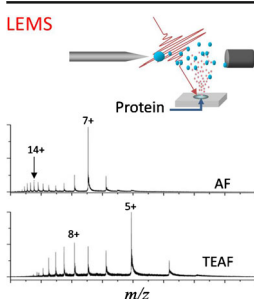


RESEARCH ARTICLE

Isolating Protein Charge State Reduction in Electrospray Droplets Using Femtosecond Laser Vaporization

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Abstract. Charge state distributions are measured using mass spectrometry for both native and denatured cytochrome *c* and myoglobin after laser vaporization from the solution state into an electrospray (ES) plume consisting of a series of solution additives differing in gas-phase basicity. The charge distribution depends on both the pH of the protein solution prior to laser vaporization and the gas-phase basicity of the solution additive employed in the ES solvent. Cytochrome *c* (myoglobin) prepared in solutions with pH of 7.0, 2.6, and 2.3 resulted in the average charge state distribution (Z_{avg}) of 7.0 ± 0.1 (8.2 ± 0.1), 9.7 ± 0.2 (14.5 ± 0.3), and 11.6 ± 0.3 (16.4 ± 0.1), respectively, in ammonium formate ES solvent. The charge distribution shifted from higher charge states to lower charge states when the ES solvent

contained amines additives with higher gas-phase basicity. In the case of triethyl ammonium formate, Z_{avg} of cytochrome *c* (myoglobin) prepared in solutions with pH of 7.0, 2.6, and 2.3 decreased to 4.9 (5.7), 7.4 ± 0.2 (9.6 ± 0.3), and 7.9 ± 0.3 (9.8 ± 0.2), respectively. The detection of a charge state distribution corresponding to folded protein after laser vaporized, acid-denatured protein interacts with the ES solvent containing ammonium formate, ammonium acetate, triethyl ammonium formate, and triethyl ammonium acetate suggests that at least a part of protein population folds within the electrospray droplet on a millisecond timescale.

Keywords: Femtosecond laser vaporization, Electrospray ionization, Protein conformation, Charge state distribution

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Introduction

Electrospray ionization (ESI) of proteins reveal a characteristic charge state distribution (CSD) correlating to the extent of denaturation [1]. Protein in the native configuration is typically folded, which protects the basic amino acids in the interior from charging and hence produces a narrow distribution at lower charge states in comparison to denatured protein. Denatured protein exhibits a much broader CSD at higher charge states because additional basic amino acids are exposed to the solvent environment and therefore can acquire more charge. Investigations of solvent-induced conformational change [2], acid and thermal denaturation [1, 3], and refolding of initially denatured proteins [4] suggest a strong correlation between the observed CSD and protein conformation. The role of protein structure in determining the CSD has also been

investigated using ion mobility mass spectrometry (IM-MS) measurements. The collision cross-section (CCS) of cytochrome *c* [5] and ubiquitin [6] measured as a function of charge state showed higher CCSs for unfolded protein ions. The consensus from these studies is that lower charge states correlate to more folded, native-like structure, and higher charge states correlate to a more unfolded structure.

Protein conformational change may occur during the electrospray process. Exposing ES droplets containing protein molecules to either acidic or basic vapors in the region between the spray tip and the MS inlet can either induce unfolding [7, 8] or refolding [4] of proteins. For instance, exposing ES droplets containing native proteins to acidic vapors results in a lower droplet pH, a favorable condition for protein denaturation [8]. Exposing acid-denatured proteins to basic vapor results in a bimodal CSD, indicating charge state reduction in at least a fraction of protein population, presumably folding as a result of an increase in pH of the ES droplet [4]. Without some degree of folding, a simple monotonic shift of the CSD to lower charge is expected, not the formation of a second, narrowly peaked distribution.

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In the electrospray process, the gas-phase basicity of a solvent is related to the ability to promote proton transfer from an analyte, thus reducing the overall charge. Gas-phase basicity is defined as the change in free energy when a proton attaches to an anion or neutral molecule. A solution additive with high gas-phase basicity has been found to charge-reduce, and stabilize noncovalent protein complexes [9]. Charge reduction results in the decrease in Columbic repulsion among the charged residues and thus the protein denaturation becomes difficult. Charge reducing additives have also been used to stabilize membrane protein complexes after release from detergent micelles in the gas phase [10].

The development of laser electrospray mass spectrometry (LEMS) has enabled the rapid analysis of complex mixtures at atmospheric pressure without sample preprocessing. LEMS analysis has been performed on a variety of samples, including proteins [11, 12], pharmaceuticals [13], explosives [14, 15], plant tissue [16], thermometer ions [17], and small analytes [18]. Femtosecond laser vaporization can be employed to deliver either native or denatured protein into the electrospray plume with various solvent conditions [11, 19]. This enables the delivery of native or denatured protein directly into the charged electrospray droplets, avoiding any structural changes and/or oxidation/reduction processes that might occur in the Taylor cone of the electrospray source. The gas-phase protein ions are presumably formed after the laser vaporized liquid aerosol containing protein molecules interacts with the charged ES droplets containing solution additives of interest.

In the present study, we investigate the role of solution additives with varying gas-phase basicities on the CSD of folded and unfolded cytochrome *c* and myoglobin. We separate the droplet processes from processes occurring in the solution flowing through the capillary and the Taylor cone on the capillary emitting the charged droplets, by laser vaporizing protein directly into the spray containing the solution additive of interest. Considering the solution, intermediate, and gas-phase regimes of electrospray ionization process [20], our measurements emulate the intermediate regime, from which gas-phase ions are released. We also determine whether interaction of the laser vaporized acid-denatured protein with the electrospray droplets containing additives with high gas-phase basicity induces charge reduction and protein folding within the approximately 100 ms time scale of the LEMS process. The shift in average charge states (Z_{avg}) and the respective contribution of folded and unfolded protein to the charge state distribution are calculated to determine the charge reduction and protein folding processes in LEMS measurements as a function of solution additives.

Materials and Methods

Sample Preparation

Solid samples of cytochrome *c*, myoglobin, ammonium acetate, ammonium formate, and ammonium bicarbonate, and aqueous samples of triethyl ammonium acetate, triethyl

ammonium formate, and triethyl ammonium bicarbonate were purchased from Sigma Aldrich (St. Louis, MO, USA). Aqueous formic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). For LEMS measurements, a 10^{-3} M stock solution of cytochrome *c* and myoglobin was prepared in HPLC grade water (Fisher Scientific, Pittsburgh, PA, USA). An aliquot of the stock solution was diluted in water to yield a final protein concentration of 2×10^{-4} M for native proteins. For denatured proteins, an aliquot of stock solution was diluted into water with addition of formic acid to yield a protein concentration of 2×10^{-4} M with a pH of either 2.6 or 2.3. Seven ESI aqueous solutions with or without the addition of charge reducing additives were utilized in this study. The electrospray solvent consisted of 5 mM concentration of ammonium acetate, ammonium formate, ammonium bicarbonate, triethyl ammonium acetate, triethyl ammonium formate, or triethyl ammonium bicarbonate solution. A 10 μ L aliquot of the diluted protein, either native or denatured, was spotted on a stainless steel plate and then subjected to laser vaporization into the ES solvents.

Laser Vaporization

A Ti:sapphire laser oscillator (KM Laboratories, Inc., Boulder, CO, USA) seeded a regenerative amplifier (Coherent, Inc., Santa Clara, CA, USA) for the creation of 75 fs, 0.6 mJ laser pulses centered at 800 nm. The laser, operated at 10 Hz to couple with the ES ion source, was focused to a spot size of ~ 250 μ m in diameter with an incident angle of 45° with respect to the sample using a 16.9 cm focal length lens, with an approximate intensity of 1.6×10^{13} W/cm². The steel sample plate was biased to -2.0 kV to compensate for the distortion of electric field between the capillary and the needle caused by the sample stage. The area sampled was 6.4 mm below and approximately 1 mm in front of the ES needle. Aqueous protein sample (10 μ L) deposited on the steel substrate was vaporized by the intense pulses, allowing for capture and ionization by an ES plume traveling perpendicular to the vaporized material. The flow rate for ES solvent was set at 2 μ L/min by a syringe pump (Harvard Apparatus, Holliston, MA, USA).

Mass Spectrometry and Data Analysis

The mass spectrometer used in this experiment has been previously described [21]. The ES needle was maintained at ground while the inlet capillary was biased to -4.5 kV to operate in positive ion mode. The postionized analytes were dried before entering the inlet capillary by counter-current nitrogen gas at 180 $^\circ$ C flow at 3 L/min and were mass analyzed using microTOF-Q II mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The average charge state (Z_{avg}) was calculated using Equation 1,

$$Z_{avg} = \frac{\sum_i^N q_i w_i}{\sum_i^N w_i} \quad (1)$$

where q_i is the net charge, W_i is the sum of signal intensity of the i^{th} charge state, and N is the number of charge states present in the mass spectra.

Results and Discussion

Analysis of Cytochrome *c* in Conventional Versus Charge Reducing Solution Additives

Conventional Electrospray Solvents To determine the charge state distribution as a function of acid concentration, solutions of cytochrome *c* with pH of 7.0, 2.6, and 2.3, were laser-vaporized into aqueous ES droplets, and the resulting CSDs were measured. The LEMS measurement of cytochrome *c* prepared in solution with pH 7.0 in aqueous ES reveals a CSD ranging from 5+ to 12+ and is peaked at 9+ (Fig. 1a), with an average charge state distribution (Z_{avg}) of 8.5 ± 0.2 , as listed in Table 1. As the pH of solution containing cytochrome *c* is decreased, a bimodal CSD is observed, peaked at 9+ and 17+ for pH 2.6 and 2.3, (Fig. 1b, and Supplementary Figure S1a in Supporting Information). The Z_{avg} values obtained for cytochrome *c* at pH 2.6 and 2.3 are 12.7 ± 0.1 and 14.1 ± 0.1 , respectively (Table 1). The lower charge states ranging from 7+ to 10+ contains $45 \pm 4\%$ and $24 \pm 2\%$ of the cytochrome *c* ion intensity for solution with pH 2.6 and 2.3, respectively (Table 1). The decrease of the low charge state ion intensity and the increase in Z_{avg} with decreasing pH suggests that the protein unfolds at lower solution pH.

Ammonium formate and ammonium acetate are volatile additives that are routinely used in ESI-MS to maintain the native structure of protein and protein complexes. The effect of ammonium formate ES solution on the CSD of cytochrome *c* was measured by adjusting the pH of the protein solution vaporized. The LEMS measurement of cytochrome *c* prepared in a solution with pH 7.0 vaporized into ES droplets containing ammonium formate (solution pH of 6.2) reveals mainly 7+ charge state (Fig. 1c), indicating charge reduction of the folded state of the protein in comparison with aqueous ES solvent. As the pH of the cytochrome *c* solution is decreased, laser vaporization into the ammonium formate ES solution reveals a bimodal CSD, peaked at 7+ and 14+ for pH 2.6 (Fig. 1d), and 7+ and 15+ for pH 2.3 (Supplementary Figure S1b). The bimodal distributions indicate the presence of at least two conformations of the protein. The lower charge states ranging from 6+ to 9+ contain $56 \pm 3\%$ at pH 2.6, and $28 \pm 1\%$ at pH 2.3, of the cytochrome *c* ion intensity (Table 1), indicating folded states of the protein whereas the higher charge states ranging from 10+ to 20+ indicate unfolded protein. The $\sim 20\%$ enhancement in the folded distribution between the aqueous ($\sim 45\%$) and ammonium formate ($\sim 56\%$) ES solutions suggests that some of the protein population folds in the electrospray droplets containing ammonium formate. The contribution of unfolded protein to the charge state distribution increased when the protein was laser vaporized from solution pH of 2.3 in comparison with 2.6. In this case, the Z_{avg} increased to

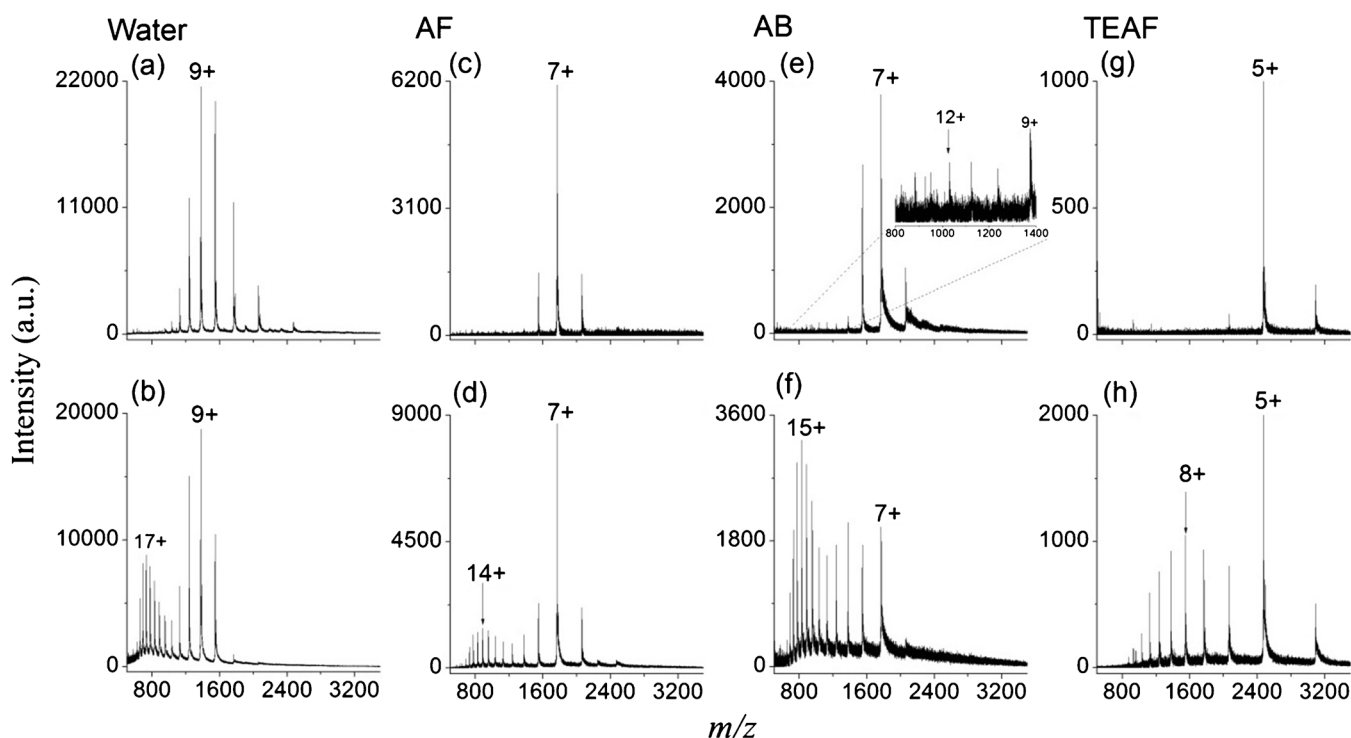


Figure 1. Representative LEMS mass spectra resulting from laser induced vaporization of cytochrome *c* at pH 7.0 (panel a, c, e, and g), and cytochrome *c* at pH 2.6 (panel b, d, f, and h) into the electro-spray solvent consisting of water (pH 6.8), 5 mM ammonium formate (AF, pH 6.2), 5 mM ammonium bicarbonate (AB, pH 8.2), and 5 mM triethyl ammonium formate (TEAF, pH 6.1), respectively

Table 1. Summary of Average Charge State Distribution (Z_{avg}) of Cytochrome *c*, and Myoglobin in Electrospray Solvent Consisting of Either Water, Ammonium Formate (AF), Ammonium Acetate (AA), Ammonium Bicarbonate (AB), Triethyl Ammonium Formate (TEAF), Triethyl Ammonium Acetate (TEAA), or Triethyl Ammonium Bicarbonate (TEAB)

Protein	pH	Average charge state (fraction folded protein)						
		Water	AF	AA	AB	TEAF	TEAA	TEAB
Cytochrome <i>c</i>	7.0	8.5±0.2	7.0±0.1	6.9±0.1	7.7±0.4	4.9±0.0	5.2±0.1	4.7±0.2
	2.6	12.7±0.1 (45±4%)	9.7±0.2 (56±3%)	9.6±0.2 (54±4%)	12.7±0.2 (15±2%)	7.4±0.2 (41±3%)	6.7±0.2 (46±4%)	7.0±0.3 (37±4%)
	2.3	14.1±0.1 (24±2%)	11.6±0.3 (28±1%)	11.5±0.3 (27±2%)	13.1±0.1 (10±1%)	7.9±0.3 (28±2%)	6.5±0.1 (47±5%)	7.1±0.1 (29±6%)
	7.0	11.8±0.3	8.2±0.1	8.0±0.1	8.2±0.3	5.7±0.0	5.9±0.0	5.5±0.1
Myoglobin	2.6	20.0±0.1 (3±2%)	14.5±0.3 (20±4%)	14.2±0.4 (19±6%)	16.6±0.1 (8±2%)	9.6±0.3 (22±4%)	9.0±0.1 (27±1%)	9.8±0.2 (12±2%)
	2.3	20.5±0.1 (2±1%)	16.4±0.1 (6±4%)	16.2±0.1 (9±3%)	18.6±0.1 (5±1%)	9.8±0.2 (19±3%)	8.8±0.3 (26±2%)	10.0±0.2 (9±3%)

The values reported in the parenthesis are the folded protein fractions of cytochrome *c* and myoglobin

11.6 ± 0.3 for cytochrome *c* as the pH was decreased, see Table 1. This indicates that the extent of protein unfolding prior to laser vaporization affects the observed protein CSD in a buffered ES solvent.

ESI-MS measurements were also performed as a control experiment in which acid-denatured cytochrome *c* was pre-mixed with similar solution additives (e.g. ammonium formate, ammonium bicarbonate, and triethyl ammonium formate) utilized in the LEMS measurement at 1:1 ratio. The mass spectra, Z_{avg} , and the fraction folded protein obtained from these measurements are reported in Supplementary Figure S2 and Supplementary Table S1.

The LEMS measurement of cytochrome *c* prepared in a solution with pH 7.0 in ammonium acetate (solution pH of 6.5) reveals mainly the 7+ charge state (Supplementary Figure S3a), with a Z_{avg} value of 6.9 ± 0.1, indicating charge reduction of the folded state of the protein. As the pH of the cytochrome *c* solution is decreased, bimodal CSDs are observed, peaked at 7+ and 13+ for pH 2.6 (Supplementary Figure S3b), and 7+ and 15+ for pH 2.3 (Supplementary Figure S3c), with a Z_{avg} value of 9.6 ± 0.2, and 11.5 ± 0.3, respectively. The lower charge states ranging from 6+ to 9+ contain 54 ± 4% at pH 2.6, and 27 ± 2% at pH 2.3, of the cytochrome *c* ion intensity. Again, the bimodal CSDs indicate the presence of at least two conformations of the protein. These measurements suggest that the addition of solution additive like ammonium acetate to an electrospray solvent can promote protein folding in the electrospray droplet and promote some degree of charge reduction.

Assessment of protein conformation was made by assuming that the CSD is a reliable indicator of either folded or unfolded structure. The assumption that there is a correlation between CSD and structure has been tested in many previous investigations [1–4, 22–24]. The use of CSD to assess solution-phase structure in the LEMS experiment further assumes that the structure in solution is preserved during laser vaporization. This has been tested through direct comparison of ESI and LEMS measurements for cytochrome *c* as a function of both sample pH before vaporization and ESI pH into which the sample is transferred [11]. Both the CSD and collision induced dissociation measurements suggest that the CSD correlates to the sample structure prior to laser vaporization. The major limitation of

using CSD as a probe of protein structure is the insensitivity to differentiating native and near native structures. Multiple structures may contribute to a particular charge state as has been demonstrated by ion mobility measurements [25, 26].

Ammonium bicarbonate is often used to maintain the near-neutral pH of the bulk solution to preserve the native structure of the protein. LEMS measurement of cytochrome *c* prepared in a solution with pH 7.0 vaporized into ES droplets containing ammonium bicarbonate (solution pH of 8.2) reveals mainly 7+ and 8+ charge states (Fig. 1e) and a small distribution ranging from 10+ to 14+ containing 13 ± 4% of the cytochrome *c* ion intensity, corresponding to unfolded states of the protein, as seen in the inset of Fig. 1e. The Z_{avg} calculated for cytochrome *c* in ES consisting of ammonium bicarbonate (Z_{avg} of 7.7 ± 0.4) was slightly higher in comparison with ammonium formate (Z_{avg} of 7.0 ± 0.1), and ammonium acetate (Z_{avg} of 6.9 ± 0.1). As the pH of solution containing cytochrome *c* is lowered, a trimodal CSD peaked at 7+, 9+, and 15+ for pH 2.6 (Fig. 1f), and a much broader CSD is observed, peaked at 15+ for pH 2.3 (Supplementary Figure S1c). The ammonium bicarbonate spray did not show a large fraction of folded cytochrome *c* at solution pH of 2.6 despite the expected higher pH (solution pH of 8.2). The calculated Z_{avg} for pH 2.6 (Z_{avg} of 12.7 ± 0.2), and pH 2.3 (Z_{avg} of 13.1 ± 0.1) were higher with the ammonium bicarbonate solution in comparison with the ammonium formate and ammonium acetate solutions. The increase in Z_{avg} for ammonium bicarbonate is possibly due to foaming of the electrospray solvent (ammonium bicarbonate) caused by $\text{CO}_2(\text{g})$ outgassing during the desolvation process via the reaction $\text{HCOOH} + \text{NH}_4^+\text{HCO}_3^- \rightarrow \text{NH}_4^+\text{HCOO}^- + \text{H}_2\text{O} + \text{CO}_2(\text{g})$ [27]. In this reaction, the counter ion (HCO_3^-) decomposes to CO_2 and H_2O in the presence of acid, creating bubbles that facilitate protein unfolding. We note that in the case of electrothermal supercharging experiment, bubble formation due to buffer decomposition was found not to be the primary reason for protein unfolding [28]. We note, however, that no acid was present in that study to induce the decomposition to H_2O and CO_2 . The presence of both an acid and $\text{CO}_2(\text{g})$ outgassing in our measurements contribute to the formation of relatively higher charge states in ES consisting of ammonium bicarbonate in comparison with ammonium formate, and ammonium acetate solution.

Solution Additives with High Gas-Phase Basicity Previous studies have shown that the use of charge reducing solution additives results in a shift in protein CSD to lower charge states [9, 29]. The electrospray measurements of globular and denatured lysozyme showed a decrease in net charging of the protein with increasing gas-phase basicity with the appearance of monomodal CSD [29, 30]. In conventional electrospray containing a solution additive with high gas-phase basicity there is the question of whether the additive assists in folding the denatured protein in solution to provide lower charge states or whether charge reduction simply occurs in the gas phase upon ionization at the end of the desolvation process [29, 31]. The charge reduction observed in a conventional ESI measurement using an additive with high gas-phase basicity is consistent with either hypothesis. The ionization of globular macromolecules in ESI-MS is believed to occur via charge residue model (CRM), where residual charge on the droplet surface is transferred to the macromolecules upon solvent evaporation [32–34]. LEMS is used here to decouple the solution-phase effects from processes occurring in the ES droplet during desolvation.

To investigate whether charge reduction occurs in the electrospray droplet, laser vaporization is used to transfer either folded or denatured proteins into the electrospray plume containing solution additives with high gas-phase basicities. Cytochrome *c* is prepared in three solutions having a pH of 7.0, 2.6, and 2.3 and was laser-vaporized into the ES droplets containing either triethyl ammonium formate (TEAF), triethyl ammonium acetate (TEAA), or triethyl ammonium bicarbonate (TEAB). The LEMS measurement of cytochrome *c* prepared in a solution with pH 7.0 vaporized into ES droplets containing TEAF (solution pH of 6.1) reveals mainly 5+ charge state (Fig. 1g) with a Z_{avg} value of 4.9, indicating enhanced charge reduction of the folded states of the protein in comparison with ammonium formate (Z_{avg} of 7.0 ± 0.1), acetate (Z_{avg} of 6.9 ± 0.1), and bicarbonate (Z_{avg} of 7.7 ± 0.4), as well as the aqueous (Z_{avg} of 8.5 ± 0.2) ES solvent. As the pH of laser vaporized cytochrome *c* solution is decreased to 2.6 and 2.3, bimodal CSDs are observed, peaked at 5+ and 8+ (Fig. 1h, and Supplementary Figure S1d). The fact that there are two distributions and not just a shifted distribution for TEAF suggests that there are both folded and unfolded states in the electrospray droplets. The lower charge states peaked at 5+ indicate significant charge reduction of the folded states, in comparison with both aqueous (peaked at 9+) and ammonium formate ES solvent (peaked at 7+). The distribution peaked at 8+ likely corresponds to the charge reduction of unfolded states of the protein. The low charge states ranging from 4+ to 6+ contain $41 \pm 3\%$ at pH 2.6, and $28 \pm 2\%$ at pH 2.3 of the cytochrome *c* ion intensity (Table 1). The fraction of folded state of cytochrome *c* (solution pH of 2.6) for TEAF ($41 \pm 3\%$) is within the standard deviation in comparison with aqueous ES ($45 \pm 4\%$), and $\sim 20\%$ lower in comparison with ammonium formate ($56 \pm 3\%$), and ammonium acetate ($54 \pm 4\%$) ES solutions. This suggests that the use of solution additive with high gas-phase basicity does not enhance protein folding in the ES droplets like the commonly used

solvent systems. However, a significant reduction in Z_{avg} was observed for cytochrome *c* (pH of 2.3) in the presence of TEAF (Z_{avg} of 7.9 ± 0.3) in comparison with aqueous (Z_{avg} of 14.1 ± 0.1), ammonium acetate (Z_{avg} of 11.5 ± 0.3), and ammonium formate (Z_{avg} of 11.6 ± 0.3) ES solution.

The LEMS measurement of cytochrome *c* prepared in solutions with pH of 7.0, 2.6, and 2.3 vaporized into ES droplets containing TEAA (Supplementary Figure S3d, e, and f) are very similar to the measurements made using TEAF. TEAA displays, however, enhanced charge reduction for the unfolded distribution in comparison with TEAF in the case of cytochrome *c*.

Cytochrome *c* solutions with pH of 7.0, 2.6, and 2.3 were laser-vaporized into ES droplets containing TEAB to measure the subsequent charge reduction and protein folding as a function of pH. LEMS measurement of cytochrome *c* prepared in a solution with pH of 7.0 in ES consisting of TEAB revealed mainly 5+ and 4+ charge states (Supplementary Figure S3g), with a Z_{avg} value of 4.7 ± 0.2 , indicating enhanced charge reduction in comparison with ammonium formate (Z_{avg} of 7.0 ± 0.1), ammonium acetate (Z_{avg} of 6.9 ± 0.1), ammonium bicarbonate (Z_{avg} of 7.7 ± 0.4), and aqueous (Z_{avg} of 8.5 ± 0.2) ES solvent. As the pH of solution containing cytochrome *c* is decreased to 2.6, a bimodal CSD is observed, peaked at 5+ and 7+ (Supplementary Figure S3h), indicating the presence of at least two conformations of the protein. The low charge states ranging from 4+ to 6+ contain $37 \pm 4\%$ of the cytochrome *c* ion intensity. The LEMS measurement of cytochrome *c* prepared in a solution with pH of 2.3 revealed a monomodal CSD distribution peaked at 7+ (Supplementary Figure S3i) indicating substantial unfolding of the protein. The reduction in Z_{avg} observed for cytochrome *c* (pH of 2.3) in the presence of triethyl ammonium bicarbonate (Z_{avg} of 7.1 ± 0.1) in comparison with ammonium bicarbonate (Z_{avg} of 13.1 ± 0.1) and aqueous ES solution (Z_{avg} of 14.1 ± 0.1) is consistent with the notion that the charge reduction scales with the gas-phase basicity of their neutral conjugates, i.e., the gas phase-basicity of triethyl amine is $221 \text{ Kcal mol}^{-1}$, ammonia is $193 \text{ Kcal mol}^{-1}$, and water is $167 \text{ Kcal mol}^{-1}$.

Analysis of Myoglobin in Conventional Versus Charge Reducing Solution Additives

Conventional Electrospray Solvents Holomyoglobin in the native state contains a heme group in the interior of the protein. The heme is released in conditions of extreme pH, high temperatures, and in the presence of organic solvents to produce apomyoglobin [35]. Here we seek to investigate whether the solution-phase conformation (holomyoglobin) is preserved upon the transfer into the gas phase, and whether the acid-denatured myoglobin can refold in the charged electrospray droplets with heme reincorporation into its hydrophobic pocket. Laser vaporization is used to transfer both the native (holomyoglobin) and acid-denatured myoglobin

(apomyoglobin) and heme into electrospray droplets containing various solution additives.

To determine the charge state distribution as a function of acid concentration, solutions of myoglobin with pH of 7.0, 2.6, and 2.3, were laser vaporized into aqueous ES droplets and the resulting CSDs were measured. The LEMS measurement of myoglobin prepared in solution with pH 7.0 in aqueous ES reveals a broad CSD ranging from 7⁺ to 15⁺ and is peaked at 12⁺ with both holo (~86%) and apo (~14%) myoglobin features (Fig. 2a). The Z_{avg} values calculated for these measurements are 11.8 ± 0.3 and 11.2 ± 0.2 for holomyoglobin and apomyoglobin, respectively, as listed in Table 1. Acid-denatured myoglobin was laser-vaporized and the LEMS measurements reveal exclusively apomyoglobin features with broad, monomodal CSDs peaked at 22⁺ and 23⁺ for solution pH of 2.6 and 2.3, respectively (Fig. 2b and Supplementary Figure S4a). The apomyoglobin features observed at higher charge states indicate the presence of unfolded states of the protein. The lower charge states ranging from 10⁺ to 13⁺ contain $3 \pm 2\%$ and $2 \pm 1\%$ of the apomyoglobin ion intensity for solution with pH 2.6 and 2.3, respectively (Table 1).

The effect of ES solution containing ammonium formate on the CSD of myoglobin in the folded or unfolded state was measured by adjusting the pH of the solution vaporized. The LEMS measurement of myoglobin prepared in a solution with pH 7.0 vaporized into ES droplets containing ammonium formate (pH 6.2) reveals mainly 8⁺ and 9⁺ charge states, corresponding to predominately holomyoglobin features (Fig. 2c)

with a Z_{avg} value of 8.2 ± 0.1 , indicating both charge reduction of the folded state of the protein in comparison with aqueous (Z_{avg} of 11.8 ± 0.3) ES solvent and that the ammonium formate maintains the heme co-factor in the hydrophobic pocket in comparison with aqueous ES solvent where ~15% of the protein is found in apomyoglobin form. This measurement suggests that the formation of apomyoglobin in the case of vaporization of myoglobin (solution pH of 7.0) into the aqueous ES solution occurs after the laser-vaporized protein interacts with the aqueous ES droplets. An alternative explanation is that apomyoglobin is vaporized but incorporates heme in the spray containing ammonium formate.

Myoglobin in a solution with pH 2.6 was vaporized into ES droplets containing ammonium formate to determine whether apomyoglobin can convert to holomyoglobin within the electrospray process. The mass spectrum revealed ~100% apomyoglobin features with bimodal CSDs, peaked at 9⁺ and 15⁺, ranging from 7⁺ to 24⁺ (Fig. 2d). This indicates that the time required for heme reincorporation into the hydrophobic pocket, upon the interaction with ammonium formate ES solvent, is longer than the LEMS measurement time transiting from the vaporization region into the vacuum of the mass spectrometer. A previous study suggested that the refolding of apomyoglobin to the globular holomyoglobin (with heme reincorporation into the hydrophobic pocket) takes hundreds of milliseconds to a second [36]. The time available during the LEMS analysis is estimated to be 100 ms [11], suggesting that there is insufficient time for heme reincorporation. The bimodal

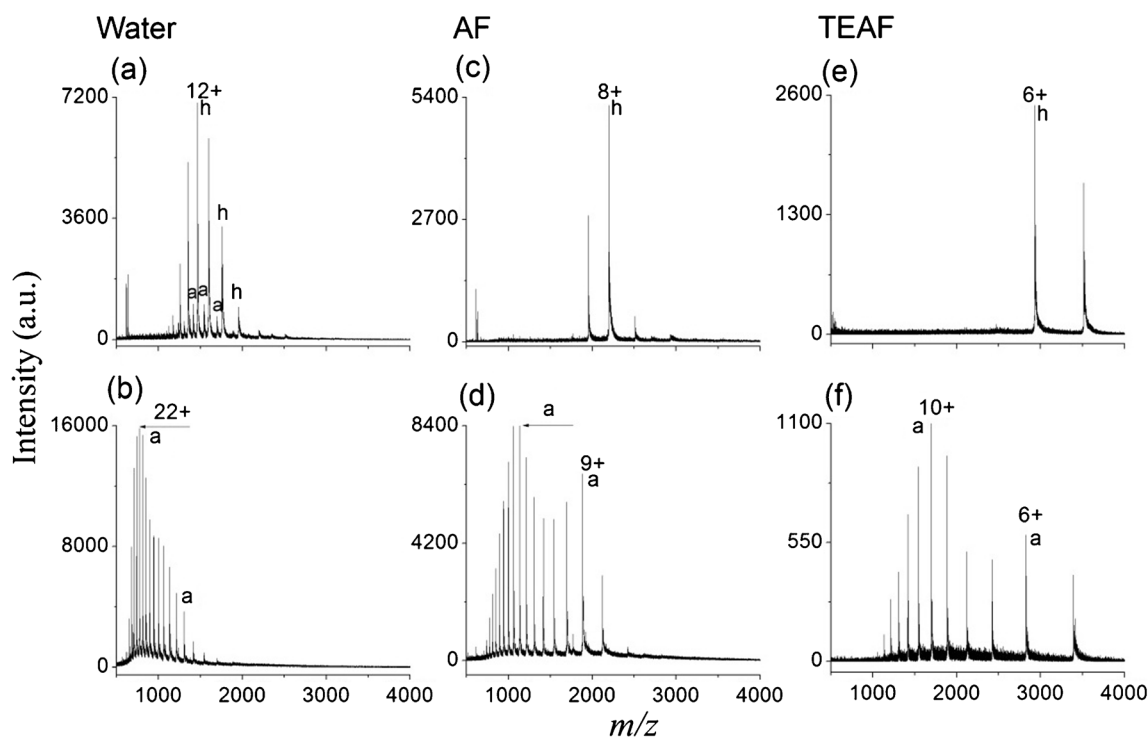


Figure 2. Representative LEMS mass spectra resulting from laser induced vaporization of myoglobin at pH 7.0 (panel a, c, and e), and myoglobin at pH 2.6 (panel b, d, and f) into the electrospray solvent consisting of water (pH 6.8), 5 mM ammonium formate (AF, pH 6.2), and 5 mM triethyl ammonium formate (TEAF, pH 6.1), respectively; ('h' represents holomyoglobin whereas 'a' represents apomyoglobin)

CSDs peaked at 9+ and 15+ suggest at least a fraction of apomyoglobin (charge states ranging from 7+ to 10+ containing $20 \pm 4\%$ of the apomyoglobin ion intensity) folds into a globular apomyoglobin conformer in the presence of the ammonium formate ES solvent. This is supported by the fact that vaporization of pH 2.6 protein solution into water only contained $3 \pm 2\%$ folded distribution. The time scale for refolding of apomyoglobin into globular apomyoglobin conformation is reported to be in the order of few microseconds [37], which is short compared with the 100 ms that the complex spends in the ES droplet. This experiment confirms that the formation of apomyoglobin occurs in the aqueous droplet for the pH 7.0 measurement and not during the vaporization process.

The LEMS measurement of myoglobin prepared in a solution with pH 2.3 vaporized into ES droplets containing ammonium formate revealed a monomodal CSD ranging from 7+ to 24+, peaked at 18+ (Supplementary Figure S4b). The charge states ranging from 7+ to 10+ contain $6 \pm 4\%$ of the apomyoglobin ion intensity while the majority of apomyoglobin features are observed at higher charge states ranging from 11+ to 24+, indicating unfolded states of the protein. The monomodal distribution suggests that the extent of charge reduction and the appearance of folded states depend either on the extent of protein unfolding prior to laser vaporization or on the pH of the ES droplet after mixing with the vaporized acidic protein solution. Similar to cytochrome *c*, ESI-MS measurements were performed as a control experiment for complete mixing of acid-denatured myoglobin with similar solution additives (e.g., ammonium formate and triethyl ammonium formate) utilized in the LEMS measurement at 1:1 ratio. The mass spectra, Z_{avg} , and the fraction folded protein obtained from these measurements are reported in Supplementary Figure S5 and Supplementary Table S1.

The LEMS measurement of myoglobin prepared in solutions with pH of 7.0, 2.6, and 2.3 vaporized into ES droplets containing ammonium acetate (Supplementary Figure S6a, b, and c) are very similar to the measurements made using ammonium formate. Similar to the measurements for cytochrome *c*, the LEMS measurements of myoglobin at lower solution pH (pH of 2.6 and 2.3) in ES solution containing ammonium bicarbonate did not result in charge reduction when compared with ammonium formate and ammonium acetate despite the higher solution pH, which is probably due to the decomposition of HCO_3^- (counter ions) to CO_2 and H_2O in the presence of acid, creating bubbles that facilitate protein unfolding.

Solution Additives with High Gas-Phase Basicity Myoglobin prepared in solutions with pH of 7.0, 2.6, and 2.3 were laser-vaporized into solution additives with gas-phase basicity to measure the subsequent charge reduction and protein folding in ES droplets. The LEMS measurement of myoglobin prepared in a solution with pH of 7.0 vaporized into ES droplets containing TEAF (solution pH of 6.1) reveals mainly 6+ and 5+ charge states (holomyoglobin features, Fig. 2e) with a Z_{avg} value of 5.7 ± 0.0 , indicating enhanced charge reduction of the

folded state of the protein in comparison with ammonium formate (Z_{avg} of 8.2 ± 0.1), ammonium acetate (Z_{avg} of 8.0 ± 0.1), ammonium bicarbonate (Z_{avg} of 8.2 ± 0.3), and aqueous (Z_{avg} of 11.8 ± 0.3) ES solvent. Laser vaporization of myoglobin prepared in solutions with pH of 2.6 and 2.3 into the same ES solvent revealed bimodal CSDs with most probable charge states at 6+ and 10+ with exclusively apomyoglobin features, as shown in Fig. 2f, and Supplementary Figure S4c. The charge states ranging from 5+ to 7+ contain $22 \pm 4\%$ and $19 \pm 3\%$ of the apomyoglobin ion intensity for solutions with pH of 2.6 and 2.3, respectively. The lower charge states peaked at 6+ indicate charge reduction, presumably of the globular conformer of apomyoglobin. The distribution peaked at 10+ likely corresponds to the charge reduction of unfolded states of the protein. The fraction of globular apo-myoglobin, calculated for myoglobin prepared in a solution with pH of 2.6 using TEAF ($22 \pm 4\%$) is greater in comparison with aqueous ES ($3 \pm 1\%$), and is within the standard deviation in comparison with ammonium acetate ($19 \pm 6\%$) and ammonium formate ($20 \pm 4\%$) ES solutions. This again suggests that the use of solution additive with high gas-phase basicity promotes charge reduction, and presumably results in some degree of protein folding within the ES droplets. The reduction in Z_{avg} observed for myoglobin (pH of 2.3) in the presence of TEAF (Z_{avg} of 9.8 ± 0.2) in comparison with ammonium formate solution (Z_{avg} of 16.4 ± 0.1) is consistent with the hypothesis that the solution additives with high gas-phase basicity can significantly reduce the protein CSD in the gas phase. The lack of heme inclusion to the protein again suggests that a refolding of apomyoglobin to holomyoglobin is limited by the time available for protein folding in LEMS measurement.

The LEMS measurement of myoglobin prepared in solutions with pH 7.0, 2.6, and 2.3 vaporized into ES droplets containing TEAA (Supplementary Figure S6 g, h, and i) are similar to the measurements made using TEAF. TEAA displays enhanced charge reduction for the myoglobin prepared in solution pH of 2.6 and 2.3 in comparison with TEAF.

Laser vaporization of myoglobin prepared in a solution with pH 7.0 into the ES droplets containing TEAB revealed mainly 6+ and 5+ charge states corresponding to holomyoglobin features (Supplementary Figure S6j) compared with 8+ and 9+ for ammonium bicarbonate solution, indicating enhanced charge reduction of the folded states of the protein. The LEMS measurement of myoglobin prepared in solutions with pH of 2.6 and 2.3 vaporized into the ES droplets containing TEAB revealed monomodal CSDs peaked at 10+ (apomyoglobin features, Supplementary Figure S6k and l) with Z_{avg} values of 9.8 ± 0.2 and 10.2 ± 0.2 , respectively. The monomodal CSD suggests substantial unfolding of the protein. A reduction in Z_{avg} was observed for myoglobin prepared in solution with pH of 2.3 in the presence of TEAB (Z_{avg} of 10.0 ± 0.2) compared with ammonium bicarbonate (Z_{avg} of 18.6 ± 0.1) and aqueous ES (Z_{avg} of 20.5 ± 0.1). This is again consistent with the ordering of the gas-phase basicities.

In addition to the shift in protein CSD, adduction of Na^+ and HCO_3^- ions to the protein ion was also observed for lower

charge states (mainly 6+ and 5+), as seen in the inset of Supplementary Figure S6j–l. The enhanced adduction is due to the lower collision energy with background gas at lower charge state in comparison with higher charge states. The peak broadening due to adduct formation was significantly reduced upon increasing the collision energy to 35 eV compared with 10 eV, suggesting the loss of an anion (HCO_3^-) and neutral (NaHCO_3) molecule from protein ion (Supplementary Figure S7d–f and Supplementary Figure S8b).

Conclusions

The use of solution additives with high gas-phase basicities resulted in greater shift in the protein CSD to the lower charge states for native and acid-denatured proteins in comparison with both aqueous and conventional solvent system. This suggests that the charge reduction, which is expected to occur in the gas phase, is dominated by proton transfer processes from multiply charged protein ions to neutral amines. We note that in addition to the charge state distribution, coupling LEMS with hydrogen/deuterium exchange, ion mobility measurement, and/or electron capture dissociation would provide alternative means of investigating the gas-phase protein conformation. We have investigated the reduction of 2,6-dichloroindophenol by L-ascorbic acid in a manner similar to previous studies [38, 39], to determine the droplet lifetime in LEMS measurement. We find that the droplet lifetime is ~ 5 ms [40], suggesting that LEMS could be used to decouple proteins and electrospray solvent systems, prior to mixing in the charged electrospray droplets to study protein folding on the millisecond timescale.

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