Molecular chaperones: containers and surfaces for folding, stabilising or unfolding proteins

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Newly solved chaperone structures include the thermosome, a group II chaperonin, and a small heat-shock protein. Novel ideas on chaperone mechanism are presented in the forced unfolding hypothesis of GroEL action. Structures of chaperone–pilin complexes reveal the mechanism of chaperone interaction in bacterial pilus assembly and there have been major advances in understanding the structure and function of Hsp100 unfoldases.

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Abbreviations

DHFR	dihydrofolate reductase
EM	electron microscopy
Hsp	heat-shock protein
MDH	malate dehydrogenase
NSF	*N*-ethylmaleimide sensitive factor
PDB	Protein Data Bank
sHsp	small Hsp

Introduction

This year marks a great loss to the molecular chaperone field and to many other important areas of structural biology, with the recent death of Paul Sigler. His enormous contributions to the understanding of chaperonins underlie much of the work reviewed here and elsewhere.

In this review, advances in studies on several different types of molecular chaperone and new insights resulting from recent structural work will be discussed. Its purpose is to survey recent highlights, not to provide a full bibliography. For those wanting more complete coverage, there are many recent reviews on chaperonins and cellular protein folding [1–8].

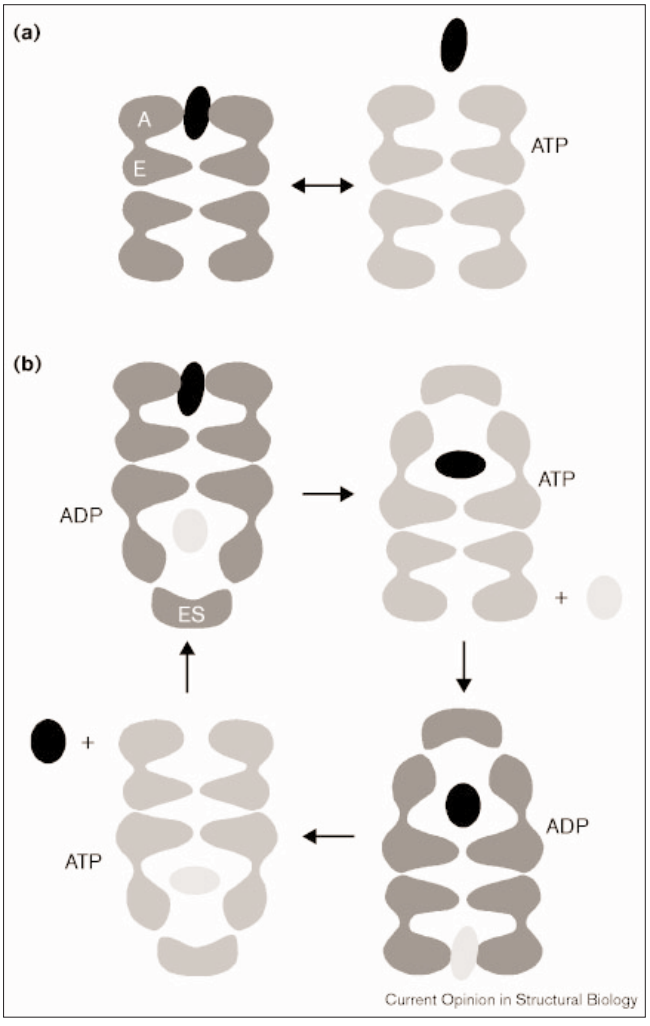
The remarkable mechanism of the GroEL–GroES chaperonin system, a ubiquitous and essential protein folding machine, is gradually becoming more clearly understood. The chaperonin oligomer forms a large, double-chambered structure, in which folding polypeptides are first bound to hydrophobic sites, then trapped inside a closed, hydrophilic chamber as the chaperonin changes conformation during its ATPase cycle. Structural, kinetic and mutagenesis studies reveal how the chaperonin binds and hydrolyses ATP, but it is still not known how the nucleotide cycle orchestrates the dramatic movements involved in its different phases of action. Structural, spectroscopic and biochemical results reveal tantalising glimpses of how peptides and non-native proteins bind to the chaperonin, but there is no complete view of the conformation of a bound protein and details of the crucial steps at the moment of release remain obscure. The structures of a group II chaperonin and a small heat-shock protein (sHsp) open up new avenues of enquiry, but do not yet explain their mechanisms of action. In contrast to these nonspecific chaperones, pilin subunit–chaperone complexes reveal highly specific interactions in the assembly of bacterial pili. The Hsp100/Clp chaperones, members of the large AAA ATPase superfamily, have a global unfolding activity and are able to rescue proteins from aggregates, in cooperation with the Hsp70 system. The first Hsp100 structure has just appeared.

Allosteric interactions in GroEL

The structural framework of chaperonins is provided by two rings of equatorial domains placed back-to-back, forming the most rigid part of the barrel-shaped oligomer [9–12] (Figure 1a). The intermediate and apical domains, which extend axially from the equatorial rings, are more mobile, but movements within a ring are concerted. Steady-state kinetic analysis revealed a hierarchical organisation, with positive cooperativity for ATP binding within rings, but negative cooperativity between rings [13]. Recent transient kinetic studies using the Phe44Tryp mutant to provide a fluorescent reporter group have shown that the nested cooperativity mechanism also applies to the ATP-induced conformational changes in GroEL [14•]. Another study using tryptophan mutants examined more rapid structural transitions induced by ATP binding and suggested that there are several distinct phases of conformational change [15•]. The kinetic findings are consistent with cryo-electron microscopy (cryo-EM) studies showing multiple conformations for different nucleotide states [11]. The multistep transition to the ATP-bound state led to the suggestion that the ATP-induced release of non-native protein substrate from the binding sites is staged, so that there is still some binding surface to retain the substrate until GroES is bound and the cavity is capped [15•]. The effects of GroEL cooperativity on protein folding rates have recently been examined [16•]. This was done by comparing the folding rates of mouse dihydrofolate reductase (DHFR) and mitochondrial malate dehydrogenase (MDH) in the presence of various GroEL mutants with altered cooperativities. The fast folding of DHFR is slowed down by increased positive cooperativity, as bound states of the protein are favoured. The slower, GroES-dependent MDH folding rate is largely independent of negative cooperativity, suggesting that the rate of encapsulation is fast relative to the rate of MDH folding.

Molecular dynamics simulations of the normal modes of vibration in GroEL probe the intrinsic flexibility of the sub-

Figure 1



The functional cycle of chaperonins. **(a)** On the left is GroEL, with high affinity for non-native protein and, on the right, GroEL–ATP, with low affinity for non-native protein. ATPase activity sets up cycles of binding and release. A, apical domain; E, equatorial domain. **(b)** ATPase cycle with GroEL, GroES (ES) and folding protein substrates. GroEL–GroES–ADP is the acceptor state for non-native protein. Subsequent ATP and GroES binding will encapsulate the protein. GroEL–GroES–ATP is the folding-active state. GroES binding alternates between rings and GroES release is stimulated by ATP binding to the opposite ring. Acceptor states are shown in dark grey and release states in light grey. The substrate is black and the second substrate in the full complex (b) is pale grey.

unit, providing an interesting view of the allosteric transitions [17•]. Simulations confirm that hinge bending and domain twisting, rather than relative motion between subunits, underlie the allosteric transitions. The results are compatible with the range of hinge angles seen in the different crystal structures and in cryo-EM studies. Relative tilting of the equatorial domains could account for the transmission of conformational signals between the rings. The effect of ATP binding is to decrease the flexibility of the equatorial domains, but to increase that of the other domains.

GroEL–substrate interactions

Figure 1 summarises the main steps in the GroE–substrate interaction. The apo-GroEL [9] and ADP-bound states (dark grey) are acceptor states, which have a higher affinity for non-native substrate. The substrate (black) is bound to hydrophobic sites on the apical domains, which surround the mouth of the cavity (Figure 1a). ATP binding (light grey) causes substrate release; the binding and release interaction is sufficient to fold some proteins. Binding to the hydrophobic sites may unfold kinetically trapped, misfolded substrates, which have a fresh chance to fold correctly upon each release [18,19]. A schematic diagram of the full GroEL–GroES system is shown in Figure 1b. In this case, the acceptor state is the open end of the GroEL–GroES–ADP complex (dark grey, with black substrate). Subsequent binding of ATP and GroES displaces the substrate from the hydrophobic binding sites and traps it inside the hydrophilic folding chamber (light grey), which is capped by GroES. The crystal structure of the bullet-shaped complex GroEL–GroES–ADP_7 revealed details of the domain movements that underlie the trapping mechanism [12]. In addition to their 60° extension towards the GroES ring, the apical domains of GroEL twist by 90° in the plane of the ring. This twist relocates the hydrophobic binding sites from their original position lining the cavity to a buried position between adjacent subunits or bound to GroES, thus removing the binding sites from the substrate. During the approximately 15 s of the ATPase cycle, the released protein subunit has a chance to fold inside the closed cavity. ATP hydrolysis followed by a new round of ATP binding in the opposite ring is required before the GroES is released, allowing the escape of the folded subunit.

It is clear that the back-to-back interaction between the two rings of GroEL plays a critical role [20,21]. A kinetic and structural study using fluorescence resonance energy transfer and cryo-EM [22•] has shown the ordered sequence of interactions between GroE complexes and substrates. Substrates first bind to the open ring of a GroEL–GroES–ADP complex. As described above, ATP and GroES binding serves to encapsulate the bound substrate. In addition, the bound ATP discharges the ligands (GroES and nucleotide substrate) from the opposite ring, which adopts a relatively closed and somewhat twisted orientation, restricting access to the hydrophobic sites [22•]. No new ligands bind to the opposite ring until the ATP is hydrolysed. When the ATP is hydrolysed, the open ring changes conformation to a more open state, with the binding sites more accessible, and a new substrate can be bound before the encapsulated one is released. The second substrate is shown in Figure 1b in light grey. One aspect that is not understood in structural terms is the existence of fast and slow pathways for GroES release [22•]. The binding of a new substrate to the open ring of the ADP-bound bullet complex accelerates GroES release, changing the rate-limiting step from GroES release to ATP hydrolysis.

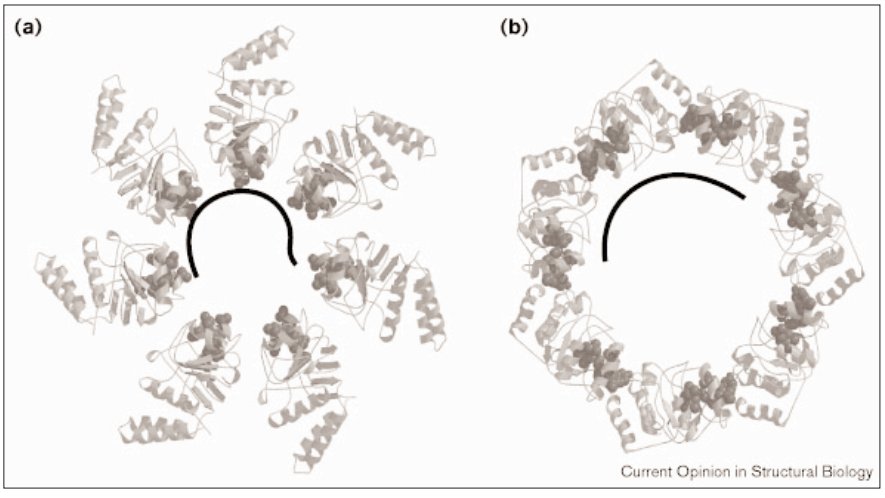
There has also been further progress on understanding the nature of peptide binding to GroEL. Chatellier *et al.* [23•] and Chen and Sigler [24•] have used phage display libraries to characterise peptides that bind to the isolated apical domain. In all these cases, the peptides bound with a similar interaction to that of the GroES mobile loop. The family of peptides bound on the apical domain surface in a hydrophobic groove formed by helices H and I, which face upwards in the GroES-bound conformation. A common feature is the packing of a hydrophobic peptide sidechain into a cavity, but there are also polar interactions. There are small local adaptations of the apical domain conformation to the different peptides, but the peptides themselves can have extended, β or α conformations [23•,25,26]. The helix H/I binding site is likely to be only part of the story, however, as an earlier mutagenesis study [27] mapped out a larger area of hydrophobic residues involved in substrate binding, including loop 199–204, which is deeper inside the GroEL cavity. Therefore, the structural information available so far covers only GroES-like binding. An interesting speculation based on a transient kinetic study [15•] is that this loop region may be involved in anchoring substrates after the initial interaction with ATP, until the cavity is sealed by GroES.

The diversity of protein folds that interact with GroEL in *Escherichia coli* has been described using 2D gel electrophoresis and mass spectrometry to identify 52 GroEL-bound proteins, out of the 10% of *E. coli* cytoplasmic proteins that were trapped on GroEL by EDTA treatment and cell lysis [28]. There was a preponderance of α-β and α-β-α sandwich folds, but it is not known whether these substrates are strictly dependent on the GroE system for their folding.

Does GroEL stretch proteins on the rack?

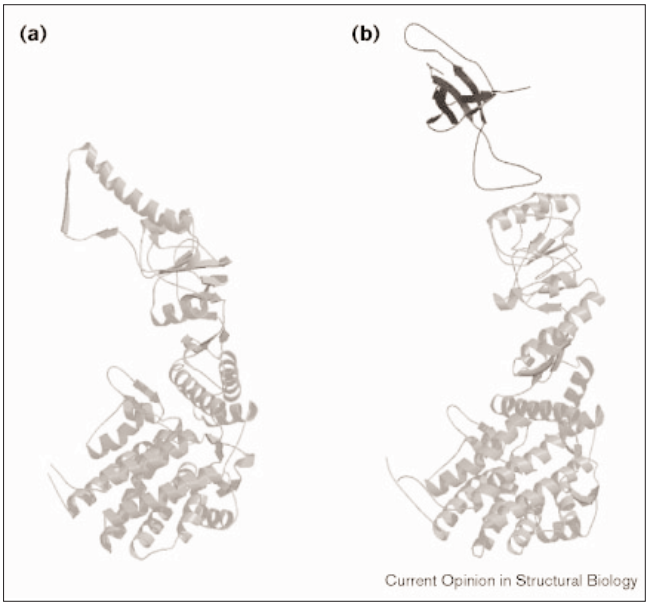
A critical event in GroEL–substrate interaction is ejection from the binding sites. It has been proposed that binding itself can cause some unfolding, by the exchange of intramolecular hydrophobic contacts with intermolecular ones [18,29,30], but different substrates appear to bind with different degrees of unfolding and there is no general agreement on this point. A very interesting hypothesis has been advanced, proposing mechanical unfolding of a substrate bound to several apical domains as they move apart during ATP and GroES binding. The hypothesis is supported by the finding that substrate interaction with GroEL–GroES–ATP (or AMPPNP) causes almost full backbone hydrogen exchange for misfolded Rubisco, implying that the interaction involves an unfolding step [31••]. Without the GroE interaction, the protected amides exchange on a much slower timescale. A single round of interaction (ATPase half-time ~15 s) is sufficient for unfolding and the kinetic measurements are compatible with full unfolding during each round of interaction. Figure 2 shows the start and end positions of the hydrophobic binding sites in the crystal structure of GroEL–GroES–ADP. The transition from the open ring to the GroES-bound state results in a greater separation of the binding sites, so that a substrate that is bound simultaneously to two or more sites will experience mechanical stress. Shtilerman *et al.* [31••] propose that this movement can mechanically unfold the bound substrate. Once released into the hydrophilic cavity, it has a fresh chance to find the correct fold during each round of interaction. Recent work from the Horwich laboratory provides direct evidence that substrates such as Rubisco bind to multiple sites. Farr *et al.* [32••] constructed a single gene containing all seven subunits of a GroEL ring in tandem and mutated binding site residues to selectively inactivate particular apical domains. This tour de force of genetic engineering reveals that a minimum of three adjacent functional binding sites are required for viability and for *in vitro* folding of Rubisco, a substrate that is strictly dependent on GroEL, GroES and ATP for its folding.

Figure 2



Mechanical unfolding on *E. coli* GroEL. **(a)** The open ring of the GroEL–GroES–ADP complex seen from the outside of the complex ([12]; PDB code 1AON). The hydrophobic binding residues [27] are shown in space-filling format. A schematic polypeptide chain is bound to three adjacent subunits. **(b)** The GroES-bound GroEL ring, seen from the GroES position. The binding sites are buried and more widely separated. The bound chain is released, but has been extended by the movement of the sites. Figures 2–6 were produced with BOBSCRIPT and RASTER3D [61–63].

Figure 3



Comparison of group II and group I chaperonin subunits. **(a)** One subunit of the thermosome, the archaeal group II chaperonin ([33••]; PDB code 1A6D). **(b)** Subunit of GroEL with a bound GroES (darker shading) [12]. There is a clear relationship between the folds of the three domains in each of the two structures, but they have somewhat different orientations for the intermediate domain. The apical domain extension (top) of the thermosome subunit plays an equivalent role to GroES.

Group II chaperonins have a built-in lid

Another recent advance in studying chaperonin structure has come from the crystal structure of the complete thermosome, a 16-subunit group II chaperonin [33••,34]. The structure solves the long-standing puzzle about the lack of a GroES homologue for group II chaperonins. Instead of a separate lid protein, extensions of the apical domains [35] join up to form a built-in lid. The rest of the fold is very similar to that of GroEL, despite the lack of sequence homology in the apical domains. The similarities extend to the aspartate in the intermediate domain that inserts into the ATP-binding site. The subunit conformations are compared in Figure 3. A surprising difference in the oligomer structures is that the thermosome subunits are in register across the intering interface, unlike GroEL, which has a staggered arrangement. This is likely to affect the allosteric properties, which are not well understood for group II chaperonins. The crystal structures only show the fully closed form, a flattened structure equivalent to the GroES–GroEL–GroES 'football' complex. Electron microscopy studies of archaeal forms of group II chaperonins have revealed an open form [36] and a bullet-shaped form, in addition to fully closed and fully open forms [37]. In the case of the eukaryotic cytosol chaperonin CCT, electron microscopy studies show some asymmetry induced by ATP binding [38].

Small heat-shock proteins – a shell, but not a container?

The sHsps comprise a more diverse family of molecular chaperone than the chaperonins. They are mostly oligomeric assemblies, with a conserved N-terminal domain related to a domain from the eye lens protein α-crystallin. They stabilise non-native proteins and prevent their aggregation, but probably release them to other chaperone systems for refolding when heat or other cellular stress conditions are relieved. They have a high capacity for protein binding. In the eye lens, α-crystallin is thought to protect other lens proteins from aggregation over the lifetime of the animal. αB-crystallin is also widespread in other tissues and is associated with stress, for example, in heart muscle and in neural tissue that is undergoing degenerative changes. α-Crystallin forms very polydisperse and disordered assemblies, but the overall structure was shown to be a hollow shell by cryo-EM [39]. The crystal structure of archaeal Hsp16.5 has been determined and reveals a stable hollow shell with an octahedral arrangement of 24 subunits [40••]. Hsp16.5 consists mainly of the α-crystallin domain, which is shown to have an immunoglobulin fold (Figure 4). An ANS-binding site that is likely to be involved in substrate binding [41] is partly exposed on the surface, but it is not obvious from the structure how the substrate is bound. The volume enclosed inside the shell is too small to account for the amount of protein that can be bound by other sHsps. Recent work on yeast Hsp26 shows that it dissociates into dimers at heat-shock temperatures, which reassemble into a completely reorganised, larger complex in the presence of denatured protein [42].

Immunoglobulin fold chaperones

In the Hsp16.5 assembly, subunit dimers exchange β strands at the edge of one β sheet (Figure 5a). This interaction may contribute to the order and stability of this sHsp, but this part of the sequence is not well conserved.

The Hsp16.5 dimer bears an interesting relationship to a different type of chaperone, that involved in the assembly of bacterial pili, for which the chaperone appears to be completely specific to its pilin substrate. The periplasmic chaperones PapD and FimC stabilise and transport subunits of their respective bacterial pili, until their export across the outer membrane and insertion into the pilus structure. Pili are surface projections responsible for host recognition and adhesion of bacteria. The Pap and Fim systems have an important role in urinary tract infections. These chaperones were known to have immunoglobulin folds [43,44], but two recent papers present a significant advance. They both report the structure of the full chaperone–substrate complex [45••,46••] (Figure 5b). Although the two structures are differently arranged, in both cases the chaperone donates an edge strand to a pilin subunit, which also has an immunoglobulin fold. Without the donated strand, the pilin subunits have an exposed hydrophobic core and are unstable.

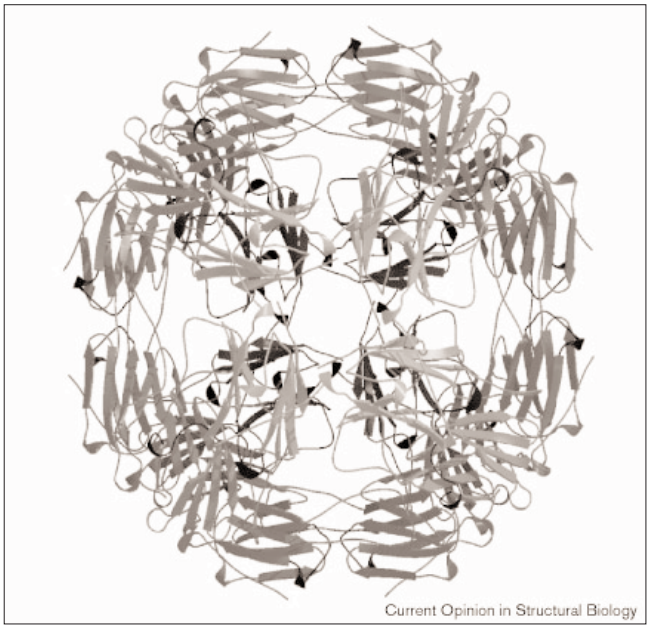
Upon assembly into the pilus rod, each pilin subunit provides the donor strand for its neighbour from a piece of disordered chain that is homologous to the chaperone donor strand. Curiously, the donated strand from the chaperone is parallel to the adjacent strand, unlike the complete immunoglobulin topology. For the assembled pilus, an assembly with antiparallel strand exchange gives a model that fits very well with the pilus dimensions seen by EM [47]. This chaperone mechanism, with its specific recognition, resembles the specific assembly of the sHsp subunits and is in marked contrast to the nonspecific promiscuous nature of chaperone–substrate recognition typical of Hsps such as Hsp70, GroEL and sHsps.

Unfoldases to the rescue

The Hsp100/Clp chaperones are members of a wider family, the AAA ATPases, with a broad spectrum of interesting functional properties [48,49,50•]. They have one or two nucleotide-binding domains containing the Walker A and B nucleotide-binding sequences and are typically hexameric rings. Unique among chaperones, they can rescue previously aggregated proteins, in cooperation with the cognate Hsp70 system [51•,52•], or can cause global unfolding of even a stably folded protein if it is tagged with the correct recognition sequence [53•]. Other members of this family have unwinding or protein dissociation roles, such as the DNA helicases, the clamp-loader subunit of a DNA polymerase complex and proteins involved in vesicle fusion, such as *N*-ethylmaleimide sensitive factor (NSF) [49,50•].

Hsp100 chaperones are often found in association with a protease ring, as in the ClpA (chaperone)–ClpP (protease) complex, analogous to the proteasome. The complex is a stack of four rings, with the double-ring protease in the middle, flanked by an ATPase ring at either end. It is thought that the substrate is first unfolded by a chaperone ring and then passed to the protease. A cryo-EM structure of the ClpA–ClpP complex shows symmetry mismatch, whereby ClpA is a hexameric ring and ClpP is a heptamer, suggesting a rotary mechanism for substrate translocation [54•]. If the green fluorescent protein, which has a very stable fold, is C-terminally tagged with the 11-residue recognition sequence, it is fully unfolded by ClpA and ATP [53•]. If ClpP is present, it is fully degraded.

Figure 4



The 24-subunit shell of the *Methanococcus jannaschi* sHsp Hsp16.5, seen along the fourfold axis ([40••]; PDB code 1SHS). The subunits at the back of the shell are shown with darker shading.

Otherwise, it is released back into solution into an equilibrium with the refolded protein.

The crystal structure of one of the NSF nucleotide-binding domains, responsible for hexamerisation, shows a wedge-shaped αβα domain with a projecting α-helical region [55•,56•]. The first Hsp100 structure from this family, that of protease-associated HslU (with one AAA module), has just appeared [57••] (Figure 6). In this case, both the chaperone (HslU is presumed to be a chaperone by analogy with other family members) and the protease (HslV) rings in the complex have sixfold symmetry. The HslU α-helical domain is larger than in the NSF domain and is connected to the wedge-shaped N-terminal domain by a flexible hinge adjacent to the nucleotide-binding site. The hinge is more closed in the presence of bound nucleotide. An inserted α-helical region (the I domain) forms a long extension from the nucleotide-binding

Figure 5

A comparison of strand interactions in immunoglobulin fold chaperones. **(a)** The Hsp16.5 dimer [40••], showing the edge strand exchange between subunit dimers. **(b)** The *E. coli* PapD–PapK complex ([45••]; PDB code 1PDK), showing the edge strand donation by the chaperone PapD (lighter shading) to the pilin subunit PapK (darker shading).

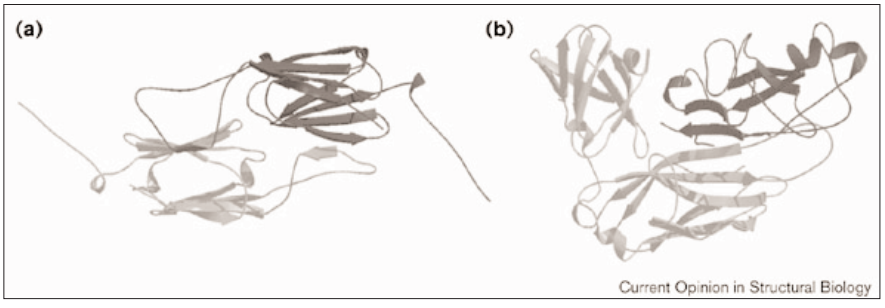
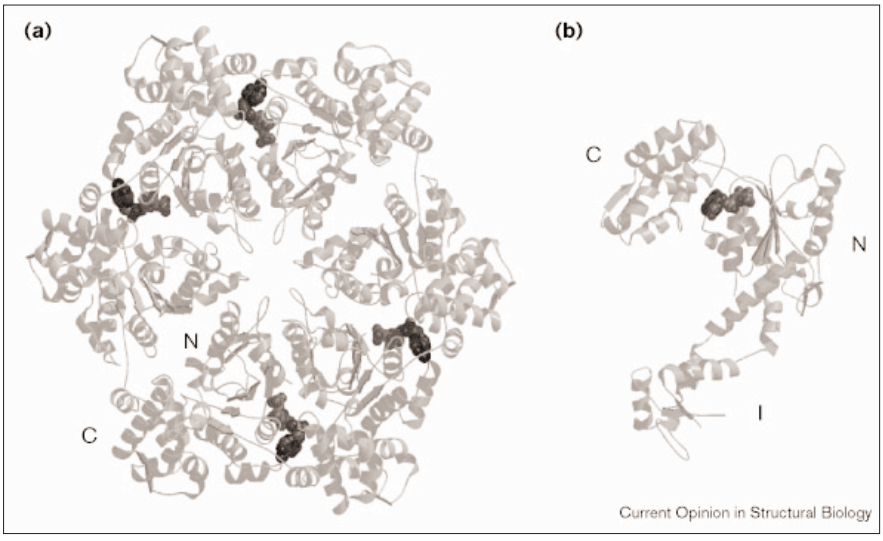


Figure 6



The structure of an AAA ATPase. The hexameric Hsp100 HslU ([57••]; PDB code 1DOO) has three domains. The N and C domains, connected by a flexible hinge region, are related to domains in previously determined AAA modules, such as the NSF oligomerisation domain. **(a)** End view of the N and C domains in the hexamer. **(b)** Side view of a complete subunit, showing the I domain extending downwards. The ATP is black and in space-filling format. In this crystal form, four subunits have bound nucleotide. The N domain contains the nucleotide-binding site and forms the main body of the hexameric ring. In the N domain, a long insertion forms the mainly helical I domain. Residues 175–209 in the I domain are disordered and are not shown. The I domain contacts the HslV protease ring, which would be below the HlsU in side view (b).

domain and this extension forms slender contacts to HslV. Comparison with a negative-stain EM study suggests that there may also be other modes of binding between HslU and HslV [58]. In the free HslU crystal forms, not all the nucleotide sites are occupied and the observation of alternate subunits with bound ligand suggests possible negative cooperativity of nucleotide binding. In the HslV–HslU complex, all the subunits have bound ligand.

Yeast Hsp104 is involved in thermotolerance and is required for the generation of the yeast prion [PSI+] [59], presumably by producing partially unfolded Sup35, which can then assemble into prion fibrils. Prion formation is inhibited by high levels of Hsp104, perhaps because the equilibrium is shifted towards unfolding at the expense of fibril formation. Rescue operations in heat-stressed yeast are performed by the combination of Hsp104 with Hsp70 and Hsp40 [51•]. In *E. coli*, a similar activity is found for ClpB acting in association with the Hsp70 system (DnaK, DnaJ and GrpE) [52•]. A recent analysis of the proteins that are dependent on this system in heat-stressed *E. coli* cells reveals a preponderance of larger proteins that would not fit inside the GroEL–GroES chamber [60].

Conclusions

In summary, there have been new developments in understanding the GroE chaperonin system, still one of the best understood of the general molecular chaperones. The multiple binding and unfolding of protein substrate, along with the large hinge rotations, has led to the intriguing proposal of mechanical unfolding as part of the mechanism. The strand donation and exchange mechanism of the specific chaperones of the bacterial pilus provide a strong contrast to the nonspecific chaperone mechanisms. Finally, novel functions of chaperones belonging to the emerging superfamily of AAA ATPases include substantial unfolding and solubilisation of aggregated proteins, rather than folding. The idea of mechanical unfolding is also plausible for the Hsp100 chaperones, as they are also ring ATPases in which the nucleotide is expected to cause conformational changes [50•]. However, they exhibit a reverse action to GroEL in the sense that it is native proteins that are recognised (by the tag sequence) and these are unfolded (pulled apart?) by the chaperone ATPase cycle.

Acknowledgements

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- •• of outstanding interest

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