RESEARCH ARTICLE

Effects of Select Anions from the Hofmeister Series on the Gas-Phase Conformations of Protein Ions Measured with Traveling-Wave Ion Mobility Spectrometry/Mass Spectrometry

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Abstract

The gas-phase conformations of ubiquitin, cytochrome *c*, lysozyme, and α -lactalbumin ions, formed by electrospray ionization (ESI) from aqueous solutions containing 5 mM ammonium perchlorate, ammonium iodide, ammonium sulfate, ammonium chloride, ammonium thiocyanate, or guanidinium chloride, are examined using traveling-wave ion mobility spectrometry (TWIMS) coupled to time-of-flight (TOF) mass spectrometry (MS). For ubiquitin, cytochrome *c*, and α -lactalbumin, adduction of multiple acid molecules results in no significant conformational changes to the highest and lowest charge states formed from aqueous solutions, whereas the intermediate charge states become more compact. The transition to more compact conformers for the intermediate charge states occurs with fewer bound H_2SO_4 molecules than HClO_4 or HI molecules, suggesting ion-ion or salt-bridge interactions are stabilizing more compact forms of the gaseous protein. However, the drift time distributions for protein ions of the same net charge with the highest levels of adduction of each acid are comparable, indicating that these protein ions all adopt similarly compact conformations or families of conformers. No significant change in conformation is observed upon the adduction of multiple acid molecules to charge states of lysozyme. These results show that the attachment of HClO_4 , HI , or H_2SO_4 to multiply protonated proteins can induce compact conformations in the resulting gas-phase protein ions. In contrast, differing Hofmeister effects are observed for the corresponding anions in solution at higher concentrations.

Key words: Ion mobility, Mass spectrometry, Hofmeister series, Adduction, Conformation

Introduction

The first reports of the effects of various salts on protein stability date back to 1888, when F. Hofmeister investigated the effectiveness of different salts on precipitat-

ing egg white proteins [1]. Since that time, an ordering of ions based on their effects on the stabilities of biomolecules in aqueous solutions has been well established, and the topic of many reviews [2–10]. The effects are more pronounced for anions than cations, with the following general order:

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Anions to the left of the series are referred to as kosmotropes, and greatly decrease protein solubility in aqueous solutions by stabilizing native conformations. At the other extreme are chaotropes, which increase protein solubility by destabilizing native conformations. This trend has been reproduced numerous times, with a few notable exceptions, including a reverse ordering of ions in some instances when the isoelectric point (pI) of the protein is several units greater than the pH of the solution [11–15]. Highly-compact, yet non-native solution conformers have been observed for numerous proteins at low pH [16–18], and in some instances, the addition of chaotropes has stabilized these “molten globules” [18].

Despite numerous studies, the molecular origins of these Hofmeister phenomena are still hotly debated. Both ion-water and ion-protein interactions are purported to play roles, but it is not well established the extent to which each of these effects contribute. A variety of techniques, including nuclear magnetic resonance [16, 17, 19–22], X-ray crystallography [11, 13, 14], X-ray absorption spectroscopy [23, 24], femtosecond (fs) mid-infrared pump-probe spectroscopy [25], sum-frequency generation spectroscopy (SFG) [6, 10, 26], Sephadex G-10 gel sieving chromatography [27, 28], Jones-Dole viscosity calculations [29], neutron [30, 31] and X-ray [32] diffraction, and molecular simulations [6, 9, 10, 19, 33–35] have been used to examine the Hofmeister effects, and conflicting evidence about the importance of ion-water versus ion-protein interactions have been reported. The extent to which ions can affect the structure of water at long distances has been a topic of debate [6, 25, 26, 33, 35–37]. Recently, both fs pump-probe spectroscopy of aqueous salt solutions [25] and SFG of aqueous solutions containing both salts and polymers [26] indicate that individual ions do not order water significantly beyond the first solvation shell. In striking contrast, recent infrared photodissociation (IRPD) experiments on ions in aqueous nanodrops have shown that sulfate dianion orders water molecules well beyond the first and second solvation shells [36], and that long-range patterning of water occurs for many different ions to various extents [37].

Few mass spectrometry (MS)-based experiments have intentionally examined nonspecific ion-protein interactions. Indeed, small cations (specifically Na^+ and K^+) [38, 39] and anions (SO_4^{2-} and PO_4^{3-}) [40] are typically considered nuisances during the analysis of biological molecules by both ESI and matrix-assisted laser desorption/ionization (MALDI)-MS, and many steps have been taken to reduce their presence in the mass spectra of proteins [39–41] and nucleic acids [38, 42, 43]. The few MS studies involving the interactions of proteins with small ions have examined more the ion's effects on overall signal [44, 45], the observed charge state distribution [46, 47], how many ions (or neutral acid molecules of the anion) adduct to the protein [48–50], or how to best minimize ion adduction [39–41]. With respect to adduction of anions to protein ions, the anions sulfate [40], perchlorate [49, 50], and iodide [48] adduct to proteins, with the extent of adduction of the latter two anions correlating well [49] or exactly [48, 50] with the number

of unmodified basic sites (arginine, lysine, histidine, and N-terminus) in the protein.

For ESI of macromolecular complexes, buffers or essential salts can be necessary to preserve the noncovalent interactions that hold the subunits together [51–53]. The retention of buffer or salt molecules on the ionized complex during droplet desolvation can complicate the determination of the complex's molecular weight. This is typically counteracted by increasing the harshness of conditions during ion desolvation, which might result in the alteration of the complex's conformation, further complicating any potential comparisons of the gas-phase conformation to that adopted by the complex in solution [54]. Addition of low volatility salts to solutions of macromolecular complexes can help preserve these complexes in compact conformations as they enter the gas phase, even after significant ion activation in the source region [55]. However, any low-volatility molecules still bound to the complex will adversely affect accurate measurement of the molecular weight of the complex. A recent study of macromolecular complexes with a variety of anions show that some anions can adduct to complexes and preserve compact conformations, yet can be readily removed by activating the ions without altering the complex ions' conformation [56].

Recent experiments with the proteins ubiquitin, cytochrome *c*, lysozyme, and insulin chain B electrosprayed from aqueous solutions containing millimolar concentrations of 11 different sodium-anion salts indicate that the levels of both acid molecule and sodium binding to each protein correlate well with the proton affinity of the anion and not with any solution properties of the anions, i.e., adduction did not follow a Hofmeister series [57]. Here, traveling wave ion mobility spectrometry (TWIMS) [58] coupled to MS is used to examine the effects of anion adduction (as multiples of the neutral acid HX) on the gas-phase conformations of ubiquitin, cytochrome *c*, lysozyme and α -lactalbumin, electrosprayed from aqueous solutions at neutral pH. The anions perchlorate, iodide, and sulfate all cause charge states of ubiquitin, cytochrome *c* and α -lactalbumin with partially-folded conformers to adopt more compact conformations. The same amount of compact conformation(s) observed for a protein ion with a specific number of bound H_2SO_4 molecules is only seen for the same protein ion with at least twice as many bound HClO_4 or HI molecules, suggesting salt-bridge interactions between the anion and protonated sites on the protein serve to compact the protein ion in the gas phase. For the highest and lowest charge states that are common to all solutions, acid binding does not result in a significant change to the observed drift time distributions. No significant change in conformation with increased adduction of molecules occurs for lysozyme, for which a reversed Hofmeister series is anticipated at pH $\sim$ 7.

Experimental

Ubiquitin ($\geq 98\%$), cytochrome *c* ($\geq 95\%$), lysozyme, the ammonium salts of chloride ($\geq 99.0\%$), perchlorate ($\geq 98.0\%$),

iodide ($\geq 99.0\%$), thiocyanate ($\geq 99.0\%$), and sulfate ($\geq 99.0\%$), and guanidinium chloride ($\geq 99.0\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification. α -lactalbumin ($\geq 85\%$) was also purchased from Sigma, and was desalted with 18 M Ω water using Bio-Rad Micro Bio Spin 6 columns (Bio-Rad Labs, Hercules, CA, USA). Solutions with a final concentration of 10 μ M protein and 5 mM ammonium salt were prepared in 18 M Ω water (Millipore, Billerica, MA, USA) for analysis. Solutions containing only 10 μ M of each individual protein were also prepared in 18 M Ω water. All aqueous solutions, with and without added salts, had pH values ~ 7 . Experiments were performed using a Synapt G2 High Definition MS (Waters, Milford, MA, USA) equipped with a Z-spray ion source. Ions were formed with nano-electrospray tips prepared by pulling borosilicate capillaries (1.00 mm o.d./0.78 mm i.d.; Sutter Instruments, Novato, CA, USA) to a tip of ~ 1 μ m i.d. on a Sutter Instruments P-87 Flaming/Brown micropipette puller. A 0.127 mm diameter platinum wire (Sigma, St. Louis, MO, USA) was inserted into the capillary in order to make electrical contact with the solution. Electrospray was initiated and maintained by applying a potential of 1.0–1.5 kV, relative to instrument ground, to the wire. The sampling and extraction cones were operated at potentials of 40 and 3 V, respectively, and the trap was operated at a flow rate of 3.00 mL/min, collision energy of 4 V and bias of 45 V.

The TWIMS mobility cell was operated with a constant wave velocity of 700 m/s, wave height of 40 V, helium flow rate of 180 mL/min, and IMS (N_2) flow rate of 90 mL/min. Pressures were approximately 2.3, 1.1×10^{-3} , 2.5×10^{-2} , and 1.1×10^{-6} mBar in the backing, source, trap, and TOF regions, respectively. The TOF analyzer was operated in resolution (“V”) mode, and all spectra were acquired for three minutes. Mass spectra were not smoothed, whereas drift time distributions were smoothed twice using the Waters Mass Lynx software built-in Savitzky Golay smooth with a 1 unit window.

Results

Unadducted Protein Conformations from Aqueous Solution

The conformations of gaseous protein ions formed by ESI are sensitive to many instrumental conditions, including ion injection energy [59–61], ion storage time prior to analysis [62–66], solvent temperature [67], and solvent composition [67]. Drift time distributions for charge states of ubiquitin ($pI=6.79$), cytochrome *c* ($pI=9.59$), lysozyme ($pI=9.32$), or α -lactalbumin ($pI=4.80$) formed from pure water, and solutions containing millimolar concentrations of the ammonium salts of sulfate, perchlorate, or iodide were measured using otherwise identical experimental conditions. Each of the salt solutions had a pH of ~ 7 , although acidification can occur during droplet evaporation [68].

TWIMS drift times can be converted to collision cross sections [54, 69, 70]. However, the most accurate calibrations for small proteins, such as the ones studied here, are for the denatured forms [70]. Even with careful control of instrumental parameters, errors up to 5% have been recently reported [70]. This uncertainty can be attributed to a number of factors, including changes in the distributions of conformers observed for a charge state with changes in the traveling wave height and velocity [71]. Recently, experiments with the *p*-methoxybenzylpyridinium ion show that ion heating can occur with increasing traveling wave height, increasing wave velocity, or decreasing pressure in the drift cell [72]. Ion heating induced by the TWAVE measurement may also cause changes in protein conformation to occur, although this effect appears to be minimal for large multi-protein complexes [73]. For these reasons, drift distributions are not plotted on a collision cross section scale. Both static-field IMS [59, 60] and TWIMS [69] data are often plotted on a normalized drift time scale, in which drift times are multiplied by the ion charge for the purposes of comparing features across different charge states. We normalized the drift times by multiplying by charge, charge squared [74], and the protocol of Ruotolo et al. [54], and a comparison is provided in the Supplemental Material. Results from multiplying drift time distributions by charge for charge states of ubiquitin (a), cytochrome *c* (b), and lysozyme (c) are shown in Figure 1.

ESI of ubiquitin from aqueous solutions results in formation of the 4+ through 9+ charge states, and the charge-normalized TWIMS arrival time distributions for these ions are shown in Figure 1a. The ubiquitin conformers have normalized drift times ranging from 35 (4+ and 5+ ions) to 75 (8+ and 9+ ions) ms. For the lowest charge states (4+ and 5+), broad, unresolved peaks centered about 47.72 and 49.75 ms are the most pronounced features. The distributions for 6+ and 7+ ions are also broad, with maxima at normalized drift times of 70.26 and 71.19 ms, respectively. For the 8+ and 9+ ions, sharp peaks centered about normalized drift times of 69.04 ms and 70.74 ms, are the only features. An increase in normalized drift times with increasing charge state has been observed for proteins using static-field IMS [59, 75, 76], where low charge state ions have collision cross section (ccs) values closest to those calculated using solution-phase structures, whereas the ions with the highest charge states have ccs values suggesting they are nearly completely devoid of secondary structure [59, 75]. The arrival time distributions for the 4+ through 9+ charge states formed by ESI out of 49% water, 49% acetonitrile, 2% acetic acid (dotted lines) are similar to those out of pure water. The 10+ through 13+ charge states were also formed out of the denaturing solution, and had greater normalized drift times than the 9+ charge state out of both solutions (Supplemental Material Figure S1).

The distributions for ubiquitin ions formed by ESI out of either aqueous or denaturing solutions do not resemble the drift profiles observed for the same charge states using

Figure 1. Charge-normalized TWIMS drift time distributions of the 4+ through 9+ charge states of ubiquitin **(a)** formed from pure water (solid lines) and 49% water, 49% acetonitrile, 2% acetic acid (dotted lines), 5+ through 9+ charge states of cytochrome *c* formed from pure water **(b)**, and 6+ through 11+ charge states of lysozyme formed from pure water **(c)**. Distributions are obtained by integrating intensities across m/z values corresponding to the protonated form of each charge state from the two-dimensional dataset. For ubiquitin, drift times corresponding to partially-folded conformers are highlighted

static-field IMS under gentle source conditions [76]. Static-field IMS distributions for ubiquitin charge states show a transition from compact to partially-folded conformers as the charge state increases from 7+ to 8+, as well as multiple features for both the 8+ and 9+ charge states. The TWIMS data indicate that a transition from more compact to more elongated conformers occurs between the 5+ and 6+ charge states, which suggests that some ion heating occurs in these TWIMS experiments. Sharp peaks with low mobilities for both the 8+ and 9+ charge states indicate a limited number of unfolded conformers with similar extents of elongation, and elongation continues to increase with increasing charge. Based on comparison of the distributions in Figure 1a to the data of Koeniger and Clemmer [76], ions with normalized drift times greater than 65 ms correspond to elongated conformations, whereas the 4+ and 5+ ions have more compact conformations.

Similar elongation of conformers was observed for cytochrome *c* (Figure 1b). These data indicate both partially-folded and elongated conformers for the 7+ and 8+ charge states, whereas the 7+ was highly compact and the 8+ exhibited both compact and partially-folded conformers at low injection energies and/or short storage times prior to injection in static-field IMS [61, 62]. For lysozyme (Figure 1c), ions with 6+ and 7+ charges have normalized drift times of ~60 ms, whereas ions with 8+ or more charges have drift times of ~80 ms. This transition from more compact to more elongated conformers as the charge state increases from 7+ to 8+ is most consistent with the distributions observed by Valentine and Clemmer at intermediate injection voltages [60].

Experiments performed on the first generation Synapt have shown that both the amplitude and frequency of the traveling wave used in the mobility cell can potentially affect protein ion conformations [71, 74]. Greater elongation was reported to occur with lower wave heights and/or higher wave velocities. In our experiments, these parameters were set to values for which loss of adduct molecules from the protein ions was minimized. It is likely that ion heating in the trap region or mobility cell of the instrument causes unfolding of some intermediate charge states of these protein ions to occur. Therefore, because differences in the conformations adopted by individual charge states are examined, data are reported on a drift time scale. Greater drift times correspond to lower mobilities and greater collision cross sections.

IMS Reduces Chemical Noise

TWIMS-MS spectra were obtained under identical experimental conditions for ubiquitin electrosprayed out of solutions containing the ammonium salts of chloride, perchlorate, iodide, sulfate, thiocyanate, or guanidinium chloride. The two-dimensional drift time (m/z) plot of 10 μ M ubiquitin electrosprayed out of an aqueous solution of 5 mM $(\text{NH}_4)_2\text{SO}_4$ is shown in Figure 2. An integrated mass spectrum (Figure 2, left), obtained by summing intensities across all drift time values, shows the most

abundant ions that are produced. A complicating factor for analyzing proteins in the presence of millimolar levels of various salts is potential mass interference from salt clusters. For example, there is considerable ion signal from cluster ions at lower m/z values, and several high abundance $[(\text{NH}_4)\text{HSO}_4]_n\text{NH}_4^+$ ions are labeled in the full mass spectrum. There are several charge state families of cluster ions in the two-dimensional spectrum, with $z=4$ clusters spanning a range of m/z values of ~ 1100 – 1900 . The extensive overlap of cluster ions with protein ions over a wide range of m/z values is a significant source of chemical noise. For example, clusters obscure detection of many ubiquitin ions with $z=4+$ charges (circled) in the full mass spectrum. As the expanded view of the full mass spectrum shows, the $4+$ ion with nine H_2SO_4 molecules attached is the only $4+$ ion that can unambiguously be identified without TWIMS separation.

By dispersing ions in the TWIMS cell prior to mass analysis, it is possible to separate protein ions from overlapping sources of chemical noise, when their mobilities are different [77–81]. Examples of extracted ion mass spectra for highlighted regions corresponding to ubiquitin $4+$, $6+$, and $8+$ ions are shown to the right of the two-dimensional plot in Figure 2. The uppermost extracted mass spectrum shows ubiquitin $4+$ ions with zero through nine adductions of neutral H_2SO_4 . Only one of these low-abundance ions can be identified in the full integrated mass spectrum. Ions corresponding to the adduction of one and two H_2SO_4 molecules to ubiquitin $4+$ have a signal-to-noise ratio of 33 in the extracted mass spectrum, whereas these ions are not observed in the full mass spectrum, illustrating the advantage of the drift separation in improving detection limits by lowering chemical noise [77–81]. $[\text{M}+4\text{H}+9\text{H}_2\text{SO}_4]^{4+}$ is the most abundant $4+$ ion. The sum of the number of acid molecules and protons for the most abundant ion is equal to the 13 basic sites in ubiquitin. This correlation between adduction and the number of basic residues has been reported previously [48–50].

Conformations of Ubiquitin $6+$ with Adducts of H_2SO_4 , HClO_4 , and HI

Drift profiles can be extracted from the two-dimensional plot in order to examine conformational changes that may occur as a result of adduction of multiple acid molecules to protein ions. Data is plotted as a function of drift time for each individual charge state, taking into account the time ions spent in the transfer TWAVE and time-of-flight MS analyzer [54, 70]. In some instances, multiple features were observed in the distribution. To simplify data presentation, peaks are labeled with Roman numerals, with increasing numerals corresponding to increasing drift times and therefore decreasing mobility and increasing collision cross section. Shoulders on a peak are designated with lowercase letters.

Drift profiles for ubiquitin $6+$ ions with between zero and five molecules of H_2SO_4 (a), HClO_4 (b), and HI (c)

adducted are shown in Figure 3. Drift distributions for $(\text{ubiquitin}+6\text{H})^{6+}$ with no adducts are broad and are similar from each of the three solutions (bottom three profiles). There is an abundant peak (labeled IIIb) with a higher mobility shoulder (IIIa). These three profiles are also remarkably similar to the distribution for $(\text{ubiquitin}+6\text{H})^{6+}$ ions formed from pure aqueous solution (Figure 1a) as are the profiles for $(\text{ubiquitin}+6\text{H})^{6+}$ from aqueous solutions containing 5 mM NH_4Cl , NH_4SCN , or guanidinium chloride (Figure 3). These data show that the conformation of the protein is not affected by 5 mM concentration of these salts to an extent observable by TWIMS-MS, i.e., changes to the conformation of the protein induced by the salts in solution, if any, are not reflected in the distributions of conformers in the gas phase. Typically, significantly higher concentrations of salts are necessary to observe Hofmeister phenomena. These solutions all have pH values of ~ 7 , but the ion concentration increases and the pH of the solution can change [68] during droplet evaporation, which makes it difficult to determine both the final pH and final concentration of each anion prior to protein ion formation.

In contrast, adduction of multiple H_2SO_4 , HClO_4 , and HI molecules does affect the conformation of the gas-phase protein ion. The drift profile for $(\text{ubiquitin}+6\text{H}+\text{H}_2\text{SO}_4)^{6+}$ has two new features, corresponding to a more-folded conformer (I), as well as an unresolved distribution of partially folded conformers (II). Adduction of two H_2SO_4 molecules to give $(\text{ubiquitin}+6\text{H}+2\text{H}_2\text{SO}_4)^{6+}$ results in feature I being the most intense peak, with very little of features II or III remaining. For ions with three or more H_2SO_4 molecules adducted, feature I is the only peak observed in the drift profile. These results indicate that adduction of two or more H_2SO_4 molecules causes these protein ions to adopt more folded conformations than observed for their bare counterparts under similar experimental conditions.

The drift profiles for ubiquitin $6+$ with multiple additions of HClO_4 or HI are similar to each another. In contrast to results for H_2SO_4 , attachment of one HClO_4 or HI molecule to the protein ion results in little observable change in conformation compared with the bare $6+$. Attachment of either two HClO_4 or two HI molecules to the protein ion results in drift profiles rather similar to that observed for the attachment of one H_2SO_4 molecule. The relative intensity of feature I is slightly greater for the adduction of two HI molecules compared with the adduction of two HClO_4 molecules, but this might be due, in part, to the more pronounced high mobility shoulder for HClO_4 (Ia). This highly compact conformation is observed exclusively for ions with two or more HClO_4 molecules adducted to the protein ion, is not observed for adduction of any number of H_2SO_4 or HI molecules, and might correspond to a non-native, molten-globule-like conformation preserved by the adduction of HClO_4 molecules. With three HI or HClO_4 molecules adducted to the protein, peak I is the most intense

Figure 2. Two-dimensional drift time(m/z) plot obtained for ubiquitin ions formed from an aqueous solution of 5 mM $(\text{NH}_4)_2\text{SO}_4$ (center), along with the integrated mass spectrum, including a zoom-in on the region spanning m/z 2000–3000 (left). Extracted mass spectra for ubiquitin ions with 4+, 6+, or 8+ charges, obtained by integrating intensities across drift times in the regions of the two-dimensional plot highlighted by white circles are shown to the right. Noise peaks are highlighted with asterisks

feature in the drift profile and features II and III are much lower in intensity. Only feature I is observed upon adduction of five HClO_4 or HI molecules to the protein ion.

For (ubiquitin+5H) $^{5+}$, which was formed out of all three solutions, and for (ubiquitin+7H) $^{7+}$, which was formed only from ammonium perchlorate and ammonium sulfate solutions (not shown), more compact conformations are observed when multiple acid molecules are adducted. As was observed for the 6+, at least twice as many HClO_4 or HI molecules adducted to the 5+ and 7+ were necessary to form a similar abundance of compact conformers compared with H_2SO_4 adducted ions. These results suggest that salt bridging or ionic interactions between the acid molecule and basic sites in the protein causes the protein ion to adopt more compact conformations in the gas phase. This compaction occurs more efficiently for dianions (SO_4^{2-}) compared with monovalent anions (ClO_4^- , Γ^-).

Molecular Adduction to Ubiquitin 4+ and 8+ Ions

To investigate how adduction of molecules affects protein ion conformations at both extremes of the conformational space (Figure 1), drift profiles for ubiquitin 4+ and 8+ ions with multiple adducts of HClO_4 , HI, and H_2SO_4 molecules were examined (Figure 4). Ubiquitin 4+ was the lowest charge state formed from all three solutions, and these ions showed extensive adduction. The distribution of (ubiquitin+4H) $^{4+}$ with no adducts formed from pure water (Figure 1) shows these ions are highly compact. Attachment of three HClO_4 , HI, or H_2SO_4 molecules results in only very subtle shifts in the peak center to longer drift times, with the greatest shift for perchlorate (<4% increase). No significant change in conformation occurs through the adduction of nine molecules, the maximum to adduct to this charge state. By

comparison, the change in drift times with molecular adduction for the 6+ ions is significantly greater (up to 30%). That no new features with shorter drift times are observed with increasing molecular adduction to the (ubiquitin+4H) $^{4+}$ suggests that these ions may already have maximally compact conformations.

Data for multiple adducts to the 8+ charge state, the highest charge state that was formed from salt solutions containing 5 mM ammonium perchlorate or ammonium sulfate are shown in Figure 4 (right). The drift distribution for the bare 8+ ion formed out of pure water (Figure 1a) shows this ion is more elongated compared to lower charge states. Addition of up to three HClO_4 or three H_2SO_4 molecules results in <3% increase in normalized drift times, indicating that there is no significant conformational change upon binding of multiple molecules. The absence of a conformational change for the 8+ upon acid molecule adduction is consistent with a higher barrier to folding into a compact form than for the 6+ and 7+, despite having a similar extent of elongation.

Cytochrome *c* 7+ with Multiple Adducts

To assess if the results for ubiquitin are general, similar experiments were conducted with cytochrome *c*, lysozyme, and α -lactalbumin. Cytochrome *c* charge states ranging from 5+ through 11+ are formed by ESI from pure aqueous solutions. The charge-normalized drift profiles for these ions span a range of normalized drift times of 50–115 ms (Figure 1b). Only charge states between 6+ and 8+ were formed from all three solutions. Distributions for cytochrome *c* 7+ ions with multiple molecules of H_2SO_4 (a), HClO_4 (b), and HI (c) adducted are shown in Figure 5. As was the case for ubiquitin, the presence of the anion in the

Figure 3. TWIMS drift time distributions of 6+ ions of ubiquitin formed from aqueous solutions of 5 mM $(\text{NH}_4)_2\text{SO}_4$ **(a)**, NH_4ClO_4 **(b)**, or NH_4I **(c)**. Distributions are shown for ions with no adducts (bottommost plots) through five adducts (uppermost), and were obtained by integrating intensities across m/z values corresponding to each addition from the two-dimensional dataset. The bottom-most plots are unadducted 6+ ions formed from aqueous solutions of NH_4Cl **(a)**, NH_4SCN **(b)**, and guanidinium chloride **(c)**

same solution as the protein has no measurable effect on the conformation of the unadducted protein ion (Figure 5, bottom plots for each set). Three distinct features are observed, and are labeled III through V.

Adduction of multiple H_2SO_4 molecules to cytochrome c 7+ results in this ion adopting more compact conformations (Figure 5a). With two H_2SO_4 molecules adducted, feature III is the dominant peak in the drift profile; the low mobility feature V is no longer observed, and the abundance of feature IV is lower. A new feature, labeled I is observed in the drift profile for the ion with four H_2SO_4 molecules adducted, as well as an unresolved distribution of partially-folded conformers (II). Feature III remains the most intense peak in the drift profile for cytochrome c 7+ ions with four H_2SO_4 molecules adducted. With six H_2SO_4 molecules adducted to the protein ion, peak I is much more abundant than feature III, and feature III is not observed with eight H_2SO_4 molecules adducted. For these ions, feature I is the only peak in the drift profile, with some high mobility ions becoming apparent (Ia). These high-mobility ions become more intense with the adduction of ten H_2SO_4 molecules to the protein ion, with a relative intensity of 44% (peak height) of feature I.

Adduction of HClO_4 (5b) or HI (5c) to cytochrome c 7+ also results in the adoption of more compact conformations compared with the unadducted ion. However, the compaction observed for a given number of HClO_4 or HI molecules is not as great as that observed for the same number of H_2SO_4 molecules, except at maximal adduction. Feature III is the most intense peak for ions with either two HClO_4 or two HI molecules adducted, with feature IV remaining at a relative intensity of ~25%, and no feature V observed at all. Further reduction of feature IV occurs when four molecules of either HClO_4 or HI are adducted. Adduction of either six HClO_4 or six HI molecules results in formation of the more folded conformers I and II, although feature III is still the dominant peak in both drift profiles. Features I and II become more defined when eight HClO_4 or HI molecules are adducted to the ion, and these drift profiles look very similar to that for cytochrome c 7+ with four H_2SO_4 molecules attached, as was the case for ubiquitin. When 10 molecules of either HClO_4 or HI are adducted, feature I is the most intense peak, and a small amount of feature III remains. The most compact feature, Ia, formed when eight or more H_2SO_4 molecules are adducted is not observed with up to 10 HClO_4 or 10 HI molecules. As observed with ubiquitin, adduction of acid molecules to cytochrome c ions with partially folded conformers results in the protein ions adopting more folded conformations, with sulfate being more efficient at compacting the protein than perchlorate or iodide.

Molecular Adduction to Cytochrome c 6+ and 8+ Ions

Drift profiles for cytochrome c 8+, the highest charge state formed from all three solutions, with varying numbers of molecules of HClO_4 , HI, or H_2SO_4 adducted are shown to the left of Figure 6. Again, profiles for the unadducted (cytochrome c +8H)⁸⁺ formed from the three different

Figure 4. TWIMS drift time distributions for ubiquitin 4+ (left) and 8+ (right) ions formed from aqueous solutions of 5 mM NH_4ClO_4 (solid), NH_4I (dotted), or $(\text{NH}_4)_2\text{SO}_4$ (filled circles), in increasing order of adduction from top to bottom

solutions are similar to the profile for this ion formed out of water alone (Figure 1b). The drift profile for the (cytochrome $c+8\text{H}$) $^{8+}$ has two features; a broad high-mobility feature (I), and a low-mobility peak (II). With one molecule added to the ion, feature II decreases in intensity, but to varying extents depending on the adducted molecule. Feature II is absent from the drift profile for cytochrome c 8+ with one HClO_4 adducted, and a slightly higher mobility feature (IIa) is observed. Both features IIa and IIb are observed when one HI molecule is adducted. There are also some changes in feature I upon adduction of one HClO_4 , HI, or H_2SO_4 molecule to the protein. This peak remains broad with one H_2SO_4 molecule adducted, whereas this peak is narrower and shifted to lower drift times with one HClO_4 or HI adducted. The distributions for adduction of two molecules to cytochrome c 8+ also show differences in the features labeled I. There are two high-mobility features in the H_2SO_4 drift profile, with feature Ib being more intense. The relative intensities of these peaks are reversed when two HI molecules are adducted, and feature Ib is the least pronounced for HClO_4 . Feature II is not observed when two HClO_4 molecules are adducted to the ion, whereas Features IIa (HI and H_2SO_4) and IIb (only HI) are still present when two molecules are adducted, but at lower relative abundances compared with those for adduction of a single molecule. The data for cytochrome c 8+ is different than those for cytochrome c 7+ and the charge states of ubiquitin. Adduction of multiple H_2SO_4 molecules to this charge state does not result in the most compaction, whereas multiple adducts of HClO_4 does. One possibility could be that features Ib and IIa are the products of native-like conformations in solution, whereas feature Ia results from the transfer of a highly folded molten-globule state to the gas phase.

Drift profiles for the cytochrome c 6+, the lowest charge state formed from all three solutions, with multiple adductions of HClO_4 , HI, and H_2SO_4 molecules are shown on the right side of Figure 6. No unadducted (cytochrome $c+6\text{H}$) $^{6+}$ is observed. The drift profile for this ion formed from pure water has a single peak, labeled II. A new conformer family is observed upon adduction of two acid molecules (I), and the relative intensities of this new feature depend on the identity of the adducted molecule. Feature I is most intense for H_2SO_4 compared with both HClO_4 and HI. Feature I is more abundant when five H_2SO_4 molecules are adducted, whereas feature II is more abundant when either five HClO_4 or HI molecules are adducted. Drift distributions for cytochrome c 6+ with ten molecules adducted are similar, with very little of feature II remaining for either HClO_4 and HI, and none for H_2SO_4 . With 15 molecules adducted to the protein, the maximum observed for this charge state, the drift profiles are indistinguishable from one another, and indicate only a single compact structure or family of structures with indistinguishable cross sections. The data for cytochrome c ions, with the exception of the 8+, suggest that the compaction observed upon maximal adduction of molecules to the protein does not depend on the molecule, and that adduction might occur through salt-bridges that compact the protein to a family of conformations beyond which further compaction is not possible. However, the intensities of intermediate conformations do depend on which molecule is adducted, suggesting different interactions between the molecule and the protein as the molecule is varied. Specifically, the compaction observed for a given number of H_2SO_4 molecules adducted is only observed upon the binding of twice as many HClO_4 or HI molecules, further supporting salt-bridge interactions as the method of interaction between the protein and the acid molecules.

Figure 5. TWIMS drift time distributions for cytochrome *c* 7+ ions formed from aqueous solutions of 5 mM $(\text{NH}_4)_2\text{SO}_4$ (a), NH_4ClO_4 (b), or NH_4I (c). Distributions are shown for ions with no adducts (bottommost) through 10 adducts (uppermost), and were obtained by integrating intensities across m/z values corresponding to each addition from the two-dimensional dataset

Lysozyme and α -Lactalbumin with Multiple Adducts of H_2SO_4 , HClO_4 , or HI

Drift profiles for lysozyme ($\text{pI}=9.32$) 7+ through 9+ ions, with varying levels of HClO_4 , HI , and H_2SO_4 adduction are shown in Figure 7a–c. The mass spectral data (not shown) confirm the disulfide bonds are intact for all solutions, and

Figure 6. TWIMS drift time distributions for cytochrome *c* 8+ (left) and 6+ (right) ions formed from aqueous solutions of 5 mM NH_4ClO_4 (solid), NH_4I (dotted), or $(\text{NH}_4)_2\text{SO}_4$ (filled circles), in increasing order of adduction from top to bottom

remain intact throughout the adduction of multiple molecules to the protein ions. For the unadducted 8+ and 9+ ions, a single conformer or unresolved family of conformers is observed. As was the case for both ubiquitin and cytochrome *c*, these distributions are indistinguishable from those observed for the bare ions out of water alone (Figure 1c). Although the unadducted 7+ is not formed from all three solutions, the profile for this ion with the addition of three molecules also has a single peak and is similar to that observed for the unadducted ion formed from pure water (Figure 1c).

Lysozyme was amongst the first proteins for which a reverse Hofmeister series was observed, i.e., chaotropes preserve native conformations whereas kosmotropes improve protein solubility [11, 13]. Valentine and Clemmer observed modest changes in the conformations of charge states of disulfide-intact lysozyme as the injection energy was varied [60]; comparison of their distributions to those in Figure 1c suggest the protein is somewhat activated in the TWIMS experiments. Despite the four disulfide bonds, this protein can undergo a significant amount of unfolding in the gas phase [60, 82, 83]. However, in these experiments, adduction of multiple acid molecules results in no significant change to the drift profiles of these ions.

α -Lactalbumin is an acidic protein ($\text{pI}=4.80$), which also has four disulfide bonds and a bound calcium ion. Results for the 6+, 7+, and 9+ charge states are shown in Figure 7d–f. The 6+ ion was formed from all three solutions, and does not change significantly upon adduction of up to four HClO_4 , HI , or H_2SO_4 molecules (7d). Results for the 9+

Figure 7. TWIMS drift time distributions for lysozyme 7+ (**a**), 8+ (**b**), and 9+ (**c**) ions, as well as α -lactalbumin 6+ (**d**), 7+ (**e**), and 9+ (**f**) ions formed from aqueous solutions of 5 mM NH_4ClO_4 (solid), NH_4I (dotted), or $(\text{NH}_4)_2\text{SO}_4$ (filled circles), in increasing order of adduction from top to bottom

ion, which adducted a maximum of three HClO_4 , HI, or H_2SO_4 molecules, were similar (7f). Adduction of acid molecules to the 7+ (7e) results in conformational changes similar to those observed for charge states of ubiquitin and cytochrome *c*. The distributions of conformations for the bare ions formed out of all three solutions differ slightly from one another. There are two features, with feature I less intense for ions formed out of the 5 mM NH_4I solution. Feature I increases with adduction of two acid molecules, with the largest increase occurring for H_2SO_4 . With five acid molecules adducted to the 7+, only feature I is observed for H_2SO_4 and HI, and a small low-mobility shoulder for HClO_4 .

With the exception of the salt concentration in the bulk solution, these experiments were designed such that a reverse Hofmeister series would be observed for lysozyme and cytochrome *c*, whereas a direct series would be observed

for ubiquitin and α -lactalbumin. Adduction of acid molecules to α -lactalbumin, ubiquitin, and cytochrome *c* results in the adoption of more compact structures, and no effect was observed for lysozyme. These proteins span a range of pI from 4.80 to 9.59, indicating that the compaction of the protein in the gas phase as a result of adduction does not depend strongly on the pI of the protein in solution. Compaction of conformations would not be anticipated for the attachment of HClO_4 or HI to ubiquitin and α -lactalbumin, considering the anions of both acids disrupt the native conformations of these proteins in solution at the pH used in these studies. Similarly, compaction would not be anticipated for the adduction of H_2SO_4 to charge states of cytochrome *c*. Thus, these results for gas-phase ions do not correlate with the Hofmeister effect in solution. A similar conclusion was recently reported for gaseous anion-protein complexes [56]. It is more likely that ion-ion interactions are

compacting the protein ions, and that the observed changes in conformation do not necessarily reflect any solution-phase characteristics of the ions or the protein.

Discussion and Concluding Remarks

The Hofmeister series is a widely accepted, albeit not fully understood, cornerstone of structural biology. Whereas molar levels of the salts studied here have varying effects on protein conformation in solution, our experiments show that addition of millimolar levels of $(\text{NH}_4)_2\text{SO}_4$, NH_4ClO_4 , NH_4I , NH_4Cl , NH_4SCN , or guanidinium chloride to the ESI solution have no measurable effect on the gas-phase conformations of charge states of ubiquitin, cytochrome *c*, lysozyme, and α -lactalbumin compared with the same charge states formed from pure aqueous solutions. Structural changes to proteins in solution are known to occur when the salts are at a concentration of several molar. Although the salt concentration in our experiments are initially three orders of magnitude lower, the anion concentration in the evaporating ESI droplet increases and the final anion molarity the protein is exposed to is unknown. Our data for unadducted protein ions formed out of solutions containing Hofmeister salts suggest that the presence of the salt in the same solution as the protein is insufficient to induce a significant conformational change in the protein, at the concentration and timescale necessary for gas-phase ion formation.

For a given number of adducted molecules, the relative intensity of compact conformers is almost always greater for H_2SO_4 than for either HClO_4 or HI . Although SO_4^{2-} stabilizes the native conformations of ubiquitin and α -lactalbumin at pH ~ 7 , observation of more compact conformations upon adduction of HClO_4 or HI , both of which are expected to disrupt native elements of secondary structure, does not correlate with the expected Hofmeister effects. Similarly, compaction of cytochrome *c* upon adduction of multiple H_2SO_4 molecules is contrary to the effects of SO_4^{2-} on this protein in solution at pH ~ 7 . With a few exceptions, the distributions observed for a given charge state with the maximum number of adducted molecules are similar for all three molecules, suggesting that similar compact conformations are formed. Anion adduction will reduce the effects of Coulomb repulsion through favorable salt-bridging, making it possible for the ion to adopt more folded conformations. Because sulfate is a dianion, it should be more efficient at stabilizing protein conformation through ionic interactions than either perchlorate or iodide, both of which are singly charged. More detailed insights into protein conformational effects induced by anion attachment, including effects of hydrogen bonding and charge, could be obtained by investigating additional anions.

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