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Anions in Electrothermal Supercharging of Proteins with Electrospray Ionization Follow a Reverse Hofmeister Series

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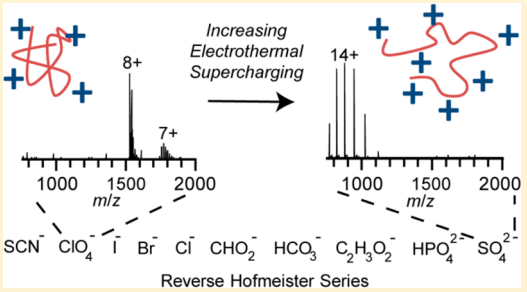
Supporting Information

ABSTRACT: The effects of different anions on the extent of electrothermal supercharging of proteins from aqueous ammonium and sodium salt solutions were investigated. Sulfate and hydrogen phosphate are the most effective anions at producing high charge state protein ions from buffered aqueous solution, whereas iodide and perchlorate are ineffective with electrothermal supercharging. The propensity for these anions to produce high charge state protein ions follows the following trend: sulfate > hydrogen phosphate > thiocyanate > bicarbonate > chloride > formate ≈ bromide > acetate > iodide > perchlorate. This trend correlates with the reverse Hofmeister series over a wide range of salt concentrations (1 mM to 2 M) and with several physical properties, including solvent surface tension, anion viscosity B-coefficient, and anion surface/bulk partitioning coefficient, all of which are related to the Hofmeister series. The effectiveness of electrothermal supercharging does not depend on bubble formation, either from thermal degradation of the buffer or from coalescence of dissolved gas. These results provide evidence that the effect of different ions in the formation of high charge state ions by electrothermal supercharging is largely a result of Hofmeister effects on protein stability leading to protein unfolding in the heated ESI droplet.



Electrospray ionization (ESI) mass spectrometry (MS) is an important tool in protein chemistry and structural biology, where it is commonly used to determine protein expression levels, to identify post-translational or induced chemical modifications,[1−4] and to investigate higher order protein and protein complex structure using a variety of techniques, such as hydrogen−deuterium exchange (HDX)[2,5−10] or photochemical oxidative labeling.[5,11−13] In native MS,[14,15] protein ions are formed from buffered aqueous solutions that typically contain ammonium acetate or ammonium bicarbonate under conditions in which the protein has a native or native-like conformation and activity. Gaseous ions formed from these solutions tend to have low charge and compact structures. Analysis of these ions can provide valuable information about protein complex stoichiometry,[16−18] protein−ligand binding,[19−21] and specific changes to protein or protein complex structure in solution.[22] In contrast, high charge state protein ions are typically formed from solutions containing organic solvents and/or acid in which the protein is unfolded. High charge state ions are advantageous because they dissociate more efficiently to form structurally useful fragments[23−29] and can be detected more efficiently with charge sensitive detectors, such as those in Fourier transform ion cyclotron resonance (FTICR) and orbitrap mass spectrometers. Higher charge state ions typically have fewer adducts, such as sodium and phosphate, which preferentially adduct to low charge state ions.[30−32] Unresolved adducts on high mass protein or protein complexes can considerably broaden mass spectral peaks, resulting in decreased sensitivity and reduced mass measuring accuracy.[33,34]

High charge states can be formed from buffered aqueous solutions in which the protein is in a native-like conformation with supercharging reagents, such as *m*-NBA or sulfolane.[8,35−45] At low concentrations in the initial solution, supercharging reagents do not measurably affect the structure of the protein, but their concentration in the ESI droplet increases as droplet evaporation occurs, and at high concentrations, these reagents chemically and/or thermally denature proteins in the droplet, resulting in the formation of high charge state protein ions.[8,37−41] Other effects, such as droplet surface tension, also play a role in supercharging from both native[37,46] and denaturing[46−48] solutions. Supercharging reagents are effective at increasing the charge of intact noncovalent protein−protein and protein−ligand complexes[35,38−41] but can also induce dissociation of complexes in the ESI droplet.[36,49]

With a newly introduced electrothermal supercharging (ETS) method, ESI mass spectra can be rapidly and reversibly switched between native and denaturing modes simply by changing the electrospray potential.[50−52] With electrothermal supercharging, protein ions are produced by ESI from aqueous buffers, typically ammonium bicarbonate (pH ∼7−8), using relatively energetic source conditions. At low spray potentials (∼0.8 kV), low charge-state distributions characteristic of native MS are produced, but at high spray potentials (∼1.3 kV), bimodal distributions of charge states dominated by a high-

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charge distribution are typically produced, where the maximum charge is similar to or greater than that formed from denaturing solutions.[51] From the high-charge ions generated during electrothermal supercharging, it is possible to obtain sequence information in top-down tandem MS experiments that is nearly identical to that obtained from high charge state ions produced from denaturing solutions. Electron transfer dissociation of cytochrome *c* 16+ ions produced from denaturing solution and by ETS from native solution results in the same sequence coverage, although there are some differences in cleavage locations and fragment ion intensities, indicating that there are subtle differences in the gas-phase conformations of the ions formed by both methods.[51]

High charge state ions in electrothermal supercharging appear to be produced as a result of protein unfolding in the ESI droplet, which is heated by the more energetic collisions with surrounding gas molecules at high spray potentials and by the relatively high inlet capillary temperatures used in these experiments.[50,51] ETS is not effective with pure water.[50] Mirza and Chait[53] suggested that salt in an electrospray solution may increase the ESI droplet lifetime, thereby allowing more time for droplet heating and thermal denaturation of proteins to occur in the heated inlet capillary region. However, different salts have only a relatively minor effect on the vapor pressure of water. For example, the vapor pressure of a 1 M sodium carbonate solution at 100 °C differs from pure water by only ~3.6%. In addition, ETS from solutions containing bicarbonate is significantly more effective than from solutions containing acetate,[50] yet the vapor pressures of 1 M sodium carbonate and 1 M sodium acetate solutions at 100 °C differ by only ~0.5%.[54] Protein denaturation is well-known to occur at water−air interfaces,[55−58] such as that occurring at the droplet surface or at a bubble surface if gaseous evolution occurs in the droplet. Konermann and co-workers[52] recently reported that myoglobin aggregation in heated ammonium bicarbonate solutions is a result of bubbles produced by bicarbonate degradation to carbon dioxide, and they proposed that formation of carbon dioxide gas bubbles during ESI droplet heating may be the cause of protein unfolding in electrothermal supercharging experiments.

The effects of various salts on protein structure and stability have been extensively investigated.[59−63] Studies done over 125 years ago by Franz Hofmeister[59] led to an ordering of anions and cations based on their propensity to cause protein aggregation or denaturation that is referred to as the Hofmeister series. The Hofmeister series depends on protein identity and experimental conditions, but the ordering of ions is typically:

cations:

$$Gdm^+ > Ca^{2+} > Mg^{2+} > Li^+ > Na^+ > K^+ > Cs^+ > NH_4^+ > N(CH_3)_4^+$$

anions:

$$SCN^- > ClO_4^- > I^- > Br^- > Cl^- > CHO_2^- > HCO_3^- > C_2H_3O_2^- > HPO_4^{2-} > SO_4^{2-}$$

with kosmotropic ions toward the right of the series that tend to precipitate (salt-out) proteins from solution and prevent protein unfolding and chaotropic ions toward the left in the series that typically increase the solubility (salt-in) of proteins and enhance protein denaturation. A reverse Hofmeister series has been observed for some proteins at low salt concentrations when the protein has a net positive charge in solution.[64−70] Both the cation and the anion of a salt in solution contribute to the stability of a protein, although anions tend to have a more significant effect than cations.[71] The detailed mechanism on how ions affect protein structure is not well understood, but both ion−protein and ion−water interactions have been implicated in the phenomenon.[60,61,69,72−76] Hofmeister effects are also associated with physical properties of aqueous electrolyte solutions and solution-phase ionic properties, such as surface tension,[62,66,71,77] ion free energy of hydration,[66,78] viscosity B-coefficient,[78] and ion surface/bulk partitioning.[77,79,80] Colussi and co-workers[81,82] reported a Hofmeister ordering of ion preference for the surface of electrospray droplets from ion abundances even for submicromolar salt solutions and found that the identity of the cation played a very small role in determining the surface activities of anions. Ruotolo and co-workers[83] reported both a direct Hofmeister series for anions and a reverse series for cations for refolding of misfolded concanavalin A tetramer using both solution-phase differential scanning calorimetry and ion mobility mass spectrometry. Effects of anion adducts on gaseous protein conformations have also been reported.[84−86]

Here, the role of the buffer in protein unfolding in electrothermal supercharging is investigated for ammonium and sodium salts with ten different anions. The effectiveness of different anions at producing electrothermal supercharging correlates well with a reverse Hofmeister series and to several solution and anion properties related to the Hofmeister series. Bubble formation upon heating does not occur appreciably from most salt solutions for which electrothermal supercharging is effective, and degassing of solutions of thermally stable salts prior to electrospray has no effect on the protein ion charge-state distributions in electrothermal supercharging. These results indicate that protein unfolding in electrothermal supercharging is predominantly caused by protein destabilization as a result of droplet heating and increasing concentration of destabilizing anions in the ESI droplet, although other factors almost certainly contribute as well.

EXPERIMENTAL SECTION

Ions were formed by nanoelectrospray (nanoESI) from solutions of 10 μM protein and 5 mM ammonium or sodium salts (≥97% purity) 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 ~1 μm with a Flaming/Brown micropipet puller (Model P-87, utter Instruments, Novato, CA, USA). All mass spectra were acquired using a Thermo LTQ (Linear Trap Quadrupole) Orbitrap with the inlet capillary heated to 250 °C. The nanoelectrospray emitter was positioned ~2 mm from the mass spectrometer inlet, and a spray potential of +1.3 kV was used to induce electrothermal supercharging. The temperature of the nanospray emitter was ~35 °C, which is well below the aqueous melting temperatures of the three proteins studied (~85 °C for cytochrome *c*,[87] >100 °C for ubiquitin,[88] and ~82 °C for β-lactoglobulin A[89]) so that no unfolding of the protein should occur in the nanospray emitter prior to droplet formation by ESI. Spectra were measured in triplicate using three different nanospray capillaries for each sample to account for tip-to-tip variability in charge-state distributions. The fraction of the protein population that is unfolded was calculated from the charge-state distribution, with the peaks

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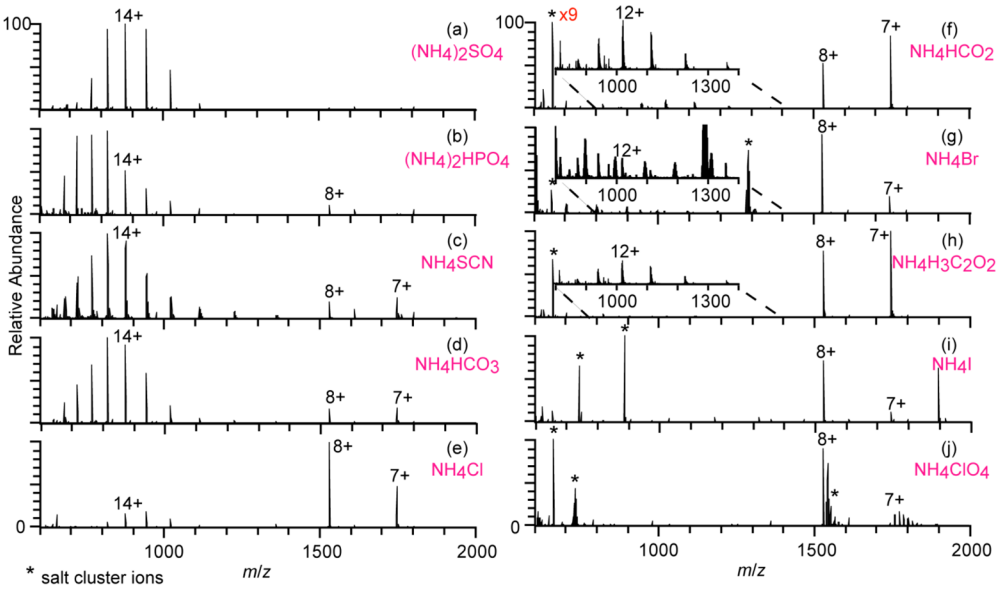

Figure 1. NanoESI mass spectra of 10 μM cytochrome *c* in different 5 mM aqueous ammonium buffers measured under electrothermal supercharging conditions (spray potential of +1.3 kV).

corresponding to the high-charge fraction of the bimodal distribution assigned to unfolded conformations (≥10+ for cytochrome *c*, ≥7+ for ubiquitin, and ≥10+ for β-lactoglobulin A).

Experiments in which bubble formation from different ammonium salt solutions was monitored over time were performed by inserting a rack of test tubes containing 2 mL of 10 μM cytochrome *c* in each solution into a 97 °C water bath and recording the results with a camera. Degassing of an ammonium sulfate buffer was done by vacuum filtration through a 0.45 μm Type HA membrane (Millipore, Billerica, MA, USA), followed by gentle stirring with a magnetic stir bar under vacuum for 10 min. Bovine cytochrome *c*, ubiquitin, and β-lactoglobulin A were purchased from Sigma (St. Louis, MO, USA) as lyophilized solids and were used without further purification.

RESULTS AND DISCUSSION

Electrothermal Supercharging with Aqueous Ammonium Salts. NanoESI mass spectra of 10 μM bovine cytochrome *c* in aqueous solutions containing 5 mM ammonium salts under electrothermal supercharging conditions are shown in Figure 1. Relatively low concentrations of ammonium salts were used to prevent signal suppression due to the formation of salt cluster ions and acid molecule adducts, the latter of which occur extensively for anions with low proton affinities, such as perchlorate, hydrogen sulfate, and iodide.[90] The effectiveness of electrothermal supercharging at producing a distribution of high charge state ions varies significantly with the identity of the anion of the ammonium salt (Figure 1, Table 1). Results for ammonium bicarbonate, which is advantageous due to a buffer capacity centered near neutral pH and to its effectiveness at ETS, is shown in Figure 1d. The charge-state distribution is bimodal, with the 7+ through 9+ ions composing a low charge-state distribution typical of cytochrome *c* formed in native ESI at low spray potential from ammonium bicarbonate solutions (Figure S-1a, Supporting Information) and indicative of compact or folded structures. The 10+ to 20+ ions form a high charge-state distribution centered around the 15+ ion and correspond to cytochrome *c* that has unfolded in the ESI droplet as a result of droplet heating at high spray potential. The average charge of cytochrome *c* from the ammonium bicarbonate solution is 13.5 ± 0.9+, and the fraction of the ion population that is unfolded is 0.87 ± 0.06. In contrast, no high charge state ions corresponding to unfolded protein are observed at low spray potential (+0.7 kV; predominantly 8+ and 7+ charge states formed; Figure S-1a, Supporting Information). The abundance of the high charge-state distribution with ETS is even greater with sulfate, hydrogen phosphate, and thiocyanate than it is with bicarbonate (Figure 1a–c, Table 1). With sulfate, 100% of the ion population is folded at low spray potential (Figure S-1b, Supporting Information), and at high spray potential, nearly the entire charge-state distribution corresponds to unfolded protein (~99%).

Ammonium acetate is an acidic buffer with a buffer capacity around ~pH 5, and it is by far the most commonly used buffer in native MS. In contrast to results for sulfate, hydrogen phosphate, thiocyanate, and bicarbonate, there is little electrothermal supercharging with ammonium acetate (Figure 1h);

Table 1. Average Fraction of the Ion Population That Is Unfolded in Electrothermal Supercharging[a]

pH	ammonium salt (5 mM)	fraction unfolded (cytochrome *c*, pI 10.5)	fraction unfolded (ubiquitin, pI 6.8)	fraction unfolded (β-lactoglobulin A, pI 5.1)
5.3	SO_4^{2-}	0.99 ± 0.01	0.91 ± 0.01	1.00 ± 0.01
8.0	HPO_4^{2-}	0.95 ± 0.01	0.83 ± 0.01	1.00 ± 0.01
5.9	SCN^-	0.90 ± 0.05	0.69 ± 0.01	0.92 ± 0.01
8.2	HCO_3^-	0.87 ± 0.06	0.60 ± 0.03	0.93 ± 0.01
5.3	Cl^-	0.27 ± 0.02	0.39 ± 0.13	0.51 ± 0.09
6.1	HCO_2^-	0.21 ± 0.06	0.27 ± 0.09	0.35 ± 0.11
5.3	Br^-	0.17 ± 0.10	0.31 ± 0.02	0.26 ± 0.26
6.3	$H_3C_2O_2^-$	0.10 ± 0.02	0.16 ± 0.09	0.34 ± 0.14
5.6	I^-	0.00 ± 0.00	0.12 ± 0.02	0.68 ± 0.27
5.7	ClO_4^-	0.00 ± 0.00	0.06 ± 0.03	0.94 ± 0.04

[a]Aqueous 5 mM ammonium salt solutions; +1.3 kV spray potential.

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the average charge and fraction unfolded of cytochrome *c* from this solution are 7.8 ± 0.1+ and 0.11 ± 0.02, respectively. Electrothermal supercharging is even less effective with iodide and perchlorate (Figure 1i,j, Table 1), where no charge states greater than 9+ are formed. The overall ranking of anions from greatest to least amount of unfolding of cytochrome *c* by electrothermal supercharging is: sulfate > hydrogen phosphate > thiocyanate > bicarbonate > chloride > formate ≈ bromide > acetate > iodide = perchlorate.

The pI of bovine cytochrome *c* is 10.5, which is well above the pH of all of the ammonium salt solutions used in these experiments, so the protein has a net positive charge in these solutions. Protein surface charge is an important factor in Hofmeister effects on protein stability and solubility in electrolyte solutions.[66,68,69,80,91,92] To determine if protein surface charge is a factor in the effectiveness of these anions in ETS, experiments with ubiquitin (pI 6.8), which has a pI intermediate in the range of solution pH values, and *β*-lactoglobulin A (pI 5.1), which has a pI below all of the pH values and thus would have a net negative charge in the initial solutions, were performed. The fraction of unfolded populations of these proteins is given in Table 1. For ubiquitin, the ordering of the efficiency of electrothermal supercharging with different anions is the same as that for cytochrome *c*. The ranking of anions for *β*-lactoglobulin A also follows the same order as for cytochrome *c*, with the fraction unfolded decreasing from sulfate to acetate, but reaches a minimum at acetate and increases again from iodide to perchlorate. Iodide and perchlorate produce no unfolding for cytochrome *c* (no high-charge ions for either salt) and almost no unfolding for ubiquitin (0.12 ± 0.02 and 0.06 ± 0.03, respectively), but significant unfolding occurs for *β*-lactoglobulin A (0.68 ± 0.27 and 0.94 ± 0.04, respectively).

To investigate the effect of salt concentration on the effectiveness of electrothermal supercharging, ETS spectra for cytochrome *c* were measured as a function of salt concentration for six different aqueous ammonium salts ranging from 1 mM to 2 M (Figure 2). The effectiveness of electrothermal supercharging increases with salt concentration for all anions. With 100 mM or greater salt concentration, all salts except acetate produce observable ETS compared to water, and at these concentrations, all but acetate and formate result in nearly 100% unfolding of cytochrome *c* with electrothermal supercharging. The ordering of anions in their effectiveness at ETS of cytochrome *c* in 5 mM salt solutions does not change over the entire range of salt concentrations studied.

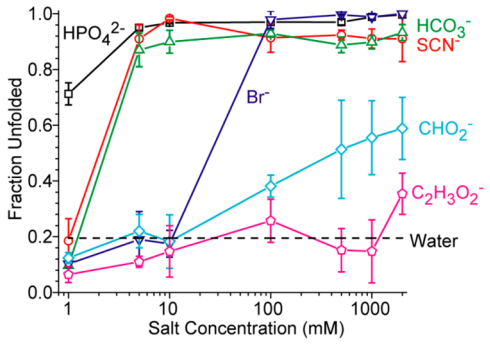

Figure 2. Fraction of the ion population corresponding to unfolded cytochrome *c* produced by electrothermal supercharging with different concentrations of ammonium salts: ammonium hydrogen phosphate (black □), thiocyanate (red ○), bicarbonate (green △), bromide (blue ▽), formate (cyan ◇), and acetate (pink ⬠) and pure water (- - -).

The ordering of anions in their effectiveness at ETS of ubiquitin is slightly different than previously reported.[50] In the previous study, the different ammonium buffers at 10 mM concentration were buffered to pH 7.0 using either acetic acid or ammonium hydroxide, and the ordering of anions at their effectiveness of electrothermal supercharging was: hydrogen phosphate (0.98 ± 0.01 of the population that is unfolded) > thiocyanate (0.95 ± 0.01) > bicarbonate (0.7 ± 0.2) > sulfate (0.6 ± 0.5) > perchlorate (no high-charge ions) ∼ acetate (no high-charge ions). The effectiveness of electrothermal supercharging in pH-adjusted solutions is a combined effect from both the cation and anion of the ammonium salt in solution and the acetate or additional ammonium added when the pH is adjusted. The buffer solutions used in this work were not adjusted for pH. The pH values of these solutions are given in Table 1 and range from 5.3 for ammonium sulfate, chloride, and bromide to 8.2 for ammonium bicarbonate. There is no correlation between the effectiveness of ETS and the solution pH, i.e., a lower pH solution does not necessarily result in more unfolding from electrothermal supercharging due to pH destabilization. Ammonium sulfate and bromide solutions have the lowest pH of the salts at 5 mM concentration, yet these salts are near opposite ends of the ordering of salts in their effectiveness at producing electrothermal supercharging. Furthermore, increasing the salt concentration, and thus increasing the buffering capacity of each solution to resist pH changes at some point during ESI droplet evaporation, does not lead to any changes in the ordering of anions in electrothermal supercharging and enhances electrothermal supercharging for all salts.

Electrothermal Supercharging with Aqueous Sodium Salts. To determine effects of the ammonium cation on these results, mass spectra with electrothermal supercharging conditions were obtained from solutions containing 10 μM ubiquitin and 5 mM sodium salts. The fraction of the ubiquitin population that is unfolded from these sodium salt solutions (Table 2) is similar to that for ubiquitin from ammonium salts (Table 1), with almost complete unfolding from sodium sulfate (0.87 ± 0.01) and sodium hydrogen phosphate (0.98 ± 0.01) solutions and minimal unfolding from sodium iodide (0.06 ± 0.01) and sodium perchlorate (0.04 ± 0.02) solutions. Thiocyanate is an exception where the fraction unfolded is 0.01 ± 0.01 with sodium as the cation, the *least* unfolding

Table 2. Average Fraction of the Ion Population That Is Unfolded in Electrothermal Supercharging[a]

pH	sodium salt (5 mM)	fraction unfolded ubiquitin in 5 mM sodium buffer
5.4	SO_4^{2-}	0.87 ± 0.01
8.4	HPO_4^{2-}	0.98 ± 0.01
6.9	SCN^-	0.01 ± 0.01
8.4	HCO_3^-	0.83 ± 0.16
6.1	Cl^-	0.23 ± 0.02
6.4	HCO_2^-	0.22 ± 0.06
5.6	Br^-	0.07 ± 0.01
6.7	$H_3C_2O_2^-$	0.03 ± 0.01
5.5	I^-	0.06 ± 0.01
5.5	ClO_4^-	0.04 ± 0.02

[a]Aqueous 5 mM sodium salt solutions; +1.3 kV spray potential.

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among all of the sodium salts, whereas the unfolded population is 0.69 ± 0.01 with ammonium as the cation, the third *most* unfolding of the ammonium salts. Overall, the similarities of these two data sets demonstrate that, in most cases, the ammonium cation plays a relatively minor role in the effectiveness of electrothermal supercharging.

Electrothermal Supercharging, The Hofmeister Series, and Related Physical Properties. The ordering of ions in the Hofmeister series depends on the relative values of the protein pI and the solution pH. Anions typically follow a direct Hofmeister ordering when a protein has a net negative charge in solution, i.e., the protein pI is below the solution pH. However, when the protein has a net positive charge in solution, i.e., the protein pI is above the solution pH, anions follow a reverse Hofmeister series at low salt concentrations (<0.3 M).[64−69] The solution pH is known in these experiments prior to ESI droplet formation, but droplet pH decreases during droplet evaporation.[93−95] The ranking of salts in their effectiveness at electrothermal supercharging correlates well with a reverse Hofmeister series. The most destabilizing anions in the reverse Hofmeister series are sulfate and hydrogen phosphate, and spectra from these ammonium and sodium salts have the most abundant high charge states corresponding to the highest fraction of the population that is unfolded from electrothermal supercharging. In contrast, the most stabilizing anions in the series are iodide, perchlorate, and thiocyanate, which produce the least unfolding from electrothermal supercharging. This suggests that the effective ESI droplet pH may be below the pI values of all three proteins prior to ion formation. Results for sodium and ammonium acetate and ammonium but not sodium thiocyanate do not follow the reverse Hofmeister series. Acetate is a kosmotrope, which typically is intermediately between bicarbonate and hydrogen phosphate in its ability to destabilize protein structure in solution. Both of the latter anions are effective at ETS, but acetate is not.

The ordering of salts from electrothermal supercharging also correlates well with several physical properties related to the ability of ions to structure water at an interface. The fraction of ubiquitin that is unfolded in ETS in ammonium and sodium salts increases with increasing solvent surface tension and anion viscosity B-coefficient and decreases with increasing anion surface/bulk partitioning coefficient (Figure 3). The increase in the surface tension of water with ammonium and sodium salts is small (~3% or less at 1 M salt concentration). Although some increase in charge would be expected due to the higher surface tension, this should be a small effect and not the primary cause of the significant increase in protein charging and bimodal charge-state distributions produced by ETS. Acetate is an outlier in the correlation between electrothermal supercharging and the reverse Hofmeister series, but it is not an exception in the correlation between ETS and these three physical properties. Although there is a correlation between the Hofmeister series and solution surface tension as well as anion surface/bulk partitioning coefficients, acetate is a known exception.[77]

An outlier to the trends in the effectiveness of anions at ETS with these three physical properties is ammonium thiocyanate (open square, Figure 3), the same anion for which the extent of unfolding from electrothermal supercharging depends strongly on the cation identity. In contrast, data for sodium thiocyanate (open triangle, Figure 3) follows the trend established by the other anions. The ammonium cation is a stronger kosmotrope

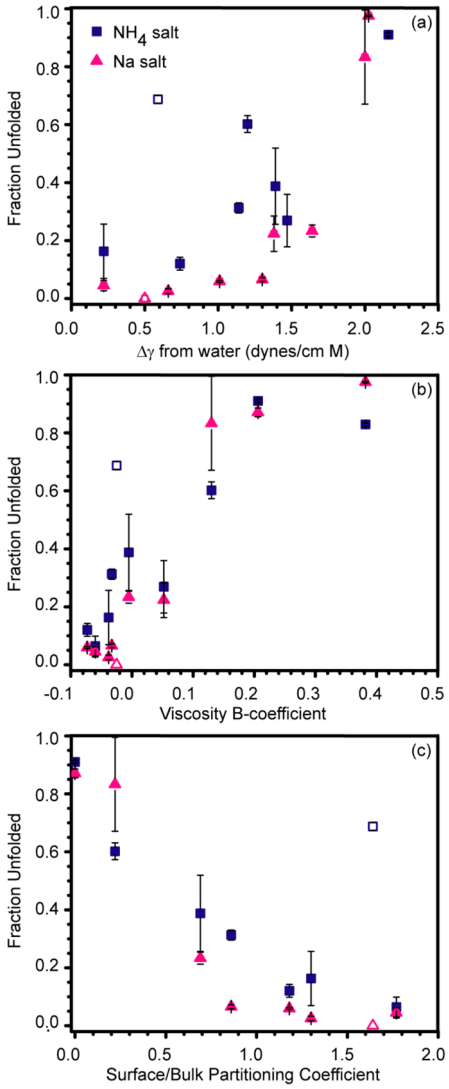

Figure 3. The fraction of the ion population corresponding to unfolded ubiquitin in electrothermal supercharging from aqueous ammonium and sodium salt solutions as a function of (a) surface tension increment (relative to pure water),[54,101] (b) anion viscosity B-coefficient,[102] and (c) anion surface/bulk partitioning coefficient (with respect to sulfate at 0.0).[77]

than sodium, and it may have a greater effect on protein stability than the chaotropic thiocyanate anion. In some instances, the cation can have a significant effect on protein stability.[96] For example, Tomé et al.[96] reported that the solubility of L-valine in 1 M ammonium sulfate is 11% *less* than that in pure water, whereas the solubility in 1 M magnesium sulfate is 10% *greater* than that in pure water. Thus, even though sulfate itself is a strong kosmotrope, when magnesium is the counterion, salting-in of a protein can occur with the sulfate anion.

The influence of the ammonium cation as a kosmotrope may also be responsible for anomalies in the β-lactoglobulin A data, in which ammonium iodide and perchlorate produce much more unfolding than expected on the basis of results for the other proteins. β-Lactoglobulin A has a net negative charge in all of the salt solutions prior to droplet formation, and the cations may associate more strongly with the negative charges on the protein, enhancing their effect on protein stability. Additionally, there could be a mixture of a direct and a reverse

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Hofmeister series for β-lactoglobulin A under the conditions in these electrothermal supercharging experiments. Although only a direct Hofmeister series is typically observed for a negatively charged protein, the solution pH in an ESI droplet decreases during droplet evaporation,[93−95] and the protein may have a net positive charge in ESI droplets from some salt solutions and a net negative charge in others.

At high salt concentrations (>0.3 M), anions typically follow a direct Hofmeister series irrespective of the protein pI relative to the solution pH. Experiments by Verhac et al.[97] show that, at both 500 mM and 1 M sodium salt concentrations, thiocyanate anion decreases the melting temperature of cytochrome *c* by ∼20 °C compared to that in pure water, whereas phosphate increases the melting temperature by ∼10 °C, in agreement with a direct Hofmeister ordering. Our results indicate that anions in electrothermal supercharging form a reverse Hofmeister series independent of the initial salt concentration. This suggests that there are likely factors in addition to the Hofmeister effect that contribute to the effectiveness of electrothermal supercharging.

Electrothermal Supercharging and Bubbles. Ammonium bicarbonate in aqueous solutions can thermally degrade to produce carbon dioxide gas, and Konermann and coworkers[52] recently proposed that protein denaturation at the surface of gas bubbles is responsible for the high charge states formed by ETS from ammonium bicarbonate solutions. In contrast, no bubbles are formed in heated ammonium acetate solutions, where little or no ETS occurs. To determine if the effectiveness of electrothermal supercharging depends significantly on bubble formation, 2 mL solutions of 10 μM cytochrome *c* in water and in 1 M salts of ammonium perchlorate, acetate, bicarbonate, hydrogen phosphate, and sulfate were inserted into a 97 °C water bath. Bubble evolution from each solution was recorded for up to 31 s (Figure S-2, Supporting Information). The ammonium bicarbonate solution starts to bubble within a second, and froth forms at the surface shortly thereafter due to protein aggregation. No significant bubbling or frothing was observed for any other solution, including ammonium hydrogen phosphate and sulfate solutions, for which the most unfolding due to electrothermal supercharging occurs for cytochrome *c* (Figure 1, Table 1).

It is possible that small bubbles may form in electrospray droplets from the coalition of dissolved gas molecules. A measure of the solubility of a gas in an aqueous electrolyte solution is given by the Setschenow constant, k_S, calculated from the following equation:

$$\log\{S/S'\} = -k_S[C]$$

where C is the electrolyte concentration, S is the solubility of the gas in the aqueous electrolyte solution, and S' is the concentration of the gas in pure water. A Setschenow constant less than one indicates a lower solubility of the gas in the electrolyte solution compared to pure water. The Setschenow constants for nitrogen and oxygen gas in aqueous sodium salt solutions are given in Table 3. The values of the Setschenow constants for both gases closely follow the ordering of anions in the reverse Hofmeister series (Table 3). The exception to this trend is perchlorate for oxygen gas, which has a Setschenow constant between that of chloride and sulfate, yet it is one of the most stabilizing anions of the reverse Hofmeister series and produces little to no electrothermal supercharging for ubiquitin and cytochrome *c*.

Table 3. Room Temperature Setschenow Constants (k_s) for Oxygen and Nitrogen Gas in Aqueous Sodium Salt Solutions Containing Hofmeister Anions

Hofmeister series	salt	k_S for oxygen gas[99]	k_s for nitrogen gas[100]
PO_4^{3-}	Na_3PO_4	0.652	
CO_3^{2-}	Na_2CO_3	0.464	0.373
SO_4^{2-}	Na_2SO_4	0.376	0.353
F^-	NaF	0.284	
	$NaClO_4$	0.160	
HCO_3^-	$NaHCO_3$		0.153
Cl^-	NaCl	0.136	0.134
Br^-	NaBr	0.131	
NO_3^-	$NaNO_3$	0.124	
I^-	NaI	0.120	
ClO_4^-			

To test if dissolved gas is a potential cause of electrothermal supercharging, a 5 mM ammonium sulfate solution was degassed by vacuum filtration followed by gentle stirring with a stir bar under vacuum for 10 min. Immediately after, a solution of 10 μM cytochrome *c* in this degassed ammonium sulfate solution was prepared, and mass spectra under electrothermal supercharging conditions were obtained (Figure S-3b, Supporting Information). The average charge and fraction unfolded of cytochrome *c* from this solution were 13.7+ and 0.99, respectively, essentially identical to the result from a solution that was not degassed (Table 1, Figure S-3a, Supporting Information). Bubble formation due to coalition of dissolved gases or gas evolution as a result of thermal decomposition of the buffer does not appear to play a significant role in electrothermal supercharging.

CONCLUSIONS

Electrothermal supercharging of proteins from aqueous solutions containing different ammonium and sodium salts was investigated. The effectiveness of ETS at producing high charge states depends strongly on the identity of the anion, with an anion ordering that closely follows a reverse Hofmeister series. This correlation with the Hofmeister series and with several physical properties related to how strongly ions influence water structure at an air/water or protein/water interface indicate that stabilization or destabilization of proteins toward thermal denaturation in the ESI droplet by these salts is likely a primary mechanism for their relative effectiveness at electrothermal supercharging. The few exceptions to the correlation in anion ordering can be rationalized by cation effects, which in some cases can have a large influence on protein stability and can change the position of the anion in the Hofmeister series. No bubbles are formed from heated ammonium sulfate and ammonium hydrogen phosphate solutions, yet ETS is most effective with these salts. ETS is equally effective from an ammonium sulfate solution that has been degassed. Both of these results indicate that bubble formation from buffer decomposition upon heating or from dissolved gases does not play a role in protein unfolding in electrothermal supercharging. Hofmeister-like effects may also play a role in other experiments in which protein unfolding occurs in ESI droplets formed from native solutions, such as during traditional supercharging with *m*-NBA or sulfolane,[37−41] or in acid denaturation when acidic vapors are introduced in the source of a mass spectrometer.[98]

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■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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