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ORIGINAL RESEARCH

Traveling-wave ion mobility-mass spectrometry reveals additional mechanistic details in the stabilization of protein complex ions through tuned salt additives

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Abstract Ion mobility–mass spectrometry is often applied to the structural elucidation of multiprotein assemblies in cases where X-ray crystallography or NMR experiments have proved challenging. Such applications are growing steadily as we continue to probe regions of the proteome that are less-accessible to such high-resolution structural biology tools. Since ion mobility measures protein structure in the absence of bulk solvent, strategies designed to more-broadly stabilize native-like protein structures in the gas-phase would greatly enable the application of such measurements to challenging structural targets. Recently, we have begun investigating the ability of salt-based solution additives that remain bound to protein ions in the gas-phase to stabilize native-like protein structures. These experiments, which utilize collision induced unfolding and collision induced dissociation in a tandem mass spectrometry mode to measure protein stability, seek to develop a rank-order similar to the Hofmeister series that categorizes the general ability of different anions and cations to stabilize gas-phase protein structure. Here, we study magnesium chloride as a potential stabilizing additive for protein structures *in vacuo*, and find that the addition of this salt to solutions prior to nano-electrospray ionization dramatically enhances multiprotein complex structural stability in the gas-phase. Based on these experiments, we also refine the physical mechanism of cation-based protein complex ion stabilization by tracking the unfolding transitions experienced by cation-bound complexes. Upon comparison with unbound proteins, we find strong evidence that stabilizing cations act to tether protein complex structure. We conclude by putting the results reported here in context, and by projecting the future applications of this method.

Keywords Ion mobility-mass spectrometry · Structural stabilization · Multiprotein complex · Collision induced unfolding · Collision induced dissociation

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Introduction

Proteins are amongst the most versatile macromolecules in living systems, and serve crucial functions in essentially all biological processes in a manner dependent upon their structures, dynamics and stabilities. Because protein assemblies are often large, heterogeneous and dynamic entities, there are numerous challenges in developing models of their high-resolution structure. Techniques such as X-ray crystallography and NMR spectroscopy have been widely and successfully used to gain atomic-level structural information on a large number of protein complexes and networks [46], but despite this success, similar analyses are difficult to perform on assemblies that exhibit high degrees of flexibility, heterogeneity and polydispersity [42,46]. Such properties are thought to be pervasive within the proteome, and are found in abundance within membrane-associated protein complexes [54], a class of protein assemblies that are among the most sought-after therapeutic targets [39]. Furthermore, since neither X-ray nor NMR techniques typically separate components during analysis, both require highly purified samples. These and other challenges highlight the need to develop new approaches aimed at multiprotein structure determination [42,46,51].

Mass spectrometry (MS) and, more recently, ion mobility-MS (IM-MS) of intact complexes is emerging as one of many alternative approaches in the field of structural proteomics [7,8,23,31,33,42,44–46,48,55,56]. It is now well established

that MS can yield insights into the composition, stoichiometry and connectivity of heterogeneous multiprotein assemblies at relatively low concentrations [2,24–26,49,59]. When combined with IM, it becomes possible to separate species not only according to their mass-to-charge ratio (m/z) but also according to their ability to traverse an ion guide containing inert gas under the influence of a weak electric field, thus yielding ion size and shape information [9,13–15,34,35,47,50,53,55]. Traveling wave IM separations specifically, that utilize a series of low-voltage 'waves' to propel ions for such size-dependant separations, have enabled most of the modern applications of IM-MS to structural biology [18,19,48,57]. IM-MS experiments, thus, provide measurements of gas-phase protein size, which when combined with detailed molecular modeling can generate 3D topology models [40,41].

Although promising, the application of IM-MS for building architectural models of multiprotein complexes calls for a general correlation between gas-phase measurements and protein structures in solution. There have been several reports of significant rearrangements of protein structure upon transfer to the gas phase [28,33]. Specifically, the processes of electrospray ionization (ESI), desolvation, transport and analysis can occur over a range of time scales and energies. As a consequence, biological molecules and assemblies can rearrange at the local residue level, unfold to more elongated conformations, and even refold to compact, yet non-native conformations [3,6,10]. Such rearrangements prompt the development of general strategies aimed at the protection of protein structure, at every level, in the absence of bulk solvent, and would have far reaching implications in characterizing the structures of gas-phase biomolecules.

While the use of gas phase additives has been reported as a means of stabilizing protein-complex ions [4,52], our group focuses on pre-ionization, additive-based approaches using Hofmeister-type salts [16,17,37], and have recently classified a large number of anions and cations in terms of their ability to stabilize multiprotein structure [20,22]. For these experiments, we use both collision induced unfolding (CIU), in which collisionally-heated ions are induced to create a series of unfolded conformations recorded by IM, and collision induced dissociation (CID), where the same collisional heating eventually leads to protein complex dissociation into highly-unfolded monomeric and stripped complex product ions captured by MS [6]. More importantly, our IM-MS data clearly show that anions and cations can differentially stabilize protein complexes through separate mechanisms, and that while the relative binding affinities of these buffer elements are likely retained in our measurements [21], the stabilization modes we observe are unique to the gas-phase. Whereas anions perform optimally as stabilizers when they bind to the protein and then dissociate from the complex to stabilize the system through 'dissociative-cooling' [22], the best cationic stabilizers are those that remain bound to the protein assembly in large numbers, even following extensive activation in the gas phase [20]. We have hypothesized that two modes of action are potentially critical in this later class of stabilizers. Cations either form multidentate interactions within proteins, enabling the tethering of disparate protein structural regions, or they act to replace highly mobile protons with less mobile cations with relatively restricted mobility, thus inhibiting the Coulombic unfolding of protein subunits, which is a critical step in the asymmetric dissociation of noncovalent protein complexes [20].

In this report, we investigate the use of magnesium chloride as an additive for stabilizing gas-phase protein structures, as this salt is composed of a cation/anion pair that previous results suggest should be strongly stabilizing for desolvated protein ions [20–22]. Our data demonstrate that both anions and cations derived from the addition of this salt in nESI solutions prior to ionization, can be used in concert to stabilize protein structures in the absence of bulk solvent to an extent not previously accessible using either constituent alone. In addition, we refine the mechanism by which cationic additives exert a stabilizing influence on gas-phase protein structure by observing the detailed structural transitions experienced by multiprotein complexes using CIU. Upon comparison with control data, we find strong evidence that stabilizing cations act to tether protein complex structure, rather than stabilize primarily through limiting charge mobility, as previously postulated.

Materials and methods

Materials

The protein tetramers avidin (egg white), and concanavalin A (ConA, jack bean), along with salts (ammonium acetate, ammonium chloride and magnesium acetate) were purchased from Sigma (St. Louis, MO). All protein samples were buffer exchanged into 100 mM ammonium acetate at pH7 using Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA) and prepared to a final concentration of 10 μM. To study the influence of different salts on protein stability without significantly altering buffer capacity or solution pH, 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 protein solutions. Final solutions contained added salt concentrations of 4 mM for ammonium chloride, 2 mM for magnesium acetate and 2 mM for magnesium chloride. The total salt and protein concentrations listed above were chosen primarily to avoid nESI-based ion suppression effects [1].

Ion mobility–mass spectrometry

Sample aliquots (5 μL) were analyzed using a quadrupole-ion mobility-time-of-flight mass spectrometry (Q-IM-ToF MS) instrument (Synapt G2 HDMS, Waters, Milford, MA). Protein ions were generated using a nESI source. The capillary of the nESI source was typically held at voltages 1.4 kV for avidin and 1.65 kV for ConA, with the source operating in positive mode. The sampling cone was operated at 50 V. The instrument settings were optimized to allow transmission of intact protein complexes and to preserve noncovalent interactions [11,27,43]. The trap traveling-wave ion guide was pressurized to contain 3.3×10^{-2}mbar of argon gas. The ion trap was run in an accumulation mode and ion lifetimes in the trap prior to IM analysis range from 0 to 50 ms in our experiments. The traveling-wave ion mobility separator was operated at a pressure of 3.5 mbar (N_2), and employed a series of DC voltage waves (40 V wave height traveling at 800–1000 m/s) to generate ion mobility separation. The ToF-MS was operated over the m/z range of 800–15000 and at a pressure of 1.6×10^{-6}mbar.

Collision induced unfolding and dissociation

Collisional activation in the ion trap traveling-wave ion guide prior to the ion mobility separator was used for CIU and CID of protein complexes in order to investigate the gas-phase stability of protein ions in the presence of different salts. This work was all performed in tandem-MS (quad selection) mode. Ions were selected in the quadrupole mass filter at a m/z corresponding to the 16^+ charge state of avidin tetramer and 20^+ of ConA tetramer. Charge states were chosen based on their intensity across each solution state interrogated, and control IM arrival time data were screened for evidence of overlapping non-tetrameric ions at the same m/z value. Each of these mass-selected ions were activated by increasing the trap collision voltage (Trap CE, as indicated in the instrument control software) which acts as a bias voltage between the quadrupole and the ion trap traveling-wave ion guide to accelerate ions to increased kinetic energies for CIU and CID experiments. For all protein-salt systems investigated here, energy-dependent arrival-time distribution profiles (CIU 'fingerprints') were constructed using 5 V stepwise increments of the trap CE. Upper voltage limits were identified as those where no further dissociation was observed.

Data analysis

All mass spectra were calibrated externally using a solution of cesium iodide (100 mg/mL) and were processed with Masslynx 4.1 software (Waters). Spectra are shown with minimal smoothing and without background subtraction. The relative abundance of mass-selected tetrameric ions (I_{tet}) was calculated as a percentage of the total intensity of all the signals observed in the mass spectra corresponding to either intact protein complex ions or their corresponding fragment ions (i.e., monomer or trimer) using Eq. 1. The relative abundance of the compact form observed for tetrameric ions separated by ion mobility (I_f), which is the only feature observed under non-activating conditions, was calculated as a percentage of the total intensity of the peaks in the arrival time distribution observed at a selected m/z value corresponding to intact tetramer using Eq. 2. These two values are used to chart the dissociation and unfolding of tetramers as a function of collision energy, respectively. The average relative standard deviation for the determination of either I_{tet} (%) or I_f (%) is 2–4 % [22].

$$I_{tet}(\%) = \frac{I_{tet}}{I_{tet} + I_{mon}} \times 100 \quad (1)$$

$$I_f(\%) = \frac{I_{folded}}{\sum I_{conformers}} \times 100 \quad (2)$$

Results and discussion

The influence of tuned salt additives on MS data

Our previous work ranked a series of Hofmeister-type anions and cations for their ability to increase the structural stability of multiprotein complexes in the absence of bulk solvent upon their addition in solution in small amounts prior to nESI using IM-MS, and we discovered that the stabilization mechanisms accessed by cations and anions are strikingly different but not mutually exclusive. Therefore, the combined effects of anions and cations can be utilized simultaneously by adding a salt comprised of highly-stabilizing cations and anions in an effort to further enhance the stability of gas-phase protein structure. It is important to note that this strategy requires the simultaneous binding of both free anions and cations to proteins in solution as a prerequisite for increased protein ion structural stability when compared to isolated anion-only or cation-only complexes.

For this study, we selected Mg^{2+} and Cl^- to comprise our stabilizing salt additive based on their comparative ability to stabilize gas-phase protein structure in previous datasets [20–22]. We applied this salt additive to 2 tetrameric protein complexes, avidin (64 kDa) and ConA (103 kDa), having large differences not only in molecular weight, but also in isoelectic point and protein structure, in order to assess its effects on protein stability and compare the addition of magnesium chloride to both magnesium and chloride adducted

proteins in isolation. MS data are shown in Fig. 1, where tetramer ions are generated from four different buffer compositions (black: control/100 % 100 mM NH_4OAc, blue: 100 mM NH_4OAc with added NH_4Cl, red: 100 mM NH_4OAc with added $Mg(OAc)_2$, purple: 100 mM NH_4OAc with added $MgCl_2$). In the absence of added salts, we observe intact avidin and ConA tetramers, with base peaks corresponding to the 16^+ and 20^+ tetramer charge states respectively (black). The charge states observed for these complexes change slightly upon addition of Cl^- and Mg^{2+} producing charge reduction and amplification when compared to control data respectively. We ascribe the observed changes in average charge state to the relative bound populations of H^+, Mg^{2+} and Cl^- found in each case, all of which can be bound as either charged species or neutralized, coupled with the invariant surface areas of the proteins studied. Additionally, the peak widths observed for mass spectra acquired from Cl^- or Mg^{2+}-containing solutions display significant broadening when compared with spectra obtained from complexes prepared in pure ammonium acetate, despite the use of similar instrument conditions in their acquisition. This peak broadening is attributed to a larger average number of Cl^- or Mg^{2+} bound to the surface of the gas-phase protein complex ions than ammonia or acetate adducts available in control solutions, owing to their differential volatility [17,36]. In the presence of $MgCl_2$ (purple), however, we notice a charge state distribution similar to control data, which can be primarily ascribed to the simultaneous binding of both Mg^{2+} and Cl^- to the protein and an averaging of their differential influence on the overall charge state observed. Further evidence of simultaneous cation and anion binding is observed in the increased breadth of the MS peaks recorded from $MgCl_2$ doped solutions, which also exhibit a larger shift in centroid molecular mass when compared with samples containing either NH_4Cl or $Mg(OAc)_2$ additives. This agrees well with our observation of resolved populations corresponding to Mg^{2+} and Cl^- both bound to small monomeric proteins (cytochrom c, ubiquitin) incubated with $MgCl_2$ (data not shown). From these MS measurements, we estimate that between 45 and 48 chloride adducts and 64–80 magnesium adducts are present simultaneously on the surface of these two protein tetramers during our subsequent gas-phase stability measurements.

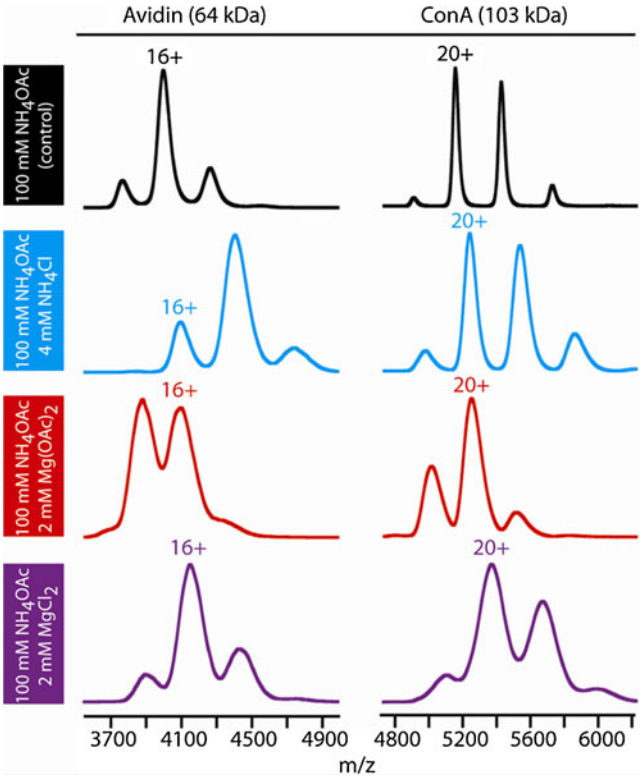

Fig. 1 nESI MS data for the avidin and ConA tetramer ions, (both 10 μm) generated from ammonium acetate-based solutions with no added salt (control, *black*), 4 mM ammonium chloride (*blue*), 2 mM magnesium acetate (*red*), or 2 mM magnesium chloride (*purple*). Spectra for each protein complex were obtained using similar IM-MS instrumental conditions

Quantifying the stabilizing effect of tuned salt additives

In order to evaluate the influence of $MgCl_2$ as a stabilizing additive for gas-phase protein structure, we performed CIU and CID stability measurements on the avidin and ConA ions incubated in the same four solution conditions as shown in Fig. 1. As a further set of control experiments, we also measured solutions where $MgCl_2$ additives were replaced with tetramethylammonium bicarbonate ($TMAHCO_3$) in equal concentrations and measured under identical instrument conditions. While our previous data identify the components of $MgCl_2$ as strongly stabilizing, the same dataset indicated that the components of $TMAHCO_3$ provided gas-phase protein ions with no measurable increase in their structural stability when added separately to solutions prior to nESI [20–22].

Plots of collision voltage versus ion intensity for compact (I_f) and intact (I_{tet}) tetramer ions recorded for the 16^+ charge state of avidin (Fig. 2a) and the 20^+ charge state of ConA are shown in Fig. 2b, and allow us to monitor protein complex unfolding and dissociation respectively. Charge states were chosen based on their intensity across each solution state interrogated, and control IM arrival time data were screened for evidence of overlapping non-tetrameric ions at the same m/z value. Both I_f and I_{tet} are observed to decrease as the collision voltage used to accelerate the ions and initiate collisional activation is increased. In addition, I_f is depleted at lower voltages than I_{tet} for ions generated from all five buffer compositions. Taken together, these results indicate that protein complexes dissociate only after the tetramer precursor ions have undergone significant unfolding in the absence of the bulk solvent, as reported previously [6,30]. Importantly, when comparing a plot of I_f (open, dashed) and I_{tet} (closed, solid), we observe that the addition of $MgCl_2$ to

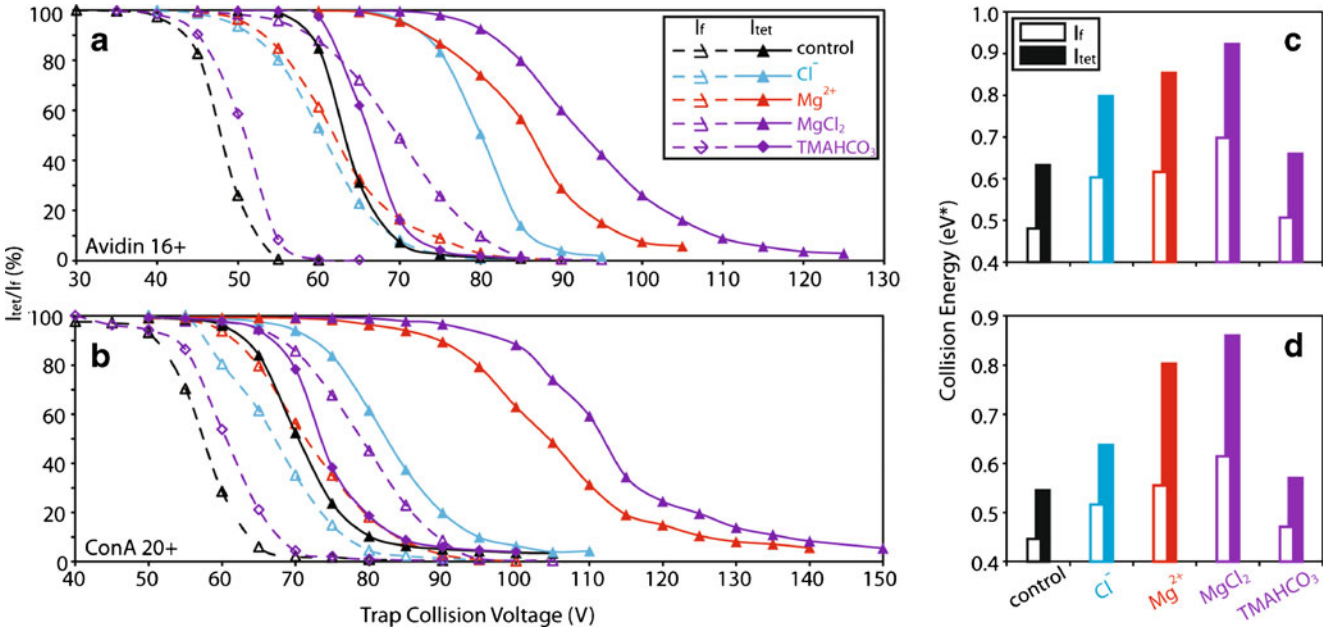

Fig. 2 Elucidating the extent of unfolding and dissociation from IM-MS. Plots of the relative intensities of tetramer ions (I_{tet}, solid lines), and the relative intensities of compact tetramer ions (I_f, dashed lines) are shown as a function of trap collision voltage for the 16^+ charge states of avidin tetramer (**a**) and the 20+ of ConA tetramer (**b**). Tetramer ions were generated from ammonium acetate-based solutions containing 10 μm protein and no added salt (control, *black*), 4 mM ammonium chloride (*blue*), 2 mM magnesium acetate (*red*), 2 mM magnesium chloride (*purple triangle*), or 2 mM tetramethylammonium (TMA) bicarbonate (*purple diamond*). Histogram plots of the collision energy (eV*) required for the 50 % dissociation (filled) and unfolding (hollow) of intact avidin (**c**) and ConA (**d**) are shown for the five buffer conditions mentioned above. Collision Energy (eV*) is a normalized version of ion kinetic energy, that takes into account both the charge on the ion and reduced mass of the ion-neutral collision complex [22]

the sample solution (purple triangle) increases the voltage values at which I_f and I_{tet} are observed to decrease, and that this change is greater than that observed for the addition of Mg^{2+} (red triangles) and Cl^- (blue triangles), respectively, and far greater than that observed for $TMAHCO_3$ (purple diamonds), which is similar to the measurements made for ions generated from pure ammonium acetate based control solutions (black).

Our results are clearly illustrated through a comparison of normalized collision energy (eV*) at which I_f and I_{tet} decrease to 50 % of their original intensity for avidin (Fig. 2c) and ConA ions (Fig. 2d) as a function of the buffer composition used for nESI. Generally, we observe that cations are stronger stabilizers than anions of gas-phase protein structure, especially when CID data are considered. This agrees well with our previous observations, which indicated that cations preferentially act to stabilize gas-phase proteins by remaining bound to the assembly at relatively high internal temperatures [20], whereas stabilizing anions mainly bind and then dissociate from protein ions to access a 'dissociative cooling' mechanism [22] which can, on its own, produce significant increases in protein ion stability. Most importantly, it is clear from our data that the simultaneous presence of both stabilizing cations and anions causes a significant increase in protein complex stability relative to the addition of either component in isolation, resulting in a 7.2–8.1 % and a 10.7–13.3 % increase in protein quaternary and tertiary structural stability respectively for avidin and ConA. In contrast, protein ions created from $TMAHCO_3$ doped solutions exhibit no significant increases in stability, resulting in dissociation and unfolding threshold values similar to control data acquired from ions generated from pure ammonium acetate. Though anticipated, this result is significant, as it indicates the differential stability we observe is predicated on the chemical character of the salts added rather than the increased degrees of freedom gained through potential $TMAHCO_3$ adduction.

The influence of tuned salt additives on protein tetramer dissociation in the gas phase

To obtain a more complete mechanistic picture of $MgCl_2$ protein ion stabilization in the gas phase, we also measured CID data for ConA tetramer ions generated from the four buffer compositions mentioned above. Figure 3b shows tandem mass spectra for 20^+ ConA tetramer ions at high collision energies (170 V acceleration). ConA tetramer ions generated from pure ammonium acetate buffer (black, control) follow the conventional asymmetric charge partitioning dissociation pathway, generating fragment ions that correspond to highly charged monomers (open square, 25.62 kDa) and lower-charge state trimers (not shown) [32]. Additionally, three

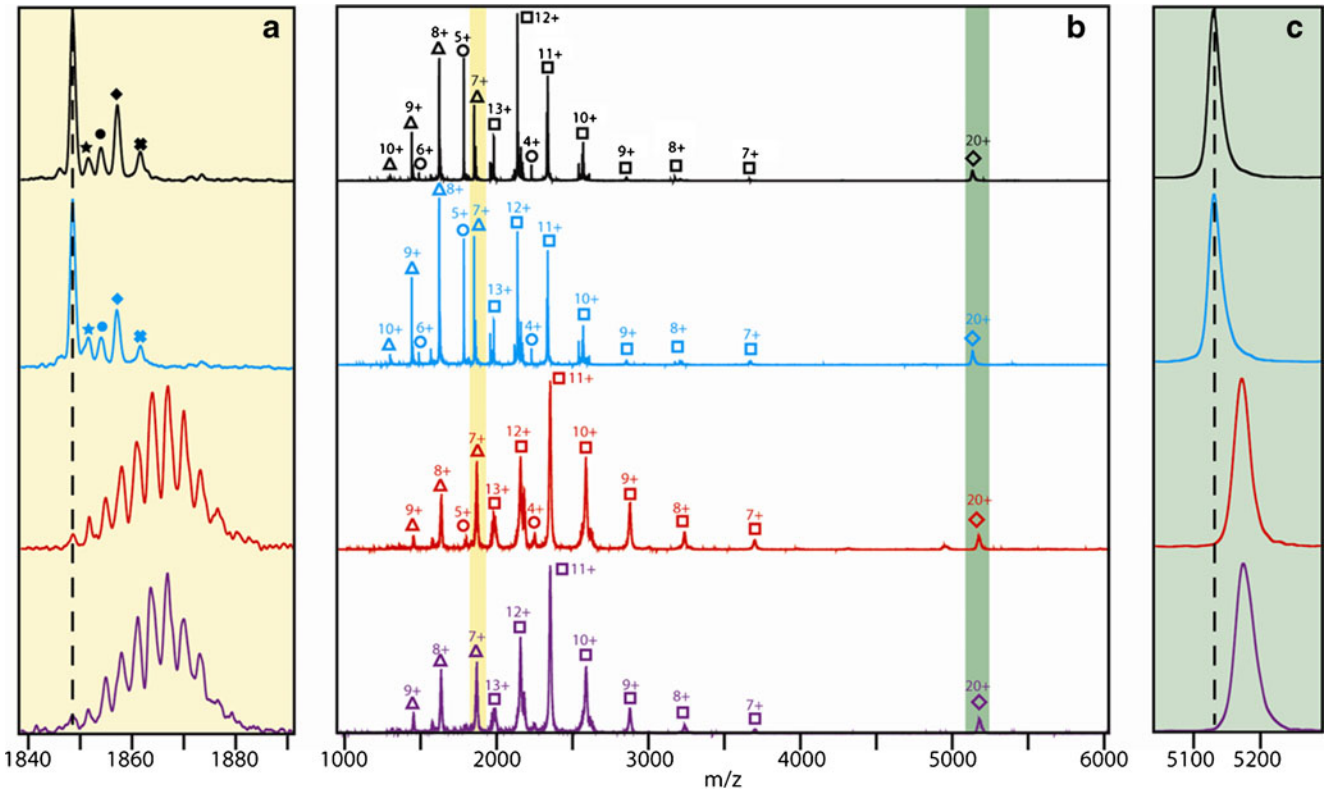

Fig. 3 CID of ConA tetramer obtained from ammonium acetate-based solutions containing 10 μM ConA and no added salt (control, *black*), 4 mM ammonium chloride (*blue*), 2 mM magnesium acetate (*red*), 2 mM magnesium chloride (*purple*). Tandem mass spectra of the 20^+ charge state of ConA tetramer created from the above four buffer solutions acquired at the highest trap collision voltages where ion transmission is observed (**b**), where tetramer precursor ions, dissociated monomer product ions, two peptide fragment ions (12.94 kDa and 8.90 kDa) are denoted by open diamond, square, triangle, and circle, respectively. The yellow box highlights the region of the mass spectrum containing the 7^+ charge state of the 12.94 kDa peptide fragment product, and this region is shown in detail in (**a**). Detailed analysis reveals a distribution of Mg^{2+} adducts resolved by MS when $Mg(OAc)_2$ or $MgCl_2$ are added to the sample solutions while there are no Cl^- adducts observed adhered to the product ions when NH_4Cl is added (**a**). Peaks corresponding to adducts arising from sodium, potassium, sodium + potassium and sodium + potassium + potassium- binding are marked with filled stars, circles, diamonds, and crosses, respectively. The green box indicates the remaining ion signal for the 20^+ tetramer precursor ion population (**c**). A mass difference of≈840 Da is recorded relative to control (intact mass=102.6 kDa, in good agreement with sequence mass), indicating the tight binding of cations at high trap CE (170 V) when the ConA tetramer is incubated with Mg^{2+}. A black dashed line marks the m/z of highest abundance (m/z=5130)

peptide fragments are also observed in the product ion spectrum for the ConA tetramer which have similar appearance energies to monomer ejection. These ions have intact masses of 12.94 kDa (open triangle), 12.68 kDa (low signal intensity, not marked), and 8.90 kDa (open circle), and likely correspond to the c-terminal half (residues 164–281), n-terminal half (residues 30–148), and a secondary n-terminal fragment (tentatively identified as the b_{82} ion in reference to the sequence order of the fragment, rather than the intact ConA monomer) resulting from decay of the 12.68 kDa fragment ion respectively (PDB ID: 2CNA), respectively.

ConA CID datasets also reveal a number of illuminating differences between cation and anion stabilized protein ions. For example, we observe that Mg^{2+} ions remain bound to the ConA tetramer in large numbers following extensive activation in the gas phase. This observation is reflected in the mass difference recorded between the ConA tetramer generated from Mg^{2+} doped solutions (red) and those created from control solutions (black, Fig. 3c). We also observe a series of resolved Mg^{2+} adducts bound to the 7^+ ions of the 12.94 kDa, C-terminal peptide fragment produced from Mg^{2+}-bound ConA tetramer (Fig. 3a, red). Conversely, MS data for Cl^- exposed tetramer indicate that chloride adducts are completely dissociated from the protein complex prior to product ion formation (Fig. 3a, c, blue). In these experiments, as observed previously, Cl^- acts to stabilize the protein complex through a "dissociative cooling" mechanism, where chloride adducts dissociate from the tetramer upon collisional activation as neutrals to carry energy away from the activated protein ions [22].

In addition to altering the structural stability of intact protein complexes, our IM-MS measurements indicate that cation addition can alter the CID pathway accessed during protein complex dissociation. For instance, the absolute number of charges transferred to the leaving monomeric protein subunits decreases slightly when ConA is incubated

with added Mg^{2+} when compared to Cl^- adducted or control samples (Fig. 3b, red). Specifically, the charge state of the most abundant monomer (open square) ions observed from Mg^{2+} bound proteins is decreased from 12^+ to 11^+ when compared to control, in combination with significantly increased signal intensity for the 7^+–9^+ monomer charge states. This charge state shift is also observed in the CID of the Mg-bound avidin tetramer (data not shown). Additionally, we observe a decrease in signal intensity of 50 % for the peptide fragments that typically result from low-energy ConA CID (open triangle and circle) compared with control datasets.

The observations above correlate well with our previous assertions regarding cation-mediated gas-phase protein ion stabilization. Our previous results have narrowed the available mechanisms of this process to two possibilities [20]. The first requires bound cations to form strong multidentate interactions within gas-phase proteins, thus enabling the tethering of different regions of the protein structure together and increasing structural stability. The second relies upon the decreased mobility of added cations within multiply-charged protein ions when compared with protons, which may work to restrict charge mobility and inhibit the Coulombic unfolding of subunits within the complex, thus limiting the asymmetric dissociation of noncovalent protein complexes. In contrast to the cation-bound protein ions measured here, the dissociation profiles measured for anion-bound protein complex ions are unchanged relative to control (Fig. 3b, blue), indicative of the complete dissociation of anion-based adducts in the early stages of collisional activation. As such, it is not surprising that our CID data for proteins incubated with $MgCl_2$ mimics the dissociation behavior of those same samples having added Mg^{2+} rather than those doped with excess Cl^-, as the former will remain bound to influence CID while the latter will not.

CIU unfolding 'fingerprints' reveal mechanistic insights in cation-bound protein stabilization

To further investigate the mechanism at work in protein ions incubated in $MgCl_2$ containing solutions, we constructed CIU unfolding 'fingerprints' for protein complex ions derived from such solutions, as well as control samples containing the constituent anion and cation components of the salt. Changes in the tertiary/secondary structures of protein ions are induced during the CIU process, leading to several structural ensembles that are stable on the millisecond timescale and can be resolved in both IM drift time and in terms of the collision energies required to drive the structural changes observed. For clarity, CIU fingerprint data is projected as a contour plot (Fig. 4) where intensities for the features observed are denoted by a color-based axis [16,30,38]. A careful analysis of CIU fingerprint data allows the nature of protein complex stabilization to be identified by observing the conformational features that are stabilized (elongated on the collision energy axis) relative to control data.

A control fingerprint for the avidin tetramer is shown Fig. 4a, highlighted with a black Y-axis. At low trap collision voltages (Trap CE), the 16^+ charge state of the avidin tetramer has a drift time of ~10 ms, which persists to a Trap CE of 52 V and is the most compact conformer for this protein observed in our experiments. At higher collision voltage (>55 V) more elongated conformations are observed that have drift times >12 ms. Three distinct conformations in addition to the most compact protein configuration are identified under our conditions, and we use a simple (I, II, III, IV) nomenclature for these conformational families, which constitute the unfolding pathway of avidin monomers within the complex. The unfolding landscape observed in our fingerprints varies substantially as a function of the buffer compositions used to prepare samples for nESI. For example, fingerprint data acquired for avidin with added Cl^- shows that the most compact conformer (I) is observed at substantially larger collision voltages (>65 V) when compared to those ions generated from pure ammonium acetate solutions, indicating that the increased stability observed in our experiments for the protein ions afforded by anions is due primarily to the enhanced stability of this compact conformer (Fig. 4a, blue Y-axis). In contrast, avidin incubated with added Mg^{2+} displays a different CIU fingerprint. Despite the similar degree to which the most compact conformer (I) is stabilized, we observe a partially unfolded conformer (II) that has a shorter drift time (~11.5 ms) and persists at higher energies (Fig. 4a, red Y-axis). This surprising observation is attributed to the development of a new partially unfolded structure that is unique to avidin samples incubated with Mg^{2+}, and thus provides evidence supporting our cation-mediated stabilization model involving the tethering of flexible regions within protein ions through strong multi-dentate interactions. This observation holds for ConA as well, where Mg-bound protein ions exhibit a more-gradual transition between conformer II→III without developing any discrete, resolved conformational populations (Fig. 4b, red Y-axis). Finally, we observe conformation IV over a significantly broadened energy range when $Mg(OAC)_2$ is added to solutions prior to nESI. This result, as observed throughout our IM-MS dataset, stands in contrast to ions incubated in the presence of excess Cl^- or pure ammonium acetate buffers, where discrete and well-resolved conformational families are observed by IM (Fig. 4b, blue and black Y-axis). In fact, the presence of excess Cl^- in nESI samples has no observable affect on the drift time axis of the CIU fingerprints recorded, only the breadth of energies over which each structure is observed is changed. It is likely that such complex transitions, as observed between conformer families II and III, and the broadened energy distributions observed for highly-unfolded conformational families, as detected in conformer family IV,

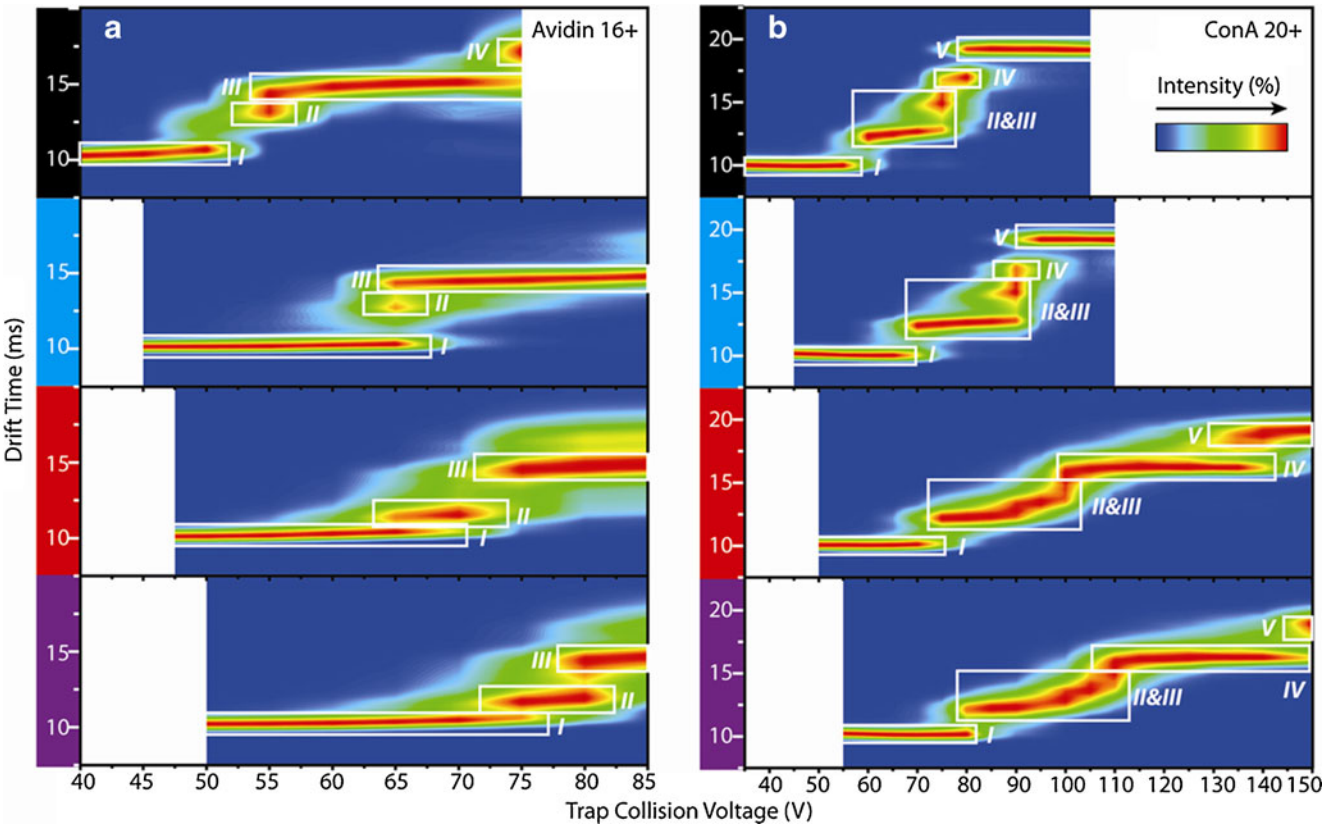

Fig. 4 CIU fingerprint contour plots are shown for 16^+ charge states of avidin tetramer (**a**) and 20^+ of concanavalin A tetramer (ConA, **b**) generated from ammonium acetate-based solutions with no added salt (control, *black* Y-axis), 4 mM ammonium chloride (*blue* Y-axis), 2 mM magnesium acetate (*red* Y-axis), or 2 mM magnesium chloride (*purple* Y-axis), where ion trap collision voltage is charted against IM drift time, and the ion intensities are denoted by a color-coded axis. The conformational forms for the tetramer are highlighted (*white box*) and labeled (I, II, III, IV, V)

constitute further evidence of cation-based tethering interactions within protein monomers (Fig. 4b, red Y-axis).

Critically, fingerprint data collected from avidin and ConA tetramer ions incubated with $MgCl_2$ displays elements from both the fingerprints of its constituent components (Fig. 4a, b, purple Y-axis). Specifically, $MgCl_2$-containing samples display a highly stabilized compact state (I) resulting primarily from the dissociation of Cl^- adducts at lower collision voltage, as well as the multi-state transitions and broadened energy profile of the highly unfolded species detected for Mg^{2+} bound protein ions. Thus, our CIU fingerprint data supports the observation that proteins and complexes incubated with $MgCl_2$ derive their increased stability through the simultaneous binding of both Mg^{2+} and Cl^- adducts, and that both dissociative cooling and tethering-type stabilization mechanisms are accessed by the resultant assemblies.

Conclusions

The correlation between solution and gas-phase protein structure has, in part, driven the development and application of IM-MS in structural biology. For example, MS measurements have been used to study bioactive peptide aggregation [12], membrane protein structure [5,58], and protein stability changes upon ligand binding [29,30]. The application of IM and MS to protein quaternary structure has rapidly developed in recent years, enabling the determination of multiprotein stoichiometry, dynamics, and 3D topology [7,56]. However, several reports have highlighted the uncontrolled distortion of protein structure in the absence of solvent, including both the general compaction of protein size and structural rearrangements that may occur upon desolvation and transfer to the gas phase. In order to facilitate the use of gas-phase measurements in the construction of native-state protein models, we herein investigated the stabilizing effect of $MgCl_2$, as this salt bears anion and cation components previously identified in isolation for their stabilizing affects. Our CIU and CID data clearly indicate that the simultaneous presence of both stabilizing cations and anions causes a significant increase in gas-phase protein quaternary and tertiary structural stability relative to their individual effects. On average, $MgCl_2$ doped samples produce gas-phase protein ions that are ~50 % more stable than those produced from pure ammonium acetate buffered

solutions, and ~10 % more stable than those samples with Mg^{2+} added alone. Furthermore, our data reveal additional details in the mechanism associated with stabilizing gas-phase protein ions through cation adduction. Specifically, through our 'CIU fingerprint' data we are able to detect evidence of frustrated protein unfolding transitions and highly-stabilized unfolded structures for Mg-bound protein ions, and both effects are likely due to multi-dentate cation-protein interactions. Samples containing added $MgCl_2$ are able to access the above mode of stabilization, along with the dissociative cooling-type stabilization associated with chloride anion adduction simultaneously, to create protein complex ions having superior structural stability. In future experiments, we plan to use the mechanistic insights presented in this report to further refine stabilizing additives for the nESI-IM-MS analysis of proteins and protein complex, thus enabling the evaluation of labile protein structures not readily amenable to gas-phase studies.

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