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Effects of Solvent on the Maximum Charge State and Charge State Distribution of Protein Ions Produced by Electrospray Ionization

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Abstract

The effects of solvent composition on both the maximum charge states and charge state distributions of analyte ions formed by electrospray ionization were investigated using a quadrupole mass spectrometer. The charge state distributions of cytochrome *c* and myoglobin, formed from 47%/50%/3% water/solvent/acetic acid solutions, shift to lower charge (higher m/z) when the 50% solvent fraction is changed from water to methanol, to acetonitrile, to isopropanol. This is also the order of increasing gas-phase basicities of these solvents, although other physical properties of these solvents may also play a role. The effect is relatively small for these solvents, possibly due to their limited concentration inside the electrospray interface. In contrast, the addition of even small amounts of diethylamine (<0.4%) results in dramatic shifts to lower charge, presumably due to preferential proton transfer from the higher charge state ions to diethylamine. These results clearly show that the maximum charge states and charge state distributions of ions formed by electrospray ionization are influenced by solvents that are more volatile than water. Addition of even small amounts of two solvents that are less volatile than water, ethylene glycol and 2-methoxyethanol, also results in preferential deprotonation of higher charge state ions of small peptides, but these solvents actually produce an *enhancement* in the higher charge state ions for both cytochrome *c* and myoglobin. For instruments that have capabilities that improve with lower m/z , this effect could be taken advantage of to improve the performance of an analysis.

Electrospray ionization (ESI) [1] is well recognized as a soft ionization method for producing gas-phase ions of large biopolymers, such as oligonucleotides, proteins, and even noncovalent biomolecular complexes [2]. In combination with mass spectrometry, molecular masses of large molecules can be measured with unprecedented accuracy [3]. The multiple charging of large analyte ions that occurs with ESI has the advantage that the masses of very large molecules can be measured using mass spectrometers with upper mass-to-charge limits. One outstanding challenge in ESI mass spectrometry is to accurately predict the observed charge state distribution of a large molecule, given its primary structure, the composition of the solvent system from which the ions are formed, instrumental conditions, etc. Several factors have been shown to influence the observed charge state distribution, including molecular conformation [4–6], acid–base chemistry both in solution and in the gas phase [7–11], solvent composition [8], instrumental factors, etc. Several models have been proposed to qualitatively account for some of these effects [12–15].

A general conclusion from several studies is that the electrospray charge state distributions of proteins formed from denaturing solution conditions are shifted to higher charge states (lower m/z) than those formed from solutions in which the protein has significant tertiary structure. This effect is widely attributed to reduced Coulomb repulsion afforded by more elongated conformations and increased accessibility of the basic residues in the protein. For example,

Chowdhury et al. [4] measured the effects of acid denaturation of cytochrome *c* on the resulting ESI mass spectra, and found a correlation between the observed charge state distributions and the solution-phase conformations of cytochrome *c*. A similar observation was made by Le Blanc et al. [5] using heat denaturation. Loo et al. [6] found that charge state distributions correlate with the denaturing capacities of different solvents.

Although there is a strong dependence of the charge state distribution observed in ESI mass spectra on the solution-phase conformation of the molecule, there is little dependence on the solution-phase charge state [7,8,11]. Wang et al. [7] electrosprayed bradykinin and gramicidin S from basic (pH ~ 11.9) and acidic (pH ~ 2.9) solutions. For bradykinin, the calculated solution equilibrium ratio, $[M + 2H]^{2+}/[M + H]^{1+}$, changes by nearly nine orders of magnitude over this pH range, whereas this ratio in the ESI mass spectra changes by only a factor of ~6.6. Thus, there is little correlation between the charge state distribution in bulk solution and the charge state distribution observed in the ESI mass spectra for this compound. Similar results were reported by Le Blanc et al. [8] who electrosprayed gramicidin S from aqueous solutions containing 0.2 M nitrogen-containing bases. They found that the ratio, $[M + 2H]^{2+}/[M + H]^{1+}$, does not depend on the solution pH, but rather this ratio decreases with increasing proton affinity of the base. These results are consistent with the higher charge state ions undergoing preferential proton transfer to the organic base. Carbeck et al. used protein charge ladders, capillary electrophoresis, and ESI mass spectrometry to show that the charge state distribution observed in ESI mass spectra does not reflect the net charge of proteins in solution [11].

Whereas predicting the charge state distribution is a challenge, more success has been obtained at predicting the absolute maximum number of charges an ion can retain. Covey et al. [15] proposed that the maximum charge state of a peptide or protein should correspond to the number of basic sites (Arg, Lys, His, and the N-terminus). In many cases, this works quite well. However, there are also very significant deviations. For example, actin and S4 ribosomal protein both have 46 basic sites, yet the maximum observed charge states reported in the literature for these proteins are 59+ and 30+, respectively [16]. Thus, actin can retain significantly more protons than basic sites, whereas the opposite is true for S4 ribosomal protein.

Schnier et al. [16] proposed a model for quantitatively calculating the maximum charge state of a protein based on the energetics and kinetics of proton transfer from the analyte ions to solvent molecules. The apparent gas-phase basicities (GB^{app}) of basic sites in the ion are calculated based on their intrinsic gas-phase basicities (GB) and the Coulomb energy from interacting charges:

$$GB^{app} \approx GB_{Intrinsic} - \sum_{i=1}^n \frac{q^2}{(4\pi\epsilon_0)\epsilon_r r_{i,t}}$$

where q is charge, ϵ_0 is the permittivity of vacuum, ϵ_r is the effective dielectric polarizability, and $r_{i,t}$ is the distance between the basic site and the i th neighboring charge. Ions electrosprayed from denaturing solutions are modeled as elongated one-dimensional strings. The GB^{app} of each charge state is calculated from the lowest energy charge configurations found using a pseudorandom walk algorithm. These calculations are described in more detail elsewhere [16]. Using this model, Schnier et al. found a good correlation between the maximum observed charge state and the first charge state with a GB^{app} below the GB of methanol. For 13 commonly electrosprayed proteins, the calculated maximum charge states agree with the experimental values reported in the literature within an average of 6%. Excellent agreement with experimental values was also obtained for a series of arginine-containing peptides using a slightly modified model in which conformations of the peptide termini were modeled more explicitly [16].

Very recently, Ridge and co-workers [17] showed that this simple point charge model for calculating GB^{app} values also produces good agreement with experimentally measured values for three highly basic peptides. The authors noted, however, that the maximum charge state of the peptide (KAP)₁₀, when electro-sprayed from water/isopropanol solution, resulted in mass spectra with a maximum charge state of 10+, whereas the maximum based on the calculated GB^{app} and the GB of isopropanol is 8+. The authors stated that “the proton affinity of the most volatile solvent does not appear to determine the maximum charge state observed.” They went on to suggest that the maximum charge state may be more dependent on the proton affinity of the less volatile solvent.

Here, we show that the proton transfer reactivity of organic solvents in aqueous/organic/acetic acid solutions, whether more or less volatile than water, does influence both the maximum and the average charge states observed in ESI mass spectra. Although clearly observable for organic solvents with relatively low GB, this effect is not as large as one would expect based on the relative values of the calculated GB^{app} of the ions and the GB of the organic solvents that one would predict based on the ion–molecule kinetics measured under the ultrahigh vacuum and long time frame conditions of FT/ICR experiments. The charge-reducing effect is more dramatic for more basic solvents. However, other factors, such as the concentration of the solvent and total number of gas-phase collisions, also influence the extent of deprotonation that occurs.

Experimental

Experiments were performed on a quadrupole mass spectrometer with an in-house-built external electrospray source. This instrument is described elsewhere [18]. Ions are generated using nanoelectrospray [19] (flow rates between 60 and 200 nL/min). The nanoelectrospray needles are made from 1.0 mm o.d./0.78 mm i.d. borosilicate capillaries pulled to a tip with an inner diameter of ~4 μ m using a micropipette puller (Sutter Instruments, Novato, CA). The electrospray is initiated by applying a potential of ~1000 V to a Pt wire inserted into the nanoelectrospray needle to within ~1 mm of the tip. The wire and nanoelectrospray needle are held in place with a patch clamp holder (WPI Instruments, Sarasota, FL). The electrospray ions are sampled from atmospheric pressure through a 12-cm long stainless steel capillary (0.50 mm i.d.) heated to a temperature of 195 °C. This temperature is higher than that of the gas that passes through the capillary. The flow rate of gas into the capillary is 1.0 L/min.

Bath gas containing various solvents is generated by passing compressed nitrogen gas through a bubbler containing the solvent of interest. The bubbler was made from a 2.0 L filtration flask containing ~0.75 L of the solvent and stoppered with a No. 6 rubber stopper. A glass tube (6 mm o.d.) passing through a hole in the stopper is used to bubble nitrogen into the liquid solvent within the flask. Polyvinyl tubing (0.63 cm i.d.) is used to transfer gas between the compressed gas regulator, the bubbler, and the electrospray source. The gas is introduced through the side of a cylindrical plastic sleeve that completely surrounds the electrospray needle and heated metal capillary. The flow of bath gas (14 L/min) is counter to the electrospray. Measurements of pH are made using an Orion Model 9 pH electrode interfaced to an Orion Model 601A *Ionanalyzer* (Orion Research, Beverly, MA). The addition of organic solvent to an aqueous solution at a level of 50% does not change the pH significantly (<0.2 pH units for methanol) [20].

Equine cytochrome *c* (>95%) and equine myoglobin (95%–100%) were purchased from Sigma Chemical (St. Louis, MO) and used without further purification. Stock solutions of the two proteins were prepared by dissolving 4.95 and 6.78 mg of cytochrome *c* and myoglobin, respectively, in 4.0 mL of water. Aliquots of these solutions were diluted by a factor of 10 in the solvent system of interest to produce solutions with analyte concentrations ~10 μ M. All

reported solution compositions are on a v/v basis. All organic solvents, acids, and bases were obtained from Aldrich Chemical (Milwaukee, WI).

The apparent GBs of charge states of cytochrome *c* (16+ through 21+) and myoglobin (27+ through 33+) were obtained from the literature [16]. All GB values for the solvent molecules were obtained from [21].

One parameter used to describe a given charge state distribution is the average charge state ($q_{average}$). This parameter is computed as follows:

$$q_{average} = \frac{\sum_{i=1}^N q_i \cdot w_i}{\sum_{i=1}^N w_i}$$

where N is the number of observed analyte charge states in a given mass spectrum, q_i is the net charge of the i th charge state, and w_i is the signal intensity of the i th charge state.

Results and Discussion

Effects of Solvent Gas-Phase Basicity

A number of experimental parameters influence both the maximum number of charges and the charge state distributions observed in ESI mass spectra of large molecules. In an attempt to reduce the number of variables that influence the observed charge states, electrospray solutions containing organic solvents were prepared such that the aqueous fraction (GB of water is 158 kcal/mol) of the electrospray solutions was kept constant at 47%. The remaining fraction of organic solvent, methanol (GB = 173 kcal/mol), acetonitrile (GB = 179 kcal/mol), or isopropanol (GB = 182 kcal/mol), was 50%. 3% acetic acid (GB = 180 kcal/mol) was added to all samples to enhance formation of high charge states and reduce effects due to protein conformation in solution. A solution with water in place of the organic solvent was also prepared.

Electrospray spectra obtained from these solutions containing cytochrome *c* and myoglobin are shown in Figures 1 and 2, respectively. From these spectra, it is apparent that the charge state distribution shifts to lower charge (higher m/z) with increasing GB of the 50% constituent solvent. For cytochrome *c*, the average charge state shifts from 16.8 to 15.6 when the 50% fraction is changed from water to isopropanol (the standard deviation in the average charge state obtained from five repetitive measurements is 0.07). This shift for myoglobin is 21.1 to 19.7. In addition to the overall shift in charge state distributions, there is a noticeable reduction in the abundance of the maximum charge state observed in these spectra. For cytochrome *c*, the 21+ ion observed with water (1.6% abundance) is not observed with the solution containing isopropanol (below detection limits of 0.6% abundance). For myoglobin, the 28+ charge state is observed for both water- (10% abundance) and isopropanol- (1% abundance) containing solutions, but the abundance of this charge state in the latter is significantly lower.

Although there is a clear shift in the charge state distribution to lower charge with increasing GB of the constituent 50% solvent fraction, the shift in maximum charge state is not as significant as would be predicted based on just the relative values of the GB of the solvent and the calculated GB^{app}s of the charge states for these two proteins. For cytochrome *c* in water- and isopropanol-containing solutions, the calculated maximum charge state based on these relative values is 27+ and 19+, respectively. Similarly, the calculated maximum charge state of myoglobin should shift from 37+ to 27+ with these two solutions. Clearly, the magnitude

of the shift in maximum charge states is only a small fraction of that predicted based solely on the GB^{app}s of the protein ions and the GB of the 50% fraction solvent.

All of these solutions contain acetic acid, which has a higher boiling point than water (118 °C) and a higher GB (180 kcal/mol) than all of the organic solvents except isopropanol. Even though its concentration is initially low in bulk solution (3%), its concentration in electrospray droplets is enhanced due to its low vapor pressure [22]. Thus, the presence of even small amounts of acetic acid may have an impact on the observed charge state distribution due to its proton-acceptor reactivity. To test this, solutions containing 2% formic acid (GB = 170 kcal/mol) were prepared and electrosprayed (2% formic acid is approximately equimolar with 3% acetic acid). Similar charge state distributions were observed, indicating that acetic acid was not acting as a significant proton acceptor at low concentrations. Note that increasing the concentration of acetic acid (up to 90%) does result in a shift to lower charge.

Diethylamine

In contrast to the small shift in charge state distributions observed for methanol-, acetonitrile- and isopropanol-containing solutions, a much more dramatic shift is observed for solutions containing even small amounts (<0.5%) of diethylamine (DEA; GB = 220 kcal/mol; boiling point 55 °C). This effect is illustrated in Figure 3 which shows electrospray mass spectra of cytochrome *c* from 47%/50%/3% water/methanol/acetic acid solutions containing 0.01% to 0.2% DEA. The maximum observed charge state with 0.2% DEA is 17+; the calculated maximum charge state based on relative apparent GBs is 6+. At higher DEA concentrations (>0.4%), the maximum charge state shifts even lower until analyte signal diminishes below detection limits. Thus, there is a strong dependence of the observed charge state on DEA concentration. However, the charge state shift is not nearly as significant as would be expected based solely on proton transfer reactivity experiments performed on an Fourier transform/ion cyclotron resonance (FT/ICR) instrument.

In addition to acting as a proton acceptor from cytochrome *c*, diethylamine also increases the pH of the solutions from which cytochrome *c* is electrosprayed. Such a pH change can also result in a change of charge state distribution to lower charge due to changes in protein conformation with varying pH [4]. In order to determine the extent to which the charge reduction is a consequence of solution pH, two separate experiments were performed. In both, cytochrome *c* (10 μM) was electrosprayed from 47%/50%/3% water/methanol/acetic acid solutions containing between 0% to 0.4% DEA. In one experiment, the pH of one set of DEA-containing solutions was adjusted to that of the solution containing no DEA (pH ~ 2.5) by adding the requisite amount of hydrochloric acid to each solution. The pH of the other set of DEA-containing solutions was not adjusted. Thus, the corresponding solutions of the two sets had identical analyte concentrations, solvent matrixes, and DEA concentrations. However, the solutions in the first set were of constant pH, whereas the pHs of the second set varied with their DEA concentrations.

Electrospray mass spectra were obtained from each of these solutions. Figure 4 shows the maximum (Figure 4a) and average (Figure 4b) charge states of cytochrome *c* obtained from these two sets of solutions as a function of DEA concentration. Both the maximum and the average charge states decrease with increasing DEA concentration for both sets of solutions. However, the reduction is greater in magnitude for the set of unaltered pH than for the constant pH set. For the set with unaltered pH, the maximum and the average charge state decreases 43% and 41%, respectively. These values for the set with constant pH are 14% and 11%, respectively. If the charge state distribution is determined solely by the charge state of the analyte in solution, i.e., solution pH, then the set of solutions with constant pH should have the same maximum and average charge state despite the varying DEA concentrations. As also shown by others [7,8,11], this is clearly not the case.

At a given pH, the ratio of protonated to neutral DEA remains nearly constant (2.7×10^8 at pH 2.5). The absolute amount of neutral DEA increases with increasing total DEA concentration for both solution sets. Thus, there is more neutral DEA available to remove protons from cytochrome *c* ions with increasing total DEA concentration for both solution sets. It should be noted that the exact pH of the droplets at the point of ion formation is not known. The droplets are more acidic than the bulk solution, both due to oxidation of water in the electrospray needle [23] and to differing rates of evaporation of the water, organic solvent, and acetic acid [22].

To further test the effects of pH, a second series of experiments were performed. Again, two sets of solutions were prepared with the same concentrations of cytochrome *c*, water, methanol, and acetic acid as before. In one set, the DEA concentration was varied from 0% to 0.4%. In the second solution set, the pH of the solution was matched to that of the corresponding solution containing the DEA by adding ammonium hydroxide. Thus, the corresponding solutions of the two sets had the same pH, but contained species with different gas-phase basicities (GB of DEA is 220 kcal/mol, GB of ammonia is 196 kcal/mol). The maximum and the average charge states from ESI mass spectra of these solutions are shown in Figure 5. Both the maximum and the average charge states of cytochrome *c* are reduced more for solutions containing DEA than for the ammonia-containing counterparts. The maximum and the average observed charge states are reduced by 48% and 42%, respectively, as the DEA concentration increases from 0% to 0.4%. These values for ammonia-containing pH analogs are only 14% and 17%, respectively. Thus, any conformational change or physical property of the protein that is solely dependent on pH cannot be causing the extra charge-reducing effect due to DEA. The most likely explanation is that the DEA is acting as an effective proton acceptor from higher charge states of cytochrome *c*. Another possible explanation is that any protonated DEA which is desorbed independently reduces the overall charge available to the protein.

Effects of Collision Rate

The results described above are entirely consistent with the solvent with the highest GB in the electrospray solution removing protons from multiply charged protein ions resulting in lower charge states in electrospray mass spectra. This observation is not consistent with the postulation by Ridge and co-workers [17] that the maximum charge state is determined by the GB of the *least* volatile solvent. If this was the case, then there should be no effect on the maximum charge state due to the addition of the various solvents that are more volatile than water. Although there is a measurable effect, the charge state shift observed with methanol, acetonitrile, and isopropanol is not as great as would be expected based on results from kinetic studies of ion–molecule reactions done under the low pressure but long time frame experiments of the FT/ICR [24].

In addition to the energetic requirements for proton transfer to occur, there are also effects due to the number of collisions. Large multiply charged ions are not efficiently deprotonated at threshold energies due to steric effects. Thus, the total number of collisions between a protein ion and the solvent also influences the extent of deprotonation that occurs. The heated metal capillary is where most gas-phase collisions occur once ions enter the electrospray interface/quadrupole mass spectrometer. This is due to the relatively high pressures within it (atmospheric pressure at the entrance) and its length (12 cm). Ions undergo ~16,000 times more collisions in the capillary than in the region between the end of the capillary and the first skimmer, and ~14 times more collisions in the region between the end of the capillary and the first skimmer than in the region between the first and second skimmers. The number of collisions that could possibly occur prior to an ion entering the electrospray interface/mass spectrometer is significantly smaller than the number inside the heated metal capillary due to the very small separation distance between the pulled electrospray capillary and the heated metal capillary (~2 mm).

An estimate of the number of gas-phase collisions that occur between multiply charged cytochrome *c* ions and solvent molecules under the conditions of this electrospray experiment was obtained using a hard spheres approximation [25]. For ions formed by electrospray ionization from a 50/50 water/organic solution, the number of collisions that occur between cytochrome *c* 15+ ions and organic solvent molecules is ~5700, 3900, and 2200 with methanol, acetonitrile, and isopropanol, respectively. A detailed description of the parameters used in these calculations is given in the Appendix. These collision numbers are an upper limit to the actual number of collisions because we assume for the purpose of this calculation that all the electrosprayed organic solvent enters the capillary and that all cytochrome *c* ions are formed prior to entering the heated metal capillary.

Although the calculated number of collisions appears large, the actual number is almost certainly much lower because not all the solvent enters the heated metal capillary and many analyte-containing droplets are not entirely desolvated until they travel a good distance into the heated metal capillary. Thus, the actual number of collisions that occur between an analyte ion and the solvent molecules is difficult to assess. In addition, not all collisions will result in the transfer of a proton because not all protonation sites are equally reactive. Only a small fraction of the protonated sites in a multiply charged protein ion have sufficiently low values of GB^{app} to undergo proton transfer at threshold energies. For example, the reduced cross section of the 20+ charge state of cytochrome *c* is estimated to be ~5% of the Langevin cross section [16]. Thus, only 1 in 20 thermal collisions would be expected to have the required energy necessary for proton transfer on the time scale of this experiment. Steric effects will reduce this fraction even further. This, combined with the limited number of actual collisions, contributes to a relatively low probability for reactive gas-phase encounters between analyte ions and solvent molecules.

Increasing the gas-phase concentration of solvent molecules does affect the maximum charge state and charge state distributions of multiply protonated ions [10]. This effect is illustrated for cytochrome *c* in Figure 6. The ions were formed by electrospray from a 47%/50%/3% water/isopropanol/acetic acid solution under conditions where the electrospray interface was encompassed by three different bath gasses: ambient atmosphere (Figure 6a), nitrogen saturated with water vapor (Figure 6b), and nitrogen saturated with isopropanol (Figure 6c). The charge state distribution of cytochrome *c* observed with the nitrogen bath gas saturated with water is similar to that obtained with the ambient atmosphere, although there is a very slight shift to higher charge. In contrast, the charge state distribution and maximum charge state are very clearly shifted to a lower charge when the nitrogen bath gas is saturated with isopropanol. The observed maximum charge state is 16+ (the maximum calculated for cytochrome *c* with isopropanol as the organic solvent is 19+). Under these conditions, there is a ~7600 fold increase in the number of collisions that can occur between cytochrome *c* ions and isopropanol. This illustrates that factors other than just the relative apparent GB of the protein ion and solvent can influence both the maximum charge state and charge state distributions. The concentration of the solvent, which influences the number of collisions, must also play a significant role. It should be noted that the addition of gas-phase isopropanol decreases the rate of evaporation of isopropanol in the electrospray droplet, resulting in an enhancement in the isopropanol concentration in the droplet relative to conditions in which gas-phase isopropanol is not present. This would result in an increased rate of deprotonation due to solvent evaporating from solvated ions.

Solvent Evaporation Versus Gas-Phase Collisions

There are two possible ways for a solvent molecule to deprotonate an ion formed by electrospray. Gas-phase collisions between desolvated proteins ions and solvent molecules occur and can result in proton transfer [24]. The other possible way for protein ions to be

deprotonated is via the desolvation process. That is, the last several solvent molecules from a solvated ion are in close contact with the charge sites in the protein. As these molecules evaporate, they can abstract a proton [16]. Clearly, both mechanisms must occur. We are not able to differentiate between these mechanisms from these experiments alone. However, these two processes can be independently studied by examining either the gas-phase ion–molecule chemistry of isolated protein ions [24,26–28] or by storing solvated ions at ultrahigh vacuum using trapping instruments, such as the Fourier-transform mass spectrometer, to directly observe the solvent evaporation process [29,30].

In the solvent evaporation process, the most volatile solvent preferentially leaves, resulting in a solvated analyte ion in which the solvent is enhanced in the least volatile component. Ridge and co-workers [17] proposed that the maximum charge state of an ion should then be determined by the GB of the least volatile solvent. Preferential solvent evaporation does result in different concentrations of the solvent in the electrospray droplet from that in the bulk. The change in concentration of the solvents can influence both the maximum charge state and charge state distributions observed by electrospray ionization (vide supra). However, the results for diethylamine reported above show that other factors, such as the GB of the most volatile component, can also have a dramatic effect on the observed charges. The results presented here indicate that both the GB and the concentration of the solvent play significant roles in the observed charge state distribution, even for solvents that are more volatile than water.

Lower Volatility Solvents

Methanol, acetonitrile, and isopropanol are all more volatile than water. To test the effects of solvents with boiling points that are higher than that of water, acidified aqueous solutions containing either 2-methoxyethanol or ethylene glycol were prepared. The GB of 2-methoxyethanol (174 kcal/mol) is similar to that of methanol (173 kcal/mol), but the standard boiling temperatures of these compounds are 124 and 65 °C, respectively. The GB of ethylene glycol (185 kcal/mol) is slightly higher than that of isopropanol (182 kcal/mol), but the boiling points of these compounds are 197 and 82 °C, respectively.

Three small peptides, Lys₂, Lys₄, and Lys₅, were electrosprayed from 47%/50%/3% water/methanol/acetic acid solutions with and without ethylene glycol. The electrospray mass spectra of each of these peptides exhibit two charge states; 2+ and 1+ for Lys₂ and 3+ and 2+ for both Lys₄ and Lys₅. Addition of ethylene glycol results in a lowering of the higher charge state (Table 1). With the addition of 12% ethylene glycol, the ratio of $(M + 2H)^{2+}/(M + H)^{1+}$ decreases 48% for Lys₂, and the $(M + 2H)^{3+}/(M + 2H)^{2+}$ ratio decreases by 80% and 91% for Lys₄ and Lys₅, respectively. These results are consistent with the ethylene glycol removing a proton preferentially from the higher charge state ions of these small peptides. Similar results were obtained with 1,*n*-diaminoalkanes (*n* = 5, 7, 9).

In striking contrast to the results obtained for the small peptides, addition of even small amounts of either 2-methoxyethanol or ethylene glycol results in an *enhancement* of the higher charge states of cytochrome *c* as well as myoglobin. This effect is illustrated in Figures 7 and 8 for ethylene glycol (up to 12%) and Figures 9 and 10 for 2-methoxyethanol (up to 50%). For cytochrome *c*, the 21+ charge state increases in abundance from 1% to 18% with the addition of 12% ethylene glycol. Similarly, the abundance of the 29+ ion of myoglobin increases from 2% to 24% with 12% ethylene glycol added. A shift in the maximum of the charge state distribution to higher charge for both proteins is also clearly observed. For myoglobin, a bimodal distribution of charge states occurs with 12% ethylene glycol. The origin of the enhancement in the abundance of the maximum charge state for these two proteins, but not for the polylysine peptides, is unclear. The apparent dependence on analyte size suggests that some conformational changes may be influencing the results for proteins. One possible explanation for this effect is that the temperature of the electrospray droplets containing the lower vapor

pressure solvents is likely to be higher than in droplets containing high vapor pressure solvents, due to lower evaporation rates for the former. But other factors are also likely playing roles. In any case, these results (as do the results of others) show that other solvent properties, besides GB, influence the observed maximum charge state and charge state distributions in electrospray ionization of large molecules.

Conclusions

The charge state distributions of two proteins, cytochrome *c* and myoglobin, shift to lower charge (higher m/z) with increasing gas-phase basicity of the 50% solvent fraction. The abundance of the maximum charge state is also reduced significantly. However, the changes in maximum charge states are only a small fraction of what would be predicted based on ion-molecule reactions between multiply charged ions and solvent molecules measured under the low pressure and long time frames of the FT/ICR experiment. This may be due to several factors, such as the solvent concentration and the number of gas-phase collisions, which affect the extent to which proton transfer occurs. It should also be noted that physical properties other than only the gas-phase basicity vary for these different solvents and that some of these properties also play a role in the observed charge state distributions.

Addition of even small amounts of solvents that have even higher gas-phase basicities results in significant shifts in both the maximum charge state and charge state distributions to lower charge. This effect is related to the propensity of the solvent to remove protons preferentially from the higher charge state ions. This can occur either by gas-phase collisions with “naked” analyte ions or it can occur in the ion desolvation process, whereby the solvent molecule removes a proton as it evaporates. These results clearly demonstrate that solvents which are more volatile than water do influence both the maximum charge state and the charge state distributions observed in electrospray ionization.

Similar results are obtained using solvents that are less volatile (higher boiling points) than water for small peptides. A surprising result from this study is that the addition of even small amounts of two higher boiling point solvents, ethylene glycol and 2-methoxyethanol, results in an *enhancement* of the higher charge states of both cytochrome *c* and myoglobin. The origin of this effect is not clear, but the apparent dependence on analyte size suggests conformational differences may be playing a role. Because the capabilities of most mass spectrometers improves at lower m/z , addition of these solvents could be useful when formation of higher charge state ions and maximum performance is desired.

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References

1. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Science 1989;246:64–71. [PubMed: 2675315]
2. Veenstra TD. Biophys Chem 1999;79:63–79. [PubMed: 17030314]and references cited therein.
3. Kelleher NL, Senko MW, Siegel MM, McLafferty FW. J Am Soc Mass Spectrom 1997;8:380–383.
4. Chowdhury SK, Katta V, Chait BT. J Am Chem Soc 1990;112:9013–9015.
5. Le Blanc JCY, Beuchemin D, Siu KWM, Guevremont R, Berman SS. Org Mass Spectrom 1991;26:831–839.
6. Loo JA, Ogorzalek Loo RR, Udseth HR, Edmonds CG, Smith RD. Rapid Commun Mass Spectrom 1991;5:101–105. [PubMed: 1666527]

7. Wang G, Cole RB. *Org Mass Spectrom* 1994;29:419–427.
8. Le Blanc JCY, Wang J, Guevremont R, Siu KWM. *Org Mass Spectrom* 1994;29:587–593.
9. Ogorzalek Loo RR, Winger BE, Smith RD. *J Am Soc Mass Spectrom* 1994;5:1064–1071.
10. Winger BE, Light-Wahl KJ, Smith RD. *J Am Soc Mass Spectrom* 1992;3:624–630.
11. Carbeck JD, Severs JC, Gao J, Wu Q, Smith RD, Whitesides GM. *J Phys Chem B* 1998;102:10596–10601.
12. Fenn JB. *J Am Soc Mass Spectrom* 1993;4:524–535.
13. Iribarne JV, Thomson BA. *J Chem Phys* 1976;64:2287–2294.
14. Dole M, Mach LL, Hines RL, Mobley RC, Ferguson LP, Alice MB. *J Chem Phys* 1968;49:2240–2249.
15. Covey TR, Bonner RF, Shushan BI, Henion J. *J Rapid Commun Mass Spectrom* 1988;2:249–256.
16. Schnier PD, Gross DS, Williams ER. *J Am Soc Mass Spectrom* 1995;6:1086–1097.
17. Sterner JL, Johnston MV, Nicol GR, Ridge DP. *J Am Soc Mass Spectrom* 1999;10:483–491.
18. Rodriguez-Cruz SE, Klassen JS, Williams ER. *J Am Soc Mass Spectrom* 1997;8:565–568.
19. Wilm M, Mann M. *Anal Chem* 1996;68:1–8. [PubMed: 8779426]
20. Popovych, O.; Tomkins, R. P. T. *Nonaqueous Solution Chemistry*; Wiley: New York, 1981; p 224.
21. Hunter, E. P.; Lias, S. G. In *NIST Chemistry Webbook, NIST Standard Reference Database Number 69*; Mallard, W. G.; Lindstrom, P. J., Eds.; National Institute of Standards and Technology: Gaithersburg, MD, March 1998.
22. Siu KWM, Guevremont R, Le Blanc JCY, O'Brien RT, Berman SS. *Org Mass Spectrom* 1993;28:579–584.
23. VanBerkel GJ, Zhou FM, Aronson JT. *Int J Mass Spectrom Ion Processes* 1997;162:55–67.
24. Williams ER. *J Mass Spectrom* 1996;31:831–842. [PubMed: 8799309]
25. Alberty, R. A.; Silbey, R. J. *Physical Chemistry*, 1st ed.; Wiley: New York, 1992; p 605.
26. Camara E, Green MK, Penn SG, Lebrilla CB. *J Am Chem Soc* 1996;118:8751–8752.
27. Gross DS, Schnier PD, Rodriguez-Cruz SE, Fagerquist CK, Williams ER. *Proc Natl Acad Sci USA* 1996;93:3143–3148. [PubMed: 8610183]
28. Ewing NP, Cassady CJ. *J Am Soc Mass Spectrom* 1999;10:928–940. [PubMed: 10497806]
29. Rodriguez-Cruz SE, Klassen JS, Williams ER. *J Am Soc Mass Spectrom* 1999;10:958–968. [PubMed: 10497808]
30. Lee SW, Freivogel P, Schindler T, Beauchamp JL. *J Am Chem Soc* 1998;120:11758–11765.
31. *CRC Handbook of Chemistry and Physics*; Lide, D. R., Ed.; CRC: Boca Raton, FL, 1996.
32. Clemmer DE, Jarrold MF. *J Mass Spectrom* 1997;32:577–592.

Appendix

The number of analyte ion and solvent molecule collisions that occur within the capillary, within the region between the end of the capillary and the first skimmer, and within the region between the first and second skimmers are calculated using a hard spheres approximation model [25] with the following assumptions and parameters.

Analyte ions were assumed to have the same speed as gas entering the capillary. Densities of organic solvents were obtained from the literature [31]. The collisional cross section of the 15 + charge state of cytochrome *c* was obtained from Clemmer and Jarrold [32].

The following additional values were used:

Molecular weight of analyte ion:	12,300 D
Solution concentration of organic solvent component:	50% (by volume)
Electrospray flow rate:	50 nL/min
Flow rate of gas into spectrometer:	1.0 L/min
Collisional cross section of ion:	2500 Å ²
Collisional diameter of solvent component:	5 Å
Capillary diameter:	0.50 mm

Capillary length:	12 cm
Temperature within capillary:	150 °C
Pressure within capillary:	760 torr
Temperature in region between capillary and first skimmer:	100 °C
Pressure in region between capillary and first skimmer:	1 torr
Length of region between capillary and first skimmer:	0.635 cm
Temperature in region between first and second skimmers:	100 °C
Pressure in region between first and second skimmers:	6×10^{-2} torr
Distance between first and second skimmers:	0.635 cm

Figure 1. Cytochrome *c* (10^{-5} M) electrosprayed from 47%/50%/3% water/solvent/acetic acid solutions, where the “solvent” was (a) water, (b) methanol, (c) acetonitrile, or (d) isopropanol. The calculated maximum charge states are 27+, 21+, 19+, and 19+, for (a), (b), (c), and (d), respectively.

Figure 2. Myoglobin (10⁻⁵ M) electrospayed from the same solution matrixes described in the caption of Figure 1. The calculated maximum charge states are 37+, 30+, 29+, and 27+ for (a), (b), (c), and (d), respectively.

Figure 3.

Electrospray mass spectra of cytochrome *c* (10^{-5} M) in 47%/50%/3% water/methanol/acetic acid sprayed from solutions containing (a) 0.01%, (b) 0.03%, and (c) 0.2% diethylamine (GB = 220 kcal/mol).

Figure 4.

(a) Maximum and (b) average charge states of cytochrome *c* as a function of concentration of diethylamine for solutions acidified to a common pH and solutions with unaltered pHs.

Figure 5.

(a) Maximum and (b) average charge states of cytochrome *c* when electrosprayed from diethylamine- (GB = 220 kcal/mol) and ammonia- (GB = 196 kcal/mol) containing solutions. The ammonia-containing solutions contained enough ammonium hydroxide such that their pHs matched those of the corresponding diethylamine-containing solutions (pHs indicated in upper x axis).

Figure 6.

Cytochrome *c* (10^{-5} M) electrosprayed from 47%/50%/3% water/methanol/acetic acid using a bath gas of (a) ambient atmosphere, (b) nitrogen bubbled through water (GB = 158 kcal/mol), and (c) nitrogen bubbled through isopropanol (GB = 182 kcal/mol).

Figure 7. Cytochrome *c* (10^{-5} M) electrosprayed from solutions containing (a) no ethylene glycol, (b) 1% ethylene glycol, and (c) 12% ethylene glycol. The base solution was 47%/50%/3% water/methanol/acetic acid. For (b) and (c), ethylene glycol was added in place of corresponding amounts of water and methanol. All three solutions contained 3% acetic acid.

Figure 8.

Myoglobin (10⁻⁵ M) electrosprayed from solutions containing (a) no ethylene glycol, (b) 1% ethylene glycol, and (c) 12% ethylene glycol. The base solution was 47%/50%/3% water/methanol/acetic acid. For (b) and (c), ethylene glycol was added in place of corresponding amounts of methanol and water. All three solutions contained 3% acetic acid.

Figure 9.

Cytochrome *c* (10^{-5} M) electrosprayed from solutions containing (a) no 2-methoxyethanol, (b) 25% 2-methoxyethanol, and (c) 50% 2-methoxyethanol. The base solution was 47%/50%/3% water/methanol/acetic acid. For (b) and (c), 2-methoxyethanol was added in place of corresponding amounts of methanol. All three solutions contained 3% acetic acid.

Figure 10.

Myoglobin (10⁻⁵ M) electrosprayed from solutions containing (a) no 2-methoxyethanol, (b) 25% 2-methoxyethanol, and (c) 50% 2-methoxyethanol. The base solution was 47%/50%/3% water/methanol/acetic acid. For (b) and (c), 2-methoxyethanol was added in place of corresponding amounts of methanol. All three solutions contained 3% acetic acid.

Table 1

Abundance ratios of charge states observed in the electrospray mass spectra of di-, tetra-, and pentapeptides of lysine. The electrospray mass spectra of each of these three peptides exhibited two predominant charge states: the 2+ and 1+ for (Lys)₂, and the 3+ and 2+ for both (Lys)₄ and (Lys)₅. The solution matrix containing no ethylene glycol was 47% water/50% methanol/3% acetic acid. The solution matrix containing ethylene glycol was 41% water/44% methanol/12% ethylene glycol/3% acetic acid

Ethylene glycol (%)	(Lys) ₂ (M + 2H) ²⁺ /(M + H) ⁺	(Lys) ₄ (M + 3H) ³⁺ /(M + 2H) ²⁺	(Lys) ₅ (M + 3H) ³⁺ /(M + 2H) ²⁺
0.0	2.4	0.66	4.9
12.0	1.2	0.13	0.45