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Effects of Anions on the Positive Ion Electrospray Ionization Mass Spectra of Peptides and Proteins†

Urooj A. Mirza and Brian T. Chait*

The Rockefeller University, 1230 York Avenue, New York, New York 10021

Positive ion electrospray ionization mass spectra of polypeptides are usually obtained from solutions that are acidified and therefore contain relatively high concentrations of anions. The present study describes an investigation of the effects of these ubiquitous anions on the positive ion electrospray ionization mass spectra of peptides and proteins. Certain anionic species in the spray solutions were observed to cause a marked decrease in the net average charge of peptide and protein ions in the mass spectra compared to the average charge measured in the absence of these anions. This charge neutralization effect was found to depend solely on the nature of the anionic species and was independent of the source of the anion (acid or salt), with the propensity for neutralization following the order: CCl_3COO^- > CF_3COO^- > CH_3COO^- ≈ Cl^-. A mechanism for the observed charge reduction effect is proposed that involves two steps. The first step occurs in solution, where an anion pairs with a positively charged basic group on the peptide. The second step occurs during the process of desolvation or in the gas phase, where the ion pair dissociates to yield the neutral acid and the peptide with reduced charge state. The different propensities for charge neutralization of the different anionic species is presumed to reflect the avidity of the anion–peptide interaction. These findings demonstrate that any attempt to correlate the distribution of charge states observed on proteins in the gas phase (by positive ion electrospray ionization mass spectrometry) with the net charge residing on the protein in solution will require that the described anion effect be taken into account. In addition, it appears that some control over the distribution of charge states on peptides and protein ions can be exercised by an appropriate choice of anion in the electrospray solution.

Electrospray ionization is a highly effective means for producing gas-phase ions from peptides and proteins in solution.[1-3] The mass spectra of these electrosprayed ions are characterized by striking distributions of peaks, where each component peak of the distribution corresponds to a different charge state of the intact polypeptide. The shapes of these charge distributions are determined by several different factors including the equilibrium state of the protein in solution prior to electrospray,[4-13] nonequilibrium phenomena leading to the production of isolated protein ions,[14-18] and subsequent events in the gas phase.[17-22]

The net charge on a given protein in solution is determined by factors intrinsic to the protein (e.g., the number, distribution, and pK_a's of ionizable amino acid residues and the three-dimensional conformation) as well as extrinsic factors (e.g., the solvent composition, pH, ionic strength, and temperature).[23] A strong correlation has been observed between the number of basic amino acid residues present in the protein and the distribution of charge states seen in its positive electrospray ionization spectrum.[1-13] The conformation of the protein in the spray solution also has a profound effect on the charge distribution of the electrosprayed ions, with denatured proteins producing on the average considerably higher charge states than the native, more tightly folded proteins.[4-13] Nonequilibrium phenomena during and following the production of electrosprayed droplets (which may include rapid changes in the solution pH) are also likely to influence the final observed distribution of charge states.[14-18] Finally, the partially or fully desolvated gas-phase protein ions may undergo charge changing reactions,[19-21] including the transfer of protons to water molecules.[22]

Positive ion electrospray ionization mass spectra of polypeptides are usually obtained from solutions that are acidified and therefore contain relatively high concentrations of anions. The present study was initiated to investigate whether these ubiquitous anions produce effects on the observed charge

* E-mail address: chait@rockvax.rockefeller.edu. FAX: (212)-327-7547.

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distributions additional to those summarized above. In particular, we discuss the effect of a series of different anions on the charge distributions observed in the positive ion electrospray ionization mass spectra of peptides and proteins.

EXPERIMENTAL SECTION

The electrospray ionization mass spectrometer and the sample preparation procedures have been described previously.[12,24] Briefly, the sample solution was pumped through a stainless steel syringe needle using a syringe pump (Harvard Model 2400-001) and electrosprayed in ambient laboratory air. The resulting highly charged droplets and solvated ions were transported into the vacuum of a quadrupole mass spectrometer (Vestec Model 201) through a 20-cm-long, 0.5-mm-i.d. heated capillary tube. The flow rate through the spray needle was 0.5 μL/min. Electrospray was performed by applying a potential of 3–5 kV to the syringe needle with respect to the capillary tube leading into the mass spectrometer vacuum. The distance between the tip of the syringe needle and the capillary tube ranged between 4 and 5 mm. The capillary leading into the mass spectrometer vacuum was sharpened by electropolishing to focus the field lines from the spray needle in order to improve the transport of charged droplets and ions into the capillary. Ionic species exiting the capillary tube, normally still somewhat solvated, are subjected to an electrostatic field defined by the potential difference ΔV between the exit of the capillary tube and a coaxial skimmer, spaced 3.3 mm apart. The pressure in the space between the capillary tube and the skimmer is not accurately known, but is estimated to be in the range of 1–10 Torr. Because of the imposed electric field and the high pressure in this region, the ionic species undergo many energetic collisions and are collisionally activated. The electrostatic field can be readily adjusted and provides a fine control over the level of collisional activation.[25] The spectra were acquired using a commercially available data system (Tecknivent Vector II) on an IBM-compatible computer. Data collection times ranged between 2 and 3 min.

Proteins and peptides used in this study were obtained from the Sigma Chemical Co. (St. Louis, MO) and were used without further purification. The catalog numbers and molecular masses (MMs) of the proteins are bovine cytochrome *c* (C-2037; MM = 12 231 Da), bovine ubiquitin (U-6253; MM = 8565 Da), equine myoglobin (M-0630; apomyoglobin MM = 16 952 Da); peptide with sequence KR-QHPGKR (L-4772; MM = 1006.2 Da), peptide with sequence VRKRTLRRL (L-2131; MM = 1197.5 Da), and bee venom mellitin (M-2272; MM = 2847.5 Da). The peptide and protein concentrations of the electrospray solutions were in the range of 10–20 μM. The measurements of pH were made with a PHM 95 pH meter (Radiometer, Copenhagen) calibrated in aqueous solutions. No corrections were applied for the pH measurements of solutions containing methanol.

RESULTS AND DISCUSSION

During an investigation of the effect of low pH on the electrospray ionization mass spectra of bovine cytochrome *c*, we observed that the average charge of the intact gas-phase protein ions *decreased* as the pH of the spray solution was decreased from 2.2 to 1.4 (Figure 1). The spectrum obtained from a solution of aqueous acetic acid (10%) at pH 2.2 (Figure 1a) gave a charge distribution with a mean[26] of +14.9 (±0.2), in accordance with our earlier findings in which we interpreted the spectrum to arise from a denatured state of the protein.[4,27] On decreasing the pH of the protein solution from 2.2 to 1.7, by the addition of a small quantity of trifluoroacetic acid (TFA) to the aqueous acetic acid (10%) solution, the mean charge decreased from +14.9 (±0.2) to +13.7 (±0.2) (Figure

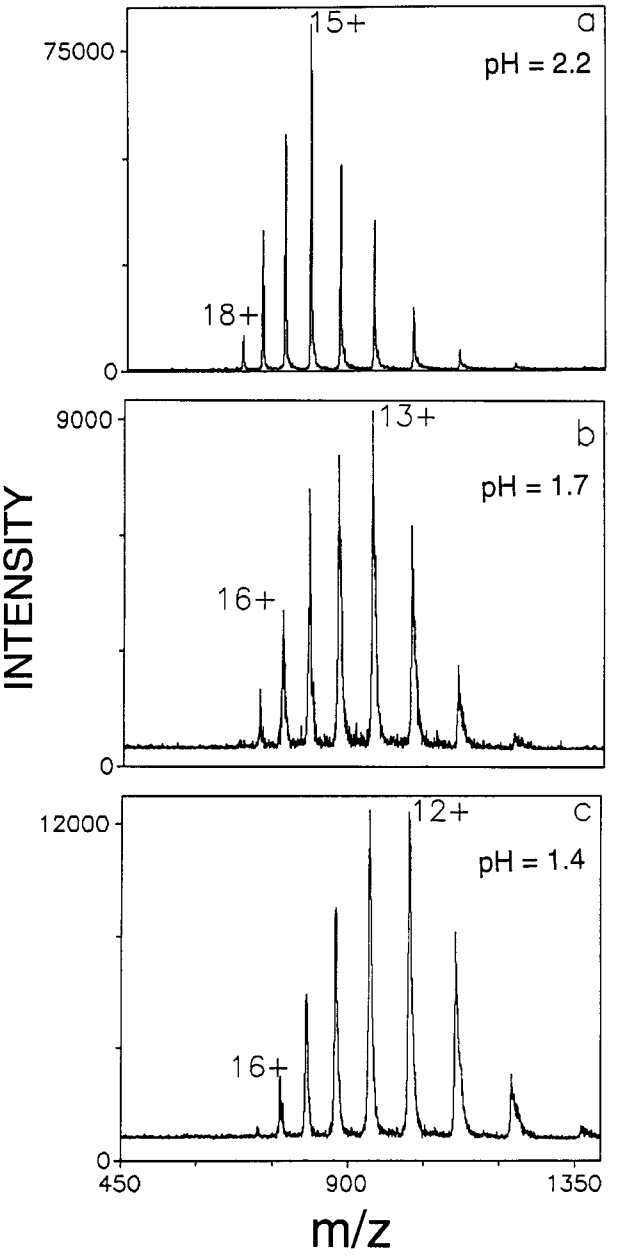

Figure 1. Positive ion electrospray ionization mass spectra of bovine cytochrome *c* obtained at three different values of the pH of the aqueous spray solution. (a) pH = 2.2 (10% acetic acid, 0% TFA); (b) pH = 1.7 (10% acetic acid, 0.3% TFA); (c) pH = 1.4 (10% acetic acid, 0.5% TFA). Protein concentration was 2 × 10^{-5} M. *n*+ designates the neutral protein with *n* attached protons.

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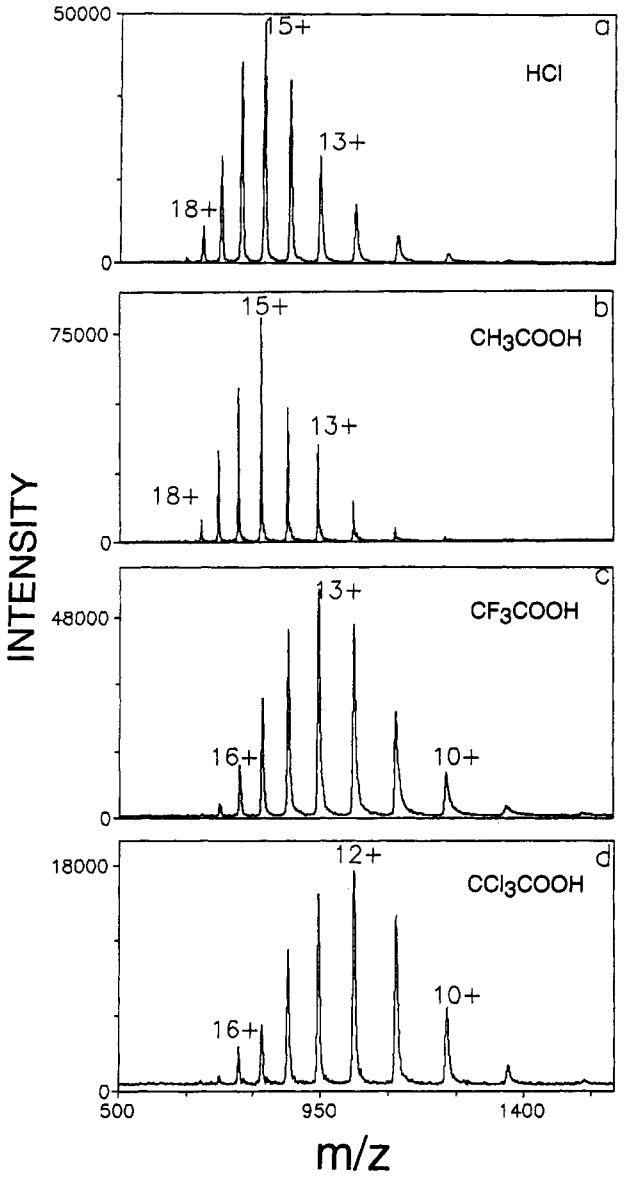

Figure 2. Positive ion electrospray ionization mass spectra of bovine cytochrome *c* obtained from aqueous/methanolic (1:1 v/v) solutions acidified to a common pH = 2.2 with (a) HCl, (b) CH_3COOH, (c) CF_3COOH, and (d) CCl_3COOH. Protein concentration was 2×10^{-5} M.

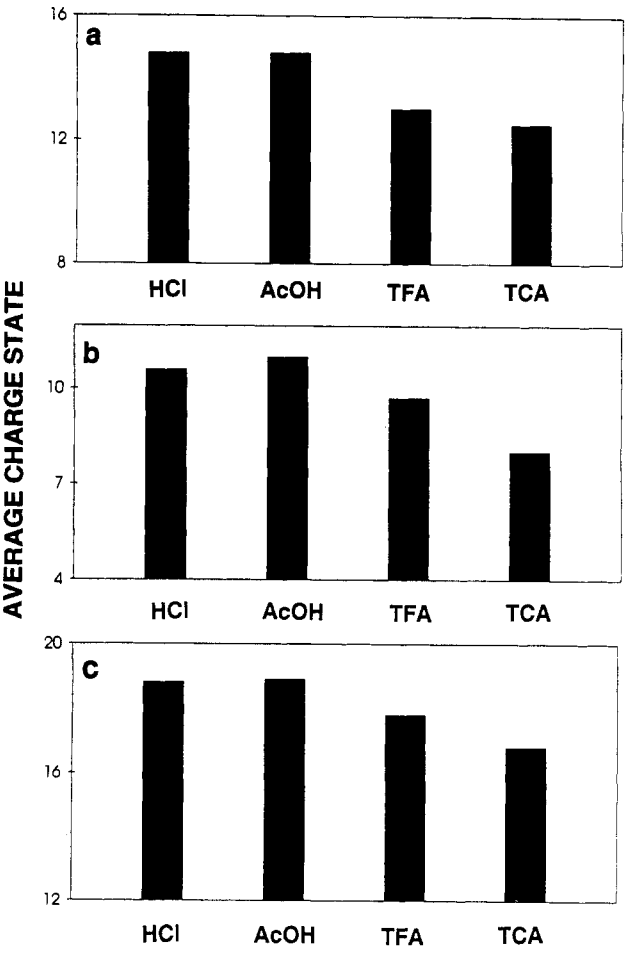

Figure 3. Bar graphs showing the average charge state observed in the positive ion electrospray ionization mass spectra of proteins as a function of the type of acid present in spray solutions maintained at pH = 2.2: (a) bovine cytochrome *c*, (b) bovine ubiquitin, (c) equine myoglobin.

1b). In addition, the mass spectrometric response also decreased and the peak widths increased. A further decrease in the pH of the protein solution to 1.4 (by further addition of TFA) brought about an additional decrease in the mean charge to a value of +12.8 (±0.2) (Figure 1c) and the virtual disappearance of the peaks resulting from the +18 and +17 charge states that were present at pH 2.2 (Figure 1a). Because a reduction in solution pH does not normally lead to a decreased probability for protonation of the ionizable amino acid side chains of the protein, the observed decrease in net average charge was not expected. We thus carried out a set of experiments designed to explore the source of the observed effect. These experiments are described below.

In comparing Figure 1, panels a–c, it should be noted that two variables have been changed simultaneously—i.e., the solution pH and the concentration of TFA (0% for Figure 1a; 0.3% for Figure 1b; and 0.5% for Figure 1c). In order to determine the relative importance of these two variables in causing the observed charge shift, we obtained electrospray ionization mass spectra of bovine cytochrome *c* with a series of four different solutions, each containing a different acid, while maintaining a constant value for the pH (and anion concentration) of these spray solutions (Figure 2). Each of these spray solutions contained 50% methanol, added to optimize the stability of the electrospray. In each case, the concentration of the acid (HCl, CH_3COOH, CF_3COOH, or CCl_3COOH) in the spray solution was adjusted to give a pH of 2.2. The mean charge on the cytochrome *c* ions measured from the different solutions were +14.8 (±0.2) (HCl), +14.8 (±0.2) (CH_3COOH), +13.0 (±0.2) (CF_3COOH), and +12.5 (±0.2) (CCl_3COOH) as summarized in bar graph form in Figure 3a. The data summarized in Figure 3a indicate that different anions have different effects on the mean charge state on the protein. The difference in mean charge on cytochrome *c* electrosprayed from the aqueous CH_3COOH solution (pH = 2.2) and the aqueous CF_3COOH solution (pH = 2.2) was 1.8 (±0.3) charge units. This value compares closely with the drop of 2.1 (±0.3) charge units observed in Figure 1, suggesting that the nature of the anion in the spray solution had a profound effect on the observed charge distributions and that the identity of the anion dominates in its effect on the mean charge over the drop in pH (over the range of 2.2–1.4 shown in Figure 1). The order of effectiveness of the various anions for producing this charge reducing effect

(see Figure 3a) is

$$CCl_3COO^- > CF_3COO^- > CH_3COO^- \approx Cl^- \qquad (I)$$

Analogous experiments performed on two other global proteins, bovine ubiquitin (Figure 3b) and equine myoglobin (Figure 3c), showed similar trends to that observed with bovine cytochrome *c*, confirming the above order of the effect of these anions.

Although the above-described experiments establish that the nature of the anions present in the spray solution influences the distribution of charge states in a predictable manner, the experiments do not establish an unambiguous mechanism for the effect. One source of ambiguity relates to possible conformational changes in proteins produced by different anions in low pH solutions. For example, Goto et al.[28–30] have shown several proteins to be maximally unfolded at pH 2.0 under conditions of low ionic strength, but upon further reduction in pH, these proteins apparently collapse into a conformational state resembling a compact "molten globule".[23] In addition, these authors demonstrated that the anions present in solution were responsible for bringing about the conformational transition and that the order of effectiveness for bringing about this transition was ferricyanide > sulfate > trichloroacetate > thiocyanate > perchlorate > iodide > nitrate > trifluoroacetate > bromide > chloride. We and others have shown previously[4–6,8–12] that conformational changes in proteins, whether produced by changes in pH, temperature, or the presence of chemical denaturants, can produce profound changes in the distribution of charge states observed from electrosprayed proteins. To exclude contributions to the present anion-dependent charge-changing phenomenon that could arise through conformational changes in the protein, we carried out a series of experiments on short peptides that are not expected to form long-lived high-order structures.

Electrospray ionization spectra were thus obtained of a short basic peptide with sequence KRQHPGKR (Figure 4) using three different acidified solutions of 50% aqueous methanol containing respectively acetic, trifluoroacetic, and trichloroacetic acid, where each solution was carefully adjusted to pH 2.2. In addition, a spectrum of the peptide was obtained from a 50% aqueous methanol solution with no added acid. Comparison of the spectra reveals that the highest degree of charging is obtained from the solution with no acid present (Figure 4a), followed by the obtained from the acetic (Figure 4b), trifluoroacetic (Figure 4c), and trichloroacetic (Figure 4d) acid solutions. The additional peaks in Figure 4c,d arise through the attachment of the acid anions to the peptide (see later). The data shown in Figure 4 (and summarized in bar graph form in Figure 5a) indicates that the changes in charge are primarily the result of changes in the major anionic species present in the different spray solutions. Analogous experiments performed with the peptide VRKRTLRRL (Figure 5b) and bee venom melittin (Figure 5c) showed similar trends, again confirming the order of the effect of anions given in eq I.

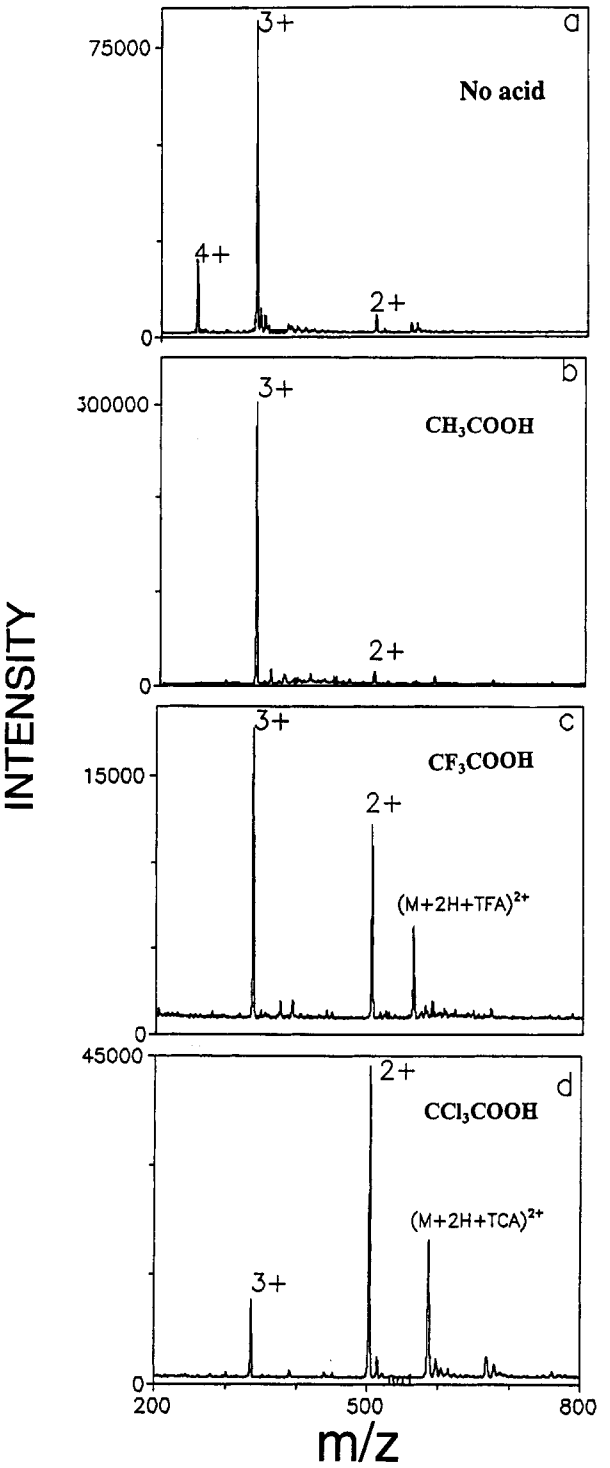

Figure 4. Positive ion electrospray ionization mass spectra of peptide KRQPHGKR obtained from spray solutions containing (a) no acid (pH = 6.0), (b) CH_3COOH (pH = 2.2), (c) CF_3COOH (pH = 2.2), and (d) CCl_3COOH (pH = 2.2). Each of these spray solutions contained 50% methanol. Peptide concentration was 2×10^{-5} M.

To further check the hypothesis that the nature of the anion present in the spray solution effects the charge state of the electrosprayed peptide ions, we again obtained mass spectra from the peptide KRQHPGKR. However, this time the spectra were obtained from three different solutions prepared by adding the sodium salts of respectively acetic, trifluoroacetic, and trichloroacetic acids to 50% aqueous methanol without the addition of acid (Figure 6). The concentrations of salt in each solution was 3 mM, and the pH was 6.3. Although the spectra contained additional peaks resulting

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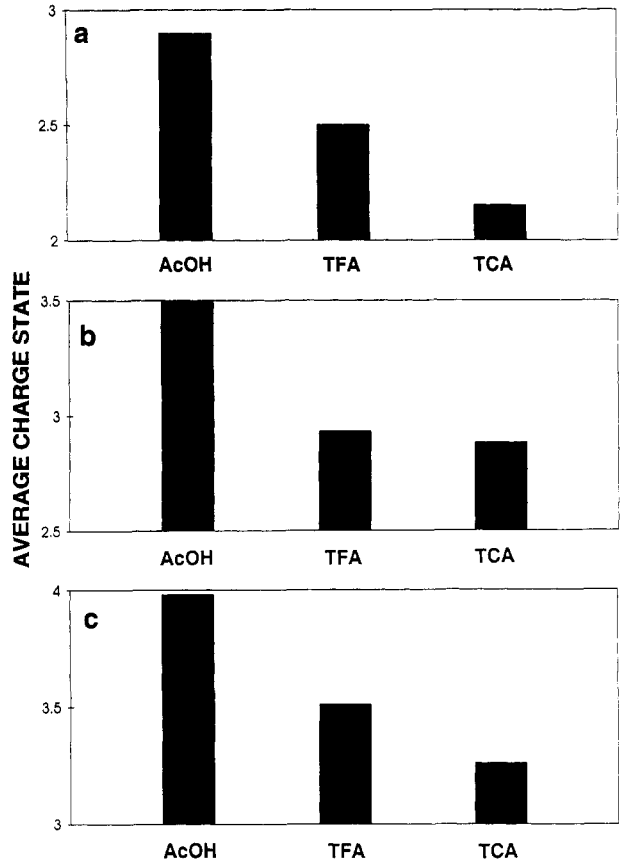

Figure 5. Bar graphs showing the average charge state observed in positive ion electrospray ionization mass spectra of peptides as a function of the type of acid present in spray solutions maintained at pH = 2.2: (a) peptide KRQPHGKR, (b) peptide VRKRTLRRL, (c) bee venom melittin.

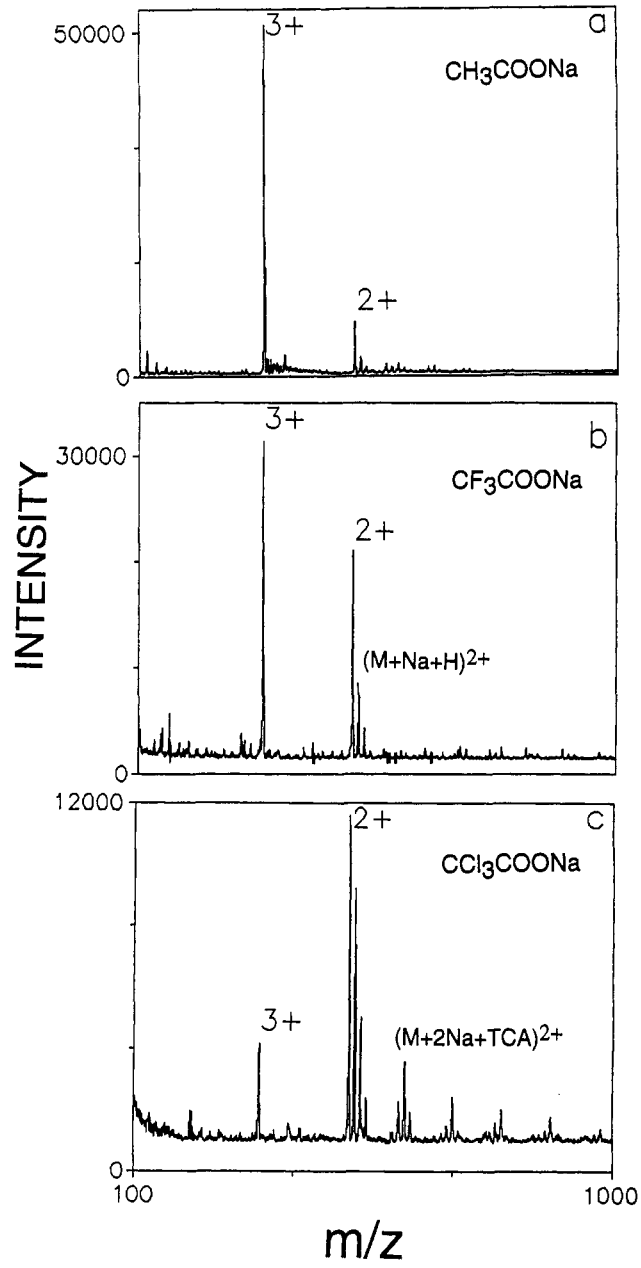

Figure 6. Positive ion electrospray ionization mass spectra of peptide KRQPHGKR obtained from spray solutions containing (a) CH_3COONa, (b) CF_3COONa, and (c) CCl_3COONa. In each case, the concentration of the salt was 3 mM and the pH was 6.3. Each of the spray solutions contained 50% methanol. Peptide concentration was 2×10^{-5} M.

from sodium adduction, the general trend in the degree of charge attachment to the peptide was very similar to that obtained with solutions containing the corresponding acids (Figure 4). Thus, the mean charge attached to the peptide ions was largest for ions electrosprayed from the solution containing no added anion (Figure 4a), followed by that obtained from solutions containing sodium acetate (Figure 6a), sodium trifluoroacetate (Figure 6b), and sodium trichloroacetate (Figure 6c), respectively. Again, attachment of the anions to the peptide was apparent, especially for the solution containing sodium trichloroacetate.

Inspection of Figures 1 and 2 reveals that the widths of the peaks arising from protonated cytochrome *c* depends on the nature of the acid present in the spray solution. The peaks observed from the protein electrosprayed from solutions containing acetic acid were narrower than those obtained from the other acidified solutions. Although we do not fully understand the origin of this peak broadening, we believe that the effect arises from the different degrees of adduction of the components of the different acid solutions to the protonated protein (see later).

In summary, the electrospray ionization mass spectra of the peptides investigated were observed to be strongly effected by the presence of certain anions in the spray solution. As with proteins, these anions brought about a marked decrease in the net average charge of the peptide ions observed in the mass spectra compared to the average charge observed in their absence. This charge reducing effect was found to depend solely on the nature of the anionic species and was independent of the source of the anion (acid or salt). The order of the anions that produce this charge neutralization effect in peptides was the same as that established above for proteins.

The results presented above lead us to propose a mechanism for the observed charge reduction effect that involves two steps. The first step occurs in solution, where an anion pairs with a positively charged basic group on the peptide or protein. The propensity for such ion pair formation follows the series shown in eq I. This series is similar to that previously noted by Goto et al.[28-30] for the tendency of anions to induce a conformational transformation of proteins from an unfolded state to a compact "molten globular" state. These authors explained the phenomenon as arising from a decrease in intramolecular charge repulsion that results from the neu-

tralization by anions of the positively charged groups on the protein. Both our present series of anions and that observed by Goto et al.[28-30] correlates with the electroselectivity series of anions toward anion-exchange resins.[31,32] The second step occurs during the process of desolvation or in the gas phase, subsequent to electrospray, where the ion pair dissociates to yield the neutral acid and the peptide with reduced charge state. This gas-phase dissociation may either occur with or without collisional activation. The process is illustrated in eq II where one protonated amino group on the peptide is shown explicitly, n is the total number of protons attached to the neutral molecule, A^- represents the anion, and HA is the neutral acid:

$$\begin{array}{l} [\text{peptide-NH}_3^+ + (n-1)\text{H}]^{n+} + \text{A}^- \\ \downarrow \\ [\text{peptide-NH}_3^+ \cdot \text{A}^- + (n-1)\text{H}]^{(n-1)+} \\ \downarrow \\ [\text{peptide-NH}_2 + (n-1)\text{H}]^{(n-1)+} + \text{HA} \end{array} \qquad \text{(II)}$$

Direct evidence for the ion pair interactions postulated above are manifested in Figures 4c, 4d, and 6c as well as in previous studies from our group.[33] Such interactions are also clearly seen in the spectrum of melittin obtained from a solution containing TFA, water, and 50% methanol (pH 2.3) (Figure 7a). This spectrum was collected under relatively gentle desolvation conditions,[24] where the temperature of the ion transport capillary was 130 °C and the potential difference ΔV between the capillary and the skimmer was 30 V. Under these gentle desolvation conditions, intense peaks are observed to arise from the adduction of the elements of one and two trifluoroacetic acid moieties to the triply charge peptide. The corresponding adducts are virtually absent from the quadruply charged peptide, presumably because adducts were either not present initially or because they were removed by the more energetic collisions experienced by the quadruply charged peptide.

Compared with Figure 7a, the spectrum shown in Figure 7b was obtained under relatively high-energy desolvation conditions (ΔV was set at 90 V; no other parameter, including the temperature of the capillary, was changed). Under these more robust desolvation conditions, the TFA adducts were removed from the triply protonated peptide and the intensity of the 3+ peptide ion peak increased relative to that of the 4+ ion peak. Similar results were obtained for adducts formed by the association of trichloroacetate anions with the peptide (data not shown). These observations provide direct evidence for the mechanism for charge neutralization proposed in eq II.

CONCLUSIONS

The positive ion electrospray ionization mass spectra of peptides and proteins were observed to be strongly effected by the presence of certain anions in the spray solution. These

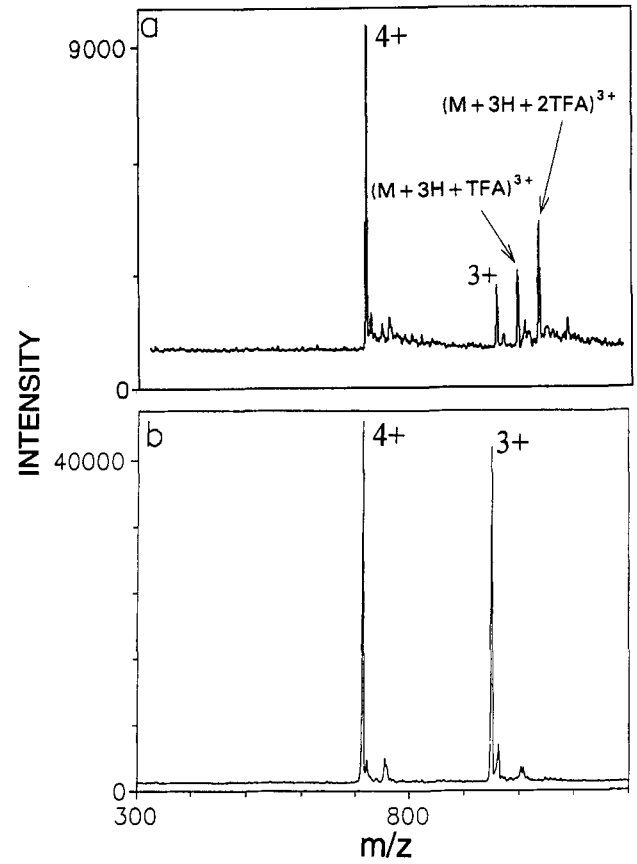

Figure 7. Positive ion electrospray ionization mass spectra of bee venom melittin obtained under two different desolvation conditions: (a) Potential difference between the transfer capillary and the skimmer, ΔV = 30 V; (b) ΔV = 90 V.

anions brought about a marked decrease in the net average charge of peptide and protein ions observed in the mass spectra compared to the average charge observed in their absence. This charge neutralization effect was found to depend solely on the nature of the anionic species and was independent of the source of the anion (acid or salt).

We propose a mechanism for the observed charge reduction effect that involves two steps. The first step occurs in solution, where an anion pairs with a positively charged basic group on the peptide. The second step occurs during the process of desolvation or in the gas phase, where the ion pair dissociates to yield the neutral acid and the peptide with reduced charge state. Different anions were observed to have different propensities for charge neutralization following the order:

$$CCl_3COO^- > CF_3COO^- > CH_3COO^- \approx Cl^-$$

This propensity for charge neutralization of the different anionic species is presumed to reflect the avidity of the anion–peptide interaction.

The present measurements do not provide information concerning the conformational transition between the unfolded state and the molten globule states of proteins. This situation is to be contrasted with our previous detection by electrospray ionization mass spectrometry of conformational transitions in solution between native and unfolded states of proteins (e.g., refs 4 and 12).

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Practical implications of the present findings are given below.

(1) The charge distribution of proteins observed in electrospray ionization mass spectra is determined by factors that include the number, distribution, and pK_a's of ionizable amino acid residues in the protein, the protein conformation, and the solution pH. Any attempt to correlate the distribution of charge states observed on proteins in the gas phase (produced by positive ion electrospray ionization mass spectrometry) with the net charge residing on the protein in solution will require that the presently described effect of anions also be taken into account.

(2) Some control over the distribution of charge states on peptide and protein ions produced by electrospray ionization can be exercised by an appropriate choice of anion in the spray solution.

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