JOURNAL OF THE ROYAL SOCIETY Interface

Development of free-energy-based models for chaperonin containing TCP-1 mediated folding of actin

Gabriel M Altschuler and Keith R Willison

J. R. Soc. Interface 2008 **5**, 1391-1408
doi: 10.1098/rsif.2008.0185

JOURNAL OF THE ROYAL SOCIETY Interface

J. R. Soc. Interface (2008) **5**, 1391–1408
doi:10.1098/rsif.2008.0185
Published online 15 August 2008

REVIEW

Development of free-energy-based models for chaperonin containing TCP-1 mediated folding of actin

Gabriel M. Altschuler[1,2] and Keith R. Willison[1,*]

[1]*Cancer Research UK Centre for Cell and Molecular Biology, Chester Beatty Laboratories, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK*
[2]*Department of Chemistry, Imperial College, London SW7 2AZ, UK*

A free-energy-based approach is used to describe the mechanism through which chaperonin-containing TCP-1 (CCT) folds the filament-forming cytoskeletal protein actin, which is one of its primary substrates. The experimental observations on the actin folding and unfolding pathways are collated and then re-examined from this perspective, allowing us to determine the position of the CCT intervention on the actin free-energy folding landscape. The essential role for CCT in actin folding is to provide a free-energy contribution from its ATP cycle, which drives actin to fold from a stable, trapped intermediate I_3, to a less stable but now productive folding intermediate I_2. We develop two hypothetical mechanisms for actin folding founded upon concepts established for the bacterial type I chaperonin GroEL and extend them to the much more complex CCT system of eukaryotes. A new model is presented in which CCT facilitates free-energy transfer through direct coupling of the nucleotide hydrolysis cycle to the phases of actin substrate maturation.

Keywords: actin; chaperonin-containing TCP-1; protein folding; chaperones; free-energy landscapes

1. INTRODUCTION

Molecular chaperone is a collective term used to group those proteins that function to assist in the folding and assembly of other proteins. The molecular chaperones that have been most widely studied are the heat inducible ATPases: the heat shock 60 (Hsp60); heat shock 70 (Hsp70); and heat shock 90 (Hsp90) protein families. The two best studied chaperone systems in eukaryotes are Hsp70 and Hsp90, the latter being a new target for anti-cancer therapy (Prodromou & Pearl 2003). The Hsp60 chaperones, often called chaperonins, are found in all three kingdoms of life. The subject of this article is the chaperonin found in eukaryotic cytosol, the chaperonin-containing TCP-1 (CCT). CCT is a uniquely complex chaperonin intimately involved in actin and tubulin cytoskeletal protein folding (Valpuesta *et al.* 2005; Horwich *et al.* 2007).

It is a commonly held view that chaperones assist folding and conformational maturation of their protein targets through complementary binding surfaces that are non-specific and non-selective, since the function of chaperones is to recognize hydrophobic amino acid side chains, normally found buried in the interior of native protein folds or present on the surfaces of protein monomers that will eventually be buried in ternary complexes (Richter & Buchner 2006). For the CCT system, perception concerning its possible roles in non-selective versus specific protein folding reactions is critical because the homologous bacterial GroEL chaperonin system does generally assist protein folding by recognizing non-native proteins via complementary hydrophobic interactions. However, we are influenced by evolutionary considerations when comparing the functions of CCT and GroEL, because they last shared a common ancestor 3 Gyr ago. CCT evolved its modern functions at the dawn of eukaryotes, 2 Gyr ago, when the archaebacterium–eubacterium fusion event occurred. CCT duplicated from a precursor, thermosome-like chaperonin, while also coming into contact with at least two novel protein folds derived by lateral gene transfer from a eubacterial symbiote, probably the donor of the mitochondrion. The novel protein folds are actin/hexokinase (Willison 1999) and the WD-40 propeller motif (Valpuesta *et al.* 2002). We know that this ancient set of CCT gene duplications was an extensive process because it is possible to detect time resolution in the phylogenetic footprint of the eight CCT subunits, which shows that the actin-specific CCT subunits are the 'youngest' (Archibald *et al.* 2002; Fares & Wolfe 2003).

*Author for correspondence (keith.willison@icr.ac.uk).

Received 7 May 2008
Accepted 18 July 2008

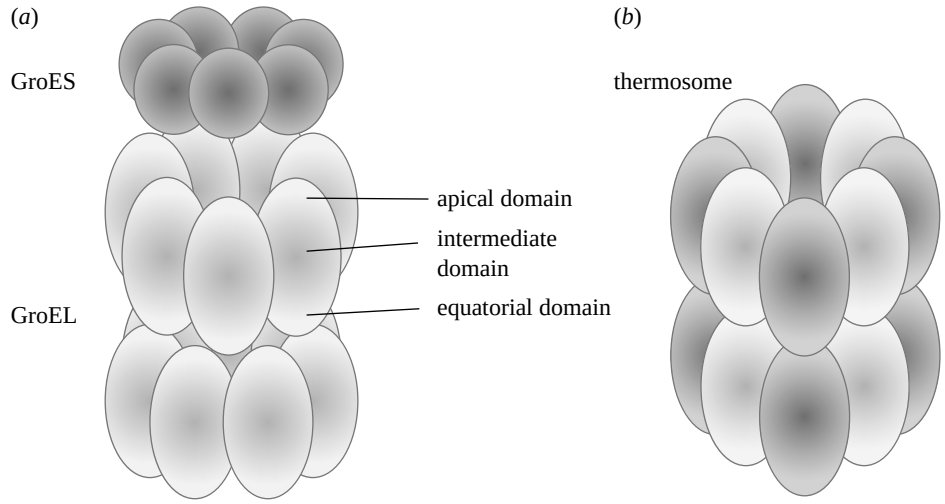

Figure 1. Chaperonin architecture. Schematic demonstrating the quaternary structure of type I and type II chaperonins based on their respective atomic structures (Braig *et al.* 1994; Ditzel *et al.* 1998). (*a*) The type I chaperonin GroEL consists of two rings of seven identical subunits. Inter-ring contacts occur via the equatorial domains, with each subunit interacting with two subunits in the opposite ring. Conformational changes relating to the identity of the nucleotide bound within the equatorial domain are transmitted via the intermediate domain to the apical domain. The apical domain interacts with the co-chaperonin GroES, consisting of seven identical subunits, which caps one end of the chaperonin during a folding cycle. (*b*) The thermosome is a type II chaperonin consisting of two rings of eight subunits that alternate between α and β isoforms (cylinder dimensions 16×15 nm). The distribution of domains within each subunit is the same as that for GroEL; however, each subunit interacts with only one subunit in the opposite ring. There is no associated co-chaperonin; however, the apical domains can adopt a conformation that effectively caps the end of the chaperonin in a similar manner to that of GroES (not shown in figure).

Genetic analysis (Kubota *et al.* 1995) and structural work on the CCT system (Llorca *et al.* 1999*a*) has revealed the exquisite selectivity of this chaperonin towards the actin and tubulin cytoskeletal proteins. This selective behaviour is difficult to reconcile with the view that CCT is orthologous in general function and mechanism to GroEL (Farr *et al.* 1997), is involved in co-translational protein folding (McCallum *et al.* 2000; Etchells *et al.* 2005) and that it folds 9–15 per cent of cytosolic proteins (Thulasiraman *et al.* 1999). The complete spectrum of CCT-interacting proteins in yeast is being catalogued and the 136 CCT-interacting proteins are being placed into categories: those that are substrates, cofactors and components of chaperone regulating networks (Dekker *et al.* 2008). These approaches should resolve the issue of the number of *bona fide* substrate proteins, those completely dependent upon CCT to reach their native states, which we think is actually rather small.

Our own studies have focused on the CCT–actin interaction because actin filaments are composed of a single monomer species unlike tubulin, which forms a complex heterodimer. We want to understand not only the mechanism of interaction between CCT and actin but also why the system has emerged. What benefits are conferred by this complex interaction? What is special and different about eukaryotic actin, folded by CCT, when we know that the prokaryotic actins can fold and assemble into filaments seemingly without the help of any molecular chaperones (Michie & Lowe 2006)? This article examines these questions from the point of view of the energetics of the mechanism and the component parts. We begin by describing chaperonin structure and then discuss recognized models for chaperonin-assisted substrate folding. This is followed by a review and an interpretation of the experimental facts established for the actin–CCT system, before we attempt to rationalize this information and develop hypothetical models for CCT folding both within and beyond the scope of the current chaperonin models.

2. CHAPERONINS

The chaperonins were identified in 1988 as a specific group of chaperones that displayed striking similarity in sequence and properties: GroEL from prokaryotes; rubisco-binding protein from plant chloroplasts; and Hsp60 from mitochondria (Hemmingsen *et al.* 1988). Their discovery, however, pre-dates this; GroEL having been found to be essential for bacteriophage λ growth in *Escherichia coli* a decade earlier (Georgopoulos & Hohn 1978). These bacterial chaperonins are characterized by an arrangement of two rings, each composed of seven protein subunits. The rings stack back-to-back, creating a central cavity, open at either end (figure 1). Each subunit has a three-domain topology. The equatorial domain includes the inter-ring contact residues and a nucleotide-binding site. The intermediate domain transmits allosteric communication between the equatorial and apical domains. The apical domain includes the binding sites for substrate recognition and also the co-chaperonin-binding sites. The binding and hydrolysis of ATP in the equatorial domains orchestrates all the concerted domain movements that propagate throughout the multimer to drive the folding cycle of the chaperonin (Horovitz & Willison 2005).

The archaebacterial thermosome and CCT chaperonins were characterized later than GroEL and clearly they constitute a separate group that shows strong sequence and structural homology (Valpuesta *et al.* 2005). The chaperonin family is now broadly separated into two types, I and II, mainly based on evolutionary

origin and structural features (figure 1). Type I chaperonins are found only in eubacteria and eukaryotic organelles of prokaryotic origin, i.e. chloroplasts and mitochondria, and each ring always consists of seven subunits. The two opposing rings are staggered so that each subunit associates with two subunits from the opposite ring. All type I chaperonins are associated with a co-chaperonin ring that binds to the top of one ring of the double-ring assembly during its functional cycle, capping that end of the central cavity and thus enclosing the protein chain to be folded. In the case of GroEL the co-chaperonin is called GroES. The type II chaperonins possess a higher degree of structural heterogeneity, being composed of one, two, three or eight distinct subunits, making up an eight-subunit ring (or nine in the case of three distinct subunits). The two rings are not staggered but are set flush so that each subunit associates with just one subunit from the opposite ring (Ditzel *et al.* 1998). In contrast to the type I chaperonins, it seems that no co-chaperonin is associated with the type II chaperonins during a folding cycle. Instead, the apical domains possess an extended apical protrusion that closes the cavity like a camera iris. The structural orientation of the apical region can change to different extents, sometimes enclosing one or even both ends of the central cavity.

3. CHAPERONIN FOLDING MECHANISMS

The established models of GroEL chaperonin action fall into two general categories, passive and active (Lin & Rye 2006). 'Passive' models describe the chaperonin as an inert form, exerting influence by preventing intermolecular interactions and confining the conformational space accessible to a substrate. 'Active' models involve specific chaperonin–substrate interactions that directly affect the conformation of bound substrate and may, during a folding cycle, be coupled to conformational rearrangements of the chaperonin. Effects on the kinetics or the thermodynamics of substrate folding may be the consequence of either of these mechanisms.

3.1. Passive models

3.1.1. Preventing aggregation. Based on the observations that many unfolded proteins are prone to self-aggregation, it is thought that the successful evolution of the chaperonins in folding proteins is, in part, due to trapping and isolating substrate monomers before aggregation can occur (Lin & Rye 2006; Horwich *et al.* 2007). This is achieved by the formation of an environment in which a single substrate protein is isolated, and therefore unaffected by potential non-productive multimolecular side reactions, the so-called Anfinsen cage (Saibil *et al.* 1993). To a first approximation, encapsulation can be thought of as equivalent to infinite dilution conditions and therefore folding to the native state occurs according to Anfinsen's hypothesis (Anfinsen 1973), via a search for the minimum free-energy configuration controlled only by the free-energy landscape of the monomeric substrate. Once folded the substrate protein is released in its native state, or if still non-native, at least still monomeric and available for further rounds of interaction with chaperonin (Burston *et al.* 1996). The initial studies on GroEL folding of rubisco (Goloubinoff *et al.* 1989), rhodanese (Mendoza *et al.* 1991) and citrate synthase (Buchner *et al.* 1991), a set of proteins with aggregation-prone unfolded states, led to the proposal that the Anfinsen cage mechanism is central to the productive folding of certain substrates by GroEL (Ellis 1994), although there must be exceptions to this general mechanism because GroEL can also help fold substrates that are too large to be encapsulated in the chaperonin cavity (Chaudhuri *et al.* 2001).

It is important to note that in this passive model the free energy of the folded state or any unfolded intermediates of the encapsulated substrate is assumed to be identical to the equivalent conformation in free solution. The folding process is determined only by the free-energy landscape of the isolated polypeptide, with all intermolecular interactions prevented. Therefore, the folding yield will increase due to prevention of off-pathway aggregation. This mechanism has recently been confirmed by the use of NMR spectroscopy for GroEL–GroES-mediated folding of human dihydrofolate reductase (DHFR) at pH6, conditions under which DHFR behaves as a stringent substrate. The folding trajectories of DHFR are the same in free solution and inside the stable cavity (Horst *et al.* 2007).

3.1.2. Confinement. The size limit for a substrate to be fully encapsulated within the chaperonin internal volume is approximately 70 kDa for GroEL/GroES (1997) and 50 kDa for the thermosome (Ditzel *et al.* 1998). For encapsulated proteins with dimensions of a similar magnitude to those of the chaperonin cavity, the ideal 'infinite dilution' approximation of the Anfinsen cage is clearly not appropriate. Non-specific steric interactions with the inner walls of the chaperone will result in a restriction of the conformational space available to the substrate. This effect, termed 'confinement' (Minton 1992), is treated as an extension of the macromolecular crowding concept (Minton 1983) and in both cases a geometric restriction of conformational freedom is imposed on the system. Confinement affects the free energy of every conformation, including transition states as well as native and unfolded states, and hence also the thermodynamics and kinetics of a folding process. Experimentally, fluorescence and circular dichroism measurements of molecules encapsulated in artificial matrices have shown that native structures are favoured in confined volumes (Eggers & Valentine 2001; Campanini *et al.* 2005; Cannone *et al.* 2005). This effect can be explained by considering the effect of confinement on the substrate free energy. Confinement reduces the conformational entropy, because the more expanded a conformation, the greater the extent to which its entropy is reduced upon confinement. In general, the native state is expected to be more compact than the unfolded state and even the molten globule state (Ptitsyn 1995). Consequently, the entropic cost associated with folding in a confined environment is less than that in bulk conditions, and folding is entropically favoured.

By contrast, the enthalpic effect due to confinement is energetically unfavourable, due to the negative effects that confinement-induced compaction has on internal bonding systems. Therefore, the change in the free energy of folding in shifting from the bulk to a confined environment may be positive or negative depending on the relative magnitudes of the entropic and enthalpic contributions. Simulations of the folding of small proteins (Klimov *et al.* 2002; Rathore *et al.* 2006) and theoretical studies (Zhou & Dill 2001) have found that the change in entropy dominates and hence, in general, confinement favours folding. In addition to favouring folding thermodynamically, confinement may also increase reaction kinetics by smoothing the folding energy landscape (Brinker *et al.* 2001; Baumketner *et al.* 2003). However, molecular dynamic (MD) simulations have demonstrated that this is only the case for certain protein motifs and is dependent on the extent of confinement (Takagi *et al.* 2003). Decreasing the available volume initially increases folding rates until an optimum volume is reached, beyond which further confinement inhibits the global conformational changes required for folding by increasing activation energy barriers and dramatically increasing the roughness of the folding landscape. Interactions between the confined substrate and solvent molecules have also been considered and they are predicted to promote transitions between conformational states by smoothing the underlying free-energy landscape (Papoian *et al.* 2004; Kovacs *et al.* 2005). In addition, the confining and ordering of solvent molecules may perturb entropy contributions to folding, thereby minimizing the significance of the hydrophobic effect. Recent MD simulation models to investigate this idea have found that the solvent confined within a hydrophobic cage has an unfolding effect. This is attributed to a 'pushing out' of confined protein towards the cage walls to minimize the free-energy penalty that would otherwise arise from disrupting solvent hydrogen bonding through surface interaction (Lucent *et al.* 2007).

The confinement concept is now recognized as a major factor in GroEL-assisted folding of substrates that are encapsulated within the chaperonin (Brinker *et al.* 2001; Weissman 2001; Baumketner *et al.* 2003; Takagi *et al.* 2003; Fan & Mark 2006; Hayer-Hartl & Minton 2006). However, recent experiments in which the volume of the GroEL cavity was reduced and resulted in slower folding of substrates are controversial and may in fact be due to effects of slowing the ATPase activity rather than confinement (Tang *et al.* 2006; Farr *et al.* 2007). Furthermore, experimental results demonstrating that conformational rearrangements of GroEL interact directly with bound substrate have demonstrated that effects other than confinement may be important (Todd *et al.* 1994; Weissman *et al.* 1994).

3.2. Active models

The above passive models of GroEL action assume that the chaperonin–substrate interactions are non-specific in the sense that the role of the chaperonin is simply to provide a geometric framework for encapsulation. However, some studies on GroEL have suggested that the chaperonin interacts more directly with certain substrates, unfolding incorrectly folded conformations before allowing refolding to the native state (Todd *et al.* 1994; Weissman *et al.* 1994). Hydrogen exchange studies on the interaction of GroEL with rubisco have observed that unfolding by GroEL occurs prior to folding and that the unfolding process is ATP dependent (Shtilerman *et al.* 1999). The iterative annealing hypothesis (Todd *et al.* 1996) suggests that a substrate may undergo a sequence of manipulations while bound to the chaperone in which incorrectly folded structures are repeatedly unfolded, each time allowing the substrate to refold until the native state is reached. Although there are technical disagreements concerning these studies (Park *et al.* 2005), the fact that GroEL makes multiple contacts with substrate, using two or more apical domains (Farr *et al.* 2000), highlights forced unfolding mechanisms that are very relevant to our thinking about CCT.

Incorrectly folded conformations represent kinetic traps; local minima on the substrate free-energy folding surface. Therefore, to achieve unfolding from these traps, GroEL must effect an unfavourable process. Initial models proposed that unfolding occurs via thermodynamic partitioning; binding of substrate to GroEL stabilizes unfolded conformations and therefore shifts the folded–unfolded equilibrium of the substrate to favour the unfolded form (Zahn & Pluckthun 1994). Alternative kinetic models, supported by observations that GroEL catalyses the unfolding rate constant of proteins when bound to the chaperonin (Itzhaki *et al.* 1995), propose that unfolding activity is due to GroEL increasing the rate rather than the yield of unfolded polypeptide chains from kinetically trapped folding intermediates. It has been suggested that the origin of this effect could be the stabilization of partially folded transition states by the hydrophobic interior surface of the GroEL ring, thus lowering the activation energy barrier for unfolding (Lin & Rye 2006).

Detection of large domain movements associated with the chaperonin folding cycles (Saibil *et al.* 1993; Llorca *et al.* 1999*a*,*b*) has motivated discussion of 'forced' folding mechanisms. These describe situations where ATP-powered chaperone domain motions are coupled to specific substrate–chaperone interactions, to drive directly an unfavourable unfolding process. In GroEL, these movements involve binding of the co-chaperone GroES followed by a large conformational change in the apical domains that rotate and twist upwards (Saibil *et al.* 1993). These domain rearrangements could result in the substrate-binding sites moving apart and away from each other, thus exerting a direct stretching force on the substrate (Shtilerman *et al.* 1999). Recently, it has been shown that the 'trans' ring of GroEL, the ring not associated with GroES, can increase folding rates through unfolding, without encapsulation (Lin *et al.* 2008). However, these experiments also showed that this unfolding effect only accounts for 10 per cent of the yield of folded protein. Encapsulation after unfolding is required to increase significantly the fraction of productive folding.

3.3. Free-energy perspective

Strictly defining whether a chaperonin is acting in a passive or active sense is a complicated issue. An alternative determinant for the nature of chaperone–substrate reaction is the manner in which the folding free-energy landscape of a substrate is affected by its interaction with the chaperone. A chaperonin acting ostensibly as an Anfinsen cage operates by introducing a large kinetic barrier that hinders access to intermolecular conformational ensembles, i.e. aggregates, while not affecting the monomeric regions of the free-energy landscape (Horst *et al.* 2007). The chaperonin-associated energy landscape is essentially a truncated version of the free solution landscape (Jahn & Radford 2005). In other cases, chaperonin interaction may alter the kinetics or thermodynamics of folding by modifying the topography of the monomer folding free-energy landscape and thus relieve kinetic or thermodynamic partitioning (Fenton & Horwich 1997). Modification of the folding landscape may result from confinement of substrate (and solvent), both considered passive modes, or through direct chaperone–substrate interactions, which are thought of as either passive or active modes depending on whether the interaction is constant or changes during the folding cycle. Forced modes are associated with the ATP cycle of the chaperonin and are represented by a series of modifications to the substrate free-energy landscape. A graphical representation of the free-energy basis of these different folding concepts is shown in figure 2 where a hypothetical free-energy landscape for a protein requiring chaperone interaction is shown. It should be noted that this representation is limited in that, for clarity, only one dimension of the folding landscape is presented. For protein folding a three-dimensional folding landscape is a more appropriate theoretical representation (Bryngelson *et al.* 1995). The one-dimensional landscape includes the fully unfolded state U, a metastable 'trapped' folding intermediate I_T, an unstable 'productive' folding intermediate I_P and the native state N. Autonomous folding on this landscape (figure 2*a*), starting from U, may produce the trapped intermediate I_T, at temperatures at which the activation energy barrier between I_T and I_P prevents further folding. In the passive, kinetic chaperone model (figure 2*b*), interaction with the chaperonin affects the topography of the substrate free-energy landscape, reducing the magnitude of this activation energy barrier and increasing the rate of formation of the productive folding intermediates, thus leading to the formation of the native state. In this situation, the new landscape is described as 'smoothed' (Brinker *et al.* 2001; Kovacs *et al.* 2005). In the thermodynamic model (figure 2*c*), the relative free energy of intermediates is altered by interaction with the chaperonin, so that previously unstable intermediates, I_P, become thermodynamically favoured and consequently populated. Release of substrate from the chaperonin surface restores the original free solution energy landscape (figure 2*a*) and allows rapid and spontaneous completion of folding from the populated, productive folding intermediate to the native state. In the forced folding mechanism (figure 2*d*), an external energy input is introduced to 'push' an unfavourable reaction over a free-energy barrier, either thermodynamic or kinetic. The chaperonin achieves this by first forming a stable complex with the trapped intermediate, I_T, yielding an initial chaperonin–substrate complex landscape. Next, a conformational change in the chaperone results in a modified energy landscape where the previously trapped intermediate is destabilized and the productive folding intermediate is favoured.

For GroEL, the extent to which these various mechanisms contribute to chaperonin folding is disputed. The short lifetime of GroES–GroEL association (approx. 20 s; Burston *et al.* 1995) relative to encapsulation-only folding lifetimes (approx. 30 min) precludes the possibility that encapsulation alone is responsible for folding while folding via forced unfolding alone has been shown to produce low yields (Lin *et al.* 2008). Consequently, it is thought that a combination of the mechanisms is deployed, whereby forced unfolding precedes release into the GroEL–GroES cavity, thus allowing rapid folding to occur from unfolded intermediates taking folding pathways that are kinetically favoured under encapsulation. The extent to which each of these kinetic and thermodynamic factors contributes must also be dependent on the particular substrate protein under study.

4. CCT AND ACTIN

4.1. The folding and unfolding pathways of actin

The study of CCT–substrate interaction, and in particular that of CCT–actin (Llorca *et al.* 1999*b*), has focused on structural characterization of chaperonin–substrate complex. Less regard has been given to understanding the underlying thermodynamics and kinetics of the folding process. Here, we explore a free-energy-based approach to the problem, initially based upon the theoretical and mechanistic constructs described in §3. A thorough understanding of the CCT folding mechanism can only be achieved once the biological and biochemical purpose of the interaction has been clarified. As shown for the GroEL-based examples, the chaperone dependence of a protein is a consequence of the character of the independent substrate free-energy landscape, from which the resultant occurrence of aggregation-prone or kinetically trapped intermediates hinders autonomous folding. Once the reason for the folding deficiency in the substrate is revealed, the free-energy contribution of the chaperone can then be understood, either within the framework of the established GroEL schemes (figure 2*b–d*) or by novel models. From this free-energy perspective, it then becomes possible to propose hypotheses to describe specific aspects of the reaction mechanism.

Actin is a globular protein, ubiquitous in eukaryotic cells, and is an obligate folding substrate of CCT (figure 3). It is one of the most abundant cellular proteins, comprising over 5 per cent of total protein mass in many cell types and as much as 20 per cent in muscle cells. Actin monomers, G-actin, assemble to a

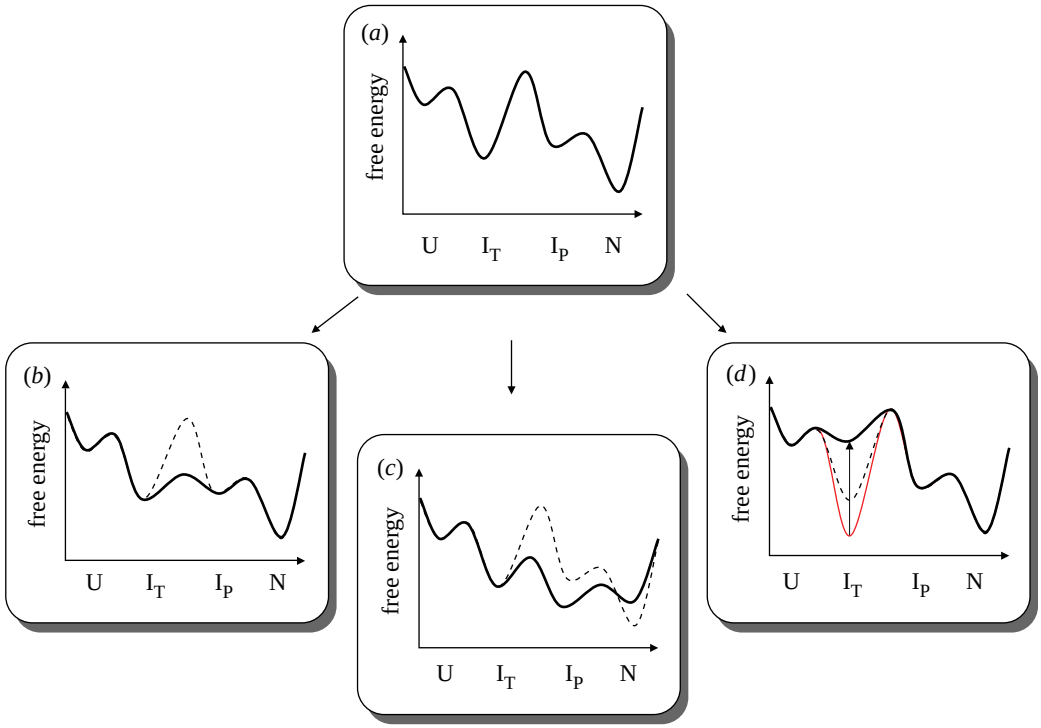

Figure 2. Free-energy representation of GroEL folding models. (*a*) One-dimensional free-energy surface of a hypothetical chaperonin substrate demonstrating relative positions of the unfolded state U, a kinetically trapped folding intermediate I_T, a productive folding intermediate with higher free energy I_P, and the native state N, the global energy minimum. Autonomous folding of the substrate from U proceeds to the local minimum I_T. A high activation energy barrier partitions this state from further folding intermediates. (*b*) The kinetic model; the chaperonin acts as a catalyst. Substrate binding reduces the kinetic energy barrier (solid line) between the intermediates I_T and I_P relative to the original energy surface (dashed line), allowing significant population of the I_P conformation, from which folding to N occurs—either bound or not bound to the chaperonin walls. (*c*) The thermodynamic model; binding to GroEL modifies the energy landscape (solid line) and stabilizes the intermediate I_P relative to I_T and N, resulting in population of I_P. Substrate is then released from the chaperonin walls and folding occurs from this productive intermediate on the original chaperonin free landscape (dashed line), increasing the probability of native state formation. (*d*) The forced unfolding model; initially, binding of substrate to chaperone is favoured due to the low free energy of the chaperonin–substrate complex (red line). Once bound, ATP-powered conformational rearrangements of the chaperone directly drive modification of the substrate energy levels to a new energy landscape (arrow to black solid line), resulting in 'unfolding' from I_T to I_P. In a similar manner to the kinetic and thermodynamic examples (*b* and *c*), release of I_P from the chaperonin walls results in spontaneous folding to the native state on the chaperonin-free landscape.

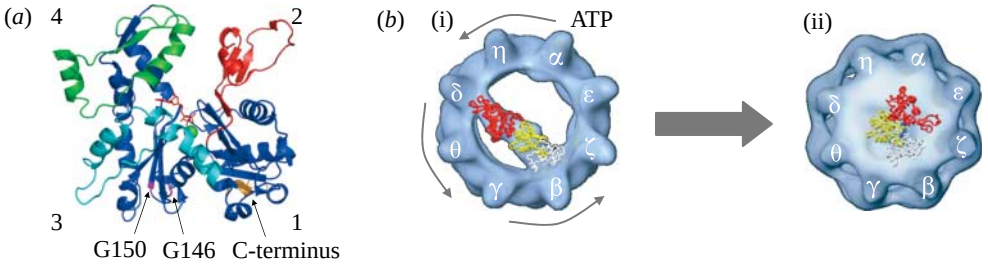

Figure 3. Structures of actin in folded and CCT-bound conformations. (*a*) Atomic structure of rabbit skeletal muscle G-actin taken from Kabsch *et al.* (1990; PDB code 1ATN) in the standard front view with the subdomains 1–4 numbered at the corners. Ca^{2+} (green sphere) and nucleotide (ball and stick) are bound in the cleft between the subdomains 3 and 4 (the large domain), and 1 and 2 (the small domain). CCT-binding sites, as identified by a β-actin peptide array and site-directed mutagenesis (Hynes & Willison 2000; McCormack *et al.* 2001*b*), are coloured; site I (red), II (green) and III (cyan). The C-terminus (gold) and the highly conserved 'hinge' residues G146 and G150 (purple) have been highlighted. (*b*) Electron microscopy reconstructions of the actin–CCT complex in the (i) absence and (ii) presence of ATP (AMPPNP), demonstrating the structural changes undergone by actin during the CCT functional cycle. The atomic structure of actin has been docked to the intra-cavity volume. The N-terminal domain (red) binds to CCT with lower affinity than the C-terminal domain (white). ATP binding to CCT induces large movements of the apical domains that give rise to a more native conformation for the bound actin. This image is reproduced from Llorca *et al.* (2001).

filamentous form, F-actin. *In vivo*, these microfilaments are involved in a range of cellular functions including cell motility, polarity, intracellular transport, tensile strength and cytokinesis. Actin is a member of an evolutionarily ancient family composed of actins and actin-related proteins (ARPs; Goodson & Hawse 2002),

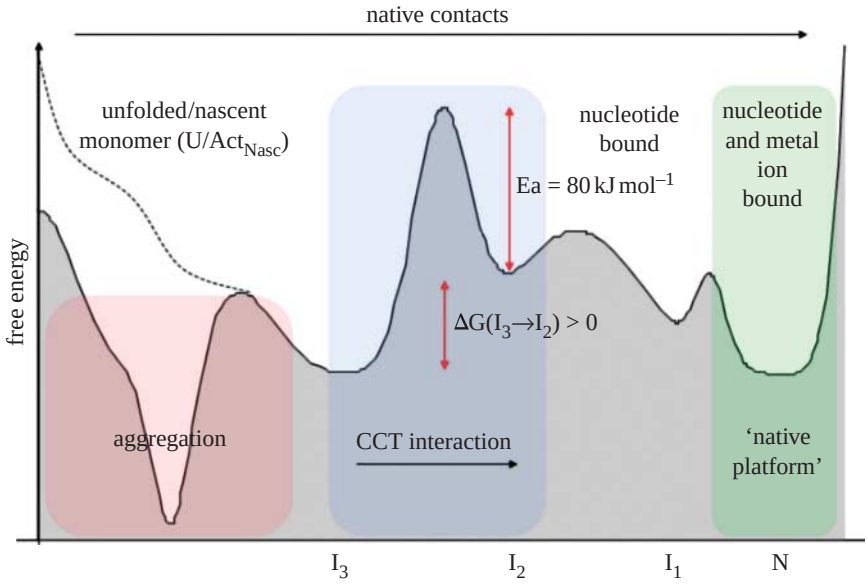

Figure 4. Proposed one-dimensional free-energy landscape for actin folding. This representation is a one-dimensional simplification of the rough multidimensional free-energy landscape for actin. States in rapid equilibrium are grouped and labelled according to the specific folding intermediates that have been experimentally identified: I_1, I_2 and I_3 (Altschuler *et al.* 2005). Monomeric, chemically unfolded or nascent actin, U/Act$_{Nasc}$, spontaneously folds to form the intermediate I_3. At moderate to high protein concentration this species irreversibly forms aggregates, which may represent the lowest free-energy actin species. At low protein concentration, I_3 is kinetically and thermodynamically isolated with respect to ligand-free folded actin I_2, and further folding of actin independent of CCT is not observed on any physiologically significant time scale. The forward folding reaction ($I_3 \rightarrow I_2$) is associated with ATP-dependent CCT interaction and activity (arrow in blue shaded area). I_2 is thermodynamically unstable with respect to the unfolded, CCT-binding, intermediate I_3. Unfolding from I_2 to I_3 is slow at room temperature ($k_u \sim 0.01$ s) due to the large activation energy of unfolding, measured as approximately 80 kJ mol^{-1} (Schuler *et al.* 2000; Altschuler *et al.* 2005). Once formed, I_2 does not bind CCT (Rohman 1999) and spontaneously binds nucleotide and cation, stabilizing actin in a conformational ensemble referred to as the 'native platform' (green shaded area).

as well as being a member of a larger, structure-based 'actin-fold' superfamily that includes hexokinase, ARPs, Hsp70s, several phosphatases, histone proteins and MreB (Bork *et al.* 1992). The bacterial protein MreB is closely related to actin in size and in both monomeric and filamentous structure and has been identified as the prokaryotic ancestor of actin (van den Ent *et al.* 2001).

The stability of native actin is completely dependent on the presence of both the cation and nucleotide that are bound in the cleft between the large and small domains of the actin fold. Release of these moieties under physiological conditions leads to spontaneous unfolding of the protein (Lehrer & Kerwar 1972; Nagy & Strzelecka-Golaszewska 1972; Kuznetsova *et al.* 1988). There has been extensive study into the mechanism of nucleotide and metal ion loss/exchange (Nowak *et al.* 1988; Gershman *et al.* 1991; Estes *et al.* 1992; Kinosian *et al.* 1993; McCormack *et al.* 2001*a*), to allow further understanding of both the process of actin polymerization and the unfolding pathway (Bertazzon *et al.* 1990; Schuler *et al.* 2000; Kuznetsova *et al.* 2002; Turoverov *et al.* 2002; Altschuler *et al.* 2005; Povarova *et al.* 2007). Actin unfolding is generally thought to occur via the sequential loss of cation and nucleotide to produce an unfolded intermediate I_2, which in turn spontaneously unfolds further to form an aggregation-prone intermediate I_3 (Altschuler *et al.* 2005). At low cation concentration, where an excess of EDTA over divalent cation ensures that cation loss is essentially irreversible, we have shown that actin unfolding occurs via the following reaction scheme (Altschuler *et al.* 2005):

$$\mathrm{N \cdot ATP \cdot Ca} \underset{\leftarrow}{\overset{k_1}{\longrightarrow}} \mathrm{I_1 \cdot} \underset{\mathrm{Ca^{2+}}}{+} \mathrm{ATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{I_2} \underset{\mathrm{ATP}}{+} \underset{\leftarrow}{\longrightarrow} \mathrm{I_3}, \qquad (4.1)$$

where N represents native G-actin and $I_{1,2,3}$ are unfolding intermediates. The intermediate I_3 is formed when actin is unfolded in the presence of EDTA or as a result of heat denaturation, moderate denaturant concentration, by dialysis from high concentration of denaturant and spontaneously during storage (Kuznetsova *et al.* 2002). At moderate to high protein concentrations, irreversible aggregation of this species occurs (Bertazzon *et al.* 1990; Kuznetsova *et al.* 1999). The $I_1 \cdot \mathrm{ATP} \rightleftharpoons I_2 + \mathrm{ATP}$ equilibrium is rapidly established relative to the other steps (Kinosian *et al.* 1993), and based on this assumption our stopped-flow fluorescence study has verified the reaction scheme and calculated the kinetic parameters (Altschuler *et al.* 2005).

I_2 can be stabilized with respect to further unfolding towards I_3 by high concentrations of sucrose (Kasai *et al.* 1965; De La Cruz & Pollard 1995) or glycerol (Altschuler 2006). Whether the osmolytes play a kinetic or thermodynamic role in this regard is unclear. This stabilized, nucleotide-free actin intermediate is regarded as quasi-folded because polymerization and DNase I-binding activity is maintained (De La Cruz *et al.* 2000). The nucleotide-binding cleft is intact and thought to adopt, at least transiently, a partially 'open'

state, similar to that observed in the profilin–actin structure (Chik *et al.* 1996), rather than the 'closed' state observed for uncomplexed ATP- or ADP-actin, in which the nucleotide-binding site is blocked (Otterbein *et al.* 2001; Graceffa & Dominguez 2003). The free-energy difference between the open and closed folded structures of nucleotide-free actin is thought to be small (Kinosian *et al.* 2004). However, it has been shown that when this cleft closure is accompanied by nucleotide binding, i.e. $I_2 \rightarrow I_1$ ATP, there is an associated decrease in free energy of 28.7 kJ mol^{-1} (Altschuler *et al.* 2005), which, considering the X-ray structural analysis, is provided predominantly through interaction of nucleotide with subdomains 3 and 4 (Nolen & Pollard 2007).

Since the transition from I_2 to I_3 is spontaneous under physiological conditions (Altschuler *et al.* 2005; Povarova *et al.* 2007), the unfolded intermediate I_3 must have a lower free energy than the nucleotide and cation-free folded conformation I_2 (figure 4). This is unusual in that it is a generally accepted dogma that the folded state of a protein resides at a free-energy minimum and that procession along the folding coordinate is associated with a decrease in free energy. In the case of actin the nucleotide-free folded state I_2 is kinetically, rather than thermodynamically, stable with respect to I_3 (figure 4). Thus, cation and nucleotide binding is required to stabilize this metastable, not completely folded conformation of actin.

In vitro unfolding/refolding assays have demonstrated that I_3 represents the conformation recognized and folded by CCT (Altschuler *et al.* 2005). This CCT-binding intermediate can also be formed by dilution out of concentrated chaotropic agents (Melki & Cowan 1994) and by *in vitro* expression in prokaryotic cell extract systems (Stemp *et al.* 2005; Pappenberger *et al.* 2006) and it is reasonable to assume that both nascent and fully unfolded actin spontaneously (re)folds to this intermediate state.

In summary, the details have been established as follows.

— Fully unfolded actin, U (upon dilution from high concentration of denaturant), and nascent actin, Ac_{Nasc} (from *in vitro* expression systems), spontaneously form the intermediate I_3.
— CCT binds and folds the intermediate I_3.
— The quasi-folded, nucleotide-free intermediate I_2 spontaneously binds nucleotide and cation to form native actin N.

These results can be represented by the following partial reaction schemes:

$$U \rightarrow I_3, \tag{4.2}$$

$$Ac_{Nasc} \rightarrow I_3, \tag{4.3}$$

$$I_2 \rightarrow I_1 \cdot ATP \rightarrow N \cdot ATP \cdot Ca^{2+}, \tag{4.4}$$

$$(ATP + Ca^{2+}) + I_3 + CCT \rightarrow \overset{ATP \quad ADP}{\underset{\cdots}{\rightleftharpoons}} \rightarrow N \cdot ATP \cdot Ca^{2+} + CCT. \tag{4.5}$$

The arrows indicate the direction of the spontaneous reaction and, with the exception of the $I_3 \rightarrow I_2$ transition, all phases of the folding pathway are indeed spontaneous and rapid under physiological conditions. Therefore, by deduction, it must be the $I_3 \rightarrow I_2$ step that requires the involvement of CCT. It can also be asserted that CCT acts on ligand-free actin, through folding I_3 to I_2, both of which are ligand-free actin polypeptide chains. Subsequent interaction of the actin intermediate with nucleotide and cation is important for the kinetic and/or thermodynamic stability of the protein but does not necessarily require further chaperone interaction. A one-dimensional free-energy landscape for actin has been constructed, which summarizes these conclusions (figure 4). This landscape depicts the relative free energies of the experimentally observed actin intermediates, I_1, I_2 and I_3 as well as the native state N, and the unfolded/nascent chain (which have been roughly grouped together in this model). At moderate protein concentrations unfolded actin irreversibly forms aggregates, which may be the lowest free-energy species (although not monomeric). This is represented as the deepest well on the free-energy landscape.

The position of the CCT-interacting form of actin on the free-energy landscape can now be understood. CCT mediates folding of the intermediate I_3 to the nucleotide-free folded I_2. This is an endothermic process with high activation energy and an overall 'uphill' free-energy gradient (figure 4). Thus, a thermodynamic role for CCT is proposed whereby the chaperonin mediates an energetically unfavourable process. There may also be a kinetic component, to lower the activation energy barrier to increase the folding reaction rate but this is not the primary effect of CCT. This suggestion can be conceptualized in the context of the various GroEL unfolding models that we have presented in figure 2. In the case of actin, I_3 is equivalent to the trapped intermediate I_T, and I_2 corresponds to the productive folding intermediate I_P. Actin folds autonomously to I_3 ($\equiv I_T$) but cannot access I_2 ($\equiv I_P$) to complete its folding to the native state, due to both kinetic and thermodynamic partitioning effects. Folding via the various models of the type in figure 2*b*,*c* or *d* may occur on CCT, to allow population of the I_2 intermediate. The subsequent release from CCT restores the kinetic partition between I_3 and I_2, establishing the metastable I_2 population. To reiterate, this 'folded', I_2 conformation of actin is subsequently thermodynamically stabilized by the binding of nucleotide and cation in the interdomain cleft, forming the native state N. The rates of both nucleotide and cation association, $I_2 \rightarrow I_1 \cdot ATP \rightarrow N \cdot ATP \cdot Ca^{2+}$, are much faster than unfolding back to I_3. Therefore, as for the case of the hypothetical protein in figure 2*a*, release of productive folding intermediate from the chaperone results in the formation of the native state. The difference in free energy between the trapped intermediate of actin I_3 and the native state N is likely to be small but has not yet been measured. An interesting peculiarity of working with actin is that it slowly, but spontaneously, unfolds from N to I_3 in storage buffers containing cation and nucleotide (Kuznetsova *et al.* 1988), suggesting that I_3 may in fact be the lowest energy state of all. Nevertheless, the kinetic barrier due to the large activation energy of unfolding I_2 to I_3 dominates the folding

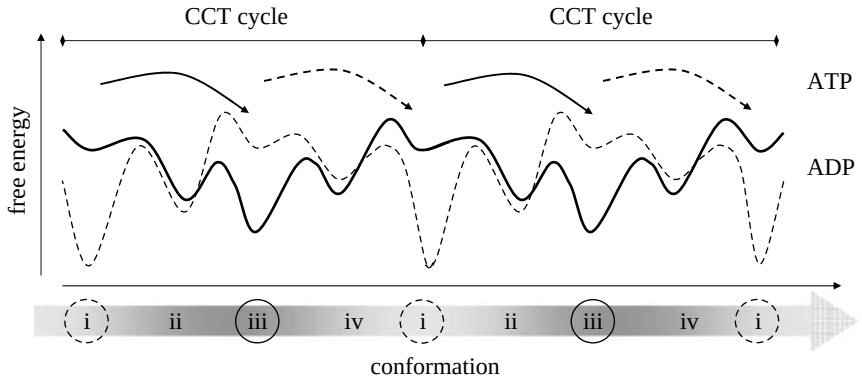

Figure 5. CCT free-energy cycle for a single ring. One-dimensional free-energy landscape of a four-state CCT folding cycle. The energy levels of the four CCT intermediates i–iv, and the transition states between them depend on the identity of the nucleotide bound to CCT: solid line, ATP bound; dashed line, ADP bound. When the chaperone is fully ATP bound, state iii has the lowest free energy (circled) and when fully ADP bound, state i has the lowest energy (dotted circled). The net free-energy change of CCT is zero through the $CCT_{ADP} \rightarrow CCT_{ATP} \rightarrow CCT_{ADP}$ cycle. However, the topography of the ATP/ADP free-energy surfaces dictates a kinetically favoured path i→ii→iii→iv→i.

landscape significantly and prevents unfolding on biologically relevant time scales. *In vivo*, it is unlikely that a significant population of monomeric, folded actin actually exists. The most stable structures are likely to be multimeric, either homo- or hetero-oligomers as in microfilaments (F-actin) or in complexes with actin-binding proteins (ABPs).

4.2. The role of CCT

The formation of I_2 from I_3, carried out during the interaction between actin and CCT, represents a thermodynamically unfavourable transition. In order for the free energy of the overall system to decrease, this process must be coupled to a thermodynamically favourable reaction. Since each assembled CCT complex is expected to be able to carry out multiple rounds of folding, the free energy of the chaperone must be the same at the start and the end of the folding cycle. The free-energy decrease required to balance the I_3–I_2 transition must be derived from an external source, which is, of course, nucleotide hydrolysis by CCT subunits. CCT contains eight potential ATP-binding sites per ring. ATP binding or hydrolysis induces conformational changes in CCT subunits, which in turn affect the conformation of the bound substrate. The substrate–chaperonin interactions provide the molecular capability for the chaperone to effect the necessary free-energy transfer between nucleotide hydrolysis and substrate folding.

During a functional cycle of CCT, the subunits undergo a series of conformational changes (Rivenzon-Segal *et al.* 2005). These are caused by the differing states of the bound nucleotide. ATP binding induces a closed conformation and ADP an open conformation (Llorca *et al.* 1999*b*). There is some dispute over the conformations adopted by the ADP–Pi and nucleotide-free states of CCT, which may represent intermediates between the open and closed forms. In order to generalize these findings we have constructed a hypothetical conformational cycle, an approach similar to that adopted for molecular motors (Astumian 2005). This provides a useful framework from which to understand how chaperonin free-energy surfaces may change depending on the nucleotide status of CCT, first in the absence and in the presence of substrate. Figure 5 shows a hypothetical one-dimensional free-energy surface for a single ring of CCT representing four possible ring conformations. It should be noted that this landscape is periodic as it represents a continuous cycle and also that it depicts the conformations of the CCT ring not the folding intermediates of the protein. The landscape topography is dependent upon whether the ring is ATP or ADP bound. The hydrolysis of ATP to ADP, or the exchange of ADP for ATP, provides the necessary free energy for transition between the two energy surfaces. Four intermediate conformations are represented, i–iv, where i is the lowest free-energy conformation for ADP-bound CCT, iii is the lowest free-energy conformation for ATP-bound CCT and ii/iv represent intermediates between these states. These can be related to the various structures identified by electron microscopy (Llorca *et al.* 1999*b*), although to maintain generality in this model they have not been directly assigned as such. The nucleotide state determines which conformation is the most stable, and the landscape asymmetry determines the order of population of the intermediate states; i→ii→iii when ATP bound and iii→iv→i when ADP bound. Therefore, switching of the nucleotide state results in the sequence of conformational changes i→ii→iii→iv→i. In this way, a thermodynamically powered folding cycle with kinetically controlled directionality is created.

Upon interaction of CCT with substrate, the cycle can be coupled to substrate free-energy changes. An unfavourable folding reaction can be driven by coupling phases that release energy ('downhill' progression along the energy landscape; figure 5) to specific transitions in substrate folding, while ensuring that phases requiring energy input (shifting between the ATP and ADP energy landscapes; figure 5) are uncoupled. Thus, CCT can direct folding while ensuring that the free energy of the chaperonin itself is the same at the start and end of a folding cycle. In reality, the folding system is more complicated than the framework presented here. The ATP-driven conformational changes in CCT occur via

an intra-ring sequential allosteric mechanism (Llorca *et al.* 2001; Rivenzon-Segal *et al.* 2005) rather than the concerted mechanism exhibited by GroEL. A sequential mode of allosteric switching provides opportunity for more complicated sequences of energy transitions to occur. Each subunit may be capable of undergoing independent cycles. Individual actin domains may first be folded via coupling to energy cycles of certain CCT subunits and global interdomain rearrangements managed subsequently by other subunit cycles.

5. CCT–ACTIN FOLDING MODELS

Here we present two models, the 'strong' and 'weak' interaction schemes via which we hypothesize that CCT couples its own energetically favourable subunit conformational changes to unfavourable phases in substrate folding, such as that described for actin in §4. Both models are approached from a free-energy perspective and, although they incorporate some mechanistic detail based on previous experimental studies, our primary aim is to present a general theoretical framework rather than define a detailed mechanism. We expect that future experimental data will provide further detail, to allow finer resolution of the mechanism.

5.1. Strong actin–CCT interaction model

There is experimental evidence that strong intermolecular interactions play a role in the course of CCT–actin folding (Hynes & Willison 2000; McCormack *et al.* 2001*b*; Neirynck *et al.* 2006; Pappenberger *et al.* 2006). Immunoprecipitation of CCT pre-loaded with unfolded actin has identified specific CCT subunits involved in mediating a specific interaction with actin and a complementary β-actin peptide array analysis has mapped the corresponding sites on actin (Hynes & Willison 2000). More recently, *in vitro* translation and CCT folding assays of a huge alanine scan series of actin mutants have revealed further information on the character of these and other discrete binding sites (Neirynck *et al.* 2006). On actin, these regions contain polar and charged residues, indicative of specific- and structured-binding sites. On CCT, the interactions occur through the apical domains of the subunits, regions known to be flexible (Pappenberger *et al.* 2002) and conformationally dependent on the character of the nucleotide bound to the equatorial region (Llorca *et al.* 1999*b*). Specific CCT subunit–actin-binding interactions result in the creation of local free-energy minima associated with intermolecular side-chain interactions, as well as long-range effects due to multivalent bonding causing realignment between substrate domains bound at different CCT sites. The free energy of a CCT-bound actin intermediate would be dependent on the extent of subunit binding, on the conformation of the bound subunit and on the actin conformation. Consequently, the chaperonin-associated actin free-energy landscape differs from the unassociated landscape model (as for the hypothetical substrate in figure 2). As the folding cycle of CCT progresses, the subunit conformations change and therefore the free-energy landscape of the actin is modified. The free energy required to effect this activity is attained by coupling CCT–actin landscape modifications to downhill phases in the CCT cycle described in figure 5, the energetically favourable transitions i→ii→iii in the ATP-bound state or iii→iv→i in the ADP-bound state. These favourable transitions are coupled to the unfavourable change in free energy required to modify the CCT-bound actin free-energy landscape. This is demonstrated by the hypothetical energy surfaces in figure 6*a*. The free-energy surface of actin is dependent upon the conformational state of CCT. As the CCT cycle progresses, the actin free-energy landscape first favours binding $I_3 + CCT \rightarrow CCT\text{-}I_3$, then chaperone-bound folding $CCT\text{-}I_3 \rightarrow CCT\text{-}I_2$, and finally release $CCT\text{-}I_2 \rightarrow I_2$. For illustration, four intermediates are shown, but this is not meant to indicate the actual number involved. It is likely that further complexity in the reaction mechanism arises due to the identity of the individual CCT subunit(s) bound and on the ATP-binding/hydrolysis activity of the subunit(s). A reaction scheme summarizing how actin folding is synchronized with the CCT cycle is shown in figure 6*b*. In addition, depending on the extent to which substrate binding affects the free energy of CCT, there may be varying degrees of 'feedback' between substrate conformation and the chaperonin cycle, introducing the possibility of substrate–chaperonin allosteric regulation, which may serve to provide greater synchronicity between the development of substrate folding and the progress of the CCT cycle. The strong model describes actin as bound to distinct CCT subunits and if ATP binding/hydrolysis occurs sequentially it follows that additional actin intermediates may be stabilized at each step of the sequential cycle. These intermediates may represent conformations corresponding to local minima along the CCT-bound I_3–I_2 coordinate and provide a means by which the free energy derived from ATP hydrolysis from each subunit results in a stepwise increase of the actin free energy while maintaining strong multivalent interactions.

Over the course of one folding cycle, the overall change in free energy is positive for actin ($I_3 \rightarrow I_2$), zero for CCT and negative for the hydrolysed nucleotide (ATP→ADP). The coupling between ATP hydrolysis, CCT free-energy transitions and the actin folding landscape is directly analogous to the aspects of the free-energy models described in figure 2. First, interaction between CCT and actin stabilizes discrete folding intermediates, in the manner of the thermodynamic model. Then, the coupling of free energy released by ATP hydrolysis to drive substrate re-arrangements occurs, modifying substrate energy levels, which is equivalent to the forced unfolding model.

5.2. Weak actin–CCT interaction model

An alternative model for actin–CCT is possible whereby folding is driven by weak intermolecular interactions. Initial capture of unfolded actin does not necessarily occur via specific interactions, as required for co-translational capture models (Spiess *et al.* 2006), and there is no subsequent strong interaction between CCT subunits and actin folding intermediates, i.e. no deep local minima on the CCT-bound actin free-energy

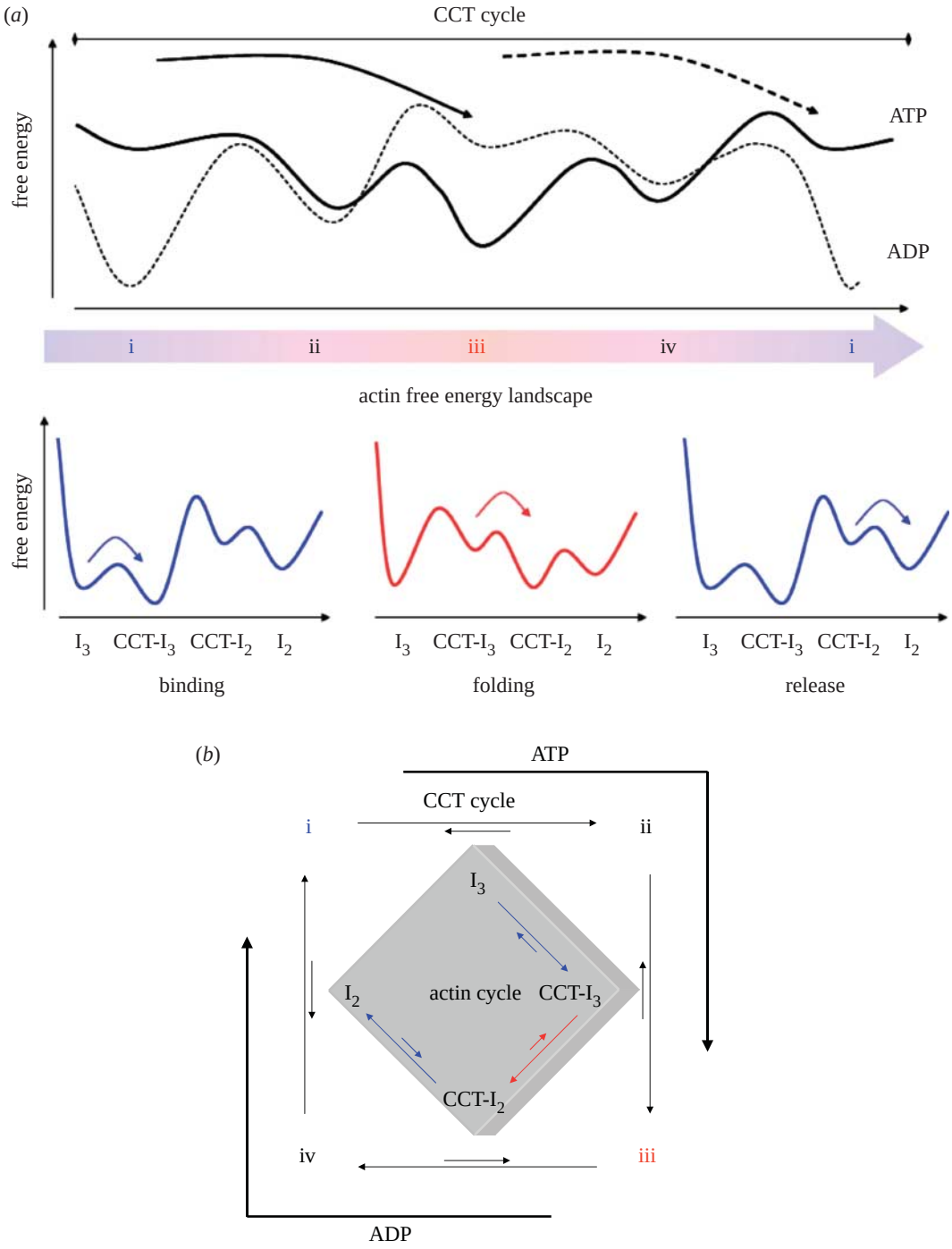

Figure 6. 'Active CCT' model of actin folding: CCT couples ATP binding and hydrolysis to the CCT-associated actin free-energy landscape. (*a*) Strong, specific interactions between actin and CCT directly couple the actin free-energy landscape to that of the CCT cycle. As CCT binds and hydrolyses nucleotide, undergoing the conformational cycle described in figure 6, the free-energy landscape of bound actin changes depending on the CCT conformation. Here, two hypothetical actin energy landscapes are described; corresponding to actin bound to CCT intermediate i (blue) and to CCT intermediate iii (red). The free energy required to modify the actin landscape between the blue and red topographies is derived from the energetically favourable, downhill phases in the CCT cycle; i→ii→iii for CCT–ATP and iii→iv→i for CCT–ADP. The blue landscape favours binding of unfolded actin I_3 and release of the folded intermediate I_2 while the red landscape favours folding CCT-I_3→CCT-I_2. The concept of alternate landscapes proposed here is similar to that of the GroEL thermodynamic model (figure 2*c*). (*b*) The consequences of the hypothetical landscapes can be summarized in a reaction scheme within the context of the CCT–ATP/ADP cycle. The entrance and exit (binding and release) points for actin within the cycle are displayed. This scheme is cyclical from the CCT point of view but linear with respect to actin, i.e. at the end of a single cycle, the CCT regains its initial state but actin has progressed along its folding coordinate.

surface. Instead, both binding domains of actin are released and explore the conformational space available within the CCT cavity, confined by non-specific intermolecular interactions. This situation nevertheless differs from that in bulk solution as the accessible volume is confined due to the nature of the inner wall

environment and the steric constraints imposed by the CCT cavity dimensions. Evidence for such a model is provided by experiments suggesting that certain CCT-interacting proteins are recognized through their hydrophobic surfaces (Spiess *et al.* 2006; Horwich *et al.* 2007). In this case, sequential binding/hydrolysis of ATP by individual subunits might serve to first release substrate into the cavity and then modify the available conformational space, increasing the confinement of the free actin domains. Confining the substrate reduces the system entropy; therefore, this requires an input of free energy, provided by the ATP-driven conformational changes of the chaperonin cycle shown in figure 5. This model can be related to elements of both the confinement model and aspects of the forced ATP-driven model. Confinement of chaperone-associated substrate alters the free-energy landscape of the initial substrate structure in a similar way to that of the GroEL/GroES cage. An active component is introduced by subsequent apical domain rearrangements that alter this confining potential to direct substrate thermal motions in a productive folding direction. However, we note that electron microscopy analysis shows that actin still remains bound to CCT even after the cavity has been closed and the actin encapsulated (Llorca *et al.* 2001), although as yet unresolved CCT–actin intermediates may involve release of one or both actin domains during the folding cycle (Farr *et al.* 1997). The multiple cycles of binding and release model for CCT–actin folding of Farr *et al.* (1997) is a weak interaction model analogous to the GroEL mechanism (Horwich *et al.* 2007).

This scenario of a sequentially changing conformational space can be considered as similar to the concept of a fluctuating Brownian ratchet (Astumian 1997), where the actin structure represents a component diffusing under Brownian motion on a potential gradient, the substrate energy landscape. A change in CCT subunit orientation, resulting in system confinement, confers an additional potential gradient on this landscape, reducing the probability that the expanded substrate structures are sampled. If this occurs in a stepwise manner, a ratchet-type system can be visualized whereby the CCT domain movements serve to confine the substrate to an increasing extent, resulting in a stepwise increase in free energy, until the point at which I_2 becomes the most stable conformation.

Greater complexity may be built into such a model by the consideration of the intra-ring sequential conformational changes that are known to occur in the CCT subunits. This could potentially allow the development of synchronicity between the oscillating ratchet potential and the characteristic time constants of specific substrate rearrangements. Through such a mechanism it would be possible for the chaperone to exert fine control over the timing of substrate folding. Recently, two-dimensional lattice models have been used to simulate folding of proteins within chaperonin cavities (Jacob *et al.* 2007). The findings support the suggestion that altering the nature of a confining potential in a sequential manner may be an important aspect in the folding of multidomain proteins.

Although the relevance of the Brownian ratchet model discussed here with regards to chaperonin-mediated protein folding is highly speculative, its relevance to biological systems is already well established. In protein systems the idea of the Brownian ratchet has been extensively discussed, predominantly not only with regard to the function of motor proteins (Cordova *et al.* 1992; Neupert & Brunner 2002; Astumian 2005; Tomkiewicz *et al.* 2007) but also in protein folding, where ATP hydrolysis of Hsp70 has been shown to drive a modified Brownian ratchet mechanism resulting in protein unfolding and membrane translocation (De Los Rios *et al.* 2006).

6. SUMMARY OF CCT–ACTIN MODEL

The above discussion on actin thermodynamics has established that CCT facilitates the thermodynamically unfavourable $I_3 \rightarrow I_2$ transition on the actin folding landscape. It is hypothesized that CCT accomplishes this through hydrolysis of ATP and acts as a conduit for conversion of the free energy from nucleotide to substrate. Two alternative models have been presented here to explain how this is achieved, the strong and weak binding models. The essential difference between these two models lies in whether there is an ATP-driven 'power stroke' affecting the thermodynamic balance between the states, from which energetic relaxation occurs, or whether hydrolysis of ATP drives conformational changes in CCT, biasing the thermal motion of the non-specifically bound unfolded actin domains and increasing the frequency with which folded states are sampled. It has been demonstrated that CCT interacts with actin via specific subunit–substrate interactions, which we believe is compelling evidence for discrete chaperone-bound folding intermediates characteristic of the strong model. However, it is possible that in some cases non-specific interactions could also affect the free-energy surface of a CCT-associated substrate; these would be of particular significance if, as has been suggested for CCT–actin folding, the substrate is released into the chaperone cavity at some stage during the folding cycle (Farr *et al.* 1997). Actin and tubulin are both multidomain proteins and it is possible that the structural elements of these substrates may develop in separate stages of their folding cycles via differing contributions from the two models. One appealing hypothesis is that strong CCT interactions control the interdomain alignment, while elements of the intra-domain structure may be formed through confining, weak interactions.

7. PRESERVED FOLDING ENERGY AND F-ACTIN DYNAMICS

What are the implications of the CCT-mediated actin folding mechanism for the structure and function of F-actin? Reisler & Egelman (2007) have collated many disparate observations concerning biochemical and structural properties of F-actin and argued convincingly that F-actin should not be considered to be a single state but rather a dynamic ensemble of many

states. Transitions between states are probably coupled to rotations and tilts of subunits, which occur readily despite the apparently severe structural constraint of a constant axial rise of every subunit in the filament of approximately 27.3 Å. Put succinctly, in its breathing modes F-actin changes its shape more than its length.

The emerging view of time-correlated motions and protein function is that active enzymatic or ligand-binding states reflect configurations that pre-exist in the ensemble. In adenylate kinase, the catalytically competent closed state is occasionally sampled on microseconds–milliseconds time scales even in the absence of ligand (Henzler-Wildman *et al.* 2007). It is suggested that the F-actin depolymerizing factor, cofilin, directly modulates one of the pre-existing, internal modes of actin rather than 'inducing' a binding site (Orlova *et al.* 2004). When bound to cofilin, the actin monomer adopts a tilted state. This tilted state of actin is also observed in 'young' actin filaments at early times after polymerization, leading to the suggestion that actin filaments anneal over time into a homogeneous structure, and that filaments are disrupted by mechanisms such as cofilin binding which, therefore, appear to act as a time reversal of polymerization (Orlova *et al.* 2004). How does annealing of F-actin occur and what structural and energetic transformations are relevant?

Nucleotide binding and hydrolysis lie at the core of actin function. The nucleotide in ATP-G-actin is non-hydrolysable, but once the monomer is incorporated into a filament ATP becomes readily hydrolysable. *In vitro* ATP-G-actin is preferentially incorporated into the growing end of the filament compared to ADP-G-actin and this behaviour reflects *in vivo* mechanisms for regulating rates of filament formation through control of ATP- and ADP-G-actin monomer levels. Curiously, nucleotide is not absolutely required for filament formation because nucleotide-free G-actin monomers may be stabilized in high concentrations of sucrose and they readily polymerize into apparently normal filaments, although without the initial lag phase (De La Cruz *et al.* 2000).

The motions and dynamic properties of actin filaments *in vitro* are enhanced in the all-ADP state, compared with both the ATP and ADP–Pi states, suggesting that ATP hydrolysis may be a mechanism to cause relaxation-based changes of the actin monomer structure in the context of the filament. Filaments are 50 per cent stiffer in the ADP–Pi state than the ADP state and, given the fact that Pi release from ADP–Pi actin is rather slow *in vivo* ($t_{1/2} \sim 1$ min), this suggests that young newly polymerized filaments are stiffer than 'old' ADP filaments. This may permit the *in vivo* functions of F-actin to differ depending on the age of the relevant actin structure (Isambert *et al.* 1995).

We believe that our finding of uphill folding of actin may provide the energetic origins of the unusual dynamic properties of F-actin. We suggest that, once incorporated into a filament, actin monomers are able to re-explore the folding energy landscape between I_2 and the transition state (figure 4). The landscape need not necessarily have the same structural features as the folding landscape on CCT owing to the specific and extensive nature of the inter-actin subunit interactions in F-actin. However, it is noticeable that the CCT-binding sites on actin overlap the main monomer–monomer interaction sites in the filament (Llorca *et al.* 1999*a*, 2001; Hynes & Willison 2000; McCormack *et al.* 2001*b*). These binding sites are located in loops that are found exposed on the surface of the native actin structure and an important function of CCT may be to facilitate the folding and packing of these labile loop structures onto the core of the G-actin monomer fold. Interestingly, these loops are absent in the bacterial precursors of the modern actins (van den Ent *et al.* 2001).

Cryo-electron microscopy (Llorca *et al.* 1999*a*) and dynamic fitting analysis (Wriggers *et al.* 2004) show that subdomains 3 and 4 may be substantially structured in the I_3 intermediate, and biochemical analysis supports this model because these two subdomains can be constructed as fusion proteins and still retain their specificity of CCT binding (Llorca *et al.* 1999*a*; McCormack *et al.* 2001*b*). Although it has not been possible to structurally characterize I_3, homo-FRET data have revealed a cooperative collapse occurring in actin diluted out of denaturant, resulting in a small interdomain separation (Villebeck *et al.* 2007*a*). It may be that in this conformation, additional interactions between subdomains 2 and 4 effectively seal the interdomain cleft, preventing nucleotide access and proper formation of the nucleotide-binding site. Measurements of ATP binding to sucrose-stabilized, nucleotide-free actin show that productive binding occurs with low probability suggesting that the nucleotide cleft is mostly closed and inaccessible (De La Cruz & Pollard 1995). Further evidence for changes associated with the nucleotide state of G-actin is that the partial specific volume of ATP-G-actin is high for a globular protein ($0.744\ \mathrm{cm^3\ g^{-1}}$) compared with ADP-G-actin ($0.727\ \mathrm{cm^3\ g^{-1}}$), which is within normal range (Kikumoto *et al.* 2003). Adiabatic compressibility measurements (Kikumoto *et al.* 2003) indicate an extraordinarily soft global conformation for ATP-G-actin ($8.8\times10^{-12}\ \mathrm{cm^2\ dyne^{-1}}$) compared to a more compact mass associated with ADP-G-actin ($5.8\times10^{-12}\ \mathrm{cm^2\ dyne^{-1}}$).

Expansion of the interdomain cleft is observed on I_3 binding to CCT with subsequent CCT-bound folding leading to a recompaction of the structure and formation of the nucleotide-binding site (Llorca *et al.* 2001; Villebeck *et al.* 2007*b*). McCormack *et al.* (2001*a*) mutagenized two conserved glycine residues (G146 and G150) in the hinge region linking the two main domains of β-actin and found that the mutants fold poorly or not at all on CCT. The most severe mutation, G150P, prevents the actin folding intermediate from crossing over the CCT cavity to bind the CCT4/δ subunit, and in the pure yeast folding assay actin G150P is completely unable to be folded to the native state by CCT (Pappenberger *et al.* 2006). This supports the idea that the hinge region in actin is the centre of the energetic transitions of actin and that the structures of the large and small domains depend upon rather than control hinge conformation. Therefore, the role of CCT

could predominantly be to provide energy for inter-domain conformational rearrangements rather than folding specific domains. In simulation experiments on actin unfolding, it has been reported that opening up the interdomain cleft introduces steric clashes between the large and small domains and that these can be alleviated by the removal of the C-terminus (residues 332–375 in human β-actin; Neirynck *et al.* 2006). Furthermore, certain mutations in the C-terminus and in regions interacting with the C-terminus in the native structure have been found to inhibit actin release from CCT. Based upon these findings, a role for the C-terminus has been proposed in actin folding, whereby its correct positioning is important in the final stages of the chaperonin-mediated folding process (Neirynck *et al.* 2006). The steric hindrance associated with C-terminus positioning and nucleotide-binding cleft opening may significantly contribute to the large activation energy of unfolding $I_2 \rightarrow I_3$ (Altschuler *et al.* 2005). Extending this reasoning, a role for the C-terminus can be envisaged, which accounts for the slow kinetics of unfolding due to the steric clashing. However, the flexibility in the hinge region allows actin to undergo the rearrangements required for nucleotide exchange and other conformational changes within the F-actin filament. In this way, conformations corresponding to intermediates on the $N \rightarrow I_3$ reaction coordinate (figure 4) may be sampled on biologically relevant time scales without the occurrence of unfolding, thus allowing for the wide range of interdomain orientations of actin that have been observed by X-ray crystallography (Nolen & Pollard 2007), electron microscopy and biophysical FRET measurements (Kozuka *et al.* 2006). Orlova & Egelman (2000) showed that F-actin exists in different discrete states of twist, which persist for several seconds and postulate the existence of an energy landscape in which the discrete states are separated by significant energy barriers. Their landscape may correspond to our $N \rightarrow I_3$ reaction coordinate.

8. FUTURE DIRECTIONS

Experimental determination of the kinetics and thermodynamics of the CCT folding system as set out in this article will not be a simple task. To develop a comprehensive understanding there are two aspects that need to be investigated, the CCT cycle on its own and the cycle with substrate. First, in the absence of substrate, the kinetics of CCT nucleotide binding and hydrolysis must be related to an underlying free-energy landscape, such as that in figure 5, encompassing the conformational changes of CCT that occur during a folding cycle. Good progress has already been made in this regard, where stopped flow experiments on bovine (Kafri & Horovitz 2003) and yeast CCT have resolved both ATP-binding and hydrolysis phases using changes in intrinsic fluorescence. Taken in combination with studies on a CCT mutant in which inter- and intra-ring cooperativity is disrupted, this has provided evidence for the sequential model of ATP-induced allosteric transitions within each CCT ring and has begun to resolve intermediates within the CCT cycle (Shimon *et al.* 2008). The next step is to relate these observable transitions to free-energy changes. A complication to doing this is that the CCT free-energy surface is multidimensional, as each permutation of nucleotide states has its own associated conformational energy surface. This makes conceptualizing a traditional free-energy landscape challenging (in figure 5 just two surfaces are shown, ATP–CCT and ADP–CCT). An alternative three-dimensional landscape, such as that proposed recently by Swint-Kruse & Fisher (2008), may be helpful in this regard, where both conformational state and position on reaction coordinate are considered. In such a scheme, CCT conformation, nucleotide state and free energy would form the axes and the energy barriers between both subunit conformations and nucleotide states could be represented. In this way, preferred reaction pathways can be highlighted, which could provide a representation of the free-energy relationships between the nucleotide states and CCT conformations that underlie the observed allostery.

The second aspect requiring investigation involves understanding how the nucleotide hydrolysis cycle of CCT is related to the substrate folding cycle. Here again, developing a multidimensional landscape is essential, in order to describe how CCT conformational changes are coupled to changes in the substrate folding landscape. There may be a further level of detail whereby, as mentioned earlier, it is possible that substrate–chaperonin coupling may lead to cooperativity affecting the CCT cycle, producing a feedback loop between the chaperone and the substrate and a more complex level of synchronicity between folding and nucleotide hydrolysis of CCT to maximize folding efficiency. It has been shown that the ATP-binding site of CCT6 in yeast is highly amenable to mutagenesis without causing major phenotypic effects *in vivo* (Lin *et al.* 1997) and it may be the case that for some subunits, their role in conferring inter- and intra-ring conformational changes during the substrate folding cycle is more important than their discrete ATPase activity. Studies have begun to investigate structure–function relationships on the intra- and inter-ring ATP cooperativity in CCT (Shimon *et al.* 2008) and it will be important to understand also how different ATP-site mutations influence substrate folding.

Although resolving steady-state CCT-bound folding intermediates has been accomplished using homo-FRET (Villebeck *et al.* 2007*a*,*b*) and EM (Llorca *et al.* 2001), accurately measuring CCT folding kinetics is beyond the scope of currently reported studies. Ensemble time-resolved measurements will be useful in determining substrate-binding rates and global folding kinetics, but in order to unpick the binding, folding and release phases of the cycle and reveal heterogeneity in substrate–CCT interaction, single molecule studies will be required. A single molecule approach will also avoid problems associated with synchronizing substrate–CCT interactions, which is particularly important in this system because each chaperonin complex is capable of multiple rounds of folding and furthermore heterogeneity in unfolded substrate conformational states may be an issue. Developing models and relating any

resolved kinetic and thermodynamic parameters to CCT structure and function will require mutagenesis of the chaperonin. Until recently it has not been possible to express and purify active CCT from *in vitro* expression of individual subunits but the recently developed protocol for yeast CCT purification (Pappenberger *et al.* 2006) has allowed purification of mutant CCT for use in biophysical assays (Shimon *et al.* 2008). These types of approaches will provide the opportunity to explore further structure/function relationships in this fascinating protein folding system.

This work is funded by Cancer Research UK, Human Frontiers Science Program (RGP63/2004) and an EPSRC Platform Grant. We thank Prof. David Klug for helpful discussions. We thank both the anonymous reviewers of this work for their insightful comments.

REFERENCES

Altschuler, G. M. 2006 Protein folding studies on the actin–CCT chaperone system. PhD thesis, Institute of Cancer Research, University of London.

Altschuler, G. M., Klug, D. R. & Willison, K. R. 2005 Unfolding energetics of G-alpha-actin: a discrete intermediate can be re-folded to the native state by CCT. *J. Mol. Biol.* **353**, 385–396. (doi:10.1016/j.jmb.2005.07.062)

Anfinsen, C. B. 1973 Principles that govern the folding of protein chains. *Science* **181**, 223–230. (doi:10.1126/science.181.4096.223)

Archibald, J. M., O'Kelly, C. J. & Doolittle, W. F. 2002 The chaperonin genes of jakobid and jakobid-like flagellates: implications for eukaryotic evolution. *Mol. Biol. Evol.* **19**, 422–431.

Astumian, R. D. 1997 Thermodynamics and kinetics of a Brownian motor. *Science* **276**, 917–922. (doi:10.1126/science.276.5314.917)

Astumian, R. D. 2005 Biasing the random walk of a molecular motor. *J. Phys.: Condens. Matter* **17**, S3753–S3766. (doi:10.1088/0953-8984/17/47/008)

Baumketner, A., Jewett, A. & Shea, J. E. 2003 Effects of confinement in chaperonin assisted protein folding: rate enhancement by decreasing the roughness of the folding energy landscape. *J. Mol. Biol.* **332**, 701–713. (doi:10.1016/S0022-2836(03)00929-X)

Bertazzon, A., Tian, G. H., Lamblin, A. & Tsong, T. Y. 1990 Enthalpic and entropic contributions to actin stability: calorimetry, circular dichroism, and fluorescence study and effects of calcium. *Biochemistry* **29**, 291–298. (doi:10.1021/bi00453a040)

Bork, P., Sander, C. & Valencia, A. 1992 An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc. Natl Acad. Sci. USA* **89**, 7290–7294. (doi:10.1073/pnas.89.16.7290)

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. & Sigler, P. B. 1994 The crystal structure of the bacterial chaperonin GroEL at 2.8 A. *Nature* **371**, 578–586. (doi:10.1038/371578a0)

Brinker, A., Pfeifer, G., Kerner, M. J., Naylor, D. J., Hartl, F. U. & Hayer-Hartl, M. 2001 Dual function of protein confinement in chaperonin-assisted protein folding. *Cell* **107**, 223–233. (doi:10.1016/S0092-8674(01)00517-7)

Bryngelson, J. D., Onuchic, J. N., Socci, N. D. & Wolynes, P. G. 1995 Funnels, pathways, and the energy landscape of protein folding: a synthesis. *Proteins* **21**, 167–195. (doi:10.1002/prot.340210302)

Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X. & Kiefhaber, T. 1991 GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry* **30**, 1586–1591. (doi:10.1021/bi00220a020)

Burston, S. G., Ranson, N. A. & Clarke, A. R. 1995 The origins and consequences of asymmetry in the chaperonin reaction cycle. *J. Mol. Biol.* **249**, 138–152. (doi:10.1006/jmbi.1995.0285)

Burston, S. G., Weissman, J. S., Farr, G. W., Fenton, W. A. & Horwich, A. L. 1996 Release of both native and non-native proteins from a cis-only GroEL ternary complex. *Nature* **383**, 96–99. (doi:10.1038/383096a0)

Campanini, B., Bologna, S., Cannone, F., Chirico, G., Mozzarelli, A. & Bettati, S. 2005 Unfolding of green fluorescent protein mut2 in wet nanoporous silica gels. *Protein Sci.* **14**, 1125–1133. (doi:10.1110/ps.041190805)

Cannone, F., Bologna, S., Campanini, B., Diaspro, A., Bettati, S., Mozzarelli, A. & Chirico, G. 2005 Tracking unfolding and refolding of single GFPmut2 molecules. *Biophys. J.* **89**, 2033–2045. (doi:10.1529/biophysj.105.064584)

Chaudhuri, T. K., Farr, G. W., Fenton, W. A., Rospert, S. & Horwich, A. L. 2001 GroEL/GroES-mediated folding of a protein too large to be encapsulated. *Cell* **107**, 235–246. (doi:10.1016/S0092-8674(01)00523-2)

Chik, J. K., Lindberg, U. & Schutt, C. E. 1996 The structure of an open state of beta-actin at 2.65 A resolution. *J. Mol. Biol.* **263**, 607–623. (doi:10.1006/jmbi.1996.0602)

Cordova, N. J., Ermentrout, B. & Oster, G. F. 1992 Dynamics of single-motor molecules: the thermal ratchet model. *Proc. Natl Acad. Sci. USA* **89**, 339–343. (doi:10.1073/pnas.89.1.339)

Dekker, C. *et al.* 2008 The interaction network of the chaperonin CCT. *Embo J.* **27**, 1827–1839. (doi:10.1038/emboj.2008.108)

De La Cruz, E. M. & Pollard, T. D. 1995 Nucleotide-free actin: stabilization by sucrose and nucleotide binding kinetics. *Biochemistry* **34**, 5452–5461. (doi:10.1021/bi00016a016)

De La Cruz, E. M., Mandinova, A., Steinmetz, M. O., Stoffler, D., Aebi, U. & Pollard, T. D. 2000 Polymerization and structure of nucleotide-free actin filaments. *J. Mol. Biol.* **295**, 517–526. (doi:10.1006/jmbi.1999.3390)

De Los Rios, P., Ben-Zvi, A., Slutsky, O., Azem, A. & Goloubinoff, P. 2006 Hsp70 chaperones accelerate protein translocation and the unfolding of stable protein aggregates by entropic pulling. *Proc. Natl Acad. Sci. USA* **103**, 6166–6171. (doi:10.1073/pnas.0510496103)

Ditzel, L., Lowe, J., Stock, D., Stetter, K. O., Huber, H., Huber, R. & Steinbacher, S. 1998 Crystal structure of the thermosome, the archaeal chaperonin and homolog of CCT. *Cell* **93**, 125–138. (doi:10.1016/S0092-8674(00)81152-6)

Eggers, D. K. & Valentine, J. S. 2001 Molecular confinement influences protein structure and enhances thermal protein stability. *Protein Sci.* **10**, 250–261. (doi:10.1110/ps.36201)

Ellis, R. J. 1994 Molecular chaperones. Opening and closing the Anfinsen cage. *Curr. Biol.* **4**, 633–635. (doi:10.1016/S0960-9822(00)00140-8)

Estes, J. E., Selden, L. A., Kinosian, H. J. & Gershman, L. C. 1992 Tightly-bound divalent cation of actin. *J. Muscle Res. Cell Motil.* **13**, 272–284. (doi:10.1007/BF01766455)

Etchells, S. A. *et al.* 2005 The cotranslational contacts between ribosome-bound nascent polypeptides and the subunits of the hetero-oligomeric chaperonin TRiC probed by photocross-linking. *J. Biol. Chem.* **280**, 28 118–28 126. (doi:10.1074/jbc.M504110200)

Fan, H. & Mark, A. E. 2006 Mimicking the action of GroEL in molecular dynamics simulations: application to the refinement of protein structures. *Protein Sci.* **15**, 441–448. (doi:10.1110/ps.051721006)

Fares, M. A. & Wolfe, K. H. 2003 Positive selection and subfunctionalization of duplicated CCT chaperonin subunits. *Mol. Biol. Evol.* **20**, 1588–1597. (doi:10.1093/molbev/msg160)

Farr, G. W., Scharl, E. C., Schumacher, R. J., Sondek, S. & Horwich, A. L. 1997 Chaperonin-mediated folding in the eukaryotic cytosol proceeds through rounds of release of native and nonnative forms. *Cell* **89**, 927–937. (doi:10.1016/S0092-8674(00)80278-0)

Farr, G. W., Furtak, K., Rowland, M. B., Ranson, N. A., Saibil, H. R., Kirchhausen, T. & Horwich, A. L. 2000 Multivalent binding of nonnative substrate proteins by the chaperonin GroEL. *Cell* **100**, 561–573. (doi:10.1016/S0092-8674(00)80692-3)

Farr, G. W., Fenton, W. A. & Horwich, A. L. 2007 Perturbed ATPase activity and not "close confinement" of substrate in the cis cavity affects rates of folding by tail-multiplied GroEL. *Proc. Natl Acad. Sci. USA* **104**, 5342–5347. (doi:10.1073/pnas.0700820104)

Fenton, W. A. & Horwich, A. L. 1997 GroEL-mediated protein folding. *Protein Sci.* **6**, 743–760.

Georgopoulos, C. P. & Hohn, B. 1978 Identification of a host protein necessary for bacteriophage morphogenesis (the groE gene product). *Proc. Natl Acad. Sci. USA* **75**, 131–135. (doi:10.1073/pnas.75.1.131)

Gershman, L. C., Selden, L. A. & Estes, J. E. 1991 High affinity divalent cation exchange on actin. Association rate measurements support the simple competitive model. *J. Biol. Chem.* **266**, 76–82.

Goloubinoff, P., Christeller, J. T., Gatenby, A. A. & Lorimer, G. H. 1989 Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfoleded state depends on two chaperonin proteins and Mg–ATP. *Nature* **342**, 884–889. (doi:10.1038/342884a0)

Goodson, H. V. & Hawse, W. F. 2002 Molecular evolution of the actin family. *J. Cell Sci.* **115**, 2619–2622.

Graceffa, P. & Dominguez, R. 2003 Crystal structure of monomeric actin in the ATP state. Structural basis of nucleotide-dependent actin dynamics. *J. Biol. Chem.* **278**, 34 172–34 180. (doi:10.1074/jbc.M303689200)

Hayer-Hartl, M. & Minton, A. P. 2006 A simple semiempirical model for the effect of molecular confinement upon the rate of protein folding. *Biochemistry* **45**, 13 356–13 360. (doi:10.1021/bi061597j)

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W. & Ellis, R. J. 1988 Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* **333**, 330–334. (doi:10.1038/333330a0)

Henzler-Wildman, K. A. *et al.* 2007 Intrinsic motions along an enzymatic reaction trajectory. *Nature* **450**, 838–844. (doi:10.1038/nature06410)

Horovitz, A. & Willison, K. R. 2005 Allosteric regulation of chaperonins. *Curr. Opin. Struct. Biol.* **15**, 646–651. (doi:10.1016/j.sbi.2005.10.001)

Horst, R., Fenton, W. A., Englander, S. W., Wuthrich, K. & Horwich, A. L. 2007 Folding trajectories of human dihydrofolate reductase inside the GroEL GroES chaperonin cavity and free in solution. *Proc. Natl Acad. Sci. USA* **104**, 20 788–20 792. (doi:10.1073/pnas.0710042105)

Horwich, A. L., Fenton, W. A., Chapman, E. & Farr, G. W. 2007 Two families of chaperonin: physiology and mechanism. *Annu. Rev. Cell Dev. Biol.* **23**, 115–145. (doi:10.1146/annurev.cellbio.23.090506.123555)

Hynes, G. M. & Willison, K. R. 2000 Individual subunits of the eukaryotic cytosolic chaperonin mediate interactions with binding sites located on subdomains of beta-actin. *J. Biol. Chem.* **275**, 18 985–18 994. (doi:10.1074/jbc.M910297199)

Isambert, H., Venier, P., Maggs, A. C., Fattoum, A., Kassab, R., Pantaloni, D. & Carlier, M. F. 1995 Flexibility of actin filaments derived from thermal fluctuations. Effect of bound nucleotide, phalloidin, and muscle regulatory proteins. *J. Biol. Chem.* **270**, 11 437–11 444. (doi:10.1074/jbc.270.19.11437)

Itzhaki, L. S., Otzen, D. E. & Fersht, A. R. 1995 Nature and consequences of GroEL–protein interactions. *Biochemistry* **34**, 14 581–14 587. (doi:10.1021/bi00044a037)

Jacob, E., Horovitz, A. & Unger, R. 2007 Different mechanistic requirements for prokaryotic and eukaryotic chaperonins: a lattice study. *Bioinformatics* **23**, i240–i248. (doi:10.1093/bioinformatics/btm180)

Jahn, T. R. & Radford, S. E. 2005 The Yin and Yang of protein folding. *FEBS J.* **272**, 5962–5970. (doi:10.1111/j.1742-4658.2005.05021.x)

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F. & Holmes, K. C. 1990 Atomic structure of the actin:DNase I complex. *Nature* **347**, 37–44. (doi:10.1038/347037a0)

Kafri, G. & Horovitz, A. 2003 Transient kinetic analysis of ATP-induced allosteric transitions in the eukaryotic chaperonin containing TCP-1. *J. Mol. Biol.* **326**, 981–987. (doi:10.1016/S0022-2836(03)00046-9)

Kasai, M., Nakano, E. & Oosawa, F. 1965 Polymerization of actin free from nucleotides and divalent cations. *Biochim. Biophys. Acta* **94**, 494–503. (doi:10.1016/0926-6585(65)90058-0)

Kikumoto, M., Tamura, Y., Ooi, A. & Mihashi, K. 2003 Partial specific volume and adiabatic compressibility of G-actin depend on the bound nucleotide. *J. Biochem.* **133**, 687–691. (doi:10.1093/jb/mvg088)

Kinosian, H. J., Selden, L. A., Estes, J. E. & Gershman, L. C. 1993 Nucleotide binding to actin. Cation dependence of nucleotide dissociation and exchange rates. *J. Biol. Chem.* **268**, 8683–8691.

Kinosian, H. J., Selden, L. A., Gershman, L. C. & Estes, J. E. 2004 Non-muscle actin filament elongation from complexes of profilin with nucleotide-free actin and divalent cation-free ATP-actin. *Biochemistry* **43**, 6253–6260. (doi:10.1021/bi036117s)

Klimov, D. K., Newfield, D. & Thirumalai, D. 2002 Simulations of beta-hairpin folding confined to spherical pores using distributed computing. *Proc. Natl Acad. Sci. USA* **99**, 8019–8024. (doi:10.1073/pnas.072220699)

Kovacs, I. A., Szalay, M. S. & Csermely, P. 2005 Water and molecular chaperones act as weak links of protein folding networks: energy landscape and punctuated equilibrium changes point towards a game theory of proteins. *FEBS Lett.* **579**, 2254–2260. (doi:10.1016/j.febslet.2005.03.056)

Kozuka, J., Yokota, H., Arai, Y., Ishii, Y. & Yanagida, T. 2006 Dynamic polymorphism of single actin molecules in the actin filament. *Nat. Chem. Biol.* **2**, 83–86. (doi:10.1038/nchembio763)

Kubota, H., Hynes, G. & Willison, K. 1995 The chaperonin containing t-complex polypeptide 1 (TCP-1). Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. *Eur. J. Biochem.* **230**, 3–16. (doi:10.1111/j.1432-1033.1995.tb20527.x)

Kuznetsova, I. M., Khaitlina, S., Konditerov, S. N., Surin, A. M. & Turoverov, K. K. 1988 Changes of structure and intramolecular mobility in the course of actin denaturation. *Biophys. Chem.* **32**, 73–78. (doi:10.1016/0301-4622(88)85035-X)

Kuznetsova, I. M., Biktashev, A. G., Khaitlina, S. Y., Vassilenko, K. S., Turoverov, K. K. & Uversky, V. N. 1999 Effect of self-association on the structural organization of partially folded proteins: inactivated actin. *Biophys. J.* **77**, 2788–2800.

Kuznetsova, I. M., Stepanenko, O. V., Povarova, O. I., Biktashev, A. G., Verkhusha, V. V., Shavlovsky, M. M. & Turoverov, K. K. 2002 The place of inactivated actin and its kinetic predecessor in actin folding-unfolding. *Biochemistry* **41**, 13 127–13 132. (doi:10.1021/bi026412x)

Lehrer, S. S. & Kerwar, G. 1972 Intrinsic fluorescence of actin. *Biochemistry* **11**, 1211–1217. (doi:10.1021/bi00757a015)

Lin, Z. & Rye, H. S. 2006 GroEL-mediated protein folding: making the impossible, possible. *Crit. Rev. Biochem. Mol. Biol.* **41**, 211–239. (doi:10.1080/10409230600760382)

Lin, P., Cardillo, T. S., Richard, L. M., Segel, G. B. & Sherman, F. 1997 Analysis of mutationally altered forms of the Cct6 subunit of the chaperonin from *Saccharomyces cerevisiae*. *Genetics* **147**, 1609–1633.

Lin, Z., Madan, D. & Rye, H. S. 2008 GroEL stimulates protein folding through forced unfolding. *Nat. Struct. Mol. Biol.* **15**, 303–311. (doi:10.1038/nsmb.1394)

Llorca, O., McCormack, E. A., Hynes, G., Grantham, J., Cordell, J., Carrascosa, J. L., Willison, K. R., Fernandez, J. J. & Valpuesta, J. M. 1999*a* Eukaryotic type II chaperonin CCT interacts with actin through specific subunits. *Nature* **402**, 693–696. (doi:10.1038/45294)

Llorca, O., Smyth, M. G., Carrascosa, J. L., Willison, K. R., Radermacher, M., Steinbacher, S. & Valpuesta, J. M. 1999*b* 3D reconstruction of the ATP-bound form of CCT reveals the asymmetric folding conformation of a type II chaperonin. *Nat. Struct. Biol.* **6**, 639–642. (doi:10.1038/10689)

Llorca, O., Martin-Benito, J., Grantham, J., Ritco-Vonsovici, M., Willison, K. R., Carrascosa, J. L. & Valpuesta, J. M. 2001 The 'sequential allosteric ring' mechanism in the eukaryotic chaperonin-assisted folding of actin and tubulin. *Embo J.* **20**, 4065–4075. (doi:10.1093/emboj/20.15.4065)

Lucent, D., Vishal, V. & Pande, V. S. 2007 Protein folding under confinement: a role for solvent. *Proc. Natl Acad. Sci. USA* **104**, 10 430–10 434. (doi:10.1073/pnas.0608256104)

McCallum, C. D., Do, H., Johnson, A. E. & Frydman, J. 2000 The interaction of the chaperonin tailless complex polypeptide 1 (TCP1) ring complex (TRiC) with ribosome-bound nascent chains examined using photo-cross-linking. *J. Cell Biol.* **149**, 591–602. (doi:10.1083/jcb.149.3.591)

McCormack, E. A., Llorca, O., Carrascosa, J. L., Valpuesta, J. M. & Willison, K. R. 2001*a* Point mutations in a hinge linking the small and large domains of beta-actin result in trapped folding intermediates bound to cytosolic chaperonin CCT. *J. Struct. Biol.* **135**, 198–204. (doi:10.1006/jsbi.2001.4385)

McCormack, E. A., Rohman, M. J. & Willison, K. R. 2001*b* Mutational screen identifies critical amino acid residues of beta-actin mediating interaction between its folding intermediates and eukaryotic cytosolic chaperonin CCT. *J. Struct. Biol.* **135**, 185–197. (doi:10.1006/jsbi.2001.4389)

Melki, R. & Cowan, N. J. 1994 Facilitated folding of actins and tubulins occurs via a nucleotide-dependent interaction between cytoplasmic chaperonin and distinctive folding intermediates. *Mol. Cell Biol.* **14**, 2895–2904.

Mendoza, J. A., Rogers, E., Lorimer, G. H. & Horowitz, P. M. 1991 Chaperonins facilitate the *in vitro* folding of monomeric mitochondrial rhodanese. *J. Biol. Chem.* **266**, 13 044–13 049.

Michie, K. A. & Lowe, J. 2006 Dynamic filaments of the bacterial cytoskeleton. *Annu. Rev. Biochem.* **75**, 467–492. (doi:10.1146/annurev.biochem.75.103004.142452)

Minton, A. P. 1983 The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences. *Mol. Cell Biochem.* **55**, 119–140. (doi:10.1007/BF00673707)

Minton, A. P. 1992 Confinement as a determinant of macromolecular structure and reactivity. *Biophys. J.* **63**, 1090–1100.

Nagy, B. & Strzelecka-Golaszewska, H. 1972 Optical rotatory dispersion and circular dichroic spectra of G-actin. *Arch. Biochem. Biophys.* **150**, 428–435. (doi:10.1016/0003-9861(72)90059-8)

Neirynck, K., Waterschoot, D., Vandekerckhove, J., Ampe, C. & Rommelaere, H. 2006 Actin interacts with CCT via discrete binding sites: a binding transition-release model for CCT-mediated actin folding. *J. Mol. Biol.* **355**, 124–138. (doi:10.1016/j.jmb.2005.10.051)

Neupert, W. & Brunner, M. 2002 The protein import motor of mitochondria. *Nat. Rev. Mol. Cell Biol.* **3**, 555–565. (doi:10.1038/nrm878)

Nolen, B. J. & Pollard, T. D. 2007 Insights into the influence of nucleotides on actin family proteins from seven structures of Arp2/3 complex. *Mol. Cell* **26**, 449–457. (doi:10.1016/j.molcel.2007.04.017)

Nowak, E., Strzelecka-Golaszewska, H. & Goody, R. S. 1988 Kinetics of nucleotide and metal ion interaction with G-actin. *Biochemistry* **27**, 1785–1792. (doi:10.1021/bi00405a060)

Orlova, A. & Egelman, E. H. 2000 F-actin retains a memory of angular order. *Biophys. J.* **78**, 2180–2185.

Orlova, A., Shvetsov, A., Galkin, V. E., Kudryashov, D. S., Rubenstein, P. A., Egelman, E. H. & Reisler, E. 2004 Actin-destabilizing factors disrupt filaments by means of a time reversal of polymerization. *Proc. Natl Acad. Sci. USA* **101**, 17 664–17 668. (doi:10.1073/pnas.0407525102)

Otterbein, L. R., Graceffa, P. & Dominguez, R. 2001 The crystal structure of uncomplexed actin in the ADP state. *Science* **293**, 708–711. (doi:10.1126/science.1059700)

Papoian, G. A., Ulander, J., Eastwood, M. P., Luthey-Schulten, Z. & Wolynes, P. G. 2004 Water in protein structure prediction. *Proc. Natl Acad. Sci. USA* **101**, 3352–3357. (doi:10.1073/pnas.0307851100)

Pappenberger, G., Wilsher, J. A., Roe, S. M., Counsell, D. J., Willison, K. R. & Pearl, L. H. 2002 Crystal structure of the CCTgamma apical domain: implications for substrate binding to the eukaryotic cytosolic chaperonin. *J. Mol. Biol.* **318**, 1367–1379. (doi:10.1016/S0022-2836(02)00190-0)

Pappenberger, G., McCormack, E. A. & Willison, K. R. 2006 Quantitative actin folding reactions using yeast CCT purified via an internal tag in the CCT3/gamma subunit. *J. Mol. Biol.* **360**, 484–496. (doi:10.1016/j.jmb.2006.05.003)

Park, E. S., Fenton, W. A. & Horwich, A. L. 2005 No evidence for a forced-unfolding mechanism during ATP/GroES binding to substrate-bound GroEL: no observable protection of metastable Rubisco intermediate or GroEL-bound Rubisco from tritium exchange. *FEBS Lett.* **579**, 1183–1186. (doi:10.1016/j.febslet.2005.01.013)

Povarova, O. I., Kuznetsova, I. M. & Turoverov, K. K. 2007 Different disturbances—one pathway of protein unfolding. Actin folding-unfolding and misfolding. *Cell Biol. Int.* **31**, 405–412. (doi:10.1016/j.cellbi.2007.01.025)

Prodromou, C. & Pearl, L. H. 2003 Structure and functional relationships of Hsp90. *Curr. Cancer Drug Targets* **3**, 301–323. (doi:10.2174/1568009033481877)

Ptitsyn, O. B. 1995 Molten globule and protein folding. *Adv. Protein Chem.* **47**, 83–229. (doi:10.1016/S0065-3233(08)60546-X)

Rathore, N., Knotts, T. A. & de Pablo, J. J. 2006 Confinement effects on the thermodynamics of protein folding: Monte Carlo simulations. *Biophys. J.* **90**, 1767–1773. (doi:10.1529/biophysj.105.071076)

Reisler, E. & Egelman, E. H. 2007 Actin structure and function: what we still do not understand. *J. Biol. Chem.* **282**, 36 133–36 137. (doi:10.1074/jbc.R700030200)

Richter, K. & Buchner, J. 2006 Hsp90: twist and fold. *Cell* **127**, 251–253. (doi:10.1016/j.cell.2006.10.004)

Rivenzon-Segal, D., Wolf, S. G., Shimon, L., Willison, K. R. & Horovitz, A. 2005 Sequential ATP-induced allosteric transitions of the cytoplasmic chaperonin containing TCP-1 revealed by EM analysis. *Nat. Struct. Mol. Biol.* **12**, 233–237. (doi:10.1038/nsmb901)

Rohman, M. J. 1999 Biochemical characterisation of chaperonin containing TCP1 (CCT). PhD thesis, University of London.

Saibil, H. R. *et al.* 1993 ATP induces large quaternary rearrangements in a cage-like chaperonin structure. *Curr. Biol.* **3**, 265–273. (doi:10.1016/0960-9822(93)90176-O)

Schuler, H., Lindberg, U., Schutt, C. E. & Karlsson, R. 2000 Thermal unfolding of G-actin monitored with the DNase I-inhibition assay stabilities of actin isoforms. *Eur. J. Biochem.* **267**, 476–486. (doi:10.1046/j.1432-1327.2000.01023.x)

Shimon, L., Hynes, G. M., McCormack, E. A., Willison, K. R. & Horovitz, A. 2008 ATP-induced allostery in the eukaryotic chaperonin CCT is abolished by the mutation G345D in CCT4 that renders yeast temperature-sensitive for growth. *J. Mol. Biol.* **377**, 469–477. (doi:10.1016/j.jmb.2008.01.011)

Shtilerman, M., Lorimer, G. H. & Englander, S. W. 1999 Chaperonin function: folding by forced unfolding. *Science* **284**, 822–825. (doi:10.1126/science.284.5415.822)

Spiess, C., Miller, E. J., McClellan, A. J. & Frydman, J. 2006 Identification of the TRiC/CCT substrate binding sites uncovers the function of subunit diversity in eukaryotic chaperonins. *Mol. Cell* **24**, 25–37. (doi:10.1016/j.molcel.2006.09.003)

Stemp, M. J., Guha, S., Hartl, F. U. & Barral, J. M. 2005 Efficient production of native actin upon translation in a bacterial lysate supplemented with the eukaryotic chaperonin TRiC. *Biol. Chem.* **386**, 753–757. (doi:10.1515/bc.2005.088)

Swint-Kruse, L. & Fisher, H. F. 2008 Enzymatic reaction sequences as coupled multiple traces on a multidimensional landscape. *Trends Biochem. Sci.* **33**, 104–112. (doi:10.1016/j.tibs.2007.12.001)

Takagi, F., Koga, N. & Takada, S. 2003 How protein thermodynamics and folding mechanisms are altered by the chaperonin cage: molecular simulations. *Proc. Natl Acad. Sci. USA* **100**, 11 367–11 372. (doi:10.1073/pnas.1831920100)

Tang, Y. C., Chang, H. C., Roeben, A., Wischnewski, D., Wischnewski, N., Kerner, M. J., Hartl, F. U. & Hayer-Hartl, M. 2006 Structural features of the GroEL-GroES nano-cage required for rapid folding of encapsulated protein. *Cell* **125**, 903–914. (doi:10.1016/j.cell.2006.04.027)

Thulasiraman, V., Yang, C. F. & Frydman, J. 1999 *In vivo* newly translated polypeptides are sequestered in a protected folding environment. *Embo J.* **18**, 85–95. (doi:10.1093/emboj/18.1.85)

Todd, M. J., Viitanen, P. V. & Lorimer, G. H. 1994 Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding. *Science* **265**, 659–666. (doi:10.1126/science.7913555)

Todd, M. J., Lorimer, G. H. & Thirumalai, D. 1996 Chaperonin-facilitated protein folding: optimization of rate and yield by an iterative annealing mechanism. *Proc. Natl Acad. Sci. USA* **93**, 4030–4035. (doi:10.1073/pnas.93.9.4030)

Tomkiewicz, D., Nouwen, N. & Driessen, A. J. 2007 Pushing, pulling and trapping-modes of motor protein supported protein translocation. *FEBS Lett.* **581**, 2820–2828. (doi:10.1016/j.febslet.2007.04.015)

Turoverov, K. K., Verkhusha, V. V., Shavlovsky, M. M., Biktashev, A. G., Povarova, O. I. & Kuznetsova, I. M. 2002 Kinetics of actin unfolding induced by guanidine hydrochloride. *Biochemistry* **41**, 1014–1019. (doi:10.1021/bi015548c)

Valpuesta, J. M., Martin-Benito, J., Gomez-Puertas, P., Carrascosa, J. L. & Willison, K. R. 2002 Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. *FEBS Lett.* **529**, 11–16. (doi:10.1016/S0014-5793(02)03180-0)

Valpuesta, J. M., Carrascosa, J. L. & Willison, K. R. 2005 Structure and function of the cytosolic chaperonin CCT. In *Protein folding handbook* (eds J. Buchner & T. Kiefhaber), pp. 725–755. Weinheim, Germany: Wiley-VCH.

van den Ent, F., Amos, L. A. & Lowe, J. 2001 Prokaryotic origin of the actin cytoskeleton. *Nature* **413**, 39–44. (doi:10.1038/35092500)

Villebeck, L., Moparthi, S. B., Lindgren, M., Hammarstrom, P. & Jonsson, B. H. 2007*a* Domain-specific chaperone-induced expansion is required for beta-actin folding: a comparison of beta-actin conformations upon interactions with GroEL and tail-less complex polypeptide 1 ring complex (TRiC). *Biochemistry* **46**, 12 639–12 647. (doi:10.1021/bi700658n)

Villebeck, L., Persson, M., Luan, S. L., Hammarstrom, P., Lindgren, M. & Jonsson, B. H. 2007*b* Conformational rearrangements of tail-less complex polypeptide 1 (TCP-1) ring complex (TRiC)-bound actin. *Biochemistry* **46**, 5083–5093. (doi:10.1021/bi062093o)

Weissman, J. S. 2001 The ins and outs of GroEL-mediated protein folding. *Mol. Cell* **8**, 730–732. (doi:10.1016/S1097-2765(01)00359-8)

Weissman, J. S., Kashi, Y., Fenton, W. A. & Horwich, A. L. 1994 GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell* **78**, 693–702. (doi:10.1016/0092-8674(94)90533-9)

Willison, K. R. 1999 Composition and function of the eukaryotic cytosolic chaperonin containing TCP-1. In *Molecular chaperones and folding catalysts: regulation, cellular function and mechanism* (ed. B. Bukau), pp. 555–573. Amsterdam, The Netherlands: Harwood Academic Publishers.

Wriggers, W., Chacón, P., Kovacs, J. A., Tama, F. & Birmanns, S. 2004 Topology representing neural networks reconcile biomolecular shape, structure, and dynamics. *Neurocomputing* **56**, 365–379. (doi:10.1016/j.neucom.2003.09.007)

Xu, Z., Horwich, A. L. & Sigler, P. B. 1997 The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex. *Nature* **388**, 741–750. (doi:10.1038/41944)

Zahn, R. & Pluckthun, A. 1994 Thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL. II. GroEL recognizes thermally unfolded mature beta-lactamase. *J. Mol. Biol.* **242**, 165–174. (doi:10.1006/jmbi.1994.1567)

Zhou, H. X. & Dill, K. A. 2001 Stabilization of proteins in confined spaces. *Biochemistry* **40**, 11 289–11 293. (doi:10.1021/bi0155504)