 1989). The so-called substrate-binding domains of other hsp70 molecules remain to be determined.

Although the crystallization of the ATPase fragment of the bovine uncoating ATPase was reported (DELUCA-FLAHERTY et al. 1988), the solution of the structure of an hsp70 protein by X-ray crystallography is still awaited. Preliminary circular dichroism studies of bovine hsc73 and the bacterial DnaK indicate a high content of $\alpha$-helical structure of both proteins (SADIS et al. 1990).

## 2.2 Physiological Functions of hsp70 Proteins

The fact that hsp70 proteins were found associated with several cellular and viral proteins in an ATP-dependent manner suggests that direct protein–protein interactions are an essential theme in their function. A first hint at the physiological role of heat shock proteins resulted from determination of their cellular distribution after heat shock (for a review see LINDQUIST and CRAIG 1988). Several heat shock proteins became concentrated in the nucleolar region, including hsp70 proteins (WELCH and SUHAN 1985). They were concentrated in the granular region, the site of preribosomes. Other heat shock proteins, such as hsp110, were found in the fibrillar region, the site of nuclear chromatin (SUBJECK et al. 1983). Nucleoli are heat sensitive and exhibit an altered morphology after heat shock. The recovery of nucleolar morphology occurred more rapidly in the presence of a constitutively expressed hsp70 in the cell, even when synthesis of other heat shock proteins was blocked (PELHAM 1984). This indicates an involvement of the protein in repair processes of RNP structures.

Interaction of hsp70 proteins has been described in the literature with a large number of diverse proteins including: clathrin (UNGEWICKELL 1985; CHAPPELL et al. 1986); the cellular proto-oncogene p53 (PINHASI-KIMHI et al. 1986); certain trans-activating proteins such as E1a of adenovirus (WALTER et al. 1987); malfolded or incompletely assembled proteins in the ER (with BiP, see below); and, as in the case of the prokaryotic hsp70 homologue, DnaK, some proteins involved in phage lambda replication. Recently, immunoprecipitation studies with hsp70-specific antibodies in human cells revealed cell cycle-specific interactions with a number of unidentified proteins (MILARSKI et al. 1989). Interestingly, in these studies coimmunoprecipitation of two different hsp70 proteins was observed. This is consistent with the previously described copurification of these proteins over several steps (WELCH and FERAMISCO 1985) and might suggest a possible functional interaction between these proteins or with identical cellular proteins.

The investigations performed to date show that the hsp70 proteins can be released from their substrates in an ATP-dependent manner in vitro. Nonhydrolyzable ATP analogues have no effect, indicating that ATP hydrolysis is necessary. However, it is not entirely clear whether ATP also has a role in the dissociation of complexes in vivo. Recently, decreased protease sensitivity of BiP was observed after adding adenine nucleotides like ATP and ADP, but not after adding nonhydrolyzable ATP analogues. This may point to a role of the nucleotides in stabilization or induction of different conformations of BiP in the absence of ATP hydrolysis (KASSENBROCK and KELLY 1989). Furthermore, covalent modifications of hsp70 proteins, such as ADP ribosylation, methylation at lysine and arginine residues, or phosphorylation at serine and threonine residues, may be involved in the regulation of their function (WANG and LAZARIDES 1984; HENDERSHOT et al. 1988).

A general model for the action of hsp70 has been proposed that accounts for its ATP-dependent association with other proteins (LEWIS and PELHAM 1985; PELHAM 1986, 1988). Hydrophobic sequences, which are normally buried inside proteins, are exposed after partial denaturation during heat shock or during other cellular processes such as translocation across membranes (see below). This results in an increased tendency for aggregation or misfolding. According to the model, hsp70 proteins prevent or disrupt wrong protein–protein interactions by binding to the exposed regions. This would be reversed with the aid of ATP hydrolysis. Repeated cycles of binding and release could thus repair damage after heat shock or prevent aggregation of proteins. Therefore, the model postulates a general affinity of heat shock proteins for denatured proteins. Although participation of hydrophobic interactions in the binding of hsp70 proteins appears possible, direct evidence for this does not exist.

Recently, an in vitro assay for studying the interaction of hsp70 proteins with model substrates was described (FLYNN et al. 1989). As a first approximation for a native or unfolded protein synthetic peptides were found to bind to purified BiP or hsp70. The ATPase activity of hsp70 and BiP was stimulated by the presence of peptides and caused dissociation of the complexes. Two important observ-

ations were made in these studies. First, BiP can bind peptides without added ATP, whereas ATP hydrolysis is necessary for the release. This is consistent with the identification of deletion mutants of human hsp70, which cannot bind ATP but can associate with nucleoli (MILARSKI and MORIMOTO 1989). Second, there was no clear correlation between the overall hydrophobicity of the peptide and its binding affinities. The assay only allows the testing of water-soluble peptides; thus, the affinities for hydrophobic peptides could not be determined. The different affinities of BiP and hsp70 for the various peptides tested may suggest the existence of sites specific for certain sequences or secondary structures rather than for unspecific epitopes. Thus, binding of BiP and hsp70 to peptide segments on the outer surface of a folded protein appears in principle to be possible. This may result in disaggregation or (partial) unfolding of proteins. On the other hand, the free energy of binding could also be used to stabilize conformations which favor certain folding pathways, reduce the tendency for aggregation, or allow membrane translocation (FLYNN et al. 1989).

### 2.2.1 DnaK—The Prokaryotic Homologue

Among the prokaryotic heat shock proteins identified so far (GEORGOPOULOS et al. 1990), only one, namely DnaK, is homologous to eukaryotic hsp70 protein. The sequence identity to *Drosophila* hsp70 protein is 48% (BARDWELL and CRAIG 1984), and to yeast Ssa1 protein, 49.8% (CRAIG et al. 1989). Mutations in the *dnaK* gene were found to lead to a block in bacteriophage $\lambda$ DNA replication at all temperatures (GEORGOPOULOS 1977; SUNSHINE et al. 1977). Later on DnaK was characterized as a heat shock protein encoded in the heat shock regulon of *E. coli* (BARDWELL and CRAIG 1984). DnaK is an abundant, consitutively expressed protein with an apparent molecular weight of 70 000. The purified protein (ZYLICZ and GEORGOPOULOS 1984) possesses a weak ATPase activity (with a turnover number of about one ATP per minute) and can be autophosphorylated at threonine residues (ZYLICZ et al. 1983). Its enzymatic activities are well studied (CEGIELSKA and GEORGOPOULOS 1989; DALIE et al. 1990). The ATPase is DNA independent, but is modulated by $\lambda$O and $\lambda$P proteins in vitro and in vivo (ZYLICZ et al. 1983). In contrast to ATP binding, ATP hydrolysis and the autophosphorylating activity depend on divalent cations (CEGIELSKA and GEORGOPOULOS 1989; DALIE et al. 1990). Interestingly, $Ca^{2+}$ ions which inhibit the ATPase, stimulate the autophosphorylation activity, indicating a regulatory role of $Ca^{2+}$ (CEGIELSKA and GEORGOPOULOS 1989). The existence of a highly conserved calmodulin-like binding domain in various members of the hsp70 family may be of relevance in this context (STEVENSON and CALDERWOOD 1990).

The role of DnaK in bacteriophage $\lambda$ replication has been investigated in detail. Besides $\lambda$O and $\lambda$P proteins several host proteins are necessary for initiation of DNA replication, including three heat shock proteins, DnaK, DnaJ, and GrpE (reviewed by GEORGOPOULOS et al. 1990). From biochemical and electron microscopic data it became apparent that DnaK participates in an ordered assembly and partial disassembly of the initiation complex, leading to

localized DNA unwinding (LIBEREK et al. 1988; ALFANO and MCMACKEN 1989a, b; DODSON et al. 1989). In the first step dimeric λO proteins bind specifically to oriλ (about 60 molecules of λO monomers per oriλ; LIBEREK et al. 1988). Complexes of λP protein and the *E. coli* DnaB interact with the resulting nucleosome-like structure. After binding of DnaJ to this prepriming nucleoprotein structure addition of DnaK leads to the complete initiation complex. In the presence of ATP DnaK and DnaJ heat shock proteins cause a partial dissociation of the initiation complex. Thereby, the helicase DnaB is activated in the presence of *E. coli* single strand binding proteins and initiates localized unwinding of the DNA template.

The specific retention of λO and λP proteins on DnaK affinity columns (LIBEREK et al. 1988) strongly suggests an interaction between DnaK and these proteins. The λP proteins were found to bind to the DnaK affinity column in a salt-resistant manner, suggesting the involvement of hydrophobic interactions, and could be released, at least partially, by ATP hydrolysis. A salt-resistant interaction of DnaK was also observed with another heat shock protein in *E. coli*, the GrpE protein. The binding could be reversed by ATP hydrolysis (ZYLICZ et al. 1987).

In summary, assembly of the initiation complex during phage λ DNA replication requires DnaK. There is also evidence for an involvement of DnaK in cellular DNA synthesis (SAKAKIBARA 1988). Furthermore, participation of DnaK in reactions other than DNA replication has been reported, e.g., phosphorylation of tRNA synthetases (WADA et al. 1986). The same subset of *E. coli* heat shock proteins seems to be involved in some of these processes, namely DnaK, DnaJ, and GrpE, which are all essential for bacterial growth.

Very recently, first evidence for a role of DnaK in stabilizing precursor proteins destined for secretion was reported (PHILLIPS and SILHAVY 1990). Overproduction of DnaK resulted in increased export of a protein consisting of the signal sequence and the amino terminal region of maltose-binding protein fused to β-galactosidase. A critical role of DnaK in intracellular traffic of precursors was proposed, similar to eukaryotic hsp70 proteins (see below). In addition to DnaK, several other proteins have been suggested to exert such a chaperone function in *E. coli*, namely SecB, trigger factor, and GroEL (COLLIER et al. 1988; CROOKE and WICKNER 1987; CROOKE et al. 1988; BOCHKAREVA et al. 1988).

### 2.2.2 Catalysis of Clathrin Depolymerization by hsc70

Secreted proteins are transported in specialized coated vesicles from the trans-Golgi to the plasma membrane. The coat consists of the protein clathrin, which forms a latticed cage. Before the vesicle fuses with its target membrane the coat has to dissassemble. The constitutively expressed hsp70, the hsc70, is involved in this process.

Early studies provided evidence for an ATP-dependent enzyme-catalyzed mechanism for clathrin depolymerization (PATZER et al. 1982). The uncoating ATPase was purified based on its ability to release clathrin triskelions from the coat (SCHLOSSMAN et al. 1984). A two-step process was proposed for the uncoating reaction (ROTHMAN and SCHMID 1986). After binding of the uncoating

ATPase to the cage, depending on the presence of ATP and clathrin light chains, ATP hydrolysis would cause a conformational change ("displacement") of a portion of a triskelion, exposing a previously buried site. By binding of the uncoating ATPase to this site, facilitated by ATP or nonhydrolyzable ATP analogues, this conformation would be stabilized ("capture"). After attachment to three points of the triskelion a complex consisting of a clathrin triskelion and three bound enzymes is released.

Immunological cross-reactivity, peptide mapping and two-dimensional gel analysis identified the uncoating ATPase as a constitutively synthesized member of the hsp70 family, called hsc70 (UNGEWICKELL 1985; CHAPPELL et al. 1986).

So far there is no direct evidence that the uncoating reaction is catalyzed by hsc70 in vivo. In view of the high abundance of the uncoating ATPase it is quite possible that disassembly of clathrin coats is not the only function of this heat shock protein. However, the fundamental property of the protein, the ATP-dependent disassembly of protein–protein complexes, would agree with the general view on the function of hsp70 proteins.

### 2.2.3 Role of Cytosolic hsp70 Proteins in Membrane Translocation of Proteins

A large number of proteins of the eukaryotic cell must be translocated across membranes to reach their functional locations in the various cellular organelles. In many cases this process occurs when polypeptide chain synthesis has been completed (posttranslational translocation). These precursor proteins differ from their native counterparts in several properties: In most cases they contain amino terminal presequences which are cleaved off during or after transit through organelle membranes. Furthermore, precursor proteins usually assume a conformation which is rather different from that of the mature form. A particular requirement for translocation appears to be that precursor proteins are in an unfolded state when traversing the membrane. In a key experiment EILERS and SCHATZ (1986) studied the import into mitochondria of a fusion protein consisting of the presequence of subunit IV of cytochrome oxidase and mouse dihydrofolate reductase (DHFR). Import could be blocked by methotrexate, a substrate analogue, which stabilizes the tertiary structure of DHFR. A similar block of import was obtained when the metallothionein domain was incorporated into a related fusion and a stable tertiary structure was induced by addition of copper (CHEN and DOUGLAS 1987). Conversely, destabilization of tertiary structure by urea or point mutations made import of mitochondrial precursor proteins more efficient. Recent experiments with translocation intermediates spanning both mitochondrial membranes suggest that during import into mitochondria proteins have to undergo extensive unfolding (SCHEYER and NEUPERT 1985; RASSOW et al., unpublished results).

Besides a certain lack of secondary and tertiary structure the presence of ATP seems to be a prerequisite for translocation across membranes. ATP dependency has been found with the import of proteins into the ER,

peroxisomes, chloroplasts, mitochondria, and the nucleus. In view of the requirement for ATP and an unfolded conformation of precursor proteins it was proposed several years ago that enzymes (so-called unfoldases) may participate in unfolding using the energy of ATP hydrolysis (ROTHMAN and KORNBERG 1986). On the other hand, a role of hsp70 proteins in folding and assembly of proteins in vivo in an ATP-dependent fashion has been suggested (PELHAM 1986). These proposals have stimulated investigations of a possible role of hsp70 proteins in membrane translocation.

Indeed, recent studies have presented genetic as well as biochemical evidence for a function of hsp70 proteins in posttranslational translocation of proteins across membranes of the ER and mitochondria. By fractionating yeast cytosol on DEAE cellulose, CHIRICO et al. (1988) identified two activities which together stimulate the import of prepro-α-factor into yeast microsomes. One activity, which was insensitive to *N*-ethylmaleimide (NEM), a sulfhydryl alkylating reagent, was purified using a GTP and an ATP agarose column. It consisted of two members of the yeast hsp70 family, namely Ssa1 and Ssa2. The two proteins are 98% homologous and differ only slightly in their isoelectric point. Whereas *ssa1*$^-$ and *ssa2*$^-$ single mutants lacked any phenotype, double mutants were temperature sensitive for growth, indicating a similar function of the proteins. Ssa1 and Ssa2 proteins had a stimulatory effect on prepro-α-factor import into yeast microsomes in the presence of yeast postribosomal supernatant from the mutant cells. In related experiments import of δ-pyrroline-5-carboxylate-dehydrogenase into yeast mitochondria was found to be stimulated by Ssa1/Ssa2 proteins in the presence of yeast postribosomal supernatant (MURAKAMI et al. 1988). In both studies the activity of the postribosomal supernatant was abolished by NEM treatment, suggesting the involvement of an NEM-sensitive activity besides Ssa1/Ssa2 proteins. Very recently, hsp70 was reported also to stimulate protein import into chloroplast (WAEGEMANN et al. 1990). Thus, a common requirement for hsp70 proteins in protein import into different organelles appears to exist.

The function of hsp70 proteins in the transfer of proteins into the ER was studied further using another heterologous cell free system. The transport of the precursor of M13-phage coat protein (procoat) into dog pancreas microsomes (WIECH et al. 1987) was stimulated by hsc70 (ZIMMERMANN et al. 1988). An increased proteinase K resistance of the procoat protein in the presence of ATP suggested a physical interaction with the heat shock protein.

Studies using yeast mutant strains depleted of *hsp70* genes provided valuable additional information (DESHAIES et al. 1988a, b). A strain lacking *ssa1*, *ssa2*, and *ssa4* genes had been found to be rescued by an *ssa1* gene on a single copy plasmid. The *ssa1* gene was fused to the yeast *gal1* promoter (WERNER-WASHBURNE et al. 1987). Thus, expression could be regulated by growing cells in the presence or absence of galactose. The effect of *ssa1* depletion on import of prepro-α-factor into the ER and of $F_1\beta$-ATPase into mitochondria was tested in vivo. After shifting of cells from galactose medium to a glucose medium, prepro-α-factor and $F_1\beta$-ATPase accumulated in the cytosol. Proteolytic

processing of the signal sequences did not occur. After partial purification on ATP-agarose the Ssa1 protein stimulated the transport of prepro-α-factor into yeast microsomes.

In conclusion, these observations suggest a general role of the hsp70 proteins in intracellular protein transport. hsp70 proteins may protect proteins from improper folding and interactions and thereby maintain their translocation competence until they reach their final compartment. A role in unfolding of precursor proteins also seems possible although there exists no experimental evidence for such a reaction. The rate of import of urea-denatured precursor proteins in the yeast ER could be increased by adding Ssa1/Ssa2 proteins (CHIRICO et al. 1988). Therefore, hsp70 may slow down refolding of unfolded proteins rather than catalyze unfolding. The localization of hsp70 proteins in different cellular compartments may suggest that they affect precursor proteins in a similar fashion on both sides of an organelle membrane. For further understanding of the functions of hsp70 proteins direct studies of their interaction with precursor proteins will be necessary.

### 2.2.4 BiP—The hsp70 Homologue in the Endoplasmic Reticulum

The ER contains an hsp70 homologue, which was initially discovered as a protein bound to unassembled immunoglobulin heavy chains; it was therefore called immunoglobulin heavy chain *bi*nding *p*rotein (BiP; HAAS and WABL 1983). Cloning and DNA sequencing of the *BiP* genes of a variety of mammals revealed a high degree of evolutionary conservation (> 98% amino acid identity) and a close relationship (about 60% amino acid identity) to cytoplasmic 70-kD heat shock proteins (MUNRO and PELHAM 1986). The intracellular location of BiP is determined by two signal sequences. A cleavable, hydrophobic signal sequence directs the protein to the ER, whereas the carboxy terminal tetrapeptide KDEL is believed to be responsible for retention of the protein in the lumen of the ER (MUNRO and PELHAM 1987).

BiP differs from other hsp70 proteins with regard to some important features. As shown by sequence analysis, BiP is identical to a 72-kD glucose-regulated protein (initially called grp78), which is not induced by heat (MUNRO and PELHAM 1986; LEE 1987). In contrast to the mammalian BiP, the recently identified yeast homologue (kar2) is induced seven fold by heat (ROSE et al. 1989; NORMINGTON et al. 1989). The rate of BiP synthesis increased after glucose starvation and in the presence of a variety of other substances, including tunicamycin, glucosamine, 2-desoxyglucose, amino acid analogues, and $Ca^{2+}$ ionophores (LEE 1987). The different stress conditions may result in accumulation of malfolded proteins in the ER which have been found to increase the rate of synthesis of glucose-regulated proteins, including BiP (KOZUTSUMI et al. 1988). From these studies it seems likely that malfolding rather than underglycosylation (CHANG et al. 1987) is the primary signal for the induction of BiP, since not all inhibitors of *N*-glycosylation tested affected the rate of BiP synthesis.

The signal cascade leading to increased BiP synthesis is only partly understood. Posttranslational modifications, including phosphorylation of serine and threonine residues and ADP ribosylation, were suggested to play a role in regulating the synthesis (HENDERSHOT et al. 1988). Conditions leading to increased synthesis of BiP resulted in a decrease in posttranslational modifications. On the other hand, binding of BiP to cellular proteins may be influenced by these modifications (HENDERSHOT et al. 1988). Modified and unmodified BiP coexist in the same compartment, but no modification of BiP molecules associated with other proteins was detected. A possible conclusion is that only the unmodified BiP is responsible for the stress response. In agreement with this, after inhibition of N-glycosylation in a mouse hepatoma cell line only the non-ADP-ribosylated form accumulated (LENO and LEDFORD 1989).

As shown by MUNRO and PELHAM (1986), BiP binds immunoglobulin heavy chains in pre-B cells in an ATP-reversible manner. Binding of BiP to various other proteins was also reversed by ATP hydrolysis, e.g., to nonglycosylated yeast invertase and prolactin containing incorrect disulfide bonds (KASSENBROCK et al. 1988), malfolded and mutant viral glycoproteins (MACHAMER and ROSE 1988; HURTLEY et al. 1989), and hydrophilic peptides (FLYNN et al. 1989). Apparently, BiP has the potential to interact with unassembled or incorrectly folded proteins. BiP possesses a peptide-dependent ATPase activity (KASSENBROCK and KELLY 1989; FLYNN et al. 1989) characterized by a low turnover number and a high affinity for ATP. The decreased sensitivity of BiP to proteolytic degradation in the presence of ATP or ADP suggests that adenine nucleotides may stabilize special conformations of BiP (KASSENBROCK and KELLY 1989). Notably, the existence of a so far unidentified ATP pool in the ER has to be assumed.

Although the precise function of BiP in the ER is unknown, several possibilities are discussed below which are not mutually exclusive.

1. *Retention of proteins in the ER*: The association of BiP with aberrant polypeptides and unassembled immunoglobulin heavy chains might suggest that BiP is part of a quality control system in the ER (HURTLEY and HELENIUS 1989) which only allows the secretion of functional proteins. First evidence for the importance of correct folding and assembly of proteins for secretion came from experiments which uncovered a correlation between acquisition of native structure and secretion efficiency (extensively discussed by ROSE and DOMS 1988) best studied in the case of abnormally glycosylated proteins (GETHING et al. 1986; DORNER et al. 1987; MACHAMER and ROSE 1988; GALLAGHER et al. 1988). The role of BiP could be to retain the misfolded proteins. The inhibition of N-glycosylation increased the association with BiP, as shown for immunoglobulin heavy chains (BOLE et al. 1986) and several human serum glycoproteins (DORNER et al. 1987). The extent and stability of BiP association were inversely correlated with secretion efficiency. In a more direct approach, DORNER et al. (1988) showed that in CHO cells, expressing plasminogen activator, reduction of BiP levels by introducing antisense RNA led to an increased secretion of the heterologous glycoprotein.

2. *Assembly of oligomeric proteins in the ER*: The interaction of monomeric heavy chains with BiP prior to their association with light chains (HAAS and WABL 1983; BOLE et al. 1986) may point to a role of BiP as an assembly factor (PELHAM 1989a). Heavy chains associated with BiP remained soluble until light chains were expressed. Assembled immunoglobulins were secreted. Proteins which oligomerize in the ER without association with BiP may assemble spontaneously or may interact with other factors as suggested for the T cell receptor (BONIFACINO et al. 1988). However, studies with mutant heavy chains argue against a role of BiP in the assembly of immunoglobulins (HENDERSHOT et al. 1987). Heavy chains lacking the $c_H1$ domain were not found in association with BiP. The detection of some completely assembled immunoglobulins and the increased rate of secretion, both shown for these mutant heavy chains, could mean that BiP does not assist immunoglobulin assembly, but might prevent the secretion of unassembled heavy chains. On the other hand, the retention of unassembled subunits or assembly intermediates is clearly not a general function of BiP since, with the exception of BiP–heavy chain complexes, stable interactions with incompletely oligomerized proteins have not been detected so far (HURTLEY and HELENIUS 1989).
3. *Folding of proteins in the ER*: Another role of BiP was suggested in the maturation of the VSV-G glycoprotein which forms homotrimers in the ER. In contrast to influenza virus hemagglutinin (HURTLEY et al. 1989), BiP was found associated with monomers of the VSV-G protein shortly after synthesis. Dissociation occurred as the subunits underwent folding (HURTLEY and HELENIUS 1989). No interaction with BiP could be detected by the time trimers were formed in the ER. Mutant forms of VSV-G, which do not fold or trimerize correctly (DOMS et al. 1988), aggregated and were found in association with BiP. Consistent with the kinetics of association, BiP may serve as a folding factor of VSV-G rather than an assembly or retention factor (HURTLEY and HELENIUS 1989). However, it is questionable whether a generalization can be made; e.g., BiP association was not detected in the case of nascent prolactin chains in a cell free translocation system (KASSENBROCK et al. 1988). Still, the possibility exists that BiP may assist specific protein folding in the ER.
4. *Translocation of proteins into the ER*: The identification of the yeast *BiP* gene (ROSE et al. 1989; NORMINGTON et al. 1989; NICHOLSON et al. 1990) allows the examination of BiP function using genetic techniques. At nonpermissive temperature a temperature-sensitive BiP mutant failed to import proteins into the ER (VOGEL et al. 1990). In the absence of BiP function imported precursor proteins might remain bound to a component of the secretory machinery or might aggregate. Both effects would result in inactivation of the translocation machinery resulting in a block of transport into the ER. On the other hand, with translocation-competent proteoliposomes import of preprolactin was found to occur in the absence of BiP, albeit with a low efficiency (NICCHITTA and BLOBEL 1990). However, as discussed by the authors, BiP binding to a polypeptide during membrane translocation may stabilize a conformation that facilitates import and may therefore increase the transloc-

ation rate. Such a function of BiP would resemble that proposed for cytosolic hsp70 proteins in maintaining a transport-competent conformation of translocated proteins. The essential character of BiP function in translocation might be missed in these in vitro experiments due to the limited efficiency of the reconstituted system.

## 3 The GroEL/hsp60 Family

All prokaryotic and eukaryotic cells investigated so far contain a heat shock protein with a molecular mass of about 60 kD. The first member of this family, the GroEL protein from *E. coli*, was purified and characterized several years ago (HENDRIX 1979; HOHN et al. 1979). Other members were identified in chloroplasts (BARRACLOUGH and ELLIS 1980; PUSHKIN et al. 1982) and mitochondria (MCMULLIN and HALLBERG 1987, 1988). Sequence analysis shows a considerable conservation between the different proteins (about 46%–54% sequence identity; HEMMINGSEN et al. 1988; READING et al. 1989). This homology can be easily explained in terms of the endosymbiotic origin of mitochondria and chloroplasts. So far, identification of a GroEL/hsp60 homologue in the cytosol has not been reported.

In addition to the sequence similarity and immunological cross-reactivity the different members of the hsp60 family have several properties in common. All are constitutively expressed but can be induced by heat shock. Molecular weights and the isoelectric points are almost identical. A close similarity in quarternary structure is obvious (as discussed in detail below). Finally, all members of this family possess a weak ATPase activity, which may be important for their function (HEMMINGSEN et al. 1988).

In view of the strong conservation a common function of these proteins is conceivable. All members of the hsp60 family are thought to assist proteins in the process of acquiring their native structure (HEMMINGSEN et al. 1988). Similar to hsp70 proteins, hsp60 proteins may act as molecular chaperones. The term "chaperonin" was proposed to define them as a third class of chaperone proteins besides nucleoplasmin and the hsp70 protein family (HEMMINGSEN et al. 1988; ELLIS and HEMMINGSEN 1989; ELLIS et al. 1989).

### 3.1 The Rubisco Subunit Binding Protein of Chloroplasts

Studies on the synthesis of the oligomeric chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) have revealed the association of newly synthesized large subunits with proteins of about 60 kD, termed Rubisco subunit binding protein (BARRACLOUGH and ELLIS 1980). Both were found in a protein particle with an apparent molecular mass of 720 kD and a sedimentation coefficient of 29S (CANNON et al. 1986; ROY and CANNON 1988). There is strong

evidence that Rubisco subunit binding protein, absent from native Rubisco, acts as a molecular chaperone during the assembly of Rubisco.

### 3.1.1 Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (Rubisco)

Rubisco is the most abundant protein in chloroplasts. In higher plants and in most photosynthetic prokaryotes it is a hexadecamer composed of eight large subunits with a moelcular mass of 52 kD and eight small subunits with a molecular mass of 15 kD (MIZIORKO and LORIMER 1983; GATENBY and ELLIS 1990). large subunits, which contain the catalytic site, are encoded by the chloroplast *rbcL* gene, whereas the small subunits are nuclear encoded, synthesized as larger precursors in the cytosol, and processed after import into the chloroplast stroma (SMITH and ELLIS 1979). The small subunits are necessary for enzymatic activity, but their exact function is unclear so far. In the prokaryote *Rhodospirillum rubrum* a dimeric Rubisco exists which comprises two large subunits only (TABITA and MCFADDEN 1974).

Rubisco catalyzes $CO_2$ fixation, the rate-limiting step in photosynthesis. In addition, it is the key enzyme of photorespiration which reduces the efficiency of $CO_2$ fixation. Therefore, major efforts were undertaken to manipulate the enzyme in order to increase the net rate of photosynthesis by recombinant DNA techniques. However, large subunits of the higher plant enzyme, when expressed in *E. coli*, formed insoluble aggregates. Thus, after coexpression of large and small Rubisco subunits from higher plants, only low levels of assembly and enzyme activity were observed (GATENBY et al. 1987). This hampered attempts to analyze enzyme structure and function by site-directed mutagenesis experiments.

### 3.1.2 Structure and Properties

The Rubisco subunit binding protein is one of the most abundant proteins in the stroma of chloroplasts. It consists of two types of subunits with molecular masses of 61 kD ($\alpha$) and 60 kD($\beta$). These were initially assumed to occur in a stoichiometry of $\alpha_6\beta_6$ (MUSGROVE et al. 1987); however, more recently the subunits were proposed to be identical to a previously identified 14-meric protein (PUSHKIN et al. 1982; HEMMINGSEN et al. 1988). It has not finally been proven, however, that the binding protein is a hetero-oligomer. The possibility of homo-oligomeric isoforms has not been ruled out. The subunits differ in a number of properties, including antigenicity, peptide pattern obtained after limited proteolysis, isoelectric point, and amino terminal amino acid sequence. In rape subunits $\alpha$ and $\beta$ have about 50% sequence identity (GATENBY and ELLIS 1990). Both subunits are nuclear encoded and translated as precursors with indistinguishable apparent molecular weight (ELLIS and VAN DER VIES 1988). After import into chloroplasts and proteolytic processing they assemble in the stroma compartment.

The protein possesses ATPase activity (CHAUDHARI et al. 1987). As first reported by BLOOM et al. (1983) addition of Mg-ATP in equimolar concentrations causes reversible dissociation of the binding protein into monomeric subunits (MUSGROVE et al. 1987). The disassembly was highly specific for ATP. Other nucleotides together with equimolar amounts of $Mg^{2+}$ ions had no effect. The dissociated subunits were neither stably phosphorylated nor adenylated (HEMMINGSEN and ELLIS 1986).

Immunological studies with an antibody raised against the pea binding protein led to the identification of related proteins in extracts of spinach, tobacco, wheat, and barley leaf extracts and castor bean endosperm. The occurrence of the Rubisco subunit binding protein correlates with the distribution of Rubisco in different plant tissues (HEMMINGSEN and ELLIS 1986; ELLIS and VAN DER VIES 1988).

### 3.1.3 The Role of Rubisco Subunit Binding Protein in the Assembly of Rubisco

The molecular details of the assembly pathway of Rubisco are largely unknown. On the basis of structural and evolutionary considerations dimerization of folded large subunits was proposed to be a common, conserved step in assembly of dimeric and hexadecameric Rubisco (GOLOUBINOFF et al. 1989a). After oligomerization of dimers to an octameric structure eight small subunits associate polarily, thereby forming the active hexadecameric enzyme.

On the basis of kinetic studies of its association with large subunits the hypothesis was advanced that the Rubisco subunit binding protein is required for the correct assembly of Rubisco (BARRACLOUGH and ELLIS 1980; ROY and CANNON 1988, for a review). Addition of antiserum against binding protein to extracts of pea chloroplasts led to inhibition of holoenzyme formation. This indicates that all assembly-competent large subunits transiently associate with the binding protein (CANNON et al. 1986).

However, the mode of action of Rubisco subunit binding protein is not entirely clear so far. It may affect assembly of Rubisco at varlous stages. Kinetic studies suggest that large subunits interact with binding proteins before assembly with small subunits occurs (GATENBY et al. 1988). Addition of Mg-ATP to stromal extracts of pea chloroplasts resulted in dissociation of the complex between Rubisco large subunit and Rubisco subunit binding protein. On the other hand, an association of Rubisco subunit binding protein was also found with small subunits (GATENBY et al. 1988). This would suggest a role of Rubisco subunit binding protein for folding of small subunits or for holoenzyme formation.

In summary, there is clear evidence for an involvement of the binding protein in the assembly of Rubisco; however, its exact role remains to be determined.

## 3.2 GroE Proteins

### 3.2.1 General Properties

The prokaryotic member of the hsp60 family, the GroEL protein, belongs to the most abundant proteins in *E.* coli and several other bacteria. It is encoded in the GroE operon, which is part of the *E. coli* heat shock regulon. When temperature is elevated from 37 °C to 46 °C the expression of the encoded proteins is increased four- to fivefold. The transcript of about 2100 nucleotides contains two open reading frames. Besides the GroEL protein (molecular weight of 52259, as estimated from the DNA sequence), a second polypeptide, the GroES protein (molecular weight of 10368), is encoded in this operon (HEMMINGSEN et al. 1988). The apparent molecular weights of the two proteins determined by denaturing gel electrophoresis are about 65000 and 15000 respectively. The GroEL protein forms an oligomeric complex which contains 14 monomers arranged in a double-ring with sevenfold rotational symmetry (HENDRIX 1979; HOHN et al. 1979). Upon gel filtration or centrifugation analysis the GroES protein displays a molecular mass of about 80 kD (CHANDRASEKHAR et al. 1986). This suggests an oligomeric structure for this protein, too. Biochemical as well as genetic evidence exists for an interaction of GroEL and GroES proteins. The GroEL protein possesses a weak ATPase activity (HENDRIX 1979) which can be inhibited by GroES (CHANDRASEKHAR et al. 1986). Furthermore, partial cosedimentation of purified GroES protein with GroEL in a glycerol gradient suggests a physical interaction. Interestingly, ATP and $MgCl_2$ are necessary for this interaction (CHANDRASEKHAR et al. 1986). Under similar conditions GroES binds to immobilized GroEL on an affinity matrix. The identification of intergenic suppressors of *groES* mutations mapping in the *groEL* gene strongly support these biochemical data (TILLY and GEORGOPOULOS 1982).

### 3.2.2 Function

GroE proteins were originally identified as host genes necessary for bacteriophage T4 morphogenesis (GEORGOPOULOS et al. 1972). Besides the inability to propagate bacteriophages some mutant alleles of both *groEL* and *groES* result in a temperature-sensitive growth of the host (WADA and ITIKAWA 1984). Recently, it was shown by a genetic approach that GroEL and GroES proteins are necessary for bacterial growth at all temperatures (FAYET et al. 1989). The actual level of GroE proteins can determine the maximal growth temperature (KUSUKAWA and YURA 1988). This suggests a more general role of the GroE proteins for cell function. Although their exact mode of action is unclear so far, strong evidence has accumulated during the last few years for an involvement of the GroE proteins in the following cellular processes.

1. *Morphogenesis of bacteriophages*: Even before their identification as heat shock proteins the involvement of the GroE proteins in the morphogenesis of bacteriophages was well established. Both proteins are required for head

assembly of the phage λ (for review, see FRIEDMAN et al. 1984) and tail assembly of T5 phages (TILLY and GEORGOPOULOS 1982). In addition, the GroEL protein has been shown to be necessary for T4 head assembly (GEORGOPOULOS et al. 1972). In all cases the GroE proteins seem to act in early steps of morphogenesis. Examination of the structures formed during λ infection of *groEL* mutants suggests that this protein may be involved in the oligomerization of the phage B protein to a dodecamer. This ring-like structure is located at the vertex of the λ head to which the λ tail becomes attached.

2. *DNA replication*: The identification of extragenic suppressors of a given mutation can be used to identify functional interactions between different proteins. Overexpression of a DNA fragment containing *groEL* and *groES* was found to restore the temperature-sensitive phenotype of *dnaA* mutations (FAYET et al. 1986; JENKINS et al. 1986). The effect could only be observed in the presence of the DnaA protein, thus excluding a bypass mechanism. Therefore, a direct interaction of the proteins has been proposed. The allele specificity of suppression supports this conclusion. A mutation in the *rpoA* gene of *E. coli*, which encodes a subunit of the RNA polymerase, can suppress a temperature-sensitive mutation in the *groES* gene (WADA et al. 1987). Furthermore, GroEL can rescue a temperature-sensitive mutation in a gene for *E. coli* single strand binding proteins (Ssb), suggesting an interaction between Ssb proteins and GroEL (RUBEN et al. 1988). It is interesting to note in this context that the levels of GroE proteins were observed to increase with shorter generation times (PEDERSEN et al. 1978). In summary, an involvement of GroE proteins in DNA replication appears to be established; however, their specific function(s) remain to be determined.
3. *Translocation of proteins across membranes*: Posttranslational export of proteins from a prokaryotic cell requires the secreted proteins to be present in a conformation that is conducive to translocation (RANDALL and HARDY 1986). Several proteins, including SecB, trigger factor, and GroEL, have been proposed to assist newly synthesized proteins in acquiring or maintaining such a translocation-competent conformation. They may prevent either foliding into a stable native structure or malfolding and aggregation.

   Evidence for interaction of GroEL with newly synthesized proteins came from photo-cross-linking experiments (BOCHKAREVA et al. 1988). After such cross-linking newly synthesized, plasmid-encoded secretory β-lactamase (and plasmid-encoded chloramphenicol acetyltransferase) sedimented during ultracentrifugation as a 20S particle. Depletion of *E. coli* extracts from GroEL by affinity chromatography showed that the particle corresponds to GroEL. Denatured but not native myoglobin caused a competitive inhibition of the cross-linking reaction. This observation suggests an interaction of GroEL with unfolded β-lactamase. Only in the presence of GroEL was the export competence of newly synthesized β-lactamase conserved during preincubation. Therefore, the GroEL protein was proposed to stabilize a secretion-competent conformation and exert a chaperone function

analogous to SecB and trigger factor (BOCHKAREVA et al. 1988; KUSUKAWA et al. 1989). As shown recently, the overproduction of GroEL in *E. coli* facilitates the export of a hybrid protein consisting of the signal sequence and the amino terminal region of the maltose-binding protein fused to β-galactosidase (PHILLIPS and SILHAVY 1990).

Employing isolated GroEL and precursor proteins a stable and reversible association of GroEL with the precursors of two secreted proteins, proOmpA and prePhoE, was observed (LECKER et al. 1989). Analysis of the complexes by sucrose gradient centrifugation suggested a 1:1 stoichiometry. In contrast, no interaction of GroEL was seen with soluble cytoplasmic proteins or with mature secreted proteins. However, the significance of these in vitro experiments is not entirely clear: in temperature-sensitive GroEL and GroES mutants only the processing of β-lactamase was slowed down; there was no effect on the secretion of other proteins, including proOmpA (KUSUKAWA et al. 1989). Thus, in the bacterial cell a certain substrate specificity for precursor proteins may exist, e.g., SecB and GroE proteins may differ in this respect.

The studies on GroEL binding to precursor proteins were carried out in the absence of ATP (LECKER et al. 1989). ATP is apparently not necessary for association of the precursor proteins with GroEL. The complexes dissociate after adding ATP (BOCHKAREVA et al. 1988). Since the interaction of GroEL and GroES was only observed in the presence of ATP (CHANDRASEKHAR et al. 1986), a role of the GroES protein in the release of GroEL-associated proteins was assumed (KUSUKAWA et al. 1989). Among different GroES mutants analyzed in respect of processing kinetics in vivo only one affected the export of β-lactamase, suggesting that a specific domain is important for the function of GroES (KUSUKAWA et al. 1989).

The nature of the interaction between GroEL and precursor proteins remains unknown. The specific association with unfolded proteins might point to hydrophobic interactions, in analogy to the hsp70 proteins. As emphasized by LECKER et al. (1989), however, the interaction of GroEL with proOmpA, an integral membrane protein, whose sequence lacks long regions of consecutive apolar residues, might mean that regions other than apolar ones may be recognized.

4. *Protein folding and assembly of oligomeric proteins*: Increasing evidence has accumulated that GroE proteins may assist proteins in the acquisition of their native structure, i.e., act as molecular chaperones. After expression of dimeric or hexadecameric prokaryotic Rubisco in *E. coli* it was shown that overexpression of GroE proteins in *E. coli* resulted in an increased assembly of Rubisco (GOLOUBINOFF et al. 1989a). An influence of GroEL on the transcription rate or the protein stability was excluded. Analysis of GroES and GroEL mutants showed that both GroE proteins are necessary.

In subsequent experiments purified components were used to study the role of GroE proteins in the reconstitution of dimeric prokaryotic Rubisco in vitro (GOLOUBINOFF et al. 1989b). After denaturation with either urea or guanidinium

chloride and acid inactivation, no spontaneous reactivation could be observed under the conditions of the assay. In contrast, in the presence of GroEL, GroES, and Mg-ATP efficient reconstitution occurred. In a first step GroEL bound unfolded Rubisco large subunits independently of the presence of GroES and Mg-ATP. The urea-denatured as well as the acid-denatured protein, which was shown to contain a secondary structure, was able to form a binary complex with the GroEL tetradecamer. The subsequent dissociation step depended on GroES and Mg-ATP. In the presence of nonhydrolyzable analogues no effective reconstitution was observed, suggesting that the energy of ATP hydrolysis is required. Optimal reconstitution was observed at equimolar concentrations of GroEL and GroES. GroES may mediate the ATP-dependent release of the protein, perhaps by inducing a conformational change. The inhibition of the ATPase activity of GroEL by GroES (CHANDRASEKHAR et al. 1986) may have a regulatory function in these processes.

In conclusion, the reconstitution experiments with dimeric rubisco indicate that GroE proteins are involved in folding and assembly of large subunits. However, a role in folding and/or assembly of small subunits of hexadecameric Rubiscos of higher plants cannot be excluded. An interaction of GroEL with small subunits was in fact suggested by the observation that small subunits copurified with GroEL after expression in *E. coli* (LANDRY and BARTLETT 1989).

Several observations are consistent with a more general role of GroE proteins in folding and assembly of proteins. First, the homologous proteins, hsp60 in mitochondria and α-subunit of the Rubisco binding protein in chloroplast, could substitute for GroEL in the reconstitution experiments with Rubisco. So far, no GroES homologue has been identified in mitochondria and chloroplasts. In view of the conservation of GroEL structure and function it seems reasonable to assume that a protein with a function analogous to GroES does exist in these organelles. Furthermore, indirect evidence that GroE proteins assist various proteins in the acquisition of their native structure came from genetic experiments. Overexpressed GroE proteins could suppress many, but not all mutations in different genes of the *ilv-* and *his* operon of *Salmonella typhimurium* (VANDYK et al. 1989). In addition, heat-sensitive folding mutants of gene 9 of *Salmonella* phage P22 could be rescued by GroE proteins at the restrictive temperature. Thus, the formation not only of active enzymes but also of structural proteins appears to depend on GroE proteins. Interestingly, among the proteins analyzed only mutations in oligomeric ones could be suppressed by GroE proteins; the tested monomeric ones remained unaffected. Although several explanations for the suppressive effect are possible, it seems conceivable that GroE proteins affect the assembly of various proteins.

### 3.3 The Mitochondrial hsp60

The heat-shock protein hsp60 was initially found in mitochondria of *Tetrahymena* (MCMULLIN and HALLBERG 1987), and then in mitochondria from all organisms analyzed so far. It has a strong structural similarity to the bacterial

GroEL. Recent studies indicate that it plays an essential role in the folding and assembly of proteins newly imported into mitochondria.

### 3.3.1 Occurrence and Conserved Properties

Heat shock proteins of the hsp60 type were identified in the yeast *Saccharomyces cerevisiae* (64 kD), *Neurospora crassa* (60 kD; HUTCHINSON et al. 1989), *Tetrahymena thermophila* (MCMULLIN and HALLBERG 1987), *Xenopus laevis* (60 kD), *Zea mays* (62 kD), human cells (58–60 kD; MCMULLIN and HALLBERG 1988; WALDINGER et al. 1988, 1989; JINDAL et al. 1989; MIZZEN et al. 1989; MIZZEN et al. 1989), and CHO cells (58 kD; PICKETTS et al. 1989). Interestingly, the hsp60 of human lymphocytes shows a genetic polymorphism (WALDINGER et al. 1988).

The nuclear-encoded hsp60 protein is constitutively expressed and targeted to mitochondria by a positively charged amino terminal presequence. After heat shock, which causes a two- to threefold increase in expression, the protein represents about 0.3% of total cell protein. Like the bacterial GroEL homologue and the Rubisco subunit binding protein, the mitochondrial hsp60 assembles into an oligomeric structure in the cell. The protein of *Tetrahymena thermophila* sedimented in sucrose gradients as a 20–25S complex (MCMULLIN and HALLBERG 1987). Electron microscopic analysis of the *Neurospora crassa* protein revealed a structure very similar to that of the GroEL protein. The particle consists of two rings, each comprising seven subunits which are arranged in two layers (HUTCHINSON et al. 1989). The monomeric subunit may have an extended conformation. So far, all available evidence suggests that the 14 subunits are identical; thus, mitochondrial hsp60 appears to be a homo-oligomer like GroEL.

### 3.3.2 Function

Mitochondrial hsp60 resembles bacterial GroEL with respect to not only supramolecular structure but also amino acid sequence (READING et al. 1989; JOHNSON et al. 1989). Not very surprisingly they appear to be rather similar in function. There is increasing evidence from genetic as well as biochemical studies for an involvement of hsp60 in folding and/or assembly of mitochondrial proteins after their import into the matrix space (CHENG et al. 1989; OSTERMANN et al. 1989; HARTL and NEUPERT 1990, for a review).

The identification of a temperature-sensitive *hsp60* mutant in yeast, called *mif4* (for *m*itochondrial *i*mport *f*unction), allowed examination of the role of hsp60 in the import of proteins into mitochondria (CHENG et al. 1989). At nonpermissive temperature the mutant hsp60 protein became aggregated and was found in the low spin pellet of cell extracts. Under these conditions mitochondrial ornithine transcarbamylase (from humans, transformed into the yeast cells) failed to form trimers and to acquire enzyme activity and the $\beta$-subunit of the mitochondrial $F_0F_1$ ATPase failed to assemble into $F_1$ particles. Cytochrome $b_2$, a protein of the mitochondrial intermembrane space, and the Rieske Fe/S protein of complex III, normally present on the outer surface of the

inner membrane of mitochondria, did not reach their functional location. In experiments with isolated *mif4* mitochondria it was shown that membrane translocation was still possible, but the imported proteins failed to assemble. Therefore, it was suggested that hsp60 assists oligomeric proteins in the acquisition of the native structure.

In subsequent biochemical studies, a physical interaction of hsp60 with proteins freshly imported after urea denaturation into ATP-depleted mitochondria was established (OSTERMANN et al. 1989). Analysis of the folding state of such hsp60-associated proteins revealed that they were in an unfolded conformation. A fusion protein, in which amino acid residues 1–69 of the precursor of *Neurospora* $F_0$ ATPase subunit 9 were joined to the amino terminus of mouse dihydrofolate reductase (DHFR), was employed to analyze the folding reaction. The state of folding of this protein could be monitored by determining the protease sensitivity of the DHFR domain. Unfolded DHFR was digested by very low concentrations of proteases, whereas folded DHFR was resistant to rather high concentrations of proteases. After import the fusion protein was found to fold in association with hsp60 in an ATP-dependent manner (OSTERMANN et al. 1989). Folding and release of bound DHFR in the presence of ATP was also observed when mitochondria containing the hsp60 precursor protein complex were lysed with mild detergents. Studies with the membrane-permeant alkylating agent, *N*-ethylmaleimide (NEM), excluded a spontaneous folding of the DHFR after release from hsp60. In extracts of NEM-treated mitochondria, imported DHFR, bound to hsp60 in the absence of ATP, was released after readdition of ATP, but did not fold correctly. It remains to be tested whether hsp60 itself is the target of the NEM effect.

Furthermore, these experiments suggested an involvement of additional component(s). After partial purification of the fusion protein–hsp60 complex by gel filtration, readdition of Mg-ATP resulted only in a partial protease resistance of the DHFR domain, indicating an incomplete folding reaction. The DHFR remained bound to hsp60 under these conditions. Apparently, an unidentified component which did not cofractionate with the fusion protein–hsp60 complex during gel filtration was necessary for release.

In summary, these findings argue for a role of hsp60 in the folding of proteins after their import into mitochondria. hsp60 was found to be able to substitute for GroEL in assisting the reconstitution of Rubisco in in vitro experiments (GOLOUBINOFF et al. 1989b). This underlines the functional similarity of the members of the GroEL family.

## 4 Conclusions

Heat shock proteins of the hsp70 and hsp60 classes may fulfill multiple functions in the cell. There is increasing evidence that direct protein–protein interaction is a common theme of their action. The tendency of small heat shock proteins to

aggregate and the interaction of hsp90 proteins with steroid receptors and tyrosine kinases (LINDQUIST and CRAIG 1988, for a review) suggests that this may also be true for a variety of other heat shock proteins.

Heat shock proteins protect cells from the damaging effect of high temperatures and other kinds of stress. Although the resolution of aggregated proteins by heat shock proteins has not been observed in vivo or in vitro, the action of hsp70 and hsp60 proteins in the absence of stress may provide hints as to the mechanism of protection. Both families act as molecular chaperones assisting protein folding and assembly by reducing the tendency of aggregation during various cellular processes. However, hsp70 and hsp60 proteins may exert their function in different ways.

hsp70 proteins stabilize protein conformations distinct from the stably folded, native structure. These altered conformations appear to be necessary for targeting to and transportation of polypeptides across membranes, for assembly into oligomeric structures, or for interactions with other proteins. Thus the shielding of previously buried sequences by hsp70 proteins might reduce the tendency of proteins to aggregate, especially at elevated temperatures. The role of hsp70 proteins in protein folding in vivo is open. The sequence-specific recognition of proteins by BiP and hsc70 in vitro might suggest a function in protein folding (FLYNN et al. 1989). However, up to now no direct experimental data suggest that hsp70 proteins may act as "unfoldases" or as "foldases."

More direct evidence exists for the involvement of hsp60 proteins in folding and assembly of proteins. At least in some cases, GroEL-like proteins have been shown to be necessary for the acquisition of the native structure of proteins in the cell. The complex quaternary structure of hsp60 proteins (in contrast to hsp70 proteins) may be helpful in promoting folding and assembly of proteins. Thus, a general role of hsp60 for protein folding in vivo appears possible. A number of other proteins were identified which assist protein folding in vivo (ROTHMAN 1989; FISCHER and SCHMID 1990; for a review), including *cis/trans*-peptidyl-prolyl-isomerase (LANG et al. 1987) and the protein disulfide isomerase (FREEDMAN 1989). Within the thermodynamic limits chaperonins and these other proteins may assist folding at a kinetic level. A main task may be the prevention of premature folding and aggregation favored both by the high protein concentration in the cell and by high temperatures.

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