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Chaperonin Complexes Monitored by Ion Mobility Mass Spectrometry

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Abstract: The structural analysis of macromolecular functional protein assemblies by contemporary high resolution structural biology techniques (such as nuclear magnetic resonance, X-ray crystallography, and electron microscopy) is often still challenging. The potential of a rather new method to generate structural information, native mass spectrometry, in combination with ion mobility mass spectrometry (IM-MS), is highlighted here. IM-MS allows the assessment of gas phase ion collision cross sections of protein complex ions, which can be related to overall shapes/volumes of protein assemblies, and thus be used to monitor changes in structure. Here we applied IM-MS to study several (intermediate) chaperonin complexes that can be present during substrate folding. Our results reveal that the protein assemblies retain their solution phase structural properties in the gas phase, addressing a long-standing issue in mass spectrometry. All IM-MS data on the chaperonins point toward the burial of genuine substrates inside the GroEL cavity being retained in the gas phase. Additionally, the overall dimensions of the ternary complexes between GroEL, a substrate, and cochaperonin were found to be similar to the dimensions of the empty GroEL−GroES complex. We also investigated the effect of reducing the charge, obtained in the electrospray process, of the protein complex on the global shape of the chaperonin. At decreased charge, the protein complex was found to be more compact, possibly occupying a lower number of conformational states, enabling an improved ion mobility separation. Charge state reduction was found not to affect the relative differences observed in collision cross sections for the chaperonin assemblies.

Introduction

The ability of proteins to interact with other proteins and form biologically active assemblies is determined primarily by their conformation. The structural analysis of these large protein complexes is therefore essential to obtain detailed insight into biological processes. High resolution structural data on proteins and protein complexes can be obtained by methods such as nuclear magnetic resonance (NMR), X-ray crystallography, and electron microscopy (EM). However, there are still numerous complexes, especially very large and heterogeneous protein assemblies, for which these techniques are less suitable.[1,2] Therefore, there is an urgent need for additional methods to investigate protein complex conformations. The application of native mass spectrometry (native MS) in structural biology has increased tremendously in the past decade,[1,3−7] and with the recent introduction of ion mobility into native MS analyses (IM-MS), a new and exciting dimension has been added to this field of research.[9−11]

During IM-MS, ions are separated based on their drift time through a neutral gas under the influence of a weak electric field.[12] The ions are subsequently identified by time-of-flight (ToF) mass spectrometry. The drift time of the ions through the ion mobility cell depends on the protein ion conformation; a larger ion will experience more interactions with the buffer gas and will thus have a lower mobility. By conversion of the drift time into a collision cross section (ccs), a measure for the overall shape and conformation of the ion is obtained.[3,13]

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So far only a few applications of native IM-MS have been reported.[3,10,11,14,15] Some of these studies provide strong indications that the structures of protein assemblies can be preserved upon transfer to the gas phase. For example, using IM-MS, the radii of gas phase $T=3$ hepatitis B virus capsids were determined to be ~15 nm.[11] These values were in excellent agreement with the capsid radii as derived from EM reconstructions (ranging from 13.1 to 15.9 nm) and clearly highlight the unique potential of IM-MS to generate fundamental structural information on biologically active complexes. In another IM-MS study, it was shown that the observed ring-type topology of an undecameric *trp* RNA-binding attenuation protein (TRAP) was similar to the solution phase ring structure as indicated by X-ray diffraction analysis.[3]

Here, we applied IM-MS to study the structural conformations of a large set of different chaperonin complexes in the gas phase. The chaperonin complex of *Escherichia coli* is composed of tetradecameric GroEL (800 kDa) and heptameric GroES (72 kDa). Together, they aid in the folding of newly synthesized proteins.[16] *In vivo*, efficient folding of substrate proteins occurs inside the *cis* cavity of the GroEL−GroES complex in the presence of ATP-Mg. The binding of a new substrate to the free *trans* ring of the complex triggers GroES dissociation and the release of the folded protein.[16] Interestingly, GroEL also functions in conjunction with bacteriophage encoded cochaperonins like gp31 (T4) and CocO (RB49) upon host cell infection.[17]

Previously, using a native MS approach, we monitored the folding of the bacteriophage T4 capsid protein (gp23) by GroEL-gp31 and also revealed substrate specific conformational changes in the chaperonin complex.[6,7] Strikingly, the chaperonin's charge state was not significantly affected by substrate binding, irrespective of the substrate identity. We also observed that during tandem MS experiments the substrate protein remained associated to the complex while monomeric GroEL subunits were dissociated. Both observations suggest that in the gas phase the substrate is accommodated inside the chaperonin complex, thus resembling the solution phase structure. Here, we provide conclusive evidence for this hypothesis by elucidating and comparing the global gas phase structural conformations of different chaperonin assemblies using IM-MS.

Materials and Methods

Protein Preparations. GroEL was overexpressed in *Escherichia coli* strain MC1009 containing plasmid pSL6. Cells were grown in Luria−Bertani (LB) medium with 100 μg/mL ampicillin and 0.0005% (w/v) arabinose at 37 °C under vigorous aeration. GroEL was purified according to a previously described protocol, slightly modified by the introduction of an acetone precipitation step.[18,19] The major capsid protein gp23 was expressed from the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible plasmid pET2331 in *E. coli* strain BL21(DE3), a generous gift from L. Black (University of Maryland, Baltimore, USA). Gp23 was purified as described previously.[20] The GroES purification protocol[21] was modified by replacing the MonoQ-sepharose HR anion exchange chromatography at pH 5.3 for a heat-treatment step. Heat treatment was performed at 72 °C for 10 min under continuous stirring, followed by centrifugation for 2 h at 4 °C and 11 000 rpm (Sorvall rotor, SS-34 rotor). GroES was detected in the supernatant while impurities were found in the pellet. The bacteriophage T4 encoded cochaperone gp31 was overexpressed in *E. coli* strain MC1009.[22] Cells were grown in LB medium with 100 μg/mL ampicillin and 0.001% (w/v) arabinose at 37 °C under vigorous conditions. Gp31 was purified as described previously.[23] Dimeric Rubisco was kindly provided by S. M. van der Vies (VU Medical Center, The Netherlands). Purified gp5 was a kind gift from C. Teschke (University of Connecticut, USA). Mitochondrial malate dehydrogenase, lysozyme from chicken egg white, and bovine serum albumin (BSA) were purchased from Sigma (M2634, L7651, and A2153, respectively). SR1 was overexpressed in *E. coli* strain MC1009 containing plasmid pET11a-SR1 (a kind gift from N. Strippel, Max Planck Institute, Germany) and purified as described previously.[24]

Mass Spectrometry. Mass spectrometry (MS) measurements were performed in positive ion mode using an electrospray ionization quadrupole ion mobility time-of-flight (ESI-Q-IM-TOF) instrument (Synapt HDMS, Waters, UK) equipped with a Z-spray nanoelectrospray ionization source. Needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL) on a P-97 puller (Sutter Instruments, Novato, USA), coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories, Milpitas, USA) six Pirani 501 sputter coater.

To produce intact gas phase ions from large complexes in solution, the source was operated at a pressure of 6.9 mbar.[25] Mass spectra were recorded with a capillary voltage of 1.3 kV and cone voltage of 175 V. Argon was used as the collision gas in the trap (5 V) and transfer (125 V) ion guides. The pressure in the trap and transfer was set at 3.4×10^{-2} mbar. The ion mobility cell was filled with nitrogen (6.5×10^{-1} mbar), and a ramped wave height of 7−30 V and wave velocity of 250 m/s were used. The duty cycle of the ion mobility cell, to allow protein complex ion separation, was set at 51 ms. The pressure in the ToF was 2.7×10^{-6} mbar.

Sample Preparation for Mass Spectrometry. GroEL polypeptide substrate complexes were prepared as before.[6,7,26] Note that GroEL concentrations listed below are for the tetradecamer, while substrate concentrations are given as monomers. In short, the buffers of all proteins were exchanged to 50 mM ammonium acetate (pH 6.9), using ultrafiltration units with a cutoff of 10 000 Da (Millipore, Bedford, USA). GroEL−substrate complexes were formed by first unfolding the substrate in 8 M urea for 1 h at room temperature at a final substrate concentration of 25 μM. To unfold Rubisco, 10 mM dithiothreitol (DTT) was also added to prevent disulfide bridge formation. The unfolded substrate was added to a 50 mM ammonium acetate buffer pH 6.9 containing 2 μM GroEL, at chaperone/substrate ratios varying from 1:0 to 1:5. The resulting maximum concentration of 1.7 M urea did not affect the oligomeric GroEL structures. The final concentration of GroEL−substrate complexes varied between 1 and 2 μM. Excess urea was removed from the sample by filtration while changing the buffer to 50 mM

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ammonium acetate pH 6.9. To prepare GroEL-BSA unspecific clusters, BSA was added in 140-fold excess over tetradecameric GroEL. A 1 mM BSA stock solution was prepared in 50 mM ammonium acetate pH 6.9. Final concentrations in the spray solutions were 0.35 μM GroEL and 50 μM BSA in 50 mM ammonium acetate pH 6.9. When indicated, imidazole was added to the chaperonin solution to final concentrations of 5 or 10 mM, using a 100 mM stock solution of imidazole. The pH of the solution was adjusted to 6.9 using acetic acid.

Ion Mobility Calibration and Collision Cross Section Calculation Using Drift Time Values. All collision cross sections (ccs's) of the protein complexes were calibrated using proteins with known ccs's as described previously.[11,15] The analyses of the calibrant proteins (equine myoglobin, cytochrome c, and bovine ubiquitin, all purchased from Sigma) were performed at a fixed wave height (10 V) and a duty cycle time in the ion mobility cell of 18 ms. To obtain calibrated ccs's for our protein assemblies, data sets for small and large proteins were correlated taking single ring GroEL (SR) and GroEL as common points.

In short, after measuring the calibrant proteins a linear correlation between the ln of the corrected drift time and the ln of the corrected ccs is obtained. Due to this linearity, IM-MS analysis of the SR (400 kDa) and GroEL complex (800 kDa) under the exact same conditions as the calibrant proteins allows the calculation of the ccs's for each charge state observed of both assemblies. By creating a new plot between the ln of the corrected drift time and the ln of the corrected ccs, for each charge state of the SR and GroEL ions, a relationship is obtained that can be described by a linear equation. This equation is subsequently used for all ccs calculations of the analyzed chaperonin complexes. For further details on the ion mobility calibration procedure, see supplementary Tables 1 and 2 and Figures S1 and S2.

The reported ccs's for the chaperonin assemblies are obtained by IM-MS analysis performed under ion mobility settings that are optimized for large protein complexes. These optimized settings involve changing the duty cycle time in the ion mobility cell to 51 ms and using a ramped wave height from 7 to 30 V (also see details above, mass spectrometry section). Previously, we extensively investigated the effect of fixed and ramped wave heights in the ion mobility cell on ccs calibration for unknown protein complexes. Although, in general, IM-MS analysis using fixed wave heights resulted in somewhat larger ccs values being observed for the protein complexes than those obtained from IM-MS analysis using a ramped wave height, there was a clear linear correlation, showing that both methods could be used for the determination of the ccs of large protein assemblies.[11] As drift time peaks are significantly narrower for our complexes when using the ramped wave height settings, we used these conditions for all chaperonin systems investigated. Ccs's were calculated for each individual well-resolved charge state of a chaperonin assembly and subsequently averaged (supplementary Table 3). Error values given reflect the standard deviation of ccs's calculated for the individual charge states of a complex.

Drift times corrections were made for the dead time between the ion mobility cell and pusher. The ccs (Ω) is obtained from the absolute drift time according to eq 1 with calibration on known ccs's.[27] This also includes a correction for the mass and charge of the protein ion.

$$\Omega(\text{Å}^2) = K \times t_D^X \times z \times \sqrt{\frac{1}{m_{ion}} + \frac{1}{m_{gas}}} \qquad (1)$$

where Ω is the calibrated ccs, K is the calibration constant, t_D is the absolute drift time (corrected), z is the charge state of the ion, m_{ion} is the mass of the ion, and m_{gas} is the mass of the target gas used in the IM cell. The exponential factor X is determined experimentally, as is well described by Ruotolo et al.[28] (see also Supporting Information, supplementary Tables 1 and 2, Figures S1 and S2).

Crystal Cross Section Calculations Using PDB Files. Cross sections were calculated from PDB files using a program implement to a VMD structural viewer (VMD 1.8.6, http://www.ks.uiuc.edu/Research/vmd/),[29] kindly provided by A. Anishkin (University of Maryland, USA). PDB files 1KPO and 1AON were used for GroEL and GroEL-GroES, respectively. The output files give cross sections of slices through the crystal structures, in this case, every 0.2 nm. With these values the total volume is calculated to obtain the rotationally averaged radius and cross section of the chaperonin structure. In general, cross sections obtained in this way are different from ccs's, as no ion neutral collisions are taken into account. However, if the structures of individual complexes are retained in the IM-MS experiments, the relative differences in the chaperonin complex dimensions should be largely preserved.

Determining Collision Cross Sections by MOBCAL. Theoretical ccs's of GroEL and GroEL-GroES were calculated using the open source software program MOBCAL.[30,31] To perform these calculations the MOBCAL program requires modifications to the code, as described by Ruotolo et al.[28] PDB files 1KPO and 1AON were used as input files for GroEL and GroEL-GroES, respectively. MOBCAL reports on two different ccs values, calculated using both the projection approximation (PA) model and the exact hard sphere scattering (EHSS) model. In general, the PA approximation value is found to be in better agreement with the IM-MS ccs measurements.[28,32]

Results and Discussion

Overall Dimensions of Various Chaperonin Complexes. Drift time profiles and mass spectra were recorded for 14 different chaperonin complexes (with nucleotides, genuine polypeptide substrates, the unspecifically binding protein BSA, and two different cochaperones) under identical conditions on a quadrupole ion mobility ToF instrument (Synapt HDMS, Waters, UK). As an example, the mass spectrum recorded for GroEL-gp23 and the corresponding ion mobility profile are shown in Figure 1A. Calibrated ccs's were determined for each individual, well-resolved, charge state and averaged (supplementary Table 3). The similar ccs's obtained for GroEL (244 $\pm$ 3 nm^2) and GroEL-gp23 (247 $\pm$ 2 nm^2) indicate that the substrate is most likely bound inside the GroEL cavity during IM-MS analysis (Figure 1B). This assumption is strengthened by the fact that the charge state of the complex is not significantly affected by substrate binding, as was reported previously.[7] Since the empty chaperonin complex is composed of two heptameric rings, it would be possible that the ringlike topology of GroEL collapses into a more compact structure in the mass spectrometer. This phenomenon of ring collapse has been hypothesized by Ruotolo et al.[3] to occur for the undecameric TRAP complex. However, our observation that the addition of a substrate to GroEL does not increase the ccs strongly suggests that the ring structure of the chaperonin is preserved in the gas phase. Otherwise, the gp23 substrate either would have to bind to the outside surface of the chaperonin or would hamper the structural collapse of

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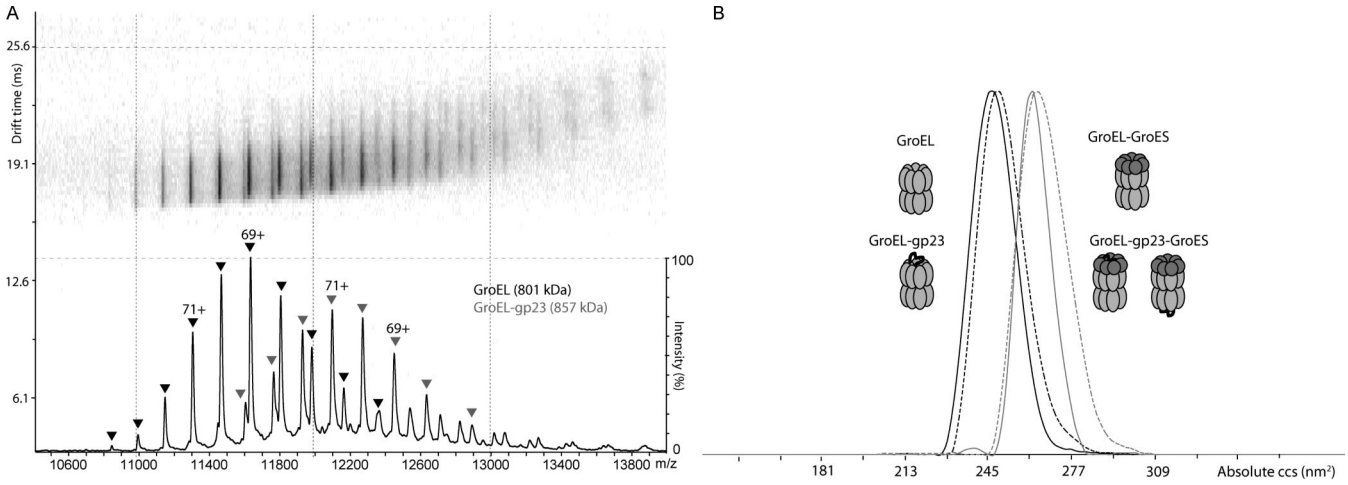

Figure 1. (A) Mass spectrum and uncorrected drift time profile of GroEL-gp23. (B) Calibrated ccs distributions of 71+ charge states of free GroEL (black, solid), GroEL-gp23 (black, dashed), GroEL-GroES (gray, solid), and GroEL-gp23-GroES (gray, dashed).

Table 1. Calibrated ccs's of Different Chaperonin Complexes[a]

chaperonin complex	mass (Da)[b]	mass increase (%)[c]	ccs (nm²)	increase ccs (%)[d]
GroEL	800 758	0	244 ± 3 (231 ± 3)	0(0)
GroEL $(ADP)_{14}$	806 736	0.7	235 ± 2	−3.7
GroEL $(ADP\ Mg)_{14}$	807 150	0.8	237 ± 1	−2.9
GroEL $(ATP)_{14}$	808 472	1.0	237 ± 1	−2.9
GroEL $(ATP\ Mg)_{14}$	808 892	1.0	237 ± 1	−2.9
GroEL-lysozyme	816 861	2.0	241 ± 1	−1.2
GroEL-MDH	836 546	4.4	242 ± 2	−0.9
GroEL-gp23	856 687	7.0	247 ± 2	1.2
GroEL-gp5	847 970	5.9	245 ± 2	0.0
GroEL-Rubisco	855 488	6.8	244 ± 1 (232 ± 1)	0.0 (0.0)
GroEL-BSA	867 228	8.3	256 ± 1 (246 ± 1)	4.9 (6.5)
GroEL-GroES[e]	873 478	9.1	261 ± 1 (248 ± 2)	7.0 (7.4)
GroEL-gp31[e]	884 405	10.4	262 ± 2	7.4
GroEL-gp23-GroES[e]	929 419	16.1	262 ± 1	7.4

[a] The increase in ccs is clearly not related to the addition of mass. [b] Masses listed are theoretical, the observed masses for all complexes are slightly higher. This excess mass is attributed to the trapping of water molecules, buffer salts or counterions within the protein complex, and is similar for all complexes analyzed.[41] [c] As compared to the molecular weight of free GroEL. [d] As compared to the calibrated ccs value of free GroEL. [e] These complexes are prepared in the presence of 250 μM ADP and Mg^{2+}. In brackets, calibrated ccs's are given for chaperonin complexes that are sprayed from a buffer containing imidazole.

the GroEL tetradecamer. In both cases the calculated ccs's as determined by IM-MS should have increased in the presence of an unfolded substrate polypeptide.

As a control experiment we analyzed GroEL in the presence of a 140 times excess of folded BSA, in which BSA functions as an unspecific binding protein ligand. This experiment allows us to mimic incorrect substrate binding modes to the GroEL assembly, as folded BSA is not a substrate it cannot bind inside the GroEL cage but instead will most likely attach to the outside surface of the chaperonin. The mass spectrum revealed the presence of a complex with a mass of 867865 Da, exactly the mass of one tetradecameric GroEL clustered with one BSA molecule. The ccs of the GroEL−BSA complexes showed an increase of 4.9% to 256 nm^2 ± 1 nm^2, as compared to free GroEL (supplementary Figure S3). Moreover, the GroEL−BSA cluster was seen to possess fewer charges than the free GroEL. We expected to observe lower charge states of GroEL−BSA clusters, as some basic sites present on the GroEL surface are no longer available for protonation, instead being involved in the interaction with the folded BSA ligand. For a globular protein a simple relationship predicts the number of charges it obtains during the native ESI process, when using ammonium acetate buffers.[33] Mass spectrometric analysis of folded (monomeric) BSA showed that it obtains a relatively low number of charges (on average, 15) compared to the estimated value of 20 charges (data not shown). Since the charge density for BSA is very low, the overall charge state of the GroEL−BSA cluster is also expected to decrease when BSA unspecifically attaches to the outside of the chaperonin. If BSA was to be enveloped within the GroEL cavity, the charge state distribution for GroEL would not be expected to change upon BSA binding. Overall, these results strengthen our hypothesis that genuine substrates remain buried inside the GroEL cavity in the gas phase during the IM-MS analysis and are not incorrectly binding to the outside of the chaperone assembly.

The potential preservation of the chaperonin's solution phase structure in the gas phase is furthermore confirmed by the analysis of the GroEL−GroES complex by IM-MS. The cochaperone GroES is thought to bind on top of GroEL, thereby increasing the ccs. In line with this hypothesis, the calibrated ccs for GroEL−GroES increased by 7.0%, compared to free GroEL, to 261 ± 1 nm^2 (Figure 1B).

Validation of the IM-MS Results by Molecular Modeling. To test the consistency of the IM-MS method, so that it can be used with confidence for the future analysis of proteins (or protein complexes) with unknown geometry, we compared our data with computational methods that can calculate cross sections (cs's) or ccs's based on PDB files. This provides a direct comparison between the gas phase structure of the protein complex and its structure as derived from other biophysical techniques such as NMR and X-ray crystallography. First theoretical cs's for GroEL and GroEL−GroES (using PDB files 1KPO and 1AON, respectively) were calculated using VMD. From these calculations, the increase in cs due to GroES binding to the chaperonin appears to be 7.2% (180 to 193 nm^2), in excellent agreement with our experimental IM-MS result (7.0%). The rotationally averaged diameter for GroEL determined by

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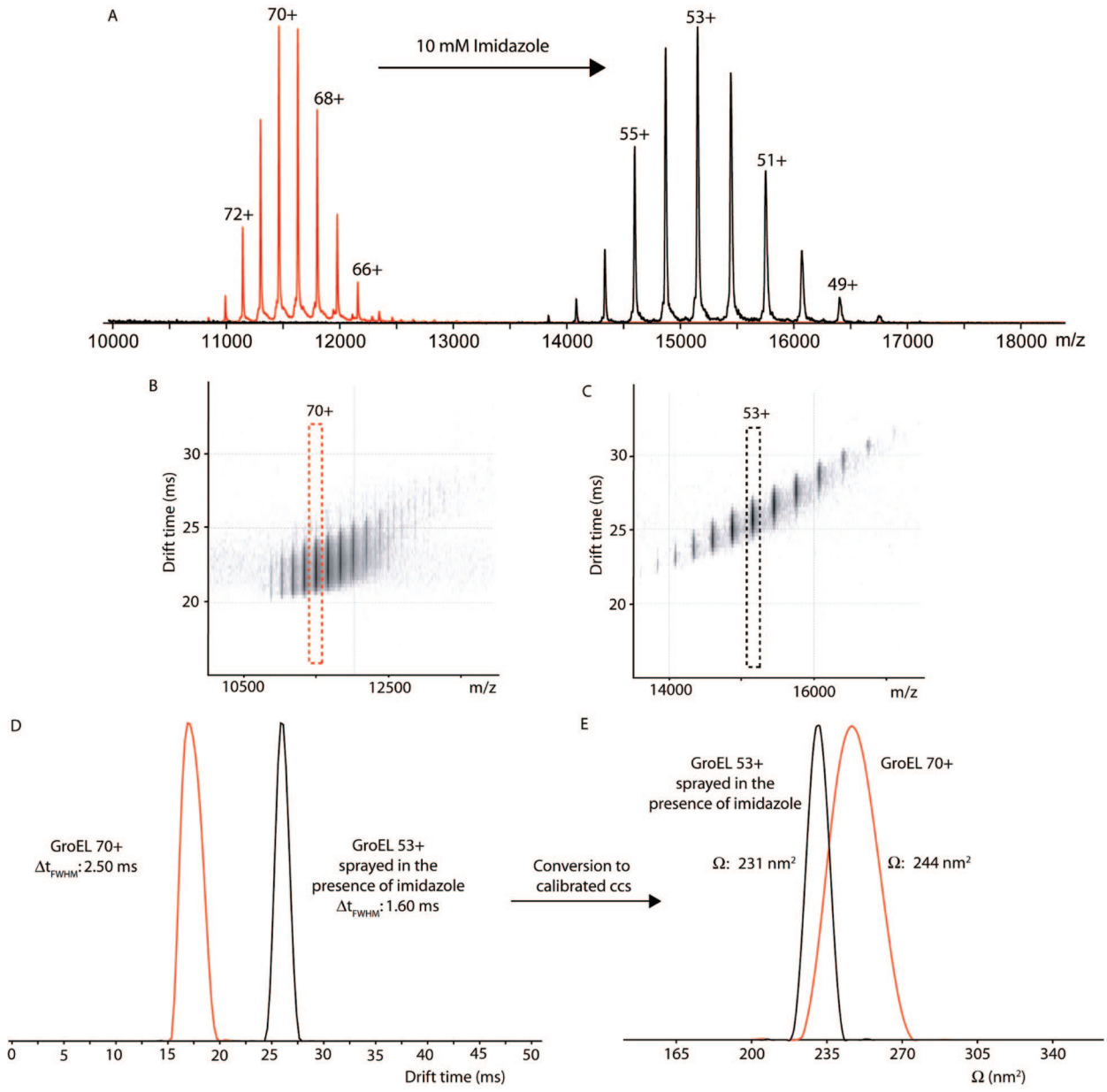

Figure 2. (A) Overlaid nano-ESI mass spectra of 5 μM GroEL sprayed from 50 mM ammonium acetate pH 6.9 (red) and 5 μM GroEL sprayed from 50 mM ammonium acetate pH 6.9 + 10 mM imidazole (black). The addition of imidazole significantly lowers the average charge state distribution of GroEL. (B) Corresponding uncorrected drift time profiles of GroEL and (C) GroEL sprayed in the presence of imidazole. The most intense charge states (boxed) were selected to create ccs distribution plots. (D) The 70+ GroEL ion peak has a resolution of 7 ($R = t_D/\Delta t_{fwhm}$), and the 53+ GroEL ion peak has a resolution of 17. Averaging over all charge states, the resolution increases from 7 to 18 for GroEL after the addition of imidazole to the spray solution. (E) Conversion of the absolute drift time into a calibrated ccs clearly shows the narrowing of the ccs distribution for GroEL ions due to the addition of imidazole to the ESI spray solution.

IM-MS is 18 nm, compared to 15 nm as determined by X-ray crystallography or cryo-EM.[34−37]

The calibrated ccs's determined by IM-MS for GroEL and GroEL-GroES are ~35% larger than the corresponding cs's as calculated by the VMD program. In general, cs's obtained in this way are different from ccs's, as no ion neutral collisions are taken into account for cs calculations. This clearly indicates the impact of charge and protein−gas interactions on the overall ccs's.

Next, we estimated ccs's using the established open source software program MOBCAL.[30,31] MOBCAL reports two different ccs values as calculated by the projection approximation (PA) model and the exact hard sphere scattering (EHSS) model. It was shown previously that for proteins the experimental ccs's derived from IM-MS analysis fall between the theoretical PA and EHSS ccs's, being closer to the PA value.[32,38] Here, the PA and EHSS model reported a ccs for GroEL of 220 and 300

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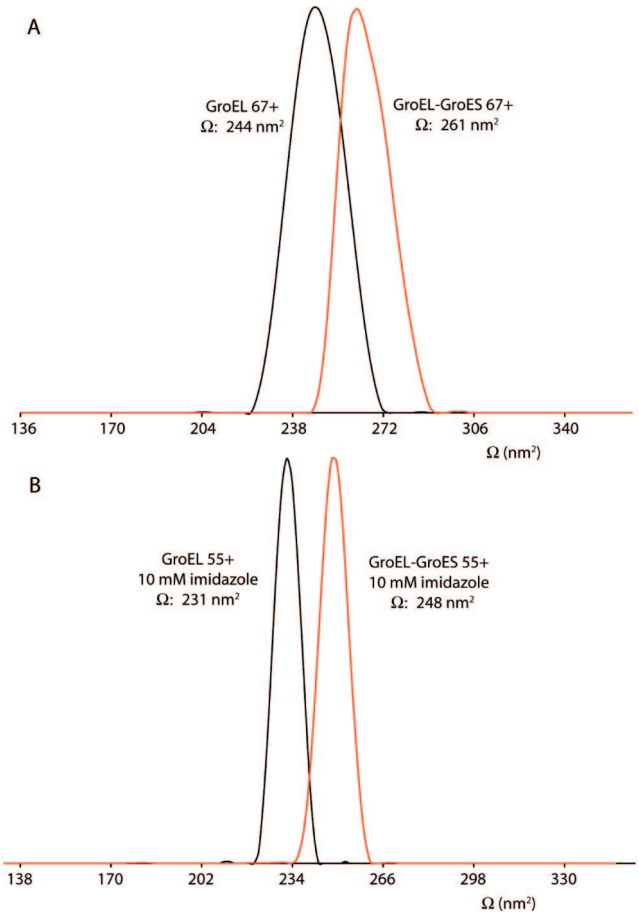

Figure 3. Calibrated ccs distribution profiles for GroEL (black) and GroEL−GroES (red). Chaperonin complexes are analyzed in (A) the presence and (B) the absence of imidazole in the spray solution. These plots show the increased resolution ($R = t_D/\Delta t_{fwhm}$ 8 to 15) in the ion mobility cell, which is very beneficial when analyzing large protein assemblies with similar ccs values. Both complexes are nearly 1 MDa, and their ccs's differ by only 7.0%. Nevertheless the two different assemblies are nearly baseline separated when electrosprayed with reduced charge.

nm^2, respectively. For GroEL−GroES, the ccs according to the PA model was 230 nm^2 and 312 nm^2 using the EHSS model. The experimental ccs's we determined for GroEL (244 ± 3 nm^2) and GroEL−GroES (261 ± 1 nm^2) were closer to the PA values, in good agreement with the previous observations.[32,38] The relative increase in ccs due to the association of GroES to GroEL is 4.5% as determined by the PA method, a somewhat smaller difference than observed by our IM-MS approach and the VMD calculations.

An important factor that can influence the ccs's as determined by IM-MS is the calibration method applied to the data. Although we carefully calibrated the IM cell and performed multiple analyses to obtain ccs values for our protein complexes, the available calibrant proteins limit the IM-MS analysis of very large protein assemblies. The proteins currently used for calibration have molecular masses up to 16 kDa and a maximum ccs of 38 nm^2.[27] Therefore, extrapolation of the calibration curve to the macromolecular complexes in our data set (~1 MDa and >200 nm^2) could possibly introduce an error in the ccs value. In addition, as mentioned previously, using a ramped wave height in the ion mobility cell for the analysis of large assemblies introduces a small calibration error, although this is compensated for by the increased resolution.[11] Finally, there is a difference in hydration of the protein complex between X-ray and IM-MS analysis that can also account for a disparity in the values.

Since we aim to study changes in the global structural conformations of these different chaperonin complexes, it is most important that the relative changes we measure by IM-MS in the gas phase appear to be consistent with information available from more contemporary in solution structural biology methods. To evaluate the ability of the IM-MS approach to distinguish possible differences in protein complex conformation, computational analyses using modeling programs like MOBCAL and VMD can certainly aid in the correct interpretation of the IM-MS data. By combining the information provided by IM-MS with computational models, more sophisticated structural models of protein assemblies can be generated.

It would be ideal to perform full docking calculations for all protein complexes, but in this case this is seriously hampered by the large size and the lack of available high-resolution structural data on most of the investigated chaperonin complexes.[28] Without this information, coarse graining molecular dynamics approaches can be helpful to generate candidate structures, but such models would at best generate fairly inaccurate models.[3,28]

Here, we confirmed the validity of the IM-MS approach for the investigation of GroEL and GroEL−GroES assemblies using two different modeling approaches. As these data appeared to be consistent with more established biophysical results, we will now proceed to use our method to study the overall shapes of other chaperonin complexes of unknown geometry.

Gas Phase Collision Cross Sections of Different Chaperonin Complexes. In Table 1 the IM-MS experimentally determined average calibrated ccs's for all analyzed chaperonin complexes are listed. Each studied GroEL−unfolded substrate polypeptide complex had a similar ccs to that of free GroEL, indicative of the burial of these genuine substrates inside the chaperone cavity, independent of the substrate identity. In contrast, unspecific binding of a folded BSA ligand to GroEL resulted in a significantly larger ccs. Therefore, we conclude that BSA does not bind inside the GroEL cavity but instead attaches to the outside surface of GroEL.

As expected, the binding of a cochaperonin on top of GroEL substantially increases the ccs, irrespective of which of the two studied cochaperonins (GroES or gp31) is bound. Gratifyingly, the binding of GroES to a pre-existing GroEL-gp23 complex increases the ccs only by the amount of GroES, again confirming the burial of the genuine gp23 substrate within the GroEL cavity (Figure 1B).

We also investigated the effect of nucleotides on the structural conformations of GroEL. It is known that transitions between the different functional folding states are regulated not only by domain movements induced by cochaperonin but also by nucleotide binding.[16,39] Previously, cryo-EM studies showed that no conformational changes were observed in GroEL by the addition of ADP, whereas ATP initiated a rearrangement of the apical domains of GroEL to a slightly more open state.[40] So the structure of the chaperonin complex is affected locally by nucleotide binding, but most likely the overall dimensions of the complex remain similar. Our data show a relatively small, but reproducible, decrease in ccs upon nucleotide binding to

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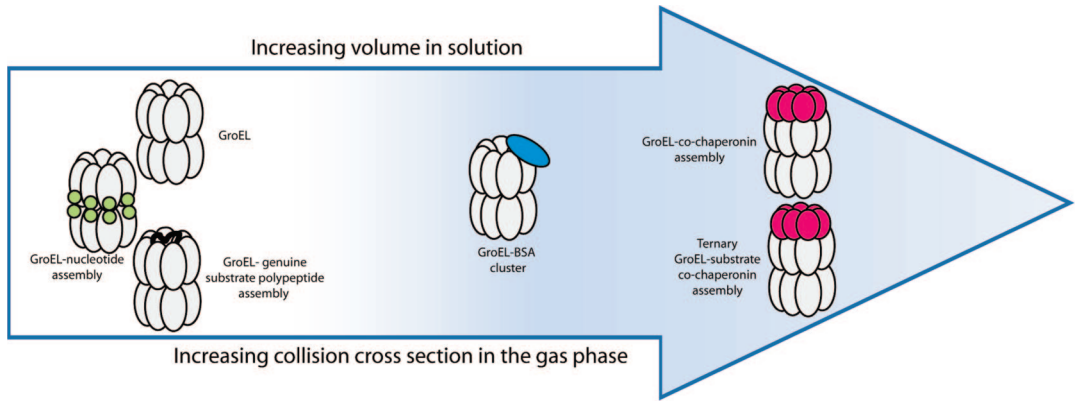

Figure 4. Schematic overview of IM-MS results. The overall changes in dimensions of the different chaperonin complexes are retained from solution phase to gas phase. In the gas phase, the volume (ccs's) slightly decreases when GroEL binds nucleotides (green circles) and remains very similar when GroEL binds a genuine substrate polypeptide (black line) as compared to GroEL. In sharp contrast, the overall dimensions of the chaperonin assembly clearly increase when a ligand (blue oval) unspecifically clusters to the outside surface of the chaperonin. When GroEL binds to a cochaperone (pink heptamer) to form a binary complex, the total volume of the assembly increases to a similar extent as that for ternary chaperonin assemblies that contain a substrate polypeptide in the folding cavity of GroEL.

GroEL, probably attributable to additional electrostatic interactions stiffening the chaperonin complex.

Effect of Charge Reduction on Protein Complex Conformation. Finally, the effect on the ccs of reducing the number of charges the chaperonin complexes obtain in the ESI process was studied. Reducing the net charge on protein ions may minimize Coulombic repulsions and thus stabilize tertiary and quaternary structures.[42,43] To reduce the charge imidazole was added to the ESI spray solution, keeping the pH at 6.9. Imidazole was chosen because of its well-described characteristics during the ESI process, and it is known not to negatively affect the preservation of intact protein complexes during the ionization process.[43]

The charge state distributions of the protein ions were strongly affected, as on average the net charge on the empty chaperonin complex was decreased by 17 charges (Figure 2A, and supplementary Figure S4). Additionally, the width of the ccs distribution of the GroEL assembly was significantly narrowed. The narrow ccs distribution for GroEL ions indicates lower conformational flexibility and an increased stability in the structure of the protein complex ion (Figure 2B−E). The extended structures that the protein complex could adopt at higher charge states are now no longer populated. When analyzing heterogeneous samples, a more uniform population of ion conformations will result in narrower drift time profiles and thus increase the possibility of separating two protein complexes that have similar ccs's.

In our analysis, multiple chaperonin complexes were present simultaneously (i.e., GroEL free or complexed to substrate and/or cochaperonin; see also Figure 1A). Figure 3 shows the improved resolution (R is here defined by $t_d/\Delta t_{fwhm}$) for a mixture of free GroEL (R increases from 7 to 18) and GroEL−GroES (R increases from 8 to 15) in the presence of 5 mM imidazole. Charge reduction thus results in an increase in resolution of more than 40%. Although this enhancement is very important, it should be noted that spraying large complexes in the presence of imidazole is quite challenging, as it somewhat compromises the ionization process.

Interestingly, the calibrated ccs values for the chaperonin complexes, as calculated from the IM-MS analysis in the presence of imidazole, are on average ~5% smaller, indicating a more compact structure (Table 1). This is in agreement with the previously reported observation that higher charge states of a protein complex resemble more extended structures with a larger ccs (see also Figure S4, which shows the correlation between charge state and ccs for GroEL).[10] Furthermore, it appears that the ccs distributions start at a similar minimum value indicating that the compact structures always exist but become significantly higher populated upon spraying the complexes from a solution containing imidazole. We primarily lose the more extended chaperonin structures that were induced by the high charge density on the protein assembly (Figure 2E).

Most interestingly is that even though the ccs's are smaller in the presence of imidazole, the measured relative increase in ccs between GroEL and GroEL−GroES is very similar (7.4%). The determined ccs for GroEL in the presence of imidazole was 231 ± 3 nm^2 and for GroEL-GroES 248 nm^2 ± 2 nm^2 (Table 1). The rotational average diameter for GroEL in the presence of imidazole is determined to be 17 nm by IM-MS. As mentioned previously, the IM-MS analysis resulted in a diameter of 18 nm for GroEL, when sprayed from ammonium acetate.

Conclusions

The generation of structural information of protein assemblies is of critical importance for the understanding of their function. Here, we used IM-MS to study the gas phase structural conformations of a range of GroEL chaperonin complexes. This relatively new approach allows the assessment of gas phase ion collision cross sections that can directly be related to the overall dimensions of the protein assembly.

The consistency of our IM-MS approach was tested by comparing our data with computational methods that can calculate cs's or ccs's, based on PDB files. The molecular modeling results showed that the gas phase ccs's of GroEL and GroEL−GroES as determined by IM-MS were consistent with structural data obtained by X-ray crystallography and NMR. Therefore, we could further use the IM-MS method to generate structural information on other chaperonin complexes of uncharacterized geometry.

All our IM-MS analyses on chaperonin complexes indicate that, in the gas phase, the genuine substrate polypeptides remain enclosed within the GroEL cavity. We found that the ccs

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determined for GroEL in complex with an unfolded substrate polypeptide is very similar to the ccs of free GroEL, independent of the substrate identity. That in the gas phase unfolded polypeptides remain bound inside the GroEL cage was further established by IM-MS analysis of unspecifically bound GroEL-BSA clusters. These clusters were generated by binding folded BSA to the chaperonin complex. In sharp contrast to the genuine GroEL−substrate complexes, the ccs of the unspecific GroEL−BSA assemblies significantly increased. This points toward the attachment of the unspecific BSA ligand to the outside surface of GroEL.

Further indications that the structural properties of protein assemblies can (partially) be retained in the gas phase come from our IM-MS analysis of GroEL in complex with cochaperones. Thus far, little structural information has been available for ternary complexes between GroEL, a substrate, and cochaperonin. Here, we show that the overall shape and dimensions of these complexes are similar to those of the empty GroEL−GroES chaperonin complex. Overall our findings are schematically summarized in Figure 4 and clearly stress that structural properties of large chaperonin complexes can be retained from solution to gas phase.

Moreover, we show that reducing the protein charge in the ESI process is advantageous for the separation of large assemblies with similar ccs's by ion mobility. Most likely Coulombic repulsions are minimized, and the number of conformational states the protein complex will occupy likely decreases. In particular, extended chaperonin structures are no longer populated. Interestingly, the charge state does not affect the relative differences in ccs values determined for the chaperonin assemblies, as again the overall dimension of the chaperonin complex is increased by 7.4% due to the complexation of GroEL to GroES.

To conclude, we show here the potential of IM-MS to generate fundamental structural information for a diverse set of chaperonin complexes. We would like to highlight the broad capabilities of this approach to study conformational changes of not only chaperone assemblies but also biologically active machineries in general.

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Supporting Information Available: Supplementary Table 1 shows the calibrated ccs's of each chaperonin complex for all individual charge states observed. The average ccs and the standard deviations are also listed. Figure S1 shows the collision cross section calibration using ubiquitin, cytochrome c, and myoglobin. Figure S2 shows the correlation and extrapolation of the small and large ions used for calibration, to obtain calibration ccs's. Figure S3 shows the IM-MS analysis of GroEL−BSA unspecific clusters. In Figure S4 the charge state of GroEL is plotted versus the calibrated ccs. This material is available free of charge via the Internet at http://pubs.acs.org.

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