Temperature Regulates Stability, Ligand Binding (Mg^{2+} and ATP) and Stoichiometry of GroEL/GroES Complexes

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ABSTRACT: Chaperonins are nanomachines that harness ATP hydrolysis to power and catalyze protein folding, chemical action that is directly linked to the maintenance of cell function through protein folding/refolding and assembly. GroEL and the GroEL-GroES complex are archetypal examples of such protein folding machines. Here, variable-temperature-electrospray ionization (vT-ESI) native mass spectrometry is used to delineate the effects of solution temperature and ATP concentrations on the stabilities of GroEL and GroEL/GroES complexes. The results show clear evidences for destabilization of both $GroEL_{14}$ and $GroES_7$ at temperatures of 50 ºC and 45 ºC, respectively, substantially below the previously reported melting temperature ($T_m \sim 70$ ºC). This destabilization is accompanied by temperature-dependent reaction products that have previously unreported stoichiometries, *viz.* $GroEL_{14}$-$GroES_x$-ATP_y, where x = 1, 2, 8 and y = 0, 1, 2, that are also dependent on Mg^{2+} and ATP concentrations. Variable-temperature native mass spectrometry reveals new insights about the stability of GroEL in response to several environmental effects: (i) temperature-dependent ATP binding to GroEL (ii) effects of temperature as well as Mg^{2+} and ATP concentrations on the stoichiometry of the GroEL-GroES complex, with Mg^{2+} showing greater effects compared to ATP; and, (iii) a change in the temperature-dependent stoichiometries of the GroEL-GroES complex ($GroEL_{14}$-$GroES_7$ vs $GroEL_{14}$-$GroES_8$) between 24 to 56 ºC. The similarities between results obtained using native MS and cryo-EM (Clare *et al.*, An expanded protein folding cage in the GroEL-gp31 complex. J Mol Biol 2006, 358, 905-11; Ranson *et al.*, Allosteric signaling of ATP hydrolysis in GroEL–GroES complexes. Nat. Struct. Mol. Biol. 2006, 13, 147-152.) underscores the utility of native MS for investigations of molecular machines as well as identification of key intermediates involved in the chaperone-assisted protein folding cycle.

Introduction

Chaperones are a class of protein complexes found in all living systems that recognize and bind non-native proteins and assist in the folding of the protein to more stable, native state(s), thereby inhibiting misfolding and aggregation. GroEL, an *E. coli* chaperone, is a model system for understanding molecular chaperones. The GroEL tetradecameric complex ($GroEL_{14}$) is comprised of two heptameric rings stacked back to back.[1] When bound to its co-chaperone, GroES, a heptameric complex that binds to the apical domain of GroEL, this complex promotes proper folding of the non-native substrate protein. While detailed mechanisms for the assembly of GroEL-GroES complex have been described,[2] largely based on the structural analysis of final products of the processes,[3] many details about the dynamics, stabilities,[4, 5] and assembly of the individual units that comprise the GroEL tetradecamer and the GroEL-GroES complex are still not fully understood. Most notably, GroEL stability, structure and function(s) are ATP-dependent, the conformational dynamics of GroEL are directly linked to ATP binding and hydrolysis, and specific domains of GroEL subunits, denoted as apical, intermediate and equatorial,[6] are known to exist as three distinct conformations and that are linked to specific orientations of the apical domain.[7] The N-terminal apical domain of each GroEL subunit is directly involved in binding substrate protein and the GroES co-chaperone. The C-terminal equatorial domains are involved in subunit-subunit interactions both within and between the heptameric GroEL rings as well as binding of nucleotide (ATP), Mg^{2+} and K^+ ions.[8] Communication between the apical and equatorial domains is largely achieved by changes in the conformation through a hinge-like motion of the intermediate domain. Collectively, the dynamics of the individual domains within these complexes act as a two-stroke folding machine wherein encapsulation of substrate by the one ring, denoted *cis* ring, followed by GroES binding initiates the folding reaction.[9-12] ATP binding drives a structural change and formation of the hydrophilic chamber that triggers protein folding, while ATP hydrolysis acts as a timer, controlling the lifetime of the GroEL-GroES folding chamber.[13]

It has been proposed that binding of ATP to the *trans* ring, following hydrolysis of ATP in the *cis* ring, triggers disassembly of the GroEL-GroES *cis* ring complex and subsequent release of GroES and the substrate protein into free solution.[14] Each cycle in this two-stroke process requires ~10-30 s,[13] regardless of whether the substrate protein has completed folding. Thus, most substrate proteins are subjected to multiple folding cycles in order to regain the native conformation required for proper function and assembly.[9]

Early mass spectrometry (MS) studies of GroEL/GroES demonstrated the utility of MS for the analysis of large protein complexes[15, 16] and that these measurements are complementary to other structural biology techniques, *e.g.,* electron microscopy, X-ray crystallography, and NMR spectroscopy. MS analyses provide unparalleled sensitivity and dynamic range for studies of conformationally heterogeneous systems, including direct analysis of reaction products formed by interactions with ligands, cofactors, and intermediates formed on the pathway from initial reactants to final products.[17, 18] Specific examples that are directly related to GroEL include work by Dyachenko *et al.* where native MS was used to investigate the thermodynamics and allostery associated with Mg^{2+}/ATP binding to GroEL[19] and van Duijin *et al.* who used native MS to study folding and assembly of gp23, the major capsid protein of bacteriophage T4, by the GroEL-gp31 complex (a 912 kDa complex).[20] Sharon[19] and Heck[20] performed their studies at different temperatures, 25 and 20 °C, respectively, and different native MS buffers, ethylenediamine diacetate (EDDA) and ammonium acetate (AmA), respectively. Sharon specifically noted differences for ATP binding and charge state distributions for GroEL using AmA buffers compared to solutions of EDDA.[19] Here, we report data obtained using variable-temperature ESI (vT-ESI) native mass spectrometry that provides additional evidences on the stability and dynamics of the GroEL and GroEL-GroES complex. In an earlier report, we described results from variable-temperature ESI native mass spectrometry that we interpreted as evidence for conformational drift associated with ATP binding to GroEL.[21] Note, vT-ESI-MS experiments report on changes in the protein/protein complex that occur in the solution contained within the ESI emitter vis-à-vis processes that occur following the transition from solution to the gas phase. **Figure 1** contains a plot of the average charge (Z_{avg}) for $GroEL_{14}$ (solid black trace) and $GroEL_{14}$-ATP_n (n = 1 -9) bound states of the GroEL tetradecamer complex versus temperature of the ESI solution; using these solution conditions complexes with n > 9 are not observed. For the apo-GroEL tetradecamer (solid black trace), Z_{avg} shifts from 64.8 to 64.3 as temperature is increased from 8 to 18 °C, followed by an increase in Z_{avg} to 65.8 between 18 to 26 °C. At temperatures greater than 26 °C, Z_{avg} for $GroEL_{14}$ hovers around 66. Note that Z_{avg} for the $GroEL_{14}$-ATP_n (n = 1-9) products are also temperature-dependent. Changes in Z_{avg} for n = 1- 4 are small, but decreasing, and changes for n = 5 and 6 are larger as are the changes for n = 7-9. Note also that Z_{avg} for n = 5 and 6 are shift relative to 1-4. The observed changes in Z_{avg} and apparent binding affinities for ATP were attributed to changes in the solvent accessible surface area (SASA) for the apo-GroEL and GroEL-ATP_n complexes owing to temperature- and ATP-dependent conformation changes.[21] It is important to note the overall similarities temperature-dependent Z_{avg} the $GroEL_{14}$ and $GroEL_{14}$-ATP_n, are consistent with changes in SASA of the complexes that have been noted for other systems[22-24] and with well-known cold- and heat-induced folding/refolding reactions of proteins and protein complexes.[25]

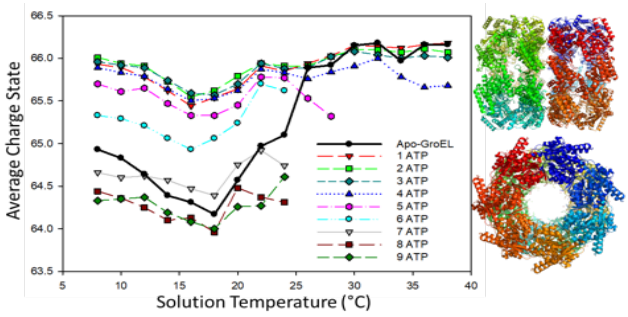

Figure 1. Plot showing temperature-dependent changes in average charge (Z_{avg}) of $GroEl_{14}$ and $GroEL_{14}$-ATP_n (n = 1-9). Solution conditions are 1 μM GroEL, 200 mM ammonium acetate, and 125 μM ATP. Note that Z_{avg} for n = 1-4 are similar, but larger changes in Z_{avg} are observed for n = 5 – 9. At T > 24 ºC the ion abundances for n = 6 - 9 are below the detection threshold. Also, the abundance of n = 5 is begins to decline at T > 24 ºC. This behavior is very different from that obtained using diethylenediammonium acetate buffer (*vide infra*).

In the work presented below, the effects of temperature and ATP binding on the stability and conformation of GroEL, GroES, and GroEL/GroES complexes are investigated using variable-temperature electrospray ionization (vT-ESI) native mass spectroemtry.[21, 26, 27] Conformational changes induced by ATP binding as well as cold- and heat-induced changes in stabilities are directly linked to changes in Gibbs energy.[22, 23, 28, 29] The vT-ESI source is used to control the temperature of the protein-containing solution in the ESI emitter; consequently, the observed changes in stability/conformational are occurring in the solution prior to ESI ionization. While we cannot completely rule out that some reactions may occur in the confined environment of the nano-droplets formed by ESI, evaporative cooling of the nanodroplets has been shown to be an effective mechanism for kinetically-trapping (freeze drying)[30] ions during the transition from solution to the gas-phase.[30, 31] The relationships between ESI freeze drying and cryo-EM has been noted previously[18] and is supported by detection of intermediates and final products that are similar to those previously reported by Ranson *et al.* using cryo-EM.[2] The similarities between results obtained using native MS and cryo-EM,[2, 32] underscore the potential utility of native MS for investigations of molecular machines as well as identification of key intermediates that may be involved in the chaperone-assisted protein folding cycle.

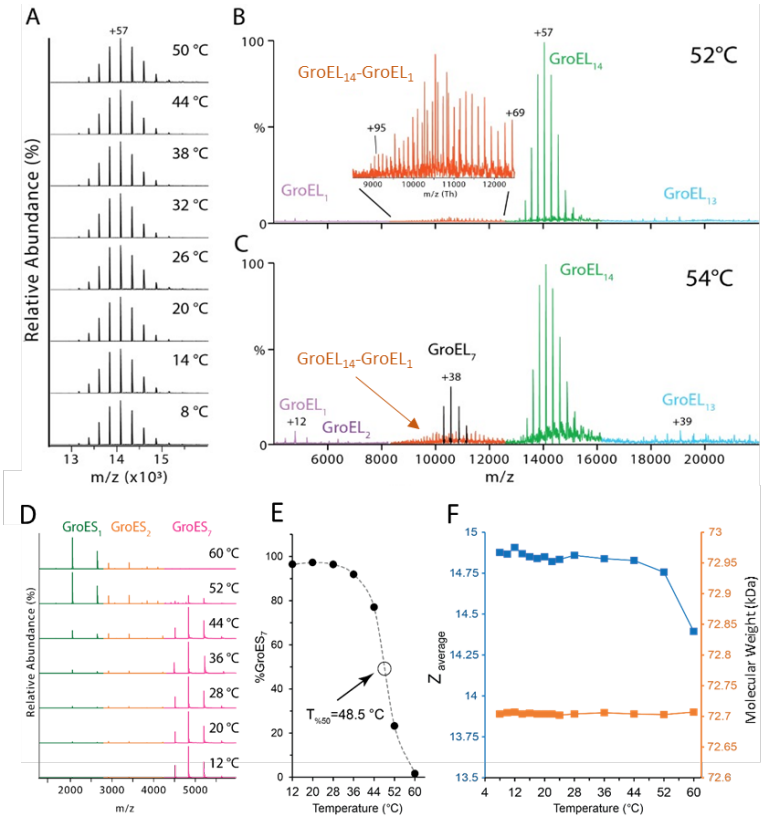

Figure 2. **Effects of temperature on stability of GroEL$_{14}$ and GroES$_7$.** (A) Charge state distribution of intact GroEL tetradecamer (1.2 μM) in 200 mM EDDA at T = 8 - 50 °C. Mass spectrum of GroEL$_{14}$ acquired at (B) T = 52 °C and (C) at T = 54 °C**.** (D) Mass spectra of GroES$_7$ (7 μM, heptamer) in 200 mM EDDA at T = - 60 °C. (E) Change in the relative abundance of GroES$_7$ upon change in temperature. (F) The average charge state and measured molecular weight of GroES$_7$ as a function of temperature shown in blue and orange axes, respectively.

Results

Thermal stability of GroEL and GroES as determined by vT-ESI native MS

Cooling and heating EDDA buffered solutions of GroEL contained in the ESI emitter over a temperature range from 8 – 50 °C did not result in changes in the average charge state (Z_{avg}) or the measured m/z values of GroEL$_{14}$ ions (**Figure 2**); however, evidence for T-dependent stabilities of GroEL$_{14}$ are observed at T > 52 °C (see inset in **Figure 2B**). The increased abundances of GroEL$_7$ ions (**Figure 2C**) relative to that for GroEL$_{14}$-GroEL$_1$ (note arrow in **Figure 2C**) are attributed to solution-phase thermal decomposition of GroEL$_{14}$ (see SI **Figure S1**) While we do not rule out a priori the possibly that these ions are formed by gas phase collision-induced dissociation (CID) (vide infra), further increases in abundances of GroEL$_7$ at 54 ºC support a solution-phase thermal activation mechanism (**Figure 2C**). The sensitivity of the relative abundances of the GroEL$_7$ to an increase of 2 °C in solution temperature denotes a large shift in stability of the GroEL$_{14}$ in this temperature range. Note also that the assignment of GroEL$_7$ ions is supported by excellent agreement between isotopically averaged mass determined experimentally and the value that is calculated, 400.47 kDa vs. 400.40 kDa, respectively. At temperatures greater than 52 ºC ions in the range of 8000-12000 m/z are observed and are assigned to a GroEL$_{15}$ complex; GroEL$_{14}$-GroEL$_1$ complex (MW ~858.3 ± 1 kDa, theoretical: 858.0 kDa). The charge states for these ions are very complex, comprised of a broad distribution of charge states that appear to correspond to unfolded GroEL monomers.

The effects of heating and cooling the ESI solutions on the stability of GroES$_7$ were also investigated (**Figure 2D**). The GroES complex is the most abundant species observed in the range of 12 < T < 28 °C, but at T > 44 °C the monomer and dimer ions are the most abundant signals observed in the mass spectra. Duckworth and co-workers assigned the fragment ions to gas phase collisional activation reactions;[33] however, based on a series of studies, we are confident that under these experimental conditions these product ions result from reactions that occur in the solution.[26, 34] The midpoint in the melting curve (**Figure 2E**) is used to estimate the melting point (T_m) of ~ 48.5 °C, (see experimental section) which is significantly lower than the T_m of ~74 °C reported by Boudker *et al.*[35] It is important to note, however, that their ΔCp vs. T curve revealed evidence for a low temperature endotherm at ~40 ºC, which agrees well with our T_m for GroES$_7$. Geels *et al.*[36] and Dyachenko *et al.*[37] have reported T_m data for GroES using mass spectrometry, and they report similar T_m values (~70 ºC), but both of these studies were performed using ammonium acetate buffer. Moreover, the DSC and vT-ESI results reveal strong evidence for T-dependent instability, and the vT-ESI results suggest that this transition may serve as the early steps leading to disassembly of GroES$_7$ complex. In both experiments it appears that these are reversible even for solutions that have been heated to 90 °C. These results and the lower charge states (Z_{avg} = 4.66 and 6.19, respectively) suggest that the GroES monomers and dimers do not undergo irreversible unfolding upon heating in EDDA buffered solutions; consequently, T_m values determined by CD spectroscopy, which report on the 2º structure, and melting in ammonium acetate do not reveal these low T-dependent melting transitions. On the other hand, native MS, reports T_m on the basis of changes in the SASA, which is indicative of changes in 3° structure of the protein complex.

Temperature-dependent ATP binding to the GroEL$_{14}$ Complex

Sharon and co-workers previously reported thermochemical data (K_a) for ATP binding to $GroEL_{14}$.[19] While their measurements were performed on samples incubated at 25 °C, the actual temperature of the solution in the ESI emitter was not specified. Owing to our observed temperature-dependent stabilities for $GroEL_{14}$ in EDDA buffered solutions, we investigated the effects of temperature on ATP binding to GroEL for vT-ESI solutions with different Mg^{2+} and ATP concentrations. Galan *et al.* observed a low temperature endotherm of GroEL by DSC that they proposed as evidence for conformation changes in the $GroEL_{14}$ complex, specifically modifications of tertiary and quaternary structure and minor changes in secondary structure.[38] Similar conformational changes were also observed using fluorescence.[39] VT-ESI native MS can potentially provide additional evidence for conformational changes as well as how such changes affect ATP binding.

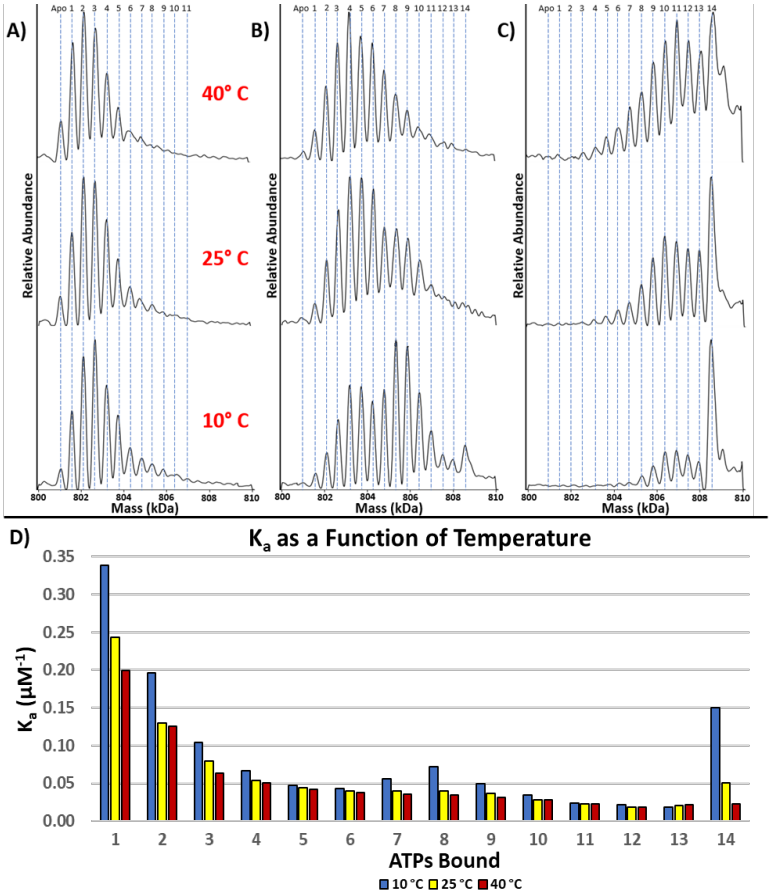

Figure 3. **ATP binding is favored at low temperatures.** Mass spectra of deconvoluted charge state of GroEL (0.5 μM, tetradecamer) in 200 mM EDDA and 1 mM Mg^{2+} incubated with ATP (A) 10 μM, (B) 25 μM and (C) 50 μM at different temperatures. (D) calculated association constants (K_a) for binding of ATP to GroEL over 3 temperatures (10 °C, 25 °C, and 40 °C). Binding is more favorable at lower temperatures for ATP_{1-3}, ATP_{7-9}, and ATP_{14} and affinity decreases with increase in solution temperature.

Addition of 10 µM ATP to a solution of $GroEL_{14}$ (solution "A", see **Table 1**) produced $GroEL_{14}$-ATP_{1-11} (**Figure 3A**) at low temperatures, but higher solution temperatures binding of ATP become less favorable (see **Figure 3A**). Increasing the concentration of ATP (**Figure 3B**) to 25 µM (solution "B", **Table 1**) yields $GroEL_{14}$-ATP_n, where n varies from 1 to 14 at 10 °C, but as T is increased, the abundances of all these products decrease, esp. at n > 7. Clearly lower solution temperatures are more favorable for ATP binding, and under these conditions the complexes also carry lower Z_{avg}, which is consistent with an ATP-induced conformational change of $GroEL_{14}$.[38] Z_{avg} trends as a function of temperature for GroEL-ATP_n in EDDA are consistent with trends seen earlier in **Figure 1**; stepwise addition of ATP induces a small decrease in Z_{avg} with each ATP bound. A similar temperature dependence is observed for solutions containing 50 µM ATP ("solution C", **Table 1**). Under these conditions ATP binding to the second GroEL ring is even more favored at 10 °C, whereas at 40 °C the more abundant signals correspond to GroEL-ATP_{3-6} (**Figure 3B**). While ATP and ADP are not distinguishable at the mass resolution used in this study. the affinity of GroEL for the binding of ATP is an order of magnitude greater than for the binding of ADP.[40, 41] The ATPase activity of GroEL will also be negligible without the presence of K^+.[42, 43]

The higher relative abundance for ATP_{2-3} (**Figure 3B**) and $ATP_{9-10(7+2-3)}$ (**Figure 3C**) reveals that binding of up to three ATP molecules at each ring is more favorable. Interestingly, Chapman *et al.* previously suggested that binding of three ATP molecules is required for successful substrate binding and release.[44] The macroscopic association constants (K_a) for temperature-dependent ATP-GroEL binding (10, 25, and 40 °C) are shown in Figure 3D. These K_a values show that ATP is bound with a higher affinity at lower temperatures and the affinity diminishes at higher solution temperature. The initial (e.g., ATPs 1-3) and last binding affinities are most affected by temperature (**Figure 3D**). The binding of the 14th ATP draws particular interest due to the relative binding affinity compared to the other affinities directly preceding it. These K_a values are consistent with positive cooperativity reported by Yifrach & Horovitz[41] for intra-ring-ATP binding – possibly reflected by the dramatic increase in relative abundance for the $GroEL_{14}$-ATP_{14} at low temperatures.

Ligand- and Temperature-dependent stabilities of the GroEL/GroES complexes

Results for temperature-dependent ATP-GroEL binding prompted further investigations on how temperature influences the binding of GroES to $GroEL_{14}$. As noted previously[19] mass spectra acquired from EDDA-buffered solutions give well resolved peaks and are less congested, thereby greatly simplifying assignment of mass spectral signals. The mass spectra obtained from EDDA-buffered solutions containing a range of concentrations of $GroEL_{14}$, $GroES_7$, Mg^{2+} and ATP (**Table 1**) were examined. The mass spectrum obtained from solution "D" contain ions corresponding to $GroEL_{14}$-$GroES_{14}$-ATP_n, where n = 12 – 14,

presumably the "football-shaped (FS)" structure.[45, 46] Lorimer suggested that K^+ facilitates ATP turnover and promotes formation of the "bullet-shaped" (BS) structure, $GroEL_{14}$-$GroES_7$-ADP, and serves as the acceptor state in the substrate refolding cycle.[47] Here, we avoided the effects of K^+ because this also further complicates assignment of the products observed in the mass spectrum. On the basis of preliminary data acquired from an EDDA solution containing K^+ (800 uM) we can report that that K^+ significantly increases ATP binding but the stoichiometry of the resulting GroEL-GroES complexes does not change.

Table 1. The effects Mg^{2+} and ATP concentrations on ATP binding and stoichiometry of GroEL-ES complex at 24 ºC in 200 mM EDDA.

Solution "X"	GroEL (nM)	Mg^{2+} (μM)	ATP (μM)	GroES (μM)	ATP/GroEL	Mg^{2+}/GroEL	$GroEL_x$-$GroES_y$-ATP_n
A	500	1000	10	-	20	2000	$GroEL_{14}$- ATP_{1-4}
B	500	1000	25	-	50	2000	$GroEL_{14}$- ATP_{1-14}
C	500	1000	50	-	100	2000	$GroEL_{14}$- ATP_{3-14}
D	260	225	110	12	420	850	$GroEL_{14}$-$GroES_{14}$-ATP_{14}
E	120	25	50	12	420	200	$GroEL_{14}$-$GroES_{7-8}$-ATP_8
F	600	500	50	12	80	850	$GroEL_{14}$-$GroES_{7-8}$-ATP_8
G	600	25	50	12	80	40	$GroEL_{14}$-$GroES_{0-2}$-ATP_{0-2}
H	500	-	-	12	-	-	ND[a]

[a] Not detected.

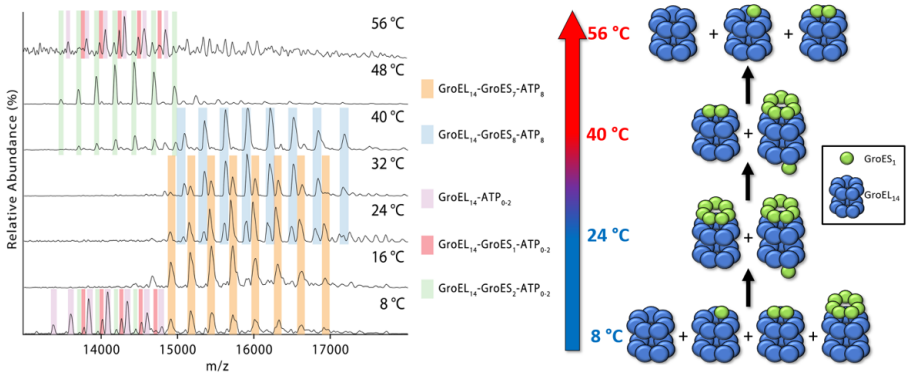

Figure 4. **Mass spectra showing the region correspoinding to the GroEL-GroES complexes formed in solution E** (**Table 1** solution "E" 120 nM GroEL, 12 μM GroES, 25 μM Mg^{2+}, 50 μM ATP in 200 mM EDDA). The accompanying illustration summarizes the observed products formed upon heating solution "E" from 8 ° to 56 °C. Lower order $GroEL_{14}$-$GroES_n$ products are initially formed at lower solution temperatures, whereas $GroEL_{14}$-$GroES_7$ and $GroEL_{14}$-$GroES_8$ products are detected at higher temperatures. At elevated temperatures the observed species revert back to the lower mass complexes seen at cold solution temperatures.

The mass spectra shown in **Figure 4** were acquired from solution "E" (25 μM Mg^{2+} and 50 μM ATP) at temperatures between 8 °C and 56 °C. At the lowest temperature (8 °C) the spectra contain abundant ions corresponding to $GroEL_{14}$-ATP_{0-2} (violet), $GroEL_{14}$-$GroES_1$-ATP_{0-2} (magenta), $GroEL_{14}$-$GroES_2$-ATP_{0-2} (green), $GroEL_{14}$-$GroES_7$-ATP_8 (orange). Increasing the temperature to 16 °C results in formation of $GroEL_{14}$-$GroES_7$-ATP_8, and at 24 and 32 °C the major products correspond to $GroEL_{14}$-$GroES_7$-ATP_8 and $GroEL_{14}$-$GroES_8$-ATP_8 (blue); however, the ratios of these two products change in the range of 24 to 32 °C. At T > 32 °C the signal for these complexes is diminished, possibly a result of disassembly, first to form $GroEL_{14}$-$GroES_2$-ATP_{0-2} followed by formation of the initial products observed at 8º C. The assignment of ATP_8 is consistent with measured mass differences for $GroEL_{14}$-$GroES_7$ and $GroEL_{14}$-$GroES_8$ masses and the measured mass of the $GroEL_{14}$-$GroES_7$ complex, which corresponded to 8 ATP molecules.

Mass spectra obtained for solution "F", which has a higher concentration of ATP and Mg^{2+} than solution "D", is most similar to the mass spectrum obtained (at 24 °C) using solution "E" (**Figure 5A**); however, the ratios of $GroEL_{14}$-$GroES_7$-ATP_8 and $GroEL_{14}$-$GroES_8$-ATP_8 are different. These results underscore the importance of both Mg^{2+} and ATP in determining the stoichiometry of GroEL-GroES complexes. The $GroEL_{14}$-$GroES_1$ and $GroEL_{14}$-$GroES_2$ product ions (magenta and green, respectively) from vT-ESI of solutions containing low Mg^{2+}/$GroEL_{14}$ and ATP/$GroEL_{14}$ ratios (solutions "E" and "F", respectively) is interesting. Further decrease in the ratio of Mg^{2+}/$GroEL_{14}$ (solution "G" relative to solution "F" (**Table 1**)) results in a mass spectrum (**Figure 5B**) that contains the ions $GroEL_{14}$-ATP_{0-2}, $GroEL_{14}$-$GroES_1$-ATP_{0-2}, and $GroEL_{14}$-$GroES_2$-ATP_{0-2}, and low abundances of $GroEL_{14}$-$GroES_x$-ATP_y (x = 7 or 8 and y = 8). The ions at higher m/z values are identical to ions observed from solution "F", whereas the ions at lower *m/z* correspond to GroEL-$GroES_y$ (y = 1 and 2). These results suggest that binding of $GroES_7$ to the GroEL-ATP complex is directly linked to the concentrations of both ATP and Mg^{2+} ions. The binding of individual GroES subunits to GroEL has not been reported and seems counterintuitive. The binding of individual GroES subunits could also explain the formation of $GroEL_{14}$-$GroES_8$ ions in solution "E". Presumably, the $GroEL_{14}$-$GroES_8$ complex has a single GroES subunit bound to the *trans* ring and an intact $GroES_7$ bound to the cis ring. VT-ESI analysis of solution "F" is similar to solution "E" in terms of increased abundances of GroEL-ATP_{1-2} and $GroEL_{14}$-$GroES_2$-ATP_2 at 8 °C and ~50 °C, respectively. Collectively, the results suggest that these experimental conditions alter the stability/dynamics of the $GroES_7$ and/or the positioning of GroES with the apical domain of the GroEL complex.

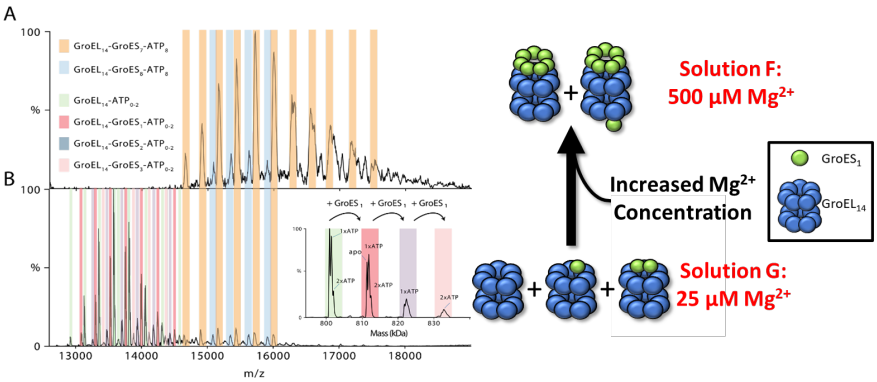

Figure 5. **Mg^{2+} regulates the stoichiometry of GroEL-ES complex.** (A) Mass spectra of solution "F" and (B) solution "G", collected at 12 k resolution (HCD = 200 V) at 24 °C.

The role of Mg^{2+} and ATP concentration on the stoichiometry of GroEL-GroES complex, specifically formation of sub-stoichiometric GroES complexes is illustrated by the data shown in **Figure 5.** The spectrum shown in **Figure 5B** was obtained from solution "G" (25 µM Mg^{2+} and 50 µM ATP), and the spectrum shown in **Figure 5A** was taken following incubation of $GroEL_{14}$ with $GroES_7$ in solution "F" containing 500 µM Mg^{2+} and 50 µM ATP (**Table 1**). These solution conditions produced several GroEL-GroES intermediates, the most abundant being $GroEL_{14}$-$GroES_1$-ATP_{0-7}, $GroEL_{14}$-$GroES_2$-ATP_{0-6} and $GroEL_{14}$-$GroES_3$-ATP_{0-6}. The increase in numbers of bound nucleotides but no detected products corresponding to $GroEL_{14}$-$GroES_7$ complex corroborates the role of Mg^{2+} in driving the reaction. Moreover, GroEL-GroES complexes were not detected from solutions of $GroEL_{14}$ and $GroES_7$ in the absence of ATP and Mg^{2+} (solution "H", **Table 1**). Boudker *et al.* previously reported Mg^{2+}-dependent stability for $GroES_7$ (K_d ~ 0.5 mM),[35] and Sakane *et al.* reported reduced stabilities for $GroES_7$ at low concentrations.[48] Possible explanations by which sub-stoichiometric GroEL-GroES complexes might be formed, and how this might be affected by the stability of the $GroES_7$ complex are discussed below.

Discussion

The vT-ESI GroEL studies were limited to a single native MS buffer EDDA owing to greater stability and structural integrity when compared to that for the more commonly used native MS buffer, ammonium acetate.[8,49] The charge states produced from ammonium acetate solutions differ from those from EDDA, and the data shown in **Figure 1** and **Figure 2** reveal differences in thermal stability of GroEL in the different buffers.[8] The upregulation of heat shock proteins and chaperonins raises questions of how the GroEL-GroES system responds to both cooling and heating conditions. Heat-induced folding increases conformational entropy and the associated changes in the dielectric constant of water,[50] which favors entropic unfolding as evidenced by changes in the solvent-accessible surface area (SASA) (**Figure 1**).[51] Cold-induced changes in conformation are associated with reorganization (hydrophobic effect) of the solvent molecules surrounding the protein. Our results show that both GroEL and GroES are stable up to ~44 °C in EDDA, but this is limited to a relatively narrow range of concentrations. The discrepancies between the previously reported thermal stabilities of GroEL (T_m ~67 °C)[52] and GroES, (T_m ~76.4 °C)[35] and our results are most likely due to differences in solution conditions, viz. salt composition and concentration. Moreover, the previously reported T_m were determined using biased techniques, *viz.* CD spectroscopy, which measures changes in 2° structure; native MS reports changes in Z_{avg}, related to changes in SASA, as well as T-dependent changes in ligand binding. GroEL, GroES, and GroEL/GroES complexes are highly dynamic, and even small changes in the solution conditions, *viz*. chemical potential (Gibbs free energy) of the local environment (T, P, pH, osmolytes, chemical chaperones, concentration of GroEL/GroES), strongly affect conformation, stability and possibly the behavior of these nanomachines. This explanation is consistent with the previously reported low temperature endotherm in the DSC profiles detected at ATP concentrations of 0.25 and 0.50 mM;[38] the authors note "existence of a conformational state of GroEL with modified 3°/4° structures having increased exposure of hydrophobic surfaces, but minor rearrangements of its 2° structure."

We show that in EDDA solutions, $GroEL_{14}$ becomes unstable above 50 °C and unfolding of the monomer resembles previously reported self-chaperonin activity of GroEL[53] even though the size of GroEL monomer ($GroEL_1$) is near the limit of substrate size reported previously, ~70 kDa.[54] This self-protection mechanism in response to heat shock stress is evidenced by our observation of a highly stable octamer in ammonium acetate that most likely consisted of a single ring GroEL and a trapped unfolded subunit that cannot be dissociated in the gas phase (data not shown). We conjecture that such high stability plays a role in the capability of this chaperone to regain its functionality after removal of heat stress. The self-assembly and monomer unfolding can be connected to irreversibility reported from thermodynamics measurements of GroEL.[38]

Disassembly of $GroES_7$ at elevated temperatures (T > 44 °C) can be attributed to the increased coulombic repulsion of negatively charged residues, Glu50 and Glu53, localized on the roof of the $GroES_7$ complex and near the subunit-subunit interface.[55, 56] The charge state distributions and Z_{avg} of unfolded subunits are not detected by native MS experiments upon heating up to 90 °C, suggesting that dissociation of subunits precedes the backbone unfolding. Supporting evidence for this statement is the observed reversibility of assembly upon cooling the solution. The native MS studies performed at sub-ambient temperature (T < 24 °C) do not reveal changes in quaternary structures of $GroEL_{14}$ and $GroES_7$ which might be expected for assembly

mechanisms involving purely hydrophobic interactions; however, for GroEL, hydrogen bonds and salt bridges are known to provide stabilization of the complexes.[52, 57-60]

Impact of ions and small molecules on GroEL-GroES function:

Physiological function of molecular chaperones such as GroEL are ATP- and Mg^{2+} K^{+}-dependent,[11, 61] and nucleotide hydrolysis serves as the energy source to power specific conformational changes.[13, 62] Temperature can significantly influence the thermodynamics and kinetics of small molecule binding. High resolution native MS and vT-ESI afford means to study these binding events with unprecedented detail at concentration levels that mimic physiological conditions, including experimental conditions that allow final products to be analyzed. On the other hand, native MS affords the ability to investigate chaperones over a range of experimental conditions that may reveal previously hidden details and the presence and roles of intermediates that are either off- or on-pathway to final products. As a single example, the fact that ATP binding is diminished at elevated temperatures provides a clue regarding the contribution of ionic interactions, with a major role of the phosphate group compared to the hydrophobic moiety (adenosine).[62] The formation of ADP, which is expected to increase the heterogeneity of the system, is inevitable in our experiments; however, because ADP is similar to ATP in thermochemistry, it does not interfere with the vT-ESI of GroEL-GroES complex. In fact, we have previously shown that ADP binding to GlnK is enthalpically driven and disfavored at elevated temperatures.[28] Moreover, we cannot rule out the contribution of conformational dynamics of GroEL, mainly local destabilization of nucleotide binding and the observed changes in ATP binding at elevated temperatures. Nevertheless, our data not only provide insight into effect of temperature on ATP binding but also on ATP-induced conformational change on GroEL. The observed decrease in the Z_{avg} of $GroEL_{14}$-ATP complex compared to apo-$GroEL_{14}$ is consistent with structural rearrangement or conformational drift that has been previously reported.[63]

T-dependent GroEL-GroES interactions

Cold-induced disassembly of protein complexes is associated with a decreased entropic penalty for ordering water near hydrophobic residues.[25] The disassembly of $GroEL_{14}$-$GroES_7$, the bullet (BS) structure, at T < 8 °C points to a major contribution of hydrophobic interactions, mainly engagement of GroES "mobile loop" with apical domain of GroEL.[64] Previously, Todd *et al.*[65] have shown the fast release of $GroES_7$ from $GroEL_{14}$-ADP complex at 4 °C, corroborating the role of hydrophobic interactions in this binding process.

The higher stability of the football (FS) structure ($GroEL_{14}$-$GroES_{14}$) compared to the BS complex at low and high temperatures suggests that the apo ring might compromise the stability of GroEL ring capped with $GroES_7$. In other words, the structural changes in the free ring upon heating or cooling the solution destabilizes the subunit-subunit interactions in the GroES-bound ring to disrupt GroES binding. Additional evidence for compromised inter-ring stability of the BS complex is the observation of single ring GroEL ($GroEL_7$) in the vT-ESI of $GroEL_{14}$ (**Figure 2**). Our data suggest that heat-induced unfolding of GroEL subunits destabilizes the subunit-subunit interactions between two rings. In fact, our results show that subunit unfolding precedes the appearance of single ring and suggests that unfolding of GroEL subunits in one ring disfavors intra-ring interactions. Such ring splitting of $GroEL_{14}$ has been previously reported under heat shock conditions,[66] and the importance of such effects serves as the basis for planned future studies – the target being how $GroES_7$ binding to both rings seems to inhibit such conformational changes and increases the stability of complex over a broader range of temperatures. The fact that the FS structure is fully loaded with ATP molecules might also enhance the stability of $GroEL_{14}$-$GroES_{14}$ complex. Furthermore, the resistance of the FS structure against cold and hot treatment points out to the limited dynamics of this structure and in contrast to GroEL biological function. On the other hand, previous studies have shown that ADP-bound BS structure is the acceptor state of GroEL-GroES system and is more physiologically relevant.[67]

The observation of intermediates, such as $GroEL_{14}$-$GroES_{1\text{-}2}$, upon both cooling and heating of the BS structure is an unexpected result as is the observation of $GroEL_{14}$-$GroES_8$ at temperatures between 24 - 40 °C. The increased abundances of these complexes at elevated temperatures can be justified based on temperature- and concentration-dependent stabilities of $GroES_7$.[35, 48] The formation of $GroEL_{14}$-$GroES_x$ (x = 1,2) at both low (8 °C) and high temperatures (up to 56 °C) can be explained by temperature-dependent disassembly of the $GroES_7$ complex, and this is supported by the 0.5 mM K_d reported by Sakane et al.[48] and Boudker et al.,[35] which raises yet another possible explanation. Specifically, that a destabilized GroES binds to the apical domain of GroEL through a limited number of GroES subunits and the exposed subunits then reorganize and disassemble. Evidences for this hypothesis are the reported instabilities previously reported[35, 48]which are further supported by the effects of Mg^{2+} concentration shown in **Figure 5**. Lastly, these intermediates are only observed in Mg^{2+}- or ATP-deprived solution, which corroborates their transient nature.

The number of bound ATPs required for GroEL and GroES interaction as well as substrate refolding has been previously investigated by Chapman *et al.*[44] In their study, ATP binding to a GroEL mutant (I493C) was inhibited in the presence of the cyclopentane-carboxamide derivative molecule, EC3016. It was shown that only 50% of W2M5 complex (W: wild type and M: mutant) is capable of binding GroES in the presence of an inhibitor, and at least three ATP molecules are required for GroES binding. Here we observed binding of 1-3 individual GroES subunits to GroEL in solutions where Mg^{2+} and ATP

concentrations are below physiological concentration (~2-3 mM and ~3 mM, respectively[68]). We discount the possibility that these products are formed by dissociation of a $GroES_7$ that is bound to the GroEL apical domain; however, we also detected a correlation in the number of ATP molecules bound to GroEL and the stoichiometry of GroEL- GroES - ATP_n complex. For n = 1-3, up to two GroES subunits are able to interact with GroEL and increasing Mg^{2+}/ATP concentrations increases ATP binding, $6 < n < 9$ as well as binding of intact GroES ($GroES_7$). We propose that binding of ATP_{1-4} can allow for rotation of the apical domain of GroEL to recruit individual GroES subunits but not enough to allow for binding intact GroES.[43] Specifically, binding of intact co-chaperone requires specific amounts of Mg^{2+} and in its absence binding of ATP_n molecules ($n > 5$) is not sufficient to capture the native GroES complex. Previously, Azem *et al.* showed that Mg^{2+} not only increases ATP binding and hydrolysis but also increase the dynamics of GroEL.[61]

The X-ray structure of $GroES_7$ provides more insight into observations of individual subunits of GroES binding to GroEL. Hunt *et al.* discovered an unexpected asymmetry in terms of subunit-subunit interactions in $GroES_7$ structure as well as interfacial residues.[55] In fact, R47 and K55 residues form salt bridges with neighboring residues in only two subunits and these stabilizing interactions are missing in the other five subunits. This structural heterogeneity[37] might also explain the observation of monomer and dimer ions in native mass spectrum of $GroES_7$ (**Figure 3**) and previous studies.[33, 37] The energy requirement for disassembly of the GroES complex can be compensated by the binding energy to $GroEL_{14}$. Previous studies have shown that both ATP and GroES binding are necessary for apical domain movement and for transition from substrate-binding mode (hydrophobic surface exposed) to the folding-active state (hydrophilic chamber).[69, 70] The role of GroES binding in this transition suggests an alternative mechanism under extreme conditions wherein sequential binding of GroES subunits to GroEL may indeed become favorable. These factors may also have implications in refolding of multi-domain proteins where $GroES_n$ binding ($n < 7$) could allow for partial or full release of a single GroES subunit, providing the gap necessary for a piece of polypeptide to extrude to the exterior of the GroEL-GroES complex.[71, 72]

The vT-ESI data offer insight into thermal stability of $GroEL_{14}$-$GroES_{1-3}$ complexes. The higher abundance of $GroES_1$ and $GroES_8$ at 32 °C < T < 40 °C (**Figure 5**) indicates that binding of a single GroES subunit to a GroEL ring is thermodynamically stable, whereas increasing temperature favors binding of two subunits (~44 °C). Above this temperature both GroEL and GroES become unstable and GroEL-GroES interactions are lost. At low temperatures, also due to the hydrophobic nature of the GroEL-GroES interaction, these intermediates are no longer formed. Overall, these results showcase the power of native high-resolution MS and vT-ESI to investigate the role of nucleotide and small molecules in chaperonins and their interaction with co-chaperone. Future studies with substrate can shed light on the thermal dependency of protein chaperones and substrate interactions and cofactors roles in driving the refolding reaction.

Experimental Data and Materials

Materials:

All reagents including magnesium acetate and ethylenediamine diacetate (EDDA) were purchased from Sigma Aldrich (St. Louis, MO). GroEL and GroES were over-expressed in *E. coli* as described previously.[73] Adenosine triphosphate sodium (ATP) was purchased from Jena Bioscience and dissolved in deionized water (Barnstead Easy Pure II, Thermo Scientific). Sample aliquots were stored at -20 °C and freshly diluted with water then added to protein prior to analysis. Protein concentration was measured using Bradford assay and are for tetradecameric and heptameric proteins unless otherwise mentioned. Fresh GroEL (6 μM, 10 μL) and GroES (60 μM, 10 μL) were diluted three-fold with EDDA and buffer exchanged into buffer using Micro Biospin P-6 gel column (BioRad).

Native mass spectrometry:

Mass spectra of proteins were collected on a Q Exactive UHMR (Ultra-High Mass Range) Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific, CA) with the mass range of 350-80000 m/z. The instrument was calibrated using CsI (2 mg/ml in 50:50 IPA: H_2O) in positive mode. Capillary voltage varied from 1.0 - 1.6 kV for the highest signal and better desolvation. To obtain better resolved peaks, in-source trapping and activation (150 - 250 V) was used; higher collision energies are important for solution with higher Mg^{2+}/ATP concentration. To increase the mass accuracy and ion abundances, HCD activation was also used (150-250 V) for ATP binding studies or to eject individual GroES subunits in GroEL-GroES complexes. The temperature of the instrument ion source was maintained at 100 °C for all the vT-ESI experiments. The temperature of the instrument ions source was varied to promote better desolvation andincrease in ion signals. The temperature of the vT-ESI source was controlled as described previously.[74] For melting experiments the response factors for multiple oligomeric states of the complexes in question were assumed to be identical, meaning that the detection of all signals for all *m/z* values were assumed to be unbiased. Mass spectra analyses were performed using UniDec[75] to determine the average charge state and experimental molecular weights. The Z_{avg} for $GroEL_{14}$-ATP_n was calculated by averaging the Z_{avg} for individual ATP bindings.

ATP Association Constants:

The K_a values for the binding of ATPs 1-14 by $GroEL_{14}$ were calculated using an ATP titration study. The solution conditions were 500 nM $GroEL_{14}$ in 200 mM EDDA buffer with 1 mM MgAc. ATP concentrations used in the study were: 0, 0.2, 0.4, 0.6, 0.8, 1, 2.5, 5, 10, 15, 20, 25, 30, 40, and 50 μM. Each concentration was analyzed at 3 different temperatures (10 °C, 25 °C, and 40 °C). The solution temperature was allowed to equilibrate for a minimum of 3 min before the measurement was conducted. Mass spectra were deconvoluted using UniDec and incorporated the 4 most abundant charge states of the $GroEL_{14}$-ATP_n distributions in each spectrum for the deconvolution. Macroscopic association constants (K_a) for ATP binding to $GroEL_{14}$ were calculated using a sequential ligand binding model.[19]

vT-ESI-MS experiments:

The temperature of the solution contained in the ESI emitter is controlled using a vT-ESI apparatus that has been described previously.[49] Briefly, the ESI emitter is positioned within an aluminum block and the temperature of the Al block is heated/cooled using a 3-tier Peltier chip controlled by a thermoelectric cooler (Meanwell LRS-100 24). A ceramic rod also transferred heat efficiently from emitter. Borosilicate glass capillary tips were pulled in house using Sutter 1000 and ions were generated via a platinum wire inserted into the capillary. The temperature of solution in vT-ESI was calibrated using a T-Type thermocouple (Physitemp Clifton, NJ) inserted into the capillary.

Conclusion

Variable-temperature ESI native mass spectrometry was used to investigate the effects of ATP and GroES binding to on the stability of the GroEL complex. This approach complements similar studies performed using differential scanning calorimetry (DSC), the traditional method used for studies of protein heat capacities (ΔC_p) and determinations of melting temperature (T_m). The sensitivity and dynamic range of native MS afford the ability to detect previously hidden products, non-native states as well as reaction intermediates, including changes in the structure/conformation of the protein complex.[76] Temperature-dependent changes in structure/conformation originate from changes of both secondary (2^o), tertiary (3^o), and/or quaternary (4^o) structure of protein complexes. T_m values for a given protein or protein complex may vary depending on solution conditions, solvent, pH, pressure, ionic strength, and presence/absence of other solutes, all of which contribute to the Gibbs energy of the system. Oftentimes low energy endotherms, changes in ΔC_p that occur at temperatures below the T_m, are observed owing to formation of misfolded states and/or subtle changes in conformation, changes in 3^o structure ("conformational drift") under conditions whereby 2^o structure is retained. The changes in Z_{avg} obtained using vT-ESI native MS also reflect temperature-dependent and ATP-dependent conformation changes of GroEL and the GroEL-GroES complexes that arise from changes in the solvent accessible surface area (SASA) of the proteins that accompany ATP binding. Galan *et al*.[38] reported low temperature endotherms associated with ATP binding to GroEL using DSC; however, as noted above, the origin(s) of the ΔC_p signals are difficult to assign from such measurements.

In summary, this work demonstrates the utility of vT-ESI and high-resolution native mass spectrometry to investigate the role of co-factor and co-chaperone molecules in stability of chaperonins using a broad range of experimental conditions. Our results specifically shed light on the thermal stability of GroEL, GroES and GroEL-GroES intermediates resulting from cooling and heating the solution. Although evidences for thermal instabilities of GroEL and GroEL/GroES complexes have been observed previously,[38, 76] the specific effects have not been fully characterized. Thermodynamic studies, inclusive of ΔG, ΔH and $T\Delta S$ that are currently underway, can reveal even greater insights on the formation of these complexes and for ATP binding that may provide greater details and may provide even further understanding of the GroEL/GroES operational mechanism. Nonetheless, this study clearly illustrates the utility vT-ESI native mass spectrometry for analysis of biologically complex systems. The presence of intermediates and their role/impact on the operation of the GroEL molecular machine are still open to debate, especially the potential role that the effects note in this study might impact our views of folding/refolding of non-native substrate proteins.

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Author Contributions

The collection, interpretation and writing of the manuscript represent contribution from all authors, and all authors have given approval to the final version of the manuscript.

Supporting Information

Mass spectral data showing effect of temperature on $GroEL_{14}$-$GroEL_1$ and $GroEL_7$. This material is available free of charge at http//pubs.acs.org.

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Temperature Regulates Stability, Ligand Binding (Mg^{2+} and ATP) and Stoichiometry of GroEL/GroES Complexes

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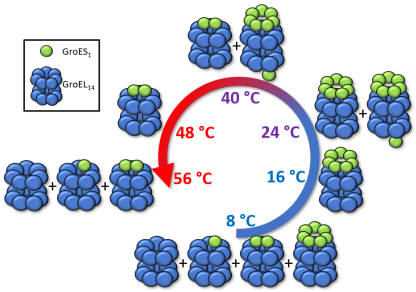
TOC graphic



The stoichiometry of the GroEL-GroES complex varies with temperature as well as concentration of both ATP and Mg^{2+}

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Supporting Information:

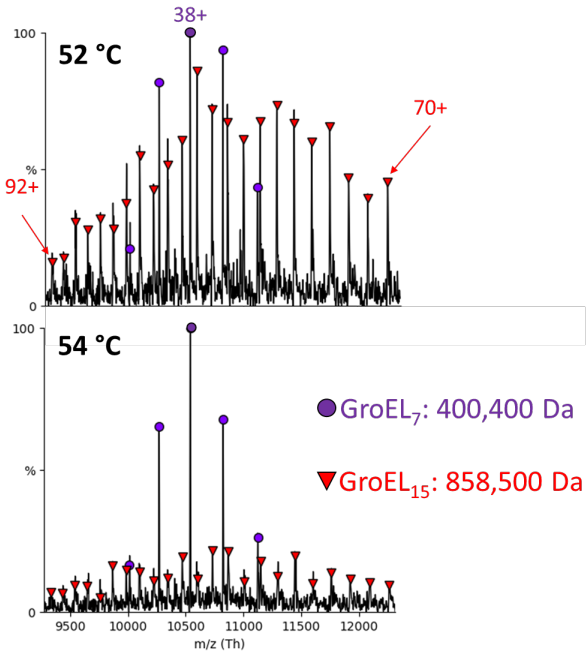

Figure S1. **Effects of temperature on GroEL$_{14}$-GroEL$_1$ and GroEL$_7$.** The mass spectral data presented in this figure are from the data presented in **Figure 2** but only focus on the GroEL$_{14}$-GroEL$_1$ and GroEL$_7$ oligomeric states. The solution conditions remain the same between the top and the bottom spectra but are maintained at different solution temperatures (52 and 54 °C). At higher solution temperatures the GroEL$_7$ becomes more abundant signaling that GroEL$_{14}$ may be dissociating into heptameric rings. The GroEL$_{14}$-GroEL$_1$ species is present at both temperatures and is thought to be the capture of a GroEL$_1$ monomer by GroEL$_{14}$. The GroEL$_{14}$-GroEL$_1$ charge state distribution is broad and would be consistent with the capture of an unfolded monomer. GroEL$_7$ charge states are consistent with a native-like heptamer that is present in solution. The rise in the GroEL$_7$ abundance seemingly attenuates the GroEL$_{14}$-GroEL$_1$ signals in the 54° C spectrum. However, when compared to the overall relative abundance for the entirety of each spectrum (**Figures 2B** and **2C**), the relative abundance of each species is actually increasing. This shift in observed oligomeric states of GroEL$_{14}$ points to a temperature-dependent transition in solution-phase stability of the complex. As the GroEL$_{14}$ complex dissociates it begins populating other non-native oligomeric states.