Binding of ATP to GroEL: a Case for Variable-Temperature Native Mass Spectrometry Thermodynamic Studies

Thomas Walker,[1] He Mirabel Sun,[1] Tiffany Gunnels,[3] Vicki Wysocki,[2] Arthur Laganowsky,[1] Hays Rye,[3] and David Russell[1*]

[1] Department of Chemistry, Texas A&M University, College Station, TX 77843
[2] Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210
[3] Department of Biochemistry & Biophysics, Texas A&M University, College Station, TX 77843

*Corresponding author, email: russell@chem.tamu.edu

ABSTRACT

Biomolecules exist in the ever-changing environment of solution, which defines their structure, stability, dynamics, and function. Moreover, these effects are manifested in protein folding, protein-protein, and protein-ligand interactions. Thermodynamic studies, especially changes in heat capacity (ΔC_p), are used to monitor the effects of solution temperature, concentration, ligands and/or co-solutes, but it can be challenging to relate such changes to molecular level reactions. Native mass spectrometry (nMS) has emerged as a complimentary technique to the traditional methods of structural and mechanistic characterization of biomolecules. Coupling of variable-temperature electrospray ionization (vT-ESI) to nMS affords mapping these changes to specific reactants and/or products using *m/z*-dispersion, whereas traditional thermodynamic measurements provide an ensemble-average of products. Here, we utilize vT-ESI-nMS to quantitate the thermodynamic contributions of stepwise binding of individual ATP or ADP ligands to GroEL—a tetradecamer chaperonin complex capable of binding up to 14 ATP molecules. We also show that small ions (*viz.* NH_4^+) are important contributors to the binding mechanisms of ATP and ADP to GroEL tetradecamer. The thermodynamic measurements reveal extensive enthalpy-entropy compensation (EEC) as well as increased cooperative effects for the formation of GroEL-ATP_{14}, whereas similar cooperativity for ADP binding is absent. The thermodynamic data demonstrate that ATP binding in the *cis* ring (GroEL-ATP_{1-7}) is largely entropic compared with more enthalpically driven reactions for ATP binding to the *trans* ring (GroEL-ATP_{8-14}), owing to negative inter-ring cooperativity. The overall entropic effects for ATP binding to GroEL tetradecamer are attributed to conformational changes of the GroEL tetradecamer, but the magnitude of the entropy is also attributable to reorganization of GroEL-hydrating water molecules and/or expulsion of water from the GroEL cavity. This study reveals new pathways, *viz.* nMS, for experimental studies aimed at expanding our understanding of biologically relevant chaperonin functions.

Introduction

GroEL is an 801 kDa tetradecamer chaperonin protein complex produced in *E. coli* that operates to refold misfolded proteins.[1, 2] The structure of GroEL consists of two heptameric stacked rings, and each subunit consists of three domains—apical, intermediate, and equatorial.[1, 3, 4] The apical domain is highly dynamic and is responsible for binding protein substrates and the co-chaperonin GroES. The intermediate domain acts as a hinge between the equatorial and the apical regions of each subunit, and the equatorial domain of each subunit is the least dynamic and serves as the interfacial contact between each heptameric ring.[2, 5-7] The equatorial domain also harbors the ATP binding site for each subunit.[1] While the structure, dynamics, and ATP binding of GroEL has been extensively investigated,[8-20] we recently reported results

using native mass spectrometry (nMS) that reveal new insights about stability and stoichiometry of GroEL-ATP/GroES interactions.[21] Using variable-temperature electrospray ionization (vT-ESI) we found that GroEL-ATP binding was temperature and ATP-concentration dependent, indicating that the thermodynamics of this vital interaction for the GroEL nanomachine might reveal new information as to how the GroEL nanomachine operates. This could provide new strategies for analysis of thermodynamics of individual binding reactions for multi-dentate systems which is especially challenging and remains difficult to achieve with current methods.

The binding of ATP to GroEL is known to induce structural changes in the GroEL complex, and these structural rearrangements alter the tertiary structure of the subunits.[10, 12] It has been shown via X-ray crystallography and cryogenic-electron microscopy (cryo-EM) that the binding of ATP by GroEL induces an extension and twisting of the apical domain[18] and a small "rocking" of the equatorial domain.[12, 22] The binding of ATP by GroEL is also influenced by the presence of small ions (*e.g.*, Mg^{2+} and K^{+}). Mg^{2+} is necessary for the binding of ATP and K^{+} is thought to activate the ATPase activity of GroEL.[23] However, it has been proposed that NH_4^{+} ions can act as a surrogate for K^{+} ions.[24-26]

Variables such as solution temperature, pressure, and concentration influence changes in the "native" structure of biomolecules through manipulation of fundamental thermodynamic contributions (ΔH, ΔS, and ΔG).[27-30] Changes in thermodynamic contributions are often hallmarks of changing conformational states and vice-versa.[30] The ability to measure and understand the underlying thermodynamics of protein-protein and protein-ligand interactions has been the focus of much research particularly in drug discovery efforts.[31, 32] Recent work has been done to quantify thermodynamic measurements using nMS.[33-35] Combined with nMS, vT-ESI allows for thermodynamic measurements of solution-phase structures with the benefit of mass separation.[34, 36, 37] Solution-based methods can be used to measure temperature-dependent interactions but often report on the ensemble average of ligand bound states present in solution; such is the case for GroEL where there are 14 potential states of ATP-bound GroEL. Here we report the thermodynamic measurements (ΔH, ΔS, and ΔG) of ATP and ADP binding to GroEL utilizing nMS with vT-ESI, along with studies that observe how endogenous ions affect the GroEL-ATP binding interaction.

Results and Discussion

As traditional approaches are often ensemble measurements, nMS was employed to measure sequential ATP binding reactions to the GroEL complex as evidenced by small shifts in *m/z* of the reaction products. These interactions were observed to be temperature and ATP concentration-dependent, with colder temperatures favoring ATP binding. Importantly, nMS measurements taken over multiple temperatures and ATP concentrations permit the quantitation of thermodynamic properties for all 14 ATP binding reactions.

GroEL-ATP$_n$ thermodynamic measurements

The data in **Figure 1A** show the temperature dependent nature of the GroEL-ATP$_n$ binding equilibrium. It is interesting to note that the binding of up to GroEL-ATP$_{14}$ is only observed at lower temperatures at 25 μM ATP and that binding affinity is lost at higher temperatures. Subtleties in the deconvoluted mass spectra also show potential cooperativity in binding at lower temperatures (*e.g.*, the increase in the abundance of GroEL-ATP$_{14}$ at 5 °C). The intrinsic association (K_a) equilibrium constants for the GroEL-ATP interaction are shown in **Figure 1B**. The association constants represent the sequential binding of each ATP to GroEL; each of these constants are shown at three temperatures in this plot: 5 °C, 23 °C, and 41 °C.

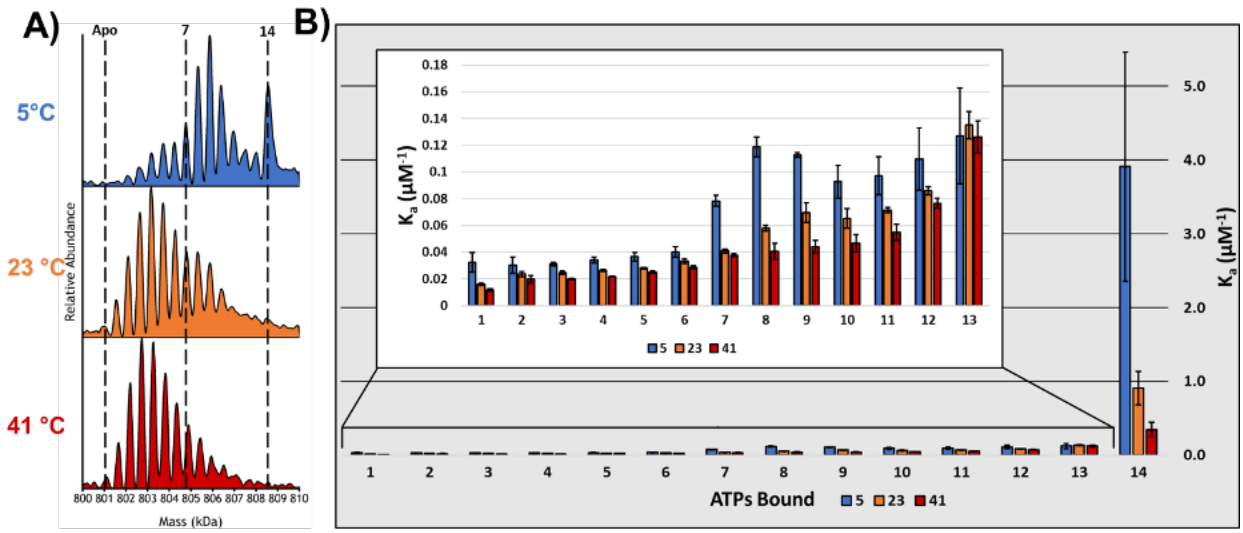


Figure 1. **A)** Deconvoluted mass spectra of 500 nM GroEL in a solution of 200 mM EDDA, 1 mM $MgAc_2$, and 25 μM ATP at 3 different temperatures. Binding affinity diminishes as solution temperature is increased. Bimodality in the ATP binding distributions is likely to be a consequence of negative cooperativity between the *cis* and *trans* rings. **B)** This bar charts shows the intrinsic K_a values calculated for the 14 ATP binding reactions for GroEL at 3 different temperatures 5 °C, 23 °C, and 41 °C. The inset shows a bar chart that expands the first 13 binding reactions so that details may be more observed easily. Binding affinity is temperature dependent and colder solution temperatures enhance the effect of inter-ring negative cooperativity, as the affinity for ATP decreases more substantially when binding in the *trans* ring begins.

The K_a values vary significantly with solution temperature with binding of ATP being favored for each reaction at 5 °C compared to 41 °C, except for GroEL-ATP_{13}. Most pronounced of all the constants, however, is GroEL-ATP_{14} where the temperature dependance is most easily observed. A similar effect for the GroEL-ATP_{14} K_a value was observed by Sharon et al., albeit their study was only conducted at room temperature.[38] A depression in the GroEL-$ATP_{8\text{-}13}$ K_a values can be seen as a hallmark of negative inter-ring cooperativity and is most apparent at low solution temperatures. The K_a values are comprised of MS data collected at various concentrations of ATP for each temperature (see methods). Deconvoluted MS data shown in **Figure 2A** display the concentration-dependent trend of ATP binding at 25 °C for 10, 25, and 50 μM ATP in EDDA.

The data in **Figure 1A** show that as solution temperature is increased, the binding affinity for ATP diminishes. It is interesting to note the behavior of the profile for the deconvoluted spectra; at colder solution temperatures, there exists a bimodality to the bound ATP distribution. The two distributions coincide with the filling of each ring of GroEL. Horovitz et al. demonstrated that the binding of ATP to GroEL can be explained through a nested-cooperativity model where each ring binds concertedly (Monod-Wyman-Changeux (MWC)) and the behavior between the rings is coupled sequentially (Koshland-Nemethy-Filmer (KNF)).[39] Binding of GroEL-ATP_{14} observed in the MS data can be interpreted as a highly cooperative reaction as the relative intensity of the GroEL-ATP_{14} signal is largely disproportionate compared to the preceding distribution of signals (GroEL-$ATP_{8\text{-}13}$). As the *trans* ring begins to bind several ATP molecules (GroEL-$ATP_{8\text{-}10}$) the cooperative effect allosterically begins to favor binding of a full ring. At higher temperatures much of this cooperative signature is lost as binding of ATP to the *cis* ring becomes favored.

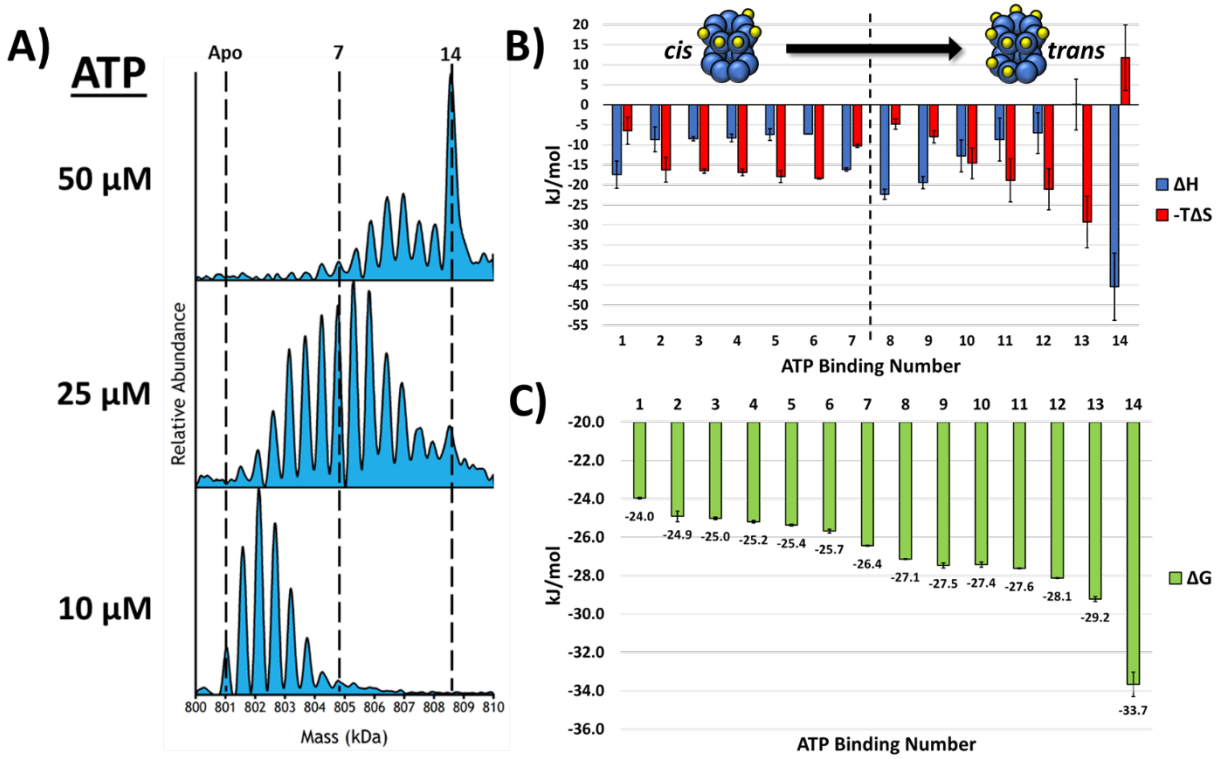


Figure 2. A) Stacked deconvoluted spectra showing the increased binding observed as the concentration of ATP is increased **B)** Bar chart showing the ΔH and -TΔS contributions at 25 °C for each of the ATP binding reactions. The *cis* ring binding reactions are mostly entropy-driven while the transition to the *trans* ring is driven enthalpically. The GroEL-ATP_{14} binding reaction is the most enthalpically favored and the least entropically favored which indicates that the structure of GroEL is becoming more rigid upon binding of GroEL-ATP_{14}. **C)** The overall Gibb's free energy associated with each of the 14 ATP binding reactions. All error bars are the standard deviation of at least 3 replicates.

From the K_a values Van't Hoff analysis was used to calculate the thermodynamic constants associated with each binding reaction. **Figure 2B** and **C** show enthalpy, entropy, and Gibb's free energy values at 25 °C. Low temperature Gibb's free energy values show the most diverse pattern in which binding of ATP becomes more favored until GroEL-$ATP_{8\text{-}9}$ (**Figure S1**). The ΔG terms increase slightly with each additional ATP bound before becoming disproportionately favored for GroEL-ATP_{14}. As the solution temperature increases, the variations in the ΔG values diminish. ΔΔG values for 41 °C – 5 °C in **Figure S2** reveal that binding for the *cis* ring (GroEL-$ATP_{1\text{-}7}$) becomes more favored as the temperature is increased when compared to the initial (GroEL-$ATP_{8\text{-}10}$) ATP binding reactions of the *trans* ring.

Binding of ATP by GroEL in EDDA is largely driven by entropy as shown in **Figure 2B**. EEC is observed for ATP binding and explains the comparative lack of ΔG fluctuation across the range of ATP binding reactions. With the exception of the first and last ATP binding reactions, binding of ATP to the *cis* ring of GroEL is largely entropically driven and is indicative of the structural rearrangement. The contributions of enthalpy and entropy begin to switch with binding of GroEL-$ATP_{7\text{-}10}$, which coincides with the filling of the *cis* ring and beginning of binding to the *trans* ring. These values switch again for GroEL-$ATP_{11\text{-}13}$ and yet again for GroEL-ATP_{14}. The binding of the ATP_{14} is the only binding reaction with any amount of unfavorable entropy. This seems to indicate that the final structural rearrangement in the *trans* ring is very ordered compared to preceding binding reactions (GroEL-$ATP_{11\text{-}13}$) and is very favored enthalpically which explains its highly favorable ΔG value.

As shown in **Figure 1**, the ATP binding affinity of GroEL is temperature dependent. **Figure S2** shows the ΔΔG plot of GroEL-ATP_n over the entire range of temperatures in this study. The relative decrease in the ΔG values for the *cis* ring binding reactions is of greater magnitude than for several of those in the *trans* ring. This trend entails the *cis* ring binding reactions are becoming more favorable compared to the *trans* ring binding reactions and that ATP binding in the *trans* ring is more allosterically disfavored. A potential structural explanation concerns the inter-ring hydrophobic contacts in the region of A109 on helix D that are extensively perturbed by the binding of ATP in the binding pocket of the equatorial domain.[4, 16] The disturbance of these contacts is theorized to be responsible for inter-ring negative cooperativity in the ATP binding interaction.[16, 22, 40] At higher temperatures increased dynamics in this region would only serve to further weaken this contact, that would in turn increase the prevalence of the negatively cooperative mechanism.

The GroEL-ATP_{1-7} binding reactions are largely driven by entropy as displayed in **Figure 2B**. The extension of the apical domain around the intermediate domain "hinge" results in the loss of salt bridge contacts (E255-K207 and R197-E386) that bind adjacent apical domains of the apo-GroEL. For the GroEL-ATP_{7-10} binding reactions the enthalpy and entropy terms switch and the binding becomes mostly enthalpically driven. The entropy-enthalpy switch may be an indication of the negative cooperativity associated with inter-ring communication when GroEL binds ATP. Saibil et al. report that as ATP binds to the *cis* ring of GroEL the equatorial domain shifts slightly which causes loss of a key hydrophobic contacts.[16, 22] The loss of these contacts is believed to be the structural progenitor of the inter-ring negative cooperativity and may explain why entropy and the overall Gibb's free energy favorability is lost with the initial binding reactions of the *trans* ring. Entropy then becomes the driving force of binding once again with GroEL-ATP_{11-13} which is most likely associated with similar structural changes seen in the *cis* ring and is consistent with observations made by Chapman et al. which report increased binding affinity when up to 3 ATP molecules are bound to a ring.[13] The GroEL-ATP_{14} binding reaction coincides with the most favorable binding and is the only binding reaction that has an associated negative entropy. The GroEL-ATP_{14} binding reaction is largely enthalpically favorable, but this may be because much of the restructuring occurs during the GroEL-ATP_{10-13} binding reactions making GroEL-ATP_{14} very favorable enthalpically but also bearing an entropic penalty.

Horovitz et al. have proposed a nested cooperativity model in which intra-ring binding is concerted (MWC) and inter-ring communication is sequential (KNF).[39] One interesting point that has arisen during our thermodynamic studies is the clear differences observed in the thermodynamic signatures for the *cis* and *trans* rings of GroEL. The *cis* ring ATP binding is governed entropically while the *trans* ring ATP binding is much more diverse in its thermodynamic contributions. The binding of GroEL-ATP_{1-7} is aided by entropy gained through the large conformational change in the apical domains, while the initial ATP binding reactions (GroEL-ATP_{8-10}) in the *trans* ring are comparatively disfavored entropically. It is not until up to 3 ATP molecules have bound to the *trans* ring that the entropy and enthalpy switch, where entropy increases in subsequent binding reactions up to GroEL-ATP_{13}.

The concerted (MWC) model of ligand binding postulates that the binding of one ligand to a protomer (subunit) induces changes in affinity for all the other protomers collectively into a relaxed or tense state.[41] In contrast, the sequential (KNF) model argues that the binding of a ligand to a subunit will only have immediate effect (positive or negative) on its neighboring subunits.[42] The thermodynamic signature for the GroEL-ATP_1 binding reaction shows that it is enthalpically driven but the GroEL-ATP_{2-6} binding reactions are driven entropically and have entropic values that are relatively unchanging. This thermodynamic pattern in conjunction with the mass spectral data suggest that binding in the *cis* ring is largely sequential in the EDDA solution. The GroEL-ATP_1 reaction sets into effect a "chain reaction" that affects adjacent

subunits via allosteric transitions. If the binding were purely concerted, then the binding distribution should be like that seen in the *trans* ring where once 2 or 3 ATP molecules are bound then the distributions heavily shift to the filled state (GroEL-ATP_{14}). These observations may be due to the difference in solution conditions used when comparing to solution-phase experiments. In these thermodynamic experiments there is no added K^+ or GroES which will ultimately change the affinity for ATP and conformation of GroEL. However, the observations made by the authors do support similar effects reported by previous investigators; specifically, the observation of cooperative binding of ATP that is non-stochastic.[5, 39, 43, 44] Therefore, it is possible that the binding of ATP to GroEL is still governed via a nested-cooperativity model, but that the *cis* ring binding is governed sequentially, *trans* ring binding is concerted, and inter-ring communication is sequential but is negatively cooperative.

GroEL-ADP_n thermodynamic measurements

Thermodynamic studies of attempted GroEL-ATP binding in ammonium acetate (AmAc) showed that the nucleotides observed in the GroEL complex were ADP and not ATP, suggesting that a substantial level of hydrolysis was occurring under these conditions. **Figure 3A** shows the post-hydrolysis ADP concentration-dependent binding of GroEL. When compared to **Figure 2A**, it is clear that GroEL binds fewer ADP than ATP (in EDDA), and signs of cooperative binding of ADP remain absent. **Figures 3B** and **C** show the thermodynamic constants for GroEL-ADP_n at 25 °C; the thermodynamic constants were only calculated up to GroEL-ADP_9 due to poor fits for the Van't Hoff plots for GroEL-$ATP_{10\text{-}14}$. The enthalpy and entropy terms switch at GroEL-$ADP_{4\text{-}5}$ displaying EEC, which causes the Gibb's free energy terms to vary only slightly. The comparison of Gibb's free energy values between ADP and ATP binding also demonstrates that ADP binds more weakly than ATP in EDDA.

Thermodynamic data for the binding of ADP in AmAc reveal distinct differences between the two nucleotides when compared to the EDDA/ATP data. The presence of EEC at 25 °C causes the ΔG values in **Figures 2C** and **3C** to remain relatively constant. However, the underlying ΔH and TΔS values show a stark mechanistic difference between the two interactions. Initial binding reactions (GroEL-$ADP_{1\text{-}4}$) for ADP-binding are driven enthalpically and not until GroEL-ADP_5 does entropy become dominant. In the EDDA/ATP data, entropy becomes dominant much sooner (GroEL-ADP_2) and several switching transitions occur as a function of sequential ATP binding. The lack of various switching reactions in the AmAc/ADP data confers an overall lack of cooperative binding of ADP; this conclusion is strengthened by the nMS data showing gaussian distribution for ADP binding at all observed ADP concentrations and by studies conducted by other researchers observing lower affinities associated with the binding of ADP.[15, 45] It has been shown that binding of ADP can cause conformational shifts in GroEL,[46, 47] which are corroborated possibly by the entropic domination seen for GroEL-$ADP_{5\text{-}9}$. However, these entropic shifts do not seem to be resultant from an allosteric transition[48] that leads to increased affinity for further ligation reactions.

As mentioned before, the existence of EEC contributes to the small fluctuations in ΔG as more ATP is bound to GroEL. EEC is a phenomenon that as ΔH or TΔS varies the other tends to compensate in an opposite direction (see **Figures 2C**, **3C**, and **S5**); this effect has been observed in thermodynamic measurements of other interactions.[49-52] The entropy term encompasses not only the conformational entropy of the structures but also the entropy of the solvent;[53] the enthalpy term is subject to the same contributions of structure and solvent.[54, 55] For example, in the GroEL system binding of ATP leads to extension of the apical domain and release of confined water, both of which are entropically favorable.[22, 44, 56] However, a more extended, labile apical domain obviates a loss of favorable binding contacts (Van der Waals, H-bonding, and salt bridging) which would be enthalpically less favorable. The resultant ΔH value for that interaction would be increased and -TΔS would "compensate" by decreasing, *i.e.,* become more favorable.

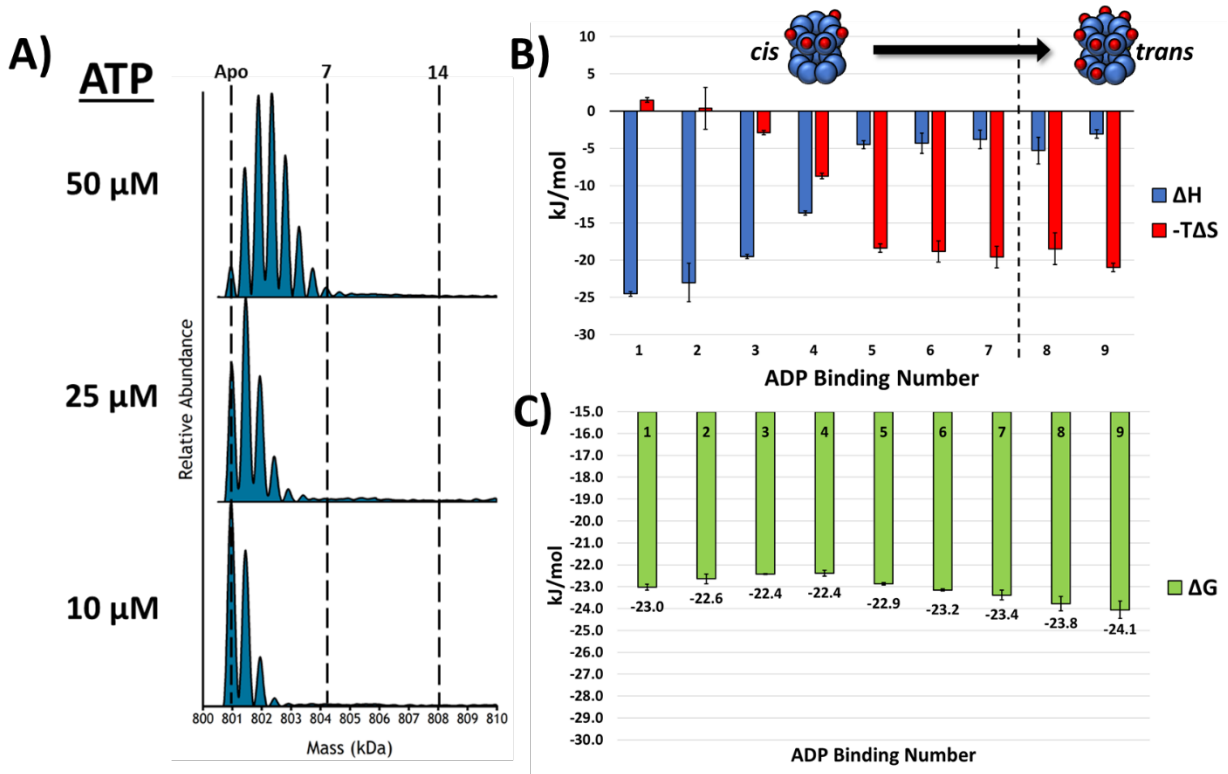


Figure 3. A) These stacked deconvoluted spectra show that as ADP concentration is increased the binding of ADP is does not show any cooperativity. **B)** Bar chart showing the ΔH and -TΔS contributions at 25 °C for each of the ADP binding reactions. The enthalpy and entropy show a singular transition from GroEL-$ADP_{4\text{-}5}$and are overall much less dynamic than the binding of ATP in EDDA. **C)** This bar chart displays the Gibb's free energy measurements for the ADP binding reactions at 25 °C. EEC is more heavily present in the ADP data set as the Gibb's free energy varies by less than 2 kJ/mol. All error bars are the standard deviation of at least 3 replicates. (**Note:** ATP was added to the solution but only ADP binding was observed.)

Effects of ions on GroEL-ATP binding

An essential ligand in the function of GroEL is the K^+ ion. However, it has been reported that NH_4^+ ions can act as substitutes for K^+ ions and activate ATPase activity of GroEL. To test this effect, ATP was added to GroEL solutions in AmAc and EDDA buffers. Observed mass shifts in AmAc experiments show that GroEL hydrolyzes most of the ATP in solution and that only signals corresponding to GroEL-ADP_n are observed (mass shifts of 460 Da corresponding to [Mg^{2+} + ADP]). When compared to experiments conducted in EDDA buffer it is clear that ATPase activity is observed in the presence of NH_4^+ (**Figure 4A**). To verify the presence of hydrolysis in AmAc a hydrolysis deficient mutant of GroEL (D398A) was introduced into the same solution composition and was found to bind only [Mg^{2+} + ATP] pairs (**Figure 4A**). A similar experiment using ADP was conducted which revealed that the binding distribution of ADP was identical regardless of whether ADP or ATP was added to the solution (**Figure S3**). **Figure 4B** shows the general mechanism of how ATP could be hydrolyzed by GroEL in an AmAc solution.

Co-solutes can contribute extensively to the chemical potential landscape available to biomolecules in solution. In the case of GroEL-ATP binding, Mg^{2+} and K^+ ions are vital to GroEL function. Mg^{2+} ions are essential for the binding of ATP to proteins and K^+ ions have been known as strong activators of ATPase activity. In previous work we have shown that the Mg^{2+} concentration impacts the stoichiometry of GroES binding to GroEL[21] (also see **Figure S4**), which illustrates the importance of Mg^{2+} concentration for proper functioning of the GroEL nanomachine. Lorimer et al. have shown that K^+ ions are necessary for the modulation of the ATPase mechanism of GroEL.[57-59] It is interesting to note that other researchers have

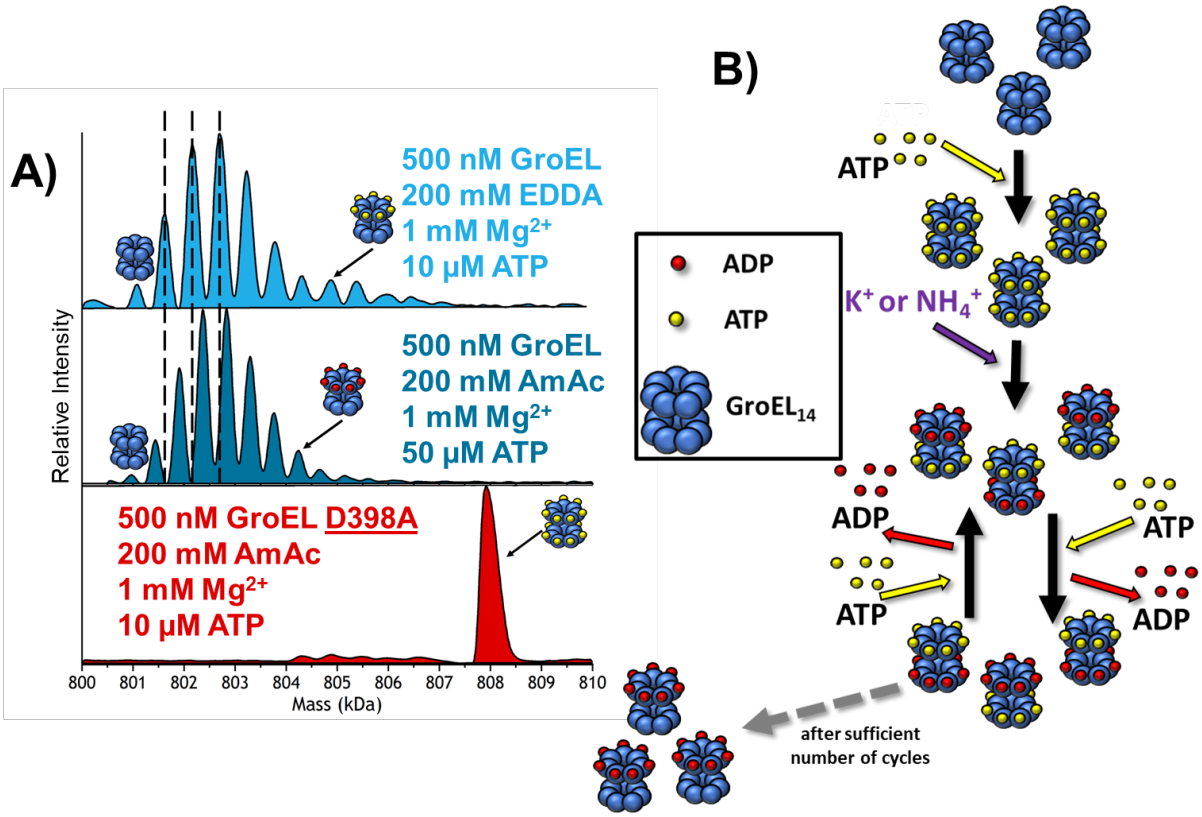


Figure 4. A) Stacked spectra showing the difference in mass shifts observed for nucleotide binding observed in EDDA and AmAc solutions. Black lines are used to aid the viewer and show that the mass shifts for EDDA are assigned to $[ATP + Mg^{2+}]_n$ while the mass shifts for AmAc conditions are assigned to $[ADP + Mg^{2+}]_n$. A hydrolysis deficient mutant (D398A) was also analyzed in an AmAc solution (red) which shows the elevated level of cooperative binding in the presence of NH_4^+ ions in the absence of hydrolysis. In AmAc solutions the wtGroEL readily hydrolyzes available ATP, thus leaving mostly ADP to bind to the complex. **B)** Simplified schematic showing the pathway of ATP hydrolysis in the presence of K^+ or NH_4^+ ions.

also reported that NH_4^+ and other monovalent cations can act as K^+ ion serrogates.[23, 24] For the GroEL-ATP_n thermodynamics data, the "traditional" nMS buffer of AmAc was avoided due to the potential for ATP hydrolysis. The data in **Figure 4A** show that in EDDA, GroEL is unable to hydrolyze any measurable amount of ATP. Conversely, in AmAc, GroEL is able to initiate turnover of ATP and hydrolyze it to form ADP. The consequence of this reaction in 200 mM AmAc buffer is that ATP to GroEL binding is not observed; only ADP binding is observed. The rate of ATP hydrolysis in AmAc is fast compared to the time scale of loading the sample and the start of data acquisition (~5 min). Even in experiments with ratios of 500:1 ATP:$GroEL_{14}$ were conducted, GroEL was able to hydrolyze enough ATP to the point where the nucleotide binding patterns were indistinguishable from experiments in which ADP was added initially (**Figure S3**). These observations are in agreement with hydrolysis rates obtained in previous GroEL-ATP hydrolysis studies.[57, 60] In the absence of hydrolysis (D398AGroEL, **Figure 4A**), the affinity of GroEL for ATP and the cooperativity of ATP binding are enhanced more so than in EDDA buffer, which lacks NH_4^+ ions. The observation of elevated cooperative binding may indicate that small ions (NH_4^+ and K^+) are allosteric ligands for GroEL functionality. Heavy metal replacement strategies in X-ray crystallographic structures have shown 3 potential binding sites for monovalent cations for each GroEL subunit.[24]

Conclusion

Here, we describe the effects of solution composition and temperature for the binding of ATP to GroEL. Mg^{2+} concentration, presence of NH_4^+ ions, and temperature were all shown to be relevant factors that

affect the GroEL-ATP interaction. The MS data support the existence of significant cooperative binding of ATP in EDDA solutions, which is enhanced further in AmAc solutions for hydrolysis deficient GroEL (D398A). The synergistic effects of monovalent cations in binding of ATP and ATPase activity remain an interesting aspect that needs to be studied further.

Using vT-nESI we were able to conduct thermodynamic measurements of ATP and ADP binding and were able to calculate enthalpy and entropy for the sequential binding of both nucleotides. The thermodynamic data show complex patterns of enthalpy-entropy compensation (EEC) for binding of ATP; such EEC patterns are somewhat lessened for the binding of ADP. In EDDA there also are different modes of ATP binding for the *cis* and *trans* rings as the thermodynamic trends for the formation of GroEL-ATP_{1-7} are distinctly different from GroEL-ATP_{8-14}. This agrees with previous findings from other researchers that propose a nested cooperativity mechanism for ATP binding. The *m/z* dispersion afforded by using MS allows for the analysis of individual binding reactions which are otherwise not observable using solution-based methods. This level of sensitivity and specificity provides a compelling case for nMS thermodynamic analysis of other systems.

Methods

Sample Preparation

GroEL tetradecamer and D398A GroEL tetradecamer were expressed and purified by the Rye research lab at the Texas A&M Department of Biochemistry and Biophysics. Aliquots of the GroEL samples were stored at -80 °C in a Tris buffer. Aliquots were buffer-exchanged into ammonium acetate (AmAc) or ethylenediamonium diacetate (EDDA) (obtained from Sigma-Aldrich) using BioRad biospin P-6 size exclusion (6000 Da cutoff) columns to remove unwanted salt contamination. Magnesium acetate ($MgAc_2$) and Na-ATP were obtained from Sigma-Aldrich and fresh solutions were prepared prior to each experiment.

Experimental

Data was collected on a Thermo Q Exactive UHMR (ultra-high mass range) mass spectrometer. Constituents for each sample were mixed immediately prior to analysis. For the thermodynamic analysis of GroEL-ATP binding solution conditions were: 1 mM $MgAc_2$, 200 mM EDDA, 500 nM GroEL (14mer), and varying concentrations of ATP. The vT-nESI device was used to modulate the temperature of the solution; more information pertaining to operation of the device can be found in previous work.[36] Solution temperatures used for this study were 5 °C to 41 °C; above 41 °C degradation products of the GroEL complex begin to become observable. The resolution setting was maintained at 25000, with 5 microscans, and injection time of 200 ms, capillary temperature was 150 °C, trap gas pressure was set to 7.0 (N_2), desolvation voltage (in-source trapping)[61] was set to -200 V, and HCD energy was set to 200 V (the latter two energy parameters only apply to EDDA buffer conditions). Under these conditions the ATP-bound states of GroEL were nearly baseline-resolved (**Figures S7** and **S9**). Thirteen solution temperatures at 8 ATP concentrations (0, 1, 5, 10, 15, 25, 35, and 50 µM ATP) were analyzed in $n \geq 3$ trials.

Data Processing

Each spectrum was deconvoluted using UniDec[62] and incorporated the 4 most abundant ATP distributions (see **Table S1** in Supporting Information for assignment statistics). The resulting relative abundances were used in a sequential binding model (**Figure S6**) to fit K_d (dissociation constant) values.[33, 63] The reciprocal of the K_d yields the K_a; the natural logs of the K_a values were plotted against inverse temperature (in K) for

Van't Hoff analysis (**Figures S8** and **S10**). The slope of the fit line is used to calculate ΔH and the y-intercept is used to calculate ΔS. Using $\Delta G = \Delta H - T\Delta S$ the Gibbs energy terms were calculated in units of $\frac{kJ}{mol}$.

Supporting Information

More thermodynamics data along with peak assignment data, examples of raw spectra, and Van't Hoff plots.

Acknowledgment

Funding for this work was provided by the National Institute of Health grants P41GM128577 (D.H.R.; V.W.) and R01GM138863 (D.H.R.; A.L.)

References

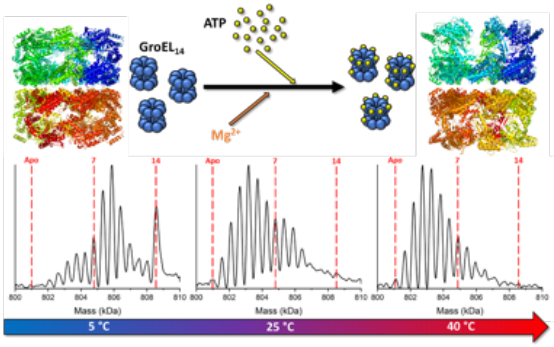
1. Boisvert, D. C. W., J.; Otwinowski, Z.; Norwich, A. L.; Sigler, P. B., The 2.4 A crystal structure of the bacterial chaperon in GroEL complexed with ATPyS. *Nat. Struct. Biol.* **1996,** *3* (2), 170-177.
2. Fei, X.; Ye, X.; LaRonde, N. A.; Lorimer, G. H., Formation and structures of GroEL:GroES2 chaperonin footballs, the protein-folding functional form. *Proc Natl Acad Sci U S A* **2014,** *111* (35), 12775-80.
3. Braig, K.; Otwinowski, Z.; Hegde, R.; Boisvert, D. C.; Joachimiak, A.; Horwich, A. L.; Sigler, P. B., The Crystal-Structure of the Bacterial Chaperonin Groel at 2.8-Angstrom. *Nature* **1994,** *371* (6498), 578-586.
4. Xu, Z.; Horwich, A. L.; Sigler, P. B., The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex. *Nature* **1997,** *388* (6644), 741-50.
5. Rye, H. S.; Burston, S. G.; Fenton, W. A.; Beechem, J. M.; Xu, Z. H.; Sigler, P. B.; Horwich, A. L., Distinct actions of cis and trans ATP within the double ring of the chaperonin GroEL. *Nature* **1997,** *388* (6644), 792-798.
6. Roh, S. H.; Hryc, C. F.; Jeong, H. H.; Fei, X.; Jakana, J.; Lorimer, G. H.; Chiu, W., Subunit conformational variation within individual GroEL oligomers resolved by Cryo-EM. *Proc Natl Acad Sci U S A* **2017,** *114* (31), 8259-8264.
7. Yang, D.; Ye, X.; Lorimer, G. H., Symmetric GroEL:GroES2 complexes are the protein-folding functional form of the chaperonin nanomachine. *Proc Natl Acad Sci U S A* **2013,** *110* (46), E4298-305.
8. Saibil, H. R.; Fenton, W. A.; Clare, D. K.; Horwich, A. L., Structure and allostery of the chaperonin GroEL. *J Mol Biol* **2013,** *425* (9), 1476-87.
9. Sigler, P. B.; Xu, Z.; Rye, H. S.; Burston, S. G.; Fenton, W. A.; Horwich, A. L., Structure and function in GroEL-mediated protein folding. *Annu Rev Biochem* **1998,** *67*, 581-608.
10. Horwich, A. L.; Farr, G. W.; Fenton, W. A., GroEL-GroES-mediated protein folding. *Chem Rev* **2006,** *106* (5), 1917-30.
11. Horovitz, A.; Reingewertz, T. H.; Cuellar, J.; Valpuesta, J. M., Chaperonin Mechanisms: Multiple and (Mis)Understood? *Annu Rev Biophys* **2022,** *51*, 115-133.
12. Ranson, N. A.; Clare, D. K.; Farr, G. W.; Houldershaw, D.; Horwich, A. L.; Saibil, H. R., Allosteric signaling of ATP hydrolysis in GroEL-GroES complexes. *Nat Struct Mol Biol* **2006,** *13* (2), 147-52.
13. Chapman, E.; Farr, G. W.; Fenton, W. A.; Johnson, S. M.; Horwich, A. L., Requirement for binding multiple ATPs to convert a GroEL ring to the folding-active state. *Proc Natl Acad Sci U S A* **2008,** *105* (49), 19205-10.

14. Terada, T. P.; Kuwajima, K., Thermodynamics of nucleotide binding to the chaperonin GroEL studied by isothermal titration calorimetry: evidence for noncooperative nucleotide binding. *Biochim Biophys Acta* **1999,** *1431* (2), 269-81.
15. Inobe, T.; Makio, T.; Takasu-Ishikawa, E.; Terada, T. P.; Kuwajima, K., Nucleotide binding to the chaperonin GroEL: non-cooperative binding of ATP analogs and ADP, and cooperative effect of ATP. *Biochim Biophys Acta* **2001,** *1545* (1-2), 160-73.
16. Ranson, N. A.; Farr, G. W.; Roseman, A. M.; Gowen, B.; Fenton, W. A.; Horwich, A. L.; Saibil, H. R., ATP-bound states of GroEL captured by cryo-electron microscopy. *Cell* **2001,** *107* (7), 869-79.
17. Motojima, F.; Yoshida, M., Discrimination of ATP, ADP, and AMPPNP by chaperonin GroEL: hexokinase treatment revealed the exclusive role of ATP. *J Biol Chem* **2003,** *278* (29), 26648-54.
18. Taniguchi, M.; Yoshimi, T.; Hongo, K.; Mizobata, T.; Kawata, Y., Stopped-flow fluorescence analysis of the conformational changes in the GroEL apical domain: relationships between movements in the apical domain and the quaternary structure of GroEL. *J Biol Chem* **2004,** *279* (16), 16368-76.
19. Chaudhry, C.; Farr, G. W.; Todd, M. J.; Rye, H. S.; Brunger, A. T.; Adams, P. D.; Horwich, A. L.; Sigler, P. B., Role of the gamma-phosphate of ATP in triggering protein folding by GroEL-GroES: function, structure and energetics. *Embo J* **2003,** *22* (19), 4877-4887.
20. Ma, J.; Karplus, M., The allosteric mechanism of the chaperonin GroEL: a dynamic analysis. *Proc Natl Acad Sci U S A* **1998,** *95* (15), 8502-7.
21. Walker, T. E.; Shirzadeh, M.; Sun, H. M.; McCabe, J. W.; Roth, A.; Moghadamchargari, Z.; Clemmer, D. E.; Laganowsky, A.; Rye, H.; Russell, D. H., Temperature Regulates Stability, Ligand Binding (Mg(2+) and ATP), and Stoichiometry of GroEL-GroES Complexes. *J Am Chem Soc* **2022,** *144* (6), 2667-2678.
22. Clare, D. K.; Vasishtan, D.; Stagg, S.; Quispe, J.; Farr, G. W.; Topf, M.; Horwich, A. L.; Saibil, H. R., ATP-triggered conformational changes delineate substrate-binding and -folding mechanics of the GroEL chaperonin. *Cell* **2012,** *149* (1), 113-23.
23. Viitanen, P. V.; Lubben, T. H.; Reed, J.; Goloubinoff, P.; O'Keefe, D. P.; Lorimer, G. H., Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K+ dependent. *Biochemistry* **1990,** *29* (24), 5665-71.
24. Kiser, P. D.; Lorimer, G. H.; Palczewski, K., Use of thallium to identify monovalent cation binding sites in GroEL. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **2009,** *65* (Pt 10), 967-71.
25. Horst, R.; Bertelsen, E. B.; Fiaux, J.; Wider, G.; Horwich, A. L.; Wuthrich, K., Direct NMR observation of a substrate protein bound to the chaperonin GroEL. *Proc Natl Acad Sci U S A* **2005,** *102* (36), 12748-53.
26. Werbeck, N. D.; Kirkpatrick, J.; Reinstein, J.; Hansen, D. F., Using (1)(5)N-ammonium to characterise and map potassium binding sites in proteins by NMR spectroscopy. *Chembiochem* **2014,** *15* (4), 543-8.
27. Privalov, P. L.; Khechinashvili, N. N., A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *J Mol Biol* **1974,** *86* (3), 665-84.
28. Wintrode, P. L.; Makhatadze, G. I.; Privalov, P. L., Thermodynamics of ubiquitin unfolding. *Proteins* **1994,** *18* (3), 246-53.
29. Privalov, P. L., Cold denaturation of proteins. *Crit Rev Biochem Mol Biol* **1990,** *25* (4), 281-305.
30. Cooper, A., Protein Heat Capacity: An Anomaly that Maybe Never Was. *The Journal of Physical Chemistry Letters* **2010,** *1* (22), 3298-3304.
31. Perozzo, R.; Folkers, G.; Scapozza, L., Thermodynamics of protein-ligand interactions: history, presence, and future aspects. *J Recept Signal Transduct Res* **2004,** *24* (1-2), 1-52.
32. Olsson, T. S.; Williams, M. A.; Pitt, W. R.; Ladbury, J. E., The thermodynamics of protein-ligand interaction and solvation: insights for ligand design. *J Mol Biol* **2008,** *384* (4), 1002-17.

33. Cong, X.; Liu, Y.; Liu, W.; Liang, X.; Russell, D. H.; Laganowsky, A., Determining Membrane Protein-Lipid Binding Thermodynamics Using Native Mass Spectrometry. *J Am Chem Soc* **2016,** *138* (13), 4346-9.
34. Qiao, P.; Schrecke, S.; Walker, T.; McCabe, J. W.; Lyu, J.; Zhu, Y.; Zhang, T.; Kumar, S.; Clemmer, D.; Russell, D. H.; Laganowsky, A., Entropy in the Molecular Recognition of Membrane Protein-Lipid Interactions. *J Phys Chem Lett* **2021,** *12* (51), 12218-12224.
35. Shi, L.; Holliday, A. E.; Shi, H.; Zhu, F.; Ewing, M. A.; Russell, D. H.; Clemmer, D. E., Characterizing intermediates along the transition from polyproline I to polyproline II using ion mobility spectrometry-mass spectrometry. *J Am Chem Soc* **2014,** *136* (36), 12702-11.
36. McCabe, J. W.; Shirzadeh, M.; Walker, T. E.; Lin, C. W.; Jones, B. J.; Wysocki, V. H.; Barondeau, D. P.; Clemmer, D. E.; Laganowsky, A.; Russell, D. H., Variable-Temperature Electrospray Ionization for Temperature-Dependent Folding/Refolding Reactions of Proteins and Ligand Binding. *Anal Chem* **2021**.
37. Laganowsky, A.; Reading, E.; Allison, T. M.; Ulmschneider, M. B.; Degiacomi, M. T.; Baldwin, A. J.; Robinson, C. V., Membrane proteins bind lipids selectively to modulate their structure and function. *Nature* **2014,** *510* (7503), 172-175.
38. Dyachenko, A.; Gruber, R.; Shimon, L.; Horovitz, A.; Sharon, M., Allosteric mechanisms can be distinguished using structural mass spectrometry. *Proc Natl Acad Sci U S A* **2013,** *110* (18), 7235-9.
39. Yifrach, O.; Horovitz, A., Nested Cooperativity in the Atpase Activity of the Oligomeric Chaperonin Groel. *Biochemistry* **1995,** *34* (16), 5303-5308.
40. Roseman, A. M.; Chen, S.; White, H.; Braig, K.; Saibil, H. R., The chaperonin ATPase cycle: mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell* **1996,** *87* (2), 241-51.
41. Monod, J.; Wyman, J.; Changeux, J. P., On the Nature of Allosteric Transitions: A Plausible Model. *J Mol Biol* **1965,** *12*, 88-118.
42. Koshland, D. E., Jr.; Nemethy, G.; Filmer, D., Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* **1966,** *5* (1), 365-85.
43. Kad, N. M.; Ranson, N. A.; Cliff, M. J.; Clarke, A. R., Asymmetry, commitment and inhibition in the GroE ATPase cycle impose alternating functions on the two GroEL rings. *J Mol Biol* **1998,** *278* (1), 267-78.
44. Gruber, R.; Mondal, T.; Horovitz, A., GroEL Allostery Illuminated by a Relationship between the Hill Coefficient and the MWC Model. *Biophys J* **2019,** *117* (10), 1915-1921.
45. Gorovits, B. M.; Horowitz, P. M., The chaperonin GroEL is destabilized by binding of ADP. *J Biol Chem* **1995,** *270* (48), 28551-6.
46. Kudryavtseva, S. S.; Pichkur, E. B.; Yaroshevich, I. A.; Mamchur, A. A.; Panina, I. S.; Moiseenko, A. V.; Sokolova, O. S.; Muronetz, V. I.; Stanishneva-Konovalova, T. B., Novel cryo-EM structure of an ADP-bound GroEL-GroES complex. *Sci Rep* **2021,** *11* (1), 18241.
47. Fei, X.; Yang, D.; LaRonde-LeBlanc, N.; Lorimer, G. H., Crystal structure of a GroEL-ADP complex in the relaxed allosteric state at 2.7 A resolution. *Proc Natl Acad Sci U S A* **2013,** *110* (32), E2958-66.
48. Cliff, M. J.; Kad, N. M.; Hay, N.; Lund, P. A.; Webb, M. R.; Burston, S. G.; Clarke, A. R., A kinetic analysis of the nucleotide-induced allosteric transitions of GroEL. *Journal of Molecular Biology* **1999,** *293* (3), 667-684.
49. Dragan, A. I.; Read, C. M.; Crane-Robinson, C., Enthalpy-entropy compensation: the role of solvation. *Eur Biophys J* **2017,** *46* (4), 301-308.
50. Zhang, J.; Jones, C. P.; Ferre-D'Amare, A. R., Global analysis of riboswitches by small-angle X-ray scattering and calorimetry. *Biochim Biophys Acta* **2014,** *1839* (10), 1020-1029.
51. Edwards, A. A.; Mason, J. M.; Clinch, K.; Tyler, P. C.; Evans, G. B.; Schramm, V. L., Altered enthalpy-entropy compensation in picomolar transition state analogues of human purine nucleoside phosphorylase. *Biochemistry* **2009,** *48* (23), 5226-38.

52. Wallerstein, J.; Ekberg, V.; Ignjatovic, M. M.; Kumar, R.; Caldararu, O.; Peterson, K.; Wernersson, S.; Brath, U.; Leffler, H.; Oksanen, E.; Logan, D. T.; Nilsson, U. J.; Ryde, U.; Akke, M., Entropy-Entropy Compensation between the Protein, Ligand, and Solvent Degrees of Freedom Fine-Tunes Affinity in Ligand Binding to Galectin-3C. *JACS Au* **2021,** *1* (4), 484-500.
53. Lumry, R.; Rajender, S., Enthalpy-entropy compensation phenomena in water solutions of proteins and small molecules: a ubiquitous property of water. *Biopolymers* **1970,** *9* (10), 1125-227.
54. Waksman, G.; Shoelson, S. E.; Pant, N.; Cowburn, D.; Kuriyan, J., Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* **1993,** *72* (5), 779-90.
55. Breiten, B.; Lockett, M. R.; Sherman, W.; Fujita, S.; Al-Sayah, M.; Lange, H.; Bowers, C. M.; Heroux, A.; Krilov, G.; Whitesides, G. M., Water networks contribute to enthalpy/entropy compensation in protein-ligand binding. *J Am Chem Soc* **2013,** *135* (41), 15579-84.
56. Macro, N.; Chen, L.; Yang, Y.; Mondal, T.; Wang, L.; Horovitz, A.; Zhong, D., Slowdown of Water Dynamics from the Top to the Bottom of the GroEL Cavity. *J Phys Chem Lett* **2021,** *12* (24), 5723-5730.
57. Grason, J. P.; Gresham, J. S.; Widjaja, L.; Wehri, S. C.; Lorimer, G. H., Setting the chaperonin timer: the effects of K+ and substrate protein on ATP hydrolysis. *Proc Natl Acad Sci U S A* **2008,** *105* (45), 17334-8.
58. Todd, M. J.; Viitanen, P. V.; Lorimer, G. H., Hydrolysis of Adenosine 5'-Triphosphate by Escherichia-Coli Groel - Effects of Groes and Potassium-Ion. *Biochemistry* **1993,** *32* (33), 8560-8567.
59. Clark, A. C.; Karon, B. S.; Frieden, C., Cooperative effects of potassium, magnesium, and magnesium-ADP on the release of Escherichia coli dihydrofolate reductase from the chaperonin GroEL. *Protein Sci* **1999,** *8* (10), 2166-76.
60. Madan, D.; Lin, Z.; Rye, H. S., Triggering protein folding within the GroEL-GroES complex. *J Biol Chem* **2008,** *283* (46), 32003-13.
61. Fort, K. L.; van de Waterbeemd, M.; Boll, D.; Reinhardt-Szyba, M.; Belov, M. E.; Sasaki, E.; Zschoche, R.; Hilvert, D.; Makarov, A. A.; Heck, A. J. R., Expanding the structural analysis capabilities on an Orbitrap-based mass spectrometer for large macromolecular complexes. *Analyst* **2017,** *143* (1), 100-105.
62. Marty, M. T.; Baldwin, A. J.; Marklund, E. G.; Hochberg, G. K.; Benesch, J. L.; Robinson, C. V., Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles. *Anal Chem* **2015,** *87* (8), 4370-6.
63. Stengel, F.; Baldwin, A. J.; Bush, M. F.; Hilton, G. R.; Lioe, H.; Basha, E.; Jaya, N.; Vierling, E.; Benesch, J. L., Dissecting heterogeneous molecular chaperone complexes using a mass spectrum deconvolution approach. *Chem Biol* **2012,** *19* (5), 599-607.

For Table of Contents Only



Synopsis

A novel native mass spectrometry method for determining the thermodynamics of ligand binding to protein complexes, *viz.* enthalpy-entropy compensation for the binding of ATP and ADP to the chaperonin GroEL.