Detection of the Intact GroEL Chaperonin Assembly by Mass Spectrometry

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We have determined mass spectrometry conditions for maintaining the intact GroEL complex in the gas phase. Using nanoflow electrospray (nano-ES) with time-of-flight (ToF) mass analysis we show that it is possible to obtain definitive charge states in the spectra of this large multiprotein complex. By applying a combination of carefully controlled parameters,[1] an aqueous solution of GroEL[2] can be induced to remain intact, giving rise to a series of peaks centered at $m/z \approx 10\,000$, Figure 1. No other well-defined species are present in contrast to previous results, obtained under the same solution conditions, in which the dominant species was monomeric GroEL.[3] The peaks at $m/z \approx 10\,000$ are assigned as +80 to +88, giving rise to a mass of 803 742 ± 616 Da for the intact GroEL complex. From the known mass of the monomer, measured previously under denaturing conditions,[3] the number of subunits in this gas-phase assembly can be determined as 14.

Given that previous ES measurements relied upon gas-phase dissociation to disrupt ligand-bound chaperonin complexes yielding monomeric GroEL and substrate protein,[3,4] it is surprising that, once present in the gas phase, the $GroEL_{14-mer}$ is remarkably stable. The $GroEL_{14-mer}$ is resistant to nozzle to skimmer dissociation in the atmospheric pressure region of the mass spectrometer. This process has led previously to dissociation of other noncovalently bound complexes.[5−7] We have found, however, that it is possible to impart sufficient internal energy to the macromolecular ions in the collision cell of the quadrupole time-of-flight mass spectrometer used in these experiments. Stepwise increases in the collision energy give rise to an intermediate charge state series centered at $m/z \approx 6000$, Figure 2. With further increase in the collision energy, no detectable peaks remain at $m/z \approx 10\,000$, the species centered at $m/z \approx 6000$ now dominates. The mass of this species is 401 556 ± 202 Da, in close agreement with the theoretical value for half of the mass of the intact complex and correlates to a GroEL heptamer. Since no other species are present, this observation demonstrates 2-fold symmetry for this complex. At the highest collision energy a well-resolved charge state series, centered at $m/z \approx 2000$, corresponding in mass to 57 287 ± 1 Da is observed. This mass is approximately one-seventh of the mass of the intermediate, demonstrating its 7-fold symmetry and illustrating the 14 subunit stoichiometry of the intact complex. Thus, these gas-phase dissociation measurements are entirely consistent with the arrangement of 14 noncovalently bound subunits in two heptameric rings.[8]

During the transfer of the GroEL complex from solution to gas-phase ions under high vacuum at the detector, these gas-phase macromolecular ions have to overcome a number of adverse effects: first charge repulsion between neighboring subunits with an average of 6 ± 1 positive charges in the case of GroEL complex and second the loss of hydrophobic interactions and unfavorable increases in entropy as solvent is stripped from the protein surface. Finally, the complex has to survive collisions with gas molecules during its flight from the ion source at atmospheric pressure to the detector under high vacuum. Previously, conditions were found whereby it was possible to preserve noncovalent assemblies containing up to six subunits on an ES-ToF instrument.[6] Using these conditions on a quadrupole ToF instrument,

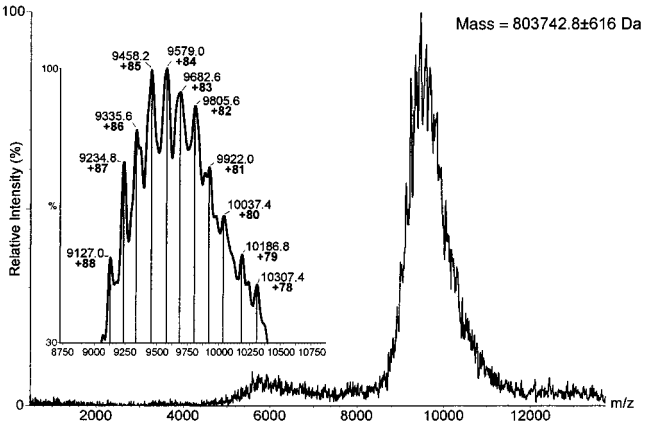

Figure 1. Nanoflow ES mass spectrum obtained from a solution of GroEL at pH 5.0 at a concentration of 1 μM. The spectrum represents the raw data after minimal smoothing and *insert* with higher smoothing and an expansion from m/z 8 750 to 10 750 with a threshold of 30% intensity. The spectrum was calibrated against cesium iodide, and the masses of the charge states were determined from the centroid of the peaks using Masslynx version 3.2 (Micromass UK Ltd.). The positions and values of the centroided data used in the mass determination are shown in the expansion. The mass was calculated from the eleven charge states labeled in the spectrum. The theoretical mass of the GroEL assembly is 800 758 Da. The difference in mass between the measured and theoretical masses could be attributed either to peptide ligands bound within the cavity of GroEL, since it is notoriously difficult to purify GroEL in the absence of ligands,[13] or to the presence of counterions.

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(1) Spectra were recorded on a Micromass Q-ToF instrument (Micromass UK Ltd.) consisting of a nanoflow electrospray source,[12] a quadrupole mass analyser, followed by a hexapole collision cell and a time-of-flight mass analyser. Ions are focused by an rF lens before transmission to the quadrupole which is used in rF-only mode as a wide band-pass filter. The ions are then conducted through the hexapole collision cell, pressurized with dry argon. Variation of the collision energy up to 140 V, induces dissociation of the macromolecular complex via collision with argon gas molecules. Ions are then pulsed into the ToF with an acceleration voltage of 8 kV at a pulse rate of 4 kHz for detection with a microchannel plate detector. Acquisition is made through a time-to-digital converter operating at 1 GHz. Mass spectra were obtained without source heating and in the absence of organic cosolvents. Mass spectrometer tuning parameters were held constant with a needle voltage of 1550 V, skimmer cone voltage of 150 V, skimmer offset of 4 V, and a collision energy of 4 V, except where stated.

(2) A GroEL stock solution (11.7 mg mL^{-1}), was kindly supplied by Y. Kawata. An aliquot was buffer-exchanged using Centricon 50 concentrators (Amicon) to give a final concentration of 100 μM in water (ELGA maxima system) adjusted to pH 5.0 with formic acid (Fisons). Solutions were kept at 4 °C during buffer exchange and prior to analysis. Stock solutions were diluted to give a final concentration of 1 μM $GroEL_{14-mer}$ in water at pH 5.0 for analysis. 1−2 μL of protein sample was introduced from a borosilicate glass needle (Clarke Electromedical Instruments) of external diameter 1.0 mm and internal diameter 0.5 mm. The capillary was pulled with a model P-97 flaming/brown micropippette puller (Clarke Electromedical Instruments) and gold-coated with a Polaron SEM coating system (Sutter Instruments Ltd.). This procedure gives rise to an internal diameter of 1−10 μm. The high viscosity of the protein solution necessitated manual breaking of the drawn end under a stereomicroscope and 2.5×10^4 Pa backing pressure during the electrospray process.

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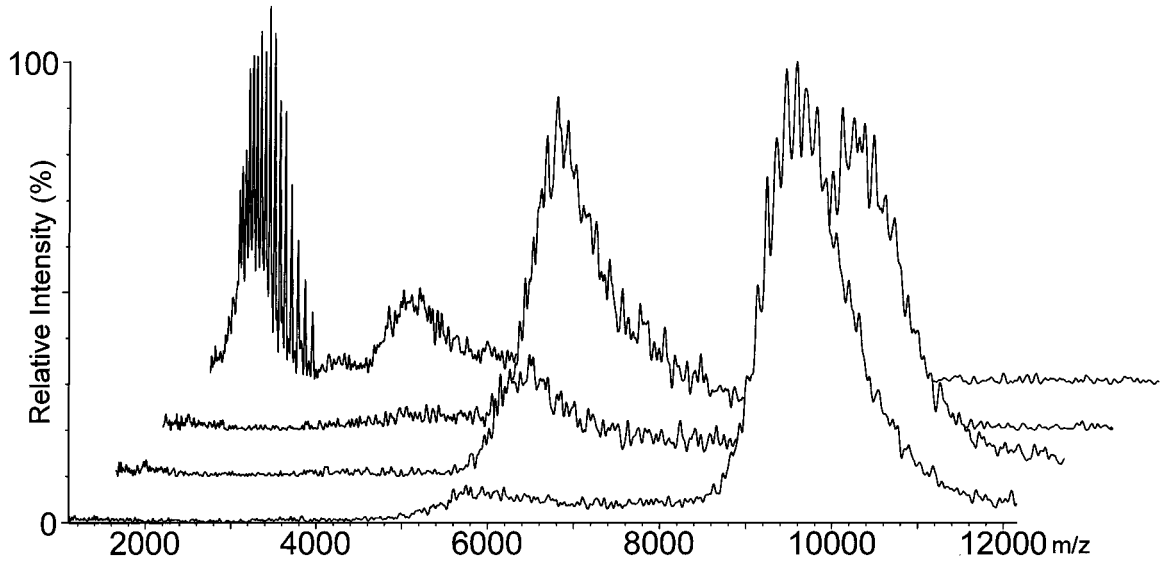

Figure 2. ES-ToF mass spectra of the collision induced dissociation of the $GroEL_{14-mer}$. Spectra were obtained at a constant pressure 9.0×10^{-3} pa in the collision cell and with increasing collision energies of 4, 13, 55, and 130 V. As the collision energy is increased, the proportion of intact $GroEL_{14-mer}$ (at *m*/*z* 10 000) decreases, while that of the single ring (*m*/*z* 6 000) was found to increase. At the highest collision energy employed no double- or single-ring GroEL remains, the only species observed being monomeric GroEL (*m*/*z* 2000). The mass of the single-ring species was calculated from three charge states. The difference in mass between the theoretical and measured masses for $GroEL_{14mer}$ is 2984 Da, while for the single-ring species this difference is 1177 Da. This suggests that during the dissociation process counterions and ligands in the central channel are lost. The measured mass of the GroEL monomer, 57 287 ± 1 Da, is higher than the theoretical mass (57 197Da), suggesting the noncovalent attachment of phosphate or sulfate ions to the monomer. The origin of the broad peak at $m/z \approx 4000$ is not know but may arise from decomposition of high *m*/*z* species in the time-of-flight analyzer.

only the monomer of GroEL was observed. This suggests that during the longer flight path of this instrument, ions either gain internal energy or have more time to dissociate. We have found that manipulation of the pressure differentials throughout the mass spectrometer is critical to maintaining the integrity of this complex in the gas phase.[9] Reducing the pressure gradient leads to collisional cooling and focusing, processes known to be important in reducing the internal energy of gas-phase ions.[10]

The observation that the GroEL complex can be maintained intact in the gas phase and induced to dissociate, through a heptameric intermediate to a monomeric subunit, is compelling evidence not only for the survival of the complex but also for the integrity of the subunit interactions. Previous experiments using prototype ES-ToF instruments[11] have shown that hexameric species with masses approaching 300 kDa can be maintained in the gas phase.[5,6] The results presented here promote mass spectrometry from the realm of small proteins and modest noncovalent complexes into the province of macromolecular assemblies with molecular masses approaching 1 000 000 Da. The mass spectra obtained demonstrate unequivocally that intact macromolecular assemblies can be maintained and their masses assigned, providing the protein sample is sufficiently homogeneous. Moreover, their masses can be measured with high accuracy and their overall topology examined. The real potential of these findings, however, lies in establishing connectivities in macromolecular complexes, critical in the postgenomic era in which the function and interactions of individual proteins will be described.

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(9) The pressures in the quadrupole region of the spectrometer were carefully balanced to allow optimal transmission of high-mass ions. A reduction of the source pumping speed in combination with an input of dry argon gas into the collision cell allowed the analyser pressure to be increased from the normal operating pressure of 8.0×10^{-4} Pa to 9.0×10^{-3} Pa. This increase in pressure also affected the pressure in the time-of-flight analyser causing an increase from to 6.8 e^{-6} to 5.5 e^{-5} Pa. Mass spectra were calibrated with CsI (Sigma Chemical Co.). Micromass Mass Lynx software version 3.1 was used for data processing.

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