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Protein Sci. 2001 10: 445-449
Access the most recent version at doi:10.1110/ps.44401

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FOR THE RECORD

Nested allosteric interactions in the cytoplasmic chaperonin containing TCP-1

GALIT KAFRI,[1] KEITH R. WILLISON,[2] AND AMNON HOROVITZ[1]

[1]Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel
[2]Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, United Kingdom

(RECEIVED October 26, 2000; ACCEPTED November 14, 2000)

Abstract

Initial rates of ATP hydrolysis by the chaperonin containing TCP-1 (CCT) from bovine testis were measured as a function of ATP concentration. Two allosteric transitions are observed: one at relatively low concentrations of ATP (<100 μM) and the second at higher concentrations of ATP. The data suggest that CCT has positive intra-ring cooperativity and negative inter-ring cooperativity in ATP hydrolysis, with respect to ATP, as previously observed in the case of GroEL. It is shown that the relatively weak positive intra-ring cooperativity found in the case of CCT may be due to heterogeneity in its subunit composition. Our results suggest that nested allosteric behavior may be common to chaperone double-ring systems.

Keywords: Protein folding; chaperonins; CCT; cooperativity; allostery

Molecular chaperones are required for protein folding, transport, and degradation in the cell. An essential family of molecular chaperones, the chaperonins, can be divided into two types: type I is found in eubacteria, mitochondria, and chloroplasts (Ranson et al. 1998; Sigler et al. 1998), and type II is found in archaea and the eukaryotic cytosol (Gutsche et al. 1999). Chaperonins are oligomeric proteins that 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). The extent of symmetry in their structure depends on the number and composition of subunits in each ring. Type I chaperonins, such as GroEL from *Escherichia coli* and mitochondrial hsp60, consist of 14 identical subunits that form two heptameric rings (Ranson et al. 1998; Sigler et al. 1998). 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). Cross-linking studies have shown that the eight different subunits of CCT rings are arranged in a fixed permutation (Liou and Willison 1997). The crystal structures of GroEL (Braig et al. 1994) and the thermosome from *Thermoplasma acidophilum* (Ditzel et al. 1998) show that they share a similar domain arrangement. Each subunit consists of three domains: (1) an equatorial domain that contains an ATP-binding site, (2) an apical domain that forms the opening of the central cavity, and (3) an intermediate domain that connects the apical and equatorial domains.

Type I chaperonins, as exemplified by GroEL, are known to mediate the in vivo folding of a large number of different proteins (Houry et al. 1999), whereas CCT is thought to be involved mainly, or perhaps exclusively, in the folding of actin and tubulin (Gao et al. 1992; Yaffe et al. 1992). Both type I and type II chaperonin-mediated protein folding are MgATP dependent (Ranson et al. 1998; Sigler et al. 1998; Gutsche et al. 1999). ATP binding and hydrolysis switch CCT (Melki and Cowan 1994) and GroEL (Staniforth et al. 1994; Yifrach and Horovitz 1996) rings between different conformations with either low or high affinity for unfolded polypeptide substrates. The ATP-induced conformational changes of GroEL (Roseman et al. 1996) are reflected in binding of ATP with positive cooperativity within rings

Reprint requests to: Amnon Horovitz, Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel; e-mail: Amnon.Horovitz@weizmann.ac.il; fax: 972-8-9344188.

Article and publication are at www.proteinscience.org/cgi/doi/10.1110/ps.44401.

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(Gray and Fersht 1991; Bochkareva et al. 1992; Jackson et al. 1993) and negative cooperativity between rings (Yifrach and Horovitz 1994, 1995). ATP-induced conformational changes of CCT have been monitored by changes in fluorescence (Melki et al. 1997); more recently, they have been visualized at 28 Å resolution by electron cryo-microscopy and single-particle reconstruction (Llorca et al. 1999). Binding of ATP to CCT was found to generate an asymmetric particle in which one ring has a slightly different conformation from the apo-ring and the other ring has undergone substantial movements in the apical and equatorial domains (Llorca et al. 1999). The structural data for the ATP-induced conformational changes in CCT and the similarities between CCT and GroEL suggest that ATP binding to CCT may also be cooperative. Kinetic evidence for cooperativity in ATP binding/hydrolysis by CCT has, however, not been reported. Here, we show that CCT undergoes two ATP-induced allosteric transitions. The data are consistent with the presence of positive intra-ring cooperativity and negative inter-ring cooperativity in ATP hydrolysis by CCT. The results are discussed in the context of the allosteric mechanism of GroEL.

Results and discussion

Initial rates of ATP hydrolysis by CCT were measured at different concentrations of ATP from 0 to 1 mM. Two allosteric transitions were observed: one at relatively low ATP concentrations (<100 μM) and the second at higher concentrations of ATP (Fig. 1). The allosteric transitions observed most likely reflect ATP-induced conformational changes in CCT and not its assembly or disassembly (Roobol et al. 1999; Dobrzynski et al. 2000). In the analysis here, we assume that the two transitions correspond to the ATP-induced allosteric switch of one ring and then the other ring of CCT. The data were fitted to a Hill-type equation for two sequential allosteric transitions. The values of the apparent ATP-binding constants of the first and second rings, K_1 and K_2, are found to be 7.6 (± 0.4) and 533 (± 22) μM, respectively. The two orders of magnitude difference in the affinity of the first and second rings for ATP are consistent with negative cooperativity between rings, as observed in the case of GroEL (Yifrach and Horovitz 1994, 1995). Previous estimates of the values of ATP-binding constants of type II chaperonins range from 5.6 μM for the *Pyrodictium occultum* (Phipps et al. 1991) and the *Pyrococcus kodakaraensis* KOD1 (Yan et al. 1997) thermosomes to 115 and 150 μM for TRiC (Martin and Cruetz 1990) and the *Methanopyrus kandleri* thermosome (Andra et al. 1998), respectively. In these earlier studies, no distinction was made between two classes of ATP-binding sites. The values of k_{cat} for ATP hydrolysis by one ring and by both rings of CCT are 0.0119 (± 0.0002) sec^{-1} and 0.0183 (± 0.0005) sec^{-1}, respectively. CCT is therefore a considerably more sluggish ATPase than GroEL, which has values of k_{cat} for ATP hydrolysis of 0.40 and 0.27 sec^{-1} by one ring and both rings, respectively (Yifrach and Horovitz 1995). The value of V_{max} for ATP hydrolysis by both rings of CCT is nearly double the value of V_{max} for ATP hydrolysis by one ring, thus indicating that in contrast with GroEL (Yifrach and Horovitz 1995), inter-ring communication in CCT has little effect on the rate of ATP hydrolysis.

The value of the Hill coefficient n for the first allosteric transition of CCT, in the presence of 50 mM K^+ ions, is found to be 2.00 (±0.25), which is lower than the value of 2.41 (± 0.13) determined for GroEL under the same conditions (Yifrach and Horovitz 1996). These values of the Hill coefficient are relatively low for assemblies of seven or eight subunits. The value of the Hill coefficient m for the second allosteric transition of CCT, in the presence of 50 mM K^+ ions, is found to be 8.52 (± 2.78). This value is close to the upper limit value of the Hill coefficient, which is equal to the total number of ATP-binding sites in a ring. In contrast, the value of the Hill coefficient for the second allosteric transition of GroEL, in the presence of 50 mM K^+ ions, was previously found to be 2.54 (± 0.56), which is considerably lower (O. Yifrach and A. Horovitz, unpubl.). The much larger value of m relative to n reflects negative cooperativity between rings, which seems to be much stronger in CCT than in GroEL. This finding is in agreement with the electron cryo-microscopy observation of an ATP-bound symmetric conformation of GroEL (White et al. 1997) but not CCT (Llorca et al. 1999) in the presence of a high ATP concentration.

Recently, we showed that one possible reason for the relatively low value of the Hill coefficient of GroEL is that the observed rate of protein folding is slower when intra-ring positive cooperativity with respect to ATP is stronger (Yifrach and Horovitz 2000). In the case of CCT, a second

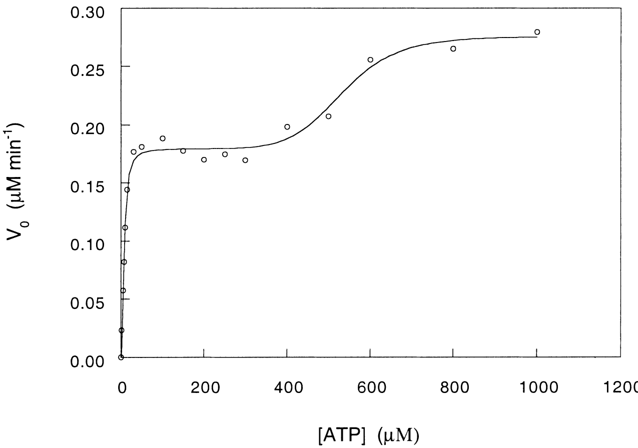

Fig. 1. Initial velocity of ATP hydrolysis by CCT at different ATP concentrations. The data were fitted to equation 4. The oligomer concentration of CCT is 250 nM.

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reason for the relatively low value of the Hill coefficient for the first transition may be differences in the intrinsic affinities for ATP of the different subunits in a ring. To explain this, let us consider, for simplicity, a heterodimer with low- and high-affinity sites ($K_1 > K_2$) for a substrate (ATP) that is in equilibrium between **T** and **R** states (L = [T] / [R]) (Fig. 2.2). In the **R** state, both sites have relatively high affinity for substrate in comparison with the **T** state ($K_{1R} > K_{1T}$ and $K_{2R} > K_{2T}$). In the case of this model, the Hill coefficient at 50% saturation (for exclusive binding to the **R** state) is given by

$$n_{50} = 2/(1 + (K_{1R}/(1+L)K_{2R})^{1/2}) \qquad (1)$$

Two extreme cases of this model are (1) a heterodimer with low- and high-affinity sites for a substrate that is found predominantly in one state and (2) a homodimer with identical sites that is in equilibrium between **T** and **R** states. In the first case ($L \to 0$), the Hill coefficient at 50% saturation is given by the following equation (Levitzki 1978):

$$n_{50} = 2/(1 + (K_1/K_2)^{1/2}) \qquad (2)$$

The value of the Hill coefficient n_{50} is <1 when $K_1 > K_2$, thus reflecting the fact that site heterogeneity cannot be distinguished from negative cooperativity. In the second case ($K_{1R} = K_{2R}$), the Hill coefficient at 50% saturation (for exclusive binding to the **R** state) is given by the following:

$$n_{50} = 2/(1 + (1/(1+L))^{1/2}) \qquad (3)$$

1. Sequential mechanism for a symmetric dimer

2. Concerted mechanism for an asymmetric dimer

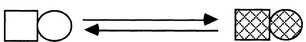

Fig. 2. Models for conformational changes in CCT. Two models for conformational changes that may account for intra-ring positive cooperativity in CCT are shown, for simplicity, for a dimer. (*1*) The subunits of an unliganded symmetric dimer undergo sequential ATP-induced transitions from a low-affinity (square) to a high-affinity (circle) state; (*2*) the subunits of an unliganded asymmetric dimer with low-affinity (square) and high-affinity (circle) sites undergo a concerted ATP-induced transition from a low-affinity (empty) to a high-affinity (hatched) state. The models can be extended for the eight sites in CCT rings.

In this case, the value of n_{50} is between 1 and 2, and negative cooperativity is never observed. The above analysis shows, therefore, that positive cooperativity in ligand binding which results from a shift in equilibrium from a low- to a high-affinity state of the protein can be partially or fully masked by site heterogeneity. That may explain why CCT with eight ATP-binding sites per ring has a Hill coefficient with a value even lower than that of GroEL with seven sites per ring. It may also explain why slight negative cooperativity is observed in the case of the archaeal chaperonin (Gutsche et al. 2000).

In conclusion, our results indicate that there is positive intra-ring cooperativity and negative inter-ring cooperativity in ATP hydrolysis by CCT, as previously found for GroEL (Yifrach and Horovitz 1994, 1995). It was suggested that positive intra-ring cooperativity in GroEL is the result of an equilibrium of each ring between a tense **T** state with low affinity for ATP and high affinity for nonfolded proteins and a relaxed **R** state with high affinity for ATP and low affinity for nonfolded proteins (Yifrach and Horovitz 1995, 1996), in accordance with the Monod-Wyman-Changeux model (Monod et al. 1965). Evidence supporting the concerted nature of the intra-ring transitions in GroEL has recently been obtained (Ma and Karplus 1998; Yifrach and Horovitz 1998; Horovitz and Yifrach 2000). In the case of CCT, it has been suggested (Lin and Sherman 1997) on the basis of genetic analysis that the allosteric transition of each ring takes place in a sequential manner, in accordance with the Koshland-Némethy-Filmer (KNF) model (Koshland et al. 1966). The interaction between rings in both GroEL and CCT requires invoking the KNF model because of the presence of inter-ring negative cooperativity. Cooperativity in CCT can therefore be described by a model in which KNF-type transitions within a ring are nested inside inter-ring KNF-type transitions (Fig. 2.1). Alternatively, the intra-ring cooperativity in CCT may reflect the combined effect of a concerted structural change and site heterogeneity (Fig. 2.2), as shown above. Our results suggest that nested allosteric behavior may be common to chaperone double-ring systems.

Materials and methods

Materials

[γ-^{32}P]ATP was purchased from Amersham; all other reagents were purchased from Sigma. CCT was purified from tubules of bovine testis (10 g) that were separated from the tunica albuginea by dissection and added to 4–8 mL of buffer A (100 mM triethanolamine at pH 7.4, 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol) containing 5 µg/mL chymostatin, 10 µg/mL leupeptin, 5 µg/mL antipain, 5 µg/mL pepstatin, 8.2 TIU aprotinin. The tubules were homogenized and centrifuged at 14,000 rpm (SA-600 rotor, Sorvall) for 30 min at 4°C. Supernatant was removed and ultracentrifuged at 54,000 rpm (Ti-60 rotor, Beckman) for 1 h at 4°C. The

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supernatant was separated on a 10%–40% sucrose gradient in 100 mM triethanolamine buffer (pH 7.4) containing 5 mM $MgCl_2$ at 24,000 rpm (SW-28 rotor, Beckman) for 17 h at 4°C. Fractions were collected, starting at the bottom of the tube, and analyzed by Western blotting using rabbit polyclonal anti-CCT serum UM1, as previously described (Hynes et al. 1995). CCT-containing fractions were combined and incubated at room temperature for 10 min with 1 mM ATP and 10 mM KCl and then loaded on a Mono Q HR 5/5 column after twofold dilution with buffer A. CCT was eluted using a 20-mL gradient of 0–0.6 M NaCl in buffer A containing 2 mM β-mercaptoethanol (buffer B). It was found to elute at 0.1–0.3 NaCl. CCT-containing fractions were combined and loaded on a 5-mL HiTrap heparin column (Pharmacia) equilibrated with buffer A. CCT was eluted using a 100-mL gradient of 0–0.8 M NaCl in buffer B. It was found to elute at 0.4–0.6 NaCl. Pure CCT fractions were combined, concentrated by Centriprep-30, and desalted using a PD-10 Sephadex column equilibrated with buffer A. Pure CCT was flash-frozen in liquid N_2 and stored at −80°C in aliquots. The concentration of the purified CCT was determined using amino acid analysis.

ATPase assays

The ATPase activity of CCT was measured as described (Horovitz et al. 1993). The reactions were started by mixing 10 μL of different concentrations of [γ-^{32}P]ATP with a 40-μL solution containing 300 μg/mL CCT in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol. Both solutions were incubated for 7 min at 25°C before mixing. The reactions were performed at 25°C and terminated after 1, 2, 3, and 4 min (for [ATP] ≤15 μM) or 2, 4, 6 and 8 min (for [ATP] >15 μM) by removing a 10-μL aliquot to 70 μL of pre-cooled stop solution containing 1 M perchloric acid and 1 mM KH_2PO_4.

Data analysis

Analysis of cooperativity in ATP hydrolysis by CCT was performed by directly fitting data of initial ATPase velocities at different ATP concentrations by using Kaleidagraph (version 2.1 Synergy Software [PCS] Inc.) to

$$V_0 = (V_{\max(1)} + V_{\max(2)}([S]/K_2)^m)/(1 + (K_1/[S])^n + ([S]/K_2)^m) \quad (4)$$

where V_0 is the observed initial rate of ATP hydrolysis, [S] is the substrate (ATP) concentration, $V_{\max(1)}$ and $V_{\max(2)}$ are the respective maximal initial rates of ATP hydrolysis by a single ring and by both rings of CCT, n and m are the respective Hill coefficients for ATP binding to the first and second rings, and K_1 and K_2 are the respective apparent binding constants of ATP for the first and second rings.

Acknowledgments

This work was supported by the Israel Science Foundation, administered by The Israel Academy of Sciences and Humanities; by the MINERVA Foundation, Germany; and by the Asher and Jeannette Alhadeff research award. A.H. is an incumbent of the Carl and Dorothy Bennett Professorial Chair in Biochemistry.

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