Mass spectrometry (MS) has revealed the composition, stoichiometry, connectivity, and dynamics of many multiprotein complexes that remain challenging for other structural biology methods. More recently, ion mobility (IM), a gas-phase separation technique that resolves protein ions according to their size and charge, coupled with MS (IM-MS) has been used to generate three-dimensional structure information from these samples. Combining information from several gas-phase techniques can overcome many of the challenges of protein structure characterization. Even though these methods are useful, their development contains certain experimental challenges. The primary problem is establishing a general correlation between gas-phase measurements and protein structures in solution. There have been several reports of significant rearrangements of protein structure upon desolvation and ionization, although recent data suggest that these examples may be in the minority. Despite these problems, general methods aimed at protecting protein structure upon the removal of bulk solvent will undoubtedly enable biomolecular structure characterization through gas-phase structural biology approaches, such as IM-MS.

Recent efforts to develop IM-MS methods use additives, both in solution prior to ionization and in the gas-phase prior to MS analysis, as a means of stabilizing protein-complex ions. We have focused on the prior-to-ionization approach, using Hofmeister-type salt additives, and have recently classified a large number of anions for their ability to stabilize multiprotein structure using measurements of both collision induced unfolding (CIU), in which ions are heated through collisions and induced to unfold, and collision induced dissociation (CID), in which increased collisional heating leads to the dissociation of assemblies into a highly unfolded monomers and stripped complexes. Our previous data revealed that anions bind to protein complexes during or prior to the nano-electrospray ionization (nESI) process and can stabilize protein ions through dissociation as neutral molecules, which act to carry away excess energy from the gas-phase protein ions. This energy release allows the protein structure to remain compact—in configurations easily correlated with X-ray and NMR datasets. Herein, we study the influence of cation-based stabilizers, compare these additives to our previous anion dataset, and find dramatic mechanistic differences between the two.

Figure 1A and B show data for tetrameric transthyretin (TTR, 55 kDa). To demonstrate the effect of different cations on TTR, a series of tandem mass spectra (showing CID, Figure 1A) and arrival-time distributions (showing CIU, Figure 1B) of the 14+ charge state of TTR are shown, acquired at a trap collision voltage of 60 V and 55 V, respectively. For each measurement, all instrument parameters are kept constant and only the cationic additive of the nESI buffer is altered. The signal corresponding to the 14+ charge state of TTR isolated for CIU/CID broadens when TTR is incubated with added cations, because it contains unresolved peaks corresponding to a range of previously described adducted forms (Supporting Information, Figure S1). In a similar fashion to anions, when cations dissociate from the protein complexes studied herein, they do so as neutral molecules (bound to acetate or hydroxide counterions). In Figure 1A, signals for 14+ TTR and 6+ to 8+ transthyretin monomer are observed at substantially different levels as a function of the cation added, while Figure 1B reveals strikingly different arrival-time distributions for 14+ cation-bound TTR, with different relative abundances for compact (I) and unfolded conformer families (II–IV). These data clearly demonstrate the differential influence of cation additives on protein dissociation and unfolding in the gas-phase.

For a more quantitative measurement of stability, we monitored CID and CIU data as a function of collision voltage (Figure S2). From these data we constructed the histograms (Figure 1C and D), which show the collision energy (eV*) values at which the intensity observed for intact (I0) and compact (Ic) tetramer ions decrease by 50%. These data include three tetrameric protein complexes other than TTR, including avidin, concanavalin A (ConA), and alcohol dehydrogenase (ADH), and dimeric β-lactoglobulin A (BLA), screened in the presence of the same ten cations. A number of general trends in protein stability are observed. First, the protein complexes studied herein undergo CIU at lower energy relative to CID, as reported previously. We note that following incubation with stabilizing cations, ADH does not appreciably undergo CIU even at the highest activation energy attainable (Supporting Information, Figure S3), therefore its dissociation data is not shown in Figure 1C. Second, cations stabilize gas-phase protein complexes to different degrees. In general, Mg++, and Ca++ have a universally stabilizing influence on I0 and Ic for all protein complexes studied herein. Conversely, cations such as K+, Rb+, and TMA+ have a negligible stabilizing effect relative to...
control data (ammonium acetate). Interestingly, Tris$^+$ exhibits a greater ability to stabilize gas-phase protein complexes than other singly charged cations studied herein. For example, for BLA, Tris$^+$ is the second-most stabilizing cation screened (behind Mg$^{2+}$). In addition, the relative stability of the five proteins studied herein are not influenced by cation additives, with BLA requiring the most energy to dissociate and TTR requiring the most energy to unfold under equivalent conditions.

Despite these similarities, we find several significant differences in the stabilization provided by cation additives when compared with our previous anion data. First, cation adducts seem to stabilize protein complexes against CID to a greater extent, on average, than equivalent anions. The collision energy at which CID occurs is raised by 31% by the average cation, while this threshold is increased by only 19% by the average anion. This argument can be extended to include the general stability afforded to complexes by the most-stabilizing cations, for which the stabilities are generally much greater than the anion-bound complexes studied to date (Supporting Information, Figure S5). Conversely, anionic adducts are generally better stabilizers of gas-phase protein unfolding than cations. Data recorded for the cation-stabilized protein complexes indicate an average CIU threshold increase of only 26%, whereas anions achieved a 36% increase in stability under similar conditions. Therefore, it is predicted that the mechanism of stabilization for cation-adducted proteins is dramatically different from that of their anion counterparts. Figure 2A shows plots of the total mass of bound cations as a function of activation voltage for TTR. Previous data for anions showed a preference for complete dissociation of protein–anion adducts at relatively low activation voltages, which stabilizes the structure through “dissociative cooling”, where bound adducts dissociate from the complex as neutral molecules to carry away excess thermal energy. The cation adducts studied herein that impart the most protein stabilization, however, tend to remain bound to the protein complex even at large activation voltage values. Also in contrast to our studies of protein–anion adducts, CIU and CID stabilities are highly correlated for cation-adducted complexes. The linear relationship between CID and CIU stability thresholds exhibits an $R^2$ value of 0.94 (Figure 2B) compared to published anion-based data ($R^2 = 0.55$). This relationship further indicates a disparity between the stabilization mechanisms for anionic and cationic additives.

Whereas anions perform optimally as stabilizers when they bind to the protein and then dissociate from the complex after relatively minimal activation, the best cationic stabilizers are those that remain bound to the protein assembly in large numbers, even following extensive activation in the gas phase. These highly stabilizing cations strongly correlate with those that have larger charge-per-unit-area values (Figure 2C and Figure S5A), in other words, the more dense the charge on the cation the better it stabilizes the protein. The larger charge-per-unit-area of these cations, which is in great excess to that of any anions that we have tested to date (Figure S5B and Table S1), presumably stabilizes these adducts in one of two ways: either by multidentate interactions within proteins, enabling the cations to more effectively tether regions of the protein structure together, or by replacing highly mobile protons with less mobile cations that restrict charge mobility and inhibit the Coulombic unfolding of subunits within the complex from charge repulsion, which is a critical step in the asymmetric dissociation of noncovalent protein complexes.

Although the cations that strongly stabilize gas-phase protein structure conform to the mechanistic discussion above, some evidence of the “dissociative-cooling” of protein

**Figure 1.** A) MS data for 14$^+$ TTR incubated with ten cations of acetate salts at 60 V trap collision voltage. The data also contain signals corresponding to 6–8$^+$ charge state monomers, resulting from CID of the complex. B) IM data for the same cations shown in (A), at 55 V trap collision voltage. Four distinct drift time features are labeled I–IV. Histogram plots of collision energy (eV*) required to dissociate (C) and unfold (D) 50% of the population of dimeric BLA and tetrameric TTR, avidin, ConA, and ADH are shown for a range of cation additives. Control data sets are also marked on the plot (NH$_4^+$).
structure is observed within our cation dataset (Figure 2A and Figure 3).

In summary, these data present the first description of additive stabilizers that cover a broad range of both cationic additives and multiprotein complexes. We observe that, in general, cations of high charge-per-unit-area stabilize proteins in a complimentary way relative to most anions. We plan to exploit this discovery in the future by using salt additives that are combined to take advantage of both cation and anion properties to improve protein structural stability. We believe that such additives are critical for IM-MS to become a reliable high-throughput method to discover multiprotein topology and structure, and as a means of elucidating the role of surfactant molecules in stabilizing gas-phase membrane protein complexes.

**Experimental Section**

Materials: Proteins Avidin (egg white), TTR (human), ConA (jack bean), ADH (yeast), BLA (bovine), and salts (acetate anion with ammonium, tetramethylammonium (TMA), sodium, potassium, rubidium, lithium, Tris (2-Amino-2-hydroxymethylpropane-1,3-diol), calcium, barium, and magnesium counterions) were purchased from Sigma (St. Louis, MO, USA). All protein samples were exchanged into 100 mM ammonium acetate buffer at pH 7 using Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA) and prepared to a final concentration of 5 mM (avidin, TTR, ConA, ADH) or 10 mM (BLA). The salts were prepared as stock solutions in 100 mM ammonium acetate at a concentration of 20 mM, each of which was then added to the protein solution. Final salt concentrations were 2 mM for avidin, TTR, ConA, ADH and 0.5 mM for BLA samples. The total salt and protein concentrations were chosen primarily to avoid ion suppression effects.

Ion mobility-mass spectrometry and CIU/CID analysis: Approximately 5 μL of sample was injected into a quadrupole ion mobility time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford MA, USA). Protein ions were generated using a nESI source and optimized to allow transmission of noncovalent protein complexes. The traveling-wave IM separator was operated at a pressure of approximately 3.5 mbar, and a 40 V wave height traveling at 800–1000 ms⁻¹ to generate IM separation. Collisional activation in the ion trap prior to IM was used to perform CIU and CID experiments. Ions were selected in the quadrupole mass filter at a m/z corresponding to 16⁺ charge state of Avidin, 19⁺ of ConA, 14⁺ of TTR, 24⁺ of ADH tetramers, and 11⁺ of BLA dimers. Charge states were chosen based on their intensity across each solution state studied, and control IM data were collected to rule out overlapping oligomers at this m/z. Trap collision voltage was varied in 5 V steps. Data analysis and normalization were carried out according to our published method. Some Figures contain axes labeled in collision energy (units of eV*), which
is a normalized version of ion kinetic energy appropriate for making stability comparisons across large mass ranges.[6]

Additional mass spectra (Figure S1), a workflow diagram (Figure S2), CID data for ADH (Figure S3), details on our data normalization procedures (Figure S4), and mechanistic information (Figure S5 & Table S1) are available in the Supporting Information.

Received: December 23, 2011
Revised: March 13, 2012
Published online: April 23, 2012

Keywords: electrospray ionization · ion mobility · mass spectrometry · proteomics · structural biology