Supercharging methods for improving analysis and detection of proteins by electrospray ionization mass spectrometry

by

Catherine Cassou Going

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Committee in charge:

Professor Evan R. Williams, chair

Professor Kristie A. Boering

Professor Robert Glaeser

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Abstract

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The characterization of mechanisms, analytical benefits, and applications of two different methods for producing high charge state protein ions in electrospray ionization (ESI) mass spectrometry (MS), or "supercharging", are presented in this dissertation. High charge state protein ions are desirable in tandem MS due to their higher fragmentation efficiency and thus greater amount of sequence information that can be obtained from them. The first supercharging method, supercharging with reagents (typically non-volatile organic molecules), is shown in this work to be able to produce such highly charged protein ions from denaturing solutions that about one in every three residues carries a charge. The high Coulomb repulsion in these ions results in these ions adopting near-linear gas-phase structures with little to no non-covalent interactions, making them ideal for efficient fragmentation in tandem MS experiments and for the minimization of gas phase HD scrambling during tandem MS. Supercharging with reagents from aqueous solutions typically produces much lower charge states as compared to that observed from a denaturing solution. However, two new reagents are presented in this work that increase protein ion charge past that from denaturing solutions when added to aqueous solutions at just 2% by volume. Increases in charge of up to 168% are reported in the presence of these reagents. The mechanism of the increases in protein ion charging with these reagents from aqueous solutions was investigated with fluorescence experiments and correlated to a destabilization of the protein structure by these reagents toward denaturation. The actual protein denaturation event likely occurs in the ESI droplet itself, consistent with previous studies of the mechanism of supercharging with reagents. Thus, efficient tandem MS of high charge states is possible from ESI of aqueous solutions in which a protein maintains its native or native-like structure and activity, enabling tandem MS analysis of protein modifications, ligand binding, or structural changes in real time. Interestingly, another application for supercharging reagents is protein desalting in the ESI droplet. Supercharging reagents bind to sodium ions, resulting in less non-specific sodium ion adduction to proteins, which can improve signal-to-noise ratios of protein ions, lower limits of detection, and enable the detection of bound ligands or specific binding of salts that might otherwise be obscured by sodium adduction. The second supercharging method, electrothermal supercharging (ETS), requires the presence of particular buffer salts rather than organic reagents to increase protein ion charge in the ESI droplet. An investigation of the effect of several different buffer salts on ETS is presented in this work, revealing that the choice of buffer salt is very important to obtaining effective ETS and that
buffer salts likely stabilize or destabilize protein structure in the ESI droplet via Hofmeister effects. The application of ETS to tandem MS of proteins produced by ESI and its utility on proteins ranging in size over an order of magnitude (8.6 kDa to 83.0 kDa) is demonstrated. Hydrogen-deuterium exchange experiments can be performed in aqueous solutions and measured continuously with ETS coupled to tandem MS for protein structure analysis in real time with a spatial resolution of 1.3 residues and without gas phase hydrogen-deuterium scrambling. This work demonstrates the wide applicability of ETS for the study of primary and higher order protein structure for small and large proteins alike.
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Chapter 1

Introduction

1.1 Bioanalytical Chemistry

Bioanalytical chemistry is a field that works toward developing devices and techniques for the purification and accurate identification and quantification of biomolecules such as proteins and DNA, as well as developing applications for analytical methods for the study of the roles these biomolecules play within a particular biological system. Concentrations of endogenous proteins in cells and blood can range over 8 orders of magnitude\(^1\) and their masses can range over 5 orders of magnitude, from 75 Da for the amino acid glycine to 40.5\(\times\)10\(^6\) Da for Tobacco Mosaic Virus capsid\(^2\) and 110.0\(\times\)10\(^6\) Da for Coliphage T4 DNA.\(^3\) Furthermore, the multitude of different post-translational modifications, sequence mutations, and conformational changes due to these modifications, make just studying the primary structure of these proteins challenging, let alone their complex interactions with each other within a biological system. In the effort to develop improved methods for quantifying proteins and studying their structure, the fields of chromatography and electrophoresis for protein separation and purification and mass spectrometry for protein identification have come to the forefront as powerful analytical techniques for molecular and cell biology.

Mass spectrometry (MS) separates molecules by their mass-to-charge \((m/z)\) ratios in the gas phase. There are many different types of mass spectrometers. Some of the most common use charge-image detection, such as Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap. Others include time-of-flight, magnetic sector, and multipole mass analyzers such as quadrupole all the way up to 22-pole.\(^4\) The high resolving power of these instruments combined with separation by chromatography and/or ion mobility prior to mass analysis enables the study of very complex mixtures of biomolecules, and the high sensitivity of these instruments with the ability to detect less than a femtomole\(^5\) of some proteins enables the study of low concentration components in those complex mixtures. Proteins and DNA are non-volatile molecules; however, they can be transferred into the gas phase as ions for analysis by mass spectrometry by electrospray ionization (ESI)\(^6\) and matrix-assisted laser desorption ionization (MALDI).\(^7, 8\) ESI and MALDI are "soft" ionization techniques, meaning they form intact gas phase ions, in contrast to techniques like electron impact ionization (EI) and chemical ionization (CI), which are higher energy techniques that can induce fragmentation of molecules.

All ions produced in this work were formed by ESI. ESI produces multiply charged protein ions from a variety of different solution conditions, including from solutions containing organic solvents and acids, in which proteins are denatured, and from buffered aqueous solutions in which proteins are in folded, native or native-like conformations. Both experimental and computational methods have been used to study how different charge states of proteins are formed, and several methods have been developed for manipulating charge-state distributions by solution phase or gas phase chemistry. The work described herein describes two types of techniques for manipulating protein charge states and the applications of these techniques to the study of the detection and analysis of proteins and complexes. Part 1 discusses advances made in supercharging with reagents for increasing protein charge and reducing non-specific cationic adducts from proteins, and Part 2 characterizes the technique of electrothermal supercharging for increasing protein ion charge from native solutions.
1.2 Electrospray Ionization of Proteins

ESI is initiated by applying a potential difference of 0.5 – 5 kV between a sample solution and the instrument. The sample is held in a capillary that has a high electric field at the tip which deforms the liquid in the tip into a cone called a Taylor cone. A liquid jet is emitted from the cone that then breaks into an aerosol of charged droplets. As a charged droplet evaporates, its radius decreases until the charge at the surface reaches the Rayleigh limit, or, the limit at which repulsive Coulomb forces exceed surface tension forces at the surface of the droplet, and the droplet undergoes fission to form progeny droplets. The charge at which the Rayleigh limit is reached is given by the following equation:

$$z_R e = 8\pi(\varepsilon_0 \gamma R^3)^{1/2}$$

(Eq. 1)

where $$z_R$$ is the integer unit of the Rayleigh charge, $$e$$ is the elementary charge, $$\varepsilon_0$$ is the permittivity of the medium, $$\gamma$$ is the surface tension of the droplet, and $$R$$ is the radius of the droplet. Progeny droplets carry away ~15% of the charge of the parent droplet, but only ~2% of the mass. Successive evaporation and fission cycles occur until all solvent is gone and only bare, gas phase ions remain.

The exact mechanism of the formation of these gas phase ions has been hotly debated for decades, and different mechanisms may exist for different analytes. The two mechanisms that have been proposed that dominate in the literature are the ion evaporation model (IEM) and the charge residue model (CRM). In the IEM, successive evaporation and fission events occur until ions desorb directly from the droplet surface when the electric field is sufficiently great to overcome the free energy of hydration of the ion. The IEM is thought to occur predominantly for salts and small molecules. In the CRM, ions are formed after a series of solvent evaporation and Rayleigh fission events until a droplet containing the ion has evaporated to dryness and any remaining charge is transferred to the analyte. The CRM is thought to dominate for large macromolecules such as proteins and large polymers, and protein ions produced from native solutions are charged at or near the Rayleigh limit predicted for a droplet of the same size as the protein determined from its crystal structure, supporting the CRM model. Somewhat recently, a combined charged residue-field emission model has been proposed, in which ion emission of charge carriers from the ESI droplet determines the charge that is transferred to the analyte in the final stages of droplet evaporation.

1.3 Factors Affecting Protein Ion Charge

A number of factors influence the number of charges observed on a protein, including solvent surface tension, various instrument parameters such as inlet capillary temperature or countercurrent gas, relative gas phase basicities of solution components, ion polarity, and protein conformation in solution. The conformation of a protein in solution prior to ESI can play a large role in determining the charge-state distribution that will be observed in the mass spectrum. If a protein is unfolded in solution, often a broad, high charge-state distribution is observed, whereas if a protein is folded in solution, a narrow, low charge-state distribution is observed. Multiple conformations of a protein in solution at one time can manifest as multi-modal charge-state distributions, and the relative abundances of each conformation can be estimated by the relative abundances of each distribution.
There are a number of advantages to forming low or high charge state protein ions. Low charge states formed from native, aqueous solutions have lower collision cross sections in the gas phase that are more similar to the cross sections predicted by crystal structures, indicating that they retain a more native-like conformation in the gas phase. Lower charge-state ions of protein complexes undergo more symmetric dissociation in the gas phase, yielding greater information about complex assembly patterns compared to higher charge states, for which commonly only monomer dissociation is observed. In many cases, lower charge states of protein complexes also dissociate predominantly along interfaces that are also the weakest in solution, giving information about solution phase stability in a gas phase measurement. Reducing the charge states of proteins to just a single charge state can dramatically simplify mass spectra of complex mixtures. High charge states are desirable in tandem MS experiments in which there is better fragmentation efficiency at higher charge states. Increasing protein ion charge moves the charge-state distribution to lower $m/z$, where there is better sensitivity and resolution, and where ions can be detected by mass spectrometers that have upper $m/z$ limits.

1.4 Manipulating Protein Charge

There are a number of methods for manipulating protein ion charge. After ESI, once bare, gas phase protein ions are formed, these ions can undergo charge reduction by ion-ion or ion-molecule reactions inside the mass spectrometer. For example, the charge states of ubiquitin (8.6 kDa), cytochrome $c$ (12.3 kDa), and myoglobin (17.6 kDa) can be reduced to just $1^+$ via proton stripping by perfluoro-1,3-dimethylcyclohexane - F$^-$ and, even more impressively, the charge states of large protein complexes such as pyruvate kinase (233 kDa) and L-glutamic dehydrogenase (336 kDa) can be reduced from 34$^+$ to 2$^+$ and from 40$^+$ to 4$, respectively, by electron transfer from p-nitrotoluene and 1,4-dicyanobenzene radical anions. Volatile molecules with gas phase basicities higher than that of the gas phase protein ion can be leaked into the mass spectrometer in order to decrease protein ion charge via proton transfer from the protein to the base. By titrating the basicities of the different molecules leaked into the mass spectrometer, one can obtain a value for the apparent gas phase basicity of the protein and even individual charge states of the protein, which can be isolated inside the mass spectrometer. In general, higher charge state ions have a lower gas phase basicity than lower charge states for a given protein, and the presence of multiple protein conformations for a given charge state can be detected by measuring more than one rate constant for proton transfer. For gas phase protein ions that have multiple adducted cations that are loosely bound, such as Cs$^+$ and tetramethylammonium$^+$, removing these adducts with collisions with an inert gas can lower the charge of the protein.

There are several methods for shifting protein charge-state distributions during the ESI droplet lifetime as well. Acidic or basic vapors can be passed over the ESI plume, changing the pH in the ESI droplet in order to acid- or base-denature a protein in the droplet, resulting in higher charge state protein ions. Fast mixing of acidic and buffered solutions within the ESI droplet and Taylor cone by using dual barrel nanospray capillaries called theta-capillaries can also be used to unfold or refold proteins in the ESI droplet and obtain higher or lower charge states, respectively. Small molecules with high basicities, such as imidazole and triethylamine, can be added into the initial ESI sample solution to form lower charge states via proton transfer during the final stages of ion desolvation.
Finally, there is a class of techniques called supercharging for increasing protein ion charge during ESI. One supercharging method increases protein ion charge via trivalent metal ion adduction. Trivalent metal ions preferentially adduct to higher charge state protein ions, adding three charges for every metal ion adducted. With just 1 mM of trivalent metal ion salt in a native ESI solution, the maximum charge state observed in the mass spectrum can be even greater than the total number of basic sites on the protein. In a second supercharging method, electrothermal supercharging, protein ion charge from native, aqueous ammonium bicarbonate buffer can be increased simply by increasing the electrospray potential. It is thought that the mechanism of the formation of these high charge states is thermal unfolding in the ESI droplet due to collisional heating of the droplet at higher ESI potentials, and this is supported by the fact that more electrothermal supercharging is observed at higher inlet capillary temperatures. In Chapters 5 and 6, a more detailed characterization of electrothermal supercharging is presented, including its performance with proteins ranging from 8.6 kDa to 83.0 kDa in size and its effectiveness when using buffer systems other than ammonium bicarbonate. Its applications to bioanalytical chemistry in top-down proteomics and improving the capabilities of mass spectrometers with upper \( m/z \) limits are also investigated. In Chapter 7, an electrothermal supercharging hydrogen-deuterium exchange method is described for obtaining residue-specific exchange kinetics in real-time when coupled to top-down proteomics.

A third supercharging method, and by far the most studied, is supercharging with reagents, which was discovered by Williams and coworkers while investigating the effects of different solvents on the charge state distributions of protein ions produced from denaturing solutions. They found that additives with high boiling points and surface tensions higher than that of a typical denaturing solution produced large increases in protein charge. The Rayleigh charge on a droplet is proportional to the square of the solvent surface tension. Thus, if these high boiling point reagents concentrate in the ESI droplet such that they constitute most of the droplet volume later in the droplet lifetime, the droplet can hold more charge, resulting in a greater amount of charge being deposited on the protein ion. Loo and coworkers then demonstrated that supercharging with reagents can also be performed from native aqueous solutions. In these experiments, only a very small amount of supercharging reagent (~1-2% by volume) is added to the aqueous protein solution, and at these low amounts, the conformation of a protein is not significantly affected. The reagents concentrate in the ESI droplet, but unlike out of denaturing solutions, the high charge-state protein ions that are formed are not due to the surface tensions of the reagents. All of the supercharging reagents that have been discovered have surface tensions below that of water, and thus the Rayleigh charge expected on these droplets should decrease upon reagent concentration compared to that for water. Rather, native supercharging must proceed by another mechanism, and there has been much debate over what this exact mechanism is (see Chapter 3 for a more detailed discussion). The most cohesive argument is for a chemical/thermal denaturation mechanism with high charge state ions being produced by protein unfolding in the ESI droplet. Supercharging reagents have been shown to destabilize protein structure in solution according to circular dichroism experiments, and these effects increase with increasing reagent concentration. Supercharging is only effective on conformationally flexible proteins, and in the absence of conformational effects, surface tension effects can be observed as a decrease in protein ion charge. In Chapter 3, further evidence supporting the mechanism of chemical/thermal denaturation by supercharging reagents is presented, and new supercharging reagents that are the most effective ever at increasing protein ion charge from native solutions are introduced.
1.5 Gas Phase Fragmentation Techniques

Proteomics experiments for determining the sequence of proteins or the locations of protein modifications such as glycosylations or phosphorylations can be performed using either a bottom-up or a top-down approach. In the bottom-up approach, the protein sample is digested with a protease such as trypsin to fragment the protein into smaller peptides, and these peptides are then separated by liquid chromatography (LC) online with ESI-MS. The peptides that elute from the LC are identified by their exact mass (LC-MS) or by fragmentation in the gas phase (LC-MS/MS). The advantages of bottom-up proteomics is that there is a theoretically unlimited range for the size of the protein of interest, and protein samples that are typically unstable in solution outside of its biological environment, such as membrane proteins, can be identified. However, during preparation for mass spectrometry, information on the presence of labile post-translational modifications can be lost, and often <100% of the total number of proteolytic fragments are recovered. In a top-down approach, there is no proteolytic fragmentation prior to electrospray. Rather, a sample is either electrosprayed directly with or without LC beforehand, and then fragmented in the gas phase in order to determine the primary sequence. The top-down approach has the benefit of preserving protein modifications prior to ESI, and has the possibility for 100% sequence identification of a protein. However, in general gas phase fragmentation techniques yield fewer ions the larger the protein, and it is useful to understand some of the different methods for fragmenting protein ions to understand why this is.

Protein ions can fragment in a number of different ways and in a number of different locations along the peptide amide bond or along sidechains. Some of the amide bond cleavage patterns are shown below and are commonly labelled as $a$, $b$, $c$, $x$, $y$, or $z$ ions:

where the charge remains on the N-terminal side of the protein for $a$, $b$, and $c$ ions, and the charge remains on the C-terminal side of the protein for $x$, $y$, and $z$ ions. The type of fragment ions formed depends on the gas phase method used to produce the fragments. Some of the first fragmentation techniques used for protein sequencing involve slow heating of the protein ions through vibrational activation, and they are collision induced dissociation (CID) and infrared multiphoton dissociation (IRMPD). In CID, protein ions are passed through a chamber of inert gas such as argon or helium by a potential applied across the chamber. The protein ions undergo collisions with the gas, converting kinetic energy to internal energy, and become vibrationally excited until the threshold energy for peptide bond cleavage is reached. In IRMPD, infrared light is used to vibrationally excite protein ion to higher vibrational states with multiple photons of infrared light and induce fragmentation along the protein backbone. CID and IRMPD produce predominantly $b$ and $y$ ions. Because these techniques involve slow vibrational heating, bonds more labile than the peptide bond also break, resulting in cleavage of side chains and dissociation of many post-translational modifications to a protein. CID is also
commonly used under more gentle conditions to fragment a protein complex into its constituent subunits rather than break the peptide bond in order to gain information on protein complex stoichiometry and assembly pattern.\textsuperscript{88, 89} Fragmentation by CID of protein complexes results in a phenomenon known as asymmetric charge partitioning, in which one monomer unit is removed from the protein carrying with it most of the charge, resulting in product ions of a highly charged monomer and a low charge (n-1)-mer.\textsuperscript{40} Another common subset of gas phase fragmentation techniques induce radical-mediated cleavage along the peptide bond, and they are electron capture dissociation (ECD)\textsuperscript{90} and electron transfer dissociation (ETD).\textsuperscript{91} In ECD, a low-energy electron (< 0.2 eV) produced from a heated filament or cathode in an FT-ICR mass spectrometer combines with a protein ion to form a radical protein ion, which subsequently fragments into primarily $c$ and $z$ ions. ETD proceeds by a similar mechanism and also produces primarily $c$ and $z$ ions, but the electron that combines with the protein is transferred via collisions from a radical anion, typically anthracene or fluoranthene. Unlike in CID or IRMPD, post-translational modifications such as phosphorylations and glycosylations are commonly retained during ECD and ETD\textsuperscript{92-96} and hydrogen-deuterium scrambling within a peptide or protein ion is minimal in ECD and ETD,\textsuperscript{97-99} suggesting that fragmentation in ECD and ETD is a non-ergodic process,\textsuperscript{90} or, it occurs so quickly that the internal energy gained upon electron-protein combination cannot be distributed throughout the protein prior to peptide bond cleavage. Others have proposed that ECD is not a non-ergodic process, however, and that fragmentation at the peptide bond is the result of redistribution of internal energy in the protein after an electron is captured near the peptide bond.\textsuperscript{100} Greater fragmentation efficiency using many of these gas phase techniques is typically observed for proteins and peptides with higher charge.\textsuperscript{42-46} In ECD and ETD, the electron capture/transfer cross section,\textsuperscript{90} which is a measure of how far away an electron or the radical anion reagent ion can be from the protein ion and still be captured or transferred, increases with the square of the charge. Higher charge state ions also have more elongated structures in the gas phase,\textsuperscript{30, 36-38} so after backbone fragmentation, there are fewer non-covalent interactions holding fragments together. Activated ion (AI) ECD or ETD involves ion heating via collisions or infrared light either prior to or subsequent to backbone fragmentation by ECD or ETD in order to improve dissociation of $c$ and $z$ ions by reducing the number of non-covalent interactions within a gas phase protein ion and thus improve overall sequence coverage.\textsuperscript{101-103} Chapter 2 demonstrates that the highest charge states of protein ions produced with supercharging reagents out of denaturing solutions can have near-linear gas phase structures with virtually no non-covalent interactions intact, making supercharging with reagents amenable to top-down fragmentation.

1.6 Salt effects on ESI performance

ESI is particularly susceptible to the deleterious effects of the presence of salts, and this can be especially troublesome in native ESI, in which salt concentrations of ~150 mM are usually used in order to mimic physiological ionic strength. The presence of even millimolar concentrations of some salts can lead to the formation of cluster ions that suppress protein ionization\textsuperscript{104-109} and can lead to extensive non-specific cation or anion adduction to proteins, thereby decreasing protein ion signal-to-noise ratios (S/N) by spreading ion signal over multiple adducted species.\textsuperscript{107, 109-113} Cole reported a decrease in lysozyme ion abundances of 330-fold in
$10^{-2}$ M CsCl solutions, and even a 30-fold decrease with $10^{-2}$ M of a volatile salt, ammonium acetate. Non-specific ion adduction is generally greater on lower charge-state ions, with the exception of trivalent metal ions for which there is greater adduction on higher charge-state ions, and for anions with low proton affinities (<300 kcal/mol). For large proteins and protein complexes for which individual adducts cannot be resolved, ion adduction can result in large errors in mass measuring accuracy and can obscure observation of specific adducts or bound ligands or cofactors. For these reasons native ESI is typically performed out of volatile buffers such as ammonium acetate or bicarbonate in order to minimize the negative impact these buffer salts can have on protein ion S/N.

Many methods have been developed for the removal of salts in solution prior to ESI, such as dialysis, ultrafiltration, and ion exchange chromatography, but these techniques are time-consuming, some taking hours to days to perform, and they can result in significant sample dilution or loss of sample, particularly for small volumes of sample. Acidifying solutions can decrease sodium ion adduction as well. McLuckey showed that less sodium adduction is observed when the pH of an ESI solution is ~3 units lower than the pI of a protein. However, acidification often destabilizes or entirely denatures a protein or protein complex in solution, and many proteins and protein complexes lose activity at lower pH, resulting in the loss of information on the native, folded and active version of the protein. A number of different additives that do not denature samples in bulk solution have been discovered for removing salt during the ESI process itself. Buffer loading is one example, in which spraying a protein solution containing sodium from, ironically, high concentrations of another salt ammonium acetate (7 M), markedly reduces sodium ion adduction in native ESI and can thereby improve S/N by ~10-fold. Such high ionic strength solutions can lead to changes in protein or protein complex structure and activity, however. For example, buffer loading with NtrC resulted in dissociation of this hexameric complex starting at a concentration of ~1 M ammonium acetate, and the catalytic rate constant for this ATPase at these concentrations is about one tenth of that observed in a more typical native ESI solution with 220 mM ammonium acetate. Ammonium buffers other than ammonium acetate can be added at much lower concentrations and are far more efficient at protein desalting. Ammonium bromide and iodide can be added at just 25 mM concentration to decrease the average number of sodium adducts to a given charge state by a factor of six and increase the abundance of the most abundant ion by a factor of six. Millimolar concentrations of amino acids such as serine that have high gas phase sodium ion affinities reduce sodium ion adduction to proteins and can be used to narrow mass spectral peaks and improve mass measuring accuracy for large protein complexes. Another approach to desalting involves adding ion chelators to sample solutions in order to sequester salt ions that would otherwise adduct to protein molecules. For example, citrate and tartrate can be used to reduce non-specific calcium adduction to proteins, while not removing specifically bound calcium ions. Chapter 4 demonstrates that supercharging reagents can be used as additives for protein ion desalting during ESI, likely by binding to sodium ions either in solution or during the final stages of the ESI droplet lifetime.

While less common, desalting can also be performed in the gas phase. For example, Dearden and coworkers showed that crown ether molecules can be leaked into a mass spectrometer to electrostatically strip adducted alkali cations from peptides during collisions with these ions. Fabris and coworkers demonstrated that a number of different highly negatively charged small molecules that are metal ion chelators, such as citrate and ethyleneglycoltetraacetate (EGTA), can strip cation adducts from negatively charged DNA and
RNA ions stored inside a mass spectrometer. McLuckey and coworkers showed that via gas phase ion-ion reactions, a cation of choice can be added to or removed from a peptide ion.

1.7 The Hofmeister Series

Salts can influence protein structure both in the solution phase and the gas phase. In solution, some proteins and protein complexes require small amounts of salt cofactors in order to fold, be active, or assemble into a complex. For example, human coagulation factor VIII depends on the presence of calcium and copper in order to be active, and the oligomeric state of the protein DnaB helicase, involved in DNA replication in *E. coli*, is dependent on the concentration of Mg<sup>2+</sup>. Salts can also indirectly affect the solution phase structure of a protein. The Hofmeister series describes a ranking of cations and anions according to their relative abilities to stabilize or salt out proteins in aqueous solutions and was discovered in 1888 by Franz Hofmeister. The Hofmeister series of ions is shown below:

Cations: N(CH<sub>3</sub>)<sub>4</sub> + > NH<sub>4</sub> + > Cs<sup>+</sup> > K<sup>+</sup> > Na<sup>+</sup> > Li<sup>+</sup> > Mg<sup>2+</sup> > Ca<sup>2+</sup> > Gdm<sup>+</sup>

Anions: citrate<sup>3-</sup> > SO<sub>4</sub><sup>2-</sup> > HPO<sub>4</sub><sup>2-</sup> > C<sub>2</sub>H<sub>5</sub>O<sub>2</sub> <sup>-</sup> > HCO<sub>3</sub> <sup>-</sup> > CHO<sub>2</sub> <sup>-</sup> > Cl<sup>-</sup> > Br<sup>-</sup> > I<sup>-</sup> > ClO<sub>4</sub> <sup>-</sup> > SCN<sup>-</sup>

with ions toward the left of the series favoring protein stabilization and salting out, and ions toward the right of the series favoring protein destabilization and salting in. In general anions are more influential than cations in the series. Guanidinium (Gdm<sup>+</sup>) is at the right end of the series, consistent with its frequent use in biochemistry as a chemical denaturant in order to measure the relative stabilities of proteins. Sulfate is at the left end of the series, and ammonium sulfate at several molar concentration is routinely used to natively salt out proteins during protein purification. While Hofmeister effects are widely studied, the exact mechanism of protein salting in or salting out in the presence of different ions is not yet fully understood. The Hofmeister series has been related to a number of different physical properties of ions in solution, such as surface tension increment, viscosity B-coefficient, free energy of hydration, polarizability, and surface/bulk partitioning, all properties related to how strongly these ions interact with water and how strongly they influence the structure of water in solution around a protein. However, there is not a single property alone that defines how an ion influences protein ion structure.

Hofmeister effects have been studied in ESI-MS as well. Infrared photodissociation (IRPD) studies on water droplets containing a single Hofmeister cation or anion have shown that the number of water solvation shells that are structured by the ion follow the Hofmeister ranking. And selective surface sampling of droplets by ESI have verified the ranking of Hofmeister salts in terms of their surface/bulk partitioning. However, the effects of Hofmeister salts on protein stability in ESI-MS have not been observed in ion mobility experiments of gas phase protein ions. Ruotolo found that protein complexes were stabilized in the gas phase by select anions from the Hofmeister series, but that the ordering of anions according to how efficient they were at stabilizing complex structure was related to their relative gas phase acidities, and to evaporative cooling when the anions desorb from the protein surface in the gas phase. Williams found that no significant differences were observed in gas phase monomeric proteins with adducts of different Hofmeister anions. In Chapter 6, investigations into buffer effects on the effectiveness of electrothermal supercharging reveal a correlation between the Hofmeister series and the degree of protein unfolding observed due to
electrothermal supercharging, suggesting the physical properties involved in the manifestation of this series are linked to the mechanism of electrothermal supercharging.
2.1 Abstract

The effectiveness of the supercharging reagents \( m \)-nitrobenzyl alcohol (\( m \)-NBA) and propylene carbonate at producing highly charged protein ions in electrospray ionization is compared. Addition of 5 \% \( m \)-NBA and 15 \% propylene carbonate increases the average charge of three proteins by \( \sim 21 \% \) or \( \sim 23 \% \), respectively, when these ions are formed from denaturing solutions (water/methanol/acetic acid). These results indicate that both reagents are nearly equally effective at supercharging when used at their optimum concentrations. A narrowing of the charge state distribution occurs with both reagents, although this effect is greater for propylene carbonate. Focusing the ion signal into fewer charge states has the advantage of improving sensitivity. The maximum charge state of ubiquitin formed with propylene carbonate is 21+, four charges higher than previously reported. Up to nearly 30 \% of all residues in a protein can be charged, and the highly charged ions of both ubiquitin and cytochrome \( c \) formed with these supercharging reagents have collisional cross sections corresponding to those calculated for highly extended near linear conformations. Under native supercharging conditions, \( m \)-NBA is significantly more effective at producing high charge states than propylene carbonate.

2.2 Introduction

Electrospray ionization (ESI) mass spectrometry (MS) is a powerful tool for protein identification and structural characterization. Protein sequence and information about posttranslational modifications can be obtained from bottom-up\(^{76, 77}\) or top-down\(^{78-80}\) tandem mass spectrometry (MS/MS). Information about higher order structure can be determined from methods such as hydrogen-deuterium exchange,\(^69, 97, 151-153\) oxidative labelling,\(^152, 154-156\) and chemical crosslinking,\(^157-161\) in which information about conformation or protein interactions is encoded in solution, and this information is read out in the gas phase using MS/MS. These methods have been used to investigate the assembly pathways and dynamics of large protein complexes with molecular weights greater than a MDa.\(^153, 160-162\) One simple indicator of protein conformation in solution is the charge-state distributions of multiply protonated ions produced by ESI. Ions formed by ESI from aqueous solutions in which a protein is folded have charge-state distributions that are typically narrow and at low charge (high \( m/z \)). In contrast, the charge-state distributions of ions formed from solutions in which a protein is unfolded, often containing organic solvents and acid, are typically broad and at high charge (low \( m/z \)). Biomodal or multimodal charge-state distributions indicate the presence of two or more distinct conformers in...
The populations of different conformers in solution have been deduced by modeling the charge-state distributions.32-35

The conformation of ions formed by ESI can differ substantially from those of the original ions in solution, and much work has gone into investigating the structural changes that can occur during the ESI process and prior to mass analysis.36,163-166 However, some elements of structural information can be retained in the gas-phase ions. Low charge ions of proteins formed from buffered aqueous solutions often have compact conformations with cross sections that are commonly compared to those calculated from crystal structures.36-38 High charge ions of proteins formed by ESI from denaturing solutions typically have more elongated conformations with larger cross sections than the lower charge state ions of the same protein formed from native aqueous solutions.30,36-38 For example, the cross sections of the 6+ through 8+ charge states of ubiquitin are similar to those calculated from molecular dynamics structures that use the crystal structure as a starting geometry.37 The collision cross section for the 13+ ion of ubiquitin, the highest cross section for this protein reported in the literature, is about two times larger than the 6+ through 8+ ions, indicating that the 13+ ion is substantially elongated.37 Molecular dynamics simulations of ubiquitin 13+ indicate that although the N-terminal half of the protein has a more compact, folded structure, the C-terminal half of the protein is likely devoid of secondary structure and is a near-linear chain.167 Although Coulomb repulsion in highly charged ions can make unfolding of compact structures in the gas phase favorable, the reactivities168-170 and cross sections171-174 of ions formed from different solutions can differ even for ions with the same charge state. This clearly indicates that ions can retain a “memory” of their solution-phase conformations even though structural changes can still occur after ion formation.

More sequence information is typically obtained in MS/MS of high charge-state ions especially in the “top-down” approach in which intact proteins are fragmented directly.43-46,93,175,176 Cross sections in electron capture42,90 and electron transfer176,177 dissociation increase with increasing ion charge, resulting in higher fragmentation efficiencies. Improved fragmentation and sequence information has also been attributed to fewer non-covalent interactions holding the protein structure together after backbone cleavage.178,179 Higher charge states can also be advantageous for improving signal in mass analyzers that use charge image detection, such as FT-ICR and Orbitrap mass spectrometers, and can lower the \( m/z \) of large proteins into a range where mass spectrometers with upper \( m/z \) limits can be used.

Manipulating the charge-state distributions of ions formed by ESI can be advantageous. The charge states of protein ions can be reduced using a variety of methods, both after ion formation as well as during ESI. The charge states of protein ions can be reduced by adding basic molecules, either in solution26-28 or in the gas phase,27,53-55 that can carry away some of the charge. Ion-ion reactions can also be effective at lowering charge. For example, the 1+ charge state of ubiquitin (8.6 kDa), cytochrome c (12.3 kDa), and myoglobin (17.6 kDa) can be produced by reacting the high charge states of these ions formed by ESI with perfluoro-1,3-dimethylocyclohexane-F anions.47-49 Alternatively, protein ions produced by ESI from solutions containing tetramethylammonium (TMA+) acetate can have multiple weakly bound TMA+ adducts. Collisional activation of these ions results in loss of TMA+ that can lead to the efficient formation of the singly protonated form of each of these proteins.58

In contrast, there are relatively few methods that can be used to substantially increase the charge states of ions. Addition of multivalent ions, such as La3+, to electrospray solutions can lead to attachment of one or more of these ions to a protein and produces a corresponding increase in charge.64 Electrothermal supercharging, a method for increasing the charging of
protein ions formed from buffered aqueous solutions, can produce nearly the same extent of charging as that obtained for the same protein ions formed by ESI from denaturing solutions. The charge on protein ions can also be increased from both denaturing and native aqueous solutions with using supercharging reagents, such as m-nitrobenzyl alcohol (m-NBA) or sulfolane. The concentrations of these high-boiling point reagents in the ESI droplets becomes enriched as desolvation occurs, and the increased concentration can produce changes to both the surface tension of the droplet, and to the protein conformation. Although the mechanism of supercharging is still debated, many experiments have shown the effects of the supercharging reagents on both surface tension and protein conformation. Sulfolane, a supercharging reagent often used from native solutions, destabilizes the folded form of myoglobin directly proportional to its concentration in solution. As solvent evaporation from the ESI droplets occurs, the concentration of sulfolane increases, resulting in an increasingly denaturing environment for proteins. The roles of both surface tension and protein conformation in supercharging were shown for two forms of the protein ribonuclease A in the same solution; a native form with four intact disulfide bonds and a reduced-alkylated form that has no disulfide bonds and is a random coil in native solutions. When these ions are formed from an aqueous ammonium acetate solution, the native ribonuclease A charge states increase with increasing m-NBA concentration, whereas the charges states of reduced-alkylated ribonuclease A decrease. The former is due to the supercharging reagent destabilizing the native protein conformation resulting in partial unfolding whereas the latter is due to a lowering of the surface tension, both phenomenon induced by the presence of m-NBA. These results show that the increased charging as a result of protein unfolding induced by the supercharging reagents can overcome the decrease in charging attributable to a decrease in droplet surface tension caused by these reagents under native conditions.

Since the initial report of the effectiveness of supercharging reagents for increasing charging in ESI, many effective supercharging reagents and applications for these reagents have been reported. Recently, Donald and coworkers showed that o-nitroanisole, ethylene carbonate, and propylene carbonate (PC) are supercharging reagents that can significantly increase protein charging from denaturing solutions. They found that 15 % propylene carbonate was more effective than 0.5 % m-NBA, although even higher concentrations of m-NBA have been shown to improve supercharging.

Here, we show that 5 % m-NBA, near the solubility limit of this compound in denaturing solutions, can produce protein charging that is within ~4 % of that obtained with 15 %, but m-NBA is a substantially more effective supercharging reagent in native aqueous solutions with or without ammonium acetate buffer. The collision cross sections for the high charge-state ions produced with these supercharging reagents in which there is a positive charge for every three to five residues indicate that these ions have highly elongated, near-linear structures in the gas phase.

2.3 Experimental

Protein ions were formed by nanoelectrospray ionization (nanoESI) from borosilicate capillaries (1.0 mm o.d./0.78 mm i.d., Sutter Instruments, Novato, CA, USA) pulled to a tip i.d. of ~1 µm with a Flaming/Brown micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA). The borosilicate capillaries were positioned ~2 mm from the inlet to the mass spectrometer, and spray was initiated by applying ~0.8 kV to a 0.127 mm diameter platinum wire
inserted into the sample solution. Data were acquired using a Thermo LTQ (Linear Trap Quadrupole) -Orbitrap™ with the inlet capillary heated to 250 °C unless otherwise noted. Both a Waters Quadrupole-Time-of-Flight (Q-TOF) Premier and a Waters Synapt G2 (Waters, Milford, MA, USA) mass spectrometer with inlet capillary temperatures at 80 °C were also used to investigate effects of the ESI interface and other instrumental conditions. Mass spectra were acquired in triplicate for each sample using three different capillaries to account for tip-to-tip variations in charge-state distributions. All proteins, supercharging reagents, methanol, acetic acid, and platinum wire were obtained from Sigma Aldrich (St. Louis, MO, USA).

The width of a distribution is given in units of charge and was calculated as the full width at half maximum of a Gaussian curve fit to a monomodal charge-state distribution, or as the sum of the full width at half maximum of two Gaussian curves fit to a bimodal charge-state distribution. Gaussian fits were done using equation 1 with Origin 9.0 (OriginLab Corporation, Northampton, MA, USA):

\[ I = A e^{-\frac{(z-z_c)^2}{2w^2}} \]  

where \( I \) is ion abundance, \( A \) is a constant, \( z \) is charge, \( z_c \) is charge at the centroid, and \( w \) is the standard deviation of the Gaussian.

Drift times in nitrogen gas were measured using travelling wave ion mobility spectrometry (TWIMS) with a wave velocity of 650 m/s, a wave height of 40 V, and helium and nitrogen flow rates of 180 and 90 mL/min, respectively. The method of Robinson and coworkers\(^\text{193}\) was used to obtain collisional cross sections from the TWIMS drift times. Briefly, TWIMS drift times, corrected for factors such as \( m/z \)-dependent flight times, are plotted as a function of cross sections measured with nitrogen gas in static drift tubes. This was done for ubiquitin 7+ – 13+, cytochrome c 14+ – 19+, and myoglobin 15+ – 24+, all produced by nanoESI from denaturing solutions consisting of methanol, water, and acetic acid (45/54/1 by volume) (see Supporting Information). These data are linear, and the best-fit line has an \( R^2 \) value of 0.9995 (Figure S-3). The average and highest deviation of these data from the line is 0.6 and 1.6 %, respectively, and these values provide some indication of the uncertainty associated with this method. Cross sections reported in the text are the average of three measurements from three different nanoESI capillaries, and the average precision of these cross sections is ~0.2 %. The uncertainty of the cross sections obtained using this method was also evaluated by using just the data for ubiquitin and myoglobin for the calibration and determining the cross sections of cytochrome c using these data. The average and maximum deviation in cross sections from the corresponding literature cross sections for all charge states of cytochrome c, is 1.2 and 1.9 %, respectively.

Cross sections in helium, calculated from low-energy structures identified by molecular dynamics simulations and reported by Bowers and coworkers\(^\text{37}\) and Clemmer and coworkers\(^\text{36}\) were converted to cross sections in nitrogen using a similar calibration procedure. Cross sections for ubiquitin 9+ – 13+, cytochrome c 14+ – 19+ and myoglobin 18+ – 24+ measured in helium were plotted as a function of cross sections measured in nitrogen (see Supporting Information, Figure S-4). These data are linear with \( R^2 =0.998 \). The average deviation of these data from the best fit line is 1.8 %.

2.4 Results and Discussion

2.4.1 Supercharging with propylene carbonate or \( m \)-NBA from denaturing solutions. The charge-state distribution of ubiquitin ions formed by nanoESI from denaturing
solution (45/54/1 methanol/water/acetic acid by volume) is relatively narrow and is centered around the 12+ charge state (Figure 1a), with an average charge and maximum charge of 11.6 ± 0.4+ and 14+, respectively (Table 1). With 5 % m-NBA, the charge-state distribution is much narrower and is centered around 13+ (Figure 1b). Both the average and maximum charge are higher: 13.2 ± 0.1+ and 16+, respectively (Table 1). With 15 % PC, the average and maximum charge is slightly higher than that obtained with 5 % m-NBA: 13.5 ± 0.3+ and 17+, respectively (Figure 1c, Table 1). Although PC is soluble at higher concentrations, there is no increase in charging with up to 50 % PC (Figure S-1). These data show that both m-NBA and PC are effective at producing ions charged well above the number of basic sites (Lys, Arg, His, and the N-terminus) in ubiquitin (13).

The charge-state distributions of cytochrome c (Figure 1d-f) and carbonic anhydrase (Figure 1g-i) also shift to significantly higher charge with 5 % m-NBA or 15 % PC, with the most abundant charge state increasing from 16+ to 22+ for cytochrome c and from 34+ to 39+ or 40+ for carbonic anhydrase. Similar charge enhancement was obtained with m-NBA and PC (Table 1), as was the case for ubiquitin. Supercharging increases the average charge of these three proteins by 21 % and 23 % for m-NBA and PC, respectively. These results indicate that both reagents are nearly equally effective at supercharging proteins from denaturing solutions when used at their optimum concentrations.

In addition to increasing charging, another advantage of supercharging reagents is that they can produce narrower charge-state distributions. Reducing the width of the charge-state distributions can increase detection limits because the protein ion signal is distributed over fewer charge states, thereby leading to increased signal-to-noise ratios (S/N) for charge states that are produced. This is particularly advantageous for larger proteins, which typically have broad charge-state distributions when ions are formed by ESI from denaturing solutions. This is also beneficial in MS/MS experiments for increasing the S/N of a mass-selected precursor. For ubiquitin, the charge-state distribution is only half the width with PC than without. The 13+ is the most abundant ion with PC, and is ~59 % of the integrated ion signal whereas the 12+ is most abundant without this supercharging reagent and is only ~42 % of the integrated ion signal. PC is slightly more effective at narrowing the distribution than m-NBA (Table 1). For carbonic anhydrase, the charge-state distribution is slightly bimodal without supercharging reagents but is monomodal with a high charge distribution with either supercharging reagent, consistent with additional unfolding of the protein occurring during ESI with these supercharging reagents.

The extent of charging obtained with ESI depends on both the type of instrument and the experimental parameters that are used. The recent supercharging results obtained by Donald and coworkers, in which higher charging was obtained with 15 % PC than with 0.5 % m-NBA, were also obtained with a Thermo LTQ mass spectrometer. Our results indicate that by increasing the m-NBA concentration to a more optimum concentration, the extent of charge enhancement obtained with both supercharging reagents is more comparable. Similar extents of supercharging with 15 % PC were obtained for both cytochrome c and carbonic anhydrase but less charging was observed for ubiquitin. A higher source capillary temperature (400 °C) was used in the previous study, which may lead to more extensive unfolding of the protein in the ESI droplet, and this effect may account for the greater charging of ubiquitin reported earlier.

Effects of capillary temperature were investigated by varying the source capillary temperature from 150 to 350 °C. The average charge of ubiquitin decreased by ~0.3+ with increasing temperature within this range, consistent with previous reports that show that more energetic source conditions can lead to a lowering of charge. These results indicate that the
capillary temperature does not have a large effect on the charge-state distributions of proteins with PC within this limited temperature range. However, other parameters, such as electrospray voltage, can play a significant role by increasing the extent of protein unfolding that can occur in the ESI droplets.65, 180

Other factors that might affect these supercharging results were investigated by repeating these experiments using a Waters Q-TOF Premier mass spectrometer, and these results are shown in Figure 2 and Table 2. The average charge state for all three proteins is about one charge lower with this instrument compared to the results on the LTQ mass spectrometer. The charge-state distributions are also broader, and bimodal in some cases (Figure 2a,d,g,h) with a lower charge distribution indicative of a more compact conformation in solution. These results may be due to more unfolding and hence higher charging that can be obtained with the more energetic source conditions of a LTQ instrument, consistent with results from electrothermal supercharging.65, 180 The average charge for the three proteins obtained using the Q-TOF instrument increases by ~20% with m-NBA and by ~24% with PC, similar to the increases in average charge observed with the LTQ instrument. This indicates that the relative change enhancement obtained with these reagents is not strongly dependent on the mass spectrometer used.

A surprising result is that the charge-state distribution of ubiquitin with PC obtained with the Q-TOF instrument is bimodal (Figure 2c), with a low abundance but very high charge distribution that extends up to 21+. This is four charge states higher than the highest value previously reported for the all protonated form of ubiquitin, and eight charge states higher than the number of basic sites on this protein. A similar phenomenon was not observed for cytochrome c or carbonic anhydrase.

2.4.2 Collision cross sections of supercharged protein ions. In order to obtain information about the structures of the highly charged ions produced with supercharging reagents, collisional cross sections were measured using travelling wave ion mobility spectroscopy (TWIMS). Drift profiles of ubiquitin 7+–21+ ions formed by nanoESI from denaturing solution without supercharging reagent (blue dashed line), with 5% m-NBA (red dashed line), and with 15% PC (black solid line) are shown in Figure 3. Collision cross sections obtained from the Figure 3 drift profiles, determined using the method of Robinson and coworkers,193 are shown in Figure 4. The drift profiles for a given charge state produced by ESI from the three different solutions and the resulting collisional cross sections are essentially indistinguishable within the accuracy of the method used to obtain these cross sections. This result is consistent with previous cross section measurements of ions formed by native supercharging.30 These results also indicate that there is only a single structure or family of unresolved structures for charge states 8+ and higher.

The highest charge state of ubiquitin for which a cross section has been reported is the 13+, and this charge state has the largest cross section that has been measured for this protein in the gas phase (26.0 nm² in nitrogen).193 For the higher charge states of ubiquitin produced here, the cross section increases even more with increasing ion charge (Figure 4). There appears to be only a slight increase in cross section between the 17+ and 19+ ions, but both the 20+ and 21+ ions continue the trend of increasing cross section observed for the lower charge states. The dashed lines in Figure 4 correspond to the collision cross sections calculated by Bowers and coworkers using an exact hard sphere scattering model (EHSS) for four different structures of ubiquitin: the A state (A), an all-helical state (H), an unfolded, high temperature state (U), and a
near linear state (L). The values for helium were converted to nitrogen values using the method described in the Experimental section. The cross section obtained for the 21+ charge state of ubiquitin is 31.4 nm², which is close to the value of 33.5 nm² calculated for the near linear state. This indicates that this highly charged ion is extraordinarily unfolded in the gas phase. Ubiquitin has 76 residues, so nearly one third of all residues is charged in the 21+ charge-state ion, resulting in high Coulomb repulsion between the charges in this ion.

The cross sections measured for the 13+ – 24+ charge states of cytochrome c formed from denaturing solutions without supercharging reagent, with 5 % m-NBA, and with 15 % PC are shown in Figure 5. The cross sections for a given charge state formed using these different solution conditions are within the uncertainty of this method except for both the 17+ and 18+ charge states. For these ions, the cross section is substantially lower when these ions are formed from denaturing solution without supercharging reagent. The cross section increases more slowly between 20+ and 22+, but then increases more quickly for the higher charge states. The dashed lines in Figure 5 are the cross sections of an all helical conformation (H), an average of random coil structures (R), and a near linear, fully extended form (L) of cytochrome c calculated by Jarrold and coworkers using EHSS and converted from helium to nitrogen values. The highest reported cross section for cytochrome c is 29.2 nm² in helium for the 20+ charge state and about 37.6 nm² in nitrogen. Our data shows that the higher charge states obtained using supercharging reagents leads to even more elongated structures. The cross section for the 24+ charge state is 40.1 nm² and 39.7 nm² with m-NBA and PC, respectively, and are indistinguishable within the uncertainty of this method. These values are less than the computed near-linear limit of 44.5 nm², but significantly higher (~32 %) than the computed all helical form with a cross section of 30.6 nm². This indicates that this ion must be highly extended with no significant extent of secondary structure. Cytochrome c has 104 residues, so ~23 % of all residues have a proton in the 24+ charge state.

The data for both ubiquitin and cytochrome c (Figures 3 and 4) show that the trend in the loss of secondary structure for the high charge state ions measured previously continues for the even higher charge states reported here. The cross sections of the highest charge states of ubiquitin (21+) and cytochrome c (24+) are on average ~31 % higher than an all helical form of these proteins and ~11 % higher than unfolded, random coil conformations that have been calculated previously. The increase in cross section between the all helical and near-linear conformations is greater for cytochrome c than for ubiquitin, possibly due to the presence of the covalently bound heme group in cytochrome c. The trend in the increase in cross section with increasing charge shows a limited range of high charge states where this increase is less pronounced. For ubiquitin and cytochrome c, this occurs from 17+ – 19+ (Figure 3) and 20+ – 22+ (Figure 4), respectively. Clemmer and coworkers also reported only a slight increase in cross section for the 18+ and 19+ charge states of cytochrome c. These transition regions may be a result of a barrier to structural change that can be surmounted by addition of charge and the resulting increase in Coulomb repulsion between charges that could unfold local regions of structure. It is energetically favorable for polarizable atoms of nearby residues to solvate charge sites in the gas phase, causing local regions of structure that enhance charge solvation. These transition regions may be attributable to overcoming a barrier to partial unfolding of these charge solvation sites resulting in more elongated protein ions.

2.4.3 Supercharging from native solutions. In order to determine the relative effectiveness of m-NBA and PC in supercharging proteins from aqueous solutions in which
proteins have a native folded structure, nanoESI mass spectra of ubiquitin formed from aqueous solutions with and without these supercharging reagents were obtained (Figure 6). In aqueous solution with no buffer, the charge-state distribution of ubiquitin without supercharging reagent (Figure 6a) is narrow, with an average and maximum charge of 6.2 ± 0.1+ and 10+, respectively. With 1.5 % m-NBA (the solubility limit of m-NBA in water) (Figure 6b) or 5 % propylene carbonate (Figure 6c), the distributions are broader and shifted to higher charge with an average charge of 9.1 ± 0.4+ and 8.1 ± 0.1+, respectively. The maximum charge state is 13+ with both supercharging reagents (Table 3). These results indicate that m-NBA is only slightly more effective at supercharging than propylene carbonate in pure water. Loo and coworkers also showed that 5 % PC can result in an increase in charge of myoglobin ions formed from pure water.\(^{75}\)

In order to determine the effect of more commonly used buffered solutions, these experiments were repeated using ~200 mM aqueous ammonium acetate with and without these supercharging reagents. The average charge state of ubiquitin formed from ammonium acetate is less than one charge lower than when these ions are formed from pure water (Figure 6d). These results are consistent with stabilization of more compact folded protein structures owing to the high ionic strength of the 200 mM ammonium acetate solution, although other factors likely play a role as well.\(^{58}\) There is a ~50 % increase in the average charge state with 1.5 % m-NBA, but, as is the case without this supercharging reagent, the average charge is ~0.5+ lower when ammonium acetate is present. In striking contrast, the average charge is significantly lower with 5 % PC and ammonium acetate present (5.8 ± 0.1+) (Figure 6f) than without the buffer present (8.1 ± 0.1+) (Figure 6c). Similarly, the maximum charge state is reduced from 13+ to 8+. The charge-state distribution with PC and ammonium acetate (Figure 6f) is similar to that obtained without supercharging reagent present (Figure 6d), indicating that very little destabilization of the protein conformation occurs with PC from a buffered aqueous solution.

Only a minor charge enhancement is obtained with 5 % PC for cytochrome \(c\) and carbonic anhydrase ions formed from 200 mM aqueous ammonium acetate solutions as well. For these proteins, the average charge state increases only by ~16 % with PC, whereas this increase is ~54 % with m-NBA. These results indicate that m-NBA is a much more effective supercharging reagent than PC when forming ions from native aqueous solutions that have high buffer concentrations.

### 2.5 Conclusions

The effectiveness of two supercharging reagents, m-NBA and propylene carbonate, on increasing charging of protein ions formed by ESI was compared. When used at their optimum concentrations in denaturing solutions, both reagents are nearly equally effective, although propylene carbonate produces slightly higher average charging and a low abundance of significantly higher charges for one of the three proteins investigated. The charge-state distribution is narrower with both reagents, although propylene carbonate is slightly more effective. This is advantageous for improving sensitivity by focusing more ion signal into fewer charge states. Under native mass spectrometry conditions, m-NBA is significantly more effective than propylene carbonate.

Up to nearly 30 % of the residues in a protein can be protonated for the highest charge states produced with these supercharging reagents. The most highly charged ions have large cross sections that are only slightly lower than those calculated for near linear conformations.
These highly extended structures should be advantageous for direct protein structural characterization by MS/MS because there should be few noncovalent bonds that would prevent dissociation from occurring after bond cleavage and these ions should have very high electron capture cross sections in electron transfer or electron capture dissociation.

2.6 Acknowledgements

The authors thank the National Institutes of Health (Grant No. R01GM097357) and the National Science Foundation (Graduate Research Fellowship for CAC; Grant No. DGE1106400) for financial support.
Table 1. Average and maximum charge and width of the charge-state distribution formed by nanoESI from denaturing solution (45/54/1 methanol, water, acetic acid by volume) without supercharging reagent, with 5 % \textit{m}-NBA, and with 15 % propylene carbonate measured using an LTQ mass spectrometer.

<table>
<thead>
<tr>
<th></th>
<th>Average Charge</th>
<th>Maximum Charge</th>
<th>Width of Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>11.6 ± 0.4+</td>
<td>14+</td>
<td>1.8 ± 0.3+</td>
</tr>
<tr>
<td>+ 5 % \textit{m}-NBA</td>
<td>13.2 ± 0.1+</td>
<td>16+</td>
<td>1.1 ± 0.1+</td>
</tr>
<tr>
<td>+ 15 % PC</td>
<td>13.5 ± 0.3+</td>
<td>17+</td>
<td>0.9 ± 0.3+</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>15.8 ± 0.2+</td>
<td>21+</td>
<td>3.8 ± 0.6+</td>
</tr>
<tr>
<td>+ 5 % \textit{m}-NBA</td>
<td>20.7 ± 0.5+</td>
<td>25+</td>
<td>4.6 ± 0.9+</td>
</tr>
<tr>
<td>+ 15 % PC</td>
<td>21.5 ± 0.1+</td>
<td>25+</td>
<td>2.4 ± 0.1+</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>33.2 ± 0.2+</td>
<td>45+</td>
<td>6.5 ± 0.8+</td>
</tr>
<tr>
<td>+ 5 % \textit{m}-NBA</td>
<td>39.0 ± 0.1+</td>
<td>48+</td>
<td>5.0 ± 0.1+</td>
</tr>
<tr>
<td>+ 15 % PC</td>
<td>39.1 ± 1.4+</td>
<td>49+</td>
<td>4.9 ± 1.6+</td>
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</tbody>
</table>
Table 2. Average and maximum charge and width of the charge-state distribution formed by nanoESI from denaturing solution (45/54/1 methanol, water, acetic acid by volume) without supercharging reagent, with 5 % m-NBA, and with 15 % propylene carbonate measured using an Q-TOF mass spectrometer.

<table>
<thead>
<tr>
<th></th>
<th>Average Charge</th>
<th>Maximum Charge</th>
<th>Width of Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>10.3 ± 0.4+</td>
<td>13+</td>
<td>5.4 ± 0.1+</td>
</tr>
<tr>
<td>+ 5 % m-NBA</td>
<td>12.2 ± 0.1+</td>
<td>15+</td>
<td>1.1 ± 0.1+</td>
</tr>
<tr>
<td>+ 15 % PC</td>
<td>13.1 ± 0.9+</td>
<td>21+</td>
<td>1.3 ± 0.1+</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>15.7 ± 0.8+</td>
<td>21+</td>
<td>5.0 ± 1.2+</td>
</tr>
<tr>
<td>+ 5 % m-NBA</td>
<td>19.6 ± 0.7+</td>
<td>24+</td>
<td>4.0 ± 0.2+</td>
</tr>
<tr>
<td>+ 15 % PC</td>
<td>19.7 ± 0.6+</td>
<td>23+</td>
<td>2.9 ± 0.1+</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>31.0 ± 0.3+</td>
<td>43+</td>
<td>13.5 ± 2.5+</td>
</tr>
<tr>
<td>+ 5 % m-NBA</td>
<td>36.1 ± 0.5+</td>
<td>47+</td>
<td>9.2 ± 0.6+</td>
</tr>
<tr>
<td>+ 15 % PC</td>
<td>37.0 ± 0.4+</td>
<td>43+</td>
<td>3.0 ± 0.2+</td>
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</table>
Table 3. Average and maximum charge of ubiquitin ions formed by nanoESI from pure water or aqueous ammonium acetate without supercharging reagent, with 1.5 % m-NBA, and with 5 % propylene carbonate measured using an LTQ mass spectrometer.

<table>
<thead>
<tr>
<th></th>
<th>Average Charge</th>
<th>Maximum Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>6.2 ± 0.1+</td>
<td>10+</td>
</tr>
<tr>
<td>+ 1.5 % m-NBA</td>
<td>9.1 ± 0.4+</td>
<td>13+</td>
</tr>
<tr>
<td>+ 5 % PC</td>
<td>8.1 ± 0.1+</td>
<td>13+</td>
</tr>
<tr>
<td>200 mM AA</td>
<td>5.4 ± 0.1+</td>
<td>6+</td>
</tr>
<tr>
<td>+ 1.5 % m-NBA</td>
<td>8.6 ± 0.4+</td>
<td>11+</td>
</tr>
<tr>
<td>+ 5 % PC</td>
<td>5.8 ± 0.1+</td>
<td>8+</td>
</tr>
</tbody>
</table>
2.8 Figures

Figure 1. NanoESI mass spectra of ubiquitin (a-c), cytochrome c (d-f), and carbonic anhydrase (g-i) in denaturing solution (45/54/1 methanol, water, acetic acid by volume) without supercharging reagent (top), with 5 % m-NBA (middle), and with 15 % propylene carbonate (bottom) measured using a LTQ mass spectrometer.
Figure 2. NanoESI mass spectra of ubiquitin (a-c), cytochrome c (d-f), and carbonic anhydrase (g-i) in denaturing solution (45/54/1 methanol, water, acetic acid by volume) without supercharging reagent (top), with 5 % m-NBA (middle), and with 15 % propylene carbonate (bottom) measured using a Q-TOF mass spectrometer.
Figure 3. TWIMS drift profiles measured in nitrogen of ubiquitin ions formed by nanoESI from denaturing solutions containing methanol/water/acetic acid without supercharging reagent (dashed blue line), with 5% m-NBA (dashed red line), or with 15% propylene carbonate (solid black line).
Figure 4. Collision cross sections measured in nitrogen of ubiquitin ions formed by ESI from denaturing solutions containing methanol/water/acetic acid without supercharging reagent (red circles), with 5 % m-NBA (green triangles), or with 15 % propylene carbonate (blue squares). Dashed lines are calculated cross sections from simulations by Bowers and coworkers for the A state (A), the all alpha helix state (H), the average of several unfolded states (U), and a near-linear state (L). The cross sections from Bowers and coworkers\textsuperscript{37} were converted from helium to nitrogen values using the method described in the Experimental section and Supporting Information.
Figure 5. Collisions cross sections measured in nitrogen of cytochrome c ions formed by ESI from denaturing solutions containing methanol/water/acetic acid without supercharging reagent (red circles), with 5 % m-NBA (green triangles), or with 15 % propylene carbonate (blue squares). Dashed lines are calculated cross sections from simulations by Jarrold and coworkers for the all alpha helix state (H), the average of several random coil states (R), and a near-linear state (L). The cross sections from Jarrold and coworkers\textsuperscript{36} were converted from helium to nitrogen values using the method described in the Experimental section and Supporting Information.
Figure 6. NanoESI mass spectra of ubiquitin in water (a-c) or 200 mM ammonium acetate (d-f) without supercharging reagent (top), with 1.5% m-NBA (middle), and with 5% propylene carbonate (bottom) measured using a LTQ mass spectrometer.
Figure S-1. Nanoelectrospray mass spectra of ubiquitin in denaturing solution (45/54/1 methanol, water, acetic acid by volume) with increasing propylene carbonate concentration.
Figure S-2. Nanoelectrospray mass spectra of ubiquitin in denaturing solution (45/54/1 methanol, water, acetic acid by volume) acquired with a capillary temperature of 150 °C, 250 °C, and 350 °C (a, b, and c, respectively), and in denaturing solution with 15% propylene carbonate with a capillary temperature of 150 °C, 250 °C, and 350 °C (d, e, and f, respectively). The inset in (d) highlights the propylene carbonate molecule adduction to ubiquitin ions at low capillary temperatures.
2.9.1 Collision cross section measurements. Drift times were converted to collision cross sections using the best-fit line equation from the calibration curve in Figure S-3. The calibration curve was created by plotting corrected Synapt G2 drift times (tD"") versus literature cross sections in nitrogen measured in static drift tubes for the 7+-13+ ions of ubiquitin, 14+-19+ ions of cytochrome c, and 15+-24+ ions of myoglobin produced by nanoESI from denaturing solutions containing methanol, water, and acetic acid (45/54/1 by volume). Corrected drift times were calculated from measured drift times using the method of Robinson and coworkers. The resulting collision cross sections for ubiquitin and cytochrome c are given in Table S-1.

![Figure S-3](image-url)

**Figure S-3.** Calibration curve for converting drift times measured on Synapt G2 to collision cross sections.
Table S-1. Collision cross sections of ubiquitin and cytochrome c ions from denaturing solution with or without supercharging reagent.

<table>
<thead>
<tr>
<th>Charge State</th>
<th>Ubiquitin Cross Section (nm²)</th>
<th>Cytochrome c Cross Section (nm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denaturing Solution +5% m-NBA +15% PC</td>
<td>Denaturing Solution +5% m-NBA +15% PC</td>
</tr>
<tr>
<td>7+</td>
<td>18.8</td>
<td>18.9</td>
</tr>
<tr>
<td>8+</td>
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<td>20.1</td>
</tr>
<tr>
<td>9+</td>
<td>21.0</td>
<td>21.1</td>
</tr>
<tr>
<td>10+</td>
<td>22.3</td>
<td>22.5</td>
</tr>
<tr>
<td>11+</td>
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</tr>
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<tr>
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<td>26.3</td>
<td>26.5</td>
</tr>
<tr>
<td>14+</td>
<td>27.4</td>
<td>27.2</td>
</tr>
<tr>
<td>15+</td>
<td>28.3</td>
<td>28.3</td>
</tr>
<tr>
<td>16+</td>
<td>28.8</td>
<td>34.3</td>
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<tr>
<td>17+</td>
<td>29.2</td>
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<tr>
<td>18+</td>
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<td>39.5</td>
</tr>
<tr>
<td>24+</td>
<td></td>
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</tbody>
</table>
**Figure S-4.** Calibration curve for converting literature helium cross sections to literature nitrogen cross sections using cross section measurements for ubiquitin, cytochrome c, and myoglobin from static drift tubes.\(^{193}\)
Chapter 3

New Supercharging Reagents Produce Highly Charged Protein Ions in Native Mass Spectrometry

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Going, C. C.; Xia, Z.; Williams, E. R. Analyst, 2015, DOI: 10.1039/C5AN01710F

3.1 Abstract

The effectiveness of two new supercharging reagents for producing highly charged ions by electrospray ionization (ESI) from aqueous solutions in which proteins have native structures and reactivities were investigated. In aqueous solution, 2-thiophenone and 4-hydroxymethyl-1,3-dioxolan-2-one (HD) at a concentration of 2% by volume can increase the average charge of cytochrome c and myoglobin by up to 163%, resulting in even higher charge states than those that are produced from water/methanol/acid solutions in which proteins are denatured. The greatest extent of supercharging occurs in pure water, but these supercharging reagents are also highly effective in aqueous solutions containing 200 mM ammonium acetate buffer commonly used in native mass spectrometry (MS). These reagents are less effective supercharging reagents than $m$-nitrobenzyl alcohol ($m$-NBA) and propylene carbonate (PC) when ions are formed from water/methanol/acid. The extent to which loss of the heme group from myoglobin occurs is related to the extent of supercharging. Results from guanidine melts of cytochrome c monitored with tryptophan fluorescence show that the supercharging reagents PC, sulfolane and HD are effective chemical denaturants in solution. These results provide additional evidence for the role of protein structural changes in the electrospray droplet as the primary mechanism for supercharging with these reagents in native MS. These results also demonstrate that for at least some proteins, the formation of highly charged ions from native MS is no longer a significant barrier for obtaining structural information using conventional tandem MS methods.

3.2 Introduction

Electrospray ionization (ESI) mass spectrometry (MS) is widely used to transfer intact proteins and large macromolecular complexes into the gas phase for structural elucidation and is routinely used for protein identification and quantitation. Solutions containing organic solvents and/or acids are typically used with ESI, owing in part to both stable ion signals and to the high charge-state ions that can be produced. The more highly charged ions produced from denaturing solutions typically have extended gas phase conformations, and can be efficiently dissociated in tandem MS experiments. ESI from aqueous solutions in which a protein is in a folded, native-like conformation, or native MS, has the advantage that information about the stoichiometries and presence of ligand binding to proteins and protein complexes can be obtained. Native MS has been used to obtain information about assembly pathways of macromolecular complexes and about subunit interactions. Aqueous solution-phase structure of folded protein conformations can be studied through hydrogen-deuterium exchange and covalent labelling techniques, such as oxidative labelling. The distribution of charge states in ESI can be indicative of the protein conformation in solution.
Charge-state distributions from native MS are often narrow and low in charge, whereas charge state distributions in denaturing MS are typically broad and high in charge. Multimodal distributions can indicate the coexistence of multiple protein conformations.

The charge states of ions produced in native MS can be increased using several different methods, often collectively referred to as “supercharging”. Trivalent metal ion supercharging uses trivalent metal ion salts in low concentration to produce nonspecific trivalent metal ion adduction, which can result in more than a 50% increase in the maximum charge of protein ions produced by ESI from aqueous solutions. Electrothermal supercharging uses an elevated spray potential in native MS to unfold proteins in the ESI droplet. This results in charge-state distributions that have nearly the same maximum and average charge as those obtained from conventional denaturing solutions, making it the most effective supercharging technique for native solutions reported to date. Supercharging with reagents can be effective at increasing charge from both denaturing and native solutions. Typically, a small concentration of supercharging reagent (1-5% by volume) is added to a sample solution. These reagents do not significantly affect protein conformation in solution prior to ESI. These reagents all have boiling points higher than that of water and become enriched in the droplet as solvent evaporation occurs. These supercharging reagents can cause chemical/thermal denaturation in the ESI droplet, although these reagents also affect other physical properties, such as the droplet surface tension, that also play a role in charging.

Many experiments have been done to elucidate factors that affect supercharging in ESI. The supercharging reagent, m-NBA, increases the charge state of native RNase A but decreases the charge state of RNase A with all of its disulfide bonds reduced when these ions are formed from the same aqueous solution. The latter protein is a random coil in solution, and the lower surface tension of m-NBA compared to that of water results in less charging. In contrast, the charging of RNase A, which is folded in solution, increases as a result of the supercharging reagents destabilizing the folded form which causes unfolding to occur in the ESI droplet. This shows that the effect of conformational changes can be greater than the effect of droplet surface tension on the extent of protein charging. Less supercharging occurs for proteins that have limited ability to unfold, such as proteins with many disulfide bonds or other chemical cross links. Other methods to unfold proteins in ESI droplets have also been demonstrated. Proteins can be made to unfold in ESI droplets by adding gaseous reagents to change droplet pH or through rapid mixing experiments using theta glass emitters. Protein folding or unfolding processes induced by rapid mixing that occur on the low microseconds time scale of small droplets produced by nanoESI can be readily investigated.

A large number of factors affect charging in electrospray ionization, and alternate mechanisms for supercharging have been proposed. Supercharging reagents can adduct onto protein ions. More adduction to higher charge states has been observed suggesting that high charge states are formed via a "direct interaction" between the reagents and the proteins. Venter and coworkers suggested that the large dipole moments of many of these reagents (ranging between 3.96 for dimethyl sulfoxide (DMSO) to 4.35 for sulfolane compared to 1.85 for water) shields adjacent charges on basic sites through solvent reorganization, enabling more charge to be deposited on the protein ions during ESI. However, Donald and coworkers investigated a large set of reagents and found no correlation between protein supercharging from denaturing solution and reagent dipole moment. Proton transfer between the protein and the reagents has been suggested as a mechanism for supercharging. However, lower charging occurs at low concentration of the supercharging reagent DMSO as a result of compaction of the
protein in solution, but supercharging occurs at higher concentrations of DMSO as a result of protein destabilization in solution. The effect of reagent concentration on the reduction or increase in charge of the same protein provides strong evidence that proton transfer reactivity does not play a role on supercharging with this reagent.

The greatest extent of charging of protein ions that have been formed from denaturing solutions with supercharging reagents is approximately one in every three residues charged, and ions with this charge density have near-linear structures in the gas phase. But supercharging from native solutions has not yet produced comparable highly charged ions. Here, results with two new supercharging reagents, 2-thiophenone and HD, are presented. These reagents produce higher charge states than previously reported reagents and can produce higher charge states than can be formed from solutions containing water/methanol/acid that are typically used to produce high charge states of peptide and protein ions.

3.3 Experimental

All mass spectra were acquired using a Thermo LTQ (Linear Trapping Quadrupole) mass spectrometer unless otherwise noted. Ions were formed by nanoelectrospray (nanoESI) from 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 micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA). A voltage of ~0.7-1.0 kV was applied to a 0.127 mm diameter platinum wire inserted into the solution in the capillary to initiate nanoESI. The nanoESI potential was adjusted to optimize protein ion signal-to-noise ratios (S/N) for each capillary and was maintained at these low voltages to prevent electrothermal supercharging. All other source instrument parameters were constant (inlet capillary temperature = 265 °C, capillary voltage = 35 V, and tube lens voltage = 120 V). Spectra were acquired in triplicate using three different capillaries to account for tip-to-tip variability in the charge-state distributions. Protein solutions at a concentration of 10 µM were prepared from lyophilized powders dissolved in water, 200 mM ammonium acetate, 200 mM ammonium bicarbonate, or denaturing solution (45/54/1 methanol/ water/ acetic acid) containing different amounts of the supercharging reagents, m-nitrobenzyl alcohol (m-NBA), sulfolane, propylene carbonate (PC), 2-thiophenone, and 4-hydroxymethyl-1,3-dioxolan-2-one (HD).

Guanidine melts of 5 µM equine cytochrome c in water, 200 mM ammonium acetate, and 200 mM ammonium bicarbonate with 0-10% supercharging reagent by volume were performed by monitoring tryptophan fluorescence intensity using a multi-mode microplate reader (Synergy H4 hybrid reader, BioTek, Winooski, VT, USA) in emission acquisition mode with 280 ± 20 nm excitation and 352 ± 10 nm emission wavelengths. Each sample was measured in triplicate in 384-well polystyrene solid black low volume flat bottom microplates (Corning, New York, NY, USA). Cytochrome c unfolding curves were fit to a two-state model \( U \rightarrow N \), where \( U \) is the unfolded state and \( N \) is the native state of the protein. The free energy of unfolding \( \Delta G_N \) was obtained by fitting the unfolding curve to a sigmoidal plot of the form:

\[
I = \frac{A}{1 + \exp\left(\frac{\Delta G_N m - [GdnC]}{RT}\right)}
\]

(Equation 1)

where \( I \) is the fluorescence intensity, \( A \) is a normalization constant, \( R \) is the gas constant, \( T \) is temperature, and \( m \) is the linear proportionality constant (average \( m = 4.0 \pm 0.7 \text{ kcal/mol/M} \)). The uncertainty in the \( \Delta G_N \) values is 0.2 kcal/mol and corresponds to the standard deviation in
ΔG_N measured for cytochrome c in water, ammonium acetate, and ammonium bicarbonate with no supercharging reagent each measured on three different days. All proteins, salts, solvents, and supercharging reagents were purchased from Sigma (St. Louis, MO, USA) and were used without further purification. The purities of the supercharging reagents are all >98%, with the exception of HD, which is ~90% pure.

3.4 Results and Discussion

3.4.1 Supercharging in aqueous solutions. With previously identified supercharging reagents, it has not been possible to produce charge states in native mass spectrometry that are comparable to or higher than those obtained from denaturing solutions containing water, methanol and acid. To illustrate the high charging obtainable with two new supercharging reagents, 2-thiophenone and HD, mass spectra of cytochrome c produced by nanoESI from pure water and with various supercharging reagents were obtained (Figure 1a-f). The charge-state distribution of cytochrome c ions produced by nanoESI from pure water is centered around 8+ (Figure 1a). An increase in average charge occurs with the known supercharging reagents, m-NBA (38%), sulfolane (43%), or PC (28%) when these reagents are used at their optimal concentrations, which is the concentration at which the greatest extent of supercharging is observed without sacrificing spray stability or protein ion signal. Significantly more charging occurs for this protein with either 2% 2-thiophenone or 2% HD (Figure 1e,f). These reagents are structural analogs of the supercharging reagents sulfolane and PC, respectively. The average charge compared to that obtained from pure water increases by ~118% with 2-thiophenone and HD, far exceeding the increases in charge observed with the conventional supercharging reagents. The maximum charge state with 2-thiophenone is 22+ and with HD is 24+. The latter values is the same as the number of basic residues (Arg, Lys, and His) in this protein. The charge state of the most abundant ion increases from 8+ in pure water to 20+ with either of these reagents.

3.4.2 Supercharging in denaturing solutions. The effectiveness of these supercharging reagents in denaturing solutions consisting of 45/54/1 methanol/water/acetic acid was evaluated, and the relative extents of supercharging obtained with these reagents is different in denaturing solutions (Figure 2) than in water (Figure 1a-f). The most effective supercharging reagents from denaturing solution are m-NBA and PC (Figure 2b,d). These reagents increase the average charge by ~43% compared to denaturing solution without any reagent, and the most abundant charge state is 23+. 2-thiophenone is the least effective supercharging reagent, increasing the average charge by only 16% with the most abundant charge state the 19+. This relatively poor supercharging with 2-thiophenone is likely due to the low concentration (0.5% by volume), above which the stability of the spray is adversely affected. With HD, there is a 40% increase in average charge with the 21+ the most abundant charge state. HD and 2-thiophenone are the most effective supercharging reagents in aqueous solution, but m-NBA and PC are superior when ions are formed from water/methanol/acid solutions.

A key finding is that the more highly charged ions can be produced with the supercharging reagents 2-thiophenone or HD in water than can be produced under typical denaturing conditions using water/methanol/acid solutions! Supercharging with 2-thiophenone or HD from water (Figure 1e,f) produces average and maximum charge states that are ~20% higher than those obtained from denaturing solutions without supercharging reagents (Figure 2a).
Moreover, the most abundant charge state is 20+ with these reagents in water compared to 15+ from a denaturing solution. Electrothermal supercharging from aqueous ammonium bicarbonate solutions produces charge-state distributions with similar extents of charging to those obtained from denaturing solution. The data with the two new supercharging reagents demonstrates for the first time that charging greater than that obtainable from denaturing solution without supercharging reagents can be obtained from aqueous solutions with either 2-thiophenone or HD.

3.4.3 Effect of supercharging reagent concentration. The extent of supercharging depends on the reagent concentration. A 2% concentration was found to be the optimal concentration for both 2-thiophenone and for HD in aqueous solutions. The average charge decreases at higher concentrations (Figure S1). For cytochrome c in water, the average charge decreases from 17.8 ± 1.1+ to 14.6 ± 1.0+ when the concentration of HD is increased from 2% to 3%. A similar decrease in charge occurs with PC in denaturing solutions above its optimal concentration of 15%. At HD concentrations higher than 2%, a significant fraction of the ion signal corresponds to cytochrome c dimer. Increasing the HD concentration from 2% to 3% increases the dimer population from 21 ± 1% to 54 ± 1% of the total protein ion abundance. This increasing prevalence of dimer population with increasing reagent concentration suggests that the supercharging reagent could be affecting the protein conformation in the ESI droplet, which can increase protein aggregation. At a concentration of 2% 2-thiophenone or HD, a significant amount of chemical noise due to cluster formation and adduction to the protein ions occurs when a quadrupole-time-of-flight (Q-TOF) mass spectrometer is used (Figure S2). This instrument has softer source conditions compared to the Thermo LTQ. Thus, a lower volume of reagent should be used when supercharging with these two new reagents on instruments with gentle source conditions for optimal protein ion signal.

3.4.4 Supercharging in buffered solutions. Buffers are typically used in native MS to increase ionic strength and mitigate pH changes, both of which can affect the native structures of proteins and protein complexes. To test the relative effectiveness of these supercharging reagents to increase the charge of protein ions formed from buffered solutions, 10 µM cytochrome c ions with the same concentration of reagents were formed by nanoESI from aqueous solutions with 200 mM ammonium acetate or 200 mM ammonium bicarbonate (Figure 1g-p). No spectra were obtained with 2-thiophenone in these ammonium buffer solutions because the electrospray was unstable. The average charge obtained for each supercharging reagent in 200 mM aqueous ammonium acetate is about 11% lower than that obtained with the same reagent in pure water. The only exception is sulfolane, for which there is a slight increase in charge. The average charge of cytochrome c produced from solutions containing HD and ammonium acetate is 15.4 ± 0.1+. This average charge is higher than that produced from a denaturing solution (14.9 ± 0.3+) and corresponds to an increase in average charge of ~123% compared to ammonium acetate without any supercharging reagent. In contrast, there is only an increase of ~57% on average for the other reagents. These increases in average charge are similar to those observed from water, suggesting that the denaturing strength of these reagents is not significantly different in pure water and ammonium acetate buffer.

In striking contrast to results in water and aqueous 200 mM ammonium acetate, supercharging with any of these reagents is ineffective in 200 mM ammonium bicarbonate. The charge-state distributions are all centered near 7+ with or without supercharging reagent, and the average charge state is nearly the same except for HD, for which the average charge is slightly
lower. These data show that more highly charged ions can be produced from solutions with low buffer concentration and that ammonium acetate is the preferred buffer.

### 3.4.5 Supercharging and noncovalent complexes

The supercharging reagents, sulfolane and DMSO, are chemical denaturants that destabilize the native structures of proteins. In addition, sulfolane and m-NBA can disrupt noncovalent interactions and cause partial or complete dissociation of protein-protein complexes. The extent to which the new supercharging reagents, 2-thiophenone and HD, disrupt noncovalent interactions compared to the standard supercharging reagents was evaluated by measuring mass spectra of myoglobin (Figure 3). The charge-state distributions of holo- and apo-myoglobin (highlighted in red) produced by nanoESI out of aqueous solutions are centered around the 8+ and 9+ charge states (Figure 3a,g,l), and holo-myoglobin is the most abundant form of these ions. An increase in charge is obtained with m-NBA (77%), sulfolane (29%), or PC (29%) in aqueous solutions. The dominant form of the protein is apo-myoglobin, not holo-myoglobin, with these reagents. In contrast, the average charge with the new supercharging reagents, 2-thiophenone and HD, is much greater. The average charge is 163% and 138% higher with 2-thiophenone and HD, respectively, and the maximum charge state increases from 11+ to 28+. The maximum charge state is close to the number of basic residues (32) in this protein. The average charge is about 10% greater than that produced from denaturing solution (18.9 ± 0.3+) for apo-myoglobin, and the maximum charge state is the same as that obtained from denaturing solution (28+). Apo-myoglobin is the dominant form of the protein observed with HD, and apo-myoglobin composes 100% of the protein ion population with 2-thiophenone.

With 200 mM ammonium acetate, there is a slight decrease in average charge for all supercharging reagents compared to the results with these reagents in pure water, again with the exception of sulfolane, for which there is a slight increase in average charge. The charge-state distribution with HD is shifted to significantly higher charge compared to that obtained with the other reagents. The average charge with HD in 200 mM ammonium acetate (18.3 ± 0.2+) is similar to that out of denaturing solution (18.9 ± 0.3+). For all reagents except PC, which shows little supercharging, apo-myoglobin is the most abundant protein species.

With ammonium bicarbonate, there is very little increase in charge with any supercharging reagent, and similar to cytochrome c, there is a decrease in the average charge with HD in this buffer. All charge-state distributions are centered around the 8+ or 9+ charge states, and holo-myoglobin is the most abundant form of myoglobin, with the exception of m-NBA.

### 3.4.6 MS evidence for protein conformational changes in ESI droplet

Apo-myoglobin is formed in solution as a result of unfolding of the F-helix in the native structure of holo-myoglobin and subsequent loss of the non-covalently bound heme group. The percentage of apo-myoglobin observed in all spectra in Figure 3 as a function of the average charge of all myoglobin species in a given mass spectrum is shown in Figure 4. These data show a trend of increasing fraction of apo-myoglobin with increasing charging obtained with the supercharging reagents. This suggests that the high charge states formed with supercharging reagents are a result of chemical destabilization of the native protein structure, which results in protein conformational changes in the ESI droplet and the formation of apo-myoglobin by loss of the heme. A decrease in supercharging as well as a decrease in protein complex dissociation in the buffered solutions suggests that the stability of the protein increases in these buffers, and that
denaturation by the supercharging reagents is less effective. Buffers, particularly phosphate buffers, are routinely used in biology to stabilize the native forms of proteins, and some proteins and protein complexes require a certain ionic strength or essential salts in order to be in their active state or to assemble.\textsuperscript{131, 133, 209-211} A similar effect was reported for a much larger complex, the homotetramer concanavalin A, where less supercharging with \textit{m}-NBA occurs with increasing ammonium acetate concentration.\textsuperscript{71} The buffer capacity increases with higher concentration, and this reduces pH changes in the ESI droplet during droplet evaporation that might also destabilize the protein structure during supercharging. This buffer capacity is highest for ammonium bicarbonate at neutral pH, and the least supercharging occurs for this buffer. The decrease in average charge with HD in ammonium bicarbonate for both cytochrome \textit{c} and myoglobin may be a result of surface tension effects. HD has a lower surface tension than water (44 ± 3 dynes/cm versus 72 dynes/cm, respectively).\textsuperscript{212} A droplet consisting of a substantial fraction of HD can hold less charge than a droplet of pure water, which can lead to lower charging in the absence of protein conformational changes.\textsuperscript{21, 70} The other reagents in this study also have lower surface tensions than water,\textsuperscript{212, 213} and the surface tension effects may be obscured by conformational changes to the protein. It has been shown that conformational effects with proteins can result in significantly larger differences in charging than surface tension effects.\textsuperscript{70}

3.4.7 Fluorescence evidence for protein conformational changes in ESI droplets. DMSO, sulfolane, and 4-vinyl-1,3-dioxolan-2-one were previously shown to be effective chemical denaturants in solution.\textsuperscript{30, 68, 213, 214} To obtain additional evidence for the role of supercharging reagents on destabilizing protein conformation \textit{in solution}, the effects of buffer and supercharging reagents on the stability of cytochrome \textit{c} was investigated with guanidine HCl melts using tryptophan fluorescence to measure protein unfolding. When cytochrome \textit{c} is in a native conformation, the single tryptophan (residue 59) is in close proximity to the heme group, which is covalently bound at residues 14 and 17, and tryptophan fluorescence is entirely quenched by the heme group.\textsuperscript{215} When unfolded, the tryptophan residue is on average further away from the heme, and tryptophan fluorescence occurs.\textsuperscript{215} Thus, tryptophan fluorescence can be used as a probe of cytochrome \textit{c} unfolding in solution. Guanidine melts were performed with between 0% and 10% by volume of PC, sulfolane, and HD in water, 200 mM ammonium acetate, and 200 mM ammonium bicarbonate. No fluorescence experiments were performed with \textit{m}-NBA because this reagent absorbs strongly up to ~400 nm, so no tryptophan fluorescence is observed with \textit{m}-NBA in solution. Experiments were not done with 2-thiophenone owing to a reaction between the reagent and guanidine that results in a black precipitate.

An example of the fluorescence data for guanidine melts with propylene carbonate in water is shown in Figure 5. With increasing propylene carbonate concentration, less guanidine is required to unfold the protein (Figure 5), indicating that PC destabilizes the native form of cytochrome \textit{c} relative to the unfolded form. The Gibbs free energies of protein folding, $\Delta G_N$, are obtained from these data (eq. 1) and show that $\Delta G_N$ becomes less negative, increasing from −6.1 kcal/mol to −4.0 kcal/mol, when the PC concentration increases from 0% to 10%. This result clearly demonstrates the extent to which PC destabilizes the native form of the protein \textit{in solution}.

$\Delta G_N$ as a function of supercharging reagent concentration for the three supercharging reagents under all buffer conditions is shown in Figure 6a-c, and all of the data is overlaid in Figure 6d. The data are fit with lines, the slope of which corresponds to the denaturing strength of a supercharging reagent under the given buffer conditions. For example, PC has a denaturing
strength of 1.8 kcal/mol/M in both water and 200 mM ammonium acetate, and the ΔG_N values show that the stability of cytochrome c is the same in ammonium acetate as in pure water. In 200 mM ammonium bicarbonate with PC, the ΔG_N values are all more negative than in ammonium acetate and pure water, even without any supercharging reagent present. This indicates that ammonium bicarbonate stabilizes cytochrome c against denaturation in solution both with and without the supercharging reagent. This significant stabilization observed with ammonium bicarbonate may be due in part to ammonium bicarbonate’s high buffer capacity and the fact that its highest buffering capacity is around pH 7, a pH at which cytochrome c is folded.\textsuperscript{216} By contrast, ammonium acetate has very poor buffer capacity at this pH, and buffers instead around pH 5, closer to the pH at which equine cytochrome c starts to unfold (pH 3) in the absence of other denaturants.\textsuperscript{217} The pH of 6 M guanidine HCl in water is 4.5, so the sample solution acidifies as the guanidine concentration increases. In all three buffer conditions, the denaturing strength of PC is about the same ~1.8 kcal/mol/M, indicating that the buffer does not affect the destabilizing effect of the supercharging reagent on the native form of the protein.

The denaturing strength of sulfolane is 1.9 to 2.0 kcal/mol/M in pure water and ammonium acetate and is similar to that of PC. These values are similar to the previously measured denaturing strength of sulfolane for myoglobin in Tris buffer (1.5 ± 0.1 kcal/mol/M).\textsuperscript{68} In ammonium bicarbonate, the denaturing strength is 0.9 kcal/mol/M, indicating that sulfolane is a less effective chemical denaturant in ammonium bicarbonate, and the protein is stabilized in this buffer both with and without sulfolane.

HD has a denaturing strength of about 1.8, 1.1 and 1.3 kcal/mol/M in water, ammonium acetate and ammonium bicarbonate, respectively. These results are consistent with those of sulfolane that show that the effectiveness of these chemical denaturants can depend on the buffer concentration and identity.

3.4.8 Stability of native proteins in solution and supercharging. The fluorescence data provide compelling evidence that the structure of cytochrome c is unaffected by the supercharging reagents in the original ESI solutions. With 10% PC, the structure of cytochrome c as monitored by fluorescence is unaffected even with ~0.5 M guanidine (Figure 5). Similarly, there is no measurable change in protein structure with up to 10% sulfolane or 10% HD without guanidine. In native supercharging, the optimal concentrations of these reagents is less than 10% (5%, 5%, and 2% for PC, sulfolane, and HD, respectively). These data indicate that the structure of cytochrome c is not significantly disrupted in the presence of these supercharging reagents in the solutions prior to ESI in native supercharging experiments.

A comparison of the mass spectra in Figure 1 and these ΔG_N data reveals a correlation between lower solution-phase stability of the native form of the protein and more effective supercharging. The charge-state distributions with PC and sulfolane are similar both in water and ammonium acetate (Figure 1), which is consistent with the similar denaturing ability of these two reagents from both of these solutions (Figure 6). For HD, the ΔG_N and denaturing strength is slightly lower in ammonium acetate than water, which is reflected in the mass spectra where there is a more substantial decrease in the most abundant charge state from 20+ to 16+ compared to that observed for PC or sulfolane. With ammonium bicarbonate, there is no supercharging observed with any reagent, consistent with the high stability of the folded form of the protein in all solutions containing ammonium bicarbonate. All mass spectra are centered around the 7+ charge state, and all ΔG_N plots with ammonium bicarbonate are similar (Figure 6d). This
qualitative correlation between the bulk solution-phase studies and the mass spectra indicate that conformational effects play a large role in the supercharging phenomenon.

The $\Delta G_N$ values are very similar for all three supercharging reagents in pure water (Figure 6d), yet the extent of supercharging differs (Figure 1b-f), with HD producing more charging than either PC or sulfolane. This indicates that in addition to the intrinsic denaturing ability of these reagents in solution, other factors play a role in the extent of supercharging observed. Some of these factors may be reagent solubility and boiling point, which influence to what extent and how quickly the reagent concentrates in the ESI droplet and the extent to which droplet heating occurs. For example, the boiling points of PC and sulfolane are similar (285 °C for sulfolane and 240 °C for PC) and are much lower than that of HD (354 °C).212 Because the boiling points of PC and sulfolane are lower than that of HD, the rate of concentration in the ESI droplet will be less, and this may produce less supercharging with these reagents than occurs with HD. Moreover, there will be less evaporative cooling with HD, and this may lead to higher ESI droplet temperatures that also promote protein unfolding. PC has a solubility limit of 17% in water, whereas sulfolane and HD are completely miscible with water. Because they are completely miscible, higher concentrations of both sulfolane and HD can occur in the evaporating droplet, consistent with more supercharging observed with sulfolane and HD than with PC. The presence of $m$-NBA has been shown previously to increase droplet lifetimes by inhibiting droplet evaporation,218 and may lead to increased protein unfolding as a result of lower evaporative cooling and longer times for unfolding to occur. Additional factors, such as surface tension, likely play a role as well in the relative effectiveness of these supercharging reagents.

### 3.5 Conclusion

Electrospray ionization in combination with two new supercharging reagents, 2-thiophenone and HD, can produce more highly charged ions from aqueous solutions in which proteins have native conformations than obtained from more traditional solutions consisting of water/methanol/acid in which proteins are unfolded. Supercharging with these new reagents in native mass spectrometry produces significantly more than a two-fold increase in average and maximum charge, and these reagents are about twice as effective at native MS supercharging than $m$-NBA, sulfolane, and PC. Both $m$-NBA and PC are still the most effective reagents for increasing protein ion charge from denaturing solution. More loss of the heme from myoglobin occurs with increasing supercharging, indicating that supercharging is a result of protein conformational changes in the electrospray droplet. The supercharging reagents PC, sulfolane and HD are effective chemical denaturants in solution, and the extent of supercharging observed with these reagents is related to the denaturing capability of the reagent and the stabilities of proteins in different buffers. Combined, these results provide compelling evidence that the primary mechanism of supercharging with these reagents in native mass spectrometry is their effectiveness at destabilizing native protein structure, resulting in unfolding of the protein in the ESI droplet.

These results demonstrate that it is possible to keep proteins in solutions in which they have native structures and reactivities, yet produce more highly charged ions than is possible with conventional solutions in which proteins are denatured. The highly charged ions produced with these reagents in native MS are almost certainly as unfolded in the gas phase as comparably charged ions produced by other methods used to form highly charged ions, such as ESI from solutions consisting of water, methanol, and acetic acid in which proteins are denatured. This
should make it possible to combine the advantages of native mass spectrometry with the capabilities of tandem mass spectrometry to obtain extensive structural information on the highly charged ions that can be produced with these reagents. This should be particularly advantageous for top-down H/D exchange methods for deducing information about protein conformations and dynamics. Continuous H/D exchange can be monitored without the need for proteolysis and denaturing conditions necessary for producing high charge states.\textsuperscript{69} Because the lifetime of the droplet in which protein denaturation occurs can be less than 27 μs,\textsuperscript{219} the potential for back-exchange in solution is eliminated.

3.6 Acknowledgements

The authors thank the National Institutes of Health (Grant No. R01GM097357) and the National Science Foundation (Graduate Research Fellowship for CAC; Grant No. DGE1106400) for financial support, and the Kuriyan Lab at the University of California, Berkeley for use of their fluorometer.
### 3.7 Tables

**Table 1. Average charge of cytochrome c in water, 200 mM ammonium acetate (AA), 200 mM ammonium bicarbonate (ABC) and denaturing solution (45/54/1 methanol, water, acetic acid by volume) with supercharging reagents added at the optimal concentration for maximal charging.**

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>AA</th>
<th>ABC</th>
<th>Denaturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Additive</td>
<td>8.2 ± 0.3+</td>
<td>6.9 ± 0.1+</td>
<td>7.4 ± 0.2+</td>
<td>14.9 ± 0.3+</td>
</tr>
<tr>
<td>1.5% <em>m</em>-NBA</td>
<td>11.3 ± 0.5+</td>
<td>10.8 ± 0.1+</td>
<td>7.6 ± 0.2+</td>
<td>21.6 ± 0.4+</td>
</tr>
<tr>
<td>5% sulfolane</td>
<td>11.7 ± 0.2+</td>
<td>12.9 ± 0.1+</td>
<td>7.4 ± 0.1+</td>
<td>20.4 ± 0.2+</td>
</tr>
<tr>
<td>5% PC</td>
<td>10.5 ± 0.2+</td>
<td>8.9 ± 0.4+</td>
<td>7.7 ± 0.1+</td>
<td>21.1 ± 0.3+</td>
</tr>
<tr>
<td>2% 2-thiophenone</td>
<td>18.5 ± 0.1+</td>
<td>--</td>
<td>--</td>
<td>17.3 ± 0.1+</td>
</tr>
<tr>
<td>2% HD</td>
<td>17.8 ± 1.1+</td>
<td>15.4 ± 0.1+</td>
<td>6.9 ± 0.1+</td>
<td>20.8 ± 0.1+</td>
</tr>
</tbody>
</table>
Table 2. Average charge of myoglobin in water, 200 mM ammonium acetate (AA), and 200 mM ammonium bicarbonate (ABC) with supercharging reagents added at the optimal concentration for maximal charging. Holo-myoglobin values are without parentheses and apo-myoglobin values are within parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>AA</th>
<th>ABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Additive</td>
<td>8.6 ± 0.5+</td>
<td>8.3 ± 0.1+</td>
<td>8.5 ± 0.4+</td>
</tr>
<tr>
<td></td>
<td>(8.9 ± 0.8+)</td>
<td>(7.5 ± 0.1+)</td>
<td>(7.9 ± 0.5+)</td>
</tr>
<tr>
<td>1.5% m-NBA</td>
<td>15.5 ± 1.5+</td>
<td>12.1 ± 0.3+</td>
<td>9.5 ± 0.5+</td>
</tr>
<tr>
<td></td>
<td>(15.2 ± 0.9+)</td>
<td>(11.5 ± 0.3+)</td>
<td>(10.4 ± 1.0+)</td>
</tr>
<tr>
<td>5% sulfolane</td>
<td>11.2 ± 0.5+</td>
<td>14.1 ± 1.4+</td>
<td>8.9 ± 0.1+</td>
</tr>
<tr>
<td></td>
<td>(10.9 ± 0.3+)</td>
<td>(12.9 ± 1.1+)</td>
<td>(8.7 ± 0.1+)</td>
</tr>
<tr>
<td>5% PC</td>
<td>10.9 ± 0.5+</td>
<td>8.4 ± 0.3+</td>
<td>9.5 ± 0.3+</td>
</tr>
<tr>
<td></td>
<td>(11.3 ± 0.7+)</td>
<td>(8.0 ± 0.5+)</td>
<td>(9.1 ± 0.2+)</td>
</tr>
<tr>
<td>2% 2-thiophenone</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>(22.6 ± 0.2+)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2% HD</td>
<td>19.5 ± 2.2+</td>
<td>18.5 ± 0.2+</td>
<td>8.2 ± 0.1+</td>
</tr>
<tr>
<td></td>
<td>(20.5 ± 0.1+)</td>
<td>(18.3 ± 0.2+)</td>
<td>(7.6 ± 0.1+)</td>
</tr>
</tbody>
</table>
Figures

Figure 1. NanoESI mass spectra of cytochrome c in water (left column), 200 mM ammonium acetate (middle column), and 200 mM ammonium bicarbonate (right column) with no supercharging reagent (a, g, l), 1.5% m-NBA (b, h, m), 5% sulfolane (c, i, n), 5% PC (d, j, o), 2% 2-thiophenone (e), and 2% HD (f, k, p).
Figure 2. NanoESI mass spectra of cytochrome c in denaturing solution (45/54/1 methanol/water/acetic acid) with no supercharging reagent (a), 5% m-NBA (b), 10% sulfolane (c), 15% PC (d), 0.5% 2-thiophenone (e), and 5% HD (f).
Figure 3. NanoESI mass spectra of myoglobin in water (left column), 200 mM ammonium acetate (middle column), and 200 mM ammonium bicarbonate (right column) with no supercharging reagent (a, g, l), 1.5% m-NBA (b, h, m), 5% sulfolane (c, i, n), 5% PC (d, j, o), 2% 2-thiophenone (e), and 2% HD (f, k, p). Apo-myoglobin is labeled with red circles.
Figure 4. The average charge of myoglobin (using an intensity weighted average of both holo- and apo-myoglobin) plotted versus the percentage of the total protein ion signal that is apo-myoglobin under all of the solution conditions shown in Figure 3.
Figure 5. Guandine melts of cytochrome c measured by tryptophan fluorescence. Data is normalized in the plot so that the maximum of the sigmoidal fit (from Equation 1) is defined as one. Guanidine concentration was increased in increments of 0.25 M, and PC concentration was increased in increments of 2.5% by volume.
Figure 6. Free energy of folding (ΔG_N) of cytochrome c as a function of supercharging reagent concentration for PC (a), sulfolane (b), HD (c), and all three reagents (d). The free energy of folding was calculated from guanidine melt data fit to Equation 1.
3.9 Supporting Information

Figure S1. NanoESI mass spectrum of cytochrome c in water with 3% HD.
Figure S2. NanoESI mass spectra of cytochrome c in water with 2% HD (a) and 2% 2-thiophenone (b) acquired using a Waters Q-TOF mass spectrometer. Adducts to cytochrome c in (a) are likely due to impurities in the HD stock, and cluster ions in (b) are likely composed of varying numbers of 2-thiophenone molecules and salts present in either the 2-thiophenone or protein stock.
Chapter 4

Desalting Protein Ions in Native Mass Spectrometry Using Supercharging Reagents

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Cassou, C. A.; Williams, E. R. Analyst, 2014, 139, 4810-4819

4.1 Abstract

Effects of the supercharging reagents $m$-NBA and sulfolane on sodium ion adduction to protein ions formed using native mass spectrometry were investigated. There is extensive sodium adduction on protein ions formed by electrospray ionization from aqueous solutions containing millimolar concentrations of NaCl, which can lower sensitivity by distributing the signal of a given charge state over multiple adducted ions and can reduce mass measuring accuracy for large proteins and non-covalent complexes for which individual adducts cannot be resolved. The average number of sodium ions adducted to the most abundant ion formed from ten small (8.6-29 kDa) proteins for which adducts can be resolved is reduced by 58% or 80% on average, respectively, when 1.5% $m$-NBA or 2.5% sulfolane are added to aqueous solutions containing sodium compared to without the supercharging reagent. Sulfolane is more effective than $m$-NBA at reducing sodium ion adduction and at preserving non-covalent protein-ligand and protein-protein interactions. Desalting with 2.5% sulfolane enables detection of several glycosylated forms of 79.7 kDa holo-transferrin and NADH bound to the 146 kDa homotetramer LDH, which are otherwise unresolved due to peak broadening from extensive sodium adduction. Although sulfolane is more effective than $m$-NBA at protein ion desalting, $m$-NBA reduces salt clusters at high $m/z$ and can increase the signal-to-noise ratios of protein ions by reducing chemical noise. Desalting is likely a result of these supercharging reagents binding sodium ions in solution, thereby reducing the sodium available to adduct to protein ions.

4.2 Introduction

The effects that different salts can have on protein stability have been known since the late 1800’s, when Franz Hofmeister reported an ordering of cations and anions based on their ability to salt in or salt out proteins, and both non-specific and specific salt-protein interactions can strongly influence protein structure and function. Phosphate and Tris buffers as well as sodium chloride are often added to aqueous protein solutions in order to stabilize native or native-like protein structure by mimicking the environment inside the cell, which has an ionic strength of ~150 mM. Specific salts and other small molecules are essential in the enzyme-cofactor or protein-ligand interactions in such varied and vital processes as electron transport, ion pumping across cell membranes, and drug interactions.

Some protein structural methods, such as X-ray crystallography and NMR, are not adversely affected by high salt concentrations, whereas high salt concentrations can be detrimental to the performance of electrospray ionization (ESI) and to a lesser extent matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS). Salts can increase baseline noise due to the formation of ionic clusters and can cause ion suppression. For example, alcohol dehydrogenase tetramer ions formed by nanoelectrospray ionization (nESI) from 50 mM ammonium acetate can be measured with excellent signal-to-noise ratios (S/N),
whereas the tetramer is undetectable from the same solution with 10 mM Tris or HEPES buffers. Similarly, addition of 10 mM CsCl to solutions of lysozyme in 1:1 methanol:water decreases the total ion abundance for the protein by 130-fold. Salt adduction to protein ions distributes the protein signal over multiple adducted ions, reducing the S/N of each protein ion, and broadens mass spectral peaks of large proteins and protein complexes for which individual adducts cannot be resolved. Peak broadening decreases mass measurement accuracy for these large proteins and can also inhibit the identification of covalent (glycosylations, phosphorylation, or other post-translational modifications) or non-covalent (specific ion or ligand binding) protein modifications. McLuckey and coworkers found that the extent of sodium ion adduction on a protein ion formed by ESI is related to the protein pI, solution pH, and charge state. There is more sodium ion adduction to low charge states, but there is more adduction of trivalent metal ions to high charge states.

To reduce the adverse effects of many salts on MS performance, salts are often removed by dialysis, ion exchange chromatography, or diafiltration prior to analysis by MS, and a myriad of products for fast filtration and online chromatographic desalting of protein solutions have been developed. However, removing even low concentrations of salts can significantly change the structure of some proteins and protein complexes. For example, NtrC4 (a σ activator protein from *Aquifex aeolicus*) requires millimolar concentrations of certain salts, e.g., Mg\(^{2+}\), BeF\(^3-\), and ADP, in order to assemble into an active hexamer. Several techniques for reducing sodium ion adduction to proteins in ESI have been developed that do not require removal of the salts from solution prior to ion formation. Buffer loading, in which high concentrations of a volatile buffer, typically ammonium acetate, is added to solution, reduces sodium ion adduction to proteins and reduces the number of salt ion clusters formed. The addition of 7 M ammonium acetate to aqueous solutions containing 20 mM NaCl results in an increase in the S/N of the most abundant ion of cytochrome *c* and ubiquitin by more than 6-fold and 11-fold, respectively, compared to solutions without the buffer added. Buffer loading also works well for proteins that require high concentrations of salt to function or assemble, like concanavalin A. Other ammonium buffer salts can also effectively reduce sodium ion adduction to proteins, and can do so at much lower concentrations. For example, 25 mM ammonium bromide added to an aqueous 1 mM NaCl solution containing ubiquitin decreases the average number of sodium ions adducted to the most abundant charge state of the protein from 6.0 to 0.4 and increases the S/N of this ion by a factor of 66. The ability of different salts to desalt proteins in the ESI droplet is related to the proton affinities of the anions, where anions with low proton affinities lead to less sodium adduction. However, anions with low proton affinities also tend to adduct to the protein as an acid molecule and form ion clusters, which can decrease the protein ion signal. Konermann and coworkers suggested that some salts, such as citrate and tartrate, may chelate ions in solution, such as calcium, that can non-specifically adduct to proteins.

Supercharging reagents can be used to produce high charge-state ions by ESI from both denaturing and native solutions. Supercharging reagents are high-boiling point compounds that are typically added in small amounts (1-2%) to sample solutions prior to ESI. For aqueous solutions, the low concentration of the supercharging reagents does not significantly affect the protein structure prior to ESI. However, the concentration of supercharging reagent increases in the droplet as solvent evaporation occurs, and the native structure of the protein can be chemically/thermally destabilized in the electrospray droplet, resulting in partial or extensive loss of folded structure.
with a concomitant increase in the number of charges on the gaseous protein ions that are formed.\textsuperscript{30,68-72} Protein-protein or protein-ligand complex dissociation may also occur as a result of this protein destabilization.\textsuperscript{30,66,68,71,72} Supercharging reagents can also increase protein charging from denaturing solutions in which the protein is initially unfolded,\textsuperscript{21,43,46,93,181,182,187,235} a result attributable to many factors, including the high surface tension of supercharging reagents compared to water-methanol-acid solutions.\textsuperscript{21} Droplets with higher surface tension can support more charge, and this high charge density can result in the formation of high charge-state ions. Loo and coworkers showed that the presence of \textit{m}-NBA or sulfolane in the spray solvent in desorption electrospray ionization (DESI) experiments performed on HPLC column effluents containing trifluoroacetic acid (TFA) decreases TFA cluster ion intensity and increases protein S/N compared to when no supercharging reagent is in the DESI solvent.\textsuperscript{108} The authors suggested that this result is likely due to the supercharging reagent binding to TFA anions, thereby inhibiting TFA cluster formation, or preventing TFA dissociation into TFA anion and a free proton in solution, reducing the amount of free TFA anion in solution.\textsuperscript{108}

Here, we show that \textit{m}-NBA and sulfolane, two of the most common supercharging reagents, reduce sodium adduction to protein ions formed by nESI from native solutions. Sulfolane is more effective than \textit{m}-NBA at reducing sodium ion adduction while still preserving non-covalent protein-protein and protein-ligand interactions. However, the use of \textit{m}-NBA to desalt protein ions can lead to up to a 7-fold increase in protein ion S/N as a result of fewer clusters and lower chemical noise. This new method for desalting protein ions in nESI can improve the mass measuring accuracy for large proteins and protein complexes and can be used to resolve different glycoforms or ligand-adducted forms of proteins that are otherwise obscured by peak broadening resulting from extensive sodium adduction. \textit{m}-NBA and sulfolane bind to sodium ions, suggesting that sodium sequestration by supercharging reagents may be the origin of the desalting effect of these supercharging reagents.

4.3 Experimental

Experiments were performed using a Waters Quadrupole-Time-of-Flight (Q-TOF) Premier (Waters, Milford, MA) mass spectrometer. Protein ions were formed by nanoelectrospray from 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 micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA) and positioned ~2 mm from the capillary inlet to the Q-TOF instrument. Nanoelectrospray was initiated by applying a potential of about +1.0 kV to a 0.127 mm diameter platinum wire inserted into the capillary and in contact with the sample solution. The nanoelectrospray potential was adjusted to optimize protein ion S/N for each tip. Values of the average number of sodium ions adducted are from four replicate measurements using four different nanospray emitters to account for tip-to-tip variation in adduction levels. All comparative S/N measurements were made using the same nanoelectrospray capillary to eliminate tip-to-tip variability, and the capillary was washed with methanol and water between each solution. All proteins were purchased as lyophilized powders from Sigma except for barnase and barstar, which were expressed in \textit{E. coli} and purified as described previously.\textsuperscript{205} Ammonium bicarbonate, sodium chloride, platinum wire, and supercharging reagents were obtained from Sigma (St. Louis, MO, USA).

4.4 Results and Discussion
**4.4.1 Effects of supercharging reagents on protein charge and sodium ion adduction.**

The average charge of ubiquitin ions formed by nESI from 5 µM ubiquitin in 10 mM ammonium bicarbonate and 1 mM sodium chloride (pH 7.8) is 5.33 ± 0.01+ (Figure 1a). There is extensive sodium ion adduction to the 5+ - 7+ charge states, with more adduction on lower charge states. The average number of sodium ions adducted to these charge states are 3.5 ± 0.1, 3.5 ± 0.1, and 2.0 ± 0.4, respectively (Table 1 and Table S-1). Mass spectra obtained from the same solution with either 1.5% m-NBA or 2.5% sulfolane are shown in Figures 1b and 1c, respectively. The average charge of ubiquitin formed from these respective solutions is 7.1 ± 0.1+ and 6.3 ± 0.1+. The average number of sodium ions adducted to the 5+ - 7+ charge states is significantly lower when either supercharging reagent is present. The average number of sodium ions adducted to the most abundant charge state decreases from 3.5 ± 0.1 in the spectrum obtained without a reagent to 1.2 ± 0.2 with m-NBA and 0.44 ± 0.03 with sulfolane. m-NBA is not soluble in aqueous solution above ~1.5%, and at the concentration used for both supercharging reagents, there should be little effect on the protein structure in solution prior to droplet formation by nESI.30, 66, 69 The presence of the 1 mM sodium chloride in these protein solutions has only a small effect on the charge of the protein ions formed with supercharging reagents. For example, the average charge of ubiquitin ions produced by nESI from solutions containing both added sodium chloride and m-NBA is only about one charge lower compared to that with the same amount of m-NBA but without sodium chloride added. The slightly lower charging with sodium chloride is likely due to stabilization of folded protein structure in the ESI droplet as a result of the higher ionic strength.

The same experiments were performed with nine other proteins for which sodium adducts could be resolved. The results are given in Table 1 for the most abundant charge state in each spectrum and in Table S-1 for every charge state. The presence of m-NBA or sulfolane in aqueous solutions containing sodium decreases the average number of sodium ions adducted to both the most abundant charge state and any given charge state in the mass spectra for all proteins. For these ten proteins, the number of sodium ions adducted to the most abundant charge state decreases by an average of 58% with m-NBA, and by 80% with sulfolane compared to solutions without supercharging reagents. Thus, adding small quantities of supercharging reagents is an effective way to desalt protein ions in aqueous solutions containing sodium and also increases the average charge state of protein ions formed from these native solutions. Sulfolane at 2.5% is more effective than 1.5% m-NBA at reducing sodium adduction to protein ions. The effectiveness of m-NBA and sulfolane at reducing sodium adduction to protein ions in nESI is protein-dependent. However, the extent of adduct reduction with supercharging reagents does not correlate well with any of several protein characteristics, such as protein size, pI, or number of disulfide bonds (Table 1).

With 2.5% sulfolane, the average charge of the proteins increases by an average of 1.0+ from solutions containing no supercharging reagent, whereas with 1.5% m-NBA, the average charge increases by 2.5+. This result is consistent with previous results that showed that sulfolane, on a per volume basis, is a less effective supercharging reagent than m-NBA, and therefore likely disrupts native protein structure to a lesser extent than m-NBA.30

The effectiveness of sulfolane at protein ion desalting without extensively perturbing protein structure is important for desalting non-covalent protein-protein or protein-ligand complexes. Nanoelectrospray of myoglobin (Figure 2, Table 1), which contains a non-covalently bound heme group, from an aqueous solution with 10 mM ammonium bicarbonate
and 1 mM NaCl results in 100% holo-myoglobin ions. Only 48% of the ion population is holo-myoglobin with 1.5% m-NBA, and the charge-state distribution is bimodal, indicating that some of the myoglobin is unfolded in the nESI droplet. In contrast, holo-myoglobin is 83% of the ion population observed with 2.5% sulfolane, and the monomodal charge-state distribution indicates that there is less perturbation of the folded form of the protein in the nESI droplet.

Protein-protein complexes, such as the 22 kDa heterodimer barnase-barstar (Figure 3, Table 2), are also preserved better with 2.5% sulfolane than with 1.5% m-NBA. A nESI mass spectrum of a 6:5 molar ratio mixture of barstar and barnase in sodiated ammonium bicarbonate solution shows the 8+ through 10+ charge-state ions of the barnase-barstar complex and ions of barstar, which is the excess reagent (Figure 3a). Because the barnase-barstar complex has a very low dissociation constant (25 ± 5 pM in aqueous ammonium bicarbonate solution, pH 7.2, 1% glycerol236), nearly all of the barnase, the limiting reagent, is present in the complex, and ions of free barnase are not observed in the mass spectrum. The most abundant charge state of the complex is 9+, and there is an average of 4.0 sodium ions adducted. In a mass spectrum of the same solution containing 1.5% m-NBA (Figure 3b), the charge-state distribution is shifted to higher charge, and the appearance of barnase ions indicates that some solution-phase unfolding and partial dissociation of the complex occur in the nESI droplet. The most abundant charge state of the complex shifts to 11+ (an average of 0.4 sodium ions adducted), and only 38% of the complex remains intact. In comparison, the charge of the most abundant ion of the complex increases to only 10+, and 84% of the barnase-barstar complex remains intact in the mass spectrum of the same solution with sodium and 2.5% sulfolane (Figure 3c). The average number of sodium ions adducted to the most abundant charge state with sulfolane is 0.6, similar to that obtained from the m-NBA solution. The examples of myoglobin and barnase-barstar illustrate that using sulfolane as a desalting agent can be particularly useful in native nESI experiments where measurement of ions from the intact complex is desired, such as when complex stoichiometry or the presence of ligand binding is being determined, although caution in interpreting these data is necessary owing to destabilization and possible dissociation of the complexes in the nESI droplet.

4.4.2 Improving mass accuracy for large proteins and protein complexes. The charge-state distributions of large proteins and protein complexes in native ESI shift to higher m/z with increasing molecular weight.11 The inability to resolve individual sodium ion adducts at high m/z can reduce mass measuring accuracy and obscure the presence of different populations of covalently and non-covalently modified forms of the protein. Robinson and coworkers showed that retention of solvent and buffer molecules by large proteins and protein complexes in ESI results in measured masses much higher than calculated masses. For example, the measured mass of the 685 kDa 20S proteasome lacking an α-subunit is ~7 kDa higher than the calculated mass.112

A native nESI mass spectrum of human holo-transtferrin (79.7 kDa) in 10 mM ammonium bicarbonate with no sodium salt added is shown in Figure 4a. Four peaks corresponding to four different glycoforms of the protein are evident in the 18-20+ charge states. These glycoforms were determined from the masses measured from a denaturing solution of the protein (Figure S-1) to correspond well with the two oligosaccharide (one diantennary and one triantennary) glycosylations to this protein that were identified by van Halbeek and coworkers237 both with and without a fucosylation site on the oligosaccharide. The glycoforms are labelled as A) two diantennary oligosaccharides; B) A with one fucosylation; C) one di- with one triantennary
oligosaccharide; and D) C with one fucosylation. The average masses measured for each of these four glycoforms formed by nESI from native solution (Figure 4a, Table 3) are on average ~6 Da higher than the measured masses of these ions formed from denaturing solution (the calculated m/z is indicated by the dashed lines in Figure 4), indicating that on average, less than one sodium ion is adducted to the protein. With 1 mM NaCl in the same solution (Figure 4b), the centroids of each peak shift to higher m/z so that the masses of ions A and C increase by ~168 Da and ~189 Da, respectively. This additional mass corresponds to an average of 8.3 and 9.3 sodium ions adducted to these two glycoforms, respectively, and the peak widths in the spectrum are broad due to sodium adduction such that the fucosylated ions B and D are no longer evident in the mass spectrum. Sodium adducts also lead to a significant increase in the baseline at higher m/z. With 1.5% m-NBA (Figure 4c), the charge-state distribution is shifted to higher charge, the most abundant charge state being 22+, and all of the peaks in the ion distribution are still broad as a result of sodium adduction. The fucosylated ions B and D are not resolved, and even ion C is evident only as a shoulder on the main A peak. Ions A and C have masses that are ~230 Da and ~300 Da higher than those formed from nESI from aqueous solutions without sodium, and ~62 Da and ~111 Da higher than those from aqueous solutions with sodium. The higher mass of these ions compared to those formed from solutions containing sodium without supercharging reagents may be due to m-NBA adduction to the protein, which has been observed before by Loo and coworkers for smaller proteins.66 With 2.5% sulfolane (Figure 4d), the most abundant charge state is 20+, and all glycosylated ions are evident in the mass spectrum, with ions A, C, and D resolved, and ion B distinguishable as a shoulder on the peak of ion A. The increase in mass for each glycosylated form of the protein is ≤~69 Da, or ~3 sodium ions. Thus, addition of sulfolane not only enables a more accurate mass measurement from a solution containing sodium, but it also reveals two additional fucosylated species present in solution that were unresolvable in the mass spectrum from this same solution without sulfolane present (Figure 4b).

A mass spectrum of the 146 kDa homotetramer of rabbit lactate dehydrogenase (LDH) formed by nESI from 10 mM ammonium bicarbonate is shown in Figure 5a (dashed lines are the calculated m/z values). The measured mass of the tetramer is 146,004 ± 18 Da, which is close to the calculated mass of 145,996 Da based on the monomer mass determined from a spectrum of LDH obtained under denaturing conditions (Figure S-2). The 8 Da mass difference corresponds to an average of ~0.4 sodium ions adducted. There is also a distribution of LDH tetramer with a mass of 146,619 ± 14 Da corresponding to bound NADH, a cofactor of LDH that has a mass of 664 Da.238 With NaCl added at 5 mM concentration to this solution (Figure 5b), the LDH tetramer peaks are broader due to sodium ion adduction, and the NADH adduct is unresolved. The measured mass of the tetramer is 146,521 ± 23 Da corresponding to bound NADH, a cofactor of LDH that has a mass of 664 Da.238 With NaCl added at 5 mM concentration to this solution (Figure 5b), the LDH tetramer peaks are broader due to sodium ion adduction, and the NADH adduct is unresolved. The measured mass of the tetramer is 146,521 ± 23 Da corresponding to bound NADH, a cofactor of LDH that has a mass of 664 Da.238
and ~4.7 sodium ions adducted, respectively. There is no data shown for LDH with 1.5% m-NBA because the protein precipitates out of solution upon addition of the supercharging reagent as evidenced by solution clouding, suggesting that the stabilities of some proteins can be affected by even low concentrations of supercharging reagents.

4.4.3 Effects on S/N and detection limits. Loo and coworkers reported binding of m-NBA to ammonium and sodium cations at low m/z, but no protonated m-NBA was observed. It is possible that supercharging reagents reduce sodium ion adduction to proteins by binding to sodium ions in solution. Mass spectra of 5 µM barstar formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl with and without supercharging reagents are shown in Figure 6. In the expanded region below m/z = 700, many clusters containing sodium, chloride, bicarbonate, and ammonium are observed without supercharging reagents (Figure 6a), resulting in significant chemical noise at high m/z where protein ion signal is often observed (Table S-3). This can lower the protein ion S/N (Table S-3). With m-NBA (Figure 6b), the dominant low m/z ions are ammonium and sodium ions complexed with one or two m-NBA molecules, but there are also some low abundance ions containing sodium, chloride, bicarbonate, and ammonium, as well as sodium chloride and sodium bicarbonate cluster ions bound to m-NBA. Thus, m-NBA appears to inhibit the formation of salt cluster ions. There are no clusters above m/z ~700 (Figure 6b, inset), resulting in very low chemical noise in the higher m/z region of the spectrum where barstar ions are observed (Table S-3). With sulfolane, sodium and ammonium complexed to one, two, or three sulfolane molecules are most abundant (Figure 6c). Clusters extend to m/z ~1600, resulting in high chemical noise in the region where protein ions are often observed that is similar to the chemical noise without supercharging reagents (Figure 6a and c insets; Table S-3).

Dearden and coworkers showed that alkali cations can be removed from peptides and small protein ions in the gas phase by crown ether molecules, leading to a concomitant decrease in the peptide or protein charge as the cations are removed. The average charge of protein ions formed from solutions containing m-NBA and sulfolane with sodium chloride is typically the same or higher than that obtained without these reagents. This indicates that protein desalting with m-NBA and sulfolane is not solely due to these supercharging reagents stripping sodium ions from proteins in the gas phase. Supercharging reagents must be influencing the amount of sodium ion adduction to proteins in solution, most likely in the nESI droplet, where enrichment of both sodium ions and supercharging reagent occurs. It is likely that sequestration of sodium ions by supercharging reagents is the cause of the reduced sodium ion adduction to protein ions observed with m-NBA and sulfolane. Other groups have reported adduction of supercharging reagents to protein ions, although no obvious adduction was observed in our study. It is plausible that the supercharging reagents may also block sites on the protein where sodium ions bind.

Addition of m-NBA or sulfolane to aqueous solutions containing sodium substantially increases the S/N of some protein ions owing to reduced chemical noise and fewer sodiated species over which protein signal is spread (Table S-3, Figure S-3). For example, the S/N of the most abundant ion of barstar formed from a 5 µM solution is 7-fold higher with m-NBA than without this supercharging reagent, and there is a 2-fold enhancement with sulfolane (Table S-3). Supercharging reagents can also decrease the limit of detection (LOD) for these protein ions formed from solutions containing sodium. The LOD for barstar from sodiated solutions, determined from protein concentration dependent measurements, is almost an order of magnitude
lower with \( m \)-NBA than without (0.02 \( \mu \)M versus 0.12 \( \mu \)M) (Table S-3). However, the effect of supercharging reagents on the S/N and detection limit is protein dependent, and an improvement was not observed for all proteins (Table S-3). This may be due to differences in the chemical and salt impurities in the protein samples.

4.5 Conclusions

Supercharging reagents can increase the charge of protein ions formed by nESI from both native and denaturing solutions, but they also have the added benefit of effectively reducing sodium ion adduction to protein and protein complex ions formed from aqueous solutions. Addition of \(<3\%\) of \( m \)-NBA or sulfolane to aqueous solutions containing millimolar concentrations of sodium can reduce sodium adduction to protein ions by an average of \(~58\%\) and \(~80\%\), respectively. The presence of \( m \)-NBA in protein solutions containing sodium reduces chemical noise caused by clusters, resulting in up to a factor of 7 enhancement in S/N for some protein ions, whereas the presence of sulfolane has little effect on protein ion S/N due to salt cluster formation that extends to high \( m/z \). Sodium adduction to both \( m \)-NBA and sulfolane occurs, and this may sequester a sufficient number of sodium ions such that sodium adduction to proteins is significantly reduced. In the future, it would be interesting to investigate whether the use of supercharging reagents for desalting protein ions formed by nESI is effective for other ions that commonly bind nonspecifically to proteins, such as potassium and calcium, or for anions, such as sulfate and phosphate, which are often added as buffers to native protein solutions.

4.6 Acknowledgements

The authors thank the National Institutes of Health (Grant No. R01GM097357) and the National Science Foundation (Graduate Research Fellowship for CAC; Grant No. DGE1106400) for financial support.
### 4.7 Tables

**Table 1.** Average charge and average number of sodium ions adducted to the most abundant protein charge state formed by nESI from aqueous solutions containing 5 µM protein, 10 mM ammonium bicarbonate, and 1 mM NaCl with no supercharging reagent, with 1.5% *m*-NBA, or with 2.5% sulfolane.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average charge</th>
<th>Average # Na adducts to (most abundant charge state)</th>
<th>Protein size</th>
<th>Protein pl</th>
<th># Disulfide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>5.33 ± 0.01</td>
<td>3.5 ± 0.1 (5+)</td>
<td>8.6 kDa</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>m-NBA</td>
<td>7.1 ± 0.1</td>
<td>1.2 ± 0.2 (7+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>6.3 ± 0.1</td>
<td>0.44 ± 0.03 (6+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barstar</td>
<td>5.5 ± 0.1</td>
<td>3.5 ± 0.4 (5+)</td>
<td>10.2 kDa</td>
<td>4.7</td>
<td>0</td>
</tr>
<tr>
<td>m-NBA</td>
<td>7.1 ± 0.1</td>
<td>1.4 ± 0.2 (7+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>5.7 ± 0.1</td>
<td>1.0 ± 0.4 (6+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>6.5 ± 0.1</td>
<td>2.5 ± 0.2 (7+)</td>
<td>12.3 kDa</td>
<td>9.6</td>
<td>0</td>
</tr>
<tr>
<td>m-NBA</td>
<td>9.42 ± 0.04</td>
<td>0.93 ± 0.01 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>8.6 ± 0.2</td>
<td>0.17 ± 0.01 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>6.9 ± 0.1</td>
<td>4.5 ± 0.2 (7+)</td>
<td>13.7 kDa</td>
<td>9.6</td>
<td>4</td>
</tr>
<tr>
<td>m-NBA</td>
<td>9.16 ± 0.03</td>
<td>1.2 ± 0.2 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>7.7 ± 0.1</td>
<td>0.5 ± 0.1 (8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>6.8 ± 0.1</td>
<td>3.3 ± 0.5 (7+)</td>
<td>14.2 kDa</td>
<td>4.8</td>
<td>4</td>
</tr>
<tr>
<td>m-NBA</td>
<td>8.9 ± 0.1</td>
<td>1.7 ± 0.1 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>7.2 ± 0.2</td>
<td>1.0 ± 0.3 (8+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>7.5 ± 0.1</td>
<td>1.7 ± 0.1 (8+)</td>
<td>14.3 kDa</td>
<td>11.4</td>
<td>4</td>
</tr>
<tr>
<td>m-NBA</td>
<td>9.9 ± 0.1</td>
<td>0.60 ± 0.03 (10+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>8.8 ± 0.3</td>
<td>0.4 ± 0.1 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>holo-Myoglobin</td>
<td>8.2 ± 0.1</td>
<td>3.9 ± 0.4 (8+)</td>
<td>17.6 kDa</td>
<td>7.4</td>
<td>0</td>
</tr>
<tr>
<td>m-NBA</td>
<td>11.2 ± 0.2</td>
<td>0.79 ± 0.02 (11+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>9.8 ± 0.4</td>
<td>0.3 ± 0.1 (10+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo-Myoglobin</td>
<td>--</td>
<td>--</td>
<td>17.0 kDa</td>
<td>7.4</td>
<td>0</td>
</tr>
<tr>
<td>m-NBA</td>
<td>12.4 ± 0.6</td>
<td>0.71 ± 0.04 (11+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>9.2 ± 0.3</td>
<td>0.28 ± 0.01 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>8.13 ± 0.04</td>
<td>4.0 ± 0.2 (8+)</td>
<td>18.3 kDa</td>
<td>4.8</td>
<td>3</td>
</tr>
<tr>
<td>m-NBA</td>
<td>10.41 ± 0.03</td>
<td>2.88 ± 0.03 (10+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfolane</td>
<td>8.5 ± 0.2</td>
<td>1.9 ± 0.2 (9+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>9.8 ± 0.1</td>
<td>4.5 ± 0.6 (10+)</td>
<td>29.0 kDa</td>
<td>6.4</td>
<td>0</td>
</tr>
<tr>
<td>m-NBA</td>
<td>11.7 ± 0.1</td>
<td>0.7 ± 0.2 (12+)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Average charge state, average number of sodium adducts to the most abundant charge state, and fractional population of barnase-barstar and free barnase formed by nESI from aqueous solutions containing 6 µM barstar, 5 µM barnase, 10 mM ammonium bicarbonate, and 1 mM NaCl with no supercharging reagent, with 1.5% m-NBA, or with 2.5% sulfolane.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average charge</th>
<th>Average # Na adducts to most abundant charge state</th>
<th>% Total protein ion population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barnase-Barstar</td>
<td>8.8+</td>
<td>4.0</td>
<td>100%</td>
</tr>
<tr>
<td>+ m-NBA</td>
<td>11.0+</td>
<td>0.4</td>
<td>38%</td>
</tr>
<tr>
<td>+ sulfolane</td>
<td>9.6+</td>
<td>0.6</td>
<td>84%</td>
</tr>
<tr>
<td>Free Barnase</td>
<td>--</td>
<td>--</td>
<td>0%</td>
</tr>
<tr>
<td>+ m-NBA</td>
<td>6.4+</td>
<td>0.7</td>
<td>62%</td>
</tr>
<tr>
<td>+ sulfolane</td>
<td>5.7+</td>
<td>0.3</td>
<td>16%</td>
</tr>
</tbody>
</table>
Table 3. Measured masses and average sodium ion adduction for four different glycoforms of holo-transferrin formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate (ABC) and 1 mM NaCl with no supercharging reagent, with 1.5% m-NBA, or with 2.5% sulfolane.

<table>
<thead>
<tr>
<th>Protein glycoforms</th>
<th>Protein mass (Da)</th>
<th>Average # Na adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM ABC</td>
<td>10 mM ABC 1 mM NaCl</td>
</tr>
<tr>
<td>A</td>
<td>79,714 ± 4</td>
<td>79,882 ± 21</td>
</tr>
<tr>
<td>B</td>
<td>79,848 ± 10</td>
<td>79,910 ± 15</td>
</tr>
<tr>
<td>C</td>
<td>80,372 ± 7</td>
<td>80,561</td>
</tr>
<tr>
<td>D</td>
<td>80,505 ± 12</td>
<td>80,567 ± 20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>10 mM ABC</th>
<th>10 mM ABC 1 mM NaCl</th>
<th>+1.5% m-NBA</th>
<th>+2.5% sulfolane</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.6 ± 0.2</td>
<td>8.3 ± 1.0</td>
<td>11.1 ± 0.6</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>0.1 ± 0.5</td>
<td></td>
<td>2.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.7 ± 0.3</td>
<td>9.3</td>
<td>14.5 ± 1.0</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>D</td>
<td>0.1 ± 0.6</td>
<td></td>
<td>3.0 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>
4.8 Figures

Figure 1. Mass spectra of ubiquitin (5 µM) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% m-NBA, and (c) with 2.5% sulfolane. Expansions showing adduction of sodium ions to the 5-7+ molecular ions are shown on the right; MI indicates the protonated molecular ion, (M+nH)⁺.
Figure 2. Mass spectra of myoglobin (5 µM) formed by nESI from aqueous solutions containing 10 mM aqueous ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% m-NBA, and (c) with 2.5% sulfolane.
Figure 3. Mass spectra of barnase-barstar (5 µM) with excess barstar formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% m-NBA, and (c) with 2.5% sulfolane.
Figure 4. Mass spectra of human holo-transferrin (79.7 kDa) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate with (a) no additional salt added, (b) 1 mM NaCl, (c) 1 mM NaCl with 1.5% m-NBA, and (d) 1 mM NaCl with 2.5% sulfolane. Expansions of the 18-21+ charge states are shown to the right. Dashed lines indicate the calculated average m/z for each glycoform without adducts.
Figure 5. Mass spectra of rabbit LDH tetramer (146 kDa) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate with (a) no additional salt added, (b) 5 mM NaCl, and (c) 5 mM NaCl with 2.5% sulfolane. Expansions of the 26+ and 24+ charge states are shown to the right. Dashed lines indicate the calculated average m/z of the tetramer without adducts.
Figure 6. Mass spectra of barstar (5 µM) formed by nESI from aqueous solutions containing 10 mM ammonium bicarbonate and 1 mM NaCl (a) with no supercharging reagent, (b) with 1.5% m-NBA, and (c) with 2.5% sulfolane. The low m/z region of the spectra are expanded. Spectra are normalized to the most abundant ion, but insets are on the same absolute scale for comparison.
4.9 Supplementary Information

Table S-1. Average charge and average number of sodium ions adducted to individual charge states calculated from mass spectra of 5 µM protein in 10 mM ammonium bicarbonate, 1 mM NaCl with no supercharging reagent, with 1.5% m-NBA, or with 2.5% sulfolane. Data for the most abundant charge state is given in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Average Charge</th>
<th>4+</th>
<th>5+</th>
<th>6+</th>
<th>7+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>5.33 ± 0.01</td>
<td>3.2 ± 0.2</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>7.1 ± 0.1</td>
<td>1.6 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Barstar</td>
<td>5.5 ± 0.1</td>
<td>2.8 ± 0.3</td>
<td>3.5 ± 0.4</td>
<td>4.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>7.1 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.29 ± 0.04</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>6.5 ± 0.1</td>
<td>0.9 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>9.42 ± 0.04</td>
<td>2.4 ± 0.5</td>
<td>4.2 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>6.9 ± 0.1</td>
<td></td>
<td>3.7 ± 0.1</td>
<td>4.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>9.16 ± 0.03</td>
<td></td>
<td></td>
<td>0.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>6.8 ± 0.1</td>
<td></td>
<td>3.1 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>8.9 ± 0.1</td>
<td></td>
<td>1.1 ± 0.3</td>
<td>1.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>7.5 ± 0.1</td>
<td></td>
<td>2.9 ± 0.2</td>
<td>2.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>9.9 ± 0.1</td>
<td></td>
<td>0.6 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>holo-Myoglobin</td>
<td>8.2 ± 0.1</td>
<td></td>
<td></td>
<td>3.5 ± 0.6</td>
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</tr>
<tr>
<td>m-NBA sulfolane</td>
<td>11.2 ± 0.2</td>
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<td></td>
<td>1.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>apo-Myoglobin</td>
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<td></td>
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<tr>
<td>m-NBA sulfolane</td>
<td>12.4 ± 0.6</td>
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<td>B-lactalbumin</td>
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<td>3.6 ± 0.2</td>
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<tr>
<td>Protein</td>
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<td>9+</td>
<td>10+</td>
<td>11+</td>
<td>12+</td>
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<tr>
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<td>0.12 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td></td>
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<tr>
<td>Barstar m-NBA sulfolane</td>
<td>1.1 ± 0.1</td>
<td>0.89 ± 0.03</td>
<td></td>
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<tr>
<td>Cytochrome c m-NBA sulfolane</td>
<td>0.6 ± 0.1</td>
<td>0.99 ± 0.04</td>
<td>0.85 ± 0.03</td>
<td>0.82 ± 0.03</td>
<td>0.59 ± 0.04</td>
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<td>Ribonuclease A m-NBA sulfolane</td>
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<td>1.2 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>α-Lactalbumin m-NBA sulfolane</td>
<td>0.5 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>1.7 ± 0.1</td>
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<td></td>
</tr>
<tr>
<td>Lysozyme m-NBA sulfolane</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.60 ± 0.03</td>
<td>0.23 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>apo-Myoglobin m-NBA sulfolane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbonic Anhydrase m-NBA sulfolane</td>
<td>3.7 ± 0.7</td>
<td>4.0 ± 0.6</td>
<td>4.5 ± 0.6</td>
<td>4.8 ± 0.8</td>
<td>6.6 ± 2.7</td>
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<td>15⁺</td>
<td>16⁺</td>
<td>17⁺</td>
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</tr>
<tr>
<td>Ubiquitin m-NBA sulfolane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barstar m-NBA sulfolane</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Cytochrome c m-NBA sulfolane</td>
<td>0.30 ± 0.02</td>
<td>0.35 ± 0.03</td>
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<tr>
<td>Ribonuclease A m-NBA sulfolane</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin m-NBA sulfolane</td>
<td></td>
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</tr>
<tr>
<td>Lysozyme m-NBA sulfolane</td>
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<tr>
<td>Holo-Myoglobin m-NBA sulfolane</td>
<td>0.44 ± 0.03</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.2 ± 0.3</td>
</tr>
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<td>Apo-Myoglobin m-NBA sulfolane</td>
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<td></td>
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<tr>
<td>B-lactalbumin m-NBA sulfolane</td>
<td>1.9 ± 0.1</td>
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<td></td>
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<td></td>
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<tr>
<td>Carbonic Anhydrase m-NBA sulfolane</td>
<td>3.0 ± 0.5</td>
<td>2.3 ± 0.1</td>
<td>1.42 ± 0.04</td>
<td>1.3 ± 0.1</td>
<td>0.82 ± 0.03</td>
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<td>0.29 ± 0.01</td>
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<tr>
<td>Ubiquitin m-NBA sulfolane</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barstar m-NBA sulfolane</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c m-NBA sulfolane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease A m-NBA sulfolane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin m-NBA sulfolane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme m-NBA sulfolane</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>holo-Myoglobin m-NBA sulfolane</td>
<td>0.6 ± 0.3</td>
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<td></td>
</tr>
<tr>
<td>apo-Myoglobin m-NBA sulfolane</td>
<td></td>
<td>0.36 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-lactalbumin m-NBA sulfolane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbonic Anhydrase m-NBA sulfolane</td>
<td>0.8 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.6</td>
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<td></td>
</tr>
</tbody>
</table>
**Figure S-1.** nESI mass spectrum of 10 μM human holo-transferrin in 60:40 methanol: water with 2% acetic acid. Four glycoforms labeled A-D are present.

**Table S-2.** Experimental masses calculated from the spectrum in Figure S-1.

<table>
<thead>
<tr>
<th>Experimental Apo-Transferrin Mass</th>
<th>Calculated Apo-Transferrin Mass</th>
<th>Glycosylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>79,584 ± 1</td>
<td>79,584 Da</td>
<td>2 Diantennary</td>
</tr>
<tr>
<td>79,731 ± 2</td>
<td>79,730 Da</td>
<td>+Fuc</td>
</tr>
<tr>
<td>80,240 ± 1</td>
<td>80,240 Da</td>
<td>1 Di, 1 Triantennary</td>
</tr>
<tr>
<td>80,386 ± 2</td>
<td>80,386 Da</td>
<td>+ Fuc</td>
</tr>
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</table>
Figure S-2. nESI mass spectrum of 10 μM rabbit LDH in 60:40 methanol: water with 2% acetic acid.
4.9.1 Effects of supercharging reagents on S/N and LOD. Signal-to-noise ratio (S/N) and limit of detection (LOD) measurements for the most abundant protein ion for barstar, ubiquitin, α-lactalbumin, and ribonuclease A are given in Table S-3 and Figure S-3. The baseline amplitude was calculated by averaging signal intensity over a width of a m/z range of 0.5 in between ions contributing to chemical noise directly next to the most abundant protein ion in a spectrum, and the root mean square (RMS) electronic noise was calculated after baseline subtraction. The S/N was calculated as the ion signal intensity divided by the total noise (including chemical noise) over a m/z range of 20 directly next to the most abundant protein ion. A linear fit to the S/N data was used to determine the LOD (where S/N = 3) for each protein. Measurements for each protein were made using the same nanoelectrospray capillary to eliminate tip-to-tip variability, and the capillary was washed in between each sample with methanol and then water to prevent cross contamination between solutions.

For barstar, ubiquitin, and α-lactalbumin, the S/N of the most abundant ion formed with 1.5% m-NBA and 2.5% sulfolane is greater than without the supercharging reagents, and for barstar and ubiquitin in particular, the S/N with m-NBA is dramatically higher than without supercharging reagent and than with sulfolane (Table S-3 and Figure S-3). This result is likely due to the high chemical noise from sulfolane-salt clusters observed in mass spectra obtained from solutions with sulfolane (Table S-3). Supercharging reagents can also decrease the LOD for these protein ions formed from solutions containing sodium. The LOD for barstar from solutions containing sodium is a factor of 10 lower with m-NBA, the LOD for ubiquitin is halved with either m-NBA or sulfolane, and the LOD for α-lactalbumin is halved with sulfolane (Table S-3). However, there is little to no improvement in S/N or LOD with supercharging reagents for other proteins, such as ribonuclease A (Table S-3 and Figure S-3). The S/N of the most abundant ribonuclease A ion decreases with supercharging reagent, and the LOD for this protein more than doubles with sulfolane. Thus, even though significant desalting occurs for all four of these proteins with m-NBA and especially with sulfolane, the effect of these reagents on protein ion S/N and LOD depends on the protein.

Table S-3. Chemical noise data for 5 μM protein nanoelectrosprayed from 10 mM ammonium bicarbonate, 1 mM NaCl with no supercharging reagent, with 1.5% m-NBA, or with 2.5% sulfolane. “N/A” indicates that there is no chemical noise intensity next to the most abundant ion.

<table>
<thead>
<tr>
<th>Protein Ion</th>
<th>Protein Ion Abundance</th>
<th>RMS Noise</th>
<th>Chemical Noise</th>
<th>Baseline</th>
<th>S/N</th>
<th>LOD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barstar</td>
<td>1210</td>
<td>36</td>
<td>N/A</td>
<td>82</td>
<td>32</td>
<td>0.12</td>
</tr>
<tr>
<td>+m-NBA</td>
<td>1750</td>
<td>8</td>
<td>N/A</td>
<td>14</td>
<td>210</td>
<td>0.02</td>
</tr>
<tr>
<td>+sulfolane</td>
<td>2329</td>
<td>8</td>
<td>87</td>
<td>52</td>
<td>65</td>
<td>0.22</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>11882</td>
<td>36</td>
<td>N/A</td>
<td>57</td>
<td>326</td>
<td>0.49</td>
</tr>
<tr>
<td>+m-NBA</td>
<td>5242</td>
<td>4</td>
<td>N/A</td>
<td>14</td>
<td>1436</td>
<td>0.24</td>
</tr>
<tr>
<td>+sulfolane</td>
<td>6676</td>
<td>5</td>
<td>86</td>
<td>49</td>
<td>586</td>
<td>0.25</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>824</td>
<td>31</td>
<td>N/A</td>
<td>47</td>
<td>25</td>
<td>0.80</td>
</tr>
<tr>
<td>+m-NBA</td>
<td>172</td>
<td>3</td>
<td>N/A</td>
<td>5</td>
<td>31</td>
<td>0.50</td>
</tr>
<tr>
<td>+sulfolane</td>
<td>450</td>
<td>3</td>
<td>23</td>
<td>13</td>
<td>35</td>
<td>0.36</td>
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<tr>
<td>Ribonuclease A</td>
<td>1282</td>
<td>6</td>
<td>64</td>
<td>42</td>
<td>103</td>
<td>0.33</td>
</tr>
<tr>
<td>+m-NBA</td>
<td>1151</td>
<td>12</td>
<td>N/A</td>
<td>18</td>
<td>94</td>
<td>0.32</td>
</tr>
<tr>
<td>+sulfolane</td>
<td>899</td>
<td>5</td>
<td>35</td>
<td>20</td>
<td>60</td>
<td>0.82</td>
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</table>
Figure S-3. Concentration dependence of the S/N of the most abundant protein ion for (a) barstar, (b) ubiquitin, (c) α-lactalbumin, and (d) ribonuclease A in 10 mM ammonium bicarbonate and 1 mM NaCl with no supercharging reagent, with 1.5% m-NBA, and with 2.5% sulfolane.
Part 2: Electrothermal Supercharging

Chapter 5

Electrothermal supercharging in mass spectrometry and tandem mass spectrometry of native proteins

Reproduced with permission from Cassou, C. A.; Sterling, H. J.; Susa, A. C.; Williams, E. R. Anal. Chem. 2013, 85, 138-146

5.1 Abstract

Electrothermal supercharging of protein ions formed by electrospray ionization from buffered aqueous solutions results in significant increases to both the maximum and average charge states compared to more conventional native mass spectrometry in which ions are formed from the same solutions but with lower spray potentials. For eight of the nine proteins investigated, the maximum charge states of protonated ions formed from native solutions with electrothermal supercharging is greater than those obtained from conventional denaturing solutions consisting of water/methanol/acid, although the average charging is slightly lower owing to contributions of small populations of more folded low charge-state structures. Under these conditions, electrothermal supercharging is slightly less effective for anions than for cations. Equivalent sequence coverage (80%) is obtained with electron transfer dissociation of the same high charge-state ion of cytochrome c formed by electrothermal supercharging from native solutions and from denaturing solutions. Electrothermal supercharging should be advantageous for combining structural studies of proteins in native environments with mass spectrometers that have limited high \(m/z\) capabilities and for significantly improving tandem mass spectrometry performance for protein ions formed from solutions in which the molecules have native structures and activities.

5.2 Introduction

Mass spectrometry (MS) is an important tool in structural biology owing to its high sensitivity, speed, and information content obtainable from complex samples. Accurate measurements of molecular mass require ionization of intact molecules, which can readily be achieved with many methods, such as the commonly used matrix assisted laser desorption/ionization\(^{239, 240}\) and electrospray ionization (ESI).\(^{6, 17}\) Protein identification and determinations of posttranslational modifications can be accomplished with various forms of tandem mass spectrometry.\(^{77, 79}\) For these measurements, the highly charged ions formed by electrospray ionization are advantageous owing to their ability to be more readily dissociated,\(^{43, 46, 93, 175}\) and also more efficiently detected in instruments, such as orbitrap and Fourier transform ion cyclotron resonance (FT/ICR) mass spectrometers, where charge-sensitive detection is used. Solutions in which the protein is denatured are commonly used for these measurements to increase the charge states formed by ESI and to enhance stable ion formation at high solution flow rates. However, information about protein activity, conformation, protein-protein binding, complex assembly and stoichiometry is lost in solutions where proteins do not have native structures.
The advent of nanoelectrospray has greatly increased the application of “native” mass spectrometry\(^88, 241\) by enhancing stable ion formation from buffered aqueous solutions in which proteins have native structures, even from solutions where high levels of essential salts that can adversely affect electrospray performance are required.\(^110\) Experiments aimed at encoding information about protein conformation, protein-substrate binding sites, allostERIC regulation, biomolecular complex structure can be done in solutions in which the protein or biomolecule complex has native structure, and mass spectrometry can be used to read out the encoded information. For example, footprinting strategies, such as oxidative footprinting,\(^152, 155, 156\) hydrogen/deuterium exchange (HDX),\(^69, 97, 99, 151, 152\) or limited proteolysis\(^242-244\) can be used to study changes in protein structure in solution. Non-native conditions are often subsequently used to produce the high charge states that are advantageous in order to read out the encoded information with MS or tandem MS. If sufficiently high charge states are produced from solutions in which the protein has native structure and activity, tandem MS analysis can be done directly via top-down fragmentation.\(^69, 245\)

Charge states formed in ESI depend on a number of factors, including solvent surface tension,\(^21, 26\) relative gas-phase basicities of sample solution components,\(^26, 107, 192, 246\) protein conformation,\(^31, 34, 35\) and various instrument parameters.\(^24, 247\) One method to increase charging in native mass spectrometry is with supercharging reagents.\(^30, 38, 66-72, 183-185\) When added to solutions at low concentrations, these supercharging reagents do not affect protein structure,\(^30, 68, 69\) but can cause chemical and/or thermal destabilization of the protein in the ESI droplet as the concentration of these low vapor pressure reagents increases. Destabilization can lead to a change in the protein structure, including partial unfolding or denaturation, which leads to higher charging,\(^30, 68-72\) although other effects, such as changes in surface tension, also play a role.\(^70\) The higher charge-state ions produced by supercharging from native solutions often have larger collisional cross sections measured by ion mobility,\(^30, 38, 69\) but this is not always the case.\(^38, 71\) For concanavalin A tetramer, there is a threshold of enhanced charging below which there is not an increase in cross sections, but above which cross sections increase significantly.\(^71\) For the SAP (serum amyloid P) 5-mer, no increase in cross section was observed with supercharging, which led the authors to conclude that no significant structural changes occurred with supercharging.\(^38\) However, differences in ion conformation are not always reflected by a difference in collision cross section.\(^248, 249\) For example, different conformers of ubiquitin that have the same charge state and collision cross section can have distinctly different rates of HDX.\(^248, 249\) Protein ions that are initially more elongated can also fold to more compact conformations in the gas phase.\(^56, 164, 175\)

Although higher charge states are normally associated with more unfolded structures, recent results with the octameric form of the \textit{Bacillus anthracis} toxin complex showed that higher charge states are formed when a specific pH induced conformational change takes place in solution, which makes the rotationally averaged size of the complex more compact both in solution and in the gas phase.\(^71\) An extended \(\beta\)-barrel domain in the structure that results in a lower rotationally averaged size of the entire complex appears to be able to accommodate more charge.

High charge-state ions can also be produced from unbuffered aqueous solutions by introducing acid or base vapor into drying gas used in an ESI interface.\(^59-61\) Protein denaturation that can occur when the pH of the ESI droplet changes can result in the formation of high charge-state ions. The use of supercharging reagents has the advantage that they are effective even...
when heavily buffered solutions that are often necessary to preserve native structure and function are used.\textsuperscript{30, 68-72}

A new electrothermal supercharging method to produce high charge-state ions from buffered aqueous solution was recently introduced.\textsuperscript{65} In this method, typical low charge-state distributions of proteins and intact complexes in buffered aqueous solutions are obtained using low ESI spray potentials, but high charge-state ions are produced from the same solutions simply by increasing the ESI spray potential. The charge-state distributions obtained with the electrothermal supercharging method can resemble those obtained from standard denaturing solutions even though the protein has a native folded structure in the original ESI solution prior to droplet formation. These results indicate that the increased charging is due to protein unfolding that occurs in the ESI droplet as a result of activation that occurs at high electric field.\textsuperscript{65}

Here, the effectiveness of electrothermal supercharging at producing high charge-state ions from native solutions is compared to charging obtained from denaturing solutions for a variety of proteins. This method can overcome instrumental $m/z$ limits in native mass spectrometry and can be used to greatly increase the sequence information obtainable from tandem MS of large protein ions formed from solutions in which the protein has native structure and activity.

### 5.3 Experimental

All proteins were purchased as lyophilized solids from Sigma (St. Louis, MO, USA) with the exception of truncated edema factor (EF\textsubscript{N}) and full length protective antigen (PA) proteins, which were a kind gift of Professor Bryan Krantz of the University of California, Berkeley, and were prepared as previously published,\textsuperscript{201} and barstar, which was expressed in \textit{E. coli} and purified as described previously.\textsuperscript{205} Solutions consisting of 10 µM protein in either “native” conditions (aqueous 100 mM ammonium bicarbonate, pH 7) or denaturing conditions (48/48/4 by volume methanol/water/acetic acid or methanol/water/ammonium hydroxide for positive and negative ions, respectively) were prepared. About 5 µL of each solution was loaded into a borosilicate capillary (1.0 mm o.d./0.78 mm i.d., Sutter Instruments, Novato, CA, USA) pulled to a tip i.d. of ~1 µm with a Flaming/Brown micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA). Nanospray was initiated by applying about ±1 kV to a platinum wire (0.127 mm diameter, Sigma, St. Louis, MO, USA) inserted into the sample solution with the nanospray emitter positioned ~3 mm from the entrance to the mass spectrometer. To obtain native mass spectra, the spray potential was about ±0.7 kV. For electrothermal supercharging, the spray potential was increased to achieve the maximum extent of electrothermal supercharging while maintaining stable ion formation.

Mass spectra were acquired in triplicate with three different ESI emitters per sample using a Thermo Linear Trap Quadrupole (LTQ)\textsuperscript{TM} (Thermo Fischer Scientific, Waltham, MA, USA) with a heated entrance capillary temperature of 275 °C. The glass windows on this instrument’s source assembly were removed for all experiments to maintain a low temperature in the air space around the nanoESI emitters to prevent protein unfolding in the emitter prior to electrospray. Mass spectra of bovine serum albumin and a mixture of PA and the dendrimer DAB-Am-16 were obtained on this same instrument, but with an entrance capillary temperature of 220 °C and 275 °C, respectively. Mass spectra from the latter samples were also acquired using a Waters Quadrupole-Time-of-Flight (Q-TOF) Premier\textsuperscript{TM} (Waters, Milford, MA, USA).
Electron transfer dissociation (ETD) experiments were done using a Thermo LTQ-Orbitrap™ (Thermo Fischer Scientific, Waltham, MA, USA) with a 20 ms reaction time with fluoranthene anion, which depleted >80% of the precursor ion abundance.

5.4 Results and Discussion

5.4.1 Electrothermal supercharging of positive ions. The extent to which electrothermal supercharging can increase the charge states of protein ions formed by nanoESI from native solutions (100 mM aqueous ammonium bicarbonate, pH 7.0) is illustrated for the protein myoglobin in Figure 1. An ESI mass spectrum obtained at a spray potential of +0.7 kV and a heated entrance capillary temperature of 275 °C is shown in Figure 1a. There are charge-state distributions corresponding to both holo- and apo-myoglobin that are centered around 8+ under these conditions, with an average charge of 8.1 ± 0.1+ and 7.6 ± 0.1+, respectively. Narrow, low charge-state distributions such as these are typically observed for protein ions formed from buffered aqueous solutions in which the protein has native or native-like structure.31 Low charge states of apo-myoglobin (23% of the total ion abundance) indicates that there is some activation of the protein from the relatively high temperature source conditions that results in a slight conformational change of the protein with loss of the non-covalently bound heme group in the gas phase.

With the spray potential at +1.4 kV and all other parameters the same, apo-myoglobin ions are predominantly formed (94% of the total ion abundance) with a charge-state distribution that is significantly broader and shifted to higher charge (Figure 1b). Charge states up to 28+ are formed, with 13+ the most abundant. The overall average charge of both forms of myoglobin is 14.8 ± 0.5+, which is nearly twice that obtained at the lower spray potential. By comparison, the charge-state distribution of myoglobin formed from a typical denaturing solution (48/48/4 by volume water/methanol/acetic acid) under the same conditions is monomodal, and corresponds to just apo-myoglobin with an average charge of 17.46 ± 0.02+. Although the relative abundances of the higher charge states are somewhat greater from the denaturing solution (Figure 1c), the maximum charge state of 28+ formed from the denaturing solution is the same as that obtained by electrothermal supercharging (Figure 1b).

The presence of apo-myoglobin ions with high charge and holo-myoglobin with low charge obtained by electrothermal supercharging under these conditions indicates the presence of populations of both folded (low charge-state distribution) and unfolded (high charge-state distribution) proteins molecules in solution prior to ion formation. The temperature of the tip of the ESI capillary is less than 40 °C with these source conditions.55 Thermal unfolding of myoglobin at neutral pH occurs around 76.5 °C (in 100 mM sodium phosphate buffer, pH 7.0),250 so there should be an insignificant population of unfolded myoglobin in the original solution prior to droplet formation. Electrothermal supercharging also occurs for ubiquitin and cytochrome c, which also have significantly higher thermal melting temperatures than the temperature of the nanoESI tip.65 These results are consistent with protein unfolding occurring in the ESI droplet prior to ion formation under these electrothermal supercharging conditions.65 A comparison between the charging obtained with electrothermal supercharging of protein ions formed from native solutions and charging obtained for the same protein ion formed from denaturing solutions for nine different proteins ranging in size from 8.6 kDa to 30.0 kDa is given in Table 1 and plotted in Figure 1d. The dashed line corresponds to a direct correlation between maximum charge or average charge of ions formed from denaturing solutions and
native solutions at either the highest nanoESI potential (high V, electrothermal supercharging), or the lowest potential (low V, native MS) at which stable electrospray could be maintained. The maximum and average charge with native MS is generally much lower than that obtained from denaturing solution (on average 41% and 36% lower than from denaturing solution, respectively), and there is a greater difference with increasing protein molecular weight, consistent with previous reports.\textsuperscript{11, 251} In striking contrast, the maximum charge state for eight of the nine protein ions formed with electrothermal supercharging is \textit{equal to or higher} than that obtained for the same protein ions formed from denaturing solution. The average charge with electrothermal supercharging is only slightly lower than that obtained from the denaturing solution owing to the broader distribution of charges formed as a result of contributions from both unfolded and folded structures. Electrothermal supercharging also produces high charge-state ions comparable to denaturing conditions for proteins that have multiple disulfide bonds, such as phospholipase A\textsubscript{2} and lysozyme, which have six and four disulfide bonds, respectively.

Unusually high charge states of EF\textsubscript{N}, a truncation mutant of anthrax edema factor, are formed under native (low V) conditions (marked by † in Figure 1d; spectra shown in Figure S-1a). The maximum and average charge are 38+ and 21.3 ± 1.0+, respectively, values which are very similar to those from denaturing conditions (42+ and 23.5 ± 0.4+, respectively) and from electrothermal supercharging conditions (47+ and 24.0 ± 0.5+, respectively). Even under the very gentle conditions of a Waters Q-TOF mass spectrometer, which has a z-spray source and inlet temperature of 80 °C, a bimodal charge-state distribution is observed (Figure S-1b), but a low charge-state distribution centered around 10+ predominates. A nearly identical spectrum is obtained with this instrument when 100 mM ammonium acetate, pH 7, is used as the native buffer solution (Supplementary Figure 1c). These data suggest that both folded and unfolded forms of EF\textsubscript{N} may be present in solution. The molten globule state of this truncation mutant EF\textsubscript{N} is only 1 kcal/mol higher in Gibbs free energy than the native form.\textsuperscript{252} Edema factor is an enzyme translocated by anthrax toxin into the cytosol of a host’s cell via a β-barrel channel the toxin forms through the cell membrane.\textsuperscript{253} This β-barrel is too narrow for folded EF\textsubscript{N} to pass, so EF\textsubscript{N} must unfold in order to enter the cell.\textsuperscript{252, 254} The ease with which this protein unfolds may account for the high charge-state distribution formed in native MS.

5.4.2 Electrothermal supercharging of negative ions. Results for the same nine proteins formed as anions under the same experimental conditions as the cations (Figure 1) are shown in Figure 2. With a spray potential of -0.7 kV, only holo-myoglobin ions are formed with an average charge of 5.7 ± 0.1-. The absence of apo-myoglobin under the same conditions as the positive ion data (Figure 1a) suggests that less activation occurs with the anions. This also appears to be the case at high spray potential (-1.4 kV) (Figure 2b), where there is a bimodal distribution with both a holo-myoglobin distribution centered around the 6- charge state and a largely apo-myoglobin distribution centered around the 14- charge state. 57 ± 5% of the total ion abundance consists of apo-myoglobin, significantly less than the 93 ± 1% under the same electrothermal supercharging conditions with positive ions (Figure 1b). Even with denaturing conditions of 48/48/4 water/methanol/ammonium hydroxide, 13 ± 2% of the ion signal corresponds to holo-myoglobin (Figure 2c). The maximum and average charge of holo-myoglobin under electrothermal supercharging are 18- and 9.9 ± 1.7-, respectively, and these values for apo-myoglobin are 20- and 14.1 ± 0.2- (Figure 2b). Electrothermal supercharging and denaturing conditions both result in a maximum charge of 20-, but electrothermal supercharging results in an average charge of 12.3 ± 0.7-, which is slightly lower than that obtained from
denaturing solution (13.1 ± 0.2-) as a result of the significant population of holo-myoglobin with low charge.

The maximum charge state and average charge from electrothermal supercharging for all nine proteins are compared to those from denaturing solution in Figure 2d. At low electrospray potentials, the maximum and average charge are much lower than those formed from denaturing solution (on average 34% and 27% lower than from denaturing solution, respectively), except for phospholipase A2, lysozyme and EFN. Lysozyme has a maximum and average charge of 7- and 5.5 ± 0.1- under denaturing conditions, which is not much higher than the values of 6- and 4.94 ± 0.04- obtained under native conditions, consistent with previous reports of low charge states centered around 6- for unreduced lysozyme formed from solutions in which the protein is non-native. The average charge of EFN under native conditions (23.4 ± 0.6-) is about equal to that from denaturing solutions (22.9 ± 0.2-) (Figure S-2a). As was the case for EFN cations, there is a distribution of high charge anions indicative of more unfolded conformers of the protein even when very soft source conditions in a Q-TOF mass spectrometer are used (Figure S-2b).

The increase in the average charge of ions from electrothermal supercharging compared to ions from native MS is 78% of that from the denaturing solution for positive ions and 40% for negative ions. Similarly, the fraction of unfolded ions (the abundances of high charge distributions divided by the total protein ion abundance) from electrothermal supercharging is 81% of that from denaturing solution for positive ions and 33% for negative ions. The overall effectiveness of electrothermal supercharging at producing high charge states and unfolding proteins during ESI is lower for negative ions than for positive ions for eight of the nine proteins under the conditions used. There is no apparent trend with protein pI (pI = 4.5-11), indicating that electrothermal supercharging is more effective for positive than negative ions for acidic and basic proteins alike. The net negative charge in native MS is lower than the net positive charge for all proteins except barstar, for which the charging is nearly the same. Only for this protein is electrothermal supercharging slightly more effective for negative compared to positive ions.

This suggests that the difference in efficiency of electrothermal supercharging for positive versus negative ions may be due to the difference in the net charge on the protein. Lower charge-state ions have less Coulombic repulsion and may require more energetic conditions to cause unfolding. The effectiveness of electrothermal supercharging has been shown previously to depend on the source capillary temperature. To determine if supercharging of negative ions may be increased at higher source capillary temperatures, spectra for cytochrome c were obtained at source capillary temperatures ranging from 150 °C to 300 °C with spray potentials between ±0.8 kV and ±1.6 kV (Figure S-3). No electrothermal supercharging is observed for anions at either 150 °C or 175 °C, but the extent of electrothermal supercharging increases significantly between 200 °C and 300 °C, where ~58% of the ions are unfolded (average charge 8.1 ± 1.6-) at the highest temperature and spray potential. This value is still significantly less than for positive ions, where ~84% of cytochrome c ions are unfolded (average charge 13.8 ± 0.5+) at the same source temperature, but it shows that the effectiveness of electrothermal supercharging can be optimized for anions, as it can for cations, by adjusting source temperatures. In contrast, the source capillary temperature has little effect on the average charge state of ions formed from denaturing solutions.

### 5.4.3 Instrument m/z limits and native MS

Charging of a protein or protein complex in native mass spectrometry generally increases as $R^{3/2}$, where $R$ is the radius of the protein approximated as a sphere, or as $M^{1/2}$, where $M$ is the protein molecular weight. 11, 18, 251, 255, 256
Thus, instruments with high \( m/z \) capabilities are typically used for measuring mass spectra of large proteins and protein complexes from buffered aqueous solutions in which these molecules have native structures. For example, an ESI mass spectrum of bovine serum albumin (583 residues, 67 kDa; aqueous 100 mM ammonium bicarbonate, pH 7) acquired using a Q-TOF mass spectrometer is shown in Figure 3a. Molecular ions with charges states ranging from 14+ to 19+ are formed. The 16+ at \( m/z = 4176 \) is the most abundant ion and the average charge is 16.4+. A mass spectrum acquired with this same solution using a Thermo LTQ mass spectrometer (+0.8 kV spray potential) has similar relative abundances of the 17+ to 19+ charge states, but the most abundant ion measured with the Q-TOF (16+) is outside the \( m/z \) range (≤ 4000) of this LTQ instrument (Figure 3 b). With electrothermal supercharging with the LTQ mass spectrometer (+1.6 kV spray potential, all other conditions identical), predominantly high charge-state ions ranging from 29+ to 65+ are formed and the average charge is 48+. There is a low abundance of 17+ and 18+ charge states, indicating that some fraction of the population remains folded under these conditions. Electrothermal supercharging shifts the charge-state distribution from the upper edge of the \( m/z \) range of the LTQ instrument to one that is almost entirely within the range of this instrument even though the protein is folded in the original solution from which these ions are formed.

The charge states of molecular ions of even larger proteins formed in native MS can be entirely outside the \( m/z \) range of many types of mass spectrometers. A mass spectrum of an equimolar mixture of the 83 kDa protective antigen (PA) protein from \textit{Bacillus anthracis} and a 1.7 kDa dendrimer poly(propyleneimine) hexadecamine generation 3.0, DAB-Am-16, measured using a Q-TOF mass spectrometer is shown in Figure 4a. PA ions with charge states ranging from 15+ to 20+ are formed with an average charge state of 18.3+. DAB-Am-16 2+ to 4+ ions are formed with an average charge of 3.0+. Even the most highly charged molecular ion of PA (20+) formed from this buffered aqueous solution has a \( m/z \) = 4192 that is outside the range of the LTQ instrument. No PA ions are observed in a mass spectrum obtained from this same solution using a LTQ instrument (Figure 4b) under native conditions (+0.8 kV spray potential). The observation of a charge-state distribution for DAB-Am-16 with an average charge state of 2.9+ (Figure 4b) indicates that that absence of PA ions in this spectrum is due to formation of molecular ion charge states that are outside the \( m/z \) range of the mass spectrometer rather than an inability to form ions from this solution under these conditions. The data in figure 4a show that the presence of the dendrimer does not suppress PA ion formation from this solution. With electrothermal supercharging with the LTQ instrument (+1.6 kV spray potential, all other conditions the same as those used for Figure 4b), a charge-state distribution of PA ions ranging from 36+ to 86+ is observed with an average charge of 60.2+. Thus, electrothermal supercharging produces more than a 3-fold gain in the number of charges for this protein. The charge-state distribution with electrothermal supercharging is centered around \( m/z \) = 1400, well within the \( m/z \) range of the mass spectrometer, despite these ions being formed from a buffered aqueous solution in which the structure of the protein was originally native prior to ESI droplet formation.

Interestingly, the charge-state distribution for DAB-Am-16 is slightly higher under native conditions (Figure 4b) than under electrothermal supercharging conditions (Figure 4c) with an average charge of 2.9+ and 2.8+, respectively. A similar result was observed for a mixture of ubiquitin and DAB-Am-16 (100 mM aqueous ammonium bicarbonate, data not shown) where a change in the spray potential from 0.7 kV to 1.7 kV resulted in a 30% increase in the average charge of ubiquitin compared to a 13% decrease in the average charge of DAB-Am-16. DAB-
Am-16 has 16 primary amine termini that can potentially be protonated in these experiments, and 5+ ions can be readily formed under some instrumental conditions from these ammonium bicarbonate solutions. In contrast to both ubiquitin and PA, DAB-Am-16 cannot undergo large structural changes, i.e., DAB-Am-16 maintains a compact, spherical structure without significant changes in solvent exposed surface area in aqueous and organic solvents and cannot unfold into a more extended structure like these globular proteins can do when denatured. Thus, the absence of a charge enhancement for DAB-Am-16 with electrothermal supercharging is consistent with the inability to cause unfolding of this molecule in the ESI droplet as opposed to the inability for this molecule to be able to carry additional charge. The slightly lower charge on DAB-Am-16 obtained with electrothermal supercharging is consistent with the higher spray potential causing more activation to the ions prior to their reaching the inlet capillary. Energetic collisions can drive endothermic proton transfer reactions between multiply protonated DAB-Am-16 and water, which is the solvent in these experiments, and result in a lowering of the net charge state of DAB-Am-16.

5.4.4 Improving dissociation from native solutions. Dissociation of low charge states of protein ions formed in native MS generally results in limited fragmentation and low sequence coverage compared to the high charge-state ions formed from denaturing solutions. For example, the most abundant cytochrome c ion formed from a native solution (+0.75 kV; Figure 5a) is 7+, less than half the number of charges as the most abundant ion formed for this protein from denaturing solutions (16+; Figure 5c). ETD (electron transfer dissociation) of the 7+ ion formed under native conditions results in mostly charge-reduced precursor ions (85% product ion abundance) and few sequence specific ions. Fragment ions $z_{17} - z_{19}$ and $z_{35}$ are formed, resulting in only 4% sequence coverage under these conditions. These results are consistent with previous ETD and ECD (electron capture dissociation) studies of low charge states of cytochrome c formed from native conditions that report little to no sequence coverage for charge states up to the 8+ and 9+ precursor ions without thermal or collisional activation prior to or subsequent to fragmentation by ETD or ECD. Reduced precursors are also formed in ETD of the 16+ ion formed from denaturing solution (11% product ion abundance), but extensive c and z fragment ions are also formed as a result of cleavages at 79 of the 99 possible backbone cleavage sites, excluding sites N-terminal to cytochrome c’s proline residues as possible cleavage sites, resulting in 80% sequence coverage. Abundant 16+ charge-state ions are also formed with electrothermal supercharging from the native solution (Figure 5b), and ETD of this ion also results in cleavage at 79 cleavage sites (80% sequence coverage) that are mostly the same as those from ETD of the 16+ ions from denaturing solution (Figure 6), suggesting the more open gas-phase structures of the 16+ ions formed from these two different solutions are similar, but not identical. No fragmentation occurs between residues 12 and 22 as a result of covalent bonds to the heme moiety at residues 14 and 17, consistent with previous ECD results for this protein. These results show that essentially the same sequence coverage can be obtained from the high charge-state ions formed by electrothermal supercharging of proteins from native solutions as that obtained from the same high charge states formed from denaturing solutions.

5.5 Conclusions

Electrothermal supercharging with ammonium bicarbonate can effectively produce high charge-state ions of proteins from buffered aqueous solutions in which the protein has a native or
native-like structure. The extent of supercharging from these buffered aqueous solutions is comparable to that obtained from denaturing solutions in which no supercharging reagents are used. Electrothermal supercharging makes it possible to combine native MS of large molecules with instruments that have limited upper \( m/z \) limits for molecular weight measurements. This method also enables greatly increased sequence information from tandem MS of ions formed from native solutions. This method could be particularly advantageous for more routinely combining structural studies of proteins in native environments with MS. For example, this method could be used in continuous H/D exchange studies by replacing acid denaturation\(^9\) or conventional supercharging reagents\(^6\) to produce high charge-state ions for tandem MS determination of where solution-phase exchange has occurred. The short lifetime of the ESI droplet essentially eliminates the opportunity for back-exchange to occur in these experiments. Information about protein complex stoichiometries can be obtained from mass measurements of intact complexes, and from the same solutions, information about the molecular sequence can be obtained from tandem MS experiments of high charge-state ions formed by electrothermal supercharging simply by increasing the spray potential. The extent of electrothermal supercharging depends on the protein, and is more effective for positive ions than for negative ions for eight of the nine proteins investigated here. The effectiveness of electrothermal supercharging depends on a number of factors, including the source temperature, physical properties of the protein, and the nature of the buffer. It is possible that other buffers or salts could be identified that would improve the charge enhancement for negative ions.

### 5.6 Acknowledgements

The authors thank the National Institutes of Health (Grant No. R01GM096097) and the National Science Foundation (Graduate Research Fellowship for CAC; Grant No. DGE1106400) for financial support, and Professor Bryan A. Krantz and Dr. Geoffrey K. Feld for their generous donation of protective antigen and edema factor samples.
### 5.7 Tables

*Table 1.* Charge states and average charge of positive and negative ions formed in nanoESI by electrothermal supercharging from native solution and from denaturing solution.

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<th>Protein</th>
<th>MW (kDa)</th>
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<th>Negative Ions</th>
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<td>Denatured</td>
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<td>14 – 4+</td>
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<td>12 – 5+</td>
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<td>28 – 8+</td>
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<td>42 – 10+</td>
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<tr>
<td>EFN</td>
<td>37 – 8-</td>
<td>21.0 ± 0.2</td>
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5.8 Figures

Figure 1. Positive ion nanoelectrospray mass spectra of 10 µM myoglobin in 100 mM ammonium bicarbonate, pH 7, at electrospray potentials of (a) +0.70 kV (native) and (b) +1.4 kV (electrothermal supercharging), and in (c) methanol/water/acetic acid (48/48/4, v/v) denaturing solution. (d) Correlation plot of the maximum and average charge state for nine proteins obtained from denaturing solution and from native and electrothermal supercharging from buffered aqueous solution. Data marked by "†" are discussed in the text.
Figure 2. Negative ion nanoelectrospray mass spectra of 10 µM myoglobin in 100 mM ammonium bicarbonate, pH 7, at electrospray potentials of (a) -0.70 kV (native) and (b) -1.4 kV (electrothermal supercharging), and in (c) methanol/water/ammonium hydroxide (48/48/4, v/v) denaturing solution. (d) Correlation plot of the maximum and average charge state for nine proteins obtained from denaturing solution and from native and eletrothermal supercharging from buffered aqueous solution. Data marked by "†" are discussed in the text.
Figure 3. Positive ion nanoelectrospray mass spectra of 10 µM bovine serum albumin (67 kDa) in 100 mM ammonium bicarbonate, pH 7, obtained (a) using a Q-TOF mass spectrometer with high m/z capabilities, and using a Thermo LTQ™ mass spectrometer with an upper m/z limit of 4,000 at (b) +0.8 kV (native) and (c) +1.6 kV (electrothermal supercharging) spray potentials.
**Figure 4.** Positive ion nanoelectrospray mass spectra of a mixture of 10 µM protective antigen (83 kDa) (PDB ID: 1ACC) and 10 µM DAB-Am-16 dendrimer (1.7 kDa) in 100 mM ammonium bicarbonate, pH 7, obtained (a) using a Q-TOF mass spectrometer with high m/z capabilities, and using a Thermo LTQ™ with an upper m/z limit of 4,000 at (b) +0.8 kV (native) and (c) +1.6 kV (electrothermal supercharging) spray potentials.
Figure 5. Positive ion nanoelectrospray mass spectra of cytochrome c formed from 100 mM ammonium bicarbonate, pH7, at (a) +0.75 kV spray potential (native) and (b) +1.3 kV spray potential (electrothermal supercharging), and from (c) a methanol/water/acetic acid solution (denaturing). ETD of (d) the 7+ ion from native solution, (e) the 16+ ion from electrothermal supercharging from the same native solution, and (f) the 16+ ion from denaturing solution resulted in sequence coverages of 4%, 80%, and 80%, respectively.
**Figure 6.** Sequence coverage map showing the cleavage sites resulting from ETD of the 16+ cytochrome c ion produced by nanoESI from native solution with electrothermal supercharging and by nanoESI from denaturing solution.
5.9 Supporting Information

**Figure S-1.** Positive ion nanoelectrospray mass spectra of 10 µM EF₅ (30 kDa) in 100 mM ammonium bicarbonate, pH 7, (a) obtained using a Thermo LTQ™ mass spectrometer at +0.7 kV spray potential, and (b) using a Waters Q-TOF mass spectrometer. (c) Mass spectrum of 10 µM EF₅ in 100 mM ammonium acetate, pH 7, obtained using a Waters Q-TOF mass spectrometer. Insets in (b) and (c) show that even under the less activating conditions used with the Q-TOF instrument, some high charge states of EF₅ are present, an indication of a population of unfolded structures in solution.
Figure S-2. Negative ion nanoelectrospray mass spectra of 10 μM EFN (30 kDa) in 100 mM ammonium bicarbonate, pH 7, obtained using (a) a Thermo LTQ™ mass spectrometer at -0.7 kV spray potential, and (b) using a Waters Q-TOF mass spectrometer. The inset shows a population of unfolded conformers of EFN formed even under optimized soft source conditions.
Figure S-3. The population of unfolded ions of cytochrome c produced from a 100 mM ammonium bicarbonate, pH 7, solution calculated by assigning the 9−6+ and 6−4− charge states as “folded” conformers and all other charge states as “unfolded” conformers. More electrothermal supercharging occurs for positive ions than for negative ions for a given heated inlet capillary temperature and spray potential.
6.1 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 to 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.

6.2 Introduction

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, and to investigate higher order protein and protein complex structure using a variety of techniques, such as hydrogen-deuterium exchange (HDX) or photochemical oxidative labeling. In native MS, 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, protein-ligand binding, and specific changes to protein or protein complex structure in solution. 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 and can be detected more efficiently with charge sensitive detectors, such as those in Fourier transform ion cyclotron resonance (FT-ICR) 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. Unresolved adducts on high mass protein or protein
complexes can considerably broaden mass spectral peaks, resulting in decreased sensitivity and reduced mass measuring accuracy.\textsuperscript{110, 112}

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 \textit{m}-NBA or sulfolane.\textsuperscript{30, 38, 66-72, 183-185}

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.\textsuperscript{30, 68-72}

Other effects, such as droplet surface tension, also play a role in supercharging from both native\textsuperscript{21, 70} and denaturing\textsuperscript{21, 181, 182} solutions. Supercharging reagents are effective at increasing the charge of intact non-covalent protein-protein and protein-ligand complexes\textsuperscript{30, 66, 68, 71, 72} but can also induce dissociation of complexes in the ESI droplet.\textsuperscript{67, 270}

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.\textsuperscript{65, 271, 272}

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-charge distribution are typically produced, where the maximum charge is similar to or greater than that formed from denaturing solutions.\textsuperscript{271}

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 \textit{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.\textsuperscript{271}

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.\textsuperscript{65, 271} ETS is not effective with pure water.\textsuperscript{65}

Mirza and Chait\textsuperscript{273} 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,\textsuperscript{65} yet the vapor pressures of 1 M sodium carbonate and 1 M sodium acetate solutions at 100 °C differ by only ~0.5%.\textsuperscript{274} Protein denaturation is well known to occur at water-air interfaces,\textsuperscript{275-278} such as occurs at the droplet surface or at a bubble surface if gaseous evolution occurs in the droplet. Konermann and coworkers\textsuperscript{272} 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 stabilization have been extensively investigated.\textsuperscript{135, 137, 220, 225, 279} Studies done over 125 years ago by Franz Hofmeister\textsuperscript{135} 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\(_3\))\(_4\)^+ (NH\(_4\))\(_4\)^+ > N(CH\(_3\))\(_4\)^+ 
Anions: SCN\(^-\) > ClO\(_4\)^- > I\(^-\) > Br\(^-\) > Cl\(^-\) > CHO\(_2\)^- > HCO\(_3\)^- > C\(_2\)H\(_3\)O\(_2\)^- > 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. 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. 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. Hofmeister effects are also associated with physical properties of aqueous electrolyte solutions and solution-phase ionic properties, such as surface
tension, ion free energy of hydration, viscosity B-coefficient, and ion surface/bulk
partitioning. Colussi and coworkers 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 coworkers 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.

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.

6.3 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 micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA). All
mass spectra were acquired using a Thermo LTQ (Linear Trap Quadrupole) Orbitrap\(^\text{TM}\) 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, >100 °C
for ubiquitin,289 and ~82 °C for β-lactoglobulin A290) 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 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 ten minutes. 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.

6.4 Results and Discussion

6.4.1 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.115 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) 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 unfolded of cytochrome c from this solution are 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). 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 1(a)-(c), Table 1). With sulfate, 100% of the ion population is folded at low spray potential (Figure S-1b), 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 1(h)); 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 1(i)-(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.138, 141, 143, 283, 284, 291 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.

The ordering of anions in their effectiveness at ETS of ubiquitin is slightly different than previously reported.65 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.

### 6.4.2 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 ($0.87 \pm 0.01$) and sodium hydrogen phosphate ($0.98 \pm 0.01$) solutions, and minimal unfolding from sodium iodide ($0.06 \pm 0.01$) and sodium perchlorate ($0.04 \pm 0.02$) solutions. Thiocyanate is an exception where the fraction unfolded is $0.01 \pm 0.01$ with sodium as the cation, the least unfolding among all of the sodium salts, whereas the unfolded population is $0.69 \pm 0.01$ 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.

### 6.4.3 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). The solution pH is known in these experiments prior to ESI droplet formation, but droplet pH decreases during droplet evaporation. 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. By 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 intermediate 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.139

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 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.295 For example, Tomé, et al.295 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 based on 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 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,292-294 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.296 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.

6.4.4 Electrothermal supercharging and bubbles. Ammonium bicarbonate in aqueous solutions can thermally degrade to produce carbon dioxide gas, and Konermann and coworkers272 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). 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:

$$\log \left\{ \frac{S}{S'} \right\} = -k_S [C]$$

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.

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 ten minutes. 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). 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). 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.

### 6.5 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, or in acid denaturation when acidic
vapors are introduced in the source of a mass spectrometer.\textsuperscript{61}

\textbf{6.6 Acknowledgements}

The authors thank the National Institutes of Health (Grant No. R01GM097357) and the National Science Foundation (Graduate Research Fellowship for CAC; Grant No. DGE1106400) for financial support.
### Table 1. Average fraction of the ion population that is unfolded in electrothermal supercharging (aqueous 5 mM ammonium salt solutions; +1.3 kV spray potential).

<table>
<thead>
<tr>
<th>pH</th>
<th>Ammonium salt (5 mM)</th>
<th>Fraction unfolded (Cytochrome c, pl 10.5)</th>
<th>Fraction unfolded (Ubiquitin, pl 6.8)</th>
<th>Fraction unfolded (β-lactoglobulin A, pl 5.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>SO₄²⁻</td>
<td>0.99 ± 0.01</td>
<td>0.91 ± 0.01</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>8.0</td>
<td>HPO₄²⁻</td>
<td>0.95 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>5.9</td>
<td>SCN⁻</td>
<td>0.90 ± 0.05</td>
<td>0.69 ± 0.01</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>8.2</td>
<td>HCO₃⁻</td>
<td>0.87 ± 0.06</td>
<td>0.60 ± 0.03</td>
<td>0.93 ± 0.01</td>
</tr>
<tr>
<td>5.3</td>
<td>Cl⁻</td>
<td>0.27 ± 0.02</td>
<td>0.39 ± 0.13</td>
<td>0.51 ± 0.09</td>
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<tr>
<td>6.1</td>
<td>HCO₂⁻</td>
<td>0.21 ± 0.06</td>
<td>0.27 ± 0.09</td>
<td>0.35 ± 0.11</td>
</tr>
<tr>
<td>5.3</td>
<td>Br⁻</td>
<td>0.17 ± 0.10</td>
<td>0.31 ± 0.02</td>
<td>0.26 ± 0.26</td>
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<tr>
<td>6.3</td>
<td>H₃C₂O₂⁻</td>
<td>0.10 ± 0.02</td>
<td>0.16 ± 0.09</td>
<td>0.34 ± 0.14</td>
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<td>5.6</td>
<td>I⁻</td>
<td>0.00 ± 0.00</td>
<td>0.12 ± 0.02</td>
<td>0.68 ± 0.27</td>
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<td>5.7</td>
<td>ClO₄⁻</td>
<td>0.00 ± 0.00</td>
<td>0.06 ± 0.03</td>
<td>0.94 ± 0.04</td>
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</table>
Table 2. Average fraction of the ion population that is unfolded in electrothermal supercharging (aqueous 5 mM sodium salt solutions; +1.3 kV spray potential).

<table>
<thead>
<tr>
<th>pH</th>
<th>Sodium salt (5 mM)</th>
<th>Fraction unfolded Ubiquitin in 5 mM sodium buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>SO$_4^{2-}$</td>
<td>0.87 ± 0.01</td>
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<tr>
<td>8.4</td>
<td>HPO$_4^{2-}$</td>
<td>0.98 ± 0.01</td>
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<tr>
<td>6.9</td>
<td>SCN$^-$</td>
<td>0.01 ± 0.01</td>
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<td>8.4</td>
<td>HCO$_3^-$</td>
<td>0.83 ± 0.16</td>
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<td>6.1</td>
<td>Cl$^-$</td>
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<td>6.4</td>
<td>HCO$_2^-$</td>
<td>0.22 ± 0.06</td>
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<tr>
<td>5.6</td>
<td>Br$^-$</td>
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<td>6.7</td>
<td>H$_3$C$_2$O$_2^-$</td>
<td>0.03 ± 0.01</td>
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<td>5.5</td>
<td>I$^-$</td>
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</tr>
<tr>
<td>5.5</td>
<td>ClO$_4^-$</td>
<td>0.04 ± 0.02</td>
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Table 3. Room temperature Setschenow constants ($k_s$) for oxygen and nitrogen gas in aqueous sodium salt solutions containing Hofmeister anions.

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<th>$k_s$ for Nitrogen Gas$^{298}$</th>
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<td>F$^-$</td>
<td>NaF</td>
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<tr>
<td></td>
<td>NaClO$_4$</td>
<td>0.160</td>
<td></td>
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<tr>
<td>HCO$_3^-$</td>
<td>NaHCO$_3$</td>
<td></td>
<td>0.153</td>
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<td>Br$^-$</td>
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<td>NO$_3^-$</td>
<td>NaNO$_3$</td>
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<td>I$^-$</td>
<td>NaI</td>
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<tr>
<td>ClO$_4^-$</td>
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</tbody>
</table>
6.8 Figures

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).
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 (■), thiocyanate (○), bicarbonate (△), bromide (▽), formate (◇), and acetate (○), and pure water (---).
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),\textsuperscript{274,299} (b) anion viscosity B-coefficient,\textsuperscript{300} and (c) anion surface/bulk partitioning coefficient (with respect to sulfate at 0.0).\textsuperscript{139}
6.9 Supplementary Information

Figure S-1. NanoESI mass spectra of 10 µM cytochrome c in 5 mM ammonium (a) bicarbonate and (b) sulfate measured under native ESI conditions with a spray potential of +0.7 kV.
Figure S-2. Bubble formation from 2 mL solutions of 10 µM cytochrome c and 1 M ammonium salts placed in a 97 °C water bath.
Figure S-3. Electrothermal supercharging nanoelectrospray mass spectra of cytochrome c in 5 mM ammonium sulfate before (a) and after (b) the solution has been degassed.
Chapter 7

Real-time H/D Exchange Kinetics of Proteins from Buffered Aqueous Solution with Electrothermal Supercharging and Top-Down Tandem Mass Spectrometry

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Going, C. C.; Xia, Z.; Williams, E. R. (to be submitted to Anal. Chem. in 2015)

7.1 Abstract

Electrothermal supercharging (ETS) with electrospray ionization produces highly charged protein ions from buffered aqueous solutions in which proteins have native folded structures. ETS increases the charge of ribonuclease A by 34% whereas only a 6% increase in charge occurs for a reduced alkylated form of this protein, which is unfolded and ~66% random coil in this solution. These results indicate that protein denaturation that occurs in the ESI droplets is the primary mechanism for ETS. ETS does not affect the extent of solution-phase hydrogen-deuterium exchange (HDX) that occurs for four proteins that have significantly different structures in solution, consistent with a droplet lifetime that is significantly shorter than observable rates of HDX. Rate constants for HDX of ubiquitin are obtained with a spatial resolution of ~1.3 residues with ETS and electron transfer dissociation of the 10+ charge-state using a single capillary containing a few μL of protein solution in which HDX continuously occurs. HDX protection at individual residues with ETS HDX is similar to that with reagent supercharging HDX and with solution phase NMR, indicating that the high spray potentials required to induce ETS do not lead to HD scrambling.

7.2 Introduction

Tandem mass spectrometry (MS) is a powerful technique for identifying proteins and for determining the identities and sites of post-translational modifications. Information about higher-order structure and dynamics of proteins in solution can also be obtained using tandem MS when combined with solution-phase labelling techniques, such as photo-oxidative labelling, selective noncovalent adduct protein probing mass spectrometry (SNAPP-MS), primary amine acetylation, and hydrogen-deuterium exchange (HDX). Of these methods, HDX is most widely used and probes primarily protein secondary structure in solution. Rates of exchange of amide hydrogen atoms along the protein backbone are obtained by MS from the 1 Da mass difference where exchange occurs. Backbone hydrogen atoms that undergo slow exchange for deuterium (on the order of several minutes to even days) are "protected" from exchange and are often involved in hydrogen bonding in alpha helix or beta sheet regions of a protein. Conversely, backbone hydrogen atoms that undergo rapid exchange (on the order of milliseconds to seconds) are likely in random coil or other highly flexible regions of a protein. HDX has the advantage that labelling at every amino acid is possible, whereas covalent modifications typically target a more limited subset of residues. However, H/D labeled sites can undergo back exchange with solvent during the time required to analyze the sample, which is typically not an issue with covalent labelling methods.

HDX is coupled to mass spectrometry in either a bottom-up or top-down approach. In the bottom-up approach, a protein sample is diluted into D2O to initiate exchange. At
incremental time points, HDX is "quenched" by adding acid to pH 2-3, where the HDX rate is at a minimum. The protein is subsequently digested with a protease, such as pepsin, which has optimal activity at pH 3, and the HDX of peptide fragments are measured using liquid chromatography (LC)-MS. One of the advantages of bottom-up HDX is that there is no limit to the size of the proteins or protein complexes that can be studied, but there is a limit to the resolution on the exchange sites that can be obtained using this approach. Information on one exchange site is lost at every proteolytic cleavage site for a given peptide fragment, and the resolution on the exchange sites is typically limited by the size of the peptides that are produced by proteolysis. Tandem MS on the peptides has been used to localize exchange to individual residues. The top-down HDX approach is similar, but does not involve proteolysis in solution prior to electrospray. After HDX is "quenched", intact protein ions are produced by ESI, and the intact protein is fragmented in the gas phase. With this approach, sites of exchange can be localized to individual amino acids. In tandem MS in either bottom-up or top-down HDX, electron capture or transfer dissociation (ECD or ETD) is typically used to minimize HD scrambling that can occur between residues in a single peptide or protein during fragmentation, which can be significant for slow-heating methods such as collision induced dissociation (CID). For example, Jorgensen and Zubarev showed that for a peptide that exchanges rapidly on one end and slowly on the other, ~5% HD scrambling occurs with ECD, but ~92% occurs with CID.

In the top-down HDX approach, it is essential to form high charge-state protein ions in order to maximize sequence coverage and the resolution of HDX information. High charge-state protein ions are more efficiently fragmented in tandem MS than low charge-state ions. Electron capture/transfer efficiencies increase with the extent of charging, and highly charged ions have more extended conformations with fewer noncovalent interactions, resulting in greater fragmentation and sequence coverage. High charge states for top-down fragmentation are typically produced by denaturing the protein prior to ESI, either by acidification during quenching of HDX, or by the introduction of organic solvents. High charge states can also be formed from aqueous solutions at neutral pH with a variety of supercharging methods. For example, supercharging with the reagent m-nitrobenzyl alcohol can be used as a "quench" step that chemically and/or thermally unfolds the protein in the ESI droplet. By using supercharging as the HDX quench and to produce high charge state ions, a continuous, real-time measurement of HDX of proteins in buffered aqueous solutions can be obtained from a single nanoESI capillary. This method saves both sample and sample manipulation, and close to single amino acid resolution on exchange rates can be obtained for a small protein.

High charge-state protein ions can be formed from aqueous buffered solutions with electrothermal supercharging (ETS). In ETS, increasing protein ion charge from aqueous ammonium bicarbonate solutions is as simple as increasing the ESI spray potential and inducing thermal denaturation of the protein in the ESI droplet. However, it is possible that HD scrambling could occur as a result of the energetic source conditions used in ETS in which both high inlet capillary temperature and high ESI potentials are used. Here, we show that ETS is significantly less effective on proteins lacking ordered structure providing additional evidence that ETS unfolds proteins in the ESI droplet. The extent of HDX incorporation in proteins is the same in native MS as ETS MS, indicating that there is no significant back exchange during protein unfolding in the ESI droplet. Top-down fragmentation coupled to ETS HDX MS shows that no measurable HD scrambling occurs during either ETS or ETD, and that ETS HDX
MS/MS data provides similar structural information at individual residues that obtained by NMR.

7.3 Experimental

Ions were formed by nanoelectrospray ionization (nanoESI) from aqueous solutions containing 100 mM ammonium bicarbonate, pH 7.8, unless otherwise specified. Borosilicate capillaries (1.0 mm o.d./0.78 mm i.d., Sutter Instruments, Novato, CA, USA) pulled to a tip i.d. of ~1 µm using a Flaming/Brown micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA) were used as nanoESI emitters, and electrospray was initiated by applying a potential of +0.8-0.9 kV for native MS and +1.3 kV for ETS MS to a platinum wire that is in contact with the aqueous solutions. The source capillary temperature was held at 250 °C, resulting in a temperature of ~35 °C at the nanoESI capillary containing the sample solution. A Thermo Linear-Trapping Quadrupole (LTQ) Orbitrap mass spectrometer equipped with electron transfer dissociation (ETD) was used to obtain mass spectral data. ETD was performed by reacting ubiquitin ions with fluoranthene anions for 30 ms. The source chamber was continuously flushed with dry nitrogen gas to reduce back exchange from incorporation of water from air into the ESI droplets. Fragment ions were identified by comparing their m/z values to those of c and z ions calculated using Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm) within an uncertainty of ± 0.005. Rate constants for HDX were obtained from the extent of deuterium incorporation as a function of time for each residue by fitting these data with an exponential function in OriginPro. Protection factors (P) are calculated as P = kint/k, where kint is the intrinsic peptide backbone exchange rate constant estimated as that for polyalanine according to the method of Englander and coworkers, and k is the measured exchange rate constant at an individual residue of ubiquitin. A value of log(P) = 0.0 is assigned for all residues for which k >1.3 min⁻¹, which corresponds to complete exchange within the standard deviation of deuterium incorporation measurements (0.06) prior to the start of data acquisition at 2.2 min.

Lyophilized samples of ribonuclease A (RNase A), holo-myoglobin, apo-myoglobin, and ubiquitin, and all solvents and salts were obtained from Sigma (Sigma, St. Louis, MO, USA). Amide-capped reduced-alkylated ribonuclease A (raRNase A) was made from RNase A as described previously, and ubiquitin was deuterated as described by Sterling et al.

7.4 Results and Discussion

7.4.1 ETS charging and protein conformation. The fidelity of HDX in solution must be maintained throughout measurements aimed at determining where exchange occurs. Heating of ESI droplets and concomitant protein unfolding in the ESI droplet that may occur during ETS could potentially result in some loss of HDX fidelity. The role of protein conformation and unfolding as the primary mechanism of high charging in ETS was investigated by comparing charging obtained for two similar proteins: one that has a native folded structure and one that is disordered and does not have higher order structure. Reduced-alkylated ribonuclease A (raRNase A) is ~66% random coil in solution at pH 6 and is entirely random coil at pH 2-3. Unmodified ribonuclease A (RNase A) has four disulfide bonds and is folded in solution with ~41% random coil at pH 6. Ions of both proteins were formed by nanoESI from aqueous 100 mM ammonium bicarbonate, pH 7.8, with native MS (+0.8 kV spray potential) and ETS MS (+1.3 kV spray potential) (Figure 1a,b black and red for RNase A and raRNase A, respectively).
With native MS, the width of the charge-state distribution for RNase A is narrow and is centered ~6+, consistent with a folded conformation in solution. In contrast, the charge-state distribution for raRNase A is broad and centered ~12+, indicative of its unfolded and largely random coil conformation in solutions around neutral pH. With ETS MS, the charge-state distribution of RNase A shifts to lower \( m/z \), and the average ion charge increases by 34% (Figure 1b; Table 1). In contrast, the charge-state distribution of raRNase A increases by only 6% (Table 1) and shifts from being centered around the 12+ charge state to the 11+ charge state (Figure 1b). Thus, a protein that is extensively disordered in solution does not undergo significant increases in charge in ETS. This indicates that the charge enhancement obtained with ETS is associated with conformational changes of proteins in the ESI droplets.

The effect of ETS on the charge of apo-myoglobin, a form of myoglobin that does not have a noncovalently attached heme group and that adopts a molten globule-like state in solution, was compared to that of the folded form of the protein with a bound heme group, holo-myoglobin. A molten globule state has more folded structure compared to that of a random coil state and retains a native-like secondary structure. However, it is a labile and dynamic state with much more conformational flexibility than a tightly folded protein. Ions of holo-myoglobin and apo-myoglobin were formed by nanoESI from ammonium bicarbonate solution under native MS and ETS MS conditions (Figure 1c,d). The charge-state distribution of holo-myoglobin produced by native MS is centered ~10+ and is predominantly holo-myoglobin (72% of the protein ion population) (Figure 1c, black). The small population of ions without the heme is due to the relatively energetic source conditions (high interface capillary temperature) necessary for optimal ETS resulting in a small amount of ETS even at low spray potentials. The temperature of the solution in the nanoESI capillary is ~35 °C, much lower than the melting temperature of myoglobin (~76.5 °C). This indicates that loss of heme does not occur prior to droplet formation by ESI. The charge-state distribution of apo-myoglobin is centered ~14+ (Figure 1c, red). The higher charge for apo-myoglobin compared to holo-myoglobin in native MS is consistent with a more labile conformation associated with a molten globule state. With ETS MS, the average charge of myoglobin formed from the holo-myoglobin solution increases by 48% (Table 1) and shifts from being predominantly holo-myoglobin to mostly apo-myoglobin (76% of the protein ion population) (Figure 1d, black). The concomitant loss of heme with increase in charge suggests that ETS causes protein denaturation and subsequent loss of the noncovalently bound heme group in the ESI droplets. For apo-myoglobin, ETS results in a smaller, but still significant increase (21%) (Table 1) in the charge (Figure 1d, red). These data indicate that unfolding and increase in charge occurs for this molten globule state of the protein, but the increases in charge that are observed are smaller than for the more rigid, folded form of the protein, holo-myoglobin. The observation that both RNase A and holo-myoglobin, which are folded proteins with native or native-like structure in aqueous solution, undergo large increases in charge with ETS, whereas raRNase A and apo-myoglobin, which do not have significant higher order structure in solution, undergo far smaller changes in charge indicates that the mechanism of electrothermal supercharging involves protein denaturation in the ESI droplet. These results are consistent with no increase in charging with ETS for a dendrimer for which unfolding or significant conformational changes are not possible.

7.4.2 ETS-HDX and protein conformation. In order to establish whether the fidelity of H/D exchange done under native conditions in solution is adversely affected by the unfolding of proteins in the electrospray droplet that occurs with ETS, HDX of the same four proteins was
investigated both with native MS and with ETS. RNase A, raRNase A, holo-myoglobin, and apo-myoglobin were diluted 1:100 to 10 µM in D_{2}O with 100 mM ammonium bicarbonate, pH 7.8, and allowed to exchange at room temperature for 60 minutes prior to electrospray. The deuterium incorporation averaged over all charge states for each protein with both native MS and with ETS is given in Table 2. The average deuterium incorporation for each protein with native MS is the same as that in ETS MS (Table 2). Conformational differences between RNase A and holo-myoglobin versus their more disordered counterparts raRNase A and apo-myoglobin, respectively, are indicated by the different number of deuterium incorporated in each (Table 2, Figure 2). For example, the deuterium incorporation of the 10+ charge-state ion of RNase A and raRNase A (Figure 2) produced in ETS MS is 143 and 195 deuterium for RNase A and raRNase A, respectively, corresponding to exchange of 60% and 77%, respectively, of the total number of backbone and fast-exchangeable sites on each protein (238 for RNase A and 254 for raRNase A). Similarly, the deuterium incorporation of the 16+ charge-state ion of apo-myoglobin produced by ETS from entirely holo- or apo-myoglobin solutions (Figure 2) is much greater for apo-myoglobin from purely apo-myoglobin solutions (+178 deuterium) than for apo-myoglobin produced from holo-myoglobin solutions (+139 deuterium), or 68% and 53%, respectively, of the 262 possible exchangeable sites. Greater deuterium incorporation for raRNase A and apo-myoglobin compared to RNase A and apo-myoglobin produced from holo-myoglobin solutions reflects the greater disorder and structural lability of the former proteins compared to the latter and demonstrates that differences in protein conformation isotopically encoded by HDX are retained during ETS MS.

The same exchange measured in native MS and with ETS indicates that the protein unfolding that occurs in the ESI droplet does not adversely affect the fidelity of the exchange that occurs in bulk solution prior to electrospray. This indicates that protein conformational changes in ETS occur more quickly than H/D exchange occurs. The lifetime of a nanoESI droplet formed under similar conditions was recently measured and is ~27 µs. For exchange to occur on this timescale would require an exchange rate constant on the order of ~6 x 10^{6} min^{-1}. This rate constant is 1000 fold higher than the intrinsic peptide backbone HDX rate constant (~1.6x10^{3} min^{-1}) under the conditions used in this experiment. Thus, conformational changes in the ESI droplet during ETS and subsequent ion formation occurs much faster than amide HDX can occur.

### 7.4.3 ETS-HDX real-time exchange rates for individual residues

The high charge states that are produced with ETS are efficiently fragmented with ETD resulting in high sequence coverage that is essential to identify individual sites of exchange. ETD of ions of ubiquitin produced with ETS was performed in order to evaluate the efficacy of this method for obtaining real-time H/D exchange rates at individual residues with top-down native MS. Exchange rate constants at individual residues of ubiquitin have been measured with NMR and top-down HDX with supercharging reagents. The charge-state distribution of ubiquitin ions produced by ETS from 200 mM ammonium bicarbonate, pH 6, is bimodal, with a low-charge distribution centered ~5+ and a high-charge distribution centered ~11+ (Figure 3a). ETD of the 10+ ion (30 ms) results in ~90% depletion of the precursor ion (Figure 3b) and cleavage of 70 of the 75 inter-residue bonds (Figure 4). There are 64 c ions and 61 z ions from which 93% sequence coverage is obtained (Figure 4). ETD does not cleave N-terminal to proline residues. Thus, only 72 inter-residue bonds can be cleaved with ETD.
In order to determine whether the activating source conditions required for ETS (high ESI interface temperature and high spray potentials) lead to HD scrambling within the protein ion, either as a result of ion formation or the ETD process itself, HD exchange was done on ubiquitin. Deuterated ubiquitin (D-ubiquitin, 92% total deuterium incorporated) was diluted 1:70 into 200 mM ammonium bicarbonate in water, pH 6 (pH adjusted with acetic acid), to a final concentration of 3 µM in order to initiate HD exchange. After dilution, the solution was loaded into the nanoESI capillary, and electrothermal supercharging was initiated within 2.2 minutes after mixing. Real time information about the exchange kinetics were obtained from ETD mass spectra of the 10+ charge state, which were acquired continuously until 21 minutes of exchange, at which point the spray potential was set to 0 kV, and all other time points were acquired by restarting ESI at those times. In contrast, HDX is quenched at specific time points in more conventional top-down HDX experiments so that multiple experiments are required in order to extract accurate rate constants.

The average deuterium incorporation for an individual residue, \( n \), determined by the difference in mass between fragment ions \( C(n-1)-C(n-2) \) or \( Z(n-2)-Z(n-1) \), versus reaction time, was used to obtain rate constants of exchange at each site. For example, the difference in the number of deuterium incorporated in \( c_2^{1+} \) and \( c_3^{1+} \) corresponds to the average number of deuterium at the backbone peptide bond for residue Phe4, and the difference in the number of deuterium incorporated in \( c_{10}^{2+} \) and \( c_{11}^{2+} \) corresponds to the average number of deuterium at the backbone peptide bond for residue Thr12 (Figure 5). Phe4 undergoes slow exchange \( (k = 3.3 \times 10^{-3} \text{ min}^{-1}) \), whereas Thr12 undergoes significantly more rapid exchange \( (k = 4.5 \times 10^{-1} \text{ min}^{-1}) \). Due to overlap between isotope distributions for ions at the same \( m/z \), single amino acid resolution of exchange rates were obtained for only 48 out of 75 residues, corresponding to an overall 1.3 residue resolution (weighted average of segment length for which HDX kinetics could be obtained).

The exchange rates for individual residues were converted to protection factors \( (P = k_{\text{int}}/k) \) and log(P) at each exchange site is shown in Figure 6. Overlaid on the top-down ETS HDX data from this work are the log(P) values from the top-down reagent supercharging HDX data measured previously,\(^{69}\) as well as the NMR HDX data from Pan and Briggs.\(^{322}\) The ETS MS data agree well with the reagent supercharging MS data and solution phase NMR data, with regions of high or low protection obtained from the ETS MS data corresponding to the same regions of high or low protection measured by the latter two methods. Regions of high protection in all three data sets correspond to regions of \( \alpha \)-helix or \( \beta \)-sheet in the crystal structure of ubiquitin (PDB: 1UBQ). For example, there is a beta sheet between residues 2-7 and a random coil between residues 8-11 in the crystal structure, and these residues have high protection factors (slow exchange) and low protection factors (fast exchange), respectively, in the MS and NMR data sets. There are some residues for which there is high protection for all three data sets in regions that are random coils in the crystal structure. This may be due to the presence of salt bridges or solvent interactions, and discrepancies between NMR or MS HDX data and ordered regions in crystal structure have been reported previously.\(^{323, 325, 326}\) The similarity of exchange rates measured using ETS MS, reagent supercharging MS and NMR along with the high correlation between exchange rates and ordered regions in the crystal structure demonstrate that there is no significant scrambling of HDX information at individual residues with ETS. These results show that real-time HDX rates of proteins can be obtained using ETS from buffered aqueous solutions in which the protein has a native structure.

7.5 Conclusions
Real-time HDX exchange kinetics that report on the secondary structure of native, folded proteins can be obtained with ETS coupled to top-down MS/MS. ETS causes proteins to unfold in the ESI droplet, the primary mechanism for higher charging with this method. Although the proteins unfold prior to ion formation, this process is sufficiently fast that essentially no back exchange can occur. Thus, information about the higher order structure of the protein is preserved as the ions are transferred into the gas phase. As demonstrated previously, ETD does not produce scrambling of HDX information within a protein in the gas phase. Thus, ETS can be effectively coupled to HDX and tandem MS to obtain residue-specific HDX information on the conformations of proteins from buffered aqueous solutions. An important advantage of this method is that the kinetic data is measured while HDX occurs, which eliminates any quenching steps that are conventionally used. Thus, HDX information on individual residues of a protein can be acquired in real-time and out of a single nanoESI capillary, meaning only a couple hundred nanograms of protein is required.

ETS typically produces higher charge states of proteins than can be produced with supercharging reagents, and under optimal conditions, ETS can produce similar charging of proteins as that obtained by ESI from solutions in which proteins are denatured. ETS is also effective on even large (~80 kDa) proteins. Thus ETS-HDX-MS/MS should be able to be extended to proteins larger than those in this work to obtain information on the secondary structure of large proteins and protein complexes or on the binding interfaces of protein complexes.

7.6 Acknowledgments

The authors thank the National Institutes of Health (Grant No. R01GM097357) and the National Science Foundation (Graduate Research Fellowship for CAC; Grant No. DGE1106400) for financial support.
7.7 Tables

Table 1. Average charge of protein ions produced by nanoESI from 100 mM ammonium bicarbonate solutions. Uncertainties are the standard deviation of three triplicate measurements.

<table>
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<th>Protein</th>
<th>Native MS (+0.8 kV)</th>
<th>ETS MS (+1.3 kV)</th>
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<tr>
<td>RNase A</td>
<td>7.1 ± 0.1+</td>
<td>9.5 ± 0.1+</td>
</tr>
<tr>
<td>raRNase A</td>
<td>11.3 ± 0.3+</td>
<td>12.0 ± 0.2+</td>
</tr>
<tr>
<td>Myoglobin *holo</td>
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<td>*15.2 ± 0.2+</td>
</tr>
<tr>
<td>apo</td>
<td>10.5 ± 0.1+</td>
<td>15.6 ± 0.3+</td>
</tr>
<tr>
<td>Apo-myoglobin</td>
<td>13.6 ± 0.4+</td>
<td>16.4 ± 0.3+</td>
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Table 2. Average deuterium incorporation of protein ions produced by nanoESI from 100 mM ammonium bicarbonate in D₂O after 60 minutes exchange. Uncertainties are the standard deviation of deuterium incorporation for each charge state.

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<th>Protein</th>
<th>Native MS (+0.8 kV)</th>
<th>ETS MS (+1.3 kV)</th>
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</thead>
<tbody>
<tr>
<td>RNase A</td>
<td>148 ± 1 D</td>
<td>145 ± 5 D</td>
</tr>
<tr>
<td>raRNase A</td>
<td>195 ± 4 D</td>
<td>196 ± 4 D</td>
</tr>
<tr>
<td>Myoglobin *holo</td>
<td>*137 ± 2 D</td>
<td>*139 ± 2 D</td>
</tr>
<tr>
<td>apo</td>
<td>137 ± 2 D</td>
<td>138 ± 3 D</td>
</tr>
<tr>
<td>Apo-myoglobin</td>
<td>176 ± 3 D</td>
<td>176 ± 3 D</td>
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7.8 Figures

Figure 1. NanoESI mass spectra of 10 µM RNase A (a,b, black), rRNase A (a,b, red), holo-myoglobin (c,d, black, *denotes that the heme is retained), and apo-myoglobin (c,d, red) in 100 mM ammonium bicarbonate acquired under native MS conditions at a potential of +0.9 kV (a,c) and under ETS MS conditions at a potential of +1.3 kV (b,d). Spectra were acquired separately and subsequently overlaid.
Figure 2. NanoESI mass spectra showing the 10+ charge state of RNase A (top left, black) and raRNase A (top right, black) and the 16+ charge state of apo-myoglobin (bottom, black) overlaid with spectra of the same charge states of each protein 60 minutes after dilution into D$_2$O. Blue and red in the bottom spectra correspond to apo-myoglobin produced from holo-myoglobin and apo-myoglobin solutions, respectively. All spectra were acquired under ETS MS conditions at +1.3 kV spray potential. The average number of deuterium incorporated for each protein is given above each arrow. An increase in sodium ion adduction is observed from D$_2$O compared to water solutions due to the presence of sodium in the D$_2$O stock.
Figure 3. NanoESI mass spectrum of ubiquitin with ETS at +1.3 kV spray potential from 200 mM ammonium bicarbonate, pH 6 (a), and ETD mass spectrum of the 10+ ion (b).
Figure 4. Map of sequence coverage of ubiquitin from ETD fragmentation of the 10+ ion.
Figure 5. Plot of deuterium incorporation for individual residues Phe4 (open squares) and Thr12 (closed squares) of ubiquitin over three hours' time.
Figure 6. Plot of the logarithm of the protection factor at individual residues of ubiquitin determined with ETS HDX-MS/MS (red circles, solid line), with supercharging HDX-MS/MS (red squares, dotted line), and with NMR (blue triangles, dashed lines). At the top are regions of secondary structure in the crystal structure of ubiquitin (PDB: 1UBQ).
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