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Review: Allostery in Chaperonins

Amnon Horovitz,[1] Yael Fridmann, Galit Kafri, and Ofer Yifrach

Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel

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Chaperonins mediate protein folding in an ATP-dependent manner. ATP binding and hydrolysis by chaperonins are subject to both homotropic and heterotropic allosteric regulation. In the case of GroEL and CCT, homotropic regulation by ATP is manifested in nested cooperativity, which involves positive intra-ring cooperativity and negative inter-ring cooperativity in ATP binding. Both types of cooperativity are modulated by various heterotropic allosteric effectors, which include nonfolded proteins, ADP, Mg^{2+}, monovalent ions such as K^+, and cochaperonins in the case of type I chaperonins such as GroEL. Here, the allosteric properties of chaperonins are reviewed and new results of ours are presented with regard to allosteric effects of ADP. The role of allostery in the reaction cycle and folding function of chaperonins is discussed.

Key Words: **nested allostery; cooperativity; GroEL; chaperonins; protein machines.**

INTRODUCTION

Discussions of allosteric proteins usually focus on relatively small proteins such as hemoglobin (Perutz, 1989). Large macromolecular assemblies can, however, display allosteric properties that are not observed in small proteins. These unusual allosteric properties arise from interactions between allosteric units within the large assembly. Particularly interesting examples for large macromolecular assemblies that display unusual allosteric properties are the chaperonins, which consist of two rings, stacked back-to-back, with a cavity at each end (Ranson *et al.*, 1998; Sigler *et al.*, 1998; Gutsche *et al.*, 1999). In chaperonins, each ring is an allosteric unit that is often found to bind and hydrolyze ATP with positive intra-ring cooperativity (Gray and Fersht, 1991; Kafri *et al.*, 2001) and negative inter-ring cooperativity (Yifrach and Horovitz, 1995; Kafri *et al.*, 2001). Chaperonins are also interesting because they belong to a class of macromolecular assemblies collectively termed "protein machines" (Alberts, 1998). In such machines, conformational changes driven by nucleoside triphosphate binding and hydrolysis lead to organized spatial and temporal function. Chaperonins mediate protein folding in a MgATP-dependent manner (Ranson *et al.*, 1998; Sigler *et al.*, 1998; Gutsche *et al.*, 1999; Thirumalai and Lorimer, 2001). Cooperativity in ATP binding and hydrolysis by chaperonins reflects the switching of rings between protein-binding and release states and is important for regulation of their reaction cycle. In addition, it has been suggested that ATP binding provides the energy for forced unfolding of GroEL-bound misfolded protein substrates, thereby providing them with further opportunity to fold correctly (Shtilerman *et al.*, 1999). Cooperativity in ATP binding is likely to increase the mechanical force applied on the bound misfolded proteins. The purpose of this article is to review various aspects of the allosteric mechanisms of chaperonins and their roles in assisted protein folding. New results are presented with regard to the allosteric effects of ADP.

NESTED COOPERATIVITY IN CHAPERONINS

Nested cooperativity arises from interactions between allosteric units within a molecule. Nested allosteric models were first put forward in order to explain certain allosteric phenomena in hemoglobin (Wyman, 1967) but they are particularly useful in the case of large oligomeric proteins with hierarchical structure such as chaperonins. Chaperonins can be divided into two types: type I, found in eubacteria, mitochondria, and chloroplasts (Ranson *et al.*, 1998; Sigler *et al.*, 1998; Thirumalai and Lorimer, 2001), and type II, found in archaea and the eukaryotic cytosol (Gutsche *et al.*, 1999). Type I chaperonins, such as GroEL from *Escherichia coli*, consist of 14 identical subunits that form two heptameric rings (Braig *et al.*, 1994). Type I (but apparently not type II) chaperonins have helper proteins, such as

[1] To whom correspondence should be addressed. Fax: ++972 8 9344188. E-mail: Amnon.Horovitz@weizmann.ac.il.

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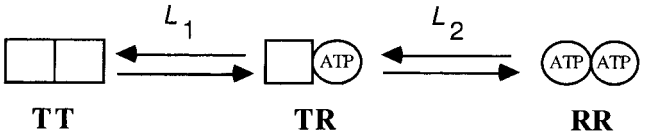

FIG. 1. Scheme for nested cooperativity in GroEL with respect to ATP. The scheme is based on both the MWC (Monod *et al.*, 1965) and the KNF (Koshland *et al.*, 1966) models of cooperativity. In the absence of ligands, GroEL is predominantly in the **TT** state. In the presence of ATP, the equilibrium is shifted toward the **TR** state (L_1 = [**TR**]/[**TT**]). A further shift in the equilibrium toward the symmetrical **RR** state occurs at higher ATP concentrations (L_2 = [**RR**]/[**TR**]). Analysis of the allosteric changes within each ring is based on the MWC model. Analysis of the interaction between rings is based on the KNF model since symmetry is broken; i.e., the changes that occur at the level of the double-ring are sequential and not concerted.

GroES from *E. coli*, that are seven-membered rings of identical subunits (Hunt *et al.*, 1996). Type II chaperonins consist of two eight- or nine-membered rings that are made up of two types of subunits in the case of the archaeal thermosome or eight different subunits in the case of the cytoplasmic eukaryotic chaperonin containing TCP-1 (CCT) (Gutsche *et al.*, 1999). The hierarchical structure of chaperonins implies that a corresponding hierarchy in their allosteric interactions may exist.

Measurements of initial rates of ATP hydrolysis by GroEL at different concentrations of ATP showed that GroEL undergoes two ATP-induced allosteric transitions and suggested a nested allosteric model for cooperativity in ATP hydrolysis by GroEL (Yifrach and Horovitz, 1995). According to this model, there are two levels of allostery in GroEL: one within each ring and the second between the two rings (Fig. 1). In the first level of allostery, each heptameric ring is in equilibrium between **T** and **R** states that interconvert in a concerted manner, in accordance with the MWC representation (Monod *et al.*, 1965). In the absence of ligands, GroEL is predominantly in the **TT** state. In the presence of ATP, the equilibrium is shifted toward the **TR** state (L_1 = [**TR**]/[**TT**]). The transition of GroEL from the **TT** state to the **TR** state is reflected by positive cooperativity in ATP binding and hydrolysis by GroEL at relatively low concentrations of ATP ($\leq$100 μM) in the absence of GroES (Gray and Fersht, 1991; Bochkareva *et al.*, 1992; Jackson *et al.*, 1993). This positive cooperativity is due to effects on binding (K-system) and not catalysis (V-system) (Bochkareva *et al.*, 1992; Jackson *et al.*, 1993) although V-system effects on the ATPase activity of GroEL are present (see below). Owing to inter-ring negative cooperativity, a further shift in the equilibrium from the **TR** state to the **RR** state (L_2 = [**RR**]/[**TR**]) takes place only at higher ATP concentrations ($>$100 μM). The second level of allostery is between the two rings of GroEL, which undergoes sequential KNF-type transitions (Koshland *et al.*, 1966) from the **TT** state via the asymmetric **TR** state to the **RR** state. The two levels of allostery in GroEL correspond, therefore, to its hierarchical double-ring structure.

Previously developed nested allosteric models include some that are based only on the MWC formalism (Robert *et al.*, 1987) and others in which KNF-type allosteric transitions are nested inside MWC transitions (Brunori *et al.*, 1986). In the model for GroEL, MWC-type interactions are nested inside KNF transitions. CCT also undergoes two ATP-induced allosteric transitions that reflect the presence of nested cooperativity (Kafri *et al.*, 2001). Genetic evidence (Lin and Sherman, 1997) and the fact that the eight different subunits of CCT rings are arranged in a fixed permutation (Liou and Willison, 1997) have led to the proposal that ATP-induced conformational changes in CCT progress sequentially around the ring in a KNF manner. Nested cooperativity in CCT may, therefore, require description only in terms of the KNF model but direct biochemical evidence for this is still lacking. Nested allosteric behavior may be common to other chaperone rings (Horwich *et al.*, 1999) but appears to be absent in the thermosome (Gutsche *et al.*, 2000).

Additional support for the nested model has come from transient kinetic analysis of the ATP-induced allosteric transitions of GroEL. Plots of the observed rate constants of the ATP-induced conformational changes of the GroEL mutants Phe44Trp (Yifrach and Horovitz, 1998a) and Tyr485Trp (Cliff *et al.*, 1999) show a bisigmoidal dependence on ATP concentration. Cliff *et al.* (1999) have extended the nested model by including several different **R** states (see below). Experimental evidence in support of the nested allosteric model for chaperonins has also come from cryo-EM work. The **TT**, **TR**, and **RR** states of wild-type GroEL and the Arg197Ala mutant have been visualized at 28-Å resolution (Roseman *et al.*, 1996; White *et al.*, 1997). Corresponding structures have also been observed in the case of the thermosome (Schoen *et al.*, 2000) in apparent conflict with the kinetic measurements (Gutsche *et al.*, 2000). In the case of CCT, ATP binding was found to generate an asymmetric particle in which one ring has a slightly different conformation from the apo-ring and the other ring (most likely the ATP-bound ring) has undergone substantial movements in the apical and equatorial domains (Llorca *et al.*, 1999). A symmetric form of CCT in which both rings have undergone ATP-induced conformational changes has not yet been reported, in agreement with the strong negative cooperativity in this system (see below). In the case of type II chaperonins, the nota-

tion of **T** and **R** is not used owing to uncertainty regarding the concerted nature of their allosteric transitions.

HILL COEFFICIENTS OF CHAPERONINS

A frequently used measure for the extent of cooperativity is the value of the Hill coefficient, n_H, which is obtained from steady-state kinetics by fitting plots of initial reaction velocity, or fractional saturation of binding sites, as a function of the ligand (substrate) concentration to the Hill equation (Hill, 1910). Values of Hill coefficients can also be determined from transient kinetics by fitting plots of the rate constants of ligand-induced allosteric transitions as a function of the ligand concentration to a Hill-type equation (Yifrach and Horovitz, 1998a). The theoretical range of values of n_H in a positively cooperative molecule with n sites is $1 < n_H \leq n$. A distinguishing feature of large allosteric systems is that $n_H \ll n$. In these systems, the low values of n_H often reflect cooperativity in ligand binding by the allosteric unit and not the full macromolecular assembly. For example, the value of n_H for *Octopus* hemocyanin, which contains 70 oxygen binding sites (seven in each of its 10 polypeptide chains), is only between 3 and 5 (van Holde *et al.*, 2000). Low values of n_H are observed also in the case of chaperonins. The value of n_H for the first allosteric transition of GroEL is about 2.5 (Gray and Fersht, 1991; Bochkareva *et al.*, 1992; Todd *et al.*, 1993; Yifrach and Horovitz, 1995). The value of n_H in the case of the first allosteric transition of CCT is only $\approx$2 (Kafri *et al.*, 2001), whereas the thermosome appears to display even slight negative cooperativity ($n_H < 1$) (Gutsche *et al.*, 2000). The low values of n_H in the case of chaperonins thus reflect cooperativity in ATP binding by the allosteric unit (which corresponds to a ring) and not the full double-ring assembly.

In the case of type II chaperonins, the low values of n_H may also be due to heterogeneity in their subunit composition. In the absence of positive cooperativity, differences in the intrinsic affinities for ATP of the different subunits in a ring will lead to apparent negative cooperativity. If positive cooperativity is present, differences in the intrinsic affinities for ATP of the different subunits in a ring will lower the value of n_H, thus partially or fully masking the positive cooperativity and, perhaps, even suggesting the existence of negative cooperativity (Kafri *et al.*, 2001). That may explain why slight negative cooperativity is observed in the case of the archaeal chaperonin (Gutsche *et al.*, 2000).

Recently, a functional explanation for the relatively low values of n_H of chaperonins was also suggested (Yifrach and Horovitz, 2000). The relationship between the extent of positive cooperativity in ATP binding by GroEL and the rate of GroE-assisted folding of mouse dihydrofolate reductase (mDHFR) was examined using mutants of GroEL with altered allosteric properties (Yifrach and Horovitz, 2000). The rate of GroEL-assisted folding of mDHFR was found to be faster when the value of n_H is lower, owing to a faster transition between the protein-acceptor **T** state and the protein-release **R** state. High values of n_H may thus lead to folding rates that are too slow on a biologically relevant time scale, thereby suggesting a functional advantage for the relatively low values of n_H of chaperonins.

INTRA-RING ALLOSTERY

A prediction of the nested allosteric model for GroEL was that the single-ring forms of this molecule undergo only one ATP-induced allosteric transition. This prediction was consistent with the observation that only one allosteric transition takes place in the presence of GroES when one ring is GroES-bound and, thus, not active under steady-state conditions (Yifrach and Horovitz, 1994). More recently, measurements of initial rates of ATP hydrolysis by SR1, a single-ring version of GroEL (Weissman *et al.*, 1995), at different concentrations of ATP up to 0.5 mM showed that SR1 undergoes only one allosteric transition (Inobe *et al.*, 2001). The prediction was also confirmed by experiments on a chimera of GroEL that is in equilibrium between the single-ring and the double-ring forms, which was generated by replacing the amino acid sequence of GroEL from position 365 to the end with the sequence of the GroEL homologue cpn60-1 from *Rhizobium leguminosarum*. This chimera was found to undergo only one ATP-induced allosteric transition when present at relatively low concentrations and, thus, predominantly in the single-ring form. At higher protein concentrations, when the equilibrium was shifted toward the double-ring form, the GroEL chimera was found to undergo two ATP-induced allosteric transitions (Erbse *et al.*, 1999). A version of SR1 containing the mutation Glu191Gly was also reported to display biphasic ATPase activity when present at a relatively high concentration (Chatellier *et al.*, 2000).

For simplicity, it was assumed in the original formulation of the nested model for GroEL (Yifrach and Horovitz, 1995) that the ATP-induced allosteric transitions of each ring are concerted (Monod *et al.*, 1965). In support of this assumption, normal mode analysis of the conformational dynamics of GroEL has shown that steric repulsions would arise if one subunit changed its conformation while its neighbors had not (Ma and Karplus, 1998; Ma *et al.*,

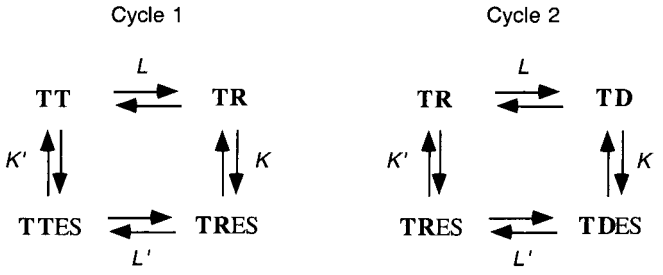

FIG. 2. Scheme for different states of GroEL in the presence of ATP, ADP, and GroES. In cycle 1, GroEL is predominantly in the **TT** state in the absence of ATP. In the presence of ATP, the equilibrium is shifted toward the **TR** state (L = [**TR**]/[**TT**]). GroES binds to the **TT** state and the ring in the **R** conformation of the **TR** state with affinities K' and K, respectively. The allosteric constant for the transition **TT**ES → **TR**ES is L'. In cycle 2, **D** designates an ADP-bound state. GroES binds to the **R** ring in the **TR** state and the **D** ring in the **TD** state with affinities K' and K, respectively. The allosteric constants for the transitions **TR** → **TD** and **TR**ES → **TD**ES are L and L', respectively. For simplicity, the notation of **T**, **D**, and **R** is used to designate the apo states and all the ADP- and ATP-bound states of one ring of GroEL, respectively.

2000). Recently, it was shown that a value of 1 for the ratio between the values of the Hill coefficients determined from steady-state data and transient kinetic data is evidence for the concerted nature of an allosteric transition (Horovitz and Yifrach, 2000). A value of about 1 for this ratio was observed for a series of GroEL mutants (Yifrach and Horovitz, 1998b), thus indicating that the allosteric transitions of GroEL are indeed concerted. Independent experimental evidence for the concerted nature of the allosteric transitions has been obtained by generating the D83C, K327C double mutant of GroEL and showing that a disulfide cross-link at these positions in only one subunit of each ring is sufficient to block its **T** → **R** allosteric transition (G. Curien and G. H. Lorimer, unpublished results). This conclusion is also supported by the recent analysis of the ATPase activity of mixed hybrids of wild-type GroEL and the D83C, K327C double mutant (Shiseki *et al.*, 2001). The nature of intra-ring allosteric transitions of type II chaperonins remains to be established.

The extent of intra-ring allostery in the GroES-bound ring of GroEL cannot be measured using steady-state kinetics but can be deduced by considering the thermodynamic cycles in Fig. 2. Owing to free energy conservation, one has $L/L' = K'/K$. In the case of cycle 1, $K' < K$ since GroES has very weak affinity for GroEL in the absence of ATP (Behlke *et al.*, 1997). It follows, therefore, that $L < L'$; i.e., GroES reduces the cooperativity of the **T** → **R** transition of the GroES-bound ring. In the case of cycle 2, $K' > K$ since GroEL has higher affinity for GroES in the presence of ATP than ADP (Jackson *et al.*, 1993; Behlke *et al.*, 1997). It follows, therefore, that $L > L'$; i.e., GroES increases the cooperativity in the structural transition involved with ATP hydrolysis. This is reflected in the quantal nature of ATP hydrolysis by GroEL in the presence of GroES (Todd *et al.*, 1994).

INTER-RING ALLOSTERY

Inter-ring negative cooperativity in ATP binding by GroEL and CCT is reflected in the fact that the midpoints of the two allosteric transitions are at different ATP concentrations. A measure of the inter-ring cooperativity is $\ln(L_1/L_2)$ (Fig. 1). Inter-ring negative cooperativity is absent when $L_1 = L_2$ as found in the case of the Arg13Gly, Ala126Val GroEL double mutant (Aharoni and Horovitz, 1996). In the case of CCT, the data have been fitted to the model-independent Hill equation and negative cooperativity is reflected in the large difference between the values of the Hill coefficients of the first and second transitions, which are about 2 and 8, respectively (Kafri *et al.*, 2001). In the case of GroEL, communication between rings is also reflected in the lower k_{cat} of ATP hydrolysis of subunits in the **R** conformation of the **RR** state relative to the **TR** state. The former state is, therefore, often designated **R′R′** (Yifrach and Horovitz, 1994). The lower k_{cat} of ATP hydrolysis by GroEL in the presence of GroES (Gray and Fersht, 1991) is another reflection of this inter-ring communication. This manifestation of communication between rings, in the presence of ATP, is absent in the Arg13Gly, Ala126Val GroEL mutant (Aharoni and Horovitz, 1996) and in CCT (Kafri *et al.*, 2001).

A common misconception is that GroES increases cooperativity in ATP hydrolysis by GroEL with respect to ATP. This misconception is due to the fact that the value of n_H for the plot of initial rates of ATP hydrolysis by GroEL, as a function of ATP concentration, is increased in the presence of GroES (Gray and Fersht, 1991). This comparison of the values of n_H is misleading since n_H in the absence of GroES is for the transition of the first ring of GroEL from **T** to **R** (i.e., for the process **TT** → **TR**), whereas n_H in the presence of GroES is mainly for the transition of the second ring of GroEL from **T** to **R** (i.e., for the process **TR**ES → **RR**ES). Instead of comparing the values of n_H in the absence and in the presence of GroES, one should compare the values of L_2 and L_2', which correspond to the transition from **T** to **R** of the second ring of GroEL, either in the absence or in the presence of GroES, respectively (Fig. 3). The value of the allosteric constant L_2' was found to be about 10^{-5} (Inbar and Horovitz, 1997), which is

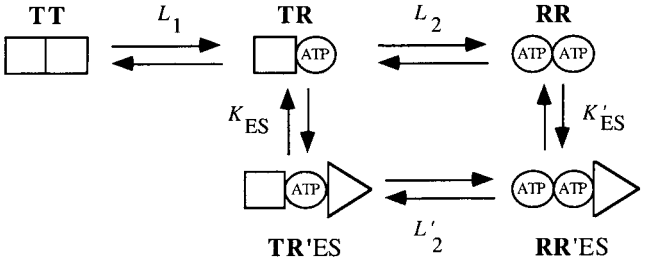

FIG. 3. Scheme for different states of GroEL in the presence of ATP and GroES. In the absence of ATP, GroEL is predominantly in the **TT** state. In the presence of ATP, the equilibrium is shifted toward the **TR** and **RR** states (L_1 = [**TR**]/[**TT**] and L_2 = [**RR**]/[**TR**]). GroES binds to the ring in the **R** conformation of the **TR** and **RR** states with affinities K_{ES} (= [**TR**′ES]/[**TR**][ES]) and K'_{ES} (=[**RR**′ES]/[**RR**][ES]), respectively. In the presence of GroES and ATP, the equilibrium is shifted toward the **RR**′ES state (L'_2 = [**RR**′ES]/[**TR**′ES]). For simplicity, the notation of **T** and **R** is used to designate all the low- and high-affinity states for ATP of one ring of GroEL. **R**′ designates the conformation of a ring bound to ATP and GroES. The symmetric football-shaped GroEL is not included in this scheme. A measure of the effect of GroES on the allosteric transition of the distal ring of GroEL is given by $\Delta\Delta G = -RT \ln (L'_2/L_2)$.

four orders of magnitude larger than the value of the allosteric constant L_2, which is about 10^{-9} (Yifrach and Horovitz, 1995). This shows that GroES promotes the **T** to **R** transition of the GroEL ring distal to GroES in the GroEL–GroES complex. This result is consistent with the observation that the affinity for nonfolded polypeptides of the distal ring (the *trans* ring) in GroEL–GroES–ATP_7 "bullet" complexes is low (Rye *et al.*, 1999). GroES may, therefore, facilitate release of protein substrates bound to that ring and thus assist (i) folding of proteins too large to be accommodated in the cavity underneath GroES and/or (ii) degradation of damaged proteins that are GroEL-bound and cannot reach the native state. In addition, promotion by GroES of the **T** to **R** transition of the GroEL ring distal to GroES may facilitate GroEL–$GroES_2$ "football" formation (Azem *et al.*, 1994b), which is not favored owing to negative inter-ring cooperativity in GroEL with respect to ATP.

Allosteric theory predicts that if one ligand affects the cooperative binding of another ligand, then the converse will also be true since the same allosteric states are involved via coupled equilibria (see, for example, Weber, 1975). Owing to free energy conservation in the cycle in Fig. 3, one has $L_2/L'_2 = K_{ES}/K'_{ES}$. From this relation and the finding that $L_2 \ll L'_2$ it follows that $K_{ES} \ll K'_{ES}$; i.e., the affinity of GroES for the ring in the **R** conformation of the **TR** state is significantly lower than for the rings in the **R** conformation of the **RR** state. The ring in the **R** conformation in the **TR** state differs, therefore, from the rings in the **RR** state both in the affinity for GroES and in the rate constant for ATP hydrolysis. The observation that ATP binding to the *trans* ring is not by itself sufficient to trigger GroES release from the *cis* ring (Rye *et al.*, 1997) is, therefore, predicted from the cycle in Fig. 3. Inter-ring allostery is, thus, critical for continuous cycling of GroEL rings between protein acceptor and release states. In its absence, as in the case of SR1 (Weissman *et al.*, 1995), release of GroES and protein substrates is blocked. The role of inter-ring allostery in type II chaperonins, which appear to function without a cochaperonin, is not known.

ALLOSTERIC EFFECTS OF ADP, K^+, AND Mg^{2+}

Cryo-EM work has shown that ADP stabilizes a (**D**) conformation of GroEL rings that is distinct from the ATP-bound (**R**) and apo (**T**) conformations (Roseman *et al.*, 1996). In addition, a specific effect of ADP on GroEL-assisted folding of tryptophanase has been observed (Mizobata *et al.*, 1992). The above findings suggest that the ADP-induced conformational changes should be reflected in positive cooperativity in ADP binding by GroEL, with respect to ADP. In contrast, it has been reported that ADP (but not ATP) binding to GroEL is noncooperative (Cliff *et al.*, 1999; Inobe *et al.*, 2001), implying that ADP binds preferentially to the **T** state or that it binds with equal affinities to the **T** and **D** states. We decided to analyze the properties of ADP as an allosteric effector by measuring its effect on the ATPase activity of GroEL. This analysis is complicated because three separate issues must be considered: (i) preferential binding of ADP to the **T**, **D**, or **R** states; (ii) competition between ADP and ATP for the same binding sites; and (iii) inter-ring communication.

Two lines of evidence indicate that ADP binds preferentially to the **R** state or the **D** state, which is presumed to have a stability that is intermediate between those of the **T** and **R** states. First, the values of n_H for the first transition **TT** → **TR** are found to decrease with increasing concentrations of ADP or ATPγS from 2.8 to about 1.5 and 1, respectively (Fig. 4A). This result suggests that ADP and ATPγS shift the equilibrium from the **TT** state to the **TD** and **TR** states, respectively. Second, in the presence of 10 but not 100 μM ATP, low concentrations of ADP stimulate the ATPase activity of GroEL, whereas high concentrations of ADP inhibit its activity (Fig. 5). This finding suggests that at low ATP concentrations, ATP binding and hydrolysis are stimulated by low concentrations of ADP that shift the equilibrium toward the **D** or **R** states, whereas high concentrations of ADP block ATP binding by competition for the same sites. Such an effect was previously observed in the case of aspartate trans-

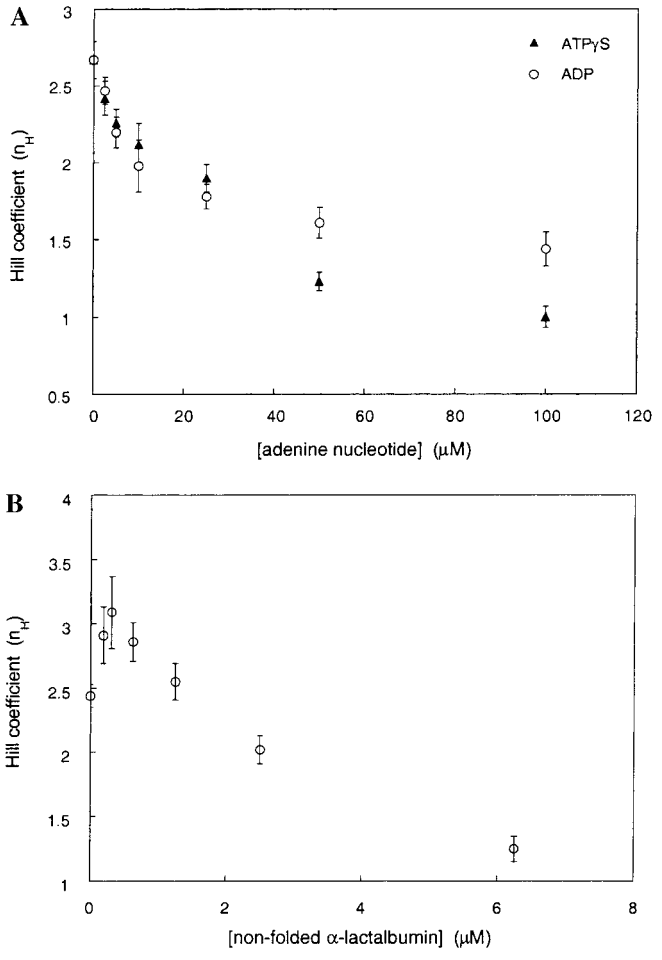

FIG. 4. Values of the Hill coefficient for the first allosteric transition of GroEL in the presence of different concentrations of ADP and ATPγS (A) or nonfolded α-lactalbumin (Yifrach and Horovitz, 1996) (B). The values of the Hill coefficients (± standard errors) were estimated by fitting to the Hill equation initial rates of ATP hydrolysis as a function of ATP concentration in the presence of different fixed concentrations of ADP, ATPγS, or nonfolded α-lactalbumin. The oligomer concentration of GroEL is 25 nM. The reactions were carried out at 25°C as described (Horovitz *et al.*, 1993) in the presence of 10 mM (A) or 50 mM K^+ ions (B).

carbamylase (Gerhart and Pardee, 1963). The results in Figs. 4A and 5 are, therefore, consistent with preferential binding of ADP to the **D** or **R** states. ADP binding may appear to be noncooperative (Cliff *et al.*, 1999; Inobe *et al.*, 2001) if the affinity of ADP for the **D** or **R** states is not much higher than for the **T** state.

Although ADP decreases the cooperativity of the transition **TT** → **TR**, it is found to increase the cooperativity of the transition **TR** → **RR** (data not shown). The opposite effect of ADP on the cooperativity of the two transitions strongly suggests that it binds preferentially to a **D** (and not **R**) state. This causes negative cooperativity between rings, with respect to ATP, to increase in the presence of increasing concentrations of ADP (Fig. 6), in agreement with transient kinetic analysis of ADP effects by Kad *et al.* (1998). Strong negative inter-ring allostery in the presence of ADP helps to ensure that the two rings of GroEL are not in phase and thus function in a reciprocal manner. Much further work is required in order to determine the thermodynamic and kinetic constants for the ADP-induced allosteric transitions of GroEL. In the case of CCT, ADP binding appears to cause little if any structural change (Llorca *et al.*, 1998). Virtually nothing is known about the thermodynamics and kinetics of ADP-induced allosteric transitions in type II chaperonins.

The ATPase activity of GroEL is dependent on K^+ or certain other monovalent ions (Viitanen *et al.*, 1990). ATP hydrolysis by type II chaperonins is also modulated by certain monovalent ions but often in a

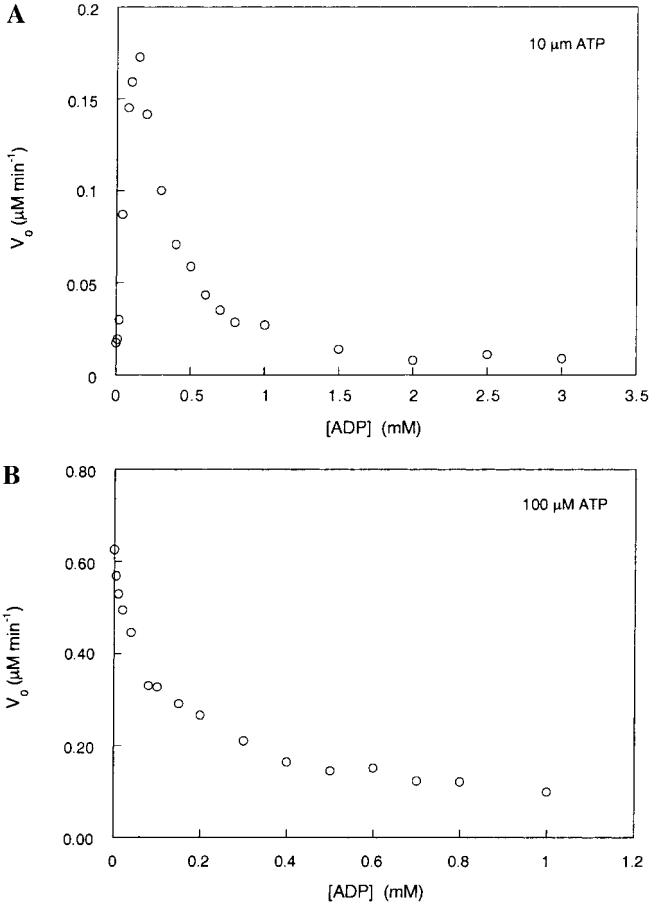

FIG. 5. Initial velocity of ATP hydrolysis by GroEL at fixed initial concentrations of 10 μM (A) or 100 μM ATP (B) in the presence of different concentrations of ADP. The oligomer concentration of GroEL is 25 nM. The reactions were carried out at 25°C as described (Horovitz *et al.*, 1993).

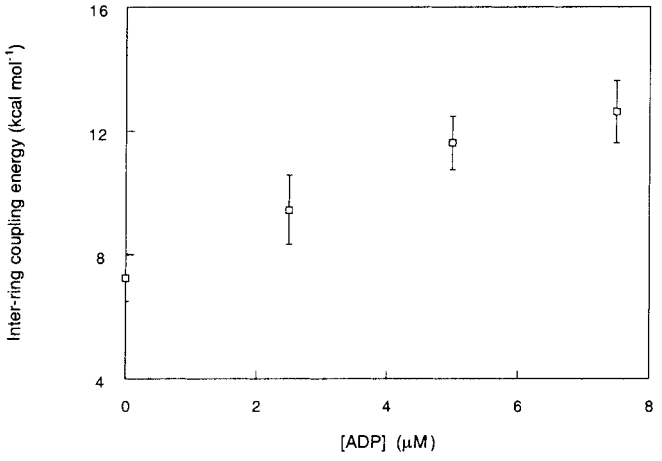

FIG. 6. Inter-ring coupling energy as a function of the concentration of ADP. Initial rates of ATP hydrolysis by GroEL were measured at different concentrations of ATP in the presence of different fixed concentrations of ADP. The data were fitted to Eq. (10) in Yifrach and Horovitz (1995). The inter-ring coupling energy was calculated using the relation $\Delta G = -RT\ln(L_1/L_2)$, where L_1 and L_2 are here the apparent allosteric constants for the first and second transitions in the presence of ADP. Experiments were carried out at 25°C in the presence of 25 nM GroEL as described (Horovitz *et al.*, 1993).

less stringent manner (Gutsche *et al.*, 1999). Cooperativity in ATP hydrolysis by GroEL, with respect to ATP, is decreased in the presence of increasing K^+ and, at low ATP concentrations, is cooperative with respect to K^+ (Todd *et al.*, 1993). In the presence of K^+, a noncooperative Mg^{2+}-induced conformational change in GroEL becomes cooperative (Clark *et al.*, 1999). Little is known about the allosteric effects of K^+ (or other monovalent ions) on the ATPase activity of type II chaperonins. The structural basis of the nucleotide-independent Mg^{2+}-induced structural change in GroEL (Azem *et al.*, 1994a; Clark *et al.*, 1999) and of the effects of monovalent ions on the ATPase activity of type I and type II chaperonins is not known.

ALLOSTERIC EFFECTS OF NONFOLDED PROTEINS

Allosteric theory is based on equilibrium assumptions that are not valid in the case of chaperone-assisted (or nonassisted) folding reactions. Such reactions are usually initiated by rapid transfer of the nonfolded protein from unfolding to refolding conditions and are, thus, far from equilibrium. In order to analyze the allosteric effects of nonfolded proteins it was necessary to find a substrate that would remain nonfolded under the folding conditions in which the ATPase reactions are carried out. α-Lactalbumin was found to be a suitable protein substrate for this purpose since, after removal of Ca^{2+} ions and reduction, it remains nonfolded under the folding conditions of the experiment. Cooperativity in ATP hydrolysis by GroEL, with respect to ATP, was measured in the absence and presence of different concentrations of nonfolded α-lactalbumin (Yifrach and Horovitz, 1996). An increasing and then decreasing relationship was found between the value of the Hill coefficient of the transition **TT** → **TR** and the concentration of nonfolded α-lactalbumin (Fig. 4B). In the case of exclusive binding of ATP to the **R** state, preferential binding of nonfolded proteins to the **T** state will shift the equilibrium in favor of the **TT** and **TR** states, thereby increasing cooperativity in ATP hydrolysis. Preferential binding of nonfolded proteins to the **R** state will shift the equilibrium in favor of the **TR** and **RR** states, thereby decreasing cooperativity in ATP hydrolysis. If, however, ATP binds preferentially but not exclusively to the **R** state, then the observed increasing and then decreasing relationship between the value of Hill coefficient of the transition **TT** → **TR** and the concentration of nonfolded α-lactalbumin is expected (Rubin and Changeux, 1966). The reason for this is that in the presence of sufficiently high concentrations of nonfolded protein, no cooperative transition takes place and ATP hydrolysis is due mainly to the **TT** state. These results showed that each ring of GroEL is in equilibrium between a **T** state, with high affinity for nonfolded proteins and low affinity for ATP, and an **R** state, with high affinity for ATP and low affinity for nonfolded proteins (Yifrach and Horovitz, 1996). This in agreement with the finding that ATP reduces the affinity of GroEL for protein substrates (Staniforth *et al.*, 1994).

The structural basis for the different affinities of the **T** and **R** states of GroEL for nonfolded proteins is fairly well understood. In the **T** state, the lining of the GroEL cavity is hydrophobic and binding of nonfolded proteins with exposed hydrophobic patches is, therefore, favored. Upon ATP binding, the lining of the GroEL cavity becomes more hydrophilic (Roseman *et al.*, 1996; White *et al.*, 1997), thereby facilitating protein release and folding. The hydrophobic–hydrophilic switch is most pronounced when the *cis* and *trans* rings of the GroEL–GroES–$(ADP)_7$ bullet complex are compared (Xu *et al.*, 1997). The rate constant of the **T** → **R** (hydrophobic to hydrophilic) transition was found to be about 1 s^{-1} in the absence of ATP and 200 s^{-1} in the presence of high concentrations of ATP (Yifrach and Horovitz, 1998a). Slower rates of the **T** → **R** transition, in the case of strong intra-ring positive cooperativity, were found to lead to slower rates of protein release and folding (Yifrach and Horovitz, 2000). Simulations of the effects of different cycling times between the **T** and the **R** states on the rate of folding have yielded similar results (Betancourt and Thirumalai, 1999).

CCT (Melki and Cowan, 1994) and the thermosome (Guagliardi *et al.*, 1994) were also found to switch from a high- to a low-affinity state for unfolded polypeptide substrates upon ATP binding. It is not clear, however, whether ATP-induced allosteric transitions in type II chaperonins involve a hydrophobic–hydrophilic switch, as observed in the case of GroEL. The role of hydrophobicity in protein substrate binding to type II chaperonins is also not fully established. Surprisingly, the cavity of the apoform of the thermosome (Ditzel *et al.*, 1998) was found to be similar to that of the *cis* ring in the GroEL–GroES–$(ADP)_7$ complex (Xu *et al.*, 1997) with respect to its hydrophilicity, perhaps owing to the crystallization conditions.

Nonfolded proteins were also found to stimulate the ATPase activity of GroEL (Martin *et al.*, 1991; Jackson *et al.*, 1993; Yifrach and Horovitz, 1996) and CCT (Melki and Cowan, 1994). In the case of GroEL, this effect is due to (i) a higher k_{cat} of GroEL rings in the **T** state and (ii) a shift in the equilibrium from the **RR** state toward the more active **TR** state. The three- to fourfold stimulation of GroEL's ATPase activity (Yifrach and Horovitz, 1996) is in good agreement with a direct measurement of the relative rate constants of ATP hydrolysis of the **T** and **R** states (G. Curien and G. H. Lorimer, unpublished results). In the case of GroEL, binding of polypeptide substrate to the *trans* ring was found to stimulate the ATP-dependent disassembly of the *cis* complex (Rye *et al.*, 1999). The mechanism of this aspect of inter-ring allostery is not clear.

STRUCTURAL BASIS OF ALLOSTERIC TRANSITIONS IN CHAPERONINS

Insight into the structural basis and dynamics of the allosteric transitions of GroEL has been derived from the crystal structure of the GroEL–GroES–$(ADP)_7$ asymmetric complex (Xu *et al.*, 1997) and its comparison with the structures of other stable states (Braig *et al.*, 1994; Boisvert *et al.*, 1996; Roseman *et al.*, 1996). The trajectory of the conformational change of a GroEL subunit was simulated (Ma *et al.*, 2000) using targeted molecular dynamics in which the ATPγS-bound structure (Boisvert *et al.*, 1996) was a starting point and the structure was "pulled" toward the conformation of the *cis* ring in the asymmetric complex (Xu *et al.*, 1997). The simulations suggested that the allosteric transition can be divided into two stages designated t to r′ and r′ to r″. The first stage begins with a downward motion of the intermediate domain, which brings Asp398 in helix M into the coordination sphere of the ATP-bound Mg^{2+} and closes off the ATP binding pocket, thus enabling hydrolysis (a similar ATP-induced conformational change takes place in the thermosome (Ditzel *et al.*, 1998)). It is completed by a small upward motion and clockwise twist of the apical domain, which leads to enlargement and a less hydrophobic surface of the cavity. The simulations show that the inter-subunit salt bridge between Arg197 and Glu386 is broken in the t to r′ transition. This finding is in agreement with a ϕ value analysis that showed that the Arg197–Glu386 interaction is broken in the transition state of the **T** → **R** reaction (Yifrach and Horovitz, 1998b).

The second stage of the transition, r′ to r″, involves an upward tilt of nearly 60° of the apical domains and a continuation of their clockwise motion, which results in an overall rotation of 90°. The simulations of Ma *et al.* (2000) suggest that the structural changes in the r′ to r″ transition are an extension (or completion) of those that take place in the t to r′ transition. This finding is in conflict with the observation of an anticlockwise rotation of the apical domains in the **R** state (White *et al.*, 1997) and with results of other simulations (de Groot *et al.*, 1999). In the simulations of Ma *et al.* (2000), the ATPγS-bound structure of the Arg13Gly, Ala126Val double mutant represents the **TT** state since surprisingly little difference was found between the crystal structures of this mutant in the absence of ATPγS (Braig *et al.*, 1994) and in complex with ATPγS (Boisvert *et al.*, 1996), in contrast with cryo-EM (Roseman *et al.*, 1996) and numerous biochemical studies. The reasons for this discrepancy may be related to crystal packing forces, the choice of an inappropriate ATP analogue, or the altered allosteric properties of the double mutant (Aharoni and Horovitz, 1996). A crystal structure of wild-type GroEL in the apo state and a high-resolution EM structure of wild-type GroEL in complex with ATP are needed in order to better characterize the t to r′ transition.

The simulations of Ma *et al.* (2000) indicate that intra-ring positive cooperativity in GroEL is due to (i) steric clashes that arise if one subunit changes conformation from t to r′ and its neighbor to the right does not and (ii) breaking of the Arg197–Glu386 interaction with both the left and the right neighboring subunits. Inter-ring negative cooperativity in GroEL is less well understood. The simulations of de Groot *et al.* (1999) showed that the modes of motion in the t to r′ and r′ to r″ transitions are coupled, in agreement with experimental results showing that the Arg197Ala mutation, for example, which reduces intra-ring positive cooperativity, also affects the inter-ring allostery. Negative allostery has been attributed to (i) preservation of the inter-ring interface, thus causing each subunit in the *trans* ring to move away from the central axis by about 2° (Xu *et al.*, 1997), and to (ii) steric clashes that arise between equatorial domains in the two

rings if they both undergo a conformational change (Ma *et al.*, 2000). It has also been suggested that the Glu434–Lys105 contact is involved in propagating structural changes across the inter-ring interface (Roseman *et al.*, 1996). None of these explanations can account for the loss of negative cooperativity, with respect to ATP, in the Arg13Gly, Ala126Val mutant. It is clear that more structural data and mutational analysis are required in order to understand the molecular basis of inter-ring allostery in GroEL. Not much is known about the mechanism of inter-ring allostery in type II chaperonins. The heterogeneity in their subunit composition and the fact that each subunit in one ring is in contact with only one subunit in the other ring (and not two subunits as in GroEL) suggest that the mechanism of inter-ring allostery in type II chaperonins is quite different from that of type I chaperonins. The presence of a different mechanism is also indicated by the lack of preservation of the inter-ring interface of CCT upon ATP binding (Llorca *et al.*, 1999).

CONCLUDING REMARKS

Allostery in hemoglobin has been studied for almost a century but is still not fully understood (Eaton *et al.*, 1999). Progress in understanding allostery in type I chaperonins represented by GroEL has been fast in comparison. However, key high-resolution structural information on certain stable states of GroEL, in particular, the ATP-bound **TR** state, and thermodynamic and kinetic data on ADP-induced transitions are still not available. The mechanism by which structural changes propagate through the molecule is still not understood, as in the case of most allosteric systems. Little is still known about the role of allostery *in vivo* (Fridmann *et al.*, 2000) and how it is affected by conditions in the cell such as macromolecular crowding. Research on all aspects of allostery in type II chaperonins in general, and CCT in particular, is still in its infancy. In the case of CCT, progress awaits development of high-level expression systems that will facilitate structure–function studies and high-resolution structural work.

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