Structural and mechanistic consequences of polypeptide binding by GroEL

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The remarkable ability of the chaperonin GroEL to recognise a diverse range of non-native states of proteins constitutes one of the most fascinating molecular recognition events in protein chemistry. Recent structural studies have revealed a possible model for substrate binding by GroEL and a high-resolution image of the GroEL–GroES folding machinery has provided important new insights into our understanding of the mechanism of action of this chaperonin. Studies with a variety of model substrates reveal that the binding of substrate proteins to GroEL is not just a passive event, but can result in significant changes in the structure and stability of the bound polypeptide. The potential impact of this on the mechanism of chaperonin-assisted folding is not fully understood, but provides exciting scope for further experiment.

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Introduction

Chaperonins are a subclass of molecular chaperones capable of mediating ATP-dependent folding of polypeptides to their native states [1]. GroEL is currently the best characterised chaperonin; it is found in the cytoplasm of *Escherichia coli* and is essential for cell viability and growth at all temperatures [2]. The complete functional cycle of GroEL is dependent on the presence of ATP and the co-chaperonin GroES [3–6]. Studies *in vitro* have established a basic model reaction scheme to describe how GroEL is able to facilitate the folding of its polypeptide substrates under conditions where the spontaneous folding reaction is non-productive or inefficient (for reviews see [1,7]; Figure 1). There is still debate on whether GroEL is an active participant in assisted folding [8] or merely acts as a cage in which folding is allowed to proceed in a protected environment [9,10]. Central to the mechanism of GroEL activity is its ability to recognise a wide spectrum of non-native polypeptides. Just how this is achieved is currently unresolved, but recent structural studies [11] have provided fascinating new clues about this aspect of the function of GroEL. Here, we review our current understanding of the nature of the molecular recognition event between GroEL and its substrate polypeptides. The consequences of this interaction are then discussed in the light of new structural information on the nature of the active GroEL–GroES folding machine [12] and from the perspective of current models for chaperonin-assisted folding.

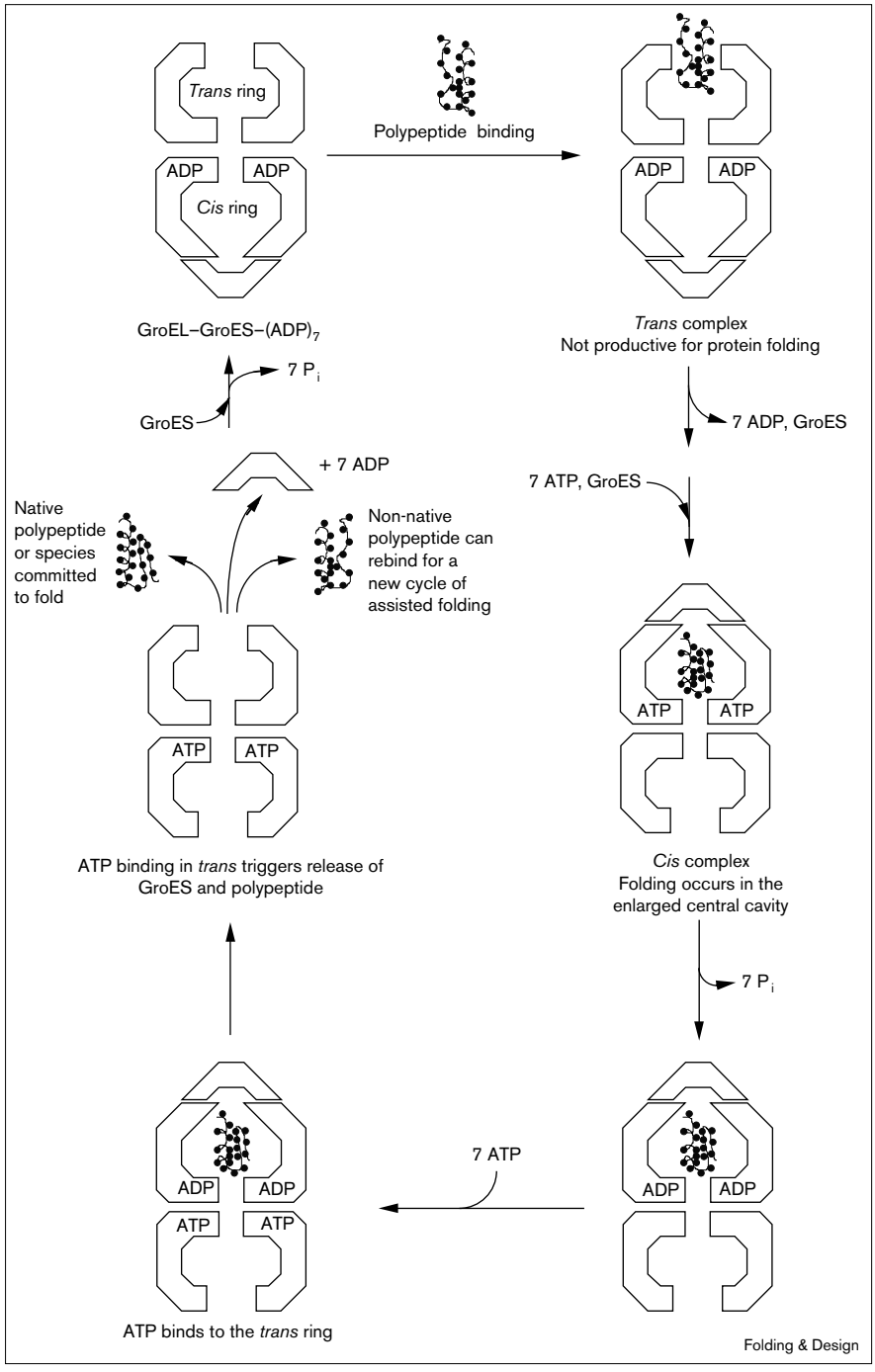
Chaperonin structure

Domain organisation

GroEL is composed of 14 identical subunits of ~57 kDa arranged in two heptameric rings stacked back-to-back ([13–15]; Figure 2). Each ring defines a central cavity ~45 Å in diameter in which substrate polypeptide is believed to bind [16–19]. Individual subunits have three domains (Figure 3), each of which has distinct functional roles. The equatorial domain is comprised of the N-terminal and C-terminal regions of the polypeptide chain and contains the ATP-binding site as well as all the residues involved in inter-ring contacts. The apical domain is made up of a central region of the polypeptide chain comprising residues 191–376 and contains the GroES and substrate polypeptide-binding sites [11,12,20]. The intermediate domain links the equatorial and apical domains, and acts as a hinge region, allowing unprecedented large-scale movements of the apical domain in response to polypeptide, GroES and nucleotide binding [12,13,18,21,22]. In contrast with GroEL, the co-chaperonin GroES is a much simpler structure, formed from seven ~10 kDa subunits, which are arranged as a dome-shaped ring following the sevenfold symmetry of the GroEL structure [23,24].

Despite the wealth of information about the structures of GroEL and GroES, and the very detailed knowledge about their interactions coupled with ATP binding and hydrolysis [3–6,12,21,25–29], many important questions remain unresolved, including the molecular nature of polypeptide recognition by GroEL and the consequences of this interaction for the mechanism of assisted protein folding. Binding of intact substrate proteins to GroEL, thus far, has only been observed directly at low resolution by small-angle neutron scattering and electron microscopy (EM) methods [17–19,30]. These techniques place the polypeptide-binding site within or at the opening of the central cavity, in the vicinity of the apical domains. Intriguingly, in the crystal structure of intact GroEL, the apical domain is the least well-defined region, appearing to contain a high degree of flexibility, with B factors as high as 150 Å^2 [14,15]. The flexibility of the apical domains might be even greater in solution as they are constrained in the GroEL

Figure 1



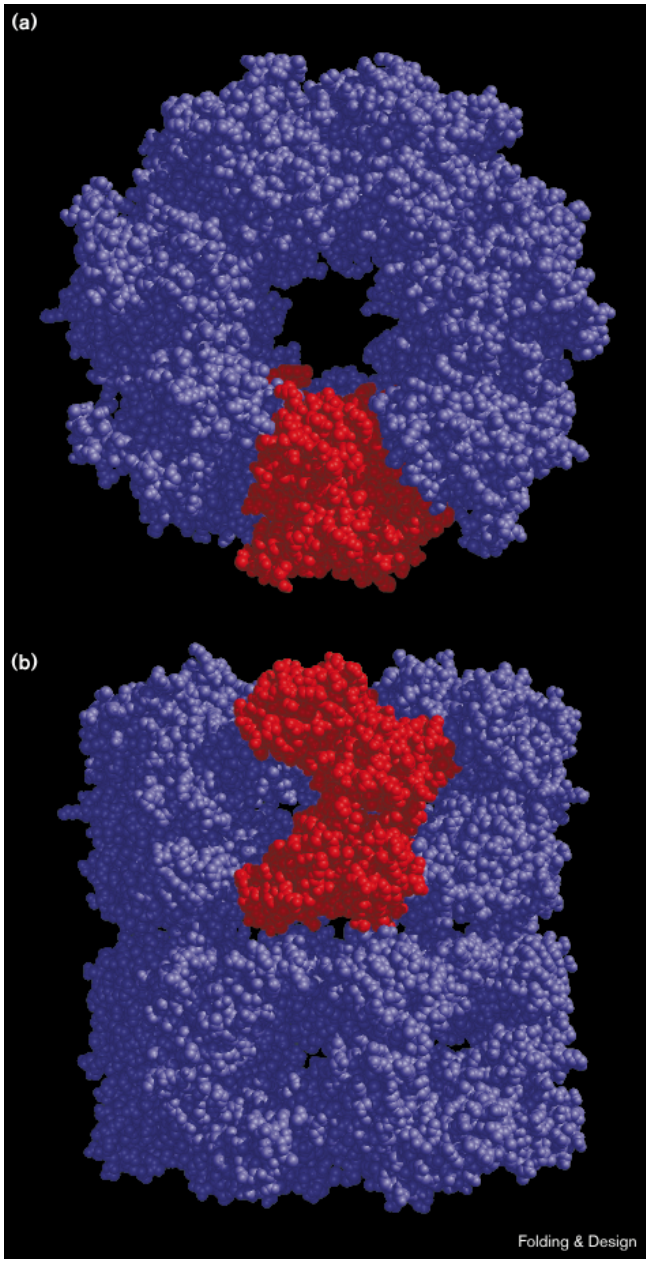
A model for chaperonin-assisted protein folding. Polypeptide is initially bound to the *trans* ring in a GroEL–GroES–$(ADP)_7$ complex. ATP binding in the *cis* ring then triggers release of nucleotides and GroES from the *trans* ring. Rebinding of GroES and ATP to the *cis* ring of GroEL encapsulates the substrate protein in a hydrophilic enlarged central cavity, in which folding can occur. Hydrolysis of ATP in the *cis* ring weakens the affinity of GroES for GroEL in this heptamer. Binding of ATP to the *trans* ring then triggers GroES to be released from the complex, and substrate polypeptide can diffuse out of the central cavity. At this stage the released substrate protein may already be native or committed to folding to the native state, or could still be non-native. In the latter case, rebinding of the substrate protein to the same GroEL molecule, or to a different GroEL, provides the substrate protein with a second chance to fold to its native state. This diagram was drawn using data and schemes from [1,5,7].

crystal lattice by packing interactions. The core of the apical domain is formed by two β sheets oriented at right angles to one another. This core is flanked by five α helices, helices H8 and H9 face the central channel, whilst the other three helices, H10, H11, and H12, are located on the opposite side of the domain (see Figure 3). Helices H11 and H12 are found towards the C-terminal region of the apical domain and appear to form a distinct sub-domain [31].

Nature of the polypeptide-binding surface

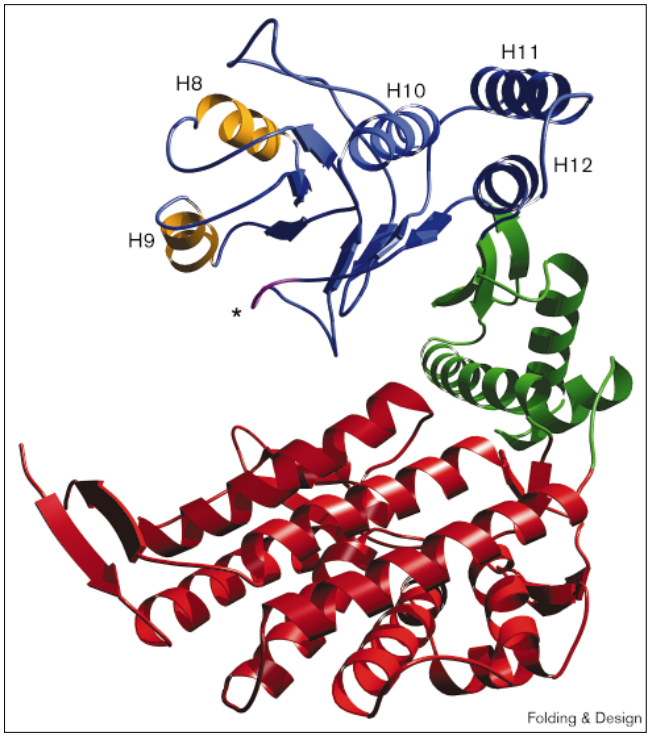
A closer inspection of the surface of the apical domains lining the central cavity in the intact GroEL crystal structure reveals several interesting features. These include a predominance of hydrophobic residues, which have long been implicated in substrate binding (Figure 4a; reviewed in [1,7]), and the absence of a well-defined groove or deep channel, common in many other interacting protein–protein

Figure 2



A space-filling view of the structure of the GroEL tetradecamer. **(a)** View from the top showing the entrance to the central cavity. **(b)** View from the side, highlighting the two heptameric rings that stack back-to-back in the GroEL 14-mer. One subunit is highlighted in red. The figure was produced using the program MIDAS [101] from the GroEL crystal structure (PDB ID: 1OEL).

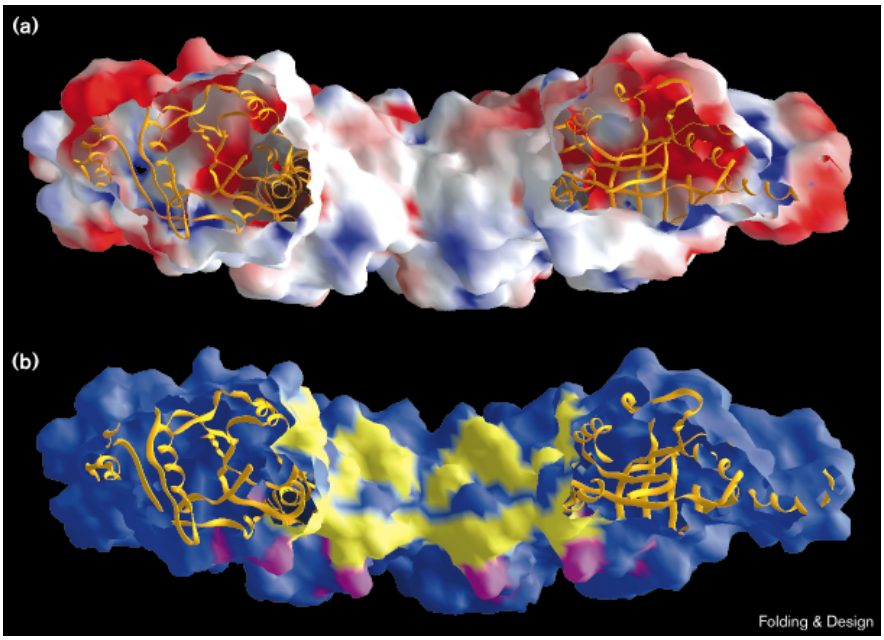
Figure 3



The structure of a GroEL subunit. The structure of a GroEL monomer coloured to highlight its three domains: the equatorial domain is coloured red, the intermediate domain is coloured green and the apical domain is coloured blue. Helices H8 and H9 in the apical domain are highlighted in orange, and the loop which contains Y199, Y203 and F204 in the apical domain is coloured magenta and marked with an asterisk. The figure was drawn using MOLSCRIPT [102].

systems, including other molecular chaperones [32–35]. The absence of a groove or channel makes the question of the nature of polypeptide binding by the chaperonin even more fascinating. Eight residues in the apical domain have been implicated in polypeptide binding by mutagenesis (Y199, Y203, F204, L234, L237, L259, V263 and V264; [20]). Interestingly, three of these residues (Y199, Y203 and F204) cluster towards the lower inner surface of the apical domain, whereas L234 and L237 (the only other two sidechains with significant surface exposure within these eight residues) form a distinct hydrophobic surface towards the upper inner surface of the domain, at the opening of the central cavity (Figure 4b). This suggests that the GroEL polypeptide-binding site is extensive (and presumably could also involve multiple apical domains) and/or that substrate binding results in large movements of this surface in an induced fit mechanism of substrate binding. A recent crystal structure of a GroEL fragment containing only residues from the apical domain, termed GroEL(191–376), has led to a more rigorous characterisation of the GroEL binding site [11]. In this work, a peptide-binding site was identified on the inner surface of the apical domain, through serendipitous binding of part of a histidine tag between adjacent molecules in the crystal. This binding site involves the eight residues implicated in polypeptide binding by mutational analysis [20] with an additional ten residues; seven of the latter have not yet been targeted by mutation (this site is described in more detail below).

Figure 4



A view of the accessible surface formed by adjacent apical domains. **(a)** The accessible surface of a section of a heptameric ring of GroEL apical domains, coloured according to electrostatic surface potential. The view shown is from the central cavity of the ring. **(b)** The same view, highlighting, in yellow and magenta, residues implicated in polypeptide binding by Fenton *et al.* [20] and Buckle *et al.* [11]. The residues coloured magenta (Y199, Y203 and F204) were implicated in polypeptide binding by mutagenesis [20], but are not directly involved in binding the histidine tag in GroEL(191–376) [11]. The residues which contribute most significantly to the upper yellow surface are I230, L234, L237, E238 and A241. Those contributing to the lower surface are Y203 (magenta) and E257, A260, T261, N265, R268, I270 and V271 (yellow). The figure was made using the program GRASP [103].

The crystal structure of a GroEL–GroES–$(ADP)_7$ complex [12], and cryo-EM images of GroEL in the presence of nucleotides and GroES [21,22], have revealed a fascinating mechanism by which substrate proteins are folded and released from the apical domain surface during chaperonin-assisted folding. This involves large-scale movements of the apical domains including a clockwise twisting of the domains relative to their position in the structure of GroEL alone (looking down the sevenfold symmetry axis), such that the apical domains undergo a rigid body movement of almost 90° looking along the sevenfold axis, and ~60° upwards relative to the equator. These movements result in the replacement of the original hydrophobic surface of the apical domain by a new, predominantly hydrophilic, surface and the formation of a much enlarged central cavity (the volume is increased by about twofold). This provides the structural basis for release of bound substrate polypeptide into the enclosed *cis* cavity, allowing folding to proceed in a protected environment ([4,6,29]; Figure 1). Binding of ATP to the *trans* ring then sends an allosteric signal [36–38] to the *cis* ring for the GroES cap to be released, allowing the substrate polypeptide to diffuse away from the chaperonin [5]. At this stage, the released polypeptide may be native, near native and committed to fold [4,6], or still non-native [39–41]. In the latter situation, the partially folded polypeptide can rebind to the same or a different GroEL molecule for a second round of assisted folding.

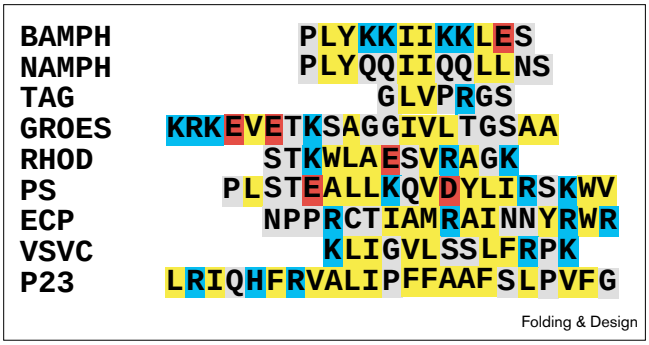
Peptide models for polypeptide binding by GroEL

Role of charge and hydrophobicity

Peptide fragments of proteins known to bind GroEL have been used to try to identify structural and sequence motifs necessary for the interaction of the substrate protein with the chaperonin. An alignment of eight peptide sequences, each of which has been demonstrated to bind to GroEL, is shown in Figure 5. Despite the fact that there is no clear sequence consensus, a clear preference for hydrophobic residues is seen, consistent with the results from studies on the binding of intact proteins to GroEL [42–45] and with the ability of the chaperonin to recognise non-native proteins. In addition, there is a distinct preference for basic amino acids, suggesting that positive charge might also be important for the interaction.

The importance of charge and hydrophobicity in polypeptide binding by GroEL was investigated recently using a series of designed synthetic peptides [46]. A systematic variation of charge and polarity in the peptides allowed a measure of how these properties influence the affinity of the peptide for GroEL. A basic amphipathic peptide bound GroEL with the highest affinity, the dissociation constant, K_d, measured was ~0.5 μM. In contrast, an acidic amphipathic variant of the peptide did not bind to the chaperonin under the same conditions, highlighting the apparent importance of both positive charge and hydrophobicity for strong binding to GroEL. These properties have also been recognised from an extensive mutational analysis of a defined region in the sequence of the small subunit of ribulose-1,5-bisphosphate carboxylase [47]. In this study, the authors concluded that high-affinity binding to GroEL was not determined by a single physical property, but was mediated through a combination of overall hydrophobicity, positive charge, and helical propensity.

Figure 5



A sequence alignment of peptides suggested to bind GroEL. The peptides are: BAMPH (a basic amphipathic peptide designed to be helical [46]); NAMPH (a designed neutral helical peptide [46]); TAG (the thrombin cleavage site in the histidine tag of GroEL(191–376) that binds to apical domains in an extended structure [11]); GROES (the GroES mobile loop that binds to GroEL in a β-hairpin structure [12,50]); RHOD (the N-terminal helix from rhodanese; transferred NOE experiments suggest that this peptide binds to GroEL in a helical conformation [48]); PS (residues 72–86 of the precursor protein of the small subunit of ribulose-1,5-carboxylase corresponding to the first helix and a β-turn in the mature protein and thought to mediate high-affinity binding of the protein to GroEL [47]); ECP (residues 19–35 from eosinophil cationic protein, which form a GroEL-binding site [104]); VSVC (a sequence from the vesicular stomatitis virus glycoprotein, VSVG; transferred NOE experiments suggest that this peptide binds to GroEL in a helical conformation [49]); and P23 (the N-terminal 23 amino acid signal sequence of pre-β-lactamase [42]). The alignment was performed using the programs CLUSTALW [105] and VISTAS [106]. Hydrophobic, basic, acidic and polar/other residues are coloured yellow, blue, red and grey, respectively.

The role of secondary structure

Several of the polypeptide sequences listed in Figure 5 correspond to helices in the structure of the intact protein from which they were excised, or are suggested to adopt a helical conformation when bound to GroEL. This raises the possibility that helical propensity might be a general feature of polypeptide binding or recognition by GroEL. The conformation of the peptide bound to the surface of GroEL has been analysed for four of the sequences shown in Figure 5. Two sequences, an N-terminal sequence from rhodanese (RHOD) and a peptide from the vesicular stomatitis virus glycoprotein (VSVC), have been shown by transferred NOE methods to adopt a helical conformation in the bound state [48,49]. In contrast, when bound to an adjacent molecule in the crystal lattice, the thrombin cleavage region of the histidine tag in the structure of GroEL(191–376) has an extended structure [11], whereas the mobile loop from GroES was predicted by transferred NOEs to form a β hairpin in the bound state [50]; the β hairpin has been confirmed in the recent crystal structure of the GroEL–GroES–$(ADP)_7$ complex [12]. Thus, it appears that the apical domain of GroEL can bind both helical and extended structures. Particularly interesting, however, is the observation that the N-terminal rhodanese peptide and the VSVC peptide are unstructured in aqueous solution [48,49], suggesting an ability of GroEL to stabilise helical conformations. Binding of the N-terminal rhodanese peptide to GroEL has also been examined using a novel hybrid-peptide system [51], in which a desired peptide sequence can be constrained in a helical conformation by disulphide bridges [52]. Under reducing conditions the constraining disulphides are broken and the peptide adopts an extended conformation. One can therefore examine the conformational dependence of peptide binding to GroEL. Hybrid peptides were designed to present either the hydrophobic or hydrophilic face of the N-terminal rhodanese helix. Interestingly, binding to GroEL was only observed for the peptide constrained in a helical conformation and exposing a hydrophobic face, fully consistent with the transferred NOE data [48] and highlighting the importance of hydrophobic surface area in the GroEL binding site. Proteolytic studies of a stable complex between GroEL and rhodanese are also consistent with these conclusions [53]. Thus, limited proteolysis of GroEL-bound rhodanese identified two fragments of the substrate protein, ~11 kDa and ~7 kDa in size, which remained stably associated with GroEL. In native rhodanese, both fragments have a homologous fold that consists of one hydrophobic and one amphipathic helix, with the ~11 kDa fragment containing an extra hydrophobic helix.

How the studies mentioned above relate to the broad spectrum of GroEL substrates and thus the general importance of helices in GroEL recognition of protein substrates is hard to judge at this stage. The observation that folding intermediates of an all β-sheet protein also interact with GroEL [54], and the identification of the bound histidine tag mentioned above in an extended structure [11], suggests that other structural motifs are probably also involved in chaperonin binding. The ability to provide a hydrophobic surface is perhaps of more importance than the precise secondary structure, and an amphipathic helix may provide a convenient way to present such a surface in small peptides.

The role of amino acid sidechains

As well as primary sequence context and backbone configuration, the role of sidechain preferences in GroEL binding has been investigated by measuring the effect of individual amino acids on the ATPase activity of GroEL [55]. In this study, the hydrophobic amino acids isoleucine, phenylalanine, leucine, tryptophan and valine were shown to have the greatest stimulatory effect on the ATPase activity of GroEL, suggesting that even the isolated amino acids bind to GroEL, the most hydrophobic having highest affinity. Notably, the less hydrophobic amino acids, threonine, proline, histidine, glutamate, and glutamine, were also observed to stimulate the ATPase activity, but with lower apparent affinity. Another interesting result from this study is the ability of both arginine and lysine to inhibit the

GroEL ATPase activity when alone, but to augment the ATPase stimulatory activity of both tyrosine and leucine when presented in a mixture. These observations are consistent with the lack of an obvious consensus primary sequence motif in the list of peptides in Figure 5. Thus, the observed promiscuity of polypeptide binding by GroEL is manifested to some extent in the variety of individual amino acids that can be bound by the chaperonin.

Stable GroEL–polypeptide complexes

Molten globules are substrates for GroEL

Early studies of stable complexes between GroEL and non-native polypeptides revealed that the bound substrate protein has properties characteristic of a molten globule state, such as proteinase sensitivity, the ability to bind ANS, and fluorescence properties intermediate between those of the native and denatured states [56,57]. Conformational preferences for strong binding to GroEL have been examined using a series of equilibrium folding intermediates of α-lactalbumin lacking one or more of its four disulphide bonds [58]. High-affinity binding to GroEL was observed for α-lactalbumin conformers, which are compact, contain few fixed tertiary contacts, have significant native-like secondary structure, and have exposed hydrophobic surface area. Interestingly, incubation of GroEL with the native state of several proteins can also result in the formation of a stable complex. For example, casein [56], pre-β-lactamase [42], and murine dihydrofolate reductase (DHFR) [59] all bind GroEL stably when incubated with the chaperonin, but their conformation when bound to GroEL in this manner has not been established in any detail. Despite close structural homology between murine DHFR and *E. coli* DHFR, only the murine DHFR forms a stable complex with GroEL when either the native or denatured state is incubated with the chaperonin [60]. Insertion of either of the two loop sequences from murine DHFR into the corresponding regions of the *E. coli* isoform conferred on *E. coli* DHFR the ability to bind GroEL [60]. It is not resolved, however, if the loops interact directly with GroEL or if the insertion of the loops has resulted in non-local structural changes that facilitate binding to GroEL.

Hydrogen exchange as a conformational probe of GroEL-bound polypeptides

More detailed information on the structure of GroEL-bound polypeptides has been generated through the use of hydrogen-exchange labelling methods in combination with NMR or electrospray ionisation mass spectrometry (ESI MS). These approaches have been used to probe the conformation of human DHFR [61,62], cyclophilin [63], mature β-lactamase [64], and a mixed disulphide derivative of bovine α-lactalbumin (BLA), termed [3SS]-BLA [65], when bound to GroEL. Although NMR reveals site-specific information about regions protected from hydrogen exchange in the bound state, exchange is monitored indirectly after release and subsequent refolding of the substrate protein and several grams of GroEL are required for a complete analysis. In contrast, in the ESI MS approach only milligram quantities of protein are required and the complex is dissociated within the mass spectrometer, preserving the level of protection in the bound substrate polypeptide and allowing its direct observation. The caveats of this method, however, are that protection as a consequence of binding to the GroEL surface cannot be easily distinguished from protection due to structure in the polypeptide chain and that residue-specific information cannot be obtained directly.

Time courses of hydrogen exchange for [3SS]-BLA [65], mature β-lactamase [64], and human DHFR [61], complexed to GroEL have been monitored by ESI MS and reveal large differences in the degree of protection of the bound substrate protein. At 4°C, GroEL-bound [3SS]-BLA exchanges almost completely with solvent within ~2 h, indicating that this GroEL-bound species is only weakly protected from exchange. As a result of aggregation, the level of hydrogen-exchange protection of free [3SS]-BLA could not be measured under the same conditions, making it impossible to observe directly if GroEL affects the stability, population distribution or conformation of the bound [3SS]-BLA species. A molten globule state of a distinct BLA species with three native disulphide bonds was used for comparison, however [66]. This species shows a similar kinetic profile of hydrogen exchange to GroEL-bound [3SS]-BLA, and a similar peak width, suggesting that the conformation of the ensemble of species in the bound state resembles closely that of the well-characterised molten globule of BLA. In contrast to these results, mature β-lactamase maintains a high level of protection from exchange when bound stably to GroEL [64]. Mature β-lactamase has been shown to bind stably to GroEL at 48°C, a temperature at which the free enzyme is fully active, but at which the GroEL-bound form is inactive [43]. Hydrogen-exchange kinetics were measured for GroEL-bound β-lactamase at 48°C and for free β-lactamase at 48°C and 25°C. Two surprising observations arose from this study. First, the kinetics of hydrogen exchange for GroEL-bound β-lactamase at 48°C resemble those of native β-lactamase at 25°C. Second, the kinetics of hydrogen exchange for free and GroEL-bound β-lactamase differ markedly at 48°C, with the free enzyme exchanging significantly faster. This suggests, therefore, that GroEL stabilises the mature protein, possibly by binding a native-like structure. The stability of GroEL-bound β-lactamase was further probed by these authors by measuring the unfolding rates of β-lactamase in the presence and absence of GroEL at 48°C. No significant difference in the unfolding rates was observed, suggesting that GroEL-bound β-lactamase retains significant structure and that the height of the transition state for unfolding from both free and GroEL-bound β-lactamase is similar.

A third ESI MS study recently examined the hydrogen-exchange protection of GroEL-bound human DHFR [61]. This study also revealed the presence of a significant degree of stable structure in the bound polypeptide, presumably in a core involving an average of 20 sites. Assuming that the protected sites are amides, their protection factors are of the order of 10^3. Interestingly, an early folding intermediate of *E. coli* DHFR contains a similar number of protected amide hydrogens to the GroEL-bound state of the human protein, with protection factors $> 10^2$ [67]. GroEL-bound human DHFR has also been investigated by hydrogen-exchange labelling monitored by NMR [62]. Using ^{15}N-labelled DHFR, a time course of amide-hydrogen occupancy was measured at specific sites allowing a relatively detailed picture of the bound substrate polypeptide. Protection factors for 42 amide hydrogens were estimated to be in the range 2–54, with the majority of well-protected amides located in the region corresponding to the central β sheet of native DHFR. Although differing greatly in magnitude, the pattern of protection found in GroEL-bound DHFR is very similar to that of native DHFR [62]. The authors used this observation, combined with the knowledge that the central β sheet of native DHFR is formed from distant elements in the primary sequence, to suggest that GroEL-bound DHFR might contain an overall native-like topology.

A very different scenario was observed with cyclophilin bound to GroEL. Cyclophilin associates with GroEL at 30°C and dissociates at lower temperatures. Zahn *et al.* [63] used the temperature dependence of complex formation to dissociate GroEL-bound cyclophilin for its subsequent analysis by NMR after a hydrogen-exchange labelling step. The authors found that under the conditions of their experiment all amides were exchanged with solvent in the presence of GroEL, but not in its absence, suggesting that binding to the chaperonin leads to a destabilisation of the entire cyclophilin secondary structure. This study was not sensitive enough to detect hydrogens with protection factors lower than ~10^3, however. GroEL-bound cyclophilin, therefore, may have retained a significant degree of structure, despite being dramatically destabilised.

Studies of the ribonuclease barnase have provided further evidence that polypeptide binding to GroEL can result in structural destabilisation. Amide-hydrogen exchange measurements of native barnase in the presence and absence of catalytic amounts of GroEL, reveal that the chaperonin catalyses the hydrogen/deuterium exchange of amide hydrogens that are deeply buried in the native barnase structure [68]. Because the amide hydrogens chosen for study only exchange with solvent upon global unfolding of the protein [69,70], the authors propose that complete unfolding of barnase occurs on the GroEL surface. In contrast with these results, ESI MS experiments indicate that significant protection persists in DHFR during iterative cycling with GroEL [61]. This suggests that the consequences of binding to the chaperonin may differ for protein substrates with different stabilities and/or topologies. Nevertheless, the results with barnase provide important evidence for a GroEL 'unfoldase' activity, which could be functionally important in that binding to GroEL would allow kinetically trapped or misfolded states renewed attempts at folding.

Mechanisms of GroEL-assisted refolding

GroEL typically retards the refolding rate of proteins that can fold efficiently in its absence. In many cases this can be explained through a competition between intramolecular refolding and intermolecular binding to GroEL [45,71]. The ability to retard and sometimes completely halt refolding has been used to identify which refolding species or intermediate states are bound most strongly by GroEL. One of the first of these studies investigated the effect of GroEL on the refolding rate of differently denatured states of lactate dehydrogenase and concluded that GroEL bound most strongly to the denatured and/or earliest intermediate state [72]. A molten-globule-like intermediate and the enzyme monomer, which appear later in the refolding pathway, do not appear to bind. The refolding rate of barnase is retarded by GroEL (the rate constant approaches a constant non-zero value in the presence of increasing concentrations of GroEL) indicating that barnase can fold whilst associated with the chaperonin [71]. In addition, because refolding rates for mutant and wild-type barnase are reduced by the same relative amount in the presence of GroEL, the interaction of refolding barnase with GroEL presumably occurs early in the refolding pathway, before the effect of the mutation on the refolding process is manifested [73]. Stopped-flow fluorescence studies of the refolding of human DHFR also supports the view that GroEL binds early refolding intermediates [62]. In this study, the authors provide evidence that an early burst phase intermediate as well as two distinct refolding intermediates, which occur later in the refolding pathway (with a time constant of 31 ms), bind to the chaperonin. GroEL also binds both early and late folding intermediates of a Fab antibody fragment [74]. The late folding intermediate (termed Dc) differs from the native state only in the arrangement of quaternary interactions between the four domains of the native Fab structure [75,76]. In addition, the unfolding rate of GroEL-bound Dc is comparable with the slow phase in unfolding of the native Fab fragment, suggesting that the structural integrity of Dc is maintained in the complex with GroEL.

Several refolding studies of proteins in the presence of GroEL have substantiated the results from peptide binding that both hydrophobicity and positive charge are important in the molecular recognition event between substrate protein and chaperonin. Refolding studies of a series of barley chymotrypsin inhibitor 2 mutants in the

presence of GroEL revealed a requirement for both hydrophobic interactions and positive charge in mediating binding to GroEL [45]. Electrostatic interactions have been shown to be important in the interaction between the molten-globule state of apo-α-lactalbumin (with four native disulphide bonds) and GroEL, where the binding constant is increased from $10^5\ M^{-1}$ to $10^6\ M^{-1}$ by an increase in salt concentration from 0.05 M to 0.25 M [77]. Both apo-α-lactalbumin and GroEL are negatively charged at neutral pH, suggesting that the increased binding constant observed at higher salt concentration is a result of electrostatic screening. The rate of binding of barnase to GroEL is also dependent on ionic strength [78]. In this case, however, high ionic strength reduces the rate of association between polypeptide and chaperonin. This almost certainly reflects the fact that barnase is positively charged and GroEL is negatively charged under the experimental conditions used. Significantly, the rate of refolding of barnase whilst complexed to GroEL is not affected greatly by ionic strength. This suggests that the interactions responsible for the retardation in the rate of barnase refolding by GroEL are non-ionic in nature, and that ionic interactions might only be important as part of the initial association event between substrate and chaperonin. Hydrophobicity and positive charge are also important features of substrate recognition by other molecular chaperones, such as DnaK [79], SecB [80], and Hsp90 [81]. Thus, although each chaperone has a different structure and mechanism for binding substrate proteins, they are related by a common requirement for positive charge and hydrophobicity for high-affinity substrate binding.

Consequences of polypeptide binding by GroEL

Examination of the substrate polypeptides for which structural information about free and GroEL-bound states is available, indicates that the result of polypeptide binding by GroEL can be both destabilisation (as in the cases of cyclophilin [63] and barnase [68]) and stabilisation (as seen for mature β-lactamase [64]). These apparently opposing effects may relate to the fact that *in vivo* the chaperonin machinery is recruited into various cellular processes as well as protein folding, such as the heat-shock response [2,82–87] and protein degradation [88]. It is intriguing to speculate that an *in vivo* requirement for structure stabilisation may be important in heat-shock response [85], although sequestering partially unfolded aggregation-prone states is also presumably important. A role *in vivo* for structure destabilisation may be to facilitate protein degradation [88], and to allow kinetically trapped or misfolded conformations renewed attempts at productive folding.

Unfoldase activity of GroEL

The idea of a chaperonin unfoldase activity [89] is particularly exciting in relation to the mechanism of GroEL-assisted folding. By capturing non-native states, GroEL can increase the proportion of unfolded states through a mass-action effect. This is distinct from an unfoldase activity that implies catalysis. Both of these activities have been observed for GroEL [68,90–92]. Catalysis of amide-hydrogen exchange by GroEL has led to the proposal that binding of polypeptide to the chaperonin can be considered as an annealing activity, in which partial unfolding/melting can be accomplished using the binding energy of complex formation [8,68,90]. The hydrogen-exchange studies involving barnase and cyclophilin were carried out with native substrate polypeptide in the presence of GroEL or GroEL and nucleotide [63,68,90]. Because barnase folds both rapidly and efficiently in the absence of GroEL [93], and cyclophilin only transiently associates with GroEL during refolding [63] (although its refolding is nevertheless facilitated by the chaperonin), the general relevance of GroEL-induced structure destabilisation to the chaperonin-assisted folding of substrate polypeptides, which require the complete GroEL–GroES–ATP system, has still to be determined.

Evidence for a GroEL-unfoldase activity relating to the complete chaperonin system has been provided by studies involving crosslinking of unfolded murine DHFR to GroEL [4]. Crosslinking was mediated using a heterobifunctional crosslinker attached to a unique cysteine residue engineered into the C-terminal region of the DHFR. Formation of native DHFR was assayed by binding of the folate antagonist methotrexate, which only interacts with the native state and a native-like folding intermediate of DHFR [94]. When GroES and ADP were incubated with the crosslinked DHFR–GroEL complex, binding of methotrexate was observed, indicating that the bound, crosslinked DHFR had attained a native-like conformation. Re-exposing the crosslinked DHFR to the polypeptide-binding regions of GroEL by subsequently releasing GroES resulted in a significant decrease in the level of bound methotrexate, indicating that re-binding had caused at least partial unfolding of the crosslinked DHFR. Although the precise nature and extent of structural perturbation caused by DHFR associating with the polypeptide-binding site of GroEL cannot be gauged, this study does suggest a genuine unfoldase activity in the context of the complete chaperonin machinery.

Inside the cis *cavity*

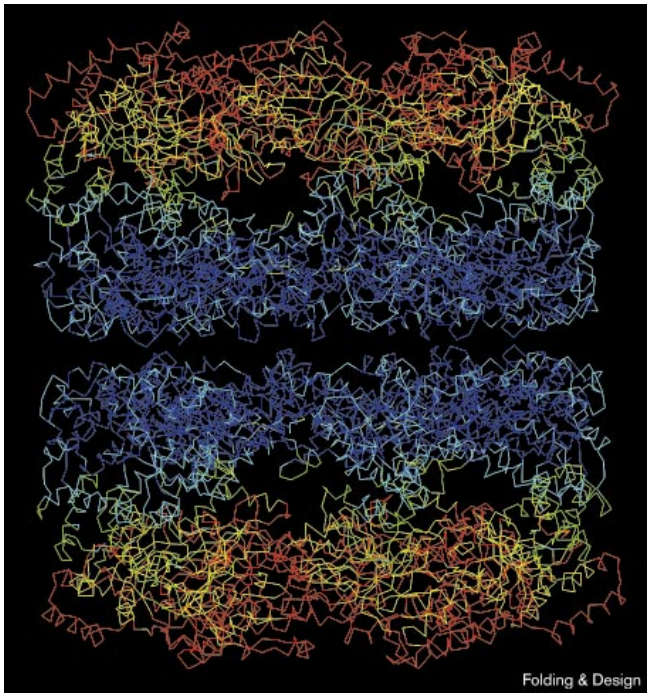
Elegant *in vitro* studies of the mechanism of GroEL-assisted folding have shown that substrate polypeptides can fold, or reach a state committed to refolding correctly, as part of a *cis* GroEL–GroES–polypeptide ternary complex [4,6,29]. Fluorescence anisotropy measurements have been used to investigate the changes in substrate polypeptide flexibility that occur within a GroEL–GroES *cis* complex [29]. Addition of GroES and ATP to a binary complex of GroEL–pyrene-labelled rhodanese results in a significant decrease in fluorescence anisotropy, indicative of an increase in substrate polypeptide flexibility. A single ring mutant

of GroEL, named SR1, gave similar results, consistent with the changes occurring within a *cis* ternary complex. The kinetics of the changes in fluorescence anisotropy are biphasic, the major phase has a half life of ~1 s, and the minor phase has a half life of ~5 s, both of which are considerably faster than the rate of release of GroES and polypeptide (half life of 15–60 s; [25,26,28,95]). Green fluorescent protein (GFP) was also used in the same study to observe how the dynamics of the substrate polypeptide are influenced within a *cis* ternary complex. Significantly, the rate of decay of fluorescence anisotropy of GFP within an SR1 *cis* ternary complex was slowed relative to free GFP, indicating a decrease in the tumbling rate of GFP trapped within the SR1–GroES cage. Taken together, these two studies suggest that substrate polypeptide is released from the walls of the apical domains in the *cis* complex, but continues to interact with them until binding of ATP to the *trans* ring triggers release of GroES and substrate polypeptide (Figure 1).

Conformational flexibility mediates substrate binding

The wide body of experimental data described in the preceding sections has demonstrated that GroEL can bind both early and late folding intermediates [62,71,72,74,92], unfolded [72,90], molten-globule-like [56–58,65], and native-like conformations [62,74], as well as helical and extended structures [11,48,49]. In general, the most common distinguishing feature of non-native states is the presence of exposed hydrophobic surface area. Combined with the fact that the proposed binding surface of GroEL is relatively featureless, predominantly hydrophobic, and extremely flexible [15,96], this might suggest that the ability of GroEL to bind a diverse range of substrate polypeptides is mediated through an induced-fit mechanism (although there is currently no clear evidence for this). It would also seem plausible that in the process of substrate binding, GroEL can influence the structure of the substrate polypeptide. How this is manifested might depend on the individual substrate protein and mirrors the fine balance of forces contributing to the stability of a particular conformation. The structure of the intact GroEL tetradecamer is shown in Figure 6, coloured according to temperature factors. There is a striking gradation in B factors with increasing distance from the equatorial domains, reaching an average value of well over 100 Å^2 for the entire apical domain. Both rigid body motion of the apical domains and local fluctuations within the domain could be responsible for the observed dynamics [15]. The high degree of flexibility inferred from the GroEL crystal structure has also been suggested by steady-state fluorescence polarisation measurements of pyrene-labelled derivatives of GroEL [96]. The observed flexibility of GroEL is dramatically reduced when unfolded rhodanese is bound to the chaperonin, consistent with an induced-fit model of polypeptide binding by GroEL.

Figure 6

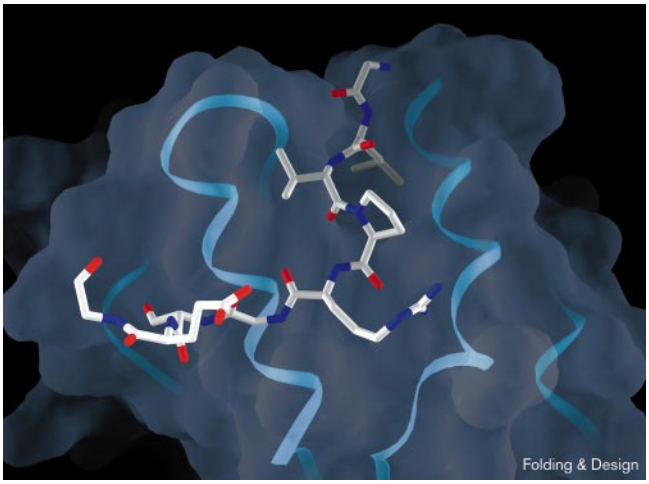


Structural temperature factors of GroEL. The GroEL tetradecamer is viewed from the side and is coloured according to the temperature factor in order to highlight the high structural mobility of the apical domains. Atoms with the lowest B values are coloured blue and those with the highest B values are coloured red.

First glimpses of an apical domain polypeptide-binding site at high resolution

The recently solved crystal structure of a GroEL fragment containing the entire apical domain sequence (residues 191–376) has provided the first detailed insights into the possible nature of substrate binding by GroEL [11]. The structure of GroEL(191–376) shows no major deviations from that of the apical domain in intact GroEL. Significantly, however, the largest differences between the two structures are found in helices H8 and H9, which face the central cavity in the intact tetradecamer (Cα rmsd values in the range 0.8–2 Å). An additional 17 residue sequence containing a histidine tag and a thrombin cleavage site was present at the N-terminus of the GroEL fragment used in the crystallographic analysis. Quite fortuitously, a part of this tag sequence from one molecule bound to a neighbouring molecule in the crystal, close to a region of the apical domain implicated by mutagenesis to be involved in polypeptide binding. Seven residues from the tag region (GLVPRGS) are visible in the electron density map. These residues adopt an extended conformation in the bound state (Figure 7). The tag sequence lies in a shallow hydrophobic surface involving helices H8 and H9. Of particular interest is the location of a leucine sidechain in the tag, which fits neatly

Figure 7



The conformation of the tag sequence GLVPRGS bound to GroEL(191–376). The peptide is shown in stick representation and helices H8 and H9, which delineate the binding surface defined by Buckle *et al.* [11], are shown as ribbons. The figure was drawn using the program GRASP [103] using coordinates kindly provided by A.R. Fersht.

into a hydrophobic pocket on the apical domain surface. The majority of contacts between the tag and the domain are nonpolar, but there are also four hydrogen bonds between the mainchain of the tag and the residues E257, N265 and T261, found in helix H9. The residues that define the binding site proposed by Buckle *et al.* [11] are shown on the surface of GroEL(191–376) in Figure 4b. Five of the eight residues in the apical domain from intact GroEL that have been shown to disrupt binding of substrate protein when subjected to mutation [20] are found in the binding site defined by Buckle *et al.* The other three residues (Y199, Y203 and F204) are inaccessible to the short tag sequence, but could be involved in binding larger substrate proteins. Ten additional residues are involved in the binding site of the tag sequence, three of which (I230, E238 and N265) do not interfere with polypeptide binding in the mutational analysis of Fenton *et al.* [20].

At present it remains to be proven conclusively if the binding site of the tag sequence is the *bona fide* GroEL substrate-binding site, but several features of it look promising. First, none of the contacts between the tag and binding site appear important in the crystal packing. Second, there is a close correlation between the residues identified by mutagenesis to be important in polypeptide binding, and those involved in binding of the tag. Finally, the binding site of GroEL(191–376) faces the central cavity in the intact GroEL structure [14,15]. But although the extended conformation observed for the bound tag sequence in the structure of GroEL (191–376) is a common mode for peptide binding in other systems [32–35,97–99], is the only known example of such a conformation in a chaperonin-bound substrate peptide (see legend to Figure 5). Although sharing some homology to other peptides known to bind GroEL (Figure 5), the tag sequence bears the strongest resemblance (57% identity) to a part of the mobile-loop sequence of GroES. The mobile loop mediates binding of GroES to GroEL and also binds in an extended conformation to the region of the apical domain involving helices H8 and H9 [12,50,100]. This raises the possibility that the tag sequence might mimic more closely GroES binding, rather than substrate binding (assuming that the two binding modes and/or sites are distinct). In support of this view, two of the residues in the tag-binding site (E238 and N265) were found, in the mutational study of Fenton *et al.* [20], to perturb only the binding of GroES to GroEL and not to affect substrate binding. More structures of GroEL–polypeptide complexes are now required to resolve this issue.

Conclusions

An extensive body of structural and functional information has led to significant advances in our understanding of the interplay between GroEL, GroES, substrate polypeptide and nucleotides in chaperonin-assisted folding. Two recent structural studies of GroEL-bound polypeptides and the GroEL–GroES–$(ADP)_7$ complex have provided us with important insights into the nature and functional consequences of this fascinating molecular recognition event. Many aspects of GroEL function are still unresolved, however, with new data and different model systems continually generating fascinating new questions about this impressive protein machinery. The first high-resolution structural model for polypeptide binding by GroEL provides us with important new clues about the location of the GroEL polypeptide-binding site and the interactions responsible for the remarkable ability of GroEL to bind a broad spectrum of substrate polypeptides and to facilitate their folding. Combined with information about domain movement and the dramatically different surface of the apical domain presented to the substrate polypeptide upon GroES binding, the nature of the 'powerstroke' of the chaperonin machinery has now been revealed. More specific issues about the nature of polypeptide binding must await further structural information, which, with the dynamic nature of the polypeptide itself and the possibility of multiple binding modes, thus far has remained elusive.

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