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Characterising electrosprayed biomolecules using tandem-MS—the noncovalent GroEL chaperonin assembly

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This contribution is dedicated to Dudley Williams on the occasion of his retirement. As a former Ph.D. student the most important advice Dudley gave to me was to focus on an interesting problem and forget about the mundane—sentiments that stood him in good stead throughout his own career and continue to influence mine.

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Abstract

It is now possible to transfer large protein complexes intact into the gas phase using nano-electrospray ionisation (ESI) and to investigate their stoichiometry in a mass spectrometer. Using as a model assembly the noncovalent 14mer (ca. 800 kDa) of the chaperonin GroEL from *Escherichia coli* we show that the measured mass of the assembly is higher than expected from the sum of the components and explore parameters of ion activation that affect this 'noncovalent mass shift'. Under optimal desolvation conditions the measured mass is ∼0.5% greater than the calculated value indicating that part of the solution environment remains attached to these ions during phase transfer. The origin of this noncovalent mass shift is explored using tandem mass spectrometry experiments. Collisional activation of the 65+ charge state of the GroEL 14mer indicates the presence of up to 100 solvent/buffer molecules, both positively and negatively charged, which are stripped during CID in the gas-filled collision cell. At high collision energies, asymmetric dissociation into highly charged monomer and 13mer complexes takes place revealing the subunit composition of the assembly. Under these conditions the noncovalent mass shift is significantly reduced (<0.04%) demonstrating the utility of this tandem approach for mass measurement of biomolecules in the gas phase.
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Keywords: Nano-electrospray; Tandem-mass spectrometry; Collision-induced dissociation; Noncovalent interactions; Protein complexes

1. Introduction

It has long been a challenge for mass spectrometry to investigate intact biomolecules in the gas phase—even more so the large and dynamic protein assemblies which constitute the molecular machinery of the living cell. Since the advent of electrospray ionisation (ESI) [1] it has become possible to ionise noncovalent complexes of ever increasing size and complexity in solution and to make them 'fly' intact in a mass spectrometer [2–4]. Of particular interest is often the presence of small molecules and ions, such as cofactors and metals, in supramolecular structures of biomolecules. Gas-phase dissociation or fragmentation in a tandem-MS experiment gives access to information about the composition and stability of these particles [5–7].

Biological mass spectrometry benefits from the recent introduction of a miniaturised version of electrospray (nano-ESI) [8], which uses spray emitters with tip diameters of only a few micrometers to disperse the liquid into very fine sprays. Due to the resulting smaller size of the initial droplets, the sensitivity is improved by at least an order of magnitude and desolvation conditions are also more gentle than in pneumatically assisted ESI [9,10]. With nano-ESI MS the structure and interactions of biological macromolecules can now be studied in near-native environments, using aqueous solutions without the addition of acids or organic solvents while adjusting the pH and ionic strength with volatile buffers such as ammonium acetate [11]. Together with recent instrumental advances [12] it is now possible to examine particles as large and complex as the ribosome (ca. 2.3 MDa) [13].

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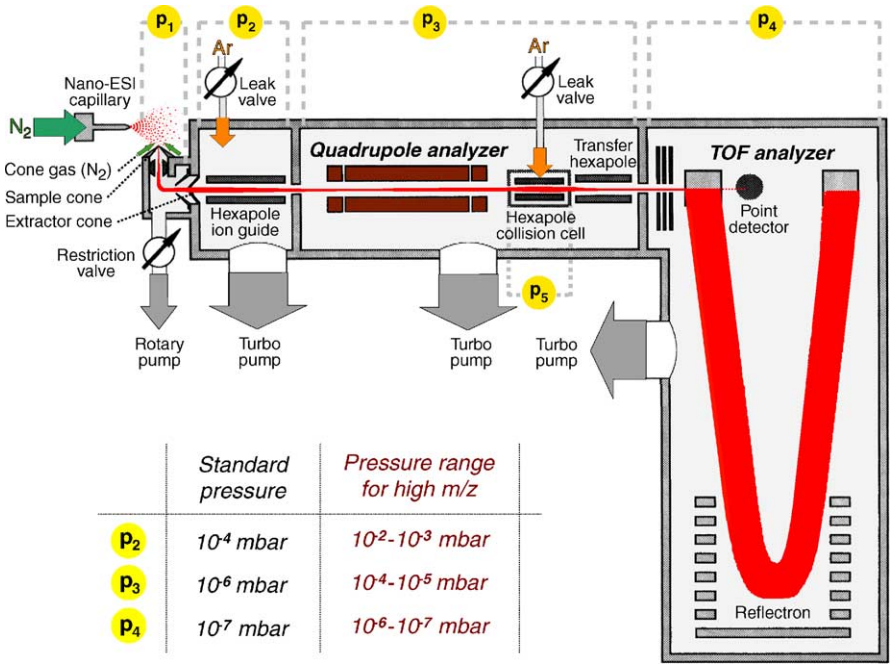

Fig. 1. Schematic layout of the Q-TOF instrument. A portion of the spray from a nanoflow capillary is sampled into vacuum through the source and ion guide stage with a Z-shape trajectory. Vacuum pressures in the instrument are controlled with a restriction valve which reduces pumping of the source region. Standard pressures and the range used for the transmission of large, noncovalent ions are indicated in the table. The instrument comprises two separate mass analysers, a quadrupole and a time-of-flight analyser. A collision cell downstream from the quadrupole can be filled with up to 3×10^{-2} mbar argon gas for CID experiments and the product ions detected in the TOF analyser.

The type of mass spectrometer most frequently employed for this research is a quadrupole-time of flight (Q-TOF) instrument. The Q-TOF combines a quadrupole mass filter with a collision cell for collision-induced dissociation (CID) of molecular ions and a subsequent time-of-flight (TOF) mass analyser. This combination facilitates tandem-MS experiments, a quasi two-dimensional approach where ions isolated from one species in the quadrupole (the parent ions) are dissociated in the collision cell and the product ion spectrum is recorded using the TOF analyser. Tandem-MS, MS/MS or MS^2 experiments as they are often known enable the elucidation of components of noncovalent biomolecular complexes [6,14]. Since multimeric protein complexes carry considerably fewer net charges per mass than individual proteins, i.e. their charge states are generally lower, the corresponding signal often appears at mass-to-charge ratios (m/z) well into several thousand. We have recently reported on a tandem mass spectrometer designed for the analysis of such high-mass ions [12]. It features a reduced quadrupole frequency for an extended m/z range and modifications which allow operation at increased pressures in the ion source, ion guide stage and collision cell (Fig. 1; see also Section 2).

Here we describe the application of these emerging methods and instrumentation to characterisation and CID of the Group I chaperonin GroEL. The characteristic double-ring toroidal structure of GroEL with seven subunits of ~57 kDa per ring assists in the efficient folding of newly synthesised and stress-denatured polypeptide chains [15,16]. Previously we have shown that it is possible to maintain the GroEL 14mer intact in the gas phase. These earlier experiments, however, were subject to the limitations of a standard quadrupole-time of flight instrument and recorded at low pH in the absence of ammonium acetate, conditions known to destabilise the assembly, facilitating the dissociation into single ring structures [17]. The results presented here for the 800 kDa assembly benefit from significant improvements in instrumentation as well as higher solution pH and presence of a volatile buffer giving rise to spectra with improved peak resolution over that reported previously [17]. In this report we investigate the presence of solution-derived molecules and ions in gas-phase ions of this large noncovalent complex. Specifically we focus on the transition of these complex ions from solution to the gas phase and discuss how their internal energy is affected by the instrument design and parameters of the mass spectrometry experiment.

2. Materials and experimental method

E. coli GroEL was expressed and purified as described previously [18], and its amino acid sequence is given by Swiss-Prot accession number P06139. For MS experiments we prepared aqueous solutions of GroEL (7 or 14 μM) in 200 mM ammonium acetate buffer (pH 7). Nano-ESI capillaries were prepared in-house from borosilicate glass tubes of 1 mm OD and 0.78 mm ID (Harvard Apparatus, Holliston, MA/USA) using a Flaming/Brown P-97 micropipette puller (Sutter Instruments, Hercules, CA/USA), and gold-coated

using an SEM sputter-coater (Polaron, Newhaven, UK). The capillary tips were cut under a stereo-microscope to give an ID of around 5 μm. Typical spray voltages were in the range of 1400–1600 V and a counter flow of nitrogen gas of 150 L/h was used.

Mass spectra were recorded on a tandem-mass spectrometer (Q-TOF, Waters, Manchester/UK) modified for high-mass operation as described previously (Fig. 1, [12]). Briefly, ions are sampled in a Z-shape trajectory through two cones (Sample and Extractor) and an ion guide into the quadrupole analyser. Voltage offsets between Sample cone, Extractor cone and ion guide control the ion acceleration and thereby the internal energy of the ions while they undergo multiple collisions with gas atoms in the intermediate vacuum stages. An additional variable voltage offset between quadrupole analyser and collision cell is used to define the collision energy of particles for CID.

3. Results

We investigated the supramolecular structure of the chaperonin GroEL from *E. coli* from a near-native environment with the high-mass Q-TOF instrument described above. Fig. 2 shows a nano-ESI mass spectrum obtained from aqueous solution buffered to pH 7 using ammonium acetate. Careful adjustment of the spray voltage and flow rate as well as acceleration voltages and pressures in the source (see below) are essential in order to obtain sufficient signal intensity while still resolving individual charge states. Around 12,000 *m/z* a group of well-resolved peaks is visible to which we assign charge states from 65+ to 72+ corresponding to a molecular mass of 804,700 ± 100 Da. Comparison with the mass for *E. coli* GroEL subunits as calculated from the amino acid sequence (57,198 Da) shows good agreement with 14 times the monomer mass (800,770 Da). It is apparent that the dominant species in this spectrum represents an intact 14mer of GroEL, in accordance with the native double-barrel shaped structure of the chaperonin (see inset in Fig. 2 [16]). Virtually no dissociation or fragmentation products are visible at lower *m/z* under these conditions. The experimentally determined mass is, in this case, about 4000 Da higher than the calculated value, which amounts to 0.5% of the total mass. The observation of a mass higher than expected from the sum of the components is a common feature in ESI mass spectra of noncovalent complexes. This 'noncovalent mass shift' is thought to be caused by molecules and ions from solution which remain attached to the complex in the gas phase—a hypothesis which we investigate further below.

Which factors determine how these ions make the transition from solution to the gas phase, and if they are transmitted intact through the vacuum of the mass spectrometer? A key parameter is the ion source pressure, as described previously [12,17,19,20]. We increased the pressure in the source of the Q-TOF (p_1, Fig. 1; the higher gas load in the source also raises p_2 in the ion guide). Fig. 3 shows how signal intensity, observed mass and peak resolution of the intact GroEL signal are affected by variation of the source pressure. While we observe no charge states due to the 14mer at pressures lower than those used to record spectrum A, their abundance increases steadily with pressure (panels B–E; virtually no monomer signal appears in any of the spectra, not shown). This demonstrates the importance of increased source pressures for the transmission of a beam of macromolecular ions and reflects the effect of collisional cooling in an ion guide as described previously [21]. While the overall charge state distribution remains almost constant with increasing pressure, the peaks shift to higher *m/z* and the peak width increases, indicating that more solution-derived matter remains bound to the gas-phase ions. Although the same acceleration potentials are used throughout the series,

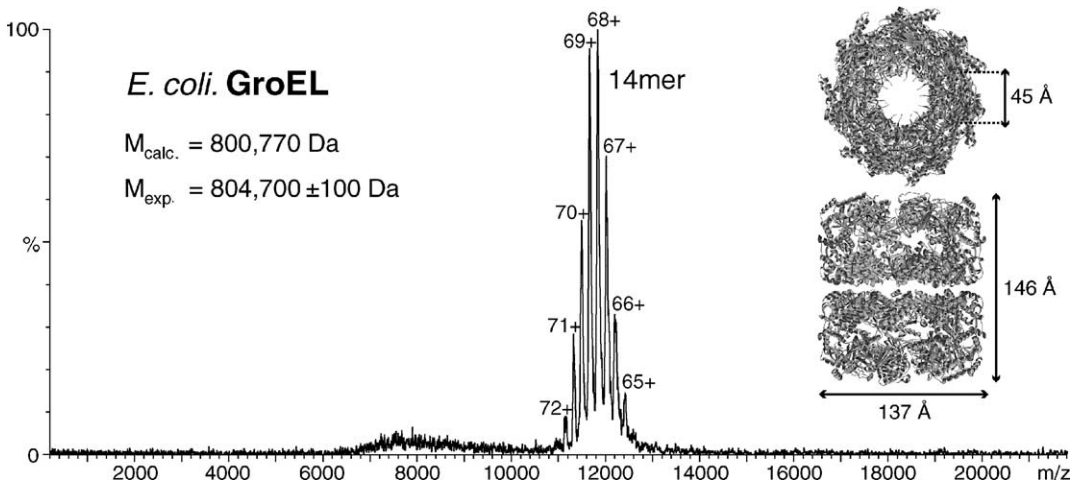

Fig. 2. Nano-ESI spectrum of intact *E. coli* GroEL 14mer. Sample and Extractor cone voltages were 150 and 50 V, respectively. Vacuum pressures with partly closed restriction valve: $p_2 = 1.1 \times 10^{-2}$ mbar, $p_3 = 8.3 \times 10^{-5}$ mbar and $p_4 = 7.1 \times 10^{-7}$ mbar, without gas in the collision cell. These conditions were also employed to record the mass spectrum in Fig. 3, panel C. Inset: X-ray structure of the *E. coli* GroEL 14mer [16].

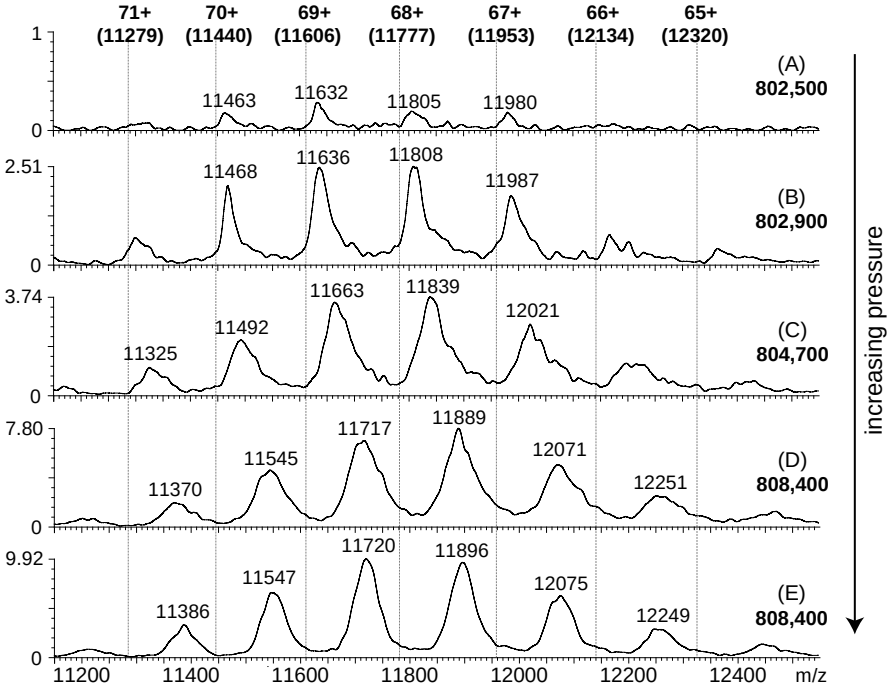

Fig. 3. Source pressure dependence of GroEL 14mer signal. The restriction valve was partly closed to raise the source pressure (p_1), but due to a higher gas load the ion guide pressure (p_2) also increases. Pressures were: (A) $p_2 = 4.4 \times 10^{-3}$ mbar, $p_3 = 3.9 \times 10^{-5}$ mbar; (B) $p_2 = 7.1 \times 10^{-3}$ mbar, $p_3 = 5.4 \times 10^{-5}$ mbar; (C) $p_2 = 1.1 \times 10^{-2}$ mbar, $p_3 = 8.3 \times 10^{-5}$ mbar; (D) $p_2 = 1.3 \times 10^{-2}$ mbar, $p_3 = 1.2 \times 10^{-4}$ mbar; (E) $p_2 = 1.6 \times 10^{-2}$ mbar, $p_3 = 1.7 \times 10^{-4}$ mbar. All other parameters are as in Fig. 2 (Fig. 3C shows an enlarged section of the spectrum in Fig. 2). The *y*-axes indicate intensities relative to the arbitrary scale in panel A. Charge states are indicated at the top of the figure with their calculated *m*/*z* values (dotted lines). Peaks are labeled with *m*/*z* values, and the mass derived from all labeled charge states in each spectrum is given on the right hand side.

a higher proportion of the ion acceleration is lost to the collision gas at higher pressures, resulting in gentler desolvation conditions. Just above the threshold pressure at which the intact GroEL 14mer is detected (Fig. 3, panels A and B), all but firmly attached ions and molecules from the solution are removed by collisional activation. Under these conditions, when peak shapes are optimal – in panel B the peak width (FWHM) is ca. 28 *m*/*z* – a noncovalent mass shift of about 2000 Da persists, however, indicating that the particles responsible for this additional mass are bound tightly to the complex.

From the data presented here it emerges that additional components adhere to large assemblies during their transit from solution to gas phase. We investigated the nature of these particles further using tandem mass spectrometry as a tool for the dissection of noncovalent complexes. Isolation of a single GroEL 14mer charge state at around 12,500 *m*/*z* in the quadrupole analyser is demonstrated in Fig. 4, panel A. For CID ions are accelerated with voltage offsets of up to 200 V into the gas-filled collision cell, where translational energy is converted into internal energy in multiple collisions. In a similar manner to desolvation and collisional cooling processes in the ion guide, heavier ions require more collisions than lighter ones to build up sufficient internal energy for dissociation to take place and for the resultant product ions to be collisionally cooled. Below 2.5×10^{-2} mbar argon pressure in the collision cell, no CID of GroEL 14mer occurs due to an insufficient number of collisions (data not shown). At 3×10^{-2} mbar and with an ion acceleration of up to 150 V (Fig. 4, panels B and C) no CID products are detected, but the isolated 65+ parent ion peak is narrower and at lower *m*/*z* than in panel A. At this intermediate voltage the complex is activated to an extent that attached ions and molecules are stripped without dissociating the protein complex. The noncovalent mass shift is significantly reduced to a value of less than 300 Da representing less than 0.04% of the total mass. The observed peak coincides with the *m*/*z* calculated from the sum of the subunits for the 65+ charge state demonstrating the utility of this approach for precise mass measurement of noncovalent assemblies.

Under these conditions of pressure and collision cell voltage additional peaks appear both higher and lower than the isolated peak which coincide with charge states of the intact 14mer. Up to four positive and interestingly also three negative charges are lost in energetic collisions with argon gas (see Fig. 4D(ii)).[1] While a charge state reduction is readily explained by loss of protons or other attached cations (presumably ammonium from the buffer or sodium impurities), the appearance of higher charge states is somewhat

[1] The small concomitant mass change upon ion detachment means that the 14mer peaks around the parent ion do not form a 'true' charge state series, but the associated slight shift of the *m*/*z* values has been neglected here.

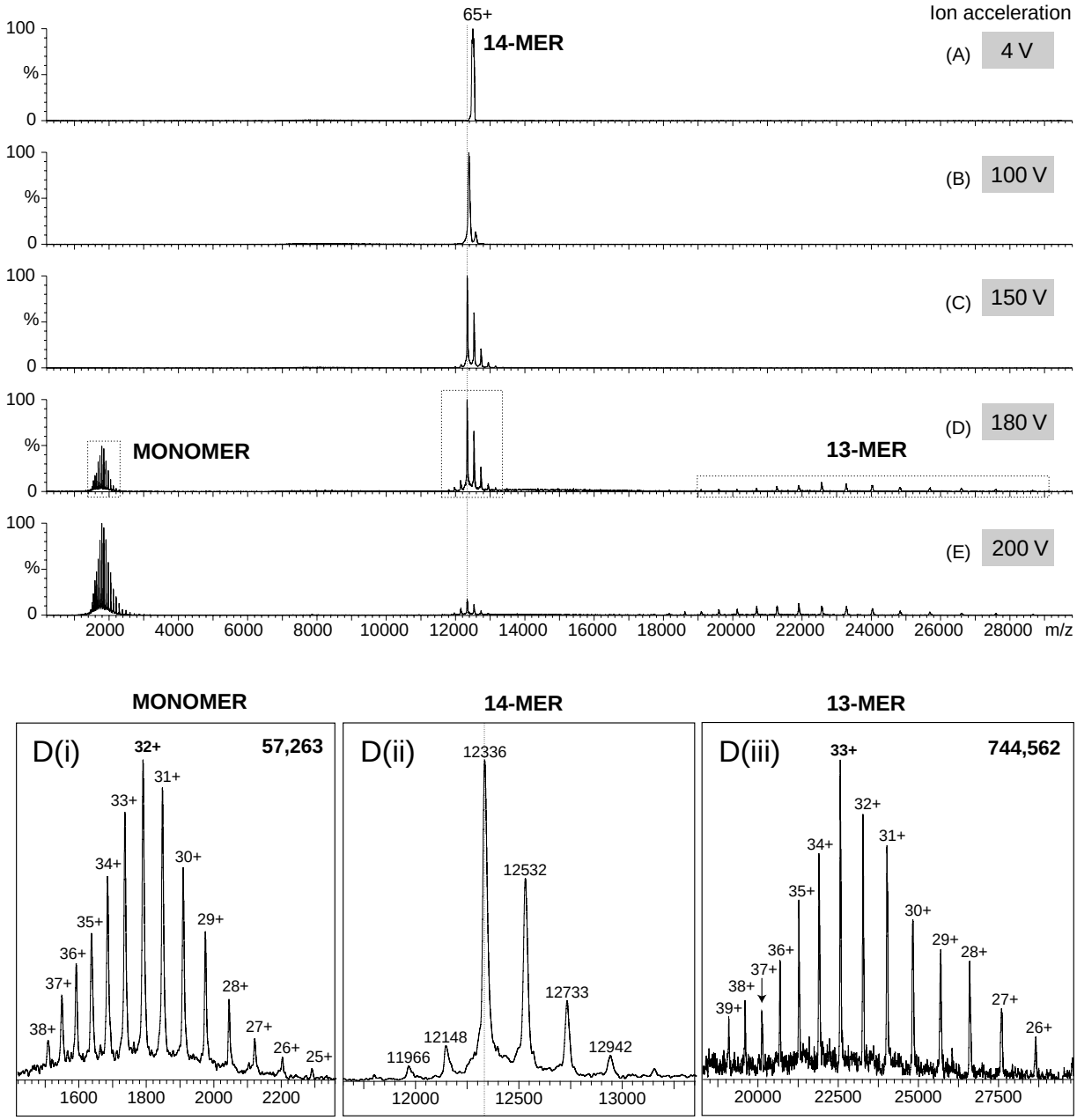

Fig. 4. Tandem-MS of the isolated 65+ charge state of GroEL 14mer at *m/z* 12,500 with 3×10^{-2} mbar argon in the collision cell. Panels A–E: the ion acceleration voltage (injection energy) into the collision cell was varied between 4 V and the maximum value, 200 V. Three insets show enlarged sections of spectrum D for the monomer (D(i)), 14mer (D(ii)) and 13mer (D(iii)) signal as well as the mass calculated for the monomer and 13mer from the charge state series (upper right corner).

unexpected. A charge gain by attachment of positive ions is energetically very unfavorable and not feasible in the gas phase. We propose therefore that loss of negative charge arises through the detachment of counter ions, presumably acetate from the buffer. This is not as unlikely as it might first appear, since the anion interacts with only a fraction of the overall positive charge of the complex (~65+) while the majority of the charge is effectively shielded by other protein subunits.

If the collision cell voltage exceeds 180 V (Fig. 4, panels D and E) dissociation products appear in the spectra at low and very high *m/z*. Two product ion distributions are apparent corresponding to highly charged GroEL monomers centred on the 32+ charge state, and stripped 13mer complexes around the 33+ charge state (Fig. 4 D(i) and D(iii)).[2] While each subunit carries on average 4.6 charges

[2] From the monomer peak series we obtain a subunit mass of 57,263 Da which is slightly higher than calculated from the amino acid sequence (57,198 Da), probably due to some unknown modification. The 65+ charge state of the 14mer parent ion appears at *m/z* 12,336, which is very close to the value calculated from 14 times the modified subunit mass (12,334.5);

in the intact 14mer complex, upon dissociation roughly half the total number of charges is concentrated on one monomer. Such highly asymmetric gas-phase dissociation is a common feature for CID of noncovalent multi-subunit complexes [14,22–24], and it resembles somewhat the asymmetric fission of electrospray droplets [25]. Since most solution-derived ions and molecules are stripped under these high-energy conditions, particularly from the highly charged monomer which is ejected from the complex during CID and the residual 13mer formed in this process, tandem-MS can delineate subunit compositions of large, heterogeneous noncovalent complexes even if their charge states in the TOF-MS spectrum are not fully resolved [12,26].

4. Discussion

It remains one of the most fascinating aspects of mass spectrometry that functional, biological entities with molecular masses of several hundred kDa or more can be ionised intact in near-native aqueous environments and gently transferred into the gas phase for analysis. This is only possible if a number of experimental conditions are fulfilled. Firstly, the solution environment needs to meet the stability criteria of the noncovalent complex under investigation such as solvent composition, pH, solution temperature and presence of other ions. Most protein complexes are stable in aqueous, dilute solutions buffered to the required pH with ammonium acetate. Secondly, a fine spray is a prerequisite to an efficient desolvation under gentle conditions. Since solvent evaporation from the droplets requires energy, the smaller the initial droplets the less collisional or thermal activation of the ions is needed. In addition, conditions for the release of complex ions from the solution in the presence of high levels of nonvolatile salt are much more favorable in smaller droplets, due to their increased surface-to-volume ratio [10]. Nano-ESI is currently the method of choice for the production of finely dispersed sprays from aqueous solutions.

Thirdly, the internal energy (temperature) of the nanodroplets, or ‘wet’ ions, must be maintained sufficiently high throughout transfer from atmosphere to vacuum in order to obtain ‘dry’, i.e. desolvated gas-phase ions. When the gas stream which carries the droplet mist enters the vacuum of the mass spectrometer through the aperture, it is subjected to rapid cooling by a molecular beam expansion [27]. In order to prevent recondensation of water vapor under these conditions and to assist with desolvation, the ion-solvent clusters have to be kept ‘warm’, either by radiative heating or collisional energy transfer from gas molecules. The latter is the more common approach in most instruments, where a cone-skimmer offset accelerates ions through a sufficiently dense gas atmosphere in the source, thus transferring energy from the gas molecules to the biomolecular complex in gentle collisions. Desolvation is a delicate process which needs to be finely balanced in order to disrupt solvent interactions while keeping the noncovalent assembly intact. In the instrument used for this study the amount of energy conferred to the biomolecular complex during desolvation is determined by the choice of acceleration voltages between the two orifices in the source region (Sample and Extractor cone), as well as the offset between the second orifice (Extractor cone) and the ion guide. When these voltages are too low the GroEL 14mer signal forms an unresolved single peak, encompassing roughly the m/z range of the charge state series seen in Fig. 2—an indication of insufficient desolvation. If on the other hand the ions are accelerated too much then noncovalent interactions are disrupted (this approach is used for cone-skimmer dissociation or in-source CID, [3,5,14]).

Lastly, a fourth factor which needs to be taken into account is the vacuum pressure in the ion guide stage of the instrument (p_2 in Fig. 1). It has been reported previously that elevated pressures are necessary for transmission of intact high-mass ions of noncovalent complexes [12,19,20]. This is attributed to a process called ‘collisional cooling’ or ‘collisional focusing’, which refers to the dampening of the speed and off-axis movement of ions in a quadrupole or hexapole ion guide operated at intermediate pressures [21,28]. Collisional cooling is essential in order to obtain a well-defined, focused ion beam from an atmospheric pressure ion source such as ESI. Ideally all ions are thermalised when they exit the ion guide. For high-mass ions though collisions become less efficient in spite of a larger collisional cross section, due to a lower centre-of-mass collision energy (see below). This can be compensated with higher pressures, increasing the number of collisions and thus facilitating transfer of high-mass ions through the ion optics.[3] The Q-TOF instrument has been fitted with a restriction valve and an argon leak valve to control pressures in the source and ion guide pumping stages, respectively (p_1 and p_2 in Fig. 1). While it would suffice to increase p_2 alone to achieve collisional cooling of high-mass ions in an ion guide [21] or ion tunnel, the off-axis sampling geometry of the gas expansion in the source also requires a higher pressure p_1 in order to transmit heavy ions into the ion guide. In all the experiments described here the ion acceleration through the first two vacuum stages has been maintained as high as possible for optimum desolvation conditions while keeping noncovalent interactions intact. If these voltages are lower, the ions need less gas pressure for collisional cooling but the noncovalent mass shift and peak width become greater (data not shown). For particles of different mass and size the optimum settings will vary, but it is important to keep in mind that

the latter is indicated by the dotted lines in Fig. 4. With this ‘revised’ monomer mass the calculated 14mer mass becomes 801,688 Da and the observed noncovalent mass shifts are accordingly smaller.

[3] Replacing nitrogen and argon with a heavier collision gas such as xenon also makes energy transfer in collisions more efficient.

there is an optimum and that the detection of intact noncovalent complexes always requires careful adjustment of the voltage and pressure gradient in the interface region of the mass spectrometer.

In tandem-MS experiments (Fig. 4) ions are heated by multiple energetic collisions to levels of internal energy at which loss of residual ion and molecule attachment, charge stripping, and eventually dissociation occurs. Theoretically the maximum energy transfer per collision between an 800 kDa protein complex and an argon atom is only on the order of 0.01% of the translational energy of the protein complex, due to the very low centre-of-mass collision energy (see similar estimate in Ref. [28]). An ion acceleration of 180 V into the collision cell (as in Fig. 4 panel D) corresponds to a kinetic energy of about 11.7 keV for the 65+ charge state of the GroEL 14mer, a figure which may at first seem to be surprisingly high. Only a small fraction of the translational energy is, however, actually converted into internal energy of the high-mass ions, due to the very low collision efficiency [24,29]. The small amount of energy transfer in a single collision illustrates the requirement for high gas pressures in the collision cell—just as for collisional cooling in the ion guide. For a rough estimate of collision numbers we consider a simple hard-sphere collision model and assume the GroEL 14mer to be a sphere with 16 nm diameter, corresponding to a collision cross section of approximately 2×10^{-16} m^2. It can be readily calculated that these ions experience tens of thousands of collisions during their passage through the argon-filled collision cell (length 0.185 m, $p_5 = 3 \times 10^{-2}$ mbar), thereby building up high levels of internal energy. Such excited gas-phase protein complexes show two different dissociation pathways: loss of attached molecules and ions at intermediate ion activation, and loss of an intact covalent subunit from the noncovalent complex at high activation.

The stripping of small particles at intermediate tandem-MS collision energies indicates that molecules and ions from the solution were previously attached to the complex. Their presence is further corroborated by the observation of charge state shifts during the activation of a tetrameric protein complex in the collision cell followed by metastable dissociation, as reported previously [30]. While the amount of additional mass which is found with gas-phase ions of large complexes depends on the desolvation conditions used, i.e. ion acceleration and gas pressures, a noncovalent mass shift persists even under optimum conditions for the preservation of near-native structures in the mass spectrometer. In our experiment, mass measurement of the intact GroEL complex yields a noncovalent mass shift of about 2000 Da under these conditions (Fig. 3, panel B). It is tempting to speculate that it could arise from cavities which are present in the quaternary structure of the protein complex. The size of the large central cavity in the GroEL 14mer is roughly 10^{-25} m^3 (see inset in Fig. 2), a volume equivalent to >3000 water molecules—hence it is possible to imagine that ~100 water or buffer molecules could remain bound, amounting to a noncovalent mass shift of 2000 Da. We cannot, however, determine at present if these ions and molecules reside in the rather hydrophobic, large cavity of the complex or, more likely, bind at subunit interfaces and in other small pockets of the protein structure where they are buried. Comparison with other more compact protein complexes also provides evidence for noncovalent mass shifts, in the range from around 100 Da up to 0.5% of the total protein mass [12,14,26,31]. It is likely that the presence of solution-derived ions and molecules in the gas-phase complex correlates with the hydrophobicity and polarity of the protein surfaces. It is also possible that these particles remain attached to specific sites on the subunit interface, forming salt bridges and solvating individual residues, thus stabilising the quaternary structure of the protein complex in the gas phase of the mass spectrometer.

5. Conclusions

We have demonstrated that noncovalent complexes of biomolecules as large as the 800 kDa *E. coli* GroEL 14mer can be transferred intact into the gas phase and their supramolecular structure investigated by tandem-MS. Experimental factors which determine the internal energy of the ions such as acceleration voltages and pressures need to be carefully optimised for a gentle phase transfer, maximising desolvation while leaving weak noncovalent interactions intact. It has been shown that the charged residue of a large noncovalent complex as it emerges after desolvation from nano-ESI droplets comprises small ions and molecules which remain bound in the gas phase. The amount of this additional mass which originates from the near-native aqueous environment of the protein in solution depends on experimental factors, but a noncovalent mass shift and peak broadening persists even under optimum desolvation conditions which preserve noncovalent interactions in the mass spectrometer.

Collision-induced dissociation of a GroEL 14mer charge state reveals the presence of up to 100 solvent and buffer molecules as well as several additional positive and negative ions in the multiply charged complex, which are stripped at intermediate collision energies. Only at very high acceleration voltages, when the high-mass ions are injected into the dense argon atmosphere of the collision cell with translational energies of more than about 11 keV, does the protein complex build up sufficient internal energy in multiple collisions for detachment of highly charged covalent subunits. This asymmetric dissociation occurs from a highly excited, 'hot' state of the protein assembly which facilitates the migration of roughly half the total charge onto the monomer which is being ejected from the activated complex. Such gas-phase dissociation is often performed to elucidate the identity and stoichiometry of subunits in a heterogeneous protein assembly.

It remains an intriguing observation that large, noncovalently bound complexes of biomolecules survive the loss of bulk solvent, and evidence is strong that they retain characteristic traits of their solution structure under appropriate conditions. While it is established that many protein assemblies known to exist in solution can be verified by observation in the gas phase of the mass spectrometer, increasingly these approaches are being applied in advance of structural data revealing subunit stoichiometry and even differences in quaternary structural arrangements [32]. We anticipate that increased knowledge of the composition of gas-phase biomolecules and a better understanding of the role of small molecules in these gas-phase structures will result from tandem mass spectrometry approaches such as those described here.

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