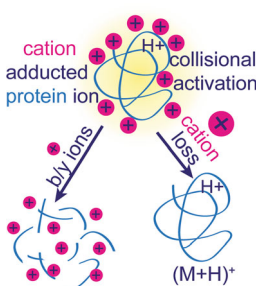


RESEARCH ARTICLE

Effects of Cations on Protein and Peptide Charging in Electrospray Ionization from Aqueous Solutions

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Abstract. The effects of eight different cations with ionic radii between 69 and 337 pm on the charging of peptides and proteins with electrospray ionization from aqueous acetate salt solutions are reported. Significant adduction occurs for all cations except NH_4^+ , and the average protein charge is lower when formed from solutions containing salts compared with solutions without salts added. Circular dichroism and ion mobility results show the protein conformations are different in pure water compared with salt solutions, which likely affects the extent of charging. The average charge of protein and peptide ions formed from solutions with Li^+ and Cs^+ , which have Gibbs solvation free energies (GSFEs) that differ by 225 kJ/mol, is similar. Lower charge states are typically formed from solutions with tetramethylammonium and tetraethylammonium

that have lower GSFE values. Loss of the larger cations that have the lowest GSFEs is facile when adducted protein ions are collisionally activated, resulting in the formation of lower analyte charge states. This reaction pathway provides a route to produce abundant singly protonated protein ions under native mass spectrometry conditions. The average protein and peptide charge with NH_4^+ is nearly the same as that with Rb^+ and K^+ , cations with similar GSFE and ionic radii. This indicates that proton transfer from NH_4^+ to proteins plays an insignificant role in the extent of protein charging in native mass spectrometry.

Keywords: Electrospray ionization, Native mass spectrometry, Protein, Peptide, Cations, Charging Mechanism, Rayleigh limit, Charge residue mechanism, Combined charge residue-field emission model

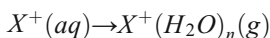
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Introduction

Electrospray ionization (ESI) is widely used to produce intact, multiply charged gas-phase macromolecular ions directly from solution for analysis by mass spectrometry (MS) [1, 2]. Multiple charging has the advantage that the mass-to-charge (m/z) ratios of the intact molecular ions are typically within the upper range of most mass spectrometers. The extent of charging depends on many factors, including solution-phase conformation [3–5], solvent surface tension [6, 7], basicities of the analyte and solvent [1, 2, 7, 8] instrumental parameters [3–5, 9], and other factors. Broad distributions of high charge states are formed from solutions in which proteins are denatured, whereas more narrow distributions of low charge states are formed from aqueous buffered solutions where proteins have native or native-like conformations. The extent of charging from either denatur-

ing or buffered aqueous solutions can be significantly increased by supercharging [6, 7, 10–19] or decreased by addition of bases either in solution [7] or in the gas phase [20, 21].

Several ESI ion formation mechanisms have been proposed to account for the observed charging, including the charge residue mechanism (CRM) [22], the ion evaporation mechanism (IEM) [23], and the combined charge residue field emission model (CCRFEM) [24], which incorporates aspects of both of the former mechanisms. Highly charged droplets that are produced by ESI can undergo solvent evaporation and Rayleigh fission to form smaller, highly charged droplets that carry away a significant fraction of the original droplet charge [25, 26]. In the IEM, gas-phase ions are produced from the ESI droplet when the electrostatic energy at the surface of the droplet overcomes the Gibbs free energy barrier for the process



where X^+ is the ion of interest and n is the number of water molecules [23, 27]. In the CRM, gas-phase ions are

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produced by solvent evaporation in the late stages of the droplet lifetime [22]. Globular protein and polymer charging is related to the Rayleigh limit charge, Z_R , of droplets roughly the same size as the analyte molecule, a result that is consistent with the CRM for ion formation under native conditions [6, 28]. In contrast, much less charge would be expected if these ions were produced by the IEM [6].

Ions, or buffers commonly used in native ESI mass spectrometry, can also affect charging of proteins and peptides, an effect related to the ionic strength as well as charge state of the ions [29–34]. Ammonium acetate is extensively used as a buffer in native ESI mass spectrometry. The extent of protein charging from aqueous ammonium acetate solution is often thought to be determined by proton transfer from NH_4^+ to the protein [2, 35–49]. It was proposed that “ NH_4^+ ions present at the surface of the shrinking water droplet, which are part of the excess charge on the droplet, are expected to attach themselves to the basic sites and ultimately lead to their protonation” [35]. However, other results indicate that cations typically adduct to acidic sites such as carboxyl groups [50, 51]. In the CCRFEM, small ions that reside at the droplet surface evaporate from the droplet at a rate determined by their solvation energy and the electric field strength at the droplet surface, whereas macromolecules are located in the interior of the droplet and ionize by the CRM [24]. Salts with low solvation energies carry away some of the droplet surface charge resulting in fewer charges available to the macromolecule [24].

Here, the effects of cations with a wide range in Gibbs solvation free energy (GSFE) values in aqueous acetate solutions on protein and peptide charge are investigated. The extent of charging trends with the GSFE, but loss of large cations with low GSFE values readily occurs from adducted molecular ions in the gas phase resulting in formation of lower charge states. This dissociation pathway results in a facile method to produce singly protonated protein ions in native mass spectrometry. These results also show that proton transfer reactions from protonated ammonia to proteins and peptides do not play a significant role in the extent of charging in native mass spectrometry.

Experimental

Data were acquired using either a Waters Q-TOF Premier hybrid mass spectrometer or a Waters Synapt G2 High Definition mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray nanoelectrospray (nano-ESI) ion source. Peptide or protein solutions (50 μM) with 25 mM acetate salts (unless otherwise noted) were prepared using Millipore Milli-Q water. The pH of all protein and peptide solutions except those in cesium acetate and rubidium acetate solutions ranges from 6.5 to 6.9. The pH of the protein and peptides in cesium and rubidium acetate solutions is 5.0, likely a result of different suppliers for these salts. Nano-ESI emitters, made by pulling borosilicate glass capillaries (1.0 mm o.d./0.78 mm i.d.; Sutter Instru-

ments, Novato, CA, USA) to a tip i.d. of approximately 1 μm with a Flaming/Brown micropipette puller (model P-87; Sutter Instruments), were filled with $\sim 5 \mu\text{L}$ of sample. A 1.0–1.5 kV potential was applied to a platinum wire (0.127 mm diameter, Sigma, St. Louis, MO, USA) that is in contact with the solution to initiate ion formation by nano-ESI. Gentle source conditions that minimized ion activation were used. For ion mobility experiments, the traveling wave ion mobility cell was operated at a He flow rate of 180 mL/min, an IMS gas (N_2) flow rate of 90 mL/min, a traveling wave velocity of 1,000 m/s, and a wave height of 40 V.

The average charge was calculated as an abundance weighted sum of all individual charge states divided by the total ion abundance for all charge states. The uncertainties correspond to the standard deviation of three replicate measurements from three separate ESI emitters. Angiotensin II, bradykinin, Substance P, bovine ubiquitin, egg white lysozyme, β -lactoglobulin, and ammonium acetate were obtained from Sigma (St. Louis, MO, USA); Substance P (free acid) from GenScript (Piscataway, NJ, USA); Substance P methyl ester from American Peptide Company (Sunnyvale, CA, USA); lithium acetate, sodium acetate, potassium acetate from Fisher Scientific (Pittsburgh, PA, USA); tetramethylammonium acetate and tetraethylammonium acetate from MP Biomedicals (Solon, OH, USA); cesium acetate and rubidium acetate from Alfa Aesar (Ward Hall, MA, USA). All chemicals were used without further purification.

Circular dichroism (CD) measurements were performed using a JASCO 810 spectropolarimeter (JASCO, Inc., Easton, MD, USA). Solutions were stirred with a Teflon stir bar in a 1.0 cm quartz cuvette at room temperature during wavelength scan analysis from 190 to 260 nm.

Results and Discussion

Effects of Cations on Protein and Peptide Charge

Ions of eight different proteins and peptides were formed by nano-ESI from aqueous solutions containing nine different acetate salt solutions to determine how cations affect the charge states that are produced. Protein and peptide ions formed from these solutions are expected to have native or native-like structures. These biopolymers have pI values between 4.8 and 12.0 [52], and the Gibbs solvation free energies of the different cations span a 475 kJ/mol range [53]. Extensive cation adduction generally occurs for all protein and peptide ions formed from these acetate salt solutions, with the most adduction occurring with sodium and potassium (Supplemental Table 1). Protonated molecular ions with no NH_4^+ adducts are produced with ammonium acetate, but with the other cations, minimal or no protonated ions are formed. For example, over 30 Na^+ adducts to the 5+ charge state of ubiquitin formed from sodium acetate solution, whereas no NH_4^+ adducts to this same charge state with ammonium acetate (Figure 1). Alkali metal acetate and alkylammonium acetate clusters are also abundant, especial-

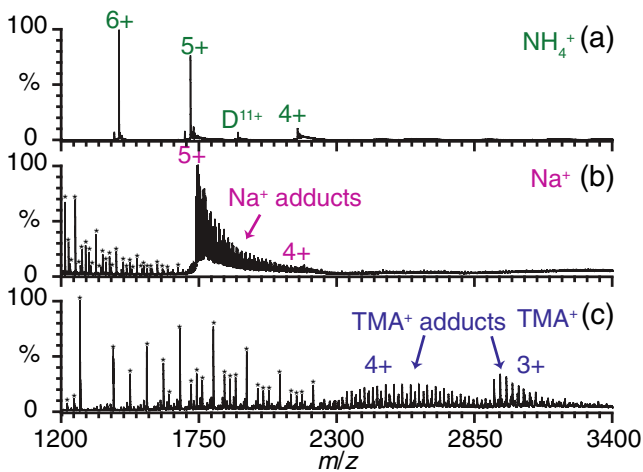


Figure 1. Nano-ESI mass spectra of 50 μM bovine ubiquitin ions formed from 25 mM aqueous solutions of (a) ammonium acetate, (b) sodium acetate, and (c) tetramethylammonium acetate; (*) indicate (b) sodium acetate or (c) tetramethylammonium acetate clusters, some of which are multiply charged

ly below $m/z \sim 1,500$ (Figure 1). Ammonium acetate clusters are not observed.

The Rayleigh limit charge of a water droplet of the same size as each ion was estimated by approximating the molecules as spheres with densities of 1 mg/mL [6, 28] ($Z_{R,m}$) and also by using gas-phase collisional cross sections (σ) [20, 54–56] of the lowest charge state reported for the molecular ions to obtain the radius approximating the ion as a sphere ($Z_{R,\sigma}$). These values, along with the maximum (Z_{max}) and average (Z_{ave}) charge of these ions formed from water without salts added and aqueous solutions with NH_4^+ or Rb^+ are given in Table 1. The charge of protein and peptide ions formed from NH_4^+ and Rb^+ acetate solutions are compared because the ionic radii of NH_4^+ and Rb^+ are similar (148 pm and 149 pm, respectively) as are their GSFEs (–285 kJ/mol and –275 kJ/mol, respectively; all values are relative to a GSFE of tetraethylammonium (TEA^+), which is arbitrarily assigned a value of zero) [53]. $Z_{R,\sigma}$ is consistently larger than $Z_{R,m}$ by an average of 27%,

and these values provide an indication of the range of uncertainties in estimating the charging predicted by the CRM. Z_{max} for protein and peptide ions formed from water is within one charge of $Z_{R,\sigma}$ except for β -lactoglobulin, where Z_{max} is much lower than $Z_{R,\sigma}$. For the biopolymer ions formed from pure water, Z_{max} is 83% of $Z_{R,\sigma}$ and 106% of $Z_{R,m}$, respectively. Lower charge states of biopolymer ions are produced from ammonium acetate and rubidium acetate solutions; Z_{max} is 75% and 69% of $Z_{R,\sigma}$ for these respective solutions, and Z_{max} is 95% and 88% of $Z_{R,m}$. These results are consistent with those of De la Mora, who reported that the Z_{max} and Z_{ave} of protein ions formed from water or ammonium acetate are within 60% to 110% of Z_R [28]. Results for dendrimers showed a correlation between ion charging in different solvents and Z_R calculated using the surface tension of the different solvents [6]. These results provide support for large globular protein ions being formed by the CRM. In contrast, ions formed by the IEM would have significantly less charge [6].

The average protein charge as a function of the relative Gibbs solvation free energy of the cations is shown in Figure 2. The dashed lines in the figures correspond to the average charge of these protein ions formed from aqueous solutions without salts added, which are higher than those from solutions containing acetate salts, although the effects of salts are minimal for β -lactoglobulin (Figure 2). The average protein ion charge decreases by $4\% \pm 1\%$ from Li^+ (GSFE is –475 kJ/mol) to Cs^+ (–250 kJ/mol), a range of 225 kJ/mol in Gibbs solvation free energy. With tetramethylammonium (TMA^+) and TEA^+ acetate, which have the lowest Gibbs solvation free energies [53], the average charge is much lower. The most significant change in protein charging occurs between Cs^+ and TMA^+ . Protein ions formed from TMA^+ and TEA^+ acetate solutions have on average 32% and 43% less charge, respectively, than those formed from Cs^+ acetate solutions. The lower charging for protein ions formed from solutions with salts could be a result of cation evaporation from the ESI droplet lowering the overall charge available for the protein as would be the case for the CCRFEM [24].

The relative ion evaporation rates for alkali metal and alkylammonium ions differ significantly and correlate with

Table 1. $Z_{R,\sigma}$ and $Z_{R,m}$ for Water Droplet the Same Size as Ion of Interest Compared with Z_{max} and Z_{ave} for Ions Formed from Aqueous Solutions Without Salts Added, or with 25 mM NH_4^+ or Rb^+

Protein/peptide	$Z_{R,\sigma}$	$Z_{R,m}$	Water, no salts added		NH_4^+		Rb^+	
			Z_{max}	Z_{ave}	Z_{max}	Z_{ave}	Z_{max}	Z_{ave}
Ubiquitin	9.5+	7.4+	10+	6.5+	6+	5.4+	5+	5.0+
Lysozyme	12+	9.4+	11+	9.4+	9+	7.7+	9+	7.5+
β -Lactoglobulin	14+	11+	10+	8.0+	9+	7.9+	8+	7.4+
Angiotensin II	3.3+	2.6+	2+	1.8+	2+	1.9+	2+	1.8+
Bradykinin	3.2+	2.6+	3+	2.0+	3+	2.0+	2+	2.0+
SubPNH ₂	3.7+	2.9+	3+	2.7+	3+	2.1+	3+	2.2+
SubPOH	3.7+	2.9+	3+	2.2+	3+	2.0+	3+	2.0+
SubPOMe	3.7+	2.9+	3+	2.9+	3+	2.1+	3+	2.2+

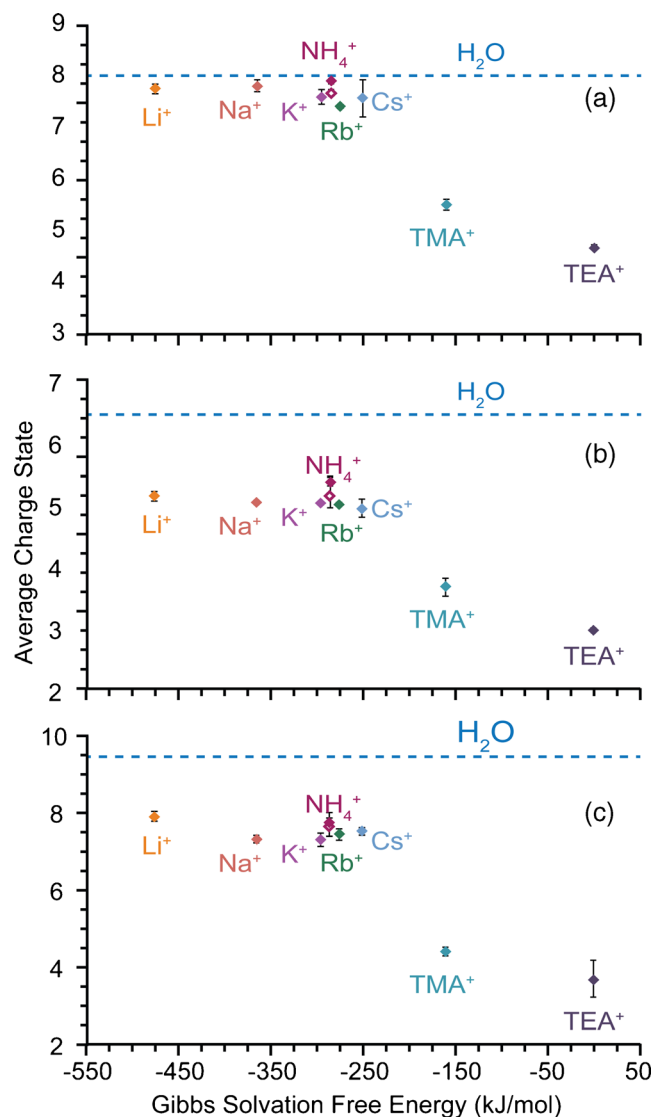


Figure 2. Average charge state of 50 μM (a) β -lactoglobulin, (b) bovine ubiquitin, and (c) egg-white lysozyme formed from 25 mM (solid diamond) or 100 mM (solid circle) acetate salt solutions and water (dashed line) plotted as a function of relative Gibbs solvation free energy of the cation (referenced to TEA^+ , which is assigned a value of zero)

the Gibbs solvation free energies (GSFEs) of the ions at low concentrations ($<10^{-5}$ M), but the difference in ion evaporation rates for the different ions is significantly less at high concentrations ($>10^{-5}$ M) and the correlation with the GSFEs of the cations is low [57–59]. The relative rates of ion evaporation for the alkali metals do not depend strongly on GSFE at high concentration, and the rate of evaporation for Li^+ is higher than that of Cs^+ [27, 58], which is the opposite of the trend reported at low salt concentrations. The lower charging of protein ions formed from Cs^+ solutions compared with that from Li^+ solutions is inconsistent with the relative ion evaporation rates reported for these alkali metal ions at high concentrations [27, 58] if the protein ions

are formed by the CCRFEM. The relative rate of ion evaporation of TEA^+ is approximately five times that of Cs^+ at high concentrations [58], which is consistent with the CCRFEM for the formation of lower protein charge from the alkylammonium acetate versus the alkali metal ion acetate solutions.

Some studies suggest that peptide ions are produced by the IEM [60–62], whereas others indicated the possibility that peptide ions are produced by the CRM [6, 62]. In contrast to proteins, the average charge for peptide ions formed from water without salts added can be lower (three different forms of Substance P), higher (angiotensin II), or similar (bradykinin) to the average charge when the alkali metal or ammonium acetate salts are added (Figure 3). The average charge of all peptide ions, except SubPNH₂, is lower when these ions are formed from TMA^+ and TEA^+ acetate solutions compared with the average charge of these ions formed from the other solutions. There is no significant dependence of the average charge of SubPNH₂ ions on the GSFE of the cations. The protein and most peptide ions investigated here follow a similar trend, in which similar charge states are formed from ammonium and alkali metal ion acetate solutions and lower charge states are formed from alkylammonium acetate solutions. The higher average charge of angiotensin II ions formed from alkali metal ion and ammonium acetate salt solutions than from pure water suggests that evaporation of small cations does not play a significant role in the charging of these ions.

Effects of Protein and Peptide Structure on Charge

The shapes of molecules in solution can significantly affect the charge of these ions in ESI. The effects of salts on the secondary structure of ubiquitin (Figure 4), β -lactoglobulin, and lysozyme (Supplemental Figures 2 and 3) were investigated using circular dichroism (CD) in the far-UV region, which probes α -helical and β -sheet content. The CD spectrum of ubiquitin in water without salts added is distinctly different from the CD spectra of ubiquitin in 25 mM ammonium acetate and 25 mM sodium acetate solutions. There is greater ellipticity at 208 nm in water, indicative of more α -helical structure [63], than when salts are present. There are more subtle differences in the CD spectra of ubiquitin in ammonium acetate and sodium acetate, indicating that the identity of the cation has only a small influence on the secondary structure of ubiquitin in these solutions. CD spectra of β -lactoglobulin and lysozyme (Supplemental Figures 2 and 3) in water without salts added and water with either ammonium acetate or sodium acetate added show similar trends. The CD signal for lysozyme in water has greater positive ellipticity between 225 and 230 nm, indicative of more α -helical structure [63] (Supplemental Figure 3) and the signal for β -lactoglobulin in water is less intense at 217 nm than the corresponding signal of β -lactoglobulin in ammonium acetate and sodium acetate.

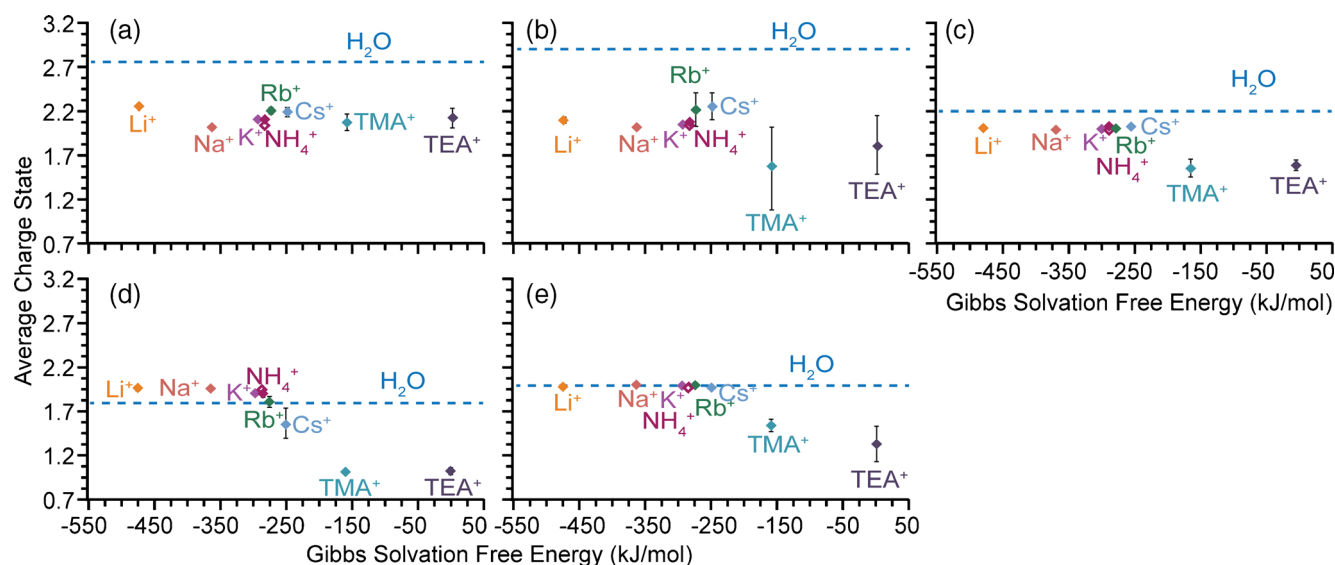


Figure 3. Average charge state of 50 μ M (a) SubPNH₂, (b) SubPOMe, (c) SubPOH, (d) angiotensin II, and (e) bradykinin formed from 25 mM (solid diamond) or 100 mM (solid diamond) acetate salt solutions and water (dashed line) plotted as a function of relative Gibbs solvation free energy of the cation (referenced to TEA⁺, which is assigned a value of zero)

These CD results show that these proteins have measurable differences in their secondary structure in water without added salts compared with when ammonium acetate or sodium acetate is in the solution and that the identity of the cation also has subtle effects on secondary structure in solution.

Ion mobility spectrometry (IMS) was also used to determine whether ubiquitin ions formed from water without salts and with ammonium acetate or sodium acetate retain different conformations in the gas phase. Arrival time distributions of $(U + 6H)^{6+}$ formed from water and from ammonium acetate and for $(U + 4Na + 2H)^{6+}$ formed from sodium acetate solutions are shown in Figure 5. The arrival time distributions of $(U + 6H)^{6+}$ formed from ammonium acetate have two broad features centered at 10 and 14 ms, whereas the arrival time distributions for the same ions formed from water has a very broad feature at 13.5 ms with

a less abundant undefined shoulder at 10 ms. The arrival time distributions of $(U + 6H)^{6+}$ formed from these two solutions show a distinct range of conformer families with the more compact conformers most abundant in ammonium acetate solution. These data clearly show that the gas-phase conformations of protonated ubiquitin 6+ formed from water and ammonium acetate solutions are different and, therefore, the solution-phase structures of these ions are most likely different as well, a result consistent with the CD measurements. The greater abundance of more compact conformers from ammonium acetate solution is also consistent with the lower charging observed from this solution.

No $(U + 6H)^{6+}$ is formed from the sodium acetate solution, but the arrival time distribution of $(U + 4Na + 2H)^{6+}$ has a

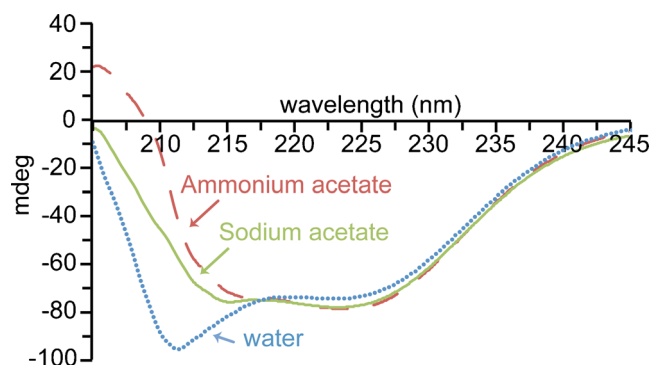


Figure 4. Circular dichroism spectra for 25 μ M ubiquitin in 25 mM sodium acetate (green line), 25 mM ammonium acetate (red dashed line), and water (blue dotted line)

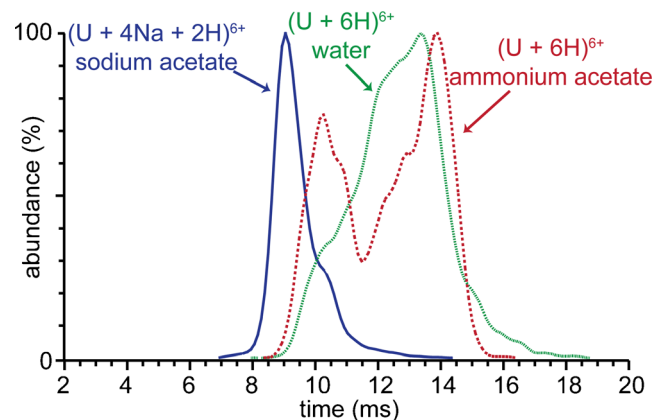


Figure 5. Ion mobility arrival time distributions of ubiquitin $(U + 6H)^{6+}$ formed from water (green dotted line), 25 mM ammonium acetate (red solid line), and $(U + 4Na + 2H)^{6+}$ formed from 25 mM sodium acetate (blue dashed line)

feature centered at 9 ms with a shoulder at 10.5 ms. This indicates that the partially sodiated ions are even more compact than the compact conformers of the fully protonated ions with the same charge state formed from water or ammonium acetate. Nonspecific cation adduction to gaseous protein ions typically results in compaction of structure compared with the protonated form, even when the ions are formed from the same solution [64]. The CD results show that the protein conformation in aqueous solutions without salts added is different than the conformation in ammonium acetate solutions. IMS results show that this conformational difference in solution translates to a difference in the conformation of gas-phase ions. The difference in solution-phase conformation of proteins in solutions without salts versus with salts added likely contributes significantly to the difference in protein charging from these solutions.

CD spectra were also obtained for angiotensin II and the three forms of SubP in water with and without salts added to determine if the salts affect the secondary structure of these peptides. The CD signal for these peptides in water without salts added is the same as that with ammonium acetate or sodium acetate added (Supplemental Figures 1 and 4), indicating that there is not a significant difference in the secondary structure of these peptides with and without salts. The C-termini of the three forms of SubP differ, and this affects the relative charging of these peptides from acetate solutions and water. This indicates that the primary structure of the peptide plays a role in the effect of cations on the extent of charging.

Effect of Internal Energy on Protein Charge

Energetic collision of multiply charged ions with gases can lower the charge states in ESI [65]. The effects of collisional activation on the average charge of ubiquitin ions formed from various acetate salt solutions and from pure water as a function of collision voltage are shown in Figure 6a. For K^+ , Rb^+ , Cs^+ , TMA^+ , and TEA^+ acetate solutions, the average charge decreases with increasing collision voltage. For example, the average charge of ubiquitin ions formed from TEA^+ acetate decreases from 2.95 ± 0.02 to 1.03 ± 0.04 , at collision voltages of 0 and 70 V, respectively. In contrast, there is a minimal decrease in the average charge of ubiquitin ions formed from water without salts added, and aqueous solutions with Li^+ , Na^+ , or NH_4^+ over the same range of collision voltages. The average charge of ubiquitin ions formed from solutions with TMA^+ and TEA^+ is lower than with the other ions at 0 V. Based on results at high collision voltage, it is likely that some collisional activation that occurs in the source, even with soft conditions, lowers the charge from that originally produced by ESI.

The decrease in average charge with increasing collision energy can be attributed to two dissociation pathways: loss of adducted cations and preferential fragmentation of the high charge ions. Loss of TEA^+ and TMA^+ from ubiquitin is facile, whereas loss of Li^+ or Na^+ does not occur prior to backbone fragmentation at higher collision energy. For exam-

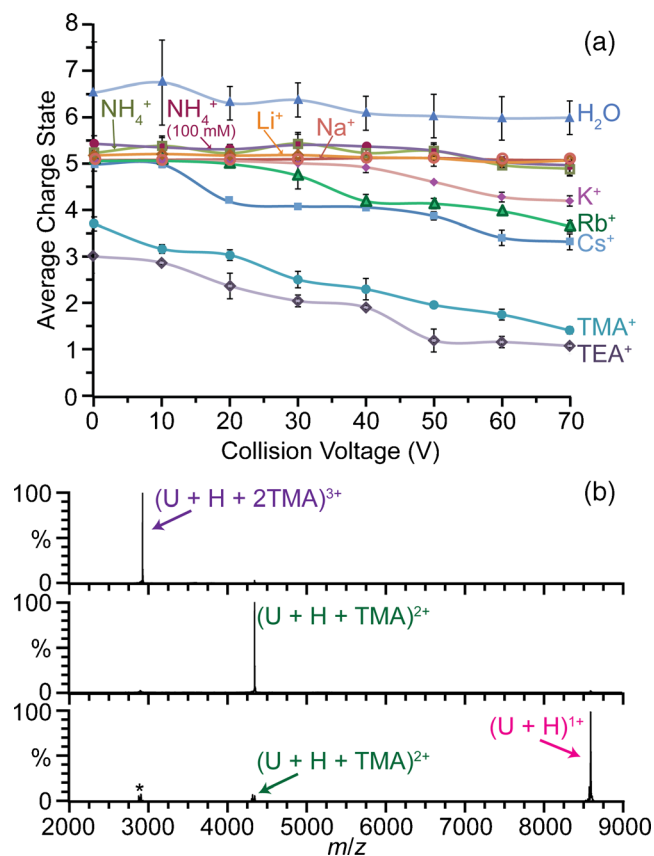


Figure 6. (a) Average charge of ubiquitin ions formed from 25 mM (unless otherwise noted) acetate salt solutions from collision voltages 0 to 70 V. (b) Collisional activation of $(U + H + 2TMA)^{3+}$ at 0 V (top) to form $(U + H + TMA)^{2+}$ at 40 V (middle) and $(U + H)^{1+}$ at 80 V (bottom); (*) indicates stable low abundance cluster ions at same m/z as precursor that are also isolated at 0 V

ple, collisional activation of $(U + 7Cs - 2H)^{5+}$ at 60 V results in the sequential loss of Cs^+ to form $(U + 6Cs - 2H)^{4+}$ and $(U + 5Cs - 2H)^{3+}$ exclusively, whereas collisional activation of $(U + 7Na - 2H)^{5+}$ at the same voltage results in b_2^+ , b_3^+ , y_3^+ , and y_4^+ fragments but no loss of Na^+ . High charge states of ubiquitin ions formed from water, ammonium acetate, sodium acetate, and lithium acetate solutions fragment to form b and y ions at collision energies of ~ 30 V and higher. In contrast, b and y ions from ubiquitin ions generated from the other acetate salt solutions start to form between 50 and 80 V. These results are consistent with the loss of the larger cations taking away internal energy and, thus, more internal energy deposition is required to produce backbone cleavage.

The binding energies of these cations to gaseous proteins and peptides are not known, but the gas phase binding energies of water molecules to these cations have been reported [66–69]. The binding energies of a water molecule to Li^+ , Na^+ , NH_4^+ , K^+ , Rb^+ , Cs^+ , TMA^+ , and TEA^+ are 142, 100, 83, 75, 67, 57, 38, and 29 kJ/mol, respectively [67–69]. This ordering is nearly the same as the GSFE values of these cations. We expect there to be a similar trend in the binding

energies of these cations to peptides and proteins. Larger cations should be bound less strongly to the peptide or protein and should be more readily lost. The higher collision voltage necessary to form *b* and *y* ions for the large cations can be attributed to the energy lost as a result of breaking the bond between the cation and protein; additional energy must be added to form *b* and *y* fragments.

Hogan et al. [24] found that the charge states of several protein ions formed by ESI from triethylammonium bicarbonate solutions are lower than the charge states of the corresponding ions formed from ammonium acetate solutions. Triethylammonium evaporates more rapidly from a droplet than ammonium [24] and could lower droplet charge and the overall charge of the protein. However, TEA⁺ adducts much less strongly to the protein ion and is easily lost as a result of gas-phase collisional activation, which also lowers the overall charge of the protein ion.

Effects of Cation Proton Transfer Reactivity on Protein and Peptide Charge

Adduction of basic molecules, such as diethylamine, in the ESI solution can result in lower charging of proteins [7, 44, 70, 71] as a result of these molecules preferentially carrying away charges as a result of proton transfer from the protein to the basic molecules. Charging of proteins and protein complexes from aqueous ammonium acetate solutions is often attributed to proton transfer from NH₄⁺ to the protein [2, 35–49]. The GSFE of NH₄⁺ (285 kJ/mol) is between that of K⁺ (295 kJ/mol) and Rb⁺ (275 kJ/mol), and the ionic radius is nearly the same as that of Rb⁺. The average charge of the protein ions formed from NH₄⁺ (25 mM solution) is higher than that of Rb⁺ and K⁺ by 5% ± 2% and 5% ± 1%, respectively. Neither Rb⁺ nor K⁺ can undergo proton transfer reactions, and this difference in protein charging for solutions containing NH₄⁺ versus Rb⁺ or K⁺ is minor. The initial pH of the rubidium acetate solutions is slightly lower than that of the ammonium and potassium solutions, but the similar results from Rb⁺ and K⁺ solutions suggest that this difference in initial solution pH has a negligible effect on the protein and peptide ion charge. Higher concentrations of ammonium acetate have little effect on protein or peptide charge. The average charge of proteins and peptides is 2% ± 1% and 1% ± 1% lower, respectively, when these ions are formed from 100 mM ammonium acetate solutions compared with 25 mM. The negligible difference in charging from solutions containing NH₄⁺ versus Rb⁺ or K⁺, and the minimal effect of NH₄⁺ concentration on protein charge indicate that proton transfer of NH₄⁺ to the protein ion is insignificant in the extent of protein charging in native mass spectrometry. However, while interaction of NH₄⁺ to deprotonated acidic sites in the protein can reduce the number of cation adducts [35, 38, 72, 73] such as sodium and proton transfer from NH₄⁺ to the acidic sites may occur, our results indicate that this does not

result in a significant change in the overall net charge on the protein ions.

Generating Singly Charged and Neutral Proteins

Low charge state ions of proteins and protein complexes are often more compact and more stable to induced conformation changes and gas phase collisional cross sections of low charge states of proteins are often similar to cross sections calculated from crystal structures [20, 74, 75]. The charge states of proteins can be reduced by several different methods, including ion-ion [76], ion-electron [77, 78] or ion-molecule reactions [21, 44]. Low charge state ions can also be readily formed by collisionally activating protein or peptide ions complexed with TMA⁺ and TEA⁺. For example, collisional activation of (U + 2TMA + H)³⁺ results in nearly exclusive formation of (U + TMA + H)²⁺ and (U + H)¹⁺ at 40 and 80 V collision energies, respectively (Figure 6b). The (U + H)¹⁺ ion can be formed from (U + TMA + H)²⁺ with a 96% yield at the latter collision voltage. Similarly, (M + H)¹⁺ of several other protein ions, including cytochrome *c*, myoglobin, and melittin can be formed from a native ESI solution using this method. Because some of the collisional energy deposited into the ion is required to break the noncovalent interaction of the cation and the protein, this method to produce singly protonated ions may not significantly perturb the structures of large proteins and protein complexes.

The number of TMA⁺ or TEA⁺ adducts is often greater than the net ion charge (Figure 1). When the number of adducts is equal to the net charge state of the ion, the protein molecule itself has a net charge of zero. Similarly, the protein molecule has a net negative charge when the number of positively charged adducts exceeds the overall net charge of the adducted protein ion. Isolating and collisionally activating these positively charged ions that are generated by ESI would lead to a negatively charged or neutral beam of protein molecules that is selected for both mass and energy.

Conclusions

The effects of different cations on the charge states of protein and peptide ions produced in electrospray ionization from aqueous acetate solutions were investigated. The charge states of three proteins formed from aqueous solutions without added acetate salts are higher than those formed from solutions with added salts, whereas the charge states of peptides can be higher, lower, or the same as those formed with added salts. Results from circular dichroism measurements show that the protein secondary structure differs in aqueous solutions without salts added compared with solutions containing either sodium or ammonium acetate. Ion mobility results show that protonated molecular ions with the same charge state formed from water and aqueous ammonium acetate solutions differ, indicating that

the solution-phase structures differ as well. These results indicate that the origin of the difference in protein charging with and without salts is an effect of differences in protein conformation in these solutions.

Collisional activation of proteins adducted to the larger cations that have low Gibbs solvation free energies produces facile loss of the cations resulting in intact protein ions with fewer charges. This dissociation pathway may account for the substantially lower protein charging from solutions containing cations such as TMA⁺ and TEA⁺. The protein ions formed from TMA⁺ and TEA⁺ solutions are less charged than those formed from ammonium or alkali metal ion solutions even at conditions where no intentional collisional activation occurs. This suggests that the CCRFEM may also contribute to the lower charging observed.

Although charging depends on the ionic strength of the aqueous solution, similar extents of protein and peptide charging containing equal concentrations of either ammonium acetate or rubidium acetate indicate that proton transfer from protonated ammonia to proteins and peptides does not play a significant role in the extent of charging in native mass spectrometry.

Formation of singly protonated protein ions from higher charge state ions that are adducted to larger cations is readily achieved by collisional activation to induce loss of the charged cation, leaving the intact singly protonated protein ion. This method can not only produce protein ions with just one charge, but should also provide a route to generating either beams of neutral protein molecules or protein anions that are selected for both mass and kinetic energy from multiply charged positive ions formed by ESI.

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