REVIEW ARTICLE Chaperonins

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The molecular chaperones are a diverse set of protein families required for the correct folding, transport and degradation of other proteins *in vivo*. There has been great progress in understanding the structure and mechanism of action of the chaperonin family, exemplified by *Escherichia coli* GroEL. The chaperonins are large, double-ring oligomeric proteins that act as containers for the folding of other protein subunits. Together with its co-protein GroES, GroEL binds non-native polypeptides and facilitates their refolding in an ATP-dependent manner. The action of the ATPase cycle causes the substrate-binding surface of GroEL to alternate in character between hydrophobic (binding/unfolding) and hydrophilic (release/folding). ATP

binding initiates a series of dramatic conformational changes that bury the substrate-binding sites, lowering the affinity for non-native polypeptide. In the presence of ATP, GroES binds to GroEL, forming a large chamber that encapsulates substrate proteins for folding. For proteins whose folding is absolutely dependent on the full GroE system, ATP binding (but not hydrolysis) in the encapsulating ring is needed to initiate protein folding. Similarly, ATP binding, but not hydrolysis, in the opposite GroEL ring is needed to release GroES, thus opening the chamber. If the released substrate protein is still not correctly folded, it will go through another round of interaction with GroEL.

INTRODUCTION TO MOLECULAR CHAPERONES

Anfinsen [1] showed that polypeptide chains can fold spontaneously to form native, compact protein structures whose three-dimensional conformation is determined by their primary structure. A caveat to this fundamental principle of biology has since been added to account for the need for helper proteins that prevent aggregation and assist folding *in vivo*. Cellular conditions of high protein concentration, temperature and ionic strength differ greatly from the artificial environment of the test-tube and are extremely unfavourable for the protein folding reaction because they favour aggregation over correct folding. These helper proteins are collectively termed the molecular chaperones and are required for the successful folding, assembly, transport and degradation of proteins within the cell [2–4]. They are abundant and ubiquitous protein families, many of which are heat-shock proteins (hsps), whose common features are an interaction with non-native protein subunits, the stabilization of protein-folding intermediates and the prevention of aggregation. These activities increase the yield of protein-folding reactions and aid recovery from cellular stress, but can also lead to protein unfolding. Despite these common features, the structures and modes of action of the molecular chaperone families are remarkably diverse. Some families are very specific in their action, whereas others are very general. Some, but not all, use the binding and hydrolysis of ATP to control the release and binding of substrate proteins.

Modes of substrate binding

It is interesting to compare modes of substrate binding in a few of the hsps for which we have models or detailed information. The diversity of chaperone–polypeptide interactions is illustrated schematically in Figure 1. The small hsps (shsps) have an

enormous capacity for substrate binding, but the structural basis of this binding remains unknown. Denatured proteins appear to bind either by coating this surface, or perhaps by being encased inside a shell formed by the shsp subunits. The latter possibility would require disassembly and reassembly of the shsp oligomers. This hsp class is not known to have ATPase activity and seems to act as a passive binder of non-native protein in response to cellular stress [5,6].

DnaK (hsp70, BiP) is the *Escherichia coli* homologue of a large and important class of hsps present in the cytosol, endoplasmic reticulum, mitochondria and chloroplasts [7]. It acts in concert with co-proteins DnaJ (involved in substrate binding and presentation) and GrpE (a nucleotide-exchange factor). Crystal structures of both the ATPase domain, which is structurally related to actin and hexokinase [8], and the substrate-binding domain [9] are separately known. The latter shows a remarkable brick-shaped domain permeated by a hole through which the bound peptide runs. An α -helix running across the top of this hole is proposed to serve as a hinged lid, allowing entry and exit of the polypeptide-chain segment that binds in a completely extended conformation. ATP binding and hydrolysis control release and binding of the substrate by an unknown mechanism involving interactions between the two domains and DnaJ.

In contrast with the previous two examples, hsp60 (GroEL, cpn60) forms a large cage-like structure with two rings of seven subunits, each surrounding a central cavity. The protein-folding intermediate binds to hydrophobic sites lining these cavities. The hsp100 family has a hexameric ring structure [10,11] and it seems plausible that the unfolded substrate might also bind in the central cavity in this case.

Some of these ATPases associate with multi-subunit proteases and play a role in protein degradation, as well as in thermotolerance. They have the capacity to dissociate protein aggregates; expression of yeast hsp104 at normal levels is required for

Abbreviations used: AMP-PNP, adenosine 5'-[β , γ -imido]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; EM, electron microscopy; (s)hsp, (small) heat-shock protein; (m)MDH, (mitochondrial) malate dehydrogenase; MR, mixed ring; SR, single ring.

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Figure 1 Substrate binding to molecular chaperones

A schematic representation of the modes of substrate binding to hsp25, hsp70 and hsp60. Non-native polypeptide is shown in pink and the various chaperone architectures in grey. For hsp25, the location of substrate binding is unknown and may be enclosed in a shell of protein subunits rather than coating the outside, as shown here.

conversion and maintenance of the psi^+ phenotype, a prion-like phenomenon involving the aggregation of the translation-termination factor sup35 [12]. Hsp90 interacts with a range of steroid receptors and kinases by an as-yet-undetermined mechanism. The recent crystal structure [13,14] of the N-terminal domain has revealed an ATP-binding site and an unexpected similarity to the fold of DNA gyrase [14].

More specific chaperones include the endoplasmic reticulum proteins calnexin/calreticulin, which bind to specific oligosaccharides and perform quality control during the processing of glycoproteins [15]. Peptidyl-prolyl isomerase (PPI) and protein disulphide-isomerase (PDI) catalyse the *cis-trans* isomerization of proline residues and the isomerization of disulphide bonds respectively. These are potentially rate-limiting steps in the folding process and several examples are now known in which their activities are required for successful protein folding [16,17]. Finally, the pro-sequences of certain proteins have been found to act as intramolecular chaperones, facilitating the acquisition of the native state before being removed by processing to yield the active protein [18]. It should be noted that this is a case of specific catalysis of a folding step, as presumably the pro-sequence has evolved to stabilize the transition state in the folding reaction of the main protein chain. This is very different from the concept of a general molecular chaperone, where stabilization of transition states is implausible for a wide range of substrates with different folds.

Scheme 1 Folding pathway of a typical oligomeric enzyme

Unfolded polypeptide chains (U) undergo a rapid hydrophobic collapse to form a compact folding intermediate (I). This folding intermediate folds productively (with a rate v_f) to form a folded, native-like monomer (F), which then dimerizes to give the native active enzyme complex (D). However, losses in the productive folding reaction arise from non-specific association (with rate v_a) of I to form aggregates (Agg). In the presence of a molecular chaperone (C), a third possible fate for the folding intermediate exists: it can form a complex with the molecular chaperone (C:I) with an affinity defined by the dissociation constant (K_d) for this interaction.

General principle of molecular chaperones in preventing aggregation

As stated above, one of the common features of the molecular chaperone family is an interaction with non-native proteins, but how can this basic affinity be translated into an increase in the efficiency of a typical folding reaction (see Scheme 1)? If the intermediates of a protein-folding reaction are sequestered by the addition of a molecular chaperone, this passive binding event alone can improve the yield of that folding reaction as long as two criteria are met: first, that the stability of the interaction between chaperone and folding intermediate is not greater than the free energy of folding, and secondly, that the loss of yield in the folding reaction is due to aggregation.

If the rate of a multimolecular aggregation process (v_a) is more dependent on the concentration of intermediate [I] than the rate of productive unimolecular protein folding (v_f), the yield of folding is increased by the reversible formation of a chaperone-intermediate (C-I) complex that lowers the concentration of folding intermediate in solution, i.e. if $v_f = k_f[I]$ and $v_a = k_a[I]^n$, then α , the partition coefficient between folding and aggregation, is given by:

$$\alpha = v_f/v_a = k_f/k_a \cdot [I]^{n-1} \quad (1)$$

If the value of [I] is high, then $\alpha \rightarrow 0$ and aggregation is favoured. Conversely, if [I] is low, then $\alpha \rightarrow 1$ and folding is favoured. Implicit in this analysis, however, is the fact that there must be a concomitant decrease in the observed rate of folding because of this drop in folding intermediate concentration [19].

If an equilibrium between bound and free states exists, then under conditions of heat shock or other cellular stress the amount of denatured protein will increase and the equilibrium will be shifted towards the bound state. Thus proteins would be stabilized by molecular chaperones in these conditions. When conditions improve, any non-native proteins that are released will have a better chance of reaching the native state, and the equilibrium will shift toward the free state.

Chaperones also deliver proteins to proteolytic complexes [20], presumably a mechanism for disposing of damaged proteins that cannot reach the native state, or proteins that have been targeted for proteolysis as part of programmed turnover or cell death.

The chaperonin family

The hsp60s and their partner proteins, the hsp10s, were named chaperonins when it was discovered that the *E. coli* protein GroEL, required for bacteriophage assembly [21], and Rubisco-

Figure 2 Structure of the GroEL oligomer and subunit

Shown on the left is the crystal structure of GroEL [35] filtered to 2.5 nm resolution, showing the double-ring structure and the location of an individual subunit within the oligomer. Shown on the right is the backbone trace of the GroEL subunit as described in the text. Based on Figure 1 of Roseman et al. [38] and reproduced from *Molecular Chaperones and Folding Catalysts*, (Bukau, B., ed.), Copyright 1998, Harwood Academic Publishers.

binding protein in chloroplasts were closely related and required for protein assembly [22]. They are involved in the ATP-dependent folding and refolding of a wide variety of structurally unrelated proteins [23,24] and are essential for cell viability at all temperatures [25,26]. Critically, they can allow protein folding to occur with both high yield and rate, suggesting that some explanation other than the passive chaperoning effect discussed earlier is required. The chaperonins are currently the molecular chaperone system for which there is the most structural and mechanistic information, and the rest of this review will concentrate on their structure and mechanism of action in assisted protein folding.

There are two subfamilies: the GroE chaperonins found in eubacteria, mitochondria and chloroplasts (Group I) and the TCP1 chaperonins found in archaeobacteria and in the eukaryotic cytosol (Group II). Although the two subfamilies have a similar cage-like structure, there are some important differences in the regions that bind substrate proteins. GroEL works with its co-protein GroES and uses an ATPase cycle to transiently create an enclosed, enlarged cavity in which protein folding takes place. No co-protein has been identified for the TCP1 subfamily, but an extra structural element built into the apical domain of TCP1 may fulfil a similar role to GroES [27,28].

GroEL (hsp60, cpn60) is a 14-mer of 58 kDa subunits in two rings, and its co-protein GroES (hsp10, cpn10) is a heptameric ring of 10 kDa subunits. The related eukaryotic cytosolic chaperonin, CCT (TCP1, TriC), contains two rings of eight subunits, derived from eight related, but distinct, gene products [29]. In the archaeobacteria, the thermosome, or TF55, has two rings of eight or nine subunits, each composed of one or two subunit types [30–32].

The chaperonins are an example of a recently emerged class of large protein complexes, which also includes the proteasome [33,34]. They form enclosed, or partially enclosed, compartments that carry out reactions on sequestered polypeptide chains. The role of the ATPase cycle has recently become well defined in this mechanism. A combination of kinetics, crystallographic studies and electron cryo-microscopic snapshots of different states has revealed the sequence of concerted hinge rotations that reorient polypeptide-binding sites and lead to massive conformational changes in the chaperonin complex.

GroEL OLIGOMER STRUCTURE AND ALLOSTERIC STATES

Structure

The structures of the GroEL oligomer and its various complexes have been determined by X-ray crystallography [35,36] and by electron cryo-microscopy [37,38]. Figure 2 shows the crystallographic structure of both the oligomer and a single subunit. The oligomer is cylindrical and formed from two heptameric rings stacked back-to-back. Each ring encloses a central cavity and there are holes in the sides of the structure. The interface between the two rings is flat, except for the two contacts (numbered 1 and 2) formed by the base of each subunit with its neighbours in the opposite ring.

On the right is shown the backbone structure of the outlined subunit with the three domains colour-coded. The termini are located close together in the equatorial domain (green), which forms the inter-ring and most of the intra-ring contacts. The inter-ring contacts are formed by salt bridges, labelled C1 and C2. This domain also contains the nucleotide-binding site, and an adenosine 5'-[γ-thio]triphosphate (ATP[S]) molecule is shown

Figure 3 Backbone alignment of the thermosome and GroEL apical domains

The thermosome (gold) and GroEL (blue) apical domains are aligned, highlighting the helical extension present in the thermosome [27,28]. The fold homology is clear in the β -sheet core of the domain. The structures were aligned by selecting equivalent residues in the conserved β -strands and performing a rigid body fit with Quanta (Molecular Simulations). The Figure was produced with GRASP [89].

bound in the site [36]. An exposed region of anti-parallel chains connects the equatorial domain to the intermediate domain (orange). This connecting region is a site of hinge rotation, labelled hinge 1. At the top of the intermediate domain, another hinge region (hinge 2) connects to the apical domain (purple), which contains the binding sites for polypeptide substrates (yellow space-filling residues) and for GroES (yellow and blue residues) [39].

An interesting variant on this structure is seen in the CCT subfamily, exemplified by the archaeobacterial thermosome. This chaperonin has two rings of eight subunits each, and rather low sequence homology with GroEL. The only detectable homology is in the equatorial domain, particularly around the ATP-binding site, and the apical domain appears to be quite divergent [29]. However, the crystal structure of the apical domain reveals that, despite the lack of sequence homology, it has a similar overall fold except for one unique and prominent feature (Figure 3) [27]. In the region that forms the hydrophobic binding site in GroEL, the thermosome apical domain has a long extension, which forms an α -helical protrusion. This extension is highly conserved in the TCP1 family and contains hydrophobic residues proposed to act as substrate-binding sites. The extensions would arch over the central channel to form a very different substrate-binding surface from that in GroE chaperonins [27] and a closed lid in the recently determined crystal structure of the intact thermosome hexadecamer is formed [28]. This difference is consistent with the observed differences in specificity of TCP1 and GroE chaperonins (for example, in the folding of luciferase [40]). Figure 3 shows an overlay of the GroEL and thermosome apical domain folds.

Scheme 2 A simplified model of nucleotide-induced allosteric transitions in GroEL

Both rings in unliganded GroEL are in the T state (represented by pink squares) with low affinity for nucleotide but high affinity for non-native protein. Binding of ATP occurs with strong positive co-operativity to one of the rings, converting it to an R state (pale pink circles) with high affinity for nucleotide and low affinity for non-native protein (a TR complex). Negative co-operativity between rings prevents ATP binding to the second ring at intermediate ATP concentrations. At higher nucleotide concentrations, the second ring is also occupied, forming an RR state (Figure adapted from Yifrach and Horovitz [46], Copyright 1994, Academic Press Ltd.)

GroEL allosteric states and nucleotide-induced conformational changes

ATP binds rapidly to GroEL to form a collision complex with a rather weak affinity ($K_d = 4$ mM [19]). This collision event is followed by conformational rearrangement, which leads to tight equilibrium binding with a K_d of approximately $10 \mu\text{M}$ [19,41–43], and occurs with strong positive co-operativity [19,44,45]. The co-operative nature of this binding establishes that GroEL can exist in (at the very least) two states: a T (or tense) state with low affinity for nucleotide and an R (or relaxed) state with high affinity for nucleotide. However, in the case of GroEL, the situation is more complex because the double-ring structure is reflected in the behaviour of its 14 nucleotide-binding sites. Tight binding of ATP occurs to only one of the two available rings with a dissociation constant (K_d) of approximately $10 \mu\text{M}$ [41–43, 46,47]. Negative co-operativity between rings also exists, resulting in different nucleotide affinities for the two rings. This tends to produce asymmetric conformations of the rings, despite their identical subunit composition (Scheme 2). At low concentrations of ATP both rings are in the tense (T) allosteric state. Owing to negative co-operativity, nucleotides first occupy one ring, converting it into the relaxed (R) state, while the nucleotide affinity of the second ring is lowered. At high concentrations of nucleotide, both rings are filled and thus converted into the R state.

T states have low affinity for ATP, but high affinity for non-native polypeptide substrates, whereas the converse is true for R states [19,48,49]. Thus the binding and hydrolysis of ATP sets up an alternating cycle in which each ring is switched between states with strong and weak affinity for a non-native protein substrate.

How is this modulation in affinity for substrate effected? To understand the role of the nucleotide cycle, we must first examine the structures of GroEL–nucleotide complexes. The location of the nucleotide-binding site was revealed by the X-ray structure of GroEL–ATP[S] [36], but this structure did not reveal the expected conformational changes in GroEL predicted by the biochemical evidence for a large change in substrate affinity [48–50]. The reasons for the oligomer remaining in the T state in this crystal structure are still unknown, but may relate to protein–protein interactions in the crystal reversing the solution conformational changes. However, low-resolution structures for several nucleotide-bound states were determined by electron cryo-microscopy and single-particle analysis of GroEL complexes in vitrified

Figure 4 Low-resolution three-dimensional structures from electron cryo-microscopy

The structures shown are: (a) GroEL, (b) GroEL-ADP, (c) GroEL-ATP, (d) GroEL-GroES-ADP and (e) GroEL-GroES-ATP. Nucleotide binding (b, c) lengthens the oligomer and twists the apical domains (see Figure 2) to different extents. The ATP form (c) has a more asymmetric structure and an altered interface between the rings. The bullet-shaped GroEL-GroES complexes (d, e) are similar to each other, but the apical domains are in different orientations in both of the rings. The extended loops linking GroES to GroEL (see Figure 8) are clearly visible (Figures from Roseman et al. [38], Copyright 1996, Cell Press).

solution [38]. The structures of GroEL, GroEL-ADP and GroEL-ATP are shown in Figure 4 (a–c). Nucleotide binding induces substantial movements of the apical domains, resulting in an overall elongation of the oligomeric structure and twisting of the apical domains to different positions. The identity of the nucleotide binding in the equatorial domain pocket has profound effects on the conformations at both hinge regions (see Figure 2). The nucleotide site is fairly close to hinge 1, but its effect on hinge 2 must be transmitted via the intermediate domain. The diversity of ring conformations obtained {including a different one for GroEL-adenosine 5'-[β , γ -imido]triphosphate (AMP-PNP) [38]} make it clear that the T and R state model is a considerable simplification and that a wide range of conformations exist. However, it is still useful to consider the mechanism in terms of this simplified model of allosteric states. The major functional consequence of the apical domain movements in these different states is that the hydrophobic binding sites originally lining the central cavity (as shown in Figure 2) are reoriented so that they become progressively occluded. This progression from unliganded, through ADP to the ATP state follows a decreasing affinity for non-native substrate [48], suggesting that this occlusion is the structural basis for the observed loss of affinity.

BINDING AND RELEASE OF SUBSTRATES

Peptide binding and the peptide-binding site

We can now consider the molecular nature of the GroEL-substrate interaction and how it is modulated by nucleotides (polypeptide binding by GroEL is comprehensively reviewed elsewhere, e.g. see [51]). The binding of non-native proteins to apo-GroEL is tight, with estimates for the dissociation constant K_d of the binding event varying between 10^{-12} and 10^{-8} M for large and small polypeptides respectively [41,52–57]. The chaperonin has been shown to bind to proteins whose native fold

is either exclusively α -helical [58] or β -sheet [59]. The most important determinant in binding is likely to be the hydrophobicity of the folding intermediate [53]. The nature of the polypeptide state that is most tightly bound has been the matter of intense debate, with evidence for both the tight binding of the most unfolded intermediates [52,60] and tight binding to molten-globule states [61,62]. It is likely that any preference is substrate-specific. For the protein barnase, it has been demonstrated convincingly that GroEL binds tightly to both fully unfolded and molten-globule states [56,57]. There is general agreement that any state with a significant exposure of hydrophobic surface will bind to the chaperonin.

Fenton et al. [39] probed the hydrophobic surface residues of GroEL by site-directed mutagenesis and found a cluster of them on the apical domain whose mutation abolished substrate binding (yellow space-filling residues in Figure 2). This occurs in the same region as that determined by the malate dehydrogenase (MDH) difference density of a GroEL-MDH complex observed by electron cryo-microscopy [37]. Subsequently, the crystal structure of the GroEL apical domain (residues 191–376) expressed in isolation with an N-terminal expression tag fortuitously showed binding of an extended stretch of seven residues in the N-terminal tag to the hydrophobic-binding site of the adjacent GroEL apical domain in the crystal lattice [63]. The binding site with the peptide is shown in Figure 5. It seems likely that this is the same binding site as that found in the intact oligomer because it covers many of the hydrophobic residues identified by Fenton et al. [39]. Although the whole site is not accessible to the N-terminal sequence, features such as the packing of a leucine side chain into a hydrophobic pocket in the binding site give credibility to this model proposed for the binding. Polar and charged groups are also involved, along with several backbone interactions, consistent with the requirement for positive charge on the substrate in addition to hydrophobicity [64,65].

Figure 5 Structure of a bound peptide on the surface of the isolated GroEL apical domain

The peptide is a hydrophobic N-terminal tag on the adjacent domain in the crystal lattice and the domain surface is coloured by convexity. A leucine side chain extends into a deep pocket (bottom right). Figure reproduced by kind permission of A. Buckle et al. [63], Copyright 1997, National Academy of Sciences, U.S.A.

Allosteric states and the release mechanism

Before considering the interaction with GroES, much of the basic mechanism can be understood by considering the tight- and weak-binding states of GroEL–nucleotide alone. Indeed, for some proteins, the presence of GroEL and ATP is sufficient to give large increases in the yield of the folding reaction [52,66,67]. As mentioned above, T states have a high affinity for protein substrates and low affinity for ATP and the converse is true for R states. A simple alternation between tight binding and release of substrate is at the core of the chaperonin mechanism. Binding serves the dual function of preventing aggregation and of unfolding kinetically trapped, misfolded states [19,41,56,68]. Subsequent ATP binding to the chaperonin releases the substrate [50,69]. Upon release, the intermolecular interactions between exposed hydrophobic sites on the substrate and the GroEL-binding surface can be replaced by intramolecular interactions: for example, burial of the hydrophobic sites and correct folding, or else further misfolding. If the substrate refolds correctly, it will not rebind to the chaperonin and subsequent hydrolysis of ATP will reset the system into the initial tight protein-binding state. If it misfolds, the cycle is repeated until the protein folds correctly [19,41,70]. Many of the proteins that require the complete GroE system for folding *in vitro* are oligomeric and so it should be noted that ‘correct folding’ will not necessarily be to the native state, but rather to a state which is committed to reaching the native state and which is a substrate for the spontaneous oligomerization reaction.

Kinetic and structural studies on the GroEL mutant Arg-197 → Ala have helped to clarify the release mechanism and the

Figure 6 Three-dimensional reconstructions of GroEL Arg-197 → Ala

Electron cryo-microscopy structures of GroEL Arg-197 → Ala TT, TR and RR states are shown as transparent surfaces (created in Bobscrip [90] and rendered in Raster 3d [91,92]). The electron density has been cut open to show the orientations of the peptide-binding sites in each state. Apical domain atomic structures were fitted into the densities, and the binding-site residues are shown space-filled in yellow (hydrophobic residues [39]) and blue (polar residues [63]). Based on work in White et al., 1997 [71].

action of ATP. The apical domain residue Arg-197 (pink residue in Figure 2) forms a salt bridge with Glu-386 on the intermediate domain of the adjacent subunit. Breaking this salt bridge by introducing the mutation results in a functional GroEL with somewhat altered kinetic properties [46]. Both positive and negative co-operativity are reduced and the ATP concentrations at which one, and then both, rings switch to the R state (Figure 3) have been defined by kinetic measurements [46]. White et al. [71] imaged this mutant at a range of ATP concentrations in order to determine the conformations corresponding to the TR and RR states. Fitting the atomic structure of the apical domain to these electron-microscopic (EM) reconstructions shows that the domains are twisted to different extents. The TR state of the mutant is similar, but not identical, to the wild-type ATP form observed previously [38], although this conformation is reached at a lower ATP concentration in the mutant. The RR state shows both rings very opened, also resembling wild-type at a very high ATP concentrations.

The R ring of the TR state shows a clockwise twist of the apical domains that tends to rotate the peptide-binding site out of the central cavity, thus making it less accessible for binding. The T ring of the TR state is twisted clockwise, closing in the ring and also tending to occlude the binding sites. The RR rings are very opened radially, in addition to the anti-clockwise twist. The effect of these movements on orientation of the peptide-binding sites is shown in Figure 6, in which the EM structures are shown as transparent rendered surfaces cut open to reveal the internal cavities. The hydrophobic-binding site residues are shown as yellow space-filling atoms and the polar/charged binding residues are in blue. The twisting and radial separation in both the TR and RR states show how the continuous band of binding surface of the TT state is disrupted and occluded in these states. An overall property of the R states is expansion of the oligomer, leading to decreased interactions between the GroEL subunits, particularly in the apical domains.

The twists in wild-type GroEL are similar, but the rotations occur in a different plane (A. M. Roseman, H. E. White, S. Chen and H. R. Saibil, unpublished work). We propose that this ATP-induced loss of accessibility/continuity is the basis for ATP-dependent substrate release in chaperonin-assisted protein folding.

FOLDING IN THE CAGE: THE GroEL–GroES COMPLEX

Structure of GroEL–GroES complexes

Perhaps the most remarkable aspect of the GroES/GroEL story is the massive conformational change caused by the binding of the co-chaperonin GroES (Figure 4d and 4e), revealed by electron cryo-microscopy [38] and by the crystal structure of the GroEL–ADP₇–GroES ternary complex [72]. Figure 7 shows the hinge rotations in the GroEL subunit bound to GroES. GroES is a heptamer of 10 kDa subunits that binds to one end of the GroEL oligomer in the presence of adenine nucleotides [73]. It has a β -barrel structure and forms a dome, which caps the opened-up apical domains of GroEL (Figure 8) [74]. GroES binding is rapid in the presence of ATP and slow in the presence of ADP. As expected from previous work [38,75], a mobile loop of GroES extends downwards and outwards to form contacts with several hydrophobic residues on GroEL (former substrate-binding residues that are rotated to the top of the apical domains). In this way, the binding of GroES creates a new type of binding site, i.e. the enlarged, enclosed *cis* cavity, with a maximum dimension of around 8 nm (80 Å). The largest substrate known to use this system is the 52 kDa large subunit of bacterial Rubisco [76] and

Figure 7 Hinge rotations in the GroEL subunit upon binding of GroES

The three domains of the GroEL subunit are coloured individually (apical in pink, intermediate in green and equatorial in blue). A schematic representation (upper left) and backbone ribbon of the atomic structure (lower left) of unliganded GroEL are shown. Upon binding of GroES and ADP (GroES not shown), a 60° opening and a 90° rotation of the apical domain is seen (upper right and lower right respectively). Reprinted by permission from Nature [72], Copyright 1997, Macmillan Magazines Ltd.

it seems likely that this is an upper limit on the size of substrate proteins imposed by the size of the *cis* cavity. Interestingly, there may be an exception to this upper limit because bacteriophage T4 expresses its own GroES homologue, Gp31, which is absolutely required for folding of the 56 kDa coat protein Gp23 [77]. Gp31 differs from its *E. coli* equivalent in having longer mobile loops and a wider internal space, which are proposed to result in the formation of an enlarged *cis* cavity [78]. This might allow the folding of a protein that would otherwise be too large to be accommodated in the *cis* cavity.

Although there is not yet a convincing image of substrate density in the *cis* cavity, it is clear from biochemical data that stringent substrates (i.e. those whose folding is absolutely dependent on the presence of GroEL, ATP and GroES) must transit through *cis* in order to fold [79–84]. The acceptor state is the open *trans* cavity of a GroEL–GroES–ADP complex. After a single round of ATP binding/hydrolysis up to half of the substrate will end up in the *cis* cavity, protected from proteolysis and aggregation [79–81]. Rye et al. [84] show the evolution of the native fold of green fluorescent protein, which develops its characteristic fluorescence while still trapped in the *cis* cavity (see below). The kinetics of GroES binding are particularly revealing. As described above, the binding of GroES to a GroEL–ATP ring is very rapid. In fact, this binding event is sufficiently fast that it

Figure 8 Crystal structure of the GroEL–GroES–ADP complex

The back half of the crystal structure of the GroEL–GroES–ADP complex [72] showing the view into the cavities. GroEL is coloured purple and GroES is green. Substrate-binding sites are shown in space-filling mode (hydrophobic residues [39] in yellow, and polar residues [63] in blue) and are rotated away from the central cavity in the *cis* ring, becoming inaccessible to protein substrate trapped in the *cis* cavity. The Figure was created using Bobscript [90].

precedes the detectable conformational change that converts GroEL from a T state into an R state [42]. This reinforces the point that the T- and R-state model must be a considerable simplification. Although GroES cannot bind to unliganded GroEL, it may be able to bind to a transient state that exists after ATP binding, but before the full nucleotide-induced conformational rearrangement takes place. Thus GroES binding is more rapid than ATP-induced substrate release and this would provide a kinetic mechanism for trapping polypeptides in the *cis* cavity.

Two especially striking new findings are revealed by the recent crystal structure of the GroEL–ADP₇–GroES complex [72]. First, there is a dramatic change in surface properties of the walls lining the enclosed cavity that accommodates the folding substrate. The hydrophobic binding sites present in the *cis* ring (Figure 8) are completely rotated into the interfaces with the neighbouring subunit and with GroES, bringing a hydrophilic surface in to line the cavity. Secondly, the intermediate domain rotates downwards, capping the nucleotide-binding cleft and inserting an aspartate (Asp-398) into the binding site, coordinating to the Mg²⁺ ion adjacent to the terminal phosphates.

The low-resolution structures of the complexes with ADP and ATP (shown in Figures 4d and 4e) have slightly different orientations of their apical domains. For the most stringent substrates, e.g. porcine heart mitochondrial (m)MDH and

Rubisco, folding is only achieved in the presence of GroEL, GroES and ATP.

Folding in the chamber, and why there are two rings

Horwich and colleagues have produced several GroEL mutants, designed on the basis of the atomic-structure findings, that have served as very effective tools in probing the mechanism of assisted protein folding. Particularly important ones are the single-ring (SR) mutants SR1, the Asp398 → Ala mutant and the combination of these mutations in SR398. The crystal structure of GroEL revealed a pair of salt bridges linking the base of each subunit to its neighbours in the opposite ring. Mutations at both of these contacts (Arg-452 → Glu, Glu-461 → Ala, Ser-463 → Ala and Val-464 → Ala) prevent association of the rings and result in SR mutants. These mutants are still able to both bind and hydrolyse ATP and to bind substrate and GroES but, remarkably, they are very poor at releasing GroES [79]. These properties were used to make stable *cis* complexes by first binding substrate to the GroEL SR and then adding nucleotide and GroES, encapsulating the substrate. Lacking the allosteric signal from ATP binding in the second GroEL ring, GroES remains tightly bound and the state of the trapped substrate could be probed both spectroscopically and by subsequent, controlled removal of the GroES by lowering the temperature or by proteolysis. This shows why GroEL has two rings; release of GroES by the *cis* ring is triggered by some change in the *trans* ring.

The crystal structure of the GroEL–GroES–ADP complex revealed the unexpected insertion of Asp-398 into the nucleotide-binding site by a downward rotation of the intermediate domain. The mutant Asp398 → Ala binds ATP, but hydrolyses it extremely slowly (half-time, $t_{1/2}$, of 20 min instead of 20 s). Combining SR1 and Asp398 → Ala mutations yielded SR398, an SR that binds GroES extremely tightly, such that it is not released by low temperature or even by incubation in 0.4 M guanidine hydrochloride. Another set of mutations, in binding-site residues 203, 337 and 349, abolishes binding of GroES and substrate. Rye et al. [84] combined the latter two constructs in a mixed-ring (MR) complex, MR2, with one Asp398 → Ala ring defective in ATP hydrolysis and the other ring with binding-site mutations rendering it incompetent in both substrate and GroES binding. Thus MR2 is forced to bind GroES and substrate only in *cis* and the *cis* ring retains its ATP.

Armed with these constructs, the Horwich group have demonstrated the following. (i) Folding takes place inside the *cis* chamber and given long enough, protein subunits will fold to the native state while trapped inside. This was demonstrated for green fluorescent protein by monitoring the evolution of the native fluorescence. (ii) For the substrates with the most stringent folding requirements, including mMDH and Rubisco, folding in *cis* requires ATP binding, but not hydrolysis. ADP and, interestingly, even AMP-PNP do not result in correct folding, showing that the requirement for ATP is extremely specific and that AMP-PNP does not mimic the ATP state sufficiently well to allow folding. (iii) There are two steps in the release of GroES: hydrolysis of the ATP in the *cis* ring first weakens the binding of GroES and primes it for release, which is then subsequently triggered by ATP binding, not hydrolysis, in the *trans* ring. This observation has subsequently been strengthened by studies on the inhibition of the GroEL ATPase cycle by ADP [85]. ATP binding to one GroEL ring cannot be hydrolysed until ADP on the other ring has dissociated.

What is it about the *cis* chamber with ATP that goes beyond that identified for ADP or AMP-PNP? At present, we only have

the detailed structure of the ADP *cis* ring and that shows a dramatic rearrangement of the surface residues. It is not known why the hydrophilic chamber in this structure is not sufficient to promote folding of stringent substrates. One possibility is that ADP or AMP-PNP binding is not sufficient to release tightly bound substrates from the hydrophobic sites, perhaps preventing this conformation from being reached in the presence of such substrates. Another possibility is that the structure is different in ATP. This is supported by the differences in apical domain orientation shown at low resolution by electron cryo-microscopy between ADP and ATP complexes, but the AMP-PNP bound structure is not very different from that in ATP [38].

Although there is excellent agreement between the crystal structures and the EM structures in many respects, there are two discrepancies. As described previously [36], the crystal structure of GroEL–ATP[S] shows very little difference from unliganded GroEL, whereas the EM structures featuring ADP, ATP and AMP-PNP show a variety of dramatic domain rotations [37,38,71]. It appears that the R state is not reached in the crystal form solved. The strong biochemical and kinetic evidence for the existence of R states make it very likely that the structure generated by the use of electron cryo-microscopy presents a truer representation of the nucleotide-bound state in solution. The other discrepancy is in the *trans* ring of the GroEL–GroES–ADP complex. The difference is very similar to that found between the ATP[S] structure and the nucleotide-bound states observed by EM. In the crystal structure, the *trans* ring is almost identical with the rings of unliganded GroEL, whereas in the EM structure the *trans* apical domains are significantly extended, as they are in the nucleotide-bound states. The significance of this discrepancy is unknown.

Why have ring structures at all?

It has been known for some time that both monomeric cpn60 from *Thermus thermophilus* and a monomeric 50 kDa cpn60 fragment can increase the yield of rhodanese refolding in the absence of GroES and ATP [86], suggesting a basic chaperoning activity independent of any ring structure. More recently, Fersht and colleagues [87,88] have shown that the isolated GroEL apical domain, discussed earlier as a structural model for polypeptide binding by GroEL, can also act in this way, although with reduced folding rates. As discussed earlier (see the Introduction) a reduction in the concentration of a folding intermediate can increase the folding yield with a concomitant decrease in the rate at which folding occurs. The isolated apical domain has a reduced affinity for substrate proteins and it appears that this affinity is poised at a point which allows the chaperonin to prevent aggregation, while still allowing productive folding. However, if isolated, monomeric cpn60 and even domains of GroEL can achieve refolding at high yield, so therefore why have large ring structures evolved at all?

Oligomeric GroEL has a much stronger affinity for non-native proteins, possibly due to the close physical proximity of seven binding sites with similar binding affinity to the isolated apical domain. The combination of binding non-native polypeptide with very high affinity and the ATP- (and GroES)-driven reduction in that binding affinity would allow a much wider range of proteins to be folded. Furthermore, as described earlier, conditions *in vivo* are extremely unfavourable to productive folding. It seems likely that the cavity is essential in the cell because it provides a protected environment for productive folding [79–81], and by effectively enforcing infinite dilution, permits folding at high rates [83,84]. This may be a key feature during stress situations *in vivo*, when the chaperonin cage may be

essential not only to handle a wide range of proteins, but to handle them quickly enough to keep the cell alive.

CONCLUSIONS

Both the binding and release of substrate and GroES are controlled by a complex cycle of hinge movements in GroEL that are promoted by the binding and hydrolysis of ATP. GroES appears to act simply as a removable lid that caps the GroEL cavity. However, the kinetics of its binding and the closed chamber it forms with GroEL provide a mechanism for ensuring that the folding protein is trapped inside a hydrophilic cavity (see Scheme 3 for a summary scheme of chaperonin-assisted protein folding

Scheme 3 Simplified reaction cycle for chaperonin-assisted protein folding

(1) Non-native protein binds to the *trans* ring of a GroEL–GroES complex. (2) End-to-end exchange of GroES (possibly through a symmetric complex with a GroES molecule bound to each of the GroEL rings) results in the encapsulation of the protein substrate in the *cis* cavity. (3) In the presence of ATP, productive folding of protein substrates can occur in the *cis* cavity. (4) Release of GroES and substrate protein (whether folded or not) results in the regeneration of the acceptor complex for non-native protein. Note that for simplicity, changes in the GroEL–nucleotide state have been omitted.

folding). The thermosome has no separate GroES, but does possess a built-in extension, which appears to play a similar role.

Many questions remain unanswered. What is the sequence of events when GroES binds and the substrate is displaced from the binding sites into the cavity? How are the large hinge rotations effected by the binding of nucleotides? A fascinating allosteric system couples the opening and closing of the GroEL cavities to its ATPase cycle. Can we map the network of interactions that define this system and provide a new structural insight into cooperativity in proteins?

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