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Article

Effects of Amino Acid Additives on Protein Stability during Electrothermal Supercharging in ESI-MS

Roshan Javanshad, Rajendra Panth, and Andre R Venter*

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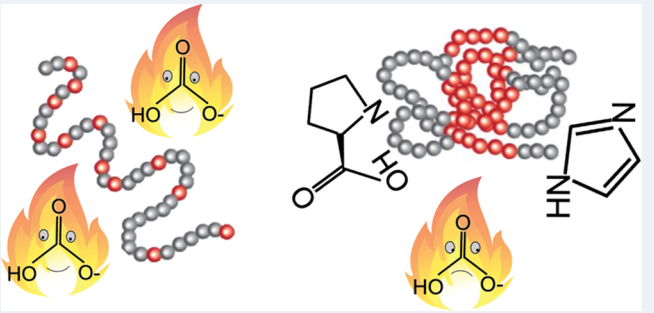
ABSTRACT: The surprising formation of highly charged protein ions from aqueous ammonium bicarbonate solution is a fascinating phenomenon referred to as electrothermal supercharging (ETS). Although the precise mechanism involved is not clearly understood, previous studies predominantly suggest that ETS is due to native protein destabilization in the presence of bicarbonate anion inside the electrospray ionization droplets under high temperatures and spray voltages. To evaluate existing hypotheses surrounding the underlying mechanism of ETS, the effects of several additives on protein charging under ETS conditions were investigated. The changes in the protein charge state distributions were compared by measuring the ratios between the intensities of *highest intensity charge states* of native and unfolded protein envelopes and shifts in the *lowest and highest observed charge states*. This study demonstrated that source temperature plays a more important role in ETS compared to spray voltage, especially when using a nebulized microelectrospray ionization source. Moreover, the effect of amino acids on ETS were generally in good agreement with the extensive literature available on the stabilization or destabilization of proteins by these additives in bulk solution. Among the natural amino acids, protein supercharging was significantly reduced by proline and glycine; however, imidazole provided the highest degree of noncovalent complex stabilization against ETS, outperforming the amino acids. Overall, our study shows that the simple addition of stabilizing reagents such as proline and imidazole can reduce the extent of apparent protein unfolding and supercharging in ammonium bicarbonate solution and provide evidence against the roles of charge depletion and thermal unfolding during ETS.



KEYWORDS: *electrothermal supercharging, protein supercharging, ammonium bicarbonate, ESI-MS*

INTRODUCTION

Native electrospray ionization mass spectrometry (ESI-MS) has emerged as a powerful tool for protein characterization by enabling detection of intact, native state proteins from purely aqueous or buffered aqueous solutions with near neutral pH. Despite being a commonly used buffer, aqueous ammonium bicarbonate solution with near neutral pH can produce highly charged protein ions with extended charge state distributions (CSDs) in ESI-MS, a hallmark characteristic of unfolded proteins, despite the native state of proteins in bulk solution. This phenomenon was first reported by Williams et al. and was named "electrothermal supercharging" due to the direct dependence of the phenomenon on temperature and electrospray voltage.[1] Previous studies proposed that electrothermal supercharging (ETS) is caused by the destabilization of protein in the presence of bicarbonate anion inside the heated electrospray droplets, resulting in extensive protein unfolding prior to ion formation.[1,2] Interestingly, an ion mobility-mass spectrometry study by Ruotolo et al. suggests that bicarbonate anion has little influence on protein complex stability during the removal from bulk solvent and in the gas phase.[3] An alternative proposed mechanism for the effect of ammonium bicarbonate on proteins suggests destabilization of the protein tertiary structure on the hydrophobic interface of carbon dioxide bubbles during the ESI process.[4] However, subsequent work challenged this hypothesis.[2] Therefore, while progress has been made in understanding protein supercharging with ammonium bicarbonate, the precise underlying mechanism remains unclear.

The present study examines the effect of solution-phase additives on protein supercharging by ammonium bicarbonate. Naturally occurring, small-molecule additives, including arginine (Arg), proline (Pro), alanine (Ala), serine (Ser), and glycine (Gly), have been broadly used to influence protein stability during different physical and chemical processes and stress-inducing conditions[5−9] such as thermal stress.[10,11] Naturally occurring amino acids are also classified as a

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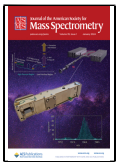

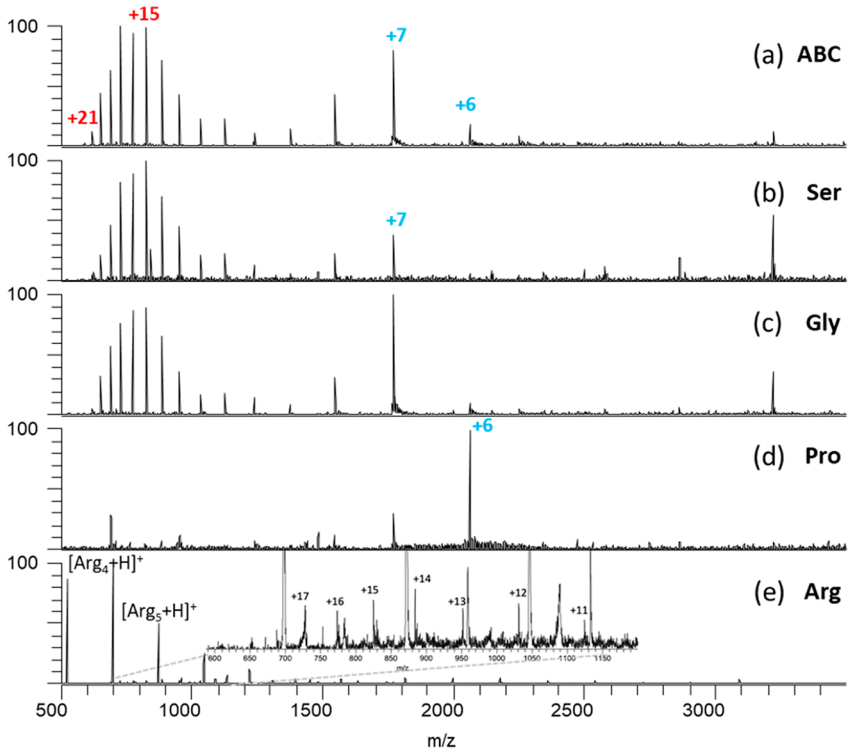

Figure 1. Representative ESI-MS spectra of cytochrome *c* in aqueous 10 mM ammonium bicarbonate (ABC) solution (a) under electrothermal supercharging (ETS) conditions (capillary temperature 200 °C and spray voltage 1.0 kV) in the presence of four different amino acids at 1 mM concentration; (b) serine, (c) glycine, (d) proline, and (e) arginine. Blue charge state labels refer to folded distributions, while Red labels refer to the charge states indicative of unfolded protein.

subgroup of osmolytes.[12,13] Osmolytes are naturally occurring small molecules that accumulate in living cells as protecting agents against stress factors. Osmolytes are compatible solutes known to benefit stabilization of protein structure, although a universally accepted mechanism of action or comprehensive understanding of specific osmolyte effects is still not fully reached after many decades since the first osmolytes were reported. Among additives used for proteins, arginine (Arg) is perhaps one of the most well-known and widely studied.[14−17] Several studies have proposed that arginine has a destabilizing effect on native protein structure.[18−20] The stabilizing effect of several other additives, including proline (Pro), alanine (Ala), serine (Ser), and glycine (Gly) and also imidazole, has been investigated, although the results of independent ESI-MS studies suggest the observed effects of these additives on the mass spectra of proteins are highly dependent on instrumentation, particularly the ionization source configuration.[21−24] The goal of the present study was to explore potential mechanisms of ETS by using solution-phase additives that are known to influence protein stability. To this end, the effect of Arg, Pro, Ala, Ser, Gly, hydroxyproline, and imidazole on protein CSDs of cytochrome *c* and ubiquitin, as well as the noncovalent complex of holo-myoglobin, was measured against different ETS conditions.

EXPERIMENTAL SECTION

Materials. Lyophilized equine heart cytochrome *c* (Cyt *c*, 95% purity) and bovine erythrocyte ubiquitin (Ubq, ≥98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Proteins were used without further purification. BioUltra grade ammonium bicarbonate (ABC) and all amino acids (≥98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). 200 μM protein stock solutions, 1.0 M ammonium bicarbonate stock solution (pH = 7.9), and 100 mM amino acid stock solutions were prepared in Milli Q water obtained from a Thermo-Barnstead Water Polisher. Final protein concentration and amino acid concentration in all working solutions were 10 μM and 1 mM respectively. The pH of aqueous ammonium bicarbonate solution was measured at room temperature with a Mettler Toledo Seven Easy pH meter (Columbus, OH) equipped with an InLab Expert pH electrode.

Instrumentation. Experiments were performed on a LTQ linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA) equipped with an electrospray emitter made from a Swagelok T-piece and two pieces of coaxial fused silica capillary tubing.[25] The outer capillary (for sheath gas) was approximately 20 mm in length with an outer diameter of 430 μm and inner diameter of 320 μm. The internal capillary (for solvent) had an outer diameter of 220 μm and an inner diameter of 50 μm. The solvent capillary extended through the T-piece and was connected to a syringe pump which delivered the working solutions for direct infusion. Spray potential was applied to the liquid junction of a stainless-steel syringe needle which delivered solvent at a flow rate of 3 μL/min, with N_2 as nebulizing gas at 100 psi. LTQ capillary voltage and tube lens voltage were set at 20 and 135 V respectively, and automatic gain control (AGC) was turned on with maximum injection time of 400 ms.

Data Analysis. Mass spectra were collected and viewed in Xcalibur Qual Browser (2.0.7). Thirty scans of direct infusion of three independent samples were collected for each solvent system. Signal intensities were calculated as the average of three independent samples, and error bars represent ± mean

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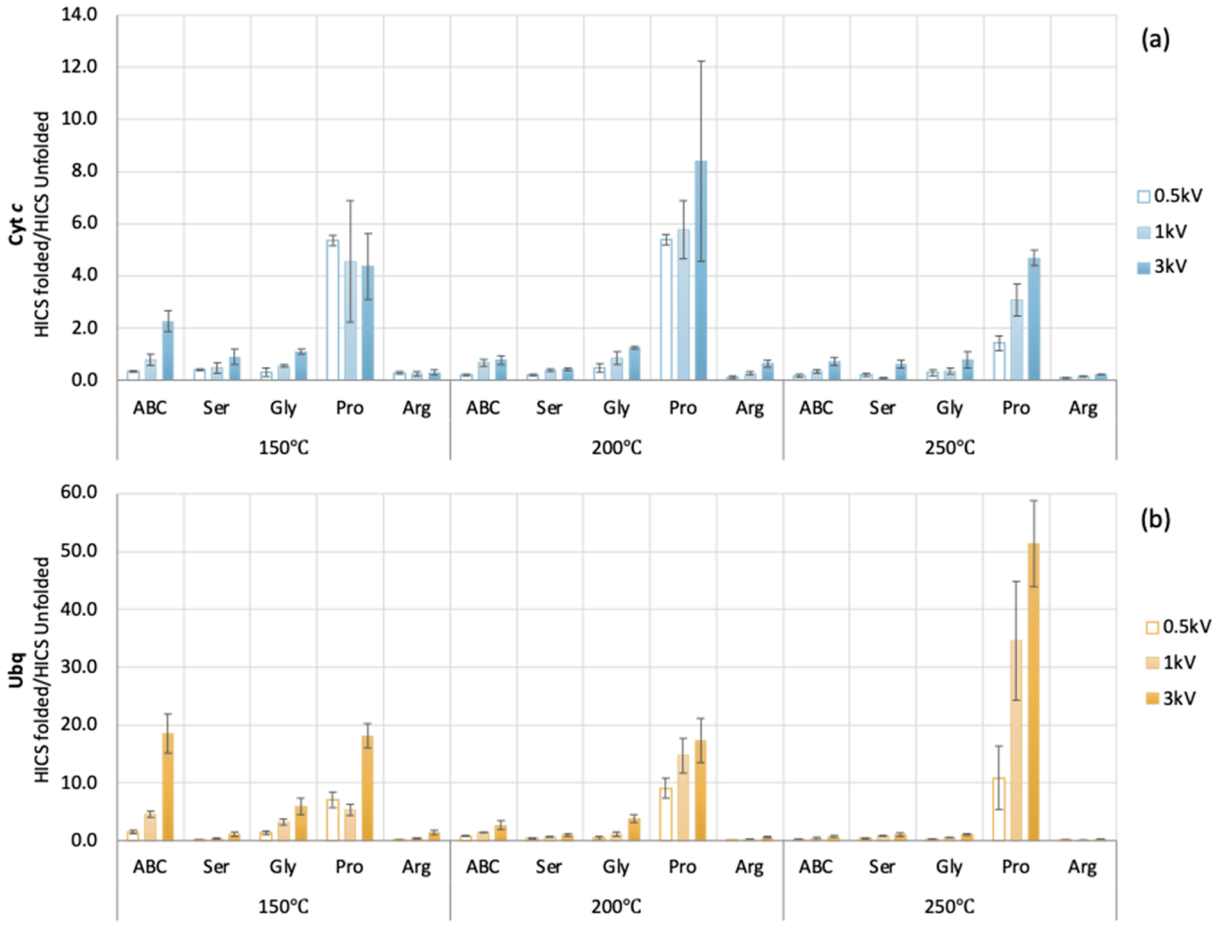

Figure 2. Effect of 1 mM amino acid additive on electrothermal supercharging under different temperatures and voltages for 10 μM (a) cytochrome *c* (Cyt *c*) and (b) ubiquitin (Ubq) in 10 mM ammonium bicarbonate (ABC), demonstrated by the conformational ratio ($HICS_{folded}/HICS_{unfolded}$), a representative measurement of apparent protein unfolding.

standard deviation of the replicate measurements at a given experimental condition.

RESULTS AND DISCUSSION

ETS is believed to be a consequence of protein destabilization in the ESI droplet.[26] We first investigated this phenomenon by testing the effect of four different amino acid osmolytes, i.e., Ser, Gly, Pro, and Arg, on protein charge state distribution (CSD) in ammonium bicarbonate solution under various ion transfer tube temperatures and spray voltages. The conformational ratio was defined as the ratio between absolute signal intensity of the highest intensity charge state (HICS) of native CSD envelope to absolute signal intensity of the HICS of unfolded CSD distribution of the model proteins, as determined and used by many previous studies.[27−30]

The representative spectra of Cyt *c* in aqueous 10 mM ABC solution under ETS conditions (200 °C capillary temperature and 1.0 kV spray potential) in the presence of 1 mM of different amino acids are shown in Figure 1.

In 10 mM ammonium bicarbonate solution, bimodal distribution of charge states represents two different populations of protein conformation. "High-charge" and "low-charge" peaks correspond to the two bell-shaped curves of the observed bimodal distributions. Based on multiple studies, including ion mobility mass spectrometry measurements, it is generally believed that Cyt *c* charge states +9 to +6 and below represent folded protein conformations, whereas charge states +9 and above represent different degrees of extended, unfolded protein conformations.[27] The extensive unfolded CSD with highest intensity charge state (HICS) of +15 and highest observed charge state (HOCS) of +21, resembling the CSD of acid-induced unfolded Cyt *c*, indicates significant unfolding of native Cyt *c* under ETS conditions in aqueous ammonium bicarbonate solution with neutral pH. The HICS of +7 and lowest observed charge state (LOCS) of +6 represent the presence of a subpopulation of native, folded Cyt *c* that "survives" ETS under these experimental conditions. The ratio between the intensity of the HICS for the native conformers subpopulation (charge state +7) and unfolded conformers subpopulation HICS (charge state +15) is approximately 0.68 ± 0.13.

The addition of 1 mM Ser to this solution resulted in subtle changes in protein CSD, as can be seen in Figure 1b. The z = +6 was the LOCS in the control, but this coalesced with the z = +7 charge state to become both HICS and LOCS for the native distribution. The ratio between signal intensity of native HICS to unfolded HICS also decreases from 0.68 ± 0.1 to 0.38 ± 0.05. It is apparent that Ser caused a shift to unfolded protein charge states. The destabilizing effect of Ser on native lysozyme and myoglobin has been reported by Chen et al.[22] and was also observed in desorption electrospray ionization (DESI-MS) of native proteins.[31,32] On the other hand, the addition of 1 mM Gly, another neutral amino acid with isoelectric point close to Ser, did not cause any changes to the

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observed HICS, HOCS, and LOCS. However, the ratio between signal intensity of native HICS to unfolded HICS increased from 0.68 to 0.86 ± 0.0.24, indicating, in Figure 1c, an increase in the folded conformer subpopulation.

The most pronounced shift in CSD of proteins under ETS conditions came from addition of 1 mM Pro and 1 mM Arg to the protein solution. A complete shift to only folded protein charge state distribution was observed with Pro, and compared to the ABC-only solution, the native charge state distribution showed a shift of HOCS to +7 and HICS to +6, as seen in Figure 1d. Arg, on the other hand, caused severe ion suppression, presumably due to the formation of protonated Arg clusters ranging from Arg_2 to Arg_{13}, due to multiple strong intermolecular interactions including hydrogen bonding and electrostatic interactions between the guanidinium groups.[33] Pro, which is less basic than Arg but more basic than Ser and Gly, also formed clusters visible in the mass spectra, although these clusters ranged only from protonated Pro_2 to Pro_4. The significant difference between Pro and Arg is that Arg has a higher gas-phase basicity than Pro yet still produced protein CSD representing unfolded protein (Figure 1e, inset) whereas Pro, with lower gas-phase basicity, produces protein CSD corresponding to folded, native protein. Therefore, the observed shifts in protein CSD under ETS conditions are not merely related to amino acid gas-phase basicity or proton affinity that could lead to *charge depletion*,[23,34] as discussed further on, and are likely a result of other factors affecting ETS during the ESI process.

We also investigated the effects of these amino acid additives on cytochrome *c* and ubiquitin under a range of capillary temperatures and spray voltages in 10 mM ammonium bicarbonate (Figure 2) and 100 mM ammonium bicarbonate (Figure S1). The average conformational ratio ($HICS_{folded}$/$HICS_{unfolded}$), a representative measurement of apparent protein unfolding, for these experiments can be found in Table S1.

In both model proteins, the effect of temperature (thermal denaturation) was more pronounced than that of spray voltage, as reported previously.[1] However, complete prevention of ETS by using low spray voltage or temperature settings was not possible in our ESI experiments, as a stable ESI signal was not achievable at sufficiently low temperatures and spray voltages when using microspray. Interestingly, Chen et al. also reported a notably higher degree of thermally induced protein unfolding in ESI droplets when using a spraying capillary with a larger inner diameter.[22,35] The more severe unfolding observed in our data compared to nano-ESI studies of ETS could be due to different mechanisms, potentially occurring synergistically: Larger droplets with longer droplet lifetimes are obtained with the nebulized micro-ESI spray compared to nanotips. Based on results by Williams and Xia, gaseous protein ions from the smaller droplets are formed outside of the mass spectrometer, whereas in larger droplets a large population of protein ions are formed inside of the mass spectrometer where thermal heating/destabilization and concomitant protein unfolding inside of the droplet occur.[26] On the other hand, larger droplets could lead to cooler ions and less thermal destabilization due to an evaporative cooling mechanism and possible hydrated ions surviving through the heated capillary. The stronger protein unfolding observed with microspray could therefore be a result of larger droplets providing more time for protein unfolding in the presence of the destabilizing bicarbonate anion under ETS conditions. Measurements of the protein melting curve in 100 mM ammonium bicarbonate compared with 100 mM ammonium acetate had shown proteins are less thermally stable in ammonium bicarbonate solution even at the same ionic strength.[26] This observation, along with the difference in 10 mM vs 100 mM ammonium bicarbonate solution (Table S1), suggests that destabilization of native protein structure by bicarbonate is the dominant contributor to protein unfolding inside the droplet, which is likely triggered more strongly by thermal destabilization and less by high electric field in the ESI. In fact, surprisingly, increasing spray voltage increased the native protein population in nebulized micro-ESI, contrary to results reported for nano-ESI. Differences between the two instrumental setups, including the use of borosilicate spray tips versus fused silica, the larger diameter of micro-ESI spray, and the presence of the nebulizing gas, could lead to the differences in observations and require further investigation.

Between the different amino acids, Pro consistently was the most successful at minimizing unfolding. In fact, the addition of Pro completely inverted the conformational ratio, as shown in Figure 2 and Figure 1d. Ser and Arg, on the other hand, increased unfolding of protein, in accordance with their known destabilizing effects. Gly showed moderate stabilization of native structure, although not as effective as Pro. Pro was especially powerful with Ubq compared to Cyt *c*. This is in accordance with previous reports where it was shown that ETS is more effective for proteins with net positive charge.[36]

None of the tested amino acids, other than Pro, were able to prevent or significantly reduce ETS at higher temperatures. Interestingly, Chen et al. also observed that Pro is the most effective amino acid in stabilization of noncovalent protein complexes in 20 μM ammonium acetate solutions on an LTQ-MS instrument at high capillary voltages.[22]

As discussed above, the most drastic factor that significantly affects ETS is the concentration of ammonium bicarbonate in solution. (Table S1). Similarly reported by Williams et al., the extend of supercharging increases with increasing concentration of ammonium bicarbonate, and at concentrations >100 mM maximum supercharging was observed.[1,37] In Figure S1 we demonstrate the efficacy of 1 mM of each amino acid in changing Cyt *c* and Ubq CSD under ETS with 100 mM ammonium bicarbonate. For comparison, the conformational ratio (intensity of the HICS of native conformers subpopulation (charge state +8) and unfolded conformers subpopulation HICS (charge state +17)) decreased by half from 0.68 ± 0.1 for Cyt *c* in 10 mM ammonium bicarbonate to 0.31 ± 0.1 in 100 mM ammonium bicarbonate (Table S1). Other than Pro, no other amino acid was able to change CSD to favor the native protein structure, even at the lowest temperature and spray voltage. Therefore, the effectiveness of the amino acid in preventing ETS is dependent on a favorable concentration ratio. However, there is a limit to the effectiveness of amino acids to overcome ETS, and the effectiveness breaks down at high concentrations of ammonium bicarbonate. Higher concentrations of amino acids lead to the formation of clusters together with ion suppression, limiting the maximum concentration that can be used with ESI-MS. At higher ETS conditions, i.e., 100 mM ABC, capillary temperature 250 °C, and spray voltage 3.0 kV, Pro was able to cause a shift toward native conformations for ubiquitin (from 0.16 to 0.31) but not for Cyt *c* (Figure S2).

A common measurement of protein stability is studying the survival of noncovalent protein complexes. Therefore, the

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effect of the most stabilizing amino acids was tested on 10 μM myoglobin in 10 mM ammonium bicarbonate. The striking stabilizing effect of Pro against ammonium bicarbonate is also evident from maintaining the noncovalent complex between heme and myoglobin in Figure 3, even under high ETS conditions (capillary temperature 250 °C and spray voltage 3.0 kV).

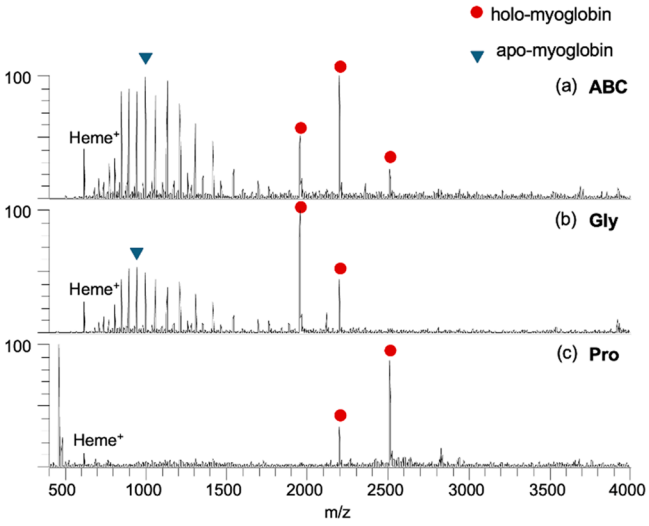

Figure 3. Representative ESI-MS spectra of myoglobin in (a) 10 mM ammonium bicarbonate (ABC) solution without any additives and in the presence of stabilizing amino acids (b) glycine and (c) proline under maximum electrothermal supercharging (ETS) conditions (capillary temperature 250 °C and spray voltage 3.0 kV).

The retention of the holo-myoglobin complex and a decrease in the ratio between heme and holo-myoglobin with Pro under ETS suggest an increase in the stability of native protein against the destabilizing effect of ammonium bicarbonate. As discussed above, previous studies and results from Table S1 have concluded that protein unfolding in ETS occurs in the droplets due to destabilization of the native protein structure mainly due to temperature. Based on previous observations in ESI-MS and bulk solution measurements,[22,38,39] we also investigated two other reportedly thermal stabilizing additives, hydroxyproline[40] and imidazole.[23,24] The result of this comparison can be found in Figure 4.

Surprisingly, L-hydroxyproline did not show any apparent stabilizing effect on proteins in ammonium bicarbonate under ETS conditions, despite a report of being a better thermal stabilizer compared to L-proline, at least in bulk solution.[40] In striking contrast, imidazole significantly reduced protein unfolding, outperforming Pro in reducing holo-mb unfolding and complex dissociation against capillary temperature up to 300 °C (Figure 4 a) and against spray voltage increase (Figure 4 b).

The stabilizing effect of Pro and imidazole, at least in native ESI-MS in ammonium acetate solution, has been attributed to charge depletion that reduces the electrostatic unfolding of protein in the ESI droplet.[23,34] It can also be speculated that charge depletion from the droplet is at least partially contributing to the formation of nativelike protein ions by reducing the number of available charges and reducing Coulombic repulsion on the protein, therefore increasing protein stability under ETS conditions. However, this hypothesis is contradictory to the effect of Arg. Arg is a

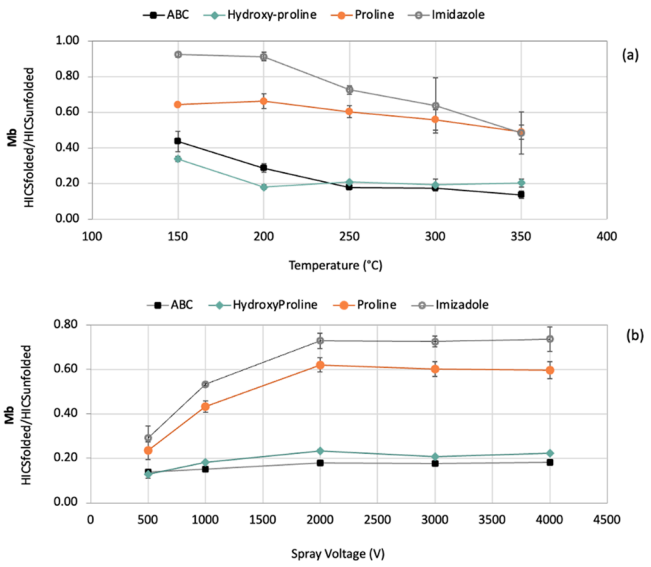

Figure 4. Conformational ratio of myoglobin (Mb) in 10 mM ammonium bicarbonate and 1 mM additive at (a) 3 kV spray voltage and different capillary temperatures and (b) 250 °C capillary temperature and different spray voltages.

more basic amino acid (gas-phase basicity = 1006.6 kJ/mol and proton affinity = 1051.1 kJ/mol), yet unfolded charge states of protein are detectable, whereas with Pro, a less basic amino acid (gas-phase basicity = 886.0 kJ/mol and proton affinity = 920.5 kJ/mol),[41] only lower protein charge states are detected. Therefore, charge depletion by the additive may not be the only mechanism behind the stabilizing effect. Without other validation methods such as ion mobility, it is not clear whether the observed protein stabilization is associated with the gas phase via a mechanism of ion cooling or occurs in solution through direct and/or indirect interactions with the protein prior to ion formation.

CONCLUSION

In the present work, the formation of highly charged protein ions from aqueous ammonium bicarbonate solution with near neutral pH during ESI-MS was explored by investigating the effect of additives known to affect protein stability. Our data demonstrates that the destabilization of the protein in ammonium bicarbonate under ETS conditions is most strongly influenced by source temperature. The effect of amino acids on ETS was generally in good agreement with the extensive literature available on the stabilization[42] or destabilization of proteins by these additives in many studies, including bulk solution or ESI/DESI experiments. A notable outlier was hydroxyproline, which showed a destabilizing effect, despite reports that hydroxyproline is more effective than proline at thermal stabilization in bulk solution. Among the tested additives, imidazole and proline were most capable of reducing protein unfolding and noncovalent complex dissociation under ETS conditions, particularly against high source temperatures.

A potentially important application of the presented results is in native mass spectrometry studies. In native-MS, volatile buffer solutions near neutral pH are invaluable. Yet, the most used volatile "buffer" in native-MS is ammonium acetate which has minimal buffering capacity at neutral pH because pK_A of acetate is 4.6.[42] Therefore, where the use of a proper volatile buffer at the right pH is important for native mass

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spectrometry analyses, ammonium bicarbonate is a better alternative. However, due to the extent of ETS and consequent protein unfolding, the use of ammonium bicarbonate for native-MS is problematic. Here we show that with the addition of stabilizing agents such as Pro and imidazole, maintaining the native conformation in ammonium bicarbonate buffer during ESI analysis is possible without significantly sacrificing spectral quality. Moreover, these additives can be used for "strengthening" noncovalent protein complexes during other stages of the mass spectrometry analysis, such as during gas-phase fragmentation, to yield more nativelike structures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.3c00377.

Effects of amino acid derivative on electrothermal supercharging under different temperatures and voltages for Cyt *c* and Ubq in ammonium bicarbonate (Figure S1, Table S1); effects of amino acid additive at higher ETS conditions for Cyt *c* and Ubq (Figure S2) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Andre R Venter − *Department of Chemistry, Western Michigan University, Kalamazoo, Michigan 49008-5413, United States;* orcid.org/0000-0001-6912-7446; Phone: +1-269-387-2420; Email: andre.venter@wmich.edu

Authors

Roshan Javanshad − *Department of Chemistry, Western Michigan University, Kalamazoo, Michigan 49008-5413, United States*

Rajendra Panth − *Department of Chemistry, Western Michigan University, Kalamazoo, Michigan 49008-5413, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/jasms.3c00377

Author Contributions

R.J. and R.P. performed all experiments. All authors contributed to the experimental design, data interpretation, and manuscript writing. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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