**RESEARCH ARTICLE**

Charging of Proteins in Native Mass Spectrometry

Anna C. Susa,[1] Zijie Xia,[1] Henry Y. H. Tang,[1,2] John A. Tainer,[2,3] Evan R. Williams[1]

[1]Department of Chemistry, University of California, Berkeley, CA 94720-1460, USA
[2]Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[3]Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

What limits protein ion charging in native MS?

ESI droplet → **Gas-phase protein ion**
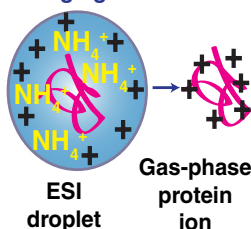
Abstract. Factors that influence the charging of protein ions formed by electrospray ionization from aqueous solutions in which proteins have native structures and function were investigated. Protein ions ranging in molecular weight from 12.3 to 79.7 kDa and pI values from 5.4 to 9.6 were formed from different solutions and reacted with volatile bases of gas-phase basicities higher than that of ammonia in the cell of a Fourier-transform ion cyclotron resonance mass spectrometer. The charge-state distribution of cytochrome c ions formed from aqueous ammonium or potassium acetate is the same. Moreover, ions formed from these two solutions do not undergo proton transfer to 2-fluoropyridine, which is 8 kcal/mol more basic than ammonia. These results provide compelling evidence that proton transfer between ammonia and protein ions does not limit protein ion charge in native electrospray ionization. Both circular dichroism and ion mobility measurements indicate that there are differences in conformations of proteins in pure water and aqueous ammonium acetate, and these differences can account for the difference in the extent of charging and proton-transfer reactivities of protein ions formed from these solutions. The extent of proton transfer of the protein ions with higher gas-phase basicity bases trends with how closely the protein ions are charged to the value predicted by the Rayleigh limit for spherical water droplets approximately the same size as the proteins. These results indicate that droplet charge limits protein ion charge in native mass spectrometry and are consistent with these ions being formed by the charged residue mechanism.

Keywords: Native mass spectrometry, ESI, Electrospray, Native ESI, Native mass spec, Native MS, Native electrospray, Electrospray ionization, Ammonium, Charging, Salts, Rayleigh limit, Charged residue mechanism, Gas-phase basicity, Apparent gas-phase basicity, Proton transfer, Combined charged residue-field emission model, Ion mobility, Circular dichroism, Mechanism, Charging mechanism, Protein ion charging

Introduction

Many factors affect the extent of charging of intact gas-phase macromolecular ions formed by electrospray ionization (ESI) [1–19], but the conformation of a molecule in solution is one of the most significant [3–5]. Broad distributions of highly charged protein ions are formed from solutions in which proteins are denatured, such as water/methanol/acid solutions. In contrast, narrower distributions of lower charge states are formed from buffered aqueous solutions in which

proteins are in native or native-like conformations [3]. Other factors, such as surface tension of the ESI droplet [6, 7], instrumental parameters [3–5, 8], supercharging methods [7, 9–13], and gaseous reactions with acid or base vapors [16–19] can also affect the extent of charging of protein ions formed by ESI. The maximum extent of charging of denatured and supercharged protein ions can be limited by the proton-transfer reactivity of the ion relative to that of the electrospray solvent or other molecules in solution [6, 18, 19].

Factors that limit the extent of charging of protein ions formed from buffered aqueous solutions by ESI are debated [15, 20–27], and several mechanisms for ion formation of protein ions from buffered aqueous solutions have been proposed [23, 28–30]. In the charged residue mechanism (CRM) for ion formation, multiply charged gas-phase ions are formed by solvent evaporation in the late stages of droplet lifetime

Correspondence to: Evan R. Williams; *e-mail:* erw@berkeley.edu

[28]. De la Mora [15] and others [7, 31, 32] showed that the extent of charging of globular macromolecular ions formed from aqueous solutions is close to the number of charges predicted by the Rayleigh limit (Z_R) for a water droplet that is approximately the same size as the macromolecule. The maximum charge of globular protein and dendrimer ions from 6 to 1400 kDa formed from aqueous solutions was between 65 and 110% of Z_R and proportional to the square root of the molecular weight, suggesting the formation of globular ions from 6 to 1400 kDa follows the CRM [15]. The maximum charge of polyethylene glycol (PEG) ions with molecular weights greater than 50 kDa exceeded Z_R, suggesting that these ions are formed from nonspherical droplets that fail to undergo Coulombic fissions because the PEG backbone is highly charged [15]. In the chain ejection model (CEM), folded native protein ions are formed by the CRM, but highly charged unfolded protein ions are ejected from the ESI droplet before complete desolvation occurs [20, 33–36].

Salts, or buffers commonly used in native ESI mass spectrometry, can also affect the extent of charging on protein ions formed from aqueous solutions [22, 37–40]. In the combined charged residue-field emission model (CCRFEM) for ion formation, small ions residing at the droplet surface evaporate from the droplet at a rate determined by the electric field strength at the droplet surface and ion solvation energies, and macromolecules located in the interior of the droplet ionize by the CRM [29]. In the CCRFEM, buffer ions with low solvation energies evaporate, carrying away charge from the ESI droplet, such that less charge remains on the macromolecules in the droplet after solvent evaporation [29]. Recently, Allen et al. [31] reported that protein ions formed from buffered aqueous ESI solutions with molecular weights less than 130 kDa were charged less than Z_R, but protein ions with molecular weights greater than 130 kDa were charged closer to Z_R. The authors suggested that this is consistent with charge-carrier emission of buffer ions limiting the charge of protein ions with molecular weights less than 130 kDa formed from buffered aqueous ESI solutions [31].

It has also been proposed that the extent of charging of proteins from native solutions is limited by proton-transfer reactivity between protein ions and commonly added salts, such as ammonium acetate or ammonium bicarbonate, which are extensively used as buffers in aqueous ESI solutions [2, 23, 26, 41–49]. Kebarle and coworkers suggested that charging of protein ions formed by ESI from aqueous ammonium salt solutions is limited by proton transfer between the protein ion and ammonium or ammonia at the surface of the ESI droplet in the final stages of solvent evaporation [23, 41, 47]. In this mechanism, NH_3 formed in the last stages of the ESI process can accept a proton from the protein resulting in less highly charged ions [41].

Adding basic molecules directly to aqueous ESI solutions can result in lower protein ion charging [49, 50] either as a result of proton-transfer reactions or competition for protons in the ionization process. Protein ions formed from aqueous solutions can be denatured by exposing the ESI droplet to gaseous acids or bases, which increase the charging as a result of the change in protein conformation [16, 17]. The proton-transfer reactivities of protein ions with volatile bases, formed by ESI from solutions in which the proteins are denatured, have been investigated experimentally [14, 18, 51–56] and modeled computationally [14, 19, 26, 41, 47, 56]. Proton-transfer reactions between protein ions and volatile basic molecules show that the apparent gas-phase basicity of high charge state ions is lower than that of low charge state ions [14, 18, 52, 56], and that proton-transfer rates between protein ions and basic molecules depend on temperature [51, 57, 58]. Conformation also affects the proton-transfer reactivity of protein ions [14, 52, 56]. The apparent gas-phase basicity of disulfide-intact lysozyme ions formed from solutions in which the protein conformation is compact is lower than that of disulfide-reduced lysozyme ions of the same charge state formed from solutions in which the protein is denatured [56].

Here, we investigate factors that limit the extent of charging of protein ions formed from buffered aqueous solutions using proton-transfer reactions with volatile molecules more basic than ammonia, circular dichroism, and ion mobility mass spectrometry. Cytochrome *c* has nearly identical secondary structure in solutions of aqueous ammonium acetate or potassium acetate, and the charge-state distributions of cytochrome *c* ions formed from these solutions are similar. Moreover, the protein ions formed from these solutions do not react with 2-fluoropyridine, which is 8 kcal/mol more basic than NH_3. These results show that charging of protein ions in native mass spectrometry is not limited by the presence of NH_4^+ or NH_3 in solution. The extent of the proton-transfer reactivities of protein ions formed from native aqueous solutions depends on conformation and how closely the ions are charged to Z_R. These results indicate that the charge on the ESI droplets limits the extent of charging of the molecular ions of proteins in native mass spectrometry.

Experimental

Proton-Transfer Reactions

Mass spectral data were acquired using a 9.4 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer that is described elsewhere [59]. Protein ions were formed from aqueous ammonium acetate, potassium acetate, or pure water solutions by nanoelectrospray ionization using borosilicate capillaries (1.0 mm o.d./0.78 mm i.d.; Sutter Instruments, Novato, CA, USA) that were pulled to a tip i.d. of 0.8 μm or 1.6 μm with a Flaming/Brown micropipette puller (Model P-87; Sutter Instruments). Tip diameters were measured with a scanning electron microscope (Hitachi TM-1000 SEM; Schaumburg, IL, USA) at the Electron Microscope Laboratory at UC Berkeley. Nanoelectrospray was initiated by applying a potential of about +0.7–1.2 kV to a 0.127 mm diameter platinum wire inserted into the capillary and in contact with the sample solution.

Volatile bases were degassed using several freeze-pump-thaw cycles and introduced into the mass spectrometer through a sapphire leak valve to a vacuum chamber pressure of 1.0×10^{-8} Torr (base pressure is $\sim 3 \times 10^{-10}$ Torr) measured using an ion gauge located remotely from the ion cell. The ion gauge was not calibrated to the pressure of the volatile basic molecules in the ion cell. Rate constants of cytochrome c ions formed from water/methanol/acetic acid and reacted with pyridine are within 35–85% of those reported by Schnier et al. [14]. Protein ions were reacted in the cell with 2-fluoropyridine (2-FP), pyridine, diethylamine (DEA), and dipropylamine (DPA) for up to 120 s.

Lyophilized protein powders of equine cytochrome c, bovine carbonic anhydrase, jack bean concanavalin A, human holo-transferrin, equine myoglobin, 2-FP, DEA, DPA, ammonium acetate, potassium acetate, and sodium acetate are from Sigma (St. Louis, MO, USA) and pyridine is from Fisher Scientific (Waltham, MA, USA). Protein solutions were prepared at 10 μM in Millipore Milli-Q water, 10 mM ammonium acetate, 10 mM potassium acetate, or 10 mM sodium acetate, except for holo-transferrin, where 200 mM ammonium acetate was used to maintain sufficient ion signal.

Ion Mobility Mass Spectrometry

Arrival time distributions and mass spectral data were acquired using a Waters Synapt G2 (Waters, Milford, MA, USA) that is located at University of California, San Francisco. The traveling wave ion mobility cell was operated with a constant wave velocity of 800 m/s, wave height of 40 V, helium flow rate of 180 mL/min, and IMS (N_2) flow rate of 90 mL/min. The time of flight mass analyzer was operated in sensitivity mode ("V"). Calculated collision cross-sections were obtained from the arrival time distributions using the procedure described by Bush et al. [60]. Cytochrome c, ubiquitin, bovine serum albumin, avidin, β-lactoglobulin, and concanavalin A were used as calibrant ions. Arrival times were assigned as the center of the full-width at half-maximum for the arrival time distribution for each ion. Average collision cross-sections, CCS_{av}, were calculated as an average weighted sum of the collision cross-sections for each charge-state distribution.

The charge-state distributions of protein ions obtained with the FT-ICR and Synapt mass spectrometers are similar, except for carbonic anhydrase ions formed from pure water. Charge states up to the 19+ and 15+ were observed with these respective instruments. The higher charging with the FT-ICR mass spectrometer is likely due to more droplet heating in this instrument, which can result in some unfolding of the protein in the droplet prior to ion formation [10].

Circular Dichroism

Circular dichroism (CD) data were acquired using a JASCO Model 815 spectropolarimeter (JASCO, Inc., Easton, MD, USA). Wavelength scans from 190 to 260 nm at 20 °C were acquired for solutions containing 10 μM cytochrome c in pure water, 10 mM ammonium acetate, potassium acetate, and sodium acetate.

Results and Discussion

Effects of Protein Conformation on Protein Ion Charging

The average charge of cytochrome c and carbonic anhydrase ions formed from pure water is higher than that from aqueous ammonium acetate (Table 1). The charge-state distribution of carbonic anhydrase ions formed from pure water is bimodal (Supplemental Figure 1), with a small distribution of higher charge state ions indicative of partially unfolded structure [3]. The conformation of proteins in aqueous solution can be affected by the presence of salts [62, 63]. To determine if these differences in charging from pure water versus aqueous ammonium salt solutions are due to salts in solution affecting the conformation of the proteins, the α-helical and β-strand content of cytochrome c and carbonic anhydrase was probed using CD. CD spectra of 10 μM cytochrome c and carbonic anhydrase in pure water, 10 mM ammonium acetate, and 10 mM potassium acetate from 200 to 260 nm are shown in Figure 1a and b. K^+ was chosen because NH_4^+ and K^+ have similar ionic radii [64], activity coefficients [65], and Gibbs solvation free energies [64]. The CD spectrum of carbonic anhydrase in pure water

Table 1. Molecular Weight, pI [61], Number of Basic Residues, Maximum Charge (Z_{max}), Average Charge (Z_{av}), Extent of Proton-Transfer with DPA, and Effective Density Values for Each Protein

	Molecular weight (kDa)	pI	Number of basic residues	Z_{max}	Z_{av}	Reaction with DPA (120 s)		Effective density (g/cm^3)
						$-\Delta Z_{max}$	$-\Delta Z_{av}$	
Cytochrome c	12.3	9.6	24	8	7.1	1.0 ± 0.0	1.1 ± 0.1	0.50
Cytochrome c (water)	12.3	9.6	24	12	8.1	3.0 ± 0.0	1.8 ± 0.1	0.44
Myoglobin	17.6	7.4	32	9	8.0	0.3 ± 0.5	0.6 ± 0.3	0.52
Carbonic anhydrase	29.1	6.8	43	12	9.8	0.7 ± 0.5	0.4 ± 0.1	0.74
Carbonic anhydrase (water)	29.1	6.8	43	19	12.9	4.3 ± 2.0	2.8 ± 0.1	0.69
Concanavalin A dimer	51.7	6.0	48	17	15.1	1.3 ± 0.5	1.5 ± 0.5	0.58
Holo-transferrin (200 mM ammonium acetate)	79.7	5.4	64	21	19.2	1.7 ± 0.5	1.4 ± 0.3	0.60

Ions Formed from 10 mM Aqueous Ammonium Acetate Solution Unless Otherwise Noted
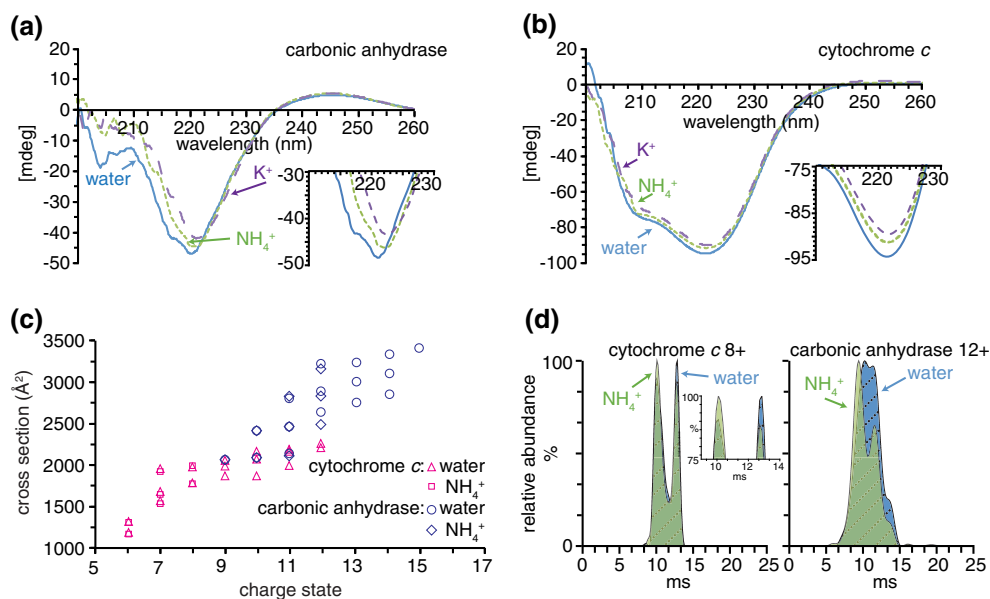
Figure 1. (**a**) CD spectra of 10 μM carbonic anhydrase, and (**b**) 10 μM cytochrome *c* in pure water (*solid blue line*), 10 mM ammonium acetate (*green dotted line*), and 10 mM potassium acetate (*purple dashed line*). (**c**) Collision cross-sections of 10 μM cytochrome *c* ions (*pink*) and carbonic anhydrase ions (*blue*) and formed from 10 mM ammonium acetate (*square, diamond*, respectively) or pure water (*triangle, circle*, respectively) as a function of charge state. (**d**) Arrival time distributions of cytochrome *c* 8+ ions and carbonic anhydrase 12+ ions formed from water (*blue*) or 10 mM ammonium acetate (*green*)

has a band at 204 nm that is absent in the CD spectra of carbonic anhydrase with ammonium or potassium acetate (Figure 1a). This band corresponds to a molar ellipticity contribution from tryptophan residues that couple with other aromatic residues [66]. This band in the spectrum of carbonic anhydrase in water but not in ammonium or potassium acetate indicates that the conformation is different in water when these two salts are not present. The CD spectra of cytochrome *c* in pure water, ammonium acetate, and potassium acetate have unresolved bands at 208 and 222 nm (Figure 1b). These bands are characteristic of a predominately α-helical protein [66]. The ellipticity at 222 nm of cytochrome *c* in pure water is slightly more negative than that of cytochrome *c* in aqueous potassium or ammonium acetate solutions. The ellipticity of a partially denatured protein can become more negative at 222 nm as a result of the loss of positive ellipticity contributions of aromatic side chains [66]. The CD spectra of carbonic anhydrase and cytochrome *c* in aqueous ammonium and potassium acetate solutions are very similar (Figure 1a, b), indicating that secondary structure of the protein in these solutions is also similar. These CD measurements indicate that the presence of salts in solution affects the secondary structure of carbonic anhydrase and to a lesser extent, cytochrome *c*, compared to that in pure water, and the secondary structure of these two proteins is similar in ammonium and potassium acetate.

The protein conformation in solution can also affect the resulting conformations of ions produced by ESI from these solutions [67–69]. Ion mobility experiments were performed to investigate if the gas-phase conformations of carbonic anhydrase and cytochrome *c* ions formed from pure water and aqueous ammonium acetate differ. The collision cross

sections of carbonic anhydrase and cytochrome *c* ions formed from pure water and aqueous 10 mM ammonium acetate as a function of charge state were obtained using traveling wave ion mobility mass spectrometry and are shown in Figure 1c. The collision cross sections for the same charge state ions formed from pure water or ammonium acetate solutions are indistinguishable within experimental error with the exception of the 12+ charge state of carbonic anhydrase. For this charge state, the cross section is slightly larger when this ion is formed from water compared to that formed from ammonium acetate (Figure 1d). The more extended conformers of the 12+ charge state of carbonic anhydrase ions formed from pure water are more abundant compared to those formed from ammonium acetate (Figure 1d).

The collisional cross sections of the 6+ through 8+ charge states of cytochrome *c* ions formed from 10 mM aqueous ammonium acetate and pure water are indistinguishable, except for the relative abundances of the the two conformers of the 8+ charge state (Figure 1d). The compact conformer of 8+ cytochrome *c* is slightly more abundant from 10 mM ammonium acetate than from pure water (Figure 1d). Higher charge states of cytochrome *c* and carbonic anhydrase ions that are formed from pure water have larger collision cross sections than the lower charge states that are formed from both ammonium acetate and pure water (Figure 1c). These ion mobility measurements show that the conformations of cytochrome *c* and carbonic anhydrase ions formed from pure water can be different than the conformation of these ions formed from 10 mM ammonium acetate. The CD measurements show that cytochrome *c* and carbonic anhydrase have different secondary structure in pure water and ammonium acetate, and this

produces a difference in the gas-phase conformations. These results show that the difference in charging of cytochrome c and carbonic anhydrase ions formed from pure water and 10 mM ammonium acetate is likely due to differences in conformations of the proteins in these solutions.
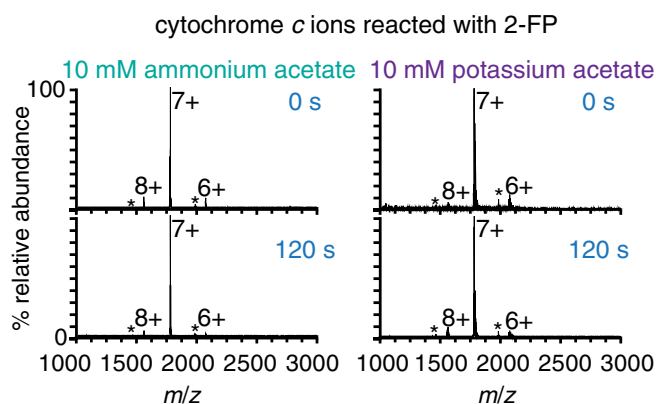
Gas-Phase Proton-Transfer Reactions Between Protein Ions and Volatile Bases

In order to determine if proton-transfer reactivity between NH_3 and proteins affects the charge of protein ions formed from aqueous ammonium acetate solutions [23, 46, 47], proton-transfer experiments for five proteins ranging in molecular weight from 12.3 to 79.7 kDa and pI values of 5.4 to 9.6 were performed with volatile bases with gas-phase basicities (GB) that are higher than that of NH_3 (196 kcal/mol). The charge-state distribution of each protein (cytochrome c, myoglobin, carbonic anhydrase, concanavalin A dimer, holo-transferrin) did not change when these ions were reacted with 2-fluoropyridine (2-FP) (GB = 204 kcal/mol) for 120 s at 1.0×10^{-8} Torr. Lower charge states of these five proteins were formed by proton transfer to pyridine, diethylamine (DEA), and dipropylamine (DPA), which have GB values of GB = 215, 221, 225 kcal/mol, respectively [70]. For example, there is no change in the charge-state distribution of concanavalin A dimer ions after reaction with 2-FP for 120 s (Figure 2). However, reaction of these ions with pyridine (120 s) results in a decrease in relative abundance of the 16+ charge state and increase in the relative abundances of the 14+ and 13+ (Figure 2). The absence of any proton transfer between the five proteins and 2-FP (204 kcal/mol) indicates that no proton-transfer reaction with NH_3 (196 kcal/mol) would occur under these same conditions. These results show that the apparent GB values for all the protein ions formed directly from aqueous ammonium acetate are more than 8 kcal/mol higher than the GB of NH_3.
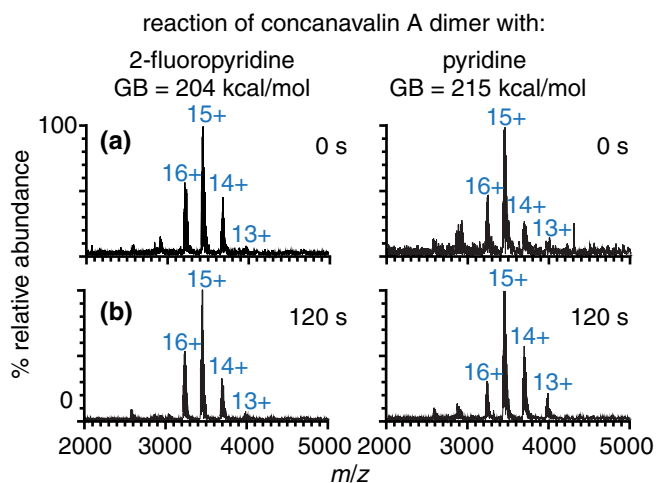
The concentration of the bases in these experiments is very low and is substantially lower than the corresponding concentration of ammonium in solution and gaseous ammonia in the ESI interface, although the long reaction times used in these experiment at least partially compensates for this difference. To evaluate if ammonium or ammonia affect protein charging from aqueous buffered ammonium acetate solutions, cytochrome c ions were formed from either 10 mM ammonium acetate or 10 mM potassium acetate. Potassium has a similar ionic radius [64], activity coefficient [65], and Gibbs solvation free energy [64] to ammonium but potassium cannot undergo proton-transfer reactions with protein ions. In addition, secondary structure of cytochrome c in ammonium and potassium acetate solutions is very similar (Figure 1a and b). The charge-state distributions of cytochrome c ions from these solutions are the same (Figure 3, top). This result provides compelling evidence that the presence of NH_4^+ or NH_3 in solution does not affect the charging of proteins in native mass spectrometry. Moreover, the charge-state distributions of ions formed from either aqueous ammonium acetate or potassium acetate solutions do not change upon reaction with 2-FP (Figure 3). This demonstrates that the apparent gas-phase basicity of proteins is greater than the gas-phase basicity of NH_3, whether or not NH_3 or NH_4^+ is present. Acetate and water can potentially proton-transfer with proteins. Different anions can affect protein charging from solutions in which proteins are denatured [71] or solutions in which proteins have native structures [72]. In these experiments, acetate and water are present in both the ammonium and potassium acetate solutions, and therefore should not affect our conclusion about NH_4^+ or NH_3 not affecting protein charge. These results clearly show that proton transfer between proteins and NH_3 does not affect the charge of ions formed from ammonium acetate solutions, and that proton transfer to ammonia does not limit the charging of proteins in native mass spectrometry.

Figure 2. Mass spectra of concanavalin A dimer ions formed from 10 mM ammonium acetate after reaction with either 2-fluoropyridine or pyridine for (**a**) 0 and (**b**) 120 s

Figure 3. Mass spectra of 10 μM cytochrome c ions formed from 10 mM ammonium acetate and 10 mM potassium acetate reacted with 2-fluoropyrine for 0 and 120 s

Charging of Protein Ions and the Rayleigh Limit

To determine if the charging of the protein ions under these conditions is close to Z_R [7, 15, 31, 32], the maximum and average charge of the protein ions, Z_{max} and Z_{av}, respectively, were compared with Z_R for water droplets of comparable sizes to the proteins. Z_R was calculated by approximating protein ions as spheres with a density of 1 g/cm^3 [7, 15, 31, 32]. For protein ions formed from aqueous ammonium acetate, Z_{av} is between 73.2 and 86.5% of Z_R, and Z_{max} is between 85.7 and 95.0% of Z_R, which is within the range of previously reported values [15, 31, 32]. However, Z_{av} and Z_{max} for protein ions formed from pure water are higher than the values for ions formed from aqueous ammonium acetate. Z_{av} for ions formed from pure water is between 93.6 and 96.1% of Z_R, and Z_{max} is between 137 and 142% of Z_R.

To determine if the difference in the conformation of the protein in pure water compared to that in aqueous ammonium acetate can account for Z_{max} greater than Z_R for protein ions formed from pure water, $Z_{R(CCS)}$ was calculated by approximating the protein ions as spheres with densities obtained from average collision cross-sections, CCS_{av}. CCS_{av} values were calculated as an average weighted sum of the collision cross-sections for each charge-state distribution from the solution from which the ions were formed. $Z_{R(CCS)}$ takes into account, in part, conformational differences of protein ions formed from pure water and aqueous ammonium acetate because protein ions with larger collision cross-sections have lower effective densities. The average effective density of protein ions formed from aqueous ammonium acetate is 0.58 g/cm^3, which is comparable to previously reported values [60]. However, the effective densities of cytochrome c and carbonic anhydrase ions formed from pure water are slightly lower than those of ions formed from aqueous ammonium acetate (Table 1).

Z_R, $Z_{R(CCS)}$, Z_{max}, and Z_{av} for protein ions formed from pure water and aqueous ammonium acetate are shown as a function of molecular weight (Supplemental Figure 2) and normalized to Z_R (Figure 4). Z_{max} and Z_{av} for protein ions formed from ammonium acetate increase with the square root of molecular weight (Supplemental Figure 2), consistent with previously reported results [15, 31, 32]. $Z_{R(CCS)}$ is greater than Z_R by an average of 41% ± 14% (Figure 4), consistent with protein ions formed from pure water that are partially elongated and not spherical. Z_{max} for protein ions formed from pure water is between 85 and 110% of $Z_{R(CCS)}$, and Z_{av} is between 56 and 75% of $Z_{R(CCS)}$. The results are consistent with the higher charging for the ions formed from pure water being a result of their less compact structures compared with those formed from ammonium acetate.

Recently, Allen et al. investigated the effects of charge carrier emission of buffer ions on the charging of protein ions formed from buffered aqueous ESI solutions by comparing Z_{av} of protein ions with Z_R [31]. The authors calculated Z_R by approximating the ions as spherical with densities of 0.6, 1.0, and 1.2 g/cm^3. For a density of 1.0 g/cm^3, Z_{av} of protein cations with molecular weights between 5.8 and 468 kDa was 60–90% of Z_R [31]. Z_{av} of protein cations with molecular weights less
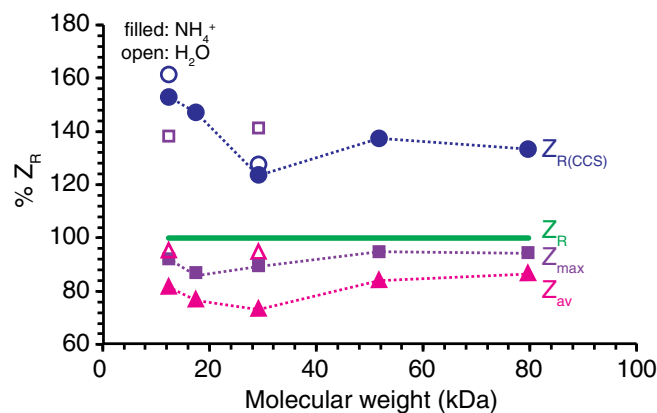

Figure 4. Z_{max} (*purple square*), Z_{av} (*pink triangles*), $Z_{R(CCS)}$ (*blue circles*) for protein ions formed from aqueous ammonium acetate (*filled*) or pure water (*open*) relative to Z_R (*green line*) as a function of molecular weight. Z_R represents the maximum number of charges on a protein predicted using the Rayleigh limit for a droplet the same size as a spherical protein with a density of 1.0 g/cm^3. $Z_{R(CCS)}$ represents the maximum number of charges predicted using the Rayleigh limit for a spherical droplet with the same radius as the CCS_{av} for the protein ions

than 130 kDa were only up to 83% of Z_R, but Z_{av} of ions greater than 130 kDa were closer to Z_R [31]. The authors suggest that this shows a molecular weight dependence of protein ions charging to Z_R that is consistent with charge-carrier emissions of buffer ions at critical field strengths limiting the charge of the smaller protein ions [31]. Here, the molecular weight range of proteins investigated is much smaller, only 12.3 to 79.7 kDa, but there is no trend between protein molecular weight and charging of protein ions to Z_R within this molecular weight range (Figure 4). Z_{max} of the protein ions formed from aqueous ammonium acetate is close to Z_R, ranging from 86 to 95% Z_R. The extent of charging is very close to that expected from the charged residue mechanism, given that factors, such as conformation, can affect charging in this simplistic assumption in determining Z_R. Our results suggest that if charge-carrier emission from aqueous ammonium acetate solutions occurs, it does not significantly affect the charge of the protein ions in native mass spectrometry.

Factors that Affect the Relative Proton-Transfer Reactivities of Protein Ions

The relative proton-transfer reactivities of the five proteins with basic molecules were investigated by comparing the change in charge-state distributions when the ions are reacted with DPA (Table 1). The relative extent of proton transfer between the five proteins investigated and DPA does not trend with the protein pI, number of basic residues, or the fraction of basic residues in the protein (Table 1, Figure 5b–e). The extent of proton transfer between protein ions and DPA is greater for ions formed from pure water than from aqueous ammonium acetate (Table 1). The proton-transfer reactivity of a protein ion is affected by both the number of charges and the ion conformation [56, 73]. The ions from pure water are more highly charged than those formed from aqueous ammonium acetate, which can increase proton-transfer reactivity but are also less
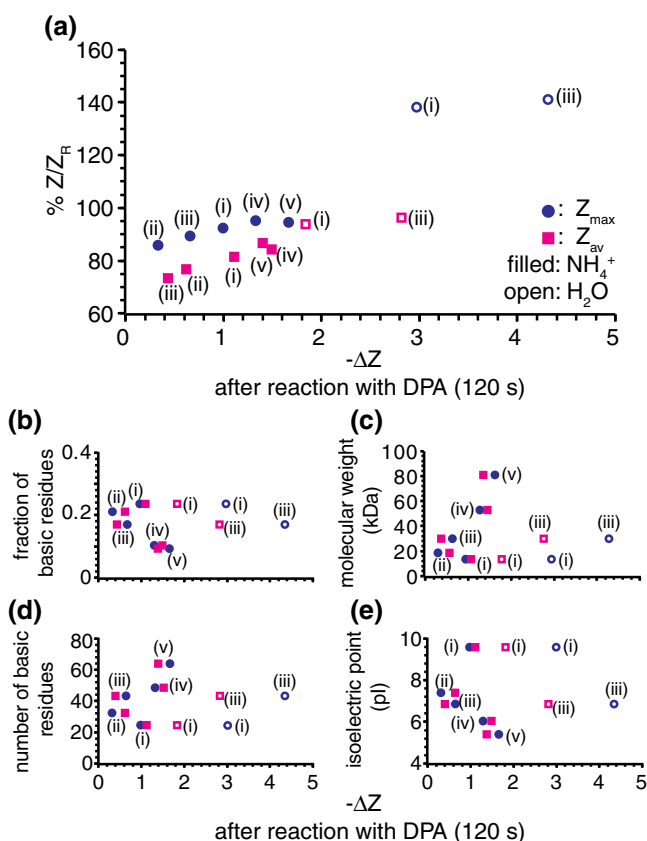
Figure 5. (a) The percent Z_{max} (circle) and Z_{av} (*square*) of Z_R, for protein ions formed from aqueous ammonium acetate (*filled markers*) or pure water (*open markers*) prior to reaction with a base as a function of the decrease in Z_{max} (*circle*) and Z_{av} (*square*) after 120 s reaction with DPA. (b) fraction of basic residues, (c) molecular weight, (d) number of basic residues, (e) isoelectric point (pI) for each protein as a function of the decrease in Z_{max} (*circle*), and Z_{av} (*square*) after 120 s reaction with DPA; (i) corresponds to cytochrome *c*, (ii) myoglobin, (iii) carbonic anhydrase, (iv) concanavalin A dimer, and (v) holo-transferrin ions, respectively

compact, which can decrease proton-transfer reactivity. Some partial collapse of the more highly charged, less compact ions may also occur in the gas phase [74], which would result in higher proton-transfer reactivity. The time scale of the ion mobility measurements and the proton-transfer reactivity experiments differ significantly, and any change in conformation in the latter may not be reflected in the ion mobility measurements.

The extent of proton transfer of protein ions with DPA trends with how closely the ions are charged to Z_R. Figure 5a shows the absolute decrease in protein ion charge, ΔZ_{max} and ΔZ_{av}, upon reaction with DPA (120 s) as a function of percent Z_{max} and Z_{av} of Z_R for the protein ions before reaction. Protein ions with the greatest Z/Z_R value undergo the most proton transfer with DPA (Figure 5a). This suggests that how closely protein ions are charged to Z_R affects the proton-transfer reactivities of the ions.

Conclusions

Factors that limit the charge of protein ions formed by ESI from aqueous solutions by ESI were investigated. The charge-state distributions of cytochrome *c* ions formed from aqueous potassium acetate or ammonium acetate are the same. Potassium has a similar Gibbs solvation free energy, activity coefficient, and ionic radius to ammonium, so any ion evaporation that occurs should be similar for both ions. However, potassium cannot undergo proton-transfer reactions with proteins. The similar charging of cytochrome *c* ions formed from potassium or ammonium acetate solutions shows that the presence of NH_4^+ or NH_3 does not affect charging in native mass spectrometry. Protein ions from these two solutions do not proton-transfer with 2-fluoropyridine, which has a gas-phase basicity that is 8 kcal/mol higher than that of ammonia, demonstrating that the apparent gas-phase basicity of protein ions formed in native mass spectrometry is greater than that of ammonia.

Protein ions formed from aqueous ammonium acetate solutions are charged closely to the values predicted by the Rayleigh limit for spherical water droplets similar in size to the proteins. The average charge of protein ions formed from aqueous ammonium acetate is between 73 and 87% of Z_R, which is within the range of previously reported values. The maximum charge of protein ions formed from aqueous ammonium acetate is between 86 and 95% of Z_R. The maximum charge of the protein ions is close to Z_R, suggesting the charge-carrier emission process in the combined charged residue-field emission model does not significantly limit the charging of these protein ions.

The extent of proton transfer between protein ions formed from aqueous solutions and molecules with much higher gas-phase basicities depends on several factors. Protein ions formed from pure water are more highly charged with less compact conformations than those formed from aqueous ammonium acetate and undergo more proton transfer with basic molecules. The extent of proton transfer between protein ions with more basic molecules is related to how closely the protein ions are charged to Z_R for spherical water droplets of comparable size to the proteins. These results are consistent with the charged residue mechanism in which ESI droplet charge limits the charge of protein ions formed from buffered aqueous solutions, when the proteins have compact folded conformations. This knowledge of the factors limiting the charging of protein ions formed from buffered aqueous solutions forms a foundation for efforts to join mass spectrometry with other solution techniques, such as high-throughput X-ray scattering, to reduce gaps between cloning library technologies and the throughput capacity of analytical platforms to characterize proteins [75].

Acknowledgments

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