SALT BRIDGES AT THE INTER-RING INTERFACE

REGULATE THE THERMOSTAT OF GroEL

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SUMMARY

The chaperonin GroEL consists of a double-ring structure made of identical subunits, and displays unusual allosteric properties due to the interaction between its constituent subunits. Cooperative binding of ATP to a protein ring allows binding of GroES to that ring, and at the same time negative inter-ring cooperativity discharges the ligands from the opposite ring, thus driving the protein-folding cycle. Biochemical and electron microscopy analysis of wild type GroEL, a single-ring mutant (SR1), and two mutants with one inter-ring salt bridge disrupted (E461K and E434K) of the chaperonin, indicate that these ion pairs form part of the interactions that allow the inter-ring allosteric signal to be transmitted. The wild type-like activities of the ion pair mutants at 25°C, are in contrast with their lack of inter-ring communication and folding activity at physiological temperatures. These salt bridges stabilize the inter-ring interface and maintain the inter-ring spacing so that functional communication between protein heptamers takes place. The characterization of GroEL hybrids containing different amounts of wild type and mutant subunits also indicates that as the number of inter-ring salt bridges increases the functional properties of the hybrids recover. Taken together, these results strongly suggest that inter-ring salt bridges form a stabilizing ring-shaped, ionic zipper that ensures inter-ring communication at the contact sites and therefore a functional protein-folding cycle. Furthermore, they regulate the chaperonin thermostat, allowing GroEL to distinguish physiological (37°C) from stress temperatures (42°C).
The chaperonin GroEL provides an interesting example of a macromolecular assembly that shows unusual allosteric properties due to the interactions between its constituent subunits (1). This tetradecameric protein binds and hydrolyzes ATP with positive intra-ring cooperativity (2-4) and negative inter-ring cooperativity (5,6). Positive cooperative binding of ATP to one protein ring promotes the conformational change required for GroES binding to the same ring. Recent studies have revealed that the intra-ring allosteric transitions in GroEL are concerted (7). Negative cooperativity in ATP binding results in an asymmetric oligomer, where the two rings of the protein adopt different conformations.

The conformational changes brought about by nucleotide binding and hydrolysis induce a spatial and temporal reorganization of the chaperonin structure, therefore controlling its functional cycle. In this context, inter-ring allosterism is critical for the continuous cycling of GroEL rings between protein accepting and releasing states, in spite of which it is less well understood than intra-ring cooperativity (8). Each subunit of a protein ring interacts with two of the opposite ring, through two contact sites (9,10). Regardless of the preservation (10) or distortion (11) of the inter-ring interface, the displacement of the equatorial domain of protein subunits in opposite rings provides most likely the structural basis for the propagation of the signal that couples ATP binding to the \textit{trans} ring with GroES dissociation from the \textit{cis} ring (12).

At the contact sites, different types of interactions (hydrophobic, hydrogen bonds, ionic) between residues belonging to interacting subunits stabilize the double ring structure of the chaperonin (9,10). Among these interactions, the salt bridges E461-R452, at the so-called right site, and E434-K105, at the left site, might be important in maintaining a functional inter-ring interface. We have characterized in this study the role of the above mentioned ionic pairs in inter-ring communication. Our results show that mutants having impaired any of
these salt bridges are not functional as folding machines at physiological temperatures due to a temperature-induced loosening of the communication between rings. This suggests that ionic pairs at the contact sites regulate the chaperonin thermostat, which is necessary to distinguish physiological (37°C) from stress conditions (42°C).
EXPERIMENTAL PROCEDURES

Protein expression and purification. GroEL mutants were produced by the homologous recombination technique (13), according to Weissman et al. (14). Polymerase chain reaction was carried out using *Pfu* polymerase (Stratagene) on the plasmid pARGRO (15). *E. coli* GroEL and GroES were purified from a pAR3 plasmid harboring strain (15) that overexpresses both proteins as described previously (16). The single-ring (SR1) and the single point double-ring (E461K and E434K) mutants were purified using the same procedure that was used with wt GroEL.

ATP Hydrolysis. ATPase activity was assayed using a spectrophotometric method that includes an ATP-regenerating system (17). The reaction mixture contained 50 mM Tris-HCl, 10 mM MgCl₂, 100 mM KCl, 0.2 mM NADH, 2 mM phosphoenolpyruvate, 15 µg/ml pyruvate kinase, and 30 µg/ml lactate dehydrogenase, pH 7.5. It was incubated at the desired temperature 10 min, and ATP (2 mM) was added and incubated 2 more min. Finally, wt GroEL, E461K, E434K (50 nM, oligomer concentration) or SR1 (100 nM, oligomer concentration) were added, and the decrease in the absorbance at 340 nm was followed during 10 min in a thermostatted Uvikon 943 spectrophotometer. The rate of ATP hydrolysis (µmoles/min) was determined from the slope of the absorbance vs. time plot, considering the extinction coefficient of NADH. The effect of GroES on the ATPase activity was measured in samples containing wt GroEL, E461K, E434K or SR1 (2:1 and 1:1 oligomer ratio, respectively).

Malate Dehydrogenase (MDH) refolding. Mitochondrial MDH (from porcine heart, Sigma Co.) was denatured in 6M urea at 37 °C during 30 min and diluted 50-fold in a medium containing the desired chaperonin specie at 25 °C, 37 °C or 42 °C. The activity of
MDH was assayed by measuring the decrease in absorption at 340 nm of NADH in 50 mM Tris-HCl, pH 7.5 with oxalacetate as substrate.

Electron Microscopy and image processing. Chaperonins (wt, E461K and E434K GroEL) were incubated with GroES (1:2 molar ratio) at 25°C, 37°C and 42 °C, during 10 min. Afterwards, ATP (2 mM) incubated at the same temperature was added to the mixture and incubated for 30 s. Samples were stained with 1% uranyl acetate and kept at the temperature of the assay. Grids were previously glow discharged and equilibrated at the corresponding temperature. Transmission electron microscopy was performed in a JEOL 1200EX-II electron microscope operated at 100 KV. 200 particles were analyzed visually for each sample and, according to their side views classified as GroES-free, asymmetric and symmetric GroEL-GroES complexes (18, 19). For image processing, micrographs of the specimens were digitized in a EIKONIX scanner with a sampling window corresponding to 3.5 Å/px and side-views were selected, aligned using a free-pattern algorithm (20) and subjected to classification using a self-organizing map algorithm (21) to remove all the non-homogeneous particles. The resolution of the average images was estimated by the spectral signal to noise ratio (SSNR) method (22) and average images were subsequently filtered to the resolution obtained.

Hybrid GroEL tetradecamers. Preparation of hybrid chaperonins was performed as recently described (7). Briefly, pure chaperonins or mixtures of wt and the above mentioned GroEL mutants at different molar ratios were denatured in 8M urea (25°C, 90 min). The unfolded proteins or protein mixtures were renatured by diluting them into buffer containing 50 mM Tris-HCl, 10 mM MgCl₂, 5 mM ATP and 0.6 M ammonium sulfate (23), and incubated 30 min at 25°C. The reconstituted tetradecamers were purified by gel filtration chromatography using a Superdex 200 column (Amersham Pharmacia Biotech) equilibrated
in 25 mM Tris-HCl, 100 mM Na$_2$SO$_4$, pH 6.8. The fractions containing the renatured tetradecamer were concentrated with Ultrafree 10-kDa filters. Isoelectrofocussing was performed using PhastGels IEF 4-6.5 on a PhastSystem (Amersham Pharmacia Biotech) that were silver stained.
RESULTS

Measurements of initial rates of ATP hydrolysis at increasing concentrations of nucleotide have shown that GroEL undergoes two different allosteric transitions that can be explained assuming a nested allosteric model (5). The first transition takes place at ATP concentrations lower than 100 μM (5), and reflects positive cooperativity in ATP binding and hydrolysis within one ring, and the second, occurring at higher nucleotide concentrations, is due to negative inter-ring cooperativity. As shown in Figure 1A, wt GroEL shows both allosteric transitions, as previously reported, at 25°C. The one corresponding to negative inter-ring cooperativity becomes less pronounced at increasing temperatures (i.e., 37°C and 42°C), suggesting that at physiological and stress temperatures communication between rings is weakened (24). As expected from a single ring particle, the transition corresponding to negative communication between rings is not observed for SR1 (Fig. 1A). At 25°C the decrease in ATPase activity for E461K (Fig. 1B) and E434K (Fig. 1C), associated with negative inter-ring cooperativity, is less pronounced than that observed for wt GroEL (Fig. 1A), and is virtually abolished at 37°C (Fig. 1B and 1C). A comparison of the data depicted in Fig. 1 indicates that the behaviour of both mutants at 25°C is similar to that of wt GroEL at 37°C, while at 37°C they resemble wt GroEL at 42°C. These results suggest that inter-ring communication is significantly weaker in both mutants, and that at physiological temperature it is not experimentally detected.

To further analyze this interpretation, the extent of inhibition of the ATPase activity of GroEL by its cochaperonin GroES was studied at the above mentioned temperatures (Figure 2). As previously reported (24), GroES inhibits the ATPase activity of GroEL by about 50-55% at 25°C and 37°C, a value that decreases to 43 % at stress temperatures (42°C). Due to
the single ring structure of SR1, GroES inhibits 77% of the chaperonin ability to hydrolyze the nucleotide at 25°C, a percentage that increases to 86% at 37°C and is maintained at 42°C. Interestingly, the inhibition at 25°C is 72% and 59% for E461K and E434K, respectively, and at 37°C and 42°C the percentage increases to values similar to those described for SR1, i.e., 80-85%. These data reinforces the idea that a defective inter-ring communication might be responsible for the temperature-induced, single-ring-like behaviour of both mutants at 37°C.

An estimation of the relative affinity of the different chaperonins used in this study for GroES was also made using electron microscopy (Table 1). All three types of GroEL were independently incubated at the desired temperature in the presence of ATP, and the percentage of each oligomer (GroES-free, asymmetric and symmetric GroEL-GroES complexes) was estimated analyzing side views of the particles. The percentage of symmetric and asymmetric complexes estimated for wt GroEL as a function of temperature suggests a slight decrease of the affinity of GroEL towards GroES at 42°C. Interestingly, the values of symmetric complexes for both mutants is twice that found for GroEL (around 70%) at 25°C and, in contrast to wt GroEL, this percentage increases up to 80-84% at 37°C and remains stable at 42°C. Therefore, electron microscopy data parallel the effect of temperature on the inhibition of the ATPase activity of these proteins by GroES. The effect of temperature on inter-ring communication was further characterized by analyzing the different proteins by electron microscopy and image processing (Fig. 3). At physiological temperatures, i.e. 37°C, the average side views of GroEL (Fig. 3A) reveal the presence of two proteins rings in close contact. In contrast, the inter-ring spacing of E434K (Fig. 3B) and E461K (Fig. 3C) GroEL is enlarged as compared with wt GroEL incubated at the same temperature, suggesting a weaker inter-ring interaction. Is important to note that the inter-ring spacing observed for both mutants at 37°C is similar to that previously described for GroEL at 45°C (24).
The above interpretation implies that in the presence of GroES and nucleotides, the efficiency of the mutants to fold stringent protein substrates, such as malate dehydrogenase, should be temperature-dependent. To prove it, the ability of the different GroEL oligomers to fold MDH was analyzed at several temperatures and compared with data obtained for wt GroEL and SR1 (Fig. 4). As previously reported, wt GroEL refolds MDH at 25°C and 37°C (Fig. 4A). The extent of refolding at 25°C (90%) is higher than at 37°C (73%), although the folding reaction is faster at the latter temperature. At 42°C the refolding yield is only 17%, suggesting that either the monomeric substrate protein that is released to the medium is not stable and able to form the active dimer, and is therefore bound by and released from the chaperonin continuously, or that the release mechanism is affected. A significant fraction of the protein substrate remains either stably or transiently bound to GroEL, as demonstrated by the recovery of active MDH (60%) when the temperature is lowered from 42°C to 25°C (not shown). The yield of MDH refolding observed for SR1 is smaller than the spontaneous one at 25°C (Fig. 4A), and negligible at 37° and 42°C (not shown). It is important to mention that in the absence of the chaperonin there is no detectable refolding of MDH at 37°C. The mutant E461K refolds MDH in a wt-like fashion at 25°C, since both the kinetics and the final extent of refolding are similar (Fig. 4B), but is unable to assist proper folding of the protein substrate at 37°C, where only around 10% of the initially denatured MDH reaches its native, active conformation. These observations also apply to the E434K mutant (Fig. 4C). As mentioned for wt GroEL, at least part of the substrate protein remains bound to the chaperonins at either 37°C or 42°C, since partial MDH refolding is observed when the samples are cooled down from 42°C to 25°C (34% for E434K and 28% for E461K) and from 37°C to 25°C (50% for E434K and 44% for E461K).
To better describe how inter-ring ionic pairs affect the transmission of the allosteric inter-ring signal, we have also characterized the behaviour of hybrids containing different amounts of wt and mutant subunits. As expected from the data presented above for pure tetradecamers, at 25 °C the MDH folding ability of the E434K- and E461K-wt GroEL hybrids are similar to that of wt GroEL (data not shown). However, at 37°C the extent of MDH refolding (see Fig. 5 for E461K-wt GroEL hybrids), and ATPase inhibition by GroES (Fig. 6) depends, although not linearly, on the wt GroEL subunit content. As the mutant and wt tetradecamers can be distinguished by isoelectrofocussing, random incorporation of E461K (Fig. 6, inset) and E434K (data not shown) subunits into the hybrid complex was confirmed by this electrophoretic technique.
DISCUSSION

The routes through which the signals travel within a protein structure in response to ligand binding is a central issue to understand the behaviour of allosteric enzymes. Allosterism has been better discussed in relatively small proteins, such as hemoglobin (25), and it has been recently modeled for large protein assemblies, such as GroEL (1,5). As compared with small proteins, GroEL displays unusual allosteric properties due to the complex interaction between its constituent subunits. Recent studies have suggested that positive intra-ring and negative inter-ring cooperativity might be connected (7). Based on EM studies it has been proposed that the inter-ring contact located in the so called left site Glu434-Lys105 is weakened upon ATP binding (26). In the right contact site, the interaction Glu461-Arg452 has been shown to be part of the heptamer-heptamer contacting surface (9,10). A recent cryo-EM study has suggested that the effect of ATP on these contact sites is different, since it shortens the distance at the right site and increases it at the left one. These data indicate that both contact sites are flexible and can rearrange in response to ligand binding (11). In this study we characterize whether these ion pairs, that are involved in inter-ring interaction, modulate GroEL function and the chaperonin’s ability to differentiate physiological from stress temperatures (24,27).

Our data clearly indicate that when any of the two salt bridges stabilizing the inter-ring interface at the contact sites is disrupted, the negative inter-ring cooperativity is weakened. This results in mutant proteins that behave at physiological temperatures like wt GroEL under stress conditions, i.e., 45°C. Previous findings on the temperature dependence of inter-ring signalling indicated that at physiological (37°C) and stress (42-45°C) temperatures, inter-ring communication is weaker than at 25°C (24,27). As a consequence of the decreased negative cooperativity between the two GroEL rings with increasing
temperatures, under stress conditions, i.e. 45°C, the release of GroES from GroEL is slowed down and therefore the chaperone system functions as a storing device which can be converted back into a folding device upon disappearance of the stress conditions (24). This functional switch avoids release of substrate proteins under conditions where aggregation rather than productive folding might take place. Therefore, GroEL has a thermostat that controls the transmission of the inter-ring allosteric signal that mediates GroES and protein substrate release.

Our observations point towards the interactions that form part of the chaperonin’s thermostat, and which allow the protein to sense the environment in order to distinguish physiological from stress conditions. As seen by electron microscopy, this mechanism seems to be related to the inter-ring spacing that increases at stress temperatures, resulting in a weaker inter-ring communication (24). In the absence of any of the above mentioned salt bridges at the inter-ring contacts, temperature destabilizes the inter-ring interface in such a way that there is no proper communication between rings at physiological temperature and therefore proper folding activity. The temperature-induced stabilization of the GroEL-GroES symmetric complexes observed for both mutants, and demonstrated by both electron microscopy and GroES-induced ATPase inhibition supports this interpretation. The formation of symmetric GroEL-GroES complexes would reduce the release of bound protein substrates from GroEL, which can be reversed once the temperature is lowered to a point where inter-ring communication is restored (i.e., 25°C). It is important to note that at 25°C a functional inter-ring communication is maintained in the mutants, their folding ability being comparable to that of wild type.

Further support for this hypothesis comes from the finding that the functional properties of the hybrid GroEL complexes depends on the wt GroEL subunit content, under
experimental conditions where the mutant tetradecamers are not functional (i.e., 37°C). This might be explained by an inter-ring salt bridge-mediated stabilization of the heptamer-heptamer interface against the thermal challenge. The well known effect of salt bridges on the stabilization of proteins against thermal denaturation (28) might be also reflected at the inter-ring interface. As expected, it is experimentally found that at 37°C an increase in the number of inter-ring ionic pairs progressively activates the GroEL hybrids, suggesting that they could exert an additive effect in maintaining the appropriate distance between contact sites for proper inter-ring communication.

The importance of salt bridges in the conversion between the allosteric states of cooperative proteins has been noticed for systems such as aspartate transcarbamoylase (29) and hemoglobin (30). It has also been suggested that in the presence of ATP, switching of intersubunit salt bridges mediates propagation of positive cooperativity around a GroEL ring, and transmission of negative cooperativity across the inter-ring interface (11,24). Our results complement these observations, since they demonstrate that in the ring-shaped molecule of GroEL, the crossed inter-subunit salt bridges at the inter-ring interface are necessary to modulate the fine tuning of GroEL’s thermostat, through stabilization of the interface by maintaining the inter-ring spacing. In summary, our data show that ionic interactions at the inter-ring interface allows GroEL to differentiate physiological from stress temperatures, a mandatory ability for a heat-shock protein.
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REFERENCES


TABLE 1. GroES binding to the chaperonins.

Wt, E434K and E461K GroEL were mixed with GroES (1:2 molar ratio) in the presence of 2 mM ATP, at the indicated temperatures.

<table>
<thead>
<tr>
<th>Complex</th>
<th>GroEL</th>
<th>E461K</th>
<th>E434K</th>
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<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>37°C</td>
<td>42°C</td>
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<tr>
<td>Asymmetric</td>
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<tr>
<td>GroEL:GroES (1:1)</td>
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<td>60</td>
<td>70</td>
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<tr>
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<tr>
<td>GroEL:GroES (1:2)</td>
<td>35</td>
<td>40</td>
<td>30</td>
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FIGURE LEGENDS.

Figure 1. Negative cooperativity in different GroEL species as a function of temperature. Effect of ATP concentration on the initial velocity of ATP hydrolysis by wt (A), E461K (B), and E434K (C) GroEL. Proteins (50 nM tetradecamer or 100 nM heptamer for SR1) were incubated at 25°C (○), 37°C (◇), or 42°C (▼), and the ATPase activity measured as described under “Experimental procedures”. For the sake of comparison data for the single-ring GroEL mutant (SR1) at 25°C is included in A (▽).

Figure 2. Effect of temperature on the inhibition of the ATPase activity of the chaperonins by GroES. wt (●), E461K (▼), E434K (▽), and SR1 (○) were mixed with GroES (1:2 and 1:1 molar ratio with double and single ring chaperonins, respectively) at the indicated temperatures and the percentage of inhibition estimated from three independent measurements.

Figure 3. Two-dimensional average images of GroEL side views incubated at 37 °C. (A) Average image of wild-type GroEL (465 particles; 28 Å resolution). (B) Average image of GroEL E434K mutant (485 particles; 26 Å resolution) (C) Average image of GroEL E461K mutant (526 particles; 26 Å resolution).

Figure 4. MDH refolding by different chaperonin species as a function of temperature. Chemically denatured MDH was diluted in reaction samples containing wt (A), E461K (B), and E434K (C) GroEL, as detailed in the experimental section, that were
previously equilibrated at 25ºC (●), 37ºC (○), and 42ºC (▼). The extent of MDH refolding by SR1 (▼) and the spontaneous refolding of the protein in the absence of chaperonin (■) at 25ºC are also included in A. At 37ºC and 42ºC the spontaneous and SR1-assisted MDH folding was negligible. MDH and GroES to chaperonin molar ratio was 2:1 and 1:1 for double- and single-ring chaperonins, respectively.

Figure 5. MDH refolding by hybrid WT:E461K. Denatured MDH was diluted in samples containing GroEL tetradecamers with the following WT:E461K ratios: 14:0 (●), 7:7 (▼), 3:11 (▼), and 0:14 (○). Experiments were performed at 37ºC. Other details as in Figure 3.

Figure 6. Functional properties of the hybrid WT:E461K. Percentage of MDH refolding (●) and ATPase inhibition by GroES (Δ) as a function of the content of wt GroEL subunits in the hybrid tetradecamer. Experiments were carried out at 37ºC, as indicated in Fig. 3. Inset, Isoelectrofocusing of wt (lane 1), hybrid WT:E461K 7:7 (lane 2) and 3:11 (lane 3) molar ratio, and E461K (lane 4) GroEL.
Fig. 5

MDH refolding (% control)

WT:E461K

14:0

7:7

3:11

0:14

Time (min)

0 10 20 30 40 50 60 70
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