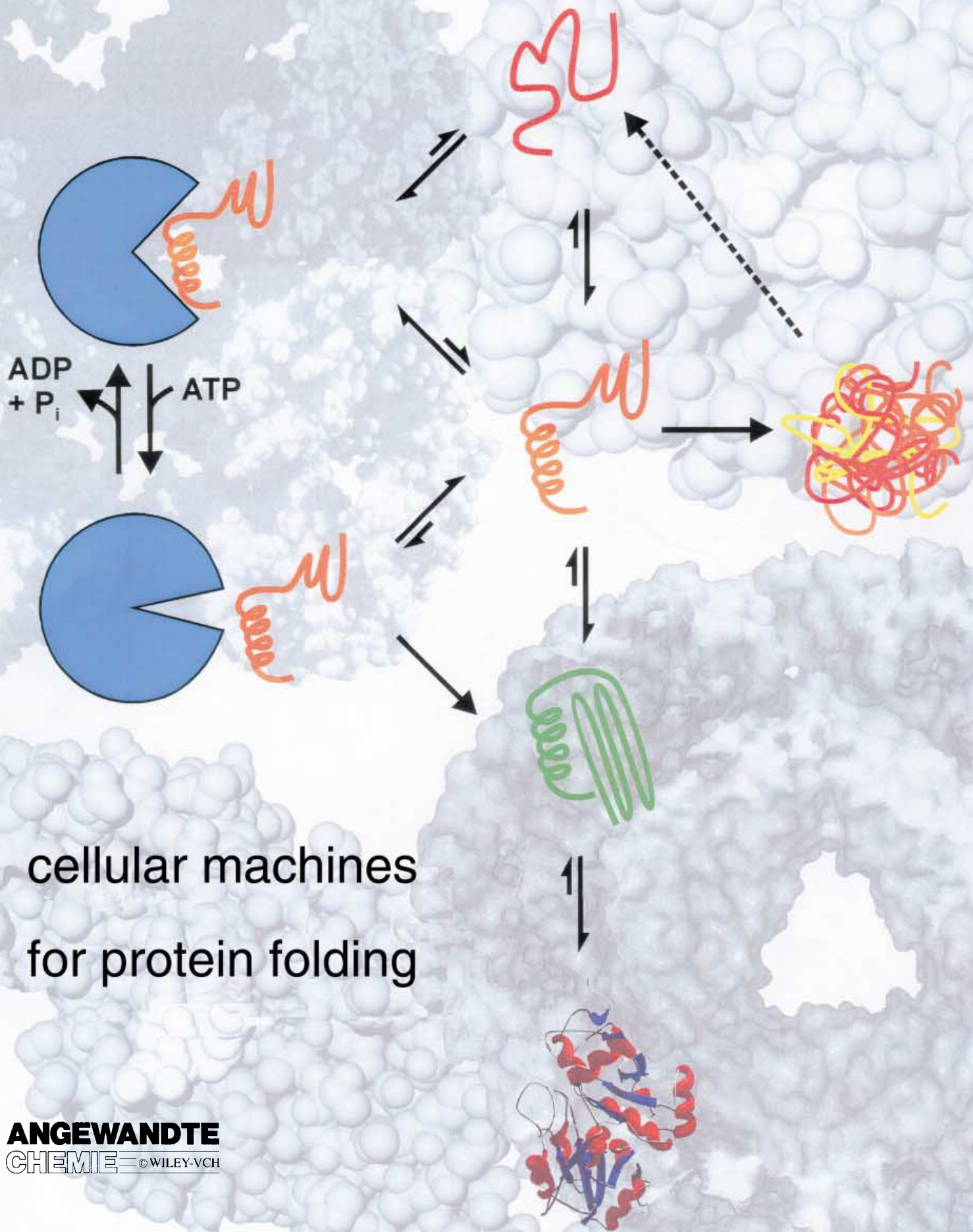


Molecular Chaperones



Molecular Chaperones—Cellular Machines for Protein Folding

Stefan Walter and Johannes Buchner*

Proteins are linear polymers synthesized by ribosomes from activated amino acids. The product of this biosynthetic process is a polypeptide chain, which has to adopt the unique three-dimensional structure required for its function in the cell. In 1972, Christian Anfinsen was awarded the Nobel Prize for Chemistry for showing that this folding process is autonomous in that it does not require any additional factors or input of energy. Based on *in vitro* experiments with purified proteins, it was suggested that the correct three-dimensional structure

can form spontaneously *in vivo* once the newly synthesized protein leaves the ribosome. Furthermore, proteins were assumed to maintain their native conformation until they were degraded by specific enzymes. In the last decade this view of cellular protein folding has changed considerably. It has become clear that a complicated and sophisticated machinery of proteins exists which assists protein folding and allows the functional state of proteins to be maintained under conditions in which they would normally unfold and aggregate. These proteins are collectively

called molecular chaperones, because, like their human counterparts, they prevent unwanted interactions between their immature clients. In this review, we discuss the principal features of this peculiar class of proteins, their structure–function relationships, and the underlying molecular mechanisms.

Keywords: chaperone proteins • protein aggregation • protein folding • protein structures • structure–activity relationships

1. Protein Folding and Molecular Chaperones

Whereas many proteins can be refolded *in vitro* under optimized conditions in good yields, the situation in a living cell is less favorable. In particular, high protein concentration and temperature promote aggregation as an undesired side reaction, competing with productive folding.^[1,2] Unlike protein assembly, which describes the ordered association of several polypeptide chains into a defined functional oligomer, aggregation is the disordered, non-specific association of polypeptide chains which leads to the formation of heterogeneous protein particles devoid of any biological function.

Considering the amount of energy the cell has already invested in the synthesis of a new polypeptide, it does not come as a surprise that strategies have evolved to promote the productive folding of a protein into its active conformation. During molecular evolution, polypeptide sequences were likely not only selected based on their biological properties, but also on whether they can fold productively. To increase the accessible conformation space, cells developed molecular

chaperones, a set of proteins that associate with unfolded polypeptides thereby preventing aggregation and promoting productive folding in an ATP-dependent manner.^[3,4]

1.1. Protein Folding In Vitro

Information transfer from DNA to mRNA and from mRNA to the polypeptide employs molecular complementarity and the genetic code. It translates the linear sequence of base triplets into a linear sequence of amino acids. The final step in the process from gene to the functional protein, protein folding, converts this linear information into a three-dimensional structure. This reaction turned out to be very complex. Although we have learned much over the past decades about the physical principles underlying the folding process,^[5–8] it is still a major challenge for biochemists to predict the structure into which a given polypeptide will fold.

The investigation of the protein-folding problem began in the 1960s with the groundbreaking experiments of Christian Anfinsen and co-workers on the reversible folding of an abundant RNA-cleaving enzyme, ribonuclease A (RNase A).^[9,10] Incubation with urea (8M) and a reducing agent resulted in an unfolded protein without any disulfide bonds or enzymatic activity. This denatured state of RNase A is thought to resemble the conformation immediately after its

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synthesis on the ribosome. When the denaturant and the reducing agent were removed by dialysis in Anfinsen's experiment, the enzyme was found to slowly regain its activity. Apparently, it had refolded *in vitro*. This observation clearly showed that the three-dimensional structure of this protein is encoded in its amino acid sequence, and that no other factors are required for structure acquisition. Thus, protein folding is an autonomous and, given the proper conditions, spontaneous process. Following the work of Anfinsen and co-workers, biochemists studying the folding properties of other small, monomeric proteins were able to confirm his observations.^[11, 12] Eventually, these results led to the notion that, in principle, every protein can be refolded *in vitro*.^[13, 14]

The native state of a protein corresponds to a fairly narrow energy minimum on the conformational energy landscape.^[5, 15] The denatured state, on the other hand, is represented by a large ensemble of conformations with high internal energy and flexibility. During the folding process, numerous noncovalent interactions are formed that require the exact positioning of the various atoms of the protein. Among these, the hydrophobic interactions seem to play an important role.^[16] Hydrophobic molecules tend to associate with each other in a polar environment for reasons of entropy and enthalpy. Accordingly, hydrophobic amino acids are predominantly found in the core of a folded protein. When biochemists began to study the folding of oligomeric proteins or of larger proteins that consist of multiple domains, it became apparent that the hydrophobic interactions are not only important in stabilizing the folded conformation, but may also have a detrimental effect.^[1] During early folding stages, many proteins form intermediates that display a considerable amount of hydrophobic surface. Protein mole-

cules can associate nonspecifically through these hydrophobic patches and ultimately aggregate. Since aggregation is a second- or higher-order reaction, protein concentration plays an important role in determining whether folding to the native state or nonspecific aggregation will predominate.^[17] For a given protein, folding *in vitro* can often be improved by optimizing the experimental conditions, including protein concentration, temperature, and pH among others.

1.2. Protein Folding In Vivo

In contrast to the situation *in vitro*, all proteins have to fold under the same set of conditions in a living cell. These conditions seem to be counterproductive for efficient folding, mainly because of the high temperature and the large number of non-native proteins present. Given the circumstances, it seems surprising that cells are usually devoid of aggregated proteins. There are two possible explanations for this observation. First, aggregation does occur *in vivo*, but its products are rapidly removed by cellular proteases. This would imply that cells waste a lot of energy to produce proteins that never become functional. Second, cells have found a strategy of minimizing the aggregation of newly synthesized proteins in the first place. This has been achieved by complex protein machinery, the chaperones, which influence the spontaneous folding reaction of proteins, thus preventing aggregation. It is important to note that these molecular chaperones do not provide specific steric information for the folding of the target protein, but rather inhibit unproductive interactions and thus allow the protein to fold more efficiently into its native structure.

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Molecular chaperones are found in all compartments of a cell where folding or, more generally, conformational rearrangements of proteins occur. Although protein synthesis is the major source of unfolded polypeptide chains, other processes can generate unfolded proteins as well. At non-physiological high temperatures or in the presence of certain chemicals, proteins can become structurally labile and may even unfold. Eventually, this would result in loss of function of the affected proteins and in the accumulation of protein aggregates. The cell responds to this threat by producing increasing amounts of specific protective proteins, a phenomenon referred to as *heat-shock response* or *stress response*.^[18] Many of these proteins were found to be molecular chaperones.

1.3. Functional Properties of Molecular Chaperones

The term molecular chaperone is used to describe a functionally related set of proteins. According to their molecular weight, molecular chaperones are divided into several classes or families. A cell may express multiple members of the same chaperone family. For example, the yeast *S. cerevisiae* produces 14 different versions of the chaperone Hsp70.^[19] Proteins from the same class of molecular chaperones often show a significant amount of sequence homology and are structurally and functionally related, whereas there are hardly any homologies between chaperones from different families. Despite this diversity, however, most molecular chaperones share common functional features.

1.3.1. Binding to Proteins That Expose Hydrophobic Surfaces

The principal property of any molecular chaperone clearly is its ability to bind unfolded or partially folded polypeptides. During the early stages of folding (Figure 1, I_{uc}) or when misfolding occurs, the hydrophobic residues of a protein are partially solvent accessible and thus render it vulnerable to aggregation. Association of these hydrophobic protein species with molecular chaperones efficiently suppresses aggregation. The low specificity of the hydrophobic interaction and the conformational flexibility of folding intermediates ensures that chaperones act promiscuously: they bind to a large variety of polypeptides that differ widely in amino acid sequence and in conformation. However, since most native proteins and many late folding intermediates (Figure 1, I_c and N) do not have hydrophobic patches, they are no longer substrates for molecular chaperones.

1.3.2. Conformational Changes of Target Proteins

In general, molecular chaperones induce conformational changes in their target proteins. One example is the controlled unfolding of a protein substrate. This unfolding activity establishes a link between chaperones and the cellular protein degradation system. Unfolding may also be of importance in protein folding (see Section 2.1.6). The formation of non-native contacts can lead to misfolded species that are trapped

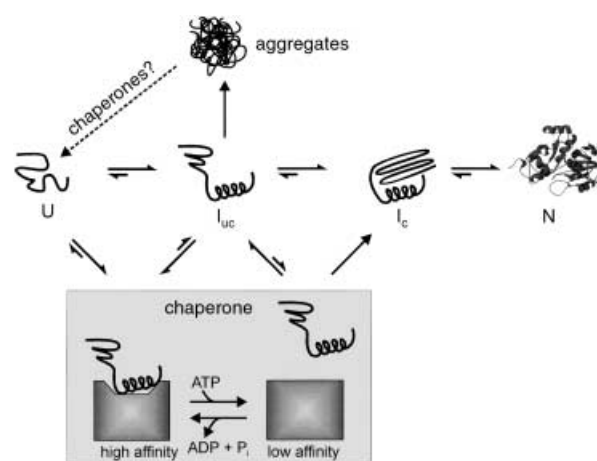


Figure 1. Chaperone-assisted protein folding. Protein biosynthesis as well as cellular stress results in the formation of unfolded polypeptides (U). These molecules fold via several intermediates (I_{uc} , I_c) with increasing structure complexity, until they reach the native, functional state (N). Some intermediates (I_{uc}) may expose hydrophobic surfaces that render them susceptible to aggregation. This reaction was thought to be irreversible, but recent results indicate that some chaperones may resolubilize aggregates. Molecular chaperones interfere with the deleterious process of aggregation by binding to species I_{uc} and U. This association not only blocks the hydrophobic patches on the bound polypeptides, but also decreases the concentration of aggregation-prone molecules, thereby slowing down aggregation. In many cases, an ATP-mediated conformation change in the chaperone triggers the dissociation of the bound polypeptide. A fraction of the released molecules may fold into a committed state (I_c), which no longer requires the assistance of the chaperone, whereas the remaining uncommitted (uc) molecules rebind and participate in another chaperone cycle. Depending on the type of chaperone, conformation changes in the polypeptide may occur during its association with the chaperone.

in local energy minima.^[20] Chaperone-mediated unfolding can disrupt these non-productive interactions and offers the polypeptide a new chance to reach its native structure.

1.3.3. Controlled Release of Bound Polypeptides

Hydrophobic interactions not only contribute to the stability of the folded structure of a protein, they are also important for the stability of oligomeric proteins and protein complexes. The contact areas often contain hydrophobic residues that become buried upon association. Thus, a protein may bind to an unfolded polypeptide or suppress its aggregation without necessarily being a chaperone. What sets molecular chaperones apart from these hydrophobic “scavengers” is their ability to form defined complexes with non-native proteins and, more importantly, to release the bound polypeptide in a controlled manner. This is usually accomplished by switching to an alternate state of the chaperone with a decreased affinity for hydrophobic polypeptides (Figure 1). According to the laws of thermodynamics, a bound protein substrate will stabilize the high-affinity state of the chaperone. Therefore some source of energy is required for switching to the low-affinity state in the presence of a bound substrate. Usually, this energy comes from the hydrolysis of ATP or the interaction with other protein components of the chaperone machinery.

2. Mechanisms of Chaperone Action

Having established the general principles of assisted protein folding, we discuss the structural and functional features of selected classes of molecular chaperones in detail in the following section.

2.1. GroE

The GroE proteins of the bacterium *E. coli* are the most extensively studied molecular chaperones.^[21–24] The *groEL* and *groES* genes encode proteins of 57 kDa and 10 kDa size, respectively, which are both required for the viability of *E. coli*.^[25] Thus, at least one essential *E. coli* protein cannot fold without assistance from the GroE chaperone.

2.1.1. Structure of the GroE Chaperone

The most striking feature of GroEL is its quaternary structure, which resembles a barrel open at both ends.^[26, 27] Fourteen subunits are assembled in two seven-membered rings, which form two separate cavities with a diameter of 45 Å (Figures 2A and B). The GroEL subunits can be dissected into three domains. The equatorial domains comprise the center part of the barrel. They bind and hydrolyze ATP and mediate all the contacts between the two rings and most of the contacts between the subunits of the same ring. The apical domains are located on the outer rims of the barrel and are responsible for binding the protein substrates and the co-chaperone GroES. The equatorial and the apical domains are connected by intermediate domains, which serve as mobile hinges that permit large structural rearrangements during the functional cycle of GroE.

The co-chaperone GroES is a dome-shaped ring-structure with a diameter of 75 Å and consists of seven subunits.^[28] An important feature of GroES is the so-called mobile loop, a stretch of 16 amino acids which mediates binding to GroEL.^[29, 30] Binding of GroES occurs at the ends of the GroEL barrel (Figure 2A), and is dependent on the presence of nucleotides, that is, ADP or ATP must be bound to the equatorial domains of the respective GroEL ring.^[31] Two types of complexes between GroES and GroEL, which differ in their stoichiometry and were aptly named “bullets” and “footballs”, have been described in the literature.^[32–36] In “bullets”, only one of the GroEL rings is associated with GroES (as in Figures 2A and B), whereas in “footballs” both GroEL rings are capped with GroES to form an apparently symmetrical particle. In the presence of ADP, “bullets” seem to be the predominant species, whereas both “footballs” and “bullets” are observed in the presence of ATP.

GroEL belongs to the family of Hsp60 chaperones, also termed “chaperonins”. For reasons of function and homology, members of this class can be divided into two groups. Group I chaperonins, such as GroEL, consist of seven subunits per ring and require a co-chaperone such as GroES. They are found in eubacteria and in the mitochondria and the chloroplasts of eukaryotic cells. Group II chaperonins consist of eight or nine subunits per ring and do not cooperate with a partner

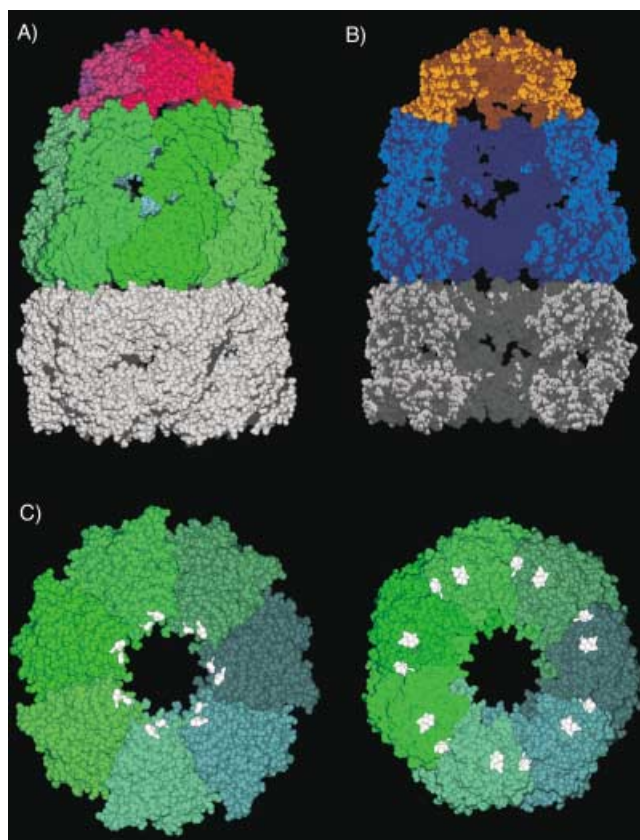


Figure 2. Structure of the GroE chaperone from *E. coli*, determined by X-ray crystallography.^[26, 27] A) Side-view of an asymmetric GroE “bullet” complex, which consists of a GroEL double-ring and a GroES single-ring. The distal GroEL ring is shown in gray, the seven subunits of the proximal GroEL ring are shown in shades of green. GroES (red) binds to the top of the proximal ring. B) Cross-section of a GroE “bullet”. Each GroEL ring encloses a cavity that serves as a folding compartment for a polypeptide substrate. Some residues of the equatorial domains have not been resolved in the crystal structure, thus giving the (wrong) impression that the two cavities are contiguous. Binding of GroES to the top GroEL ring blocks the access to the upper cavity and concomitantly induces a movement of the apical domains. The diameter of the proximal cavity increases from 45 Å to 80 Å, and its height from 73 Å to 85 Å. C) Changes in the GroEL structure upon binding of GroES. Left: the seven subunits that make up one ring of GroEL are shown in shades of green and blue. The hydrophobic amino acids in the apical domains that have been identified as important for binding the polypeptides and GroES are shown in white.^[40] In the absence of GroES, these residues coat the inside of the central cavity and account for the high affinity for unfolded polypeptides of this state. Right: upon binding of GroES, the apical domains rotate outwards by $\sim 90^\circ$. The hydrophobic patches become buried in the subunit interfaces, thus rendering the inner surface of the cavity mainly hydrophilic and causing the release of the bound polypeptide.

chaperone. They are found in the cytosol of archaea and eukaryotes. Relative to the group I chaperonins, our molecular understanding of the group II chaperonins is limited. One of the many questions that remain to be answered is whether their substrate specificity is as broad as that observed for GroEL.^[37, 38]

2.1.2. Polypeptide Binding to GroEL

GroEL recognizes a polypeptide as a potential substrate by virtue of exposed hydrophobic surfaces, which are character-

istic for unfolded and misfolded proteins. The dominance of hydrophobic interactions in polypeptide binding has been demonstrated by determining the thermodynamic properties of the binding reaction.^[39] The protein-binding site on GroEL was identified by mutational analysis,^[40] and more recently by the X-ray crystal structures of the isolated apical domain^[41] and of a complex between GroEL and a hydrophobic peptide.^[42] The peptide binds into a hydrophobic groove around the opening of the central cavity (Figure 2C). The high plasticity of the binding site allows it to undergo subtle structural rearrangements, thereby providing an optimized binding surface for individual substrates. Since the partially folded substrate is flexible as well, it is very likely that both the substrate and the apical domains will undergo structural rearrangements upon association.^[43] This explains why GroEL can bind to a wide range of partially folded proteins.

The structures of various substrate proteins have been characterized while bound to GroEL. It appears that GroEL is capable of interacting with a host of different conformations, ranging from largely unfolded polypeptides to highly structured stable folding intermediates.^[44–46] How can this structural heterogeneity be explained? From a thermodynamic point of view, there is a competition for the protein substrate between folding (i.e. formation of structure) on the one hand, and binding to GroEL on the other hand, because both are driven by the hydrophobic effect. In the case of folding, the hydrophobic residues become shielded from the solvent by forming a hydrophobic core in the protein. In the case of binding, shielding is achieved by interaction with the hydrophobic groove of the apical domains of GroEL.

For a single residue in a polypeptide substrate these options are mutually exclusive, but not necessarily for the whole protein. The structure(s) of the bound polypeptide might reflect an energy minimum that is determined by the relative size of two ΔG values, one for folding and one for binding. In essence, GroEL puts no restriction on the structure of the bound protein, as long as there is enough hydrophobic surface to interact with. This is different from Hsp70 (see below), in which the channel-like architecture of the binding site requires a locally stretched conformation of the bound polypeptide.^[47]

2.1.3. Interaction between GroEL and GroES

A key element in the functional cycle of GroE is the interaction between the two partner chaperones. Binding of the GroES co-chaperone induces major structural changes in the GroEL particle.^[27, 48] First, GroES serves as a lid that closes the cavity of the ring it is bound to, thereby encapsulating a polypeptide attached to the same ring. Second, it induces a rigid body movement of the apical domains in GroEL that completely changes the nature of the cavity: the hydrophobic patches in the apical domains which are responsible for polypeptide binding are replaced by largely polar residues (Figure 2C). Concomitantly, the affinity for hydrophobic polypeptides strongly decreases, and the bound substrate is released into the closed cavity.^[49, 50] Third, this domain movement increases the volume of the cavity from $\sim 85\,000 \text{ \AA}^3$ to $\sim 175\,000 \text{ \AA}^3$. This provides sufficient

space for the released polypeptide to undergo the conformation rearrangements required for reaching its native state. Thus, binding of GroES serves as a molecular switch that puts the chaperone from binding mode into folding mode: it stimulates the release of the bound protein into an environment that favors productive folding.^[49, 50] Because of the finite cavity volume, however, GroE-assisted folding is restricted to proteins smaller than 60 kDa.^[51] Interestingly, a protein that requires both GroEL and GroES for its folding has been identified, although its size (82 kDa) makes it too big to fit in the cavity.^[52] In this special case, however, the mechanism of GroE-mediated folding seems to be different.^[53]

2.1.4. ATP Hydrolysis by GroEL and Substrate Release

After its (partial) folding inside GroE, the protein has to exit the cavity and exert its biological function in the cell. But how can it leave while GroES is blocking its way out? Although the GroEL/GroES complex is very stable ($K_d \sim 1 \text{ nM}$), the ATPase serves as a built-in timer that controls the discharge of GroES. The seven molecules of ATP bound to the *cis* ring, that is, the ring associated with GroES and the folding polypeptide (Figure 3), are hydrolyzed at a rate of $0.25\text{--}0.5 \text{ min}^{-1}$.^[54, 55] This induces a conformation change in

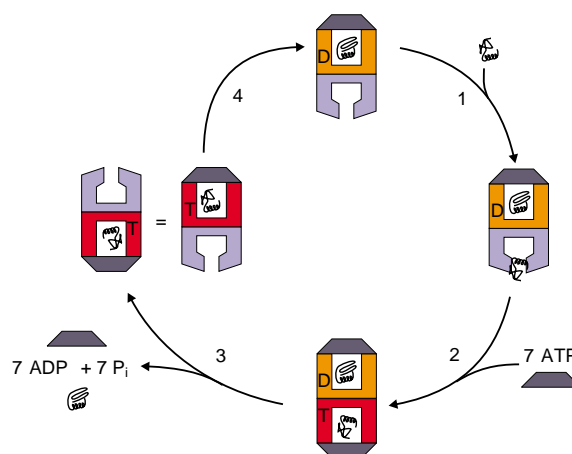


Figure 3. GroE chaperone cycle. Although GroEL is composed of two rings, the functional cycle is best described on the level of single rings, which represent the operational units of the chaperone. Although both rings are active at the same time, they are in different phases of the cycle. The processing of an individual substrate polypeptide requires two revolutions of the GroE cycle, during which the polypeptide remains associated with the same GroEL ring. For graphical reasons, however, the orientation of the GroEL molecule is reversed after step 3 (i.e. the polypeptide does not “move” to the top ring as shown). GroE-assisted folding can be dissected into 3 steps: capture, encapsulation/folding, and release. During capture (step 1; D = ADP), a hydrophobic polypeptide is prevented from aggregation by binding to GroEL. The acceptor ring (lilac) is nucleotide-free and therefore has a high affinity for the polypeptide. Binding of ATP (T) and GroES to this ring (step 2) induces a set of structural changes in GroEL (red ring). Most importantly, the affinity for the bound polypeptide is decreased and it is released into the closed cavity where it starts to fold. Subsequent hydrolysis of ATP (step 4) induces a second conformational change in GroEL (top ring, orange), which allows the *trans* ring (bottom ring, lilac) to bind polypeptide and start a new cycle. Once ATP binds to the *trans* ring (red), GroES is displaced from the *cis* ring (orange), and the substrate polypeptide is released (step 3).

GroEL that increases the affinity of the *trans* ring for ATP.^[56] Binding of ATP to the *trans* ring triggers the release of the GroES in the *cis* ring and thus allows the encapsulated protein to exit the cavity.^[57]

2.1.5. The Functional Cycle of GroE

The functional cycle of GroE-assisted protein folding (Figure 3) can be divided into three steps: capture, folding, and release.^[23] During the capture phase, a polypeptide substrate is bound to the apical domains of one GroEL ring. In the second step, binding of Mg/ATP and GroES to the same ring creates a folding-active *cis* complex by inducing major conformational changes in the GroEL tetradecamer. The polypeptide is discharged into the protected environment of the *cis* cavity and starts to fold. After 15–30 s, the ATP bound to the *cis* ring is hydrolyzed, and a second conformational change primes GroEL for the release of GroES by binding ATP to the *trans* ring. The polypeptide is released from the cavity, irrespective of its folding state.

The input of energy associated with ATP hydrolysis is used to maintain the balance sheet of the chaperone cycle. Each individual step, that is, binding of polypeptide, GroES, and ATP, is an exergonic and therefore irreversible reaction, which drives the cycle in one direction. Since the starting point in a cycle is identical to the end point, there must be an energy source to compensate for this loss of energy.

2.1.6. Unfolding of Polypeptides by GroE

An important question is how GroEL promotes the folding of proteins that are trapped in non-native conformations, a situation referred to as misfolding. It is assumed that GroEL is capable of partially unfolding these proteins, thereby setting them back on the right track to the native state.

Data from different laboratories indicate that there are several potential mechanisms that GroEL could utilize to unfold a protein. The most simple model, thermodynamic coupling, is based on the aforementioned competition between binding and folding.^[58] Since binding to GroEL requires a polypeptide to expose hydrophobic surfaces, and the amount of exposed hydrophobic surface generally decreases with the degree of folding, GroEL will preferentially bind to more unfolded conformations of a protein. Provided there is a rapid equilibrium between the various conformations of the polypeptide, GroEL will effectively unfold the protein. This capability of GroEL has been demonstrated for a variety of relatively small proteins.^[59]

The coupling mechanism, however, has one important shortcoming. It would not allow a polypeptide to escape from a kinetic trap on its folding pathway, because, according to this model, all unfolding reactions occur in free solution at their intrinsic rates. This problem may be solved by an alternative mechanism for GroEL-mediated unfolding. A stable, compact folding intermediate of the enzyme Rubisco was shown to bind to GroEL without any major structural changes. But upon addition of ATP and GroES, this intermediate became transiently unfolded,^[60] likely as a result of the movements of the apical domains which occur upon GroES binding (Fig-

ure 2C). This may exert a mechanical stress on the bound protein, thereby virtually tearing its structure apart. Importantly, this mechanism requires that the polypeptide is bound to multiple apical domains simultaneously.^[61] Active unfolding, however, could not yet be observed with other stringent substrate proteins of GroE. In the case of malate dehydrogenase (MDH), no significant structural changes are observed when the MDH/GroEL complex dissociates upon binding of GroES.^[45]

2.1.7. Folding Inside the Chaperone

Once GroES is bound, the substrate is released into the protected environment of the *cis* cavity. This gives the protein the opportunity to fold without interference from other folding polypeptides, since GroES blocks the entry to the cavity, and unfavorable side reactions such as aggregation can therefore not occur. This situation is often referred to as folding in “infinite dilution” or folding in the “Anfinsen cage”. Monomeric proteins might reach the native state during these 15–30 s, provided their folding is sufficiently fast. Alternatively, they might fold into a committed state, in which they no longer require the assistance of the GroE chaperone although they have not reached the native structure yet (Figure 1). For an oligomeric protein whose native state involves the assembly of several polypeptide chains, this committed state represents the obligatory exit point from the chaperone cycle, because further encapsulation would actually block the pathway to the native protein.^[45, 62]

Proteins that fold more slowly than the time taken for a chaperone cycle are assumed to undergo kinetic partitioning after dissociation from GroE. A fraction of the protein molecules will have reached either the native state or the committed state and will therefore no longer be a substrate for assisted folding.^[63] The remaining molecules have the chance to rebind to GroEL and participate in another chaperone cycle. As discussed above, it is possible that this step includes structural rearrangements such as unfolding of kinetically trapped species that might have formed.

An open question is whether the walls of the cavity have any effect on the folding protein, that is, whether the energy landscape that describes the folding pathway is altered by the steric restraints and the complex chemical composition of the local environment. An investigation of such an effect would require a comparison between folding inside GroEL and folding in free solution, which is possible only for proteins whose folding is not dependent on GroE.

How long is the optimal cycle time for GroE-assisted folding? Generally, longer cycle times mean that GroEL hydrolyzes less ATP per second and therefore consumes less energy. It also means that, on average, a polypeptide would spend more time in the folding cage. This would have two consequences: more GroEL molecules would be needed per cell to provide the required chaperone capacity, and it would slow the folding of fast-folding proteins. Even worse, subunits of some oligomeric proteins may adopt a conformation that is no longer capable of assembling if kept in the “Anfinsen cage” for too long.^[64] On the other hand, if the cycle is too fast, the chaperone system may become less efficient. Faster cycling

leads to an increase in energy consumption, but not necessarily to improved folding yields.

To accomplish its task as a molecular chaperone, GroEL must be capable of binding to a large variety of unfolded, misfolded, and partially folded polypeptides. Experiments with denatured cell extracts have demonstrated that about 40% of the *E. coli* proteins can interact with GroEL.^[65] However, it is unlikely that GroE participates in the folding of all these proteins, because the cellular concentration of GroEL ($\sim 1 \mu\text{M}$) is simply too small for that purpose.^[66] A number of *E. coli* proteins that interact with GroEL have been identified recently,^[67] but it is still not clear how many of them are stringently dependent on GroEL in their folding.

2.2. The Hsp70 System

The Hsp70 proteins constitute the central part of an ubiquitous chaperone system that is present in most compartments of eukaryotic cells, in eubacteria, and in many archaea. As in the case of the GroE proteins, the most extensively studied representative of this chaperone group, the DnaK protein, is from the bacterium *E. coli*. Hsp70 proteins are involved in a wide range of cellular processes, including protein folding and degradation of unstable proteins (for a discussion, see ref.^[68]). The common function of Hsp70 in these processes appears to be the binding of short hydrophobic segments in partially folded polypeptides, thereby preventing aggregation and arresting the folding process.^[19, 69]

DnaK and many other Hsp70 chaperones interact in vivo with two classes of partner proteins that regulate critical steps of its functional cycle, similar to the cooperation between GroEL and GroES: the Hsp40 and the GrpE proteins. Furthermore, additional partner proteins have been identified in the past years, especially in eukaryotic cells, and some of them link Hsp70 to other chaperone systems (see Section 2.3).

2.2.1. Structural and Functional Properties of Hsp70

Hsp70 is comprised of two functional entities: an N-terminal ATPase domain, and a smaller C-terminal peptide-binding domain. The crystal structures of both the ATPase domain and the peptide-binding domain of DnaK have been determined.^[70–72] The ATPase domain of Hsp70 (Figure 4A) is comprised of two subdomains separated by a cleft that contains the nucleotide-binding site.^[73] The nature of the bound nucleotide determines the peptide-binding properties of the C-terminal domain. In the ATP state, peptide substrates bind and dissociate very rapidly albeit with low affinity.^[74] With no nucleotide or ADP bound to the N-terminal domain, the rates of peptide binding and dissociation decrease by more than two orders of magnitude, and the affinity increases significantly. ATP hydrolysis thus serves as a molecular switch between two states of Hsp70, characterized by high dynamics/low affinity or low dynamics/high affinity. Since the structure of full-length Hsp70 has not been determined yet, we have no direct information on how communication between nucleotide binding and peptide binding occurs at a molecular level. However, the X-ray crystal structure of the peptide binding

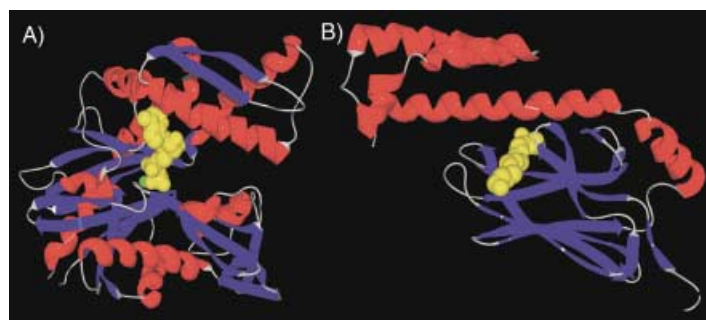


Figure 4. Structure of the DnaK chaperone from *E. coli*. A) Crystal structure of the nucleotide-binding domain of DnaK complexed with ADP.^[71] The nucleotide (yellow) and a magnesium ion (green) are bound in a deep, solvent-inaccessible crevice formed by two subdomains of the protein. The structure is very similar to that of two other ATP-binding proteins, hexokinase and actin. B) Crystal structure of the peptide-binding domain of DnaK complexed with a heptameric peptide.^[72] The peptide-binding site consists of a hydrophobic groove that is located in a subdomain formed by eight antiparallel β -strands (blue). The peptide NRLLLTG (yellow; only the backbone atoms are shown) binds to this groove in an extended conformation and is locked in place by an all-helical subdomain (red). Presumably, this conformation represents the high-affinity state of Hsp70.

domain of DnaK co-crystallized with a heptapeptide bound to its active site provides insight into how this communication might work.^[72]

2.2.2. Peptide Binding by Hsp70

The peptide-binding domain (Figure 4B) consists of two structural units, a β sandwich with a hydrophobic groove on its upper side, and a α -helical domain that sits on top of it. The peptide substrate is bound to the groove in a stretched backbone conformation (see Figure 4B). It is held in place by hydrophobic interactions and hydrogen bonds between the peptide and the β sandwich. The α -helical domain covers the top of the cleft and traps the peptide in the binding site. It is very likely that this “closed” structure corresponds to the ADP state of Hsp70, since it would readily explain the high peptide affinity and the slow exchange kinetics. How could binding of ATP to the N-terminal domain of Hsp70 increase the dynamics of peptide binding? The current model assumes that ATP binding induces a conformational change in the chaperone that removes the helical lid from the peptide binding groove, thus making it accessible to solvent.^[47]

Analyses with peptide libraries have shown that Hsp70 preferentially binds to peptides that contain hydrophobic residues.^[75–77] In proteins, this type of sequence is mainly found in the core of the folded structure, or in subunit interfaces.^[78] Furthermore, the topology of the peptide-binding site in Hsp70 requires that the bound segment is separated from the rest of the protein substrate by $\sim 10 \text{ \AA}$. This implies that the portion of the substrate that is bound to Hsp70 must be considerably unfolded and highly flexible.

Besides the specificity of polypeptide binding, its kinetics are also of importance. Aggregation is a relatively fast process that occurs on a timescale of seconds. To compete with it, complex formation between an unfolded polypeptide and Hsp70 must be equally fast. In the nucleotide-free state,

association rate constants for peptide substrates were in the range of $10-100\text{M}^{-1}\text{s}^{-1}$.^[79] If a cellular concentration of $10\mu\text{M}$ is assumed for an unfolded polypeptide, complex formation would take at least several minutes. It would thus be too slow to prevent a substrate from aggregating. In the “open” ATP-bound state of Hsp70, binding kinetics for peptides are accelerated by at least one order of magnitude. However, the capture of a polypeptide requires the conversion of this labile Hsp70/ATP/polypeptide complex into the more stable Hsp70/ADP/polypeptide form by hydrolysis of the bound ATP. But this reaction, which occurs at a rate of only $\sim 0.1\text{min}^{-1}$, is again too slow to compete with aggregation. The somewhat puzzling conclusion is that for kinetic reasons Hsp70 proteins should not be very potent suppressors of aggregation, a fact that is illustrated by the experimental observation that DnaK alone is not efficient in preventing the aggregation of non-native proteins.^[80, 81] The solution to this problem is provided by the co-chaperones Hsp40 and GrpE, and highlights their importance in the Hsp70 system.

2.2.3. Partner Proteins of Hsp70—Modulation of the Functional Cycle

Hsp40 belongs to a diverse class of proteins that consist of multiple functional domains. One of the domains, the ~ 75 -amino-acid J domain, is conserved in all Hsp40 chaperones. Mutational analysis revealed that this domain is essential for the interaction between Hsp40 and Hsp70.^[82] Its name is derived from DnaJ, the Hsp40 protein from *E. coli* that cooperates with DnaK. The most important features of Hsp40 are that it binds to peptides, and that it stimulates ATP hydrolysis of Hsp70.^[83] A current model of how Hsp40 and Hsp70 cooperate (Figure 5) assumes that a polypeptide

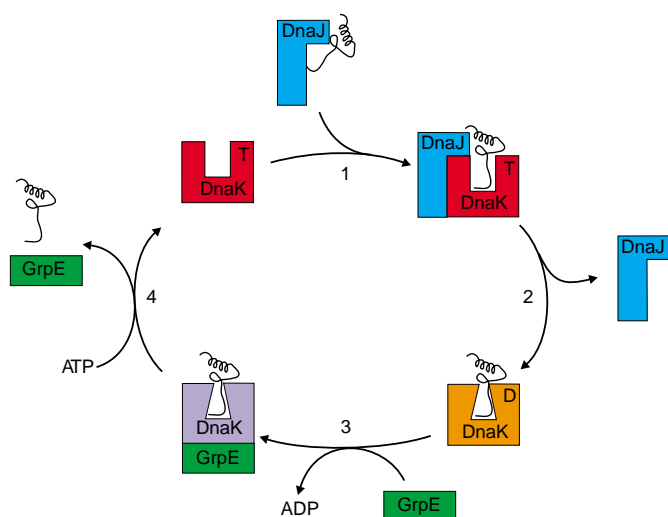


Figure 5. *E. coli* DnaK chaperone cycle. A largely unfolded polypeptide substrate (black ribbon) is captured by the co-chaperone DnaJ (blue). Upon complex formation with DnaK (red), the substrate is transferred from DnaJ to the peptide-binding site of DnaK (step 1). DnaJ-stimulated hydrolysis of ATP (T) closes the binding site (orange conformation of DnaK) and locks in the substrate, thus forming a stable protein/DnaK complex (step 2). After the dissociation of DnaJ, the bound ADP (D) is displaced by the nucleotide exchange factor GrpE, shown in green (3). Subsequent binding of ATP to DnaK releases GrpE and induces a conformational change that opens the peptide binding site (4). The polypeptide can dissociate.

substrate first binds to Hsp40.^[81] The Hsp40/protein complex then associates with Hsp70/ATP, the protein substrate is transferred to the open peptide-binding cleft of Hsp70, and locked in by the subsequent Hsp40-stimulated hydrolysis of ATP. The functional cycle of Hsp70 is completed by the exchange of ADP for ATP, which shifts the chaperone back into its dynamic mode and allows the bound polypeptide to dissociate.

In the presence of Hsp40, ATP-hydrolysis is accelerated and may no longer be the rate-limiting step. Thus, it does not come as a surprise that the second potentially slow reaction of the cycle, nucleotide exchange, can also be stimulated. The co-chaperone responsible for this is GrpE,^[84] which interacts stably with nucleotide-free Hsp70, but not with nucleotide-bound Hsp70. Accordingly, GrpE decreases the affinity of Hsp70 for nucleotides. Facilitated nucleotide exchange appears to be a two-step process (Figure 5). In the first step, GrpE binds to Hsp70 and thus displaces the bound ADP. Subsequently, the binding of ATP to Hsp70 stimulates the release of GrpE.^[85, 86] The crystal structure of GrpE bound to the nucleotide-free ATPase domain of DnaK gives us some hints as to why GrpE decreases the affinity of DnaK for nucleotides.^[71] The nucleotide-binding pocket in the complex is more open than in free DnaK, and therefore less interactions between the nucleotide and the protein are possible.

To date, there is no experimental evidence that Hsp70 chaperones can actively induce conformational changes in their target proteins as has been shown for other molecular chaperones. Furthermore, unlike GroEL, Hsp70 does not provide a favorable micro-environment for protein folding. Rather, Hsp70 seems to serve as a general acceptor for unfolded polypeptides generated by protein biosynthesis or by cellular unfolding processes. The interaction of these target proteins with Hsp70 prevents aggregation and arrests folding. What happens after their release from Hsp70 may depend on the nature of the polypeptide and on the cellular context. Some proteins may fold spontaneously into their native structure, while others may be transferred to more specialized chaperone machines such as GroE or Hsp90, or to systems involved in protein transport or degradation.

2.3. The Hsp90 Chaperone System

Eukaryotes in particular have evolved an additional chaperone machinery, Hsp90, which is far more complex than both GroE and Hsp70. First, it includes the Hsp70 system, at least during part of the chaperone cycle, and second, it comprises a large number of cofactors. More than a dozen have been identified so far.

What makes this chaperone system unique is that relative to the other chaperones, already a large number of client proteins have been identified which depend on Hsp90 to reach their functional conformation under physiological conditions. This suggests that stable complexes that allow isolation are formed between Hsp90 and its client proteins. A striking example, which highlights the influence of Hsp90 on protein folding, is the case of src kinase. This protein is an

important player in the regulation of the cell cycle and therefore constitutes an interesting target for therapeutic intervention. Screens for natural substances that inhibit src function *in vivo* led to the selection of the ansamycin geldanamycin.^[87] However, subsequent analysis showed that this potential inhibitor of src binds to Hsp90 with high affinity and high specificity.^[88] This interaction inactivates Hsp90 which in turn leads to decreased levels of active src kinase.

The number of proteins that require the Hsp90 system for reaching their functional conformation is growing steadily.^[89] But at the moment it is not known what common feature—a sequence motif or a specific structural element—is the basis for their interaction with Hsp90. Many of the substrate proteins of Hsp90 are regulatory proteins or perform key functions in proliferation. One may speculate that the Hsp90 dependence of their activity could enable an additional level of regulation.^[90]

How does Hsp90 fulfill its task? Results from *in vivo* experiments suggest that the target polypeptide passes through several Hsp90 complexes that differ in the composition of partner proteins.^[91] The conformational changes of the target protein that occur during this passage are completely unknown. In an enigmatic way, the acquisition of the functional conformation of the target protein is promoted through the interactions with different Hsp90 complexes.

2.3.1. Structural and Functional Properties of Hsp90

Hsp90 is an elongated homodimer with a dissociation constant of ~ 60 nM.^[92, 93] The dimerization site is located in the C-terminal part of the protein.^[92, 94] The three-dimensional structure of the N-terminal domain of Hsp90 (amino acids 1–215) has been solved by X-ray crystallography (Figure 6).^[95, 96] This domain exhibits a novel fold, which consists of a β sheet

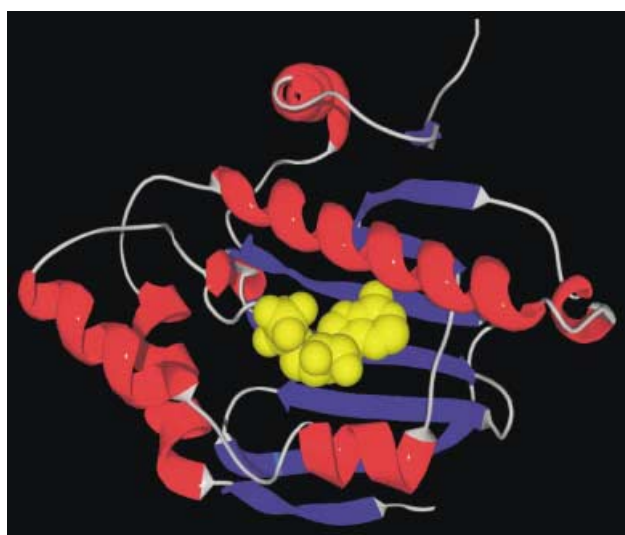


Figure 6. Structure of the N-terminal ATP-binding domain of yeast Hsp90 complexed with ADP.^[97] The domain consists of a twisted eight-stranded β -sheet with a cluster of α -helices arranged on top of it. The nucleotide (yellow) is bound in an unusual kinked conformation in a deep pocket formed by the surrounding helices and loops. This pocket also constitutes the binding site of the antitumor drug geldanamycin.^[95]

and helices arranged on top of it. It was later found that this domain contains the ATP-binding pocket.^[97] ATP is bound in an unusually kinked conformation: the adenosine ring and the ribose unit are buried in the interior of the binding pocket and the phosphate groups point to the surface. Like the GroEL and Hsp70 chaperones, Hsp90 is a weak ATPase.^[98, 99] Hsp90 from the baker's yeast *S. cerevisiae* hydrolyzes ~ 0.3 ATP min⁻¹ at the physiological temperature of the organism. ATP hydrolysis seems to be of crucial importance for Hsp90 function *in vivo*, because mutant proteins that do not hydrolyze ATP do not support the functions of Hsp90 essential for viability.^[99, 100] Recent findings suggest that the ATPase cycle is coupled to large conformational changes in the Hsp90 dimer.^[101, 102] Kinetic analysis of the ATPase reaction showed that regions outside the ATP-binding domain are important for efficient hydrolysis. It is therefore reasonable to assume that once ATP is bound, the N-terminal domain interacts with other parts of the Hsp90 molecule which may include an acceptor for the γ -phosphate group of ATP.^[102] In addition, the two N-terminal domains of the Hsp90 dimer interact in the presence of ATP.^[101] This interaction is required for coordinated and efficient ATP hydrolysis.^[93] How these ATP-induced interactions affect the conformation of the client proteins remains to be seen.

Two consequences of the ATPase cycle can be envisioned which highlight the concept of molecular chaperones as machines: 1) The movements of domains may affect the accessibility of binding sites for nonnative proteins, similar to what has been observed for GroEL. Fragmentation studies have suggested that Hsp90 contains two binding sites for nonnative proteins which differ in their specificities.^[98, 103] It may well be that these sites, which are located in different parts of the protein, cooperate in an ATP-regulated manner. 2) ATP-induced conformational changes in Hsp90 may affect the interaction with specific cofactors or partner proteins. This implies that the Hsp90 chaperone cycle is driven and regulated by ATP hydrolysis. Since the hydrolysis reaction and the formation of the different Hsp90 complexes required for the activation of client proteins occur on the same timescale,^[104] this may well be the case.

2.3.2. Partner Proteins of Hsp90

So far, only limited information is available on what factors regulate the association of Hsp90 with its numerous partner proteins. More importantly, the function of these cofactors is also largely unknown. Some of the partner proteins contain “docking modules”, the so-called tetratricopeptide repeat (TPR) motifs, which consist of arrays of helices that form a groove in which an extended peptide sequence can bind.^[105] The interacting peptide sequences have been found in the C-terminal ends of Hsp90 and Hsp70.^[106] Hop/Sti1, one of the partner proteins of Hsp90 and of central importance for the Hsp90 cycle, consists mainly of TPR motifs, in agreement with its function of bringing the Hsp70 and Hsp90 chaperones together (cf. Figure 7).^[107] In addition, Hop/Sti1 inhibits the ATPase of Hsp90 upon binding.^[108]

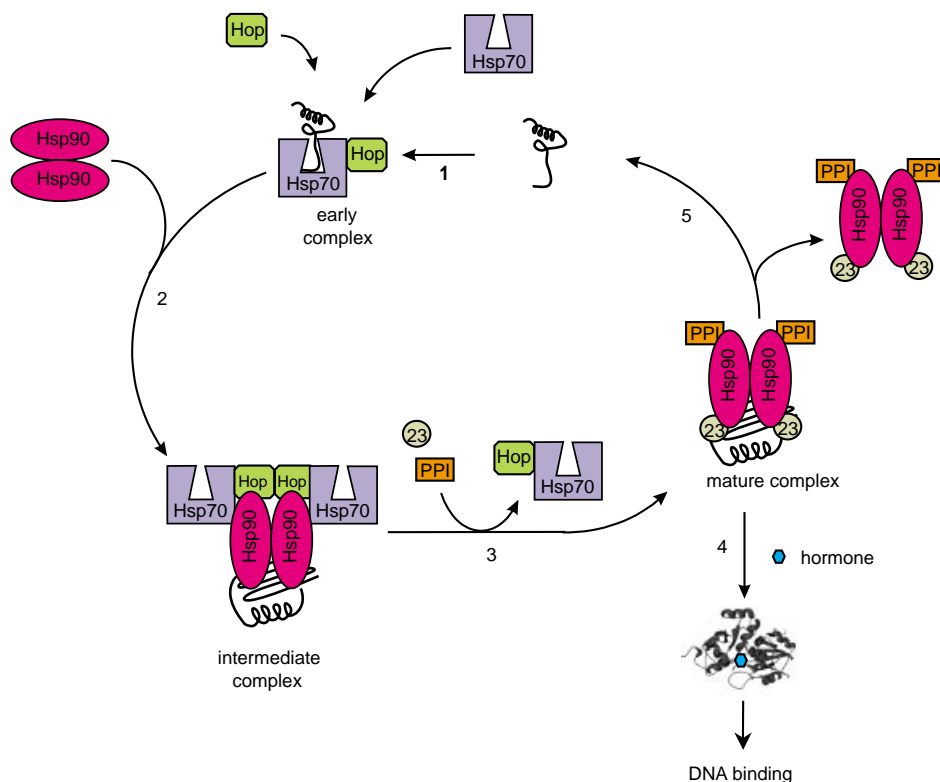


Figure 7. Hsp90 chaperone system for a steroid hormone receptor (SHR). The involvement of cofactors may change depending on the target protein. In the case of SHR, an inactive conformation of the receptor is captured by the molecular chaperone Hsp70 (step 1). The co-chaperone Hop is recruited to establish a physical connection between Hsp70 and Hsp90. Presumably, ATP hydrolysis by Hsp70 releases the bound SHR and transfers it to the Hsp90 dimer, thus resulting in the formation of the intermediate complex (step 2). Transition to the mature complex is mediated by the replacement of both Hsp70 and Hop with large prolyl isomerases (PPI) and another helper protein (p23) (step 3). Upon binding of its hormone ligand, the SHR is released from the mature complex, the receptor switches to its active conformation and migrates to the nucleus (step 4). In the absence of a hormone ligand, the receptor protein may participate in another cycle (step 5).

Other Hsp90 cofactors belong to the class of peptidyl-prolyl isomerases (PPIases), enzymes that catalyze the *cis/trans* isomerization of prolyl-peptide bonds in proteins.^[109, 110] Single-domain PPIases are involved in signal transduction and protein folding *in vivo*. In yeast there are two large Hsp90-associated PPIases, whereas in higher eukaryotes three different isoforms have been identified. In addition to the PPIase domains, they all have other domains. Interestingly, the Hsp90-associated PPIases also contain TPR motifs. It is presently unclear what the function of these enzymes is in the Hsp90 complex and how they are selected for incorporation into the Hsp90 complex. There is some evidence that the bound client protein is involved in this process.^[111] Because of their enzymatic activity one may speculate that catalysis of *cis/trans* isomerization is also important in the context of the Hsp90 complex. It has been demonstrated already that all Hsp90-associated prolyl isomerases and a small Hsp90-binding protein (p23) share the ability to bind selectively non-native proteins *in vitro*,^[112–115] which suggests that these proteins may be in direct contact with the client protein in the Hsp90 complex. Thus the Hsp90 system is a highly dynamic, reversibly assembling molecular machine with moving parts.

2.3.3. Regulation of Protein Conformation by Hsp90

How does Hsp90 affect the folding of other proteins? At present, it seems that under physiological conditions Hsp90 does not play a major general role in the *de novo* folding of proteins.^[116] However it is of critical importance for the folding of a number of proteins that are involved in key regulatory processes. There is some indirect evidence that these target proteins exist in metastable conformations. In the case of steroid hormone receptors, the ligands form part of the hydrophobic core of the protein and thus contribute significantly to the stability of the folded structure. Hsp90 may thus be required to keep steroid hormone receptors in an open state that is capable of ligand binding (Figure 7). In the absence of the chaperone, the receptor may assume an energetically more favorable collapsed state. In a number of cases, the chaperone dependence allows an additional level of regulation for the target protein which is independent of a target-specific signal. Furthermore, association with Hsp90 may generally stabilize unstable intermediate conformations

and thus increase the conformational space that proteins can successfully explore. Regulation of conformation may be the common denominator for an otherwise structurally and functionally diverse set of Hsp90 client proteins. Moreover, it seems reasonable to assume that under stress conditions Hsp90 may interact with a larger number of unfolded proteins.

2.3.4. The Hsp90 Chaperone Cycle

For regulation of the conformation, the substrate protein has to pass through three complexes, which differ in the composition of the cofactors.^[91, 104] First, the client protein seems to be bound by Hsp70 and its cofactors (Figure 7, step 1) which then form a complex with Hop/Sti1 and Hsp90 (Figure 7, step 2). It is assumed that the non-native protein is handed over from one chaperone to the other in this step, analogous to the substrate transfer from Hsp40 to Hsp70 (Figure 5).^[117] As in all processes in which Hsp70 is involved, it is unclear what conformational consequences binding and release have for the client protein. Also, it is unknown whether binding of Hsp90 client proteins to Hsp70 has to precede binding to Hsp90. This may depend on the respective client protein. *In vitro*, Hsp90 is able to directly bind to non-

native proteins.^[90, 118] In one well-studied example, the bound protein was shown to be largely folded. On the folding trajectory this conformation seems to be rather close to the native state, with significant secondary and tertiary structure, but it lacks enzymatic activity.^[119] This is in good agreement with the proposed conformation of ligand-free steroid hormone receptors described in Section 2.3.3.

Unlike the other chaperones, the formation and dissociation of a complex between Hsp90–Hsp70–Sti1/Hop and the non-native protein is only part of the cycle. Following the association of these chaperone components with a non-native protein, Hsp70 and Sti1/Hop dissociate from Hsp90 and the cofactors p23 and one of the large PPIases associate (Figure 7, step 3).^[120] The cofactor p23 binds to Hsp90 only in the presence of ATP.^[121] However, no other factors that govern the association of the Hsp70–Hsp90 complex and the formation of the Hsp90–p23–PPIase complex are currently known. From this Hsp90–p23–PPIase complex, the non-native protein is released (at least in the case of the steroid hormone receptor) in its active conformation, which is capable of ligand binding (Figure 7, steps 4 or 5).^[122] It is reasonable to assume that conformational changes in Hsp90 govern the interaction with the different components of the chaperone machinery. Thus, the partner proteins may either monitor whether Hsp90 is loaded with substrate and regulate the progress of the cycle accordingly, or they may be directly involved in changing the conformation of the target protein bound to Hsp90.

2.4. Small Heat-Shock Proteins (sHsps)

Yet another variation on the theme of protecting proteins from irreversible aggregation by reversible interaction with specialized folding factors is represented by the small heat-shock proteins (sHsps). These proteins have been found in almost all organisms studied so far. In many organisms, several different family members are present in one compartment, thus suggesting functional diversity. In comparison to the other classes of chaperones, they show a characteristic heterogeneity in sequence and size. Their common trait is a conserved C-terminal domain, the α -crystallin domain, which refers to the most prominent family member, the eye-lens protein α -crystallin.^[123]

2.4.1. Structure

All sHsps investigated up to now form oligomeric complexes, mainly of 12 to 42 subunits. The three-dimensional structure of an archaeal sHsp was studied by X-ray crystallography and revealed a hollow sphere with openings to the inside (Figure 8).^[124] The basic building block of this oligomeric structure is a dimer, which associates further to form the sphere. The C-terminal domain of the monomer is predominantly β structured; the structure of the N-terminal domain could only partly be resolved. The reconstruction of the three-dimensional structure of the eye-lens protein α -crystallin by electron microscopy confirmed the picture of a hollow spherical structure.^[125] However, whereas the archaeal sHsp

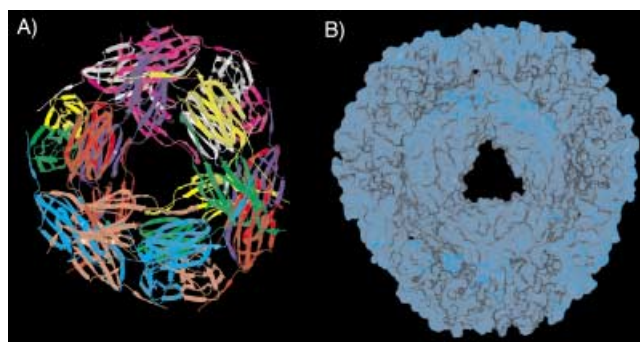


Figure 8. Structure of the small heat-shock protein from the archaeon *Methanococcus jannaschii*.^[124] A) The heat-shock protein forms a porous, hollow sphere, which consists of 24 identical subunits. The subunits are mainly β -structured. B) Cross-section. The outer diameter of the particle is ~ 120 Å, the inner diameter is ~ 65 Å, and the volume of the cavity ~ 140000 Å³. Because of its amino acid composition, the inner surface is much more hydrophobic than the outer surface.

has a rigid and well-defined quaternary structure, a range of different oligomers was detected for α -crystallin.

2.4.2. Complex Formation with Unfolded Proteins

sHsps have been included in the class of molecular chaperones because they bind specifically to unfolded proteins *in vitro* and prevent their aggregation.^[126, 127] Compared to the molecular chaperones discussed above, sHsps have a remarkable binding capacity. They are able to bind a large number of non-native proteins, possibly up to one target protein per subunit of the oligomeric sHsp complex.^[128] Furthermore, there seems to be no restriction on the size of proteins that can be bound.^[129]

A striking feature of the sHsps is that upon substrate binding, they form very large complexes of regular globular shape. In agreement with the lack of intermediates in the formation of substrate/chaperone complexes, this process was found to be highly cooperative.^[130] The simultaneous binding of non-native proteins seems to be a prerequisite for efficient and stable complex formation. The specificity of this reaction is further highlighted by the finding that binding can be saturated at a defined ratio of non-native protein to sHsp. The underlying mechanism is still unresolved. In the case of Hsp26, a sHsp from yeast, *in vitro* experiments gave insight into the regulation of the binding activity of this chaperone.^[130] At temperatures above 40 °C, the well-defined oligomeric complex dissociates into stable dimers in a completely reversible reaction. This dissociation seems to expose binding sites for non-native proteins. Remarkably, the binding of the substrate protein to the Hsp26 dimer results in the formation of large defined complexes (Figure 9). The resulting substrate/sHsp complex shows an organization that is completely different from the Hsp26 complex.

2.4.3. sHsps as Part of the Chaperone Machinery

The complexes sHsps form with non-native proteins *in vitro* are remarkably stable.^[128–131] Although the non-native protein is not released spontaneously, these complexes are not

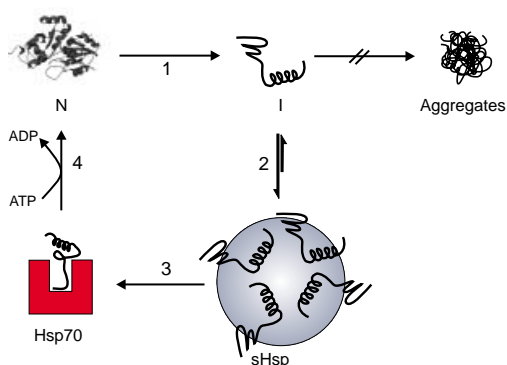


Figure 9. sHsp chaperone system for a polypeptide substrate. Under conditions of heat shock or chemical stress, some polypeptides (N) in the cell may become partially unfolded, leading to the accumulation of folding intermediates (I) (step 1). These molecules are rescued from aggregation by association with small heat-shock proteins (step 2). In the presence of Hsp70, the resulting large polypeptide/sHsp complexes can dissociate (step 3). The molecular mechanism of this stimulated release, however, is currently not known. After the transfer of the protein substrates to the Hsp70 chaperone system, the polypeptides refold in an ATP-dependent process (step 4, cf. Figure 5).

dead-end traps for the unfolded protein. As a proof of principle, it has been demonstrated that a bound enzyme can be shifted back to the native state by adding a specific ligand that stabilizes the functional conformation of the protein.^[131] However, in contrast to other chaperones, no active-release mechanism has been detected so far. Interestingly, in some organisms the expression of an sHsp and an Hsp70 chaperone is genetically linked.^[132] This and the finding that overexpression of Hsp70 has a beneficial effect on the clearance of aggregates *in vivo* suggest that sHsps may function together with other ATP-dependent members of the chaperone family.^[133] Biochemical proof for this co-operation came from *in vitro* experiments in which Hsp70 was added to preformed sHsp/substrate complexes. In the presence of ATP, Hsp70 was able to promote the folding of the protein to the native state (Figure 9).^[131] Independently, cell lysates were screened for factors that promote the refolding of sHsp-bound proteins.^[128, 134] These experiments also identified Hsp70 as the key player in the reactivation of proteins rescued from aggregation by sHsps.

The emerging picture is that sHsps function in binding non-native proteins once large quantities of unfolded proteins are formed, for example, as a consequence of stress conditions or overexpression of proteins. Binding prevents the formation of large aggregates and makes the subsequent refolding by Hsp70 or other potential ATP-dependent chaperone systems possible. This cooperation of different components of the cellular chaperone machinery thus allows two key properties of molecular chaperones, binding and folding, to be separated in space and time.

2.5. What Else Is out There?

The molecular chaperones introduced in Sections 2.1–2.4 constitute the major chaperone systems involved in protein folding. However, for the sake of clarity, we have omitted a

number of partner proteins that have been reported to interact with these chaperones. There are excellent review articles available that provide a more detailed picture for the dedicated readership.

As mentioned earlier, molecular chaperones are also important in other contexts in which protein conformations are manipulated. One of these processes is the resolubilization of protein aggregates. In the past years, some members of the Hsp100/Clp class of chaperones, namely ClpB from *E. coli* and Hsp104 from yeast, have been reported to dissolve protein aggregates. Importantly, these two chaperones require assistance from the Hsp70 system for their task.^[135, 136] Hsp100/Clp chaperones are also involved in protein degradation, during which they act as “unfoldases” and use the energy provided by ATP hydrolysis to actively unfold a target protein.^[137] Interestingly, all Hsp100/Clp chaperones form rings of six or seven subunits.

In the bacterium *E. coli*, many proteins designated for export associate with the chaperone SecB after their ribosomal synthesis.^[138–140] SecB was shown to arrest the folding of its client proteins. Subsequently, SecB guides its substrate to the translocation machinery, which transports it through the cytoplasmic membrane. Transport systems for unfolded proteins have also been found in some organelles of the eukaryotic cell, for example, in the mitochondria and in the endoplasmic reticulum.

3. Perspectives

The life cycle of a protein is marked by the conformational changes it has to undergo. Folding, trafficking, maintenance, and degradation of proteins are all processes that depend on the assistance of molecular chaperones. The past decade has witnessed an enormous increase in our understanding of these processes, and a large amount of both structural and functional data on chaperone proteins is now available. Despite these efforts, our knowledge and the picture that emerges remains sketchy. The variety of different chaperones is perplexing and at present we know only a little about their cellular function and how they accomplish their task(s) on a molecular level. There are several exciting directions in which this field is developing.

3.1. Chaperone Networks

An important question that remains to be answered is how the different classes of molecular chaperones cooperate in the cell. Examples of such chaperone networks are the cooperation between the Hsp90 system and the Hsp70 system during the activation of steroid hormone receptors (see Figure 7), or the cooperation between the Hsp70 and GroE in protein folding (Section 2.2.3). It is not clear whether the passage of a polypeptide substrate through such networks is vectorial or random.^[80, 141, 142] Another puzzling feature is the overlap in the function of the various chaperone classes. All chaperones are able to suppress the aggregation of folding proteins and are overproduced simultaneously under stress conditions.

While cells may require some functional overlap for backup purposes, much remains to be learned about the specific roles of individual classes of chaperones.

3.2. Conformational Changes of Proteins in Chaperone Complexes

In general, chaperone-mediated structural changes of polypeptides could involve folding, unfolding, and disassembly. Protein unfolding is a nonspecific process, as demonstrated by the fact that all known proteins can be unfolded by a single chemical compound, guanidine hydrochloride. Under cellular conditions, unfolding is an endergonic reaction and requires a source of energy. The function of a molecular chaperone would thus be to couple protein unfolding with the chemical energy provided by ATP hydrolysis so that the whole process becomes exergonic. Such an unfolding activity seems to be required for both protein degradation and for the recycling of misfolded proteins. A similar principle can be envisioned for the chaperone-assisted disassembly of defined protein complexes and of nonspecific aggregates. In both cases, the multiple interactions that stabilize the association of the components must be broken, which again requires the input of energy.

3.3. Chaperones and Disease

The importance of molecular chaperones for cell viability is illustrated by the fact that deletions of their genes are often lethal or cause severe cellular defects, such as reduced resistance to stress. If a specific chaperone activity is missing or reduced to subnormal levels, a disease may be the consequence. In other cases, the action of chaperones may cause a disease, as in the case of bovine spongiform encephalopathy or Creutzfeldt–Jakob syndrome, which are both characterized by the deposition of fibrillous aggregates of the prion protein (PrP) in the brain.^[143] For mammalian prion diseases, the involvement of chaperones is still speculative. In yeast, however, prion formation was shown to be critically dependent on Hsp104.^[144] Hsp104 seems to regulate the size and the number of seeds that are required for fiber polymerization and propagation. In this case, cells can be cured by the inactivation of Hsp104.^[144, 145] In addition, chaperones are emerging as molecular targets for therapy in cancer, tissue transplantation, and septic shock syndrome, among others. Depending on their respective contribution, inhibition or stimulation of chaperone activity may be required for intervention.

3.4. Molecular Chaperones in Biotechnology

The production of recombinant proteins in bacteria is often impaired by the formation of inclusion bodies, which are large aggregates that consist mainly of inactive forms of the overexpressed protein.^[146] In some cases, this problem could

be alleviated by the simultaneous overproduction of molecular chaperones.^[147–149] Molecular chaperones may thus become useful tools to optimize biotechnical processes and to establish fine-tuned “cell factories” for the production of recombinant proteins.

3.5. Chaperones and Evolution

As mentioned earlier, small proteins usually do not require the assistance of molecular chaperones for *in vitro* folding, whereas larger proteins often do. Apparently, large proteins must confer certain evolutionary advantages on the cell, despite the additional expenses required for their production. Among others, two considerations may be important in this context: 1) The conformation space that is accessible for small proteins may be limited, that is, some three-dimensional structures can only be formed by longer polypeptide chains. Since the structure and the function of a protein are intimately related, some biological functions may be unique to large proteins. 2) Large proteins often comprise multiple domains, which have specific functions such as oligomerization, substrate binding, etc. This setup allows the creation of proteins with novel properties by a simple rearrangement of existing building blocks.

Another reason why chaperones have proven useful during molecular evolution concerns the conformational stability of proteins. Enzymes require flexibility to exert their biological function, for example, for binding substrates or for responding to regulatory signals. Thus, the three-dimensional structure has been selected for *optimum* stability, and not for *maximum* stability. Typically, the free energy of folding is in the range of 20–80 kJ mol⁻¹,^[150] a value that corresponds to a small number of molecular interactions such as ion pairs or hydrogen bonds. The disadvantage of this low stability is that some proteins start to unfold at elevated temperatures. Therefore, a means is required to inhibit irreversible aggregation under these circumstances. The intimate relationship between chaperones and evolution is exemplified in a recent analysis of the function of Hsp90 during development.^[151] According to this study, this chaperone is essential to maintain the function of mutated proteins. Under stress conditions, this task can no longer be performed, and the mutations gain effect, thus resulting in new phenotypes and traits.

In summary, the analysis of chaperone function has taken a number of unexpected turns concerning both the molecular mechanisms of these ATP-driven molecular machines and their involvement in fundamental cellular processes. In contrast to their endangered human counterparts, molecular chaperones seem to be about to enter center stage. Their importance for the basic mechanisms that determine life at the molecular level is just beginning to be unveiled.

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