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Is Native Mass Spectrometry in Ammonium Acetate Really Native? Protein Stability Differences in Biochemically Relevant Salt Solutions

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Abstract

Ammonium acetate is widely used in native mass spectrometry to provide adequate ionic strength without adducting to protein ions, but different ions can preferentially stabilize or destabilize the native form of proteins in solution. The stability of bovine serum albumin (BSA) was investigated in 50 mM solutions of a variety of salts using electrospray emitters with submicron tips to desalt protein ions. The charge-state distribution of BSA is narrow (+14 – +18) in ammonium acetate (AmmAc), whereas it is much broader (+13 – +42) in solutions containing sodium acetate (NaAc), ammonium chloride (AmmCl), potassium chloride (KCl), and sodium chloride (NaCl). The average charge state and percent of unfolded protein increases in these respective solutions, indicating greater extents of protein destabilization and conformational changes. In contrast, no high charge states of either bovine carbonic anhydrase II or IgG1 were formed in AmmAc or NaCl despite their similar melting temperatures to BSA, indicating that the presence of unfolded BSA in some of these solutions is not an artifact of the electrospray ionization process. The charge states formed from the nonvolatile salt solutions do not change significantly for up to 7 min of electrospray, but higher charging occurs after 10 min consistent with solution acidification. Formation of unfolded BSA in NaAc but not AmmAc indicates that the cation identity, not acidification, is responsible for structural differences in these two solutions. Temperature-dependent measurements show both increased charging and aggregation at lower temperatures in NaCl:Tris than in AmmAc, consistent with lower protein stability in this solution. These results are consistent with the order of these ions in the Hofmeister series and indicate that data on protein stability in AmmAc may not be representative of solutions containing nonvolatile salts that are directly relevant to biology.

INTRODUCTION

Native mass spectrometry (MS) with electrospray ionization (ESI) has been widely used to characterize molecular weights, stoichiometries, ligand-binding affinities, and thermochemical stabilities of proteins and noncovalent protein-protein/protein-ligand complexes. Mass spectrometry has the advantages of low sample consumption, high sensitivity, and the ability to measure multiple analytes with different masses simultaneously.[1–3] The extent of protein charging in ESI depends on a number of factors, but conformation is the most significant contributor to the different charge states of a protein in ESI mass spectra.[4–6] Ions formed from solutions in which proteins are denatured are more highly charged than ions formed from solutions in which proteins are folded, where the charging is typically close to (within ~80%) the Rayleigh limit calculated for a water droplet of the same size.[7,8] Changes in pH,[5,9,10] temperature,[11–15] ionic strength,[16,17] and buffer identity[18–20] can all affect charge-state distributions, consistent with results from other biophysical techniques that probe protein conformation.[21–24] In combination with ion mobility spectrometry, native MS can resolve minor protein structural transitions at the level of individual conformers.[25–27]

In addition to ionic strength, protein stability and solubility depend on the identity of ions. Over 130 years ago, Hofmeister ordered ions based on their propensity to either “salt in” or “salt out” proteins in solution,[28,29] an effect attributed to both direct ion-macromolecule interactions and changes in the dynamics of the water solvation shell that can stabilize or destabilize native protein structures.[30–32] The effect of buffer identity on the thermodynamic stability of proteins can be assessed using different spectroscopic or calorimetric techniques, such as circular dichroism[33] and differential scanning calorimetry/fluorimetry.[24,33–37] With native mass spectrometry, nonvolatile salts or buffers in solution adduct onto protein ions, which can lead to

peak broadening and lower signals.[19,38] At high concentrations of nonvolatile salts, protein charge-state distributions are often unresolved, leading to limited or no mass information. For this reason, ammonium acetate is typically used in native MS. Ammonium can transfer a proton to either the protein or to acetate forming gaseous ammonia, leaving unadducted protein ions. Although ammonium acetate can provide an adequate ionic strength environment, the maximum buffer capacity is around pH 4.7 and 9.3, and its buffering capacity near physiological pH is significantly reduced.[39] Acetate also absorbs in the UV region making it difficult to use some spectroscopic structural methods from these solutions.[40] Ammonium acetate is not abundant in the cellular environment, and proteins may exhibit different biophysical characteristics in this buffer compared to physiologically relevant buffer systems that are prevalent in biochemistry.

Several methods can reduce the adverse effects of nonvolatile salts in protein mass spectra. With liquid chromatography separations, trap columns can be used to desalt protein samples before elution to the ESI source.[41] Addition of solution additives, such as low concentrations of small molecules,[42,43] ammonium iodide,[44] or high concentrations of ammonium acetate to the protein solution can reduce salt adduction.[45] Salt adduction can also be minimized during the ESI process by using submicron diameter nanoelectrospray ionization emitters.[19,38,46] Smaller initial droplet sizes are formed with emitters that have smaller tip diameters. Under conditions where the majority of the initially formed droplets do not contain a protein molecule, most of the salt is separated from the protein by the electrospray process. Submicron emitters make it possible to resolve protein charge-state distributions directly from biochemically relevant buffer solutions at physiological ionic strength with no additives or sample preparation steps.[19,38,47,48] These emitters have been used to study the structure and stoichiometry of proteins and protein complexes,[47,49–51] as well as DNA and RNA.[52–55] Submicron emitters are also useful

for preventing nonspecific aggregation that may occur during the electrospray ionization process, enabling the accurate measurement of small noncovalent complex stoichiometry[56,57] and protein-ligand binding constants by MS.[20,58–61]

The combination of submicron diameter emitters and native MS provides a sensitive readout for how solution conditions, such as buffer identity, affect protein conformation. Here, the conformation and stability of bovine serum albumin (BSA) from solutions containing ammonium acetate as well as different nonvolatile salts are investigated using submicron diameter emitters and compared to predictions of the Hofmeister series.

EXPERIMENTAL SECTION

Mass spectra were acquired using a Waters quadrupole-time-of-flight (Q-TOF) Premier mass spectrometer (Waters Corporation, Milford, MA). The extraction cone voltage, sampling cone voltage, and the collision gas (argon) flow rate was 3.0 V, 100 V, and 0.75 mL/min (~0.01 mbar), respectively. These values were chosen to maximize ion signal without causing a change in charge state or dissociation and are typical values used for native MS of larger proteins.[38,62] The backing pressure in the source was maintained at ~6 mbar. Mass spectra were acquired between m/z = 150 – 9,000 and averaged for ~20 s. Nanoelectrospray emitters were pulled from borosilicate glass capillaries (1.0 mm outer diameter, 0.78 mm inner diameter, Sutter Instrument, Novato, CA) to a final inner tip diameter of 608 nm ± 17 nm using a P-87 Flaming/Brown micropipette puller (Sutter Instrument).[63] Tip diameters were imaged using a Hitachi TM-1000 scanning electron microscope (Tokyo, Japan) at the University of California, Berkeley Electron Microscopy Laboratory. Ions were formed using an electrospray voltage of +0.8-1.3 kV applied

to a platinum wire that is inserted into the back side of the emitter and is in contact with the solution.

Variable temperature ESI (vT-ESI) experiments were performed using a homebuilt vT-ESI source similar to that of Sterling *et al.*[64] This source consists of an aluminum cylinder wrapped in resistive heating wire. The temperature is controlled using an Omega CNi-3222 temperature controller (Stamford, CT) and monitored using a thermocouple. A second thermocouple placed inside of the tip was used to calibrate the solution temperature to the temperature measured in the heating apparatus. Mass spectra were acquired continuously while increasing the solution temperature until signal was lost due to protein aggregation. The temperature of the emitter tip without heating is 29 ºC ± 1 ºC because of heat transfer from the heated mass spectrometer inlet. A mass spectrometer inlet temperature of 80 ºC was used to minimize any thermal activation that may occur in the mass spectrometer interface and is a value often used in native mass spectrometry experiments for this type of instrument.[19,65,66]

Solutions of 5 μM bovine serum albumin (BSA) were prepared in 50 mM solutions of aqueous ammonium acetate (AmmAc), sodium acetate (NaAc), sodium chloride (NaCl), potassium chloride (KCl), and ammonium chloride (AmmCl). Experiments were also performed using 40 mM salt concentrations with 10 mM Tris buffer titrated to pH 6.8 ± 0.2. Solutions of 5 μM bovine carbonic anhydrase II and bovine IgG1 were also prepared in 50 mM solutions of aqueous AmmAc and NaCl. All reagents were obtained from Sigma-Aldrich and were used without further purification.

RESULTS AND DISCUSSION

Effects of Salt Identity on Protein Charging, Structure and Stability. Mass spectra of proteins in aqueous solutions containing mM concentrations of nonvolatile salts, such as NaCl, can be readily obtained using submicron diameter emitters that significantly reduce both the extent of salt adduction onto protein ions and the abundances of large salt clusters, which can interfere with protein signal. Mass spectra of 5 μM bovine serum albumin (BSA) in aqueous solutions of 50 mM ammonium acetate (AmmAc), sodium acetate (NaAc), sodium chloride (NaCl), potassium chloride (KCl), and ammonium chloride (AmmCl) were acquired using nanoelectrospray emitters that have tip diameters of 608 nm ± 17 nm (Figure 1). In AmmAc, BSA charge states between +14 and +18 are produced with an abundance weighted average charge state (z_{av}) of 15.6 ± 0.3 (Figure 1a). The distribution of low charge states is indicative of a folded, compact structure of this protein in solution.[42,67] There is also a low abundance of dimer with *m/z* between 5,500 and 8,000. The dissociation constant of the dimer of BSA is ~10 μM, indicating that there will be some dimer in a 5 μM solution.[68] The number of analyte molecules in droplets that are initially formed using an emitter with ~600 nm tips is 1 in ~14.[56] Thus, the dimers that are observed almost certainly originate directly from the bulk solution and are not formed by nonspecific aggregation that can occur at much higher analyte concentrations or with larger emitters.

Mass spectra obtained from NaAc, AmmCl, KCl, and NaCl solutions also have low charge states, corresponding to compact BSA conformers, but high charge states ranging from +19 up to +42 are also formed (Figure 1b-1e). These higher charge state ions are indicative of more unfolded conformations of BSA in solution. The z_{av} of BSA in the NaAc, AmmCl, KCl, and NaCl solutions are higher than that from AmmAc, with values of 17.3 ± 1.3, 20.4 ± 0.8, 21.4 ±

2.2, and 22.0 ± 4.3, respectively. The different z_{av} values in these different solutions indicate that the protein structure and stability also differ in these solutions. By comparison, the z_{av} for BSA formed from a denaturing 49.5:49.5:1 water:methanol:acetic acid solution is ~44.9 (Figure S1). Results from temperature dependent MS experiments for BSA in 100 mM AmmAc show the majority of signal for the unfolded form of the protein at 63 ºC occurs between charge states of +28 to +42.[69] The higher charging both from a denaturing solution and from heated ammonium acetate solutions indicates that the unfolded structures formed at room temperature from the aqueous solutions with some salts are likely less unfolded.

The average charge of the folded form of the protein (the low charge-state distribution) is lower in solutions containing Na^+ and K^+ compared to those containing NH_4^+. The compact conformer of BSA in NaCl has a z_{av} of 15.0 ± 1.1 whereas the z_{av} in AmmCl is 16.1 ± 0.1. In contrast, the z_{av} for the unfolded form of the protein in these two solutions is nearly the same (z_{av} = 27.9 ± 4.2 and 27.2 ± 2.8 for NaCl and AmmCl, respectively). The reduction in z_{av} for the compact form but not for the unfolded form indicates that this effect is not caused by an overall reduction in droplet charge due to emission of Na^+ from the droplets prior to formation of desolvated gaseous BSA ions. The slightly lower charging of the folded form of the protein from NaCl compared to NaAc is consistent with differences in conformation in solution rather than lower charge by cation emission.

To determine the extent to which the charge states of the folded forms of other proteins are affected by different salts, mass spectra were acquired for bovine carbonic anhydrase II and bovine IgG1 in both AmmAc and NaCl solutions. Under the same conditions where a significant abundance of unfolded forms of BSA were observed from NaCl solutions, no high charge states corresponding to unfolded forms of these two other proteins were observed (Figure S2 and S3).

Moreover, the z_{av} for both proteins are similar in the two different solutions. The z_{av} of IgG1 is 23.4 and 23.0 in ammonium acetate and NaCl, respectively, and these respective values for carbonic anhydrase II are 9.4 and 9.0. The small difference in z_{av} for IgG1 and for carbonic anhydrase II between these two solutions and larger difference for BSA is consistent with a subtle compaction of the structure of BSA in the NaCl solution.

The significant extent of unfolding observed for BSA but not for IgG1 and carbonic anhydrase II in NaCl is surprising because these three proteins have similar melting temperatures.[70–72] This indicates that the high charge states of BSA are not an artifact of any droplet heating that can induce unfolding under some ESI conditions or that may occur as a result of the desalting process.[73,74] Protein-glass surface interactions can also cause unfolding for proteins with a net positive charge in solution.[49,51] The isoelectric point of BSA is ~5.1-5.5[75] so the protein is negatively charged at neutral pH. Thus, the protein should not interact with the glass surface of the electrospray capillaries. These data indicate that the unfolded forms of BSA in mass spectra from nonvolatile salt solutions are not an artifact of the ESI process and that these unfolded structures exist in the bulk solution.

The fraction of unfolded protein (% unfolded) was determined from the relative abundances of charge states above +18 to those that are +18 and lower. These values are approximate because *m*/*z* detection and transmission biases are not taken into account, but they do serve as a useful indicator of the relative stability of BSA in these different solutions. The % unfolded protein varies from 0% in AmmAc to 56% in NaCl (Table 1). Together, these data indicate that the folded form of BSA is destabilized in each of these nonvolatile salt solutions relative that in AmmAc. The shift of the folded form to lower charge states in the presence of Na^+ and K^+ indicates that these ions also affect the structure of the folded form compared to that in

AmmAc. There are high concentrations of Na^+ and K^+ in the intracellular and extracellular (130 – 150 mM)[76] environments, indicating that protein biophysical characteristics measured in AmmAc, as is done in most native MS experiments, may not provide reliable data about the stabilities and structures of proteins in more biological environments.

Variability During ESI Desalting. A complicating factor with nanoESI from solutions that contain significant concentrations of nonvolatile salts is that the spray characteristics can vary with time and between different emitters.[38,63] This can lead to different droplet sizes and variable extents of salt adduction to protein ions produced by desalting from submicron emitters. To determine the extent to which the measured z_{av} and unfolded protein are affected by variability in the ESI process during desalting, the z_{av} of BSA in NaCl was measured for the period of desalting after the appearance of protein ion signal (a single desalting event) and after initial onset of desalting for three different electrospray emitters (Table S1). The z_{av} of BSA in NaCl during a single desalting event is 21.5 ± 0.2, indicating there is little variability while desalting occurs. The z_{av} varies more significantly between three different emitters (20 ± 4). This variability may be due to minor differences in the tip diameter, tip morphology, or in positioning the emitters. Prell and coworkers showed that small changes in electrospray emitter position leads to variability in the collision-induced unfolding transitions of proteins inside the instrument.[77] The presence of both unfolded and folded BSA in the nonvolatile salt solutions indicate that the energy difference between these two forms of the protein is small. A small change in effective temperature of the droplet can lead to significant changes in the extent of unfolding and hence the z_{av} values.[73] The z_{av} and % unfolded are highly reproducible across multiple emitters from ammonium acetate solutions (15.6 ± 0.4, 0.3% ± 0.6 respectively), consistent with a greater

stability of the folded form of BSA in this solution. The variability of % unfolded is much greater in nonvolatile salt solutions, with values of 20.7% ± 11.5, 38.6% ± 1.4, 53.0% ± 14.4, 56.0% ± 21.3 in NaAc, AmmCl, KCl, and NaCl, respectively (Table 1). These data indicate that the tip-to-tip variability when desalting is likely caused by the small energy difference between the folded and unfolded forms of BSA in these nonvolatile solutions which amplifies the effects of factors that may affect stability, such as minor differences in droplet temperature. This effect is much smaller in AmmAc solutions because small changes in the stability of the folded form does not lead to unfolded structures due to the larger energy difference between these two forms of the protein.

The Hofmeister series order for these cations from most to least stabilizing is, $NH_4^+ >> K^+ > Na^+$, where K^+ and Na^+ have more propensity for "salting in" behavior or protein denaturation than does NH_4^+.[32] The z_{av} for the chloride salts follows the trend $NH_4^+ < K^+ < Na^+$, consistent with the ordering of these ions in the Hofmeister series. There is slightly more of the unfolded form with Na^+ than K^+, but there is essentially no unfolded form with NH_4^+. Anions can have a greater effect on protein stability.[32] For the anionic Hofmeister series, acetate is more stabilizing than chloride.[32] Both the z_{av} and the fraction unfolded is significantly higher with chloride than it is with acetate for the sodium salts, consistent with the ordering of these ions in the Hofmeister series. The combination of ammonium with acetate compared to chloride salts with either sodium or potassium preferentially stabilizes a folded form of BSA.

Electrochemically Induced pH Changes. Solutions can become more acidic with time during electrospray leading to protein destabilization.[9,10] BSA undergoes conformational changes below pH 4.3.[78] Differences in acidification between buffered (containing acetate) and unbuffered

solutions could also lead to differences in structure and charging from these solutions. AmmAc and NaAc have the same buffer capacity at lower pH due to the same concentration of acetate. Despite the same buffer capacity, no unfolding occurs in AmmAc throughout 6.5 min of electrospray whereas about 24% and 28% of BSA is unfolded in NaAc after 1.5 min and 6.5 min of electrospray, respectively. The similar extents of unfolding after 1.5 and 6.5 min with NaAc indicates that any pH change from an initial value of 6.8 to a value within the buffer capacity of this solution does not cause additional unfolding. The difference in the extent of unfolding at these two times is attributed to variability of desalting during electrospray. Most importantly, these results eliminate the possibility that the differences in protein stability with respect to unfolding is due to a pH change. Thus, the difference in stability can be directly attributed to the presence of Na^+ vs. NH_4^+ rather than acidification.

To determine the effects of acidification on the stability of BSA in unbuffered 50 mM NaCl solutions, mass spectra were acquired from an approximately equal ionic strength solution consisting of 40 mM NaCl and 10 mM Tris (NaCl:Tris) that is buffered at pH 6.8. Results acquired from 1 – 2 min after the start of electrospray for these two solutions are shown in Figure 2a and 2b. The z_{av} and % unfolded in 50 mM NaCl are 21.7 and 46.1% unfolded, respectively (Figure 2a). These values for the solution containing 10 mM Tris are 18.9 and 33.6% unfolded, respectively (Figure 2c). The difference in these values is within the standard deviation of z_{av} and % unfolded measured for solutions in NaCl, consistent with the higher variability in signal from solutions containing NaCl. The pH of the unbuffered solution decreases with increasing time for electrospray whereas that for the Tris solution should not until the buffer capacity is exceeded. A mass spectrum acquired after ~6.5 minutes of electrospray from 50 mM NaCl is shown in Figure 2b. The z_{av} is 21.6 and there is 52.6% unfolded population between 6 – 7 min after the start of

electrospray. For NaCl:Tris, the z_{av} of 17.3 and unfolded population of 24.7% is similar to that obtained between 1 – 2 min (Figure 2c, d), consistent with little acidification occurring in this buffered solution. The z_{av} values in nonbuffered NaCl after 6 – 7 min are nearly the same as those obtained after just 1 – 2 min. Despite the similarity in these values, there are some differences in the spectra. The z_{av} of the compact form of the population increases by ~2 charges, consistent with a slight expansion of the structure of the compact form after several minutes. The slightly higher % unfolded after 6 – 7 min is consistent with variations in desalting observed in NaAc. However, the relative abundances of some intermediate charge states (+19 – +24) increase by 73% ± 6 and there is a corresponding decrease in the relative abundance of the most highly charged ions leading to a similar overall z_{av} and % unfolded value at these two times. These results indicate that there are minor changes in the charge-state distributions that can be attributed to acidification in this unbuffered solution, but these changes do not significantly affect either the z_{av} or the % unfolded values on the time scale of these experiments.

After 10 minutes of electrospray, the z_{av} of BSA in 50 mM NaCl is 25.7 with 76.7% unfolding, consistent with a greater extent of unfolding because of acidification. Compared to NaCl, the z_{av} of BSA in 50 mM AmmAc after around 10 minutes of ESI is 15.8 with no unfolded protein, indicating that any acidification that occurs in ammonium acetate during this time frame does not affect the protein stability to a measurable extent. The pH of a 10 mM AmmAc solution has been reported to decrease from ~6.9 to ~5.6 after ~10 min of electrospray with 60 nA of current.[10] Although the current was not measured directly in these BSA experiments, an electrospray current of ~7.2 ± 0.4 nA was measured in a separate experiment using a similar potential and distance to ground. Therefore, the pH decrease should be even smaller with these lower currents and higher ionic strength (50 mM) AmmAc concentrations, consistent with no

significant change in the appearance of the mass spectra with time. However, these data indicate that some acidification does occur for the unbuffered solutions over a timescale of ~10 mins that results in minor changes in the mass spectra of unbuffered solutions. All the mass spectra shown in Figure 1 were acquired around 7 min after the start of electrospray, indicating that the increased extent of unfolding and the significant increase in the z_{av} in the unbuffered, nonvolatile solutions is not caused to a significant extent by acidification during electrospray, even though some acidification occurs and leads to small increases in these values. Therefore, based on the low currents and short time scales of our experiments, any acidification that occurred is unlikely to be significant.

Salt Identity and BSA Thermal Stability. The thermodynamic stability of the native form of BSA decreases with increasing temperature where unfolded forms can be more stable. To investigate how the thermal stability of BSA is affected by different salts, mass spectra of BSA in 50 mM AmmAc and 40 mM NaCl with 10 mM Tris buffer were acquired at 30 ºC and 50 ºC. At these temperatures, electrospray remained stable for both solutions. The temperature was subsequently increased until signal was lost due to aggregation. Mass spectra from these solutions obtained at 30 ˚C and 50 ˚C are shown in Figure 3. In AmmAc, only folded BSA is observed, but the z_{av} of the folded form increases from 15.8 (0% unfolded) to 16.5 (0% unfolded) between temperatures of 30 ˚C to 50 ˚C (Figure 3a and 3b). A similar change in z_{av} was reported for vT-ESI experiments on 2 μM BSA in 100 mM AmmAc, where higher charge states corresponding to unfolded protein only appeared at 53 ºC.[69] At temperatures above 60 ºC, signal is lost and does not return upon cooling the solution to room temperature, consistent with protein aggregation.

BSA has been reported to aggregate at temperatures above 60 ºC by a number of other biophysical techniques.[79,80]

For the NaCl:Tris solutions, there is also an increase in the charge state of the folded form of BSA between 30 ºC and 50 ºC (Figure 3c and 3d, respectively). In addition, there is an increase in the fraction of the population that is unfolded at the higher temperature. The z_{av} of BSA in NaCl increases from 18.1 with 20.0% unfolded at 30 ºC to 21.1 with 50.1% unfolded at 50 ºC, (Figure 3c and 3d, respectively). The much larger change in the z_{av} between 30 ºC and 50 ºC in NaCl vs. AmmAc (3.0 vs. 0.7, respectively) indicates a larger extent of unfolding in NaCl over the same temperature range. Protein signal is not observed above 55 ºC from NaCl solutions whereas abundant signal is observed from AmmAc solutions even at 60 ºC, consistent with a lower stability and higher propensity for unfolding and aggregation in NaCl (Figure S4). By comparison, the onset of aggregation has been reported to occur at 55 ºC for 60 μM BSA in 800 mM NaCl.[70] To confirm that aggregation occurs at a lower temperature in the two solutions, these solutions were heated to 73 ºC for 25 minutes. The AmmAc solution remained clear whereas the NaCl:Tris solution turned turbid from large aggregate formation (Figure S5). Large, insoluble aggregates can also be observed in the NaCl:Tris solution (Figure S6). These results indicate that the rate of aggregation is much faster in NaCl:Tris than AmmAc. The lower aggregation temperature of BSA in NaCl as well as the larger increase in the z_{av} indicate that BSA is destabilized in NaCl relative to AmmAc, consistent with the trends predicted by the Hofmeister series.

CONCLUSIONS

The stability of the model protein, BSA, in ammonium acetate was compared to that in solutions containing nonvolatile salts. Whereas no high charge states indicative of unfolded proteins are formed from ammonium acetate at temperatures up to 50 ºC, high charge state ions are formed from solutions of sodium acetate, ammonium chloride, potassium chloride and sodium chloride even at room temperature. Remarkably, approximately 56% of the protein is unfolded in 50 mM sodium chloride. Acidification of the solution during electrospray occurs and has a small effect on the extent of unfolding observed from some solutions, but this effect is minor and does not affect the comparisons of ammonium acetate and sodium acetate where ~21% of the protein is unfolded in the latter solution. The z_{av} of the folded form of the protein differs from the ammonium containing solutions compared to the sodium and potassium containing solutions indicating that the structure of the folded form of the protein is different when these nonvolatile salts are present in place of ammonium. In solution heating experiments, signal is lost due to aggregation at a lower temperature in sodium chloride compared to ammonium acetate solutions, consistent with a higher stability of the folded form of the protein in the latter solution. The ordering of stability of BSA in these solutions is generally consistent with the prediction of the Hofmeister series for both cations and anions. The absence of unfolded species for carbonic anhydrase and IgG1, despite their very similar melting temperatures to BSA, indicate that BSA is affected more strongly by different ion identities than other two proteins. While the salts investigated here are most relevant to native mass spectrometry and to biochemical buffers, similar experiments on a wider range of proteins and salts may provide additional insights into these effects.

Hydrogen-deuterium exchange (HDX) experiments were performed to determine if additional evidence for the presence of two different BSA conformations in NaCl could be

obtained. Both the signal heterogeneity as a result of extensive salt adduction (and HDX) combined with the time necessary to desalt ions made interpretation of these results ambiguous. Obtaining additional support for the presence of both partially unfolded and folded structures in these salt solutions using a solution-phase method that can clearly detect and quantitate absolute abundances of different conformers vs. measuring a change from one conformational form to another would be desirable.

These data indicate that ammonium acetate that is commonly used in native MS workflows to characterize the stoichiometries of protein complexes and to investigate physical properties, such as melting temperatures of proteins, may lead to higher protein stability relative to commonly used biochemical buffers. This may also affect the relative abundances of complexes with multiple stoichiometries. Proteins that are even less stable in solution, such as intrinsically disordered proteins that retain at least some secondary and even tertiary structure, will likely be significantly more influenced by experimental conditions, including choice of buffer and nanospray emitter positioning. These data highlight the importance of choosing the right mass spectrometry conditions, including buffers, for characterizing the physical properties of proteins.

SUPPORTING INFORMATION

Mass spectrum of BSA from 49.5/49.5/1 water/methanol/acetic acid; Mass spectra of carbonic anhydrase in AmmAc and NaCl Solutions; Mass spectra of IgG1 in AmmAc and NaCl solutions. Mass spectra of BSA at temperatures above 50 °C; Microscope images of BSA aggregates formed at 73 °C; Variability of the BSA z_{av} over a single desalting event, across multiple desalting events from one emitter, and from three different emitters.

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Table 1: Average Charge States (z_{av}) and Percent of Unfolded Population (% unfolded) of 5 µM BSA from Electrospray Ionization Mass Spectra Obtained from Aqueous Solutions with Different Salts (50 mM)

Salt Solution	z_{av}	% unfolded
AmmAc	15.6 ± 0.4	0.3 ± 0.6
NaAc	17 ± 1	21 ± 10
AmmCl	20.3 ± 0.8	38 ± 1
KCl	21 ± 2	53 ± 10
NaCl	23 ± 4	56 ± 20

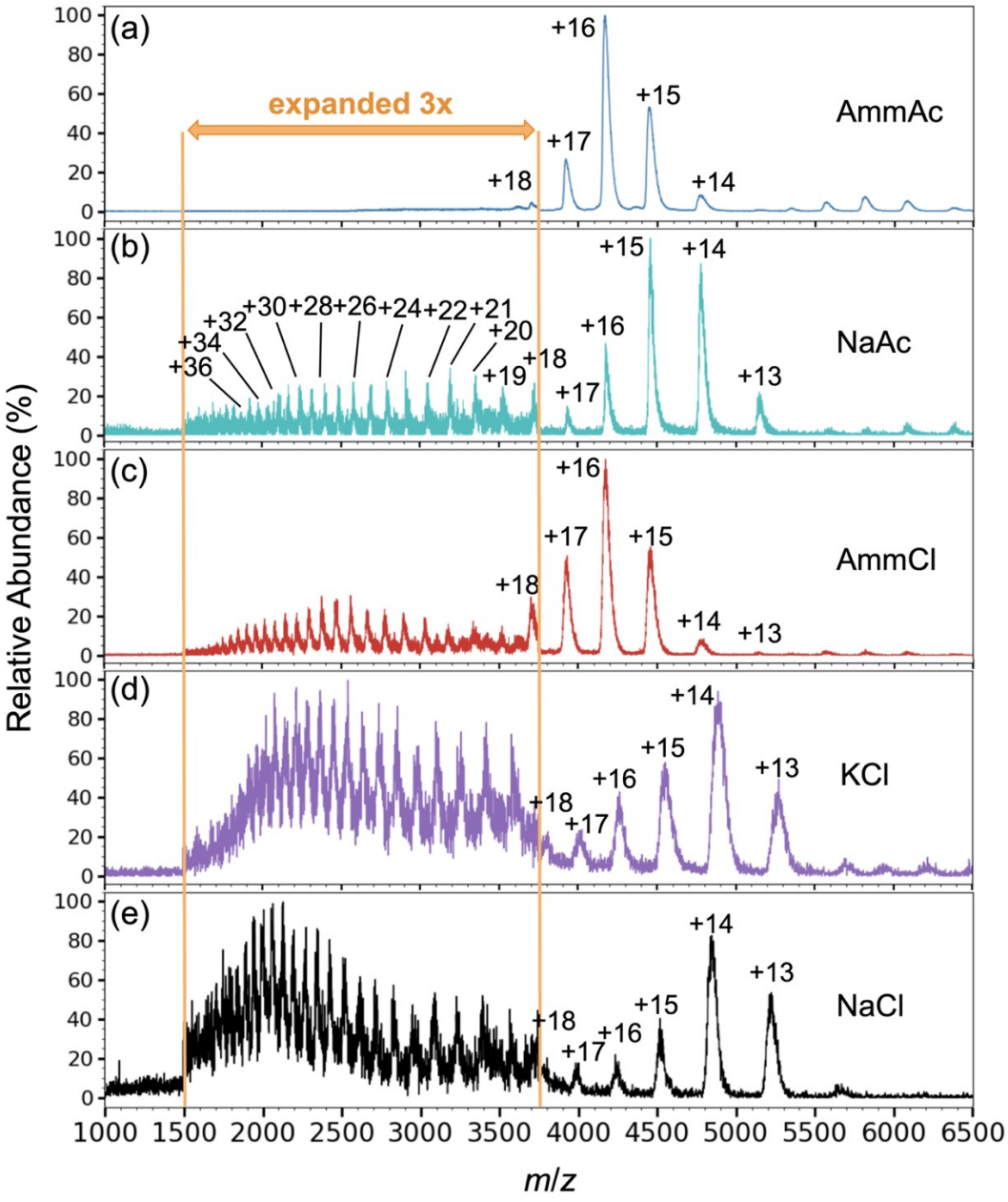


Figure 1. Electrospray ionization mass spectra of 5 μM BSA in 50 mM aqueous a) AmmAc b) NaAc c) AmmCl d) KCl and e) NaCl. The relative abundance in the *m*/*z* range between 1,500 and 3,750 is expanded by 3x. Ions corresponding to dimers occur in a *m*/*z* range between 5,500 and 8,000.

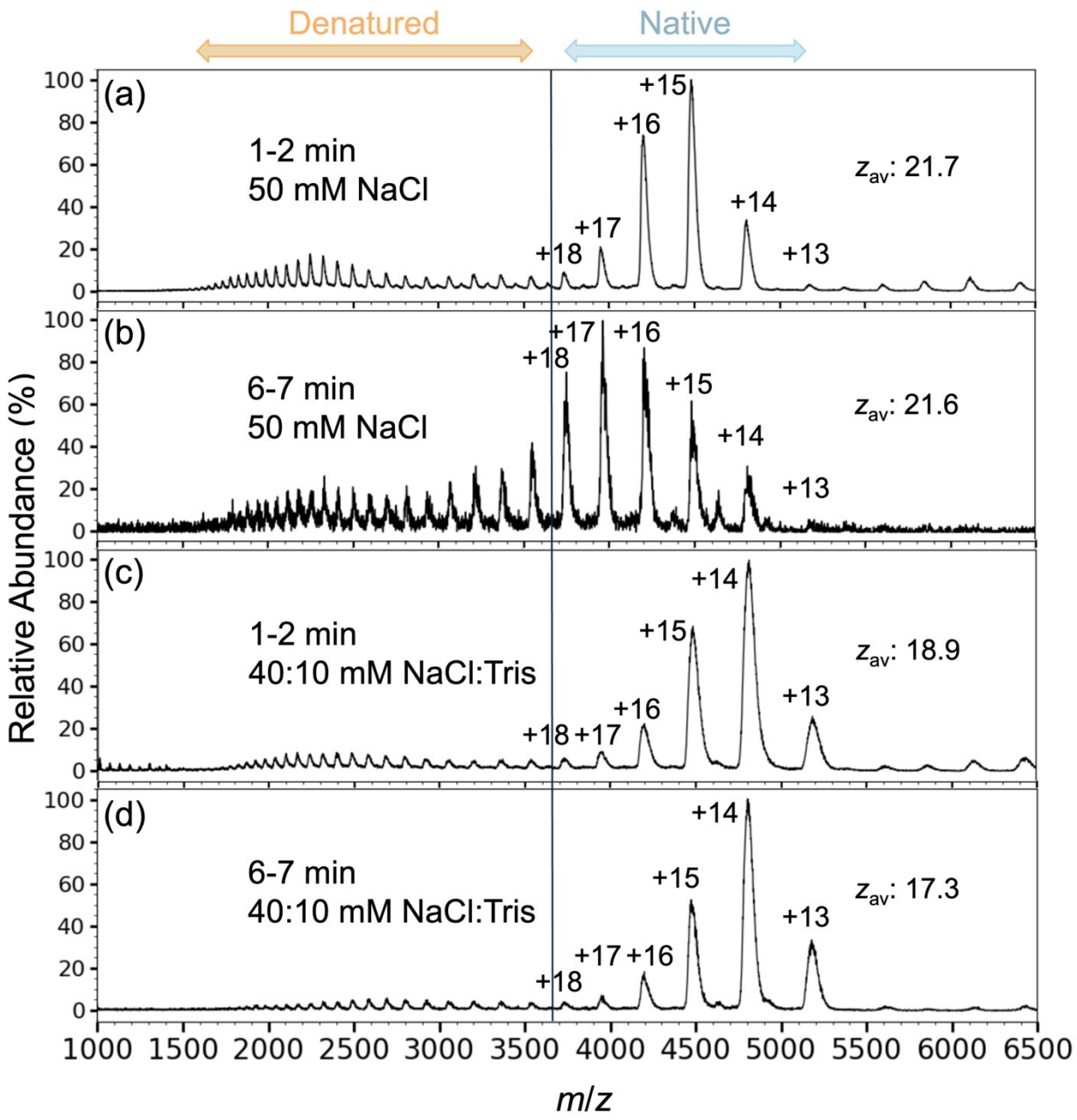


Figure 2. Electrospray ionization mass spectra of 5 μM BSA in aqueous solutions of 50 mM NaCl a) 1 – 2 min after the onset of ESI and b) 6 – 7 min after the onset of ESI, and in 40:10 mM NaCl:Tris buffer c) 1 – 2 min after the onset of ESI and d) 6 – 7 min after the onset of ESI.

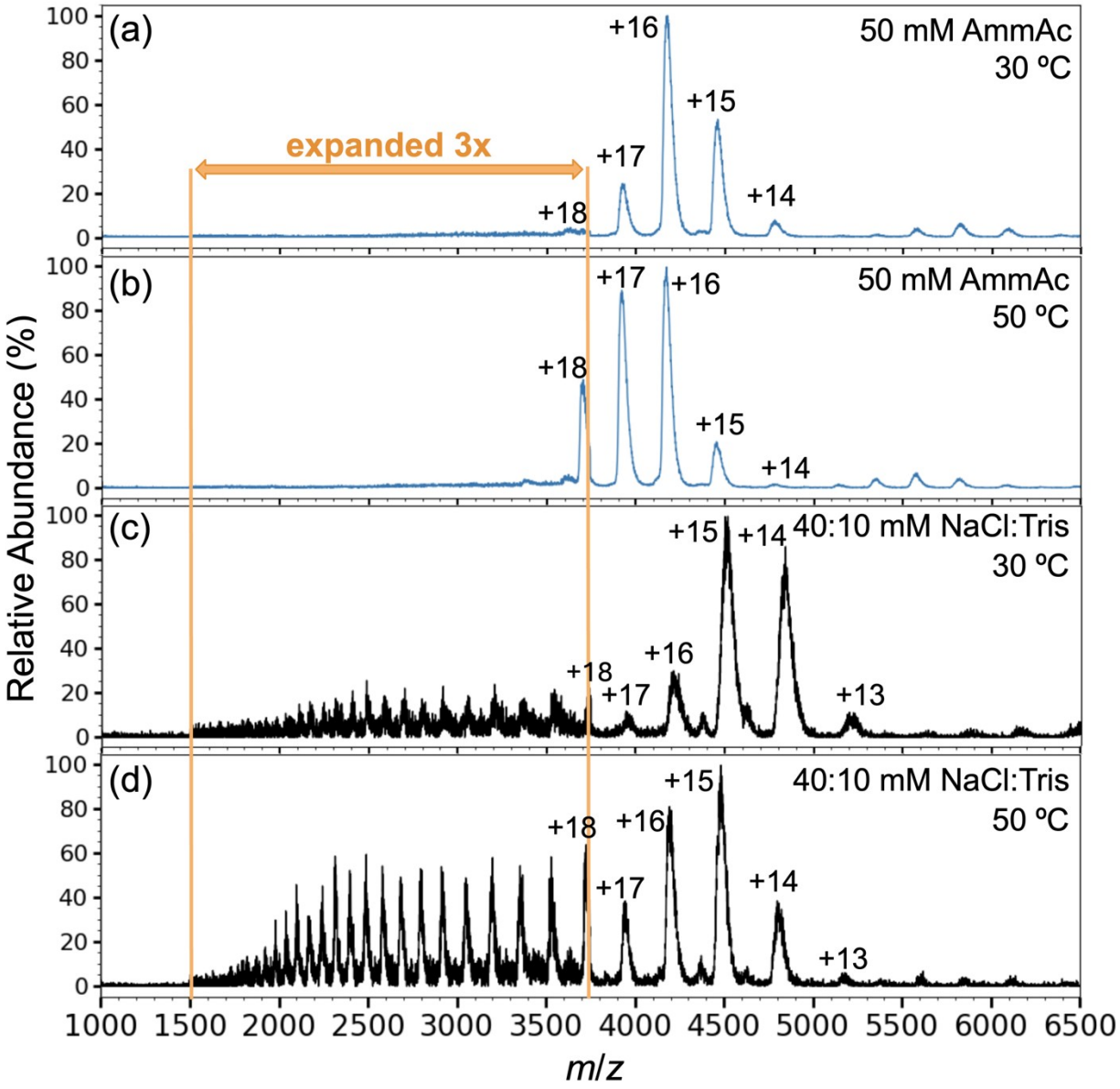
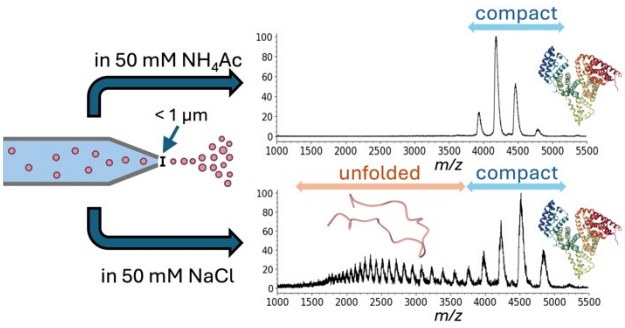


Figure 3. Mass spectra of 5 μM BSA in 50 mM AmmAc at a) 30 ºC and b) 50 ºC, and in 40:10 NaCl:Tris buffer at c) 30 ºC and d) 50 ºC.

TOC Graphic


compact
in 50 mM NH_4Ac
< 1 µm
unfolded
compact
in 50 mM NaCl
m/z
m/z