Published in final edited form as:

J Am Soc Mass Spectrom. 2017 October ; 28(10): 2001–2010. doi:10.1007/s13361-017-1732-x.

Joule heating and thermal denaturation of proteins in nano-ESI theta tips

Feifei Zhao, Sarah M. Matt, Jiexun Bu, Owen G. Rehrauer, Dor Ben-Amotz, and Scott A. McLuckey*

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907-2084
United States

Abstract

Electroosmotically-induced Joule heating in theta tips and its effect on protein denaturation were investigated. Myoglobin, equine cytochrome *c*, bovine cytochrome *c* and carbonic anhydrase II solutions were subjected to electroosmosis in a theta tip and all of the proteins were denatured during the process. The extent of protein denaturation was found to increase with the applied square wave voltage and electrolyte concentration. The solution temperature at the end of a theta tip was measured directly by Raman spectroscopy and shown to increase with the square wave voltage, thereby demonstrating the effect of Joule heating through an independent method. The electroosmosis of a solution comprised of myoglobin, bovine cytochrome *c*, and ubiquitin demonstrated that the magnitude of Joule heating that causes protein denaturation is positively correlated with protein melting temperature. This allows for a quick determination of a protein's relative thermal stability. This work establishes a fast, novel method for protein conformation manipulation prior to MS analysis and provides a temperature-controllable platform for the study of processes that take place in solution with direct coupling to mass spectrometry.

Graphical abstract

*Address reprint requests to: Dr. Scott A. McLuckey, 560 Oval Drive, Department of Chemistry, Purdue University, West Lafayette, IN 47907-2084, USA, Phone: (765) 494-5270, Fax: (765) 494-0239, mcluckey@purdue.edu.

Keywords

Joule heating; theta tip; electroosmosis; protein denaturation

Introduction

Nano-electrospray ionization (nESI) is used to generate gaseous ions of biomolecules such as proteins, carbohydrates, lipids, *et cetera*. [1, 2] Proteins normally result in multiply charged ions when subjected to electrospray ionization and charge state distributions are related to protein conformation. It is generally accepted, for example, the magnitude of charges is relatively high for unfolded conformations, which are described as high charge state distributions, while more folded conformations usually display relatively low charge state distributions. [3, 4, 5] For this reason, charge state distributions have been used in a biophysical context to monitor protein conformations using mass spectrometry. The magnitude of protein ion charge also has analytical implications. For example, high charge state ions are more efficiently detected by charge sensitive detectors like those used by Fourier transform based mass analyzers. [6] Furthermore, increasing the charge state of a protein ion can lead to improved sequence coverage in top-down analysis, [7, 8, 9] especially when electron transfer dissociation (ETD) and electron capture dissociation (ECD) are used as dissociation methods. [10] Therefore, in-source protein denaturation can be desirable for the primary structural characterization of a protein via tandem mass spectrometry.

Tertiary and quaternary protein structures are stabilized by various interactions including salt bridges, hydrogen bonding, hydrophobic interactions and van der Waals interactions. [11,12] These interactions can be affected by a variety of factors including temperature, [4, 13, 14, 15] pH, [16, 17, 18] ionic strength, [19] solvent, [20, 21, 22] surface effects, [23] as well as

instrumental parameters[24]. Most methods intended to change protein conformation involve bulk solution manipulations, such as the addition of acid, base, organic solvent, supercharging reagents[14] or other additives, as well as heating. These methods can be time consuming and require larger sample volumes. In recent years, fast conformation manipulation methods in conjunction with ESI have been developed, including vapor exposure [25, 26, 27], electrothermal denaturation[24, 28], and theta tip mixing. [29, 30, 31, 32] During vapor exposure, the ESI droplets containing the protein are allowed to interact with acidic or basic vapors added to the nitrogen curtain gas, leading to protein denaturation or refolding on the basis of pH changes. [25, 26] The electrothermal supercharging method manipulates protein conformation by changing the ionization voltage. [24] It has been reasoned that by applying a high spray voltage the droplet size is increased, thereby elongating its lifetime in the hot capillary interface and maximizing the thermal denaturation of the protein in the droplet.

Theta tips are nESI dual channel emitters that also function as micro-mixers prior to the ionization step. [29] They are pulled from theta capillaries made of borosilicate glass that contain a septum in the center dividing a capillary into two separate channels into which different solutions can be loaded. A platinum wire is placed in each channel to apply spraying and mixing voltages. By applying the same ESI voltage to both channels, the solutions are sprayed out simultaneously and subsequently mix in the Taylor cone as well as in the ensuing droplets on a sub-millisecond time scale. [29, 33] This method has been applied to study protein unfolding and folding by mixing protein solutions with acid or ammonium acetate in the theta tip Taylor cone and droplets. [29, 30, 32] Due to the short mixing time, short-lived unfolding intermediates have been observed. [29] A more recent study has shown that electroosmotic flow can be induced between channels of a theta tip when applying differential voltages in the two channels. [31] The duration and extent of mixing can be controlled by tuning the applied voltage and time of electroosmosis. The solution phase mixing overcomes the reagent volatility limitation in the vapor exposure strategy [25, 27] and does not require a special mass spectrometric interface set up. However, the mixing step can alter the protein solution pH and composition if the two sides are mixed with dissimilar solutions. This is unfavorable for reagent or pH-sensitive studies like covalent modification and HDX[34]. Here we demonstrate thermal denaturation of proteins in theta tips via Joule heating, which can be a useful way to manipulate protein conformation without altering solution composition.

Joule heating, also known as resistive or ohmic heating, arises from an electrical current passing through a conductor or semi-conductor. It is widely used in various research areas including, for example, melting point measurements, [35] controlling thermosensitive polymer behavior, [36] and facilitating chemical reactions. [37] Joule heating in electrophoretic separation has been well-studied as it has been shown to reduce separation efficiency. [38] The magnitude of the temperature change due to Joule heating in a solution is related *inter alia* to voltage, molar conductivity, and concentration via the following relationship:

$$\Delta T \sim V^2 \Lambda c \quad (1)$$

where ΔT is the temperature change ($^{\circ}\text{C}$), V is the voltage (V), Λ is the molar conductivity ($\text{S}\cdot\text{m}^2\cdot\text{mol}^{-1}$) of the electrolyte and c is the electrolyte concentration ($\text{mol}\cdot\text{L}^{-1}$). Other factors that affect the temperature change include geometric considerations, such as the radius at the tip, glass thickness, heat dissipation, etc. [38, 39] The small size of the theta tip, and the associated large resistance, implies that Joule heating is expected to produce a substantial temperature rise near the apex of a theta tip (the upper limit of which may be roughly estimated as described in the SI). Herein, we demonstrate Joule heating in a theta tip resulting from electroosmosis and take advantage of the effect to thermally denature proteins. The solution temperature was directly measured by Raman spectroscopy to establish a relationship between voltage and temperature. The influence of voltage and electrolyte concentration on the magnitude of Joule heating were investigated.

Experimental Section

Materials

Myoglobin from equine skeletal muscle, cytochrome *c* from bovine heart, cytochrome *c* from equine heart, ubiquitin from bovine erythrocytes, carbonic anhydrase II from bovine erythrocytes, and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade water was purchased from Fisher Scientific (Fair Lawn, NJ). All protein were dissolved in 0, 5 or 10 mM ammonium acetate solution at pH around 6 and the final protein concentration is 5–20 μM unless specifically noted. Proteins and chemical reagents were used without further purification.

Capillaries and tip holder

Dual channel borosilicate theta capillaries (1.5 mm O.D., 1.17 mm I.D., 0.165 mm septum thickness, 10 cm length) were purchased from Sutter Instrument Co. (Novato, CA). Theta capillaries were pulled to theta tips (O.D. 10 μm) using a Flaming/Brown micropipette puller (P-87) from Sutter Instrument Co. (Novato, CA).

Solutions were loaded into both channels of a theta tip, which was held by a theta tip holder from Warner Instruments, LLC (Hamden, CT), pictured in Supplemental Figure S-1. The original silver wires in the holder were replaced with Teflon coated platinum wires (A-M Systems, Sequim, WA) to avoid discharge between the wires at the back of the theta capillary when voltages applied to the wires were different. The two wires were inserted into each channel of a theta tip to apply voltage to each side independently.

Mass Spectrometry

A quadrupole/time-of-flight (QqTOF) tandem mass spectrometer (QStar Pulsar XL, Sciex, Concord, ON, Canada) was used to perform all mass spectrometric experiments. The experimental procedure consists of four steps: electroosmosis, ionization, dump spray, and mass analysis. In the electroosmosis step the protein solution was electrically pumped back and forth between the two channels by grounding the wire in one theta tip channel while applying 100 ms of 10 Hz square wave voltage to the wire in the opposite channel. The square wave duty cycle is 50% and the voltage is ± 100 V to ± 500 V, where “+/-” was used to indicate the switch between positive and negative voltages during an electroosmotic

cycle. Next, the ionization step was triggered (1500 V on both wires, 80 ms), during which the ions are accumulated in Q2. The dump spray step was then triggered to spray out any residual analyte that had been exposed to the electroosmosis step. For this purpose, a dump spray step of 200 ms was found sufficient to return the mass spectrum to that of the pre-osmosis step. The ion path voltages were set such that no ions were accumulated in Q2 during the dump spray step. Finally, the mass spectrum was recorded during the 150 ms mass analysis step. The power supplies and detailed trigger system is summarized in Supplemental Figure S-2.

Raman temperature measurements

Temperature measurements within the theta tip were conducted off-line using a set-up to simulate the theta tip arrangement in front of the mass spectrometer. To measure the solution temperature via Raman spectroscopy, the timing of the voltages applied to the wires in the theta tip was designed to simulate the various steps described above for the mass spectrometry experiments. The detailed triggering method is shown in Supplemental Figure S-3.

The temperature of the fluid near the apex of the theta tip was obtained non-invasively using Raman spectroscopy. The micro-Raman spectra were measured using a custom-built instrument which includes a 532 nm laser excitation (Coherent Sapphire SF CDRH 532 nm) and a TE-cooled CCD (Princeton Instruments SP2300). A 100x objective (Olympus LM Plan Fl) with a working distance of 3.4 mm was used to both focus the laser and collect the backscattered Raman signal. Laser power at the sample was set to 24.5 mW. Fine positional control was accomplished with a motorized microscope stage (Prior H101A/C). For the Raman experiments, both channels of the theta tip were filled with 5 mM ammonium acetate. Consecutive spectra with 100 ms exposure time were acquired while continuously cycling through the process of electroosmosis-spray-simulated mass analysis. To obtain the training spectra for temperature calibration, a Pyrex 9530-3 borosilicate glass capillary (1.5–1.8 mm O.D., 90 mm length) was filled with 18.2 MΩ cm ultrapure water and heated using a Physitemp TS-4MPER thermal stage to temperatures between 20 °C and 90 °C, measured using a needle thermocouple (Physitemp MT-26/4 with an Omega DP701 reader). The temperature training spectra were collected with an integration time of 5 minutes per spectrum. Two representative training spectra corresponding to 30.3 °C and 74.3 °C are shown in Figure 5 insert (b).

The shape and intensity of the OH stretching mode of water is highly temperature dependent and has been used in the past for Raman thermometry. For example, D'Arrigo et al. calibrated temperatures based on a ratio of OH stretch areas with respect to measured temperature values from a thermocouple. [40] These areas were based on an approximate isosbestic point near 3400 cm⁻¹, and the calibrated value was the ratio of the OH area to the left and to the right of that point. We employed an alternative hyperspectral procedure using self-modeling curve resolution (SMCR) to decompose the OH stretch into the two primary spectral components such that each spectrum is a linear combination of those two components. Each spectrum was first baseline subtracted in the OH stretch region using a quadratic fit to user-defined points in the baseline on either side of the OH band. Next, using

the baseline subtracted training spectra, a quadratic calibration curve relating measured temperature to a parameter representing the fractional spectral weight of the high temperature component in each measured spectrum was generated. Then for each experimental spectrum, a total least squares fit of the measured spectrum to the two SMCR components was used to quantify the fractional weight of the high temperature component, which was in turn converted to temperature using the training calibration curve. Two of these experimental spectra and their corresponding temperature values are shown in Figure 5 insert (b).

The Raman measurements were obtained asynchronously at a frame rate of about 6 fps, and subsequently synchronized with the applied voltage cycles to create plots similar to Figure 5 insert (a). Each curve of Figure 5 insert (a) includes 400 Raman measured temperature data points and approximately 110 cycles of electroosmosis-spray-simulated mass analysis process.

Results and Discussion

Electroosmosis induced protein denaturation

Bovine carbonic anhydrase II, a 29 kDa protein reported to have a melting point of 64 °C, [41] has been the subject of folding/denaturation studies under a variety of conditions and several conformational states have been noted. [42, 43, 44] Mass spectrometry studies of conventional pH induced unfolding of carbonic anhydrase II was also reported. [29] In its native state (i.e., the holo-carbonic anhydrase II (hCA II) form), a Zn^{2+} co-factor is present, [45] although the presence of Zn^{2+} has not been observed to be key to folding of this protein. When hCA II dissolved in a 5 mM NH_4OAc aqueous solution was subjected to nESI from a theta tip without an electroosmosis step, a narrow charge state distributions centered at +11 was observed containing the Zn^{2+} co-factor (Figure 1(a)). 100 ms of a 10 Hz square wave at ± 200 V resulted in the generation of higher charge states of hCA II (Figure 1(b)) with the +15 charge state being most abundant of the newly apparent charge states. The ± 200 V square wave with 50% duty cycle induced a bidirectional electroosmosis, which suppressed the bulk motion of the solutions from one channel to the other. Therefore, the small amount of denatured protein subjected to electroosmosis can be cleared up by applying a dump spray voltage for 200 ms to regain the pre-osmosis spectrum. At ± 230 V, a more extensive shift in charge states of hCA II was noted, along with low levels of apo-carbonic anhydrase II (aCA II) ions over a wide range of charge states (Figure 1(c)). In this case, the +18 charge state was most abundant of the higher charge states. The abundance pattern of the higher charge states is also suggestive of the presence of several charge state distributions. In any case, the data of Figure 1 clearly suggest that protein denaturation can take place upon electroosmosis in the theta tip and that the extent of denaturation increases with the square wave voltage.

Myoglobin is another extensively studied globular protein and has a reported melting temperature of 76 °C at neutral pH. [46] In its native state, myoglobin contains a non-covalently-bound heme ligand. It is referred to as holo-myoglobin (hMb) when the heme group is present and apo-myoglobin (aMb) when it is absent. When exposed to heat, hMb undergoes stepwise unfolding through a series of intermediates. [47] The initial stage of

unfolding involves a slight extension of the tertiary structure while preserving the heme ligand. In the following phase, a dramatic tertiary structure alteration occurs resulting in the loss of the heme ligand and generation of unfolded aMb. Further heating of the protein may lead to polymerization of aMb before precipitation. Accumulated free heme ligand can also polymerize or nonspecifically attach to aMb and hMb. [48] The inserts of Figure 2 show a selection of nESI spectra obtained as a function of square wave voltage in a theta tip. Both channels of the theta tip contained a myoglobin solution in 5 mM NH₄OAc solution. Figure 2(a) shows the spectrum of myoglobin sprayed after applying 100 ms of square wave at a voltage of ± 150 V. The spectrum, as well as those obtained at lower square wave voltages, is essentially identical to that obtained in the absence of electroosmosis (not shown) and clearly suggests that the native hMb conformation is preserved due to the retention of the heme group and the low charge state distributions centered at +8. The insert of Figure 2(b) shows the spectrum obtained after 100 ms electroosmosis induced by a 10 Hz ± 230 V square wave. At this voltage, a portion of the hMb cation population lost the heme ligand to form aMb ions in two clearly apparent charge state distributions with maxima at +14 and +9, respectively, as shown by the open, green circles in Figure 2(b). With the square wave voltage increased to ± 300 V, the original hMb charge states were further depleted and the higher charge state aMb peaks grew in relative abundance (see the insert of Figure 2(c)). Some of the lost heme ligand was observed to attach to hMb to form a complex with two heme groups, as indicated by the blue triangles. The attachment of more than one heme group to denatured hMb has been noted in solution phase studies. [49, 50] The spectra of the inserts (b) and (c) show at least two distinct charge state distributions for aMb ions, which likely reflects distinct folding states of aMb. The plot of Figure 2 shows the percentage of aMb ion signal relative to total myoglobin ion signal (aMb+hMb ions) as a function of square wave voltage with the solid line representing a sigmoidal fit to the data. While this plot does not reflect the evolution of different folding states of aMb, the percentage of aMb ions provides an overall reflection of the extent of denaturation of the protein. If the aMb ions are taken as representing any unfolded state while the hMb ions are taken as representative of the native state, the plot of Figure 2 treats myoglobin as a two-state system (i.e., folded versus unfolded). A sigmoidal shape for the percentage of the unfolded state is as a function of denaturation condition (e.g., temperature, pH, concentration of denaturant, etc.) is expected for such a scenario[51].

Similar phenomena were noted with equine cytochrome *c* (eCyt *c*), which has a reported melting temperature of 85 °C. [52] This protein contains a covalently bound heme ligand, which remains bound to the protein upon denaturation. [53] Therefore, upon heating, eCyt *c* mainly undergoes tertiary structure extension, reflected by an increase in charge states in nESI mass spectra. In this case, we use the abundance weighted average charge state of the protein as a reflection of the extent of denaturation as a function of square wave voltage in the plot of Figure 3. These results were obtained from series of nESI mass spectra derived from eCyt *c* in an aqueous solution of 5 mM NH₄OAc as a function of square wave voltage. Inserts (a)–(c) show the mass spectra obtained using 100 ms of 10 Hz square wave at voltages of ± 290 V, ± 310 V, and ± 340 V, respectively.

Collectively, the results for these proteins showed that protein denaturation under conditions of electroosmosis in a theta tip at relatively high square wave voltages is a general

phenomenon. Furthermore, the extent of denaturation was found to be both condition dependent (e.g., the magnitude of the square wave voltage) and protein dependent (e.g., higher square wave voltages were required to denature proteins with higher melting temperatures). The results are consistent with thermal denaturation as a result of Joule heating at the end of the theta tip during electroosmosis.

Influence of ammonium acetate concentration on protein denaturation

Ammonium acetate is a commonly used additive in native mass spectrometry. [54] High concentration of ammonium acetate displaces nonvolatile adducts and reduces the nonvolatile components influence. [55] It also stabilizes the native conformation of proteins in solution through ionic specific interaction, which is normally referred to as Hofmeister Effects [56, 57] Besides, the protein-ligand dissociation constant also decreases with higher ammonium acetate concentration, when the protein's isoelectric point is higher than the pH of the solution. [58] The isoelectric point of myoglobin is 6.8–7.4, while the 5 mM ammonium acetate solution pH is around 6. At this pH, ammonium acetate enhances the retention of the heme in the myoglobin binding pocket. Since holo-myoglobin stability is determined by the heme ligand affinity, [59] the high binding affinity of the heme in ammonium acetate solution can further stabilize myoglobin.

Based solely on the considerations mentioned above, an increase in ammonium acetate concentration in the theta tip under electroosmosis conditions might be expected to inhibit the extent of denaturation. However, as demonstrated in Figure 4, the extent of denaturation increases with a doubling of ammonium acetate concentration. Figure 4(a) shows a mass spectrum of myoglobin obtained using deionized water (i.e., no added ammonium acetate) after 100 ms of electroosmosis at ± 230 V. The result is consistent with the native protein (i.e., low charge state distributions of hMb ions), suggesting that denaturation does not take place to a detectable extent under such solution conditions. Using the same square wave voltage, the addition of 5 mM NH_4OAc to the protein solution results in the spectrum in Figure 2(b), which shows clear evidence for protein denaturation via the appearance of two aMb distributions. The hMb low charge state distributions remains highly abundant, however, which indicates that much of the protein in solution sampled by the mass spectrometer remains in the native state. Figure 4(b) shows the spectrum obtained using 10 mM NH_4OAc where significant myoglobin denaturation during electroosmosis was observed. The bimodal distribution of aMb peaks became dominant with only a small amount of hMb peaks remaining. The excess heme ligand formed via electroosmosis is observed to form a non-specific complex with hMb indicating further denaturation. Electroosmosis using ± 200 V of bovine cytochrome *c* (bCyt *c*) in 5 mM and 10 mM NH_4OAc solution showed the same trend, where the 10 mM NH_4OAc solution gave rise to higher bCyt *c* denaturation (Supplemental Figure S-4). These results are consistent with an increase in Joule heating due to an increase in the conductivity of the solution with increasing electrolyte concentration (see Equation 1), which overcomes any stabilization effects that might otherwise arise with increasing ammonium acetate concentration. The effect of electrolyte concentration, in addition to the voltage effect described above, provides another indirect piece of evidence for Joule heating.

Temperature measurements using Raman spectroscopy

Protein denaturation can arise in a variety of ways and therefore provides only indirect evidence for Joule heating in a theta tip during electroosmosis. We therefore examined the temperature of the solution very near to the end of the tip as a function of operating conditions. The highest resistance to current flow is expected to be at the narrowest point of the channel, which is at the end of the tip, thus the Joule heating effect is the strongest at the end of the tip. It was therefore desirable to be able to measure temperature in a small volume at or near the end of the tip to minimize error associated with the bulk solution elsewhere in the theta tip. The measurement of Raman scattering from a tightly focused laser spot, estimated to be 8–15 fL with our system, provides the needed spatial resolution. To measure the solution temperature during each step (electroosmosis, spray and mass analysis), a voltage supply and triggering system was established to mimic the procedure used in the mass spectrometry experiments, as described in Supplemental, Figure S-3. A 5 mM NH_4OAc solution was loaded into both channels of the theta tip. The duration of electroosmosis, spray, and mass analysis steps were set to 100 ms, 300 ms and 200 ms, respectively, as in the MS experiment. A 10 Hz square wave was used to induce 100 ms of electroosmosis with voltage values from ± 100 V to ± 500 V. The solution temperature was measured during this process and the resultant temperatures are shown in Figure 5. During the electroosmosis step, the solution temperature increased from room temperature to a maximum temperature. When the square wave was completed, the solution temperature cooled down and reached the starting room temperature, as shown in Figure 5 insert (a).

Figure 5 shows how the maximum solution temperature obtained during the electroosmosis step is correlated to the applied square wave heating voltage. Based on the measured temperature, applying a ± 200 V square wave to one channel increased the solution temperature to about 44 °C, while ± 300 V voltage increased the solution temperature to 51 °C. Increasing the voltage amplitude to 500 V led to a maximum temperature at 77 °C. The small size of the theta tip, and the associated large resistance, implies that Joule heating is expected to produce a substantial temperature rise near the apex of a theta tip (whose magnitude may be roughly estimated as described in the SI), although heat dissipation exists in the open system. The Raman laser measuring point is about 8 μm away from the end of the tip and the true maximum temperature at the tip apex may also be underestimated. Nevertheless, the Raman measurements directly show that the electroosmosis process gives rise to an increase in the solvent temperature that is directly related to the square wave voltage applied to induce electroosmosis.

Correlation between protein melting temperature and denaturation voltage

The melting temperature of a protein is a measure of its thermal stability towards denaturation and several reports have employed heated ESI or nESI emitters to examine thermal denaturation of proteins and protein complexes. [60, 61, 62] Therefore, the onset and extent of protein denaturation might be expected to correlate with theta tip heating voltage. Indeed, the extent of denaturation observed for the three proteins discussed above (viz., bovine CA II, myoglobin, and eCyt c) are consistent with this expectation. That is, the protein with the lowest reported melting temperature (CA II) showed extensive denaturation at the lowest square wave voltages and the protein with the highest reported melting

temperature (eCyt *c*) required the greatest square wave voltages to lead to extensive denaturation. A more reliable comparison, however, can be made with a mixture of proteins such that all experiments are conducted with the same theta tip and solution conditions, thereby ensuring that each protein is exposed to the same extent of Joule heating. To study the correlation between protein melting temperature and heating voltage, a 5 mM NH₄OAc solution containing myoglobin (melting temperature of 76 °C [46]) bovine cytochrome *c* (melting temperature of 80 °C [63]) and ubiquitin (melting temperature of 100 °C [64]) was subjected to electroosmosis in a theta tip. Since the ionization efficiencies of these three proteins are different, the concentrations of myoglobin, cytochrome *c* and ubiquitin in the mixture were adjusted to 0.11, 0.07 and 0.02 mg/mL, respectively. Figure 6(a) shows the spectrum obtained when the protein mixture was subjected to 100 ms of electroosmosis using a 10 Hz $\pm$ 200 V square wave. No change in the mass spectrum was noted relative to the spectrum obtained without electroosmosis (not shown) suggesting that none of the proteins underwent measurable denaturation. Myoglobin showed signs of denaturation (viz., the appearance of aMb ions of relatively high charge states) using a $\pm$ 230 V square wave for heating (Figure 6(b)), while the bCyt *c* and ubiquitin ions remain unchanged at this voltage. The first sign of the denaturation of bCyt *c*, as reflected by the appearance of a higher charge state distributions, is observed at $\pm$ 250 V (Figure 6(c)). The abundances of the higher charge state distributions of myoglobin and bCyt *c* were observed to increase further at $\pm$ 300 V (Figure 6(d)) and $\pm$ 500 V (Figure 6(e)). Ubiquitin, which has the highest melting temperature in the mixture, showed no charge state distribution change until $\pm$ 500 V square wave voltage was applied. Figure 6(e) shows a modest charge state shift from +5 to +6 at $\pm$ 500 V heating which suggests that the ubiquitin tertiary structure might be perturbed under these conditions. Overall, these results are fully consistent with an increase in solution temperature with increasing square wave heating voltage.

Conclusions

In this report, we demonstrate protein denaturation resulting from electroosmosis in a theta tip nano-ESI capillary. The effect is shown to arise from Joule heating via both direct and indirect evidence. Indirect evidence included an increase in the extent of protein denaturation with the magnitude of the voltage of a square wave used to effect electroosmosis. This effect was demonstrated for myoglobin, equine cytochrome *c*, and carbonic anhydrase II solutions. Joule heating is expected to increase with field strength. An increase in the extent of denaturation for myoglobin was also observed with an increase in the ammonium acetate concentration. Joule heating is expected to increase with solution conductivity. Using Raman spectroscopy temperature measurements near to the capillary tip, an increase in solution temperature, direct evidence for Joule heating, was found to correlate with the amplitude of the square wave voltage. Electroosmosis-induced Joule heating was observed to be positively correlated to protein melting temperature when a solution of a mixture of proteins of known melting temperature was subjected to a series of experiments with increasing square wave heating voltage. This work points to the development of a convenient and efficient way to modulate solution temperature in a nano-ESI theta tip prior to spraying into a mass spectrometer. It represents a flexible approach for controlled protein denaturation that does not depend on changes in solution additives or solvent composition.

With further development, this effect may serve as the basis for a method to study protein thermal stabilities on small quantities of materials and with mixtures of proteins. Given the ability to alter temperatures in a pulsed fashion on the time-scales of tenths of seconds, this effect may also prove to be useful in studying protein unfolding and refolding dynamics on such a time-scale.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health under Grant GM R37-45372. Support for F. Z. was provided by a W. Brooks Fortune Fellowship in Analytical Chemistry.

References

1. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray Ionization for Mass Spectrometry of Large Biomolecules. *Science*. 1989; 246:64–71. [PubMed: 2675315]
2. Heck AJR. Native Mass Spectrometry: a Bridge between Interactomics and Structural Biology. *Nat. Methods*. 2008; 5:927–933. [PubMed: 18974734]
3. Kaltashov IA, Eyles SJ. Studies of Biomolecular Conformations and Conformational Dynamics by Mass Spectrometry. *Mass Spectrom. Rev.* 2002; 21:37–71. [PubMed: 12210613]
4. Liu J, Konermann L. Irreversible Thermal Denaturation of Cytochrome *c* Studied by Electrospray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2009; 20:819–828. [PubMed: 19200750]
5. Kaltashov IA, Abzalimov RR. Do Ionic Charges in ESI MS Provide Useful Information on Macromolecular Structure. *J. Am. Soc. Mass Spectrom.* 2008; 19:1239–1246. [PubMed: 18602274]
6. Cassou CA, Williams ER. Anions in Electrothermal Supercharging of Proteins with Electrospray Ionization Follow a Reverse Hofmeister Series. *Anal. Chem.* 2014; 86:1640–1647. [PubMed: 24410546]
7. Reid GE, Wu J, Chrisman PA, Wells JM, McLuckey SA. Charge-State-Dependent Sequence Analysis of Protonated Ubiquitin Ions via Ion Trap Tandem Mass Spectrometry. *Anal. Chem.* 2001; 73:3274–3281. [PubMed: 11476225]
8. Jockusch RA, Schnier PD, Price WD, Strittmatter EF, Demirev PA, Williams ER. Effects of Charge State on Fragmentation Pathways, Dynamics, and Activation Energies of Ubiquitin Ions Measured by Blackbody Infrared Radiative Dissociation. *Anal. Chem.* 1997; 69:1119–1126. [PubMed: 9075403]
9. Breuker K, Oh HB, Horn DM, Cerda BA, McLafferty FW. Detailed Unfolding and Folding of Gaseous Ubiquitin Ions Characterized by Electron Capture Dissociation. *J. Am. Chem. Soc.* 2002; 124:6407–6420. [PubMed: 12033872]
10. Zubarev RA, Kelleher NL, McLafferty FW. Electron Capture Dissociation of Multiply Charged Protein Cations. A Nonergodic Process. *J. Am. Chem. Soc.* 1998; 120:3265–3266.
11. Xu D, Tsai CJ, Nussinov R. Hydrogen Bonds and Salt Bridges across Protein-Protein Interfaces. *Protein Eng.* 1997; 10:999–1012. [PubMed: 9464564]
12. Kumar S, Nussinov R. Close-Range Electrostatic Interactions in Proteins. *ChemBioChem.* 2002; 3:604–617. [PubMed: 12324994]
13. Mirza UA, Cohen SL, Chait BT. Heat-Induced Conformational Changes in Proteins Studied by Electrospray Ionization Mass Spectrometry. *Anal. Chem.* 1993; 65:1–6. [PubMed: 8380538]
14. Sterling HJ, Williams ER. Origin of Supercharging in Electrospray Ionization of Noncovalent Complexes from Aqueous Solution. *J. Am. Soc. Mass Spectrom.* 2009; 20:1933–1943. [PubMed: 19682923]

15. Gratacós-Cubarsí M, Lametsch R. Determination of Changes in Protein Conformation Caused by pH and Temperature. *Meat Sci.* 2008; 80:545–549. [PubMed: 22063364]
16. Russo N, Estrin D, Martí M, Roitberg A. pH-Dependent Conformational Changes in Proteins and Their Effect on Experimental pK_as: The Case of Nitrophorin 4. *PLoS Comput. Biol.* 2012; 8:e1002761. [PubMed: 23133364]
17. Park Y, Kim K, Lim D, Lee EK. Effects of pH and Protein Conformation on in-Solution Complexation between Bovine α -Lactalbumin and Oleic Acid: Binding Trend Analysis by Using SPR and ITC. *Process Biochem.* 2015; 50:1379–1387.
18. Thakur G, Jiang K, Lee D, Prashanthi K, Kim S, Thundat T. Investigation of pH-Induced Protein Conformation Changes by Nanomechanical Deflection. *Langmuir.* 2014; 30:2109–2116. [PubMed: 24512545]
19. Ohyashiki T, Taka M, Mohri T. The Effects of Ionic Strength on the Protein Conformation and the Fluidity of Porcine Intestinal Brush Border Membranes. *J. Biol. Chem.* 1985; 260:6857–6861. [PubMed: 3997850]
20. Iavarone AT, Jurchen JC, Williams ER. Effects of Solvent on the Maximum Charge State and Charge State Distribution of Protein Ions Produced by Electrospray Ionization. *J. Am. Soc. Mass Spectrom.* 2000; 11:976–985. [PubMed: 11073261]
21. Nemethy G, Peer WJ, Scheraga HA. Effect of Protein-Solvent Interactions on Protein Conformation. *Annu. Rev. Biophys. Bioeng.* 1981; 10:459–497. [PubMed: 7020582]
22. Yu Y, Wang J, Shao Q, Shi J, Zhu W. The Effects of Organic Solvents on the Folding Pathway and Associated Thermodynamics of Proteins: a Microscopic View. *Sci. Rep.* 2016; 6:19500. [PubMed: 26775871]
23. Mortensen DN, Williams ER. Surface-Induced Protein Unfolding in Submicron Electrospray Emitters. *Anal. Chem.* 2016; 88:9662–9668. [PubMed: 27615434]
24. Sterling HJ, Cassou CA, Susa AC, Williams ER. Electrothermal Supercharging of Proteins in Native Electrospray Ionization. *Anal. Chem.* 2012; 84:3795–3801. [PubMed: 22409200]
25. Kharlamova A, Prentice BM, Huang T, McLuckey SA. Electrospray Droplet Exposure to Gaseous Acids for the Manipulation of Protein Charge State Distributions. *Anal. Chem.* 2010; 82:7422–7429. [PubMed: 20712348]
26. Kharlamova A, DeMuth JC, McLuckey SA. Vapor Treatment of Electrospray Droplets: Evidence for the Folding of Initially Denatured Proteins on the Sub-Millisecond Time-Scale. *J. Am. Soc. Mass Spectrom.* 2012; 23:88–101. [PubMed: 22016004]
27. Girod M, Antoine R, Dugourd P, Love C, Mordehai A, Stafford G. Basic Vapor Exposure for Tuning the Charge State Distribution of Proteins in Negative Electrospray Ionization: Elucidation of Mechanisms by Fluorescence Spectroscopy. *J. Am. Soc. Mass Spectrom.* 2012; 23:1221–1231. [PubMed: 22565506]
28. Mortensen DN, Williams ER. Electrothermal Supercharging of Proteins in Native MS: Effects of Protein Isoelectric Point, Buffer, and nanoESI-Emitter Tip Size. *Analyst.* 2016; 141:5598–5606. [PubMed: 27441318]
29. Fisher CM, Kharlamova A, McLuckey SA. Affecting Protein Charge State Distributions in Nano-Electrospray Ionization via in-Spray Solution Mixing Using Theta Capillaries. *Anal. Chem.* 2014; 86:4581–4588. [PubMed: 24702054]
30. Mortensen DN, Williams ER. Investigating Protein Folding and Unfolding in Electrospray Nanodrops upon Rapid Mixing Using Theta-Glass Emitters. *Anal. Chem.* 2015; 87:1281–1287. [PubMed: 25525976]
31. Fisher CM, Hilger RT, Zhao F, McLuckey SA. Electroosmotically Driven Solution Mixing in Borosilicate Theta Glass nESI Emitters. *J. Mass Spectrom.* 2015; 50:1063–1070. [PubMed: 28338258]
32. Mortensen DN, Williams ER. Ultrafast (1 μ s) Mixing and Fast Protein Folding in Nanodrops Monitored by Mass Spectrometry. *J. Am. Chem. Soc.* 2016; 138:3453–3460. [PubMed: 26902747]
33. Mortensen DN, Williams ER. Theta-Glass Capillaries in Electrospray Ionization: Rapid Mixing and Short Droplet Lifetimes. *Anal. Chem.* 2014; 86:9315–9321. [PubMed: 25160559]
34. Englander SW. Hydrogen Exchange and Mass Spectrometry: A Historical Perspective. *J. Am. Soc. Mass Spectrom.* 2006; 17:1481–1489.

35. Blanco E, Ruso JM, Sabín J, Prieto G, Sarmiento F. Thermal Stability of Lysozyme and Myoglobin in the Presence of Anionic Surfactants. *J. Therm. Anal. Calorim.* 2007; 87:211–215.
36. Aseyev V, Hietala S, Laukkanen A, Nuopponen M, Confortini O, Prez FED, Tenhu H. Mesoglobules of Thermoresponsive Polymers in Dilute Aqueous Solutions above the LCST. *Polymer.* 2005; 46:7118–7131.
37. Upadhyay, SK. *Chemical Kinetics and Reaction Dynamics*. Springer, New York City, NY: Anamaya Publishers, New Delhi, India; 2006.
38. Grossman, PD., Colburn, JC. *Capillary Electrophoresis: Theory & Practice*. Academic Press, Inc.; Cambridge, MA: 1992.
39. Whatley, H. Basic Principles and Modes of Capillary Electrophoresis. In: Petersen, JR., Mohammad, AA., editors. *Clinical and Forensic Applications of Capillary Electrophoresis*. Humana Press Inc.; Totowa, NJ: 2001. p. 37
40. D'Arrigo G, Maisano G, Mallamace F, Migliardo P, Wanderlingh F. Raman Scattering and Structure of Normal and Supercooled Water. *J. Chem. Phys.* 1981; 75:4264–4270.
41. Sarraf N, Saboury A, Ranjbar B, Moosavi-Movahedi A. Structural and Functional Changes of Bovine Carbonic Anhydrase as a Consequence of Temperature. *Acta Biochim. Pol.* 2004; 51:665–671. [PubMed: 15448728]
42. Gudiksen KL, Urbach AR, Gitlin I, Yang J, Vazquez JA, Costello CE, Whitesides GM. Influence of the Zn(II) Cofactor on the Refolding of Bovine Carbonic Anhydrase After Denaturation with Sodium Dodecyl Sulfate. *Anal. Chem.* 2004; 76:7151–7161. [PubMed: 15595855]
43. Bushmarina NA, Kuznetsova IM, Biktashev AG, Turoverov KT, Uversky VN. Partially Folded Conformations in the Folding Pathway of Bovine Carbonic Anhydrase II: a Fluorescence Spectroscopic Analysis. *ChemBioChem.* 2001; 2:813–821. [PubMed: 11948867]
44. Semisotnov GV, Rodionova NA, Kutysenko VP, Ebert B, Blanck J, Ptitsyn OB. Sequential Mechanism of Refolding of Carbonic Anhydrase B. *FEBS Letters.* 1987; 224:9–13. [PubMed: 2824244]
45. Saito R, Sato T, Ikai A, Tanaka N. Structure of Bovine Carbonic Anhydrase II at 1.95 Å Resolution. *Acta Crystallogr.* 2004; D60:792–795.
46. Wan L, Twitchett M, Eltis L, Mauk G, Smith M. In vitro Evolution of Horse Heart Myoglobin to Increase Peroxidase Activity. *Proc. Natl. Acad. Sci.* 1998; 95:12825–12831. [PubMed: 9788999]
47. Awad ES, Deranleau DA. Thermal Denaturation of Myoglobin. I. Kinetic Resolution of Reaction Mechanism. *Biochemistry.* 1968; 7:1791–1795. [PubMed: 5689840]
48. Hargrove MS, Barrick D, Olson JS. The Association Rate Constant for Heme Binding to Globin is Independent of Protein Structure. *Biochemistry.* 1996; 35:11293–11299. [PubMed: 8784183]
49. Lee VWS, Chen Y-L, Konermann L. Reconstitution of Acid-Denatured Holomyoglobin Studied by Time-Resolved Electrospray Ionization Mass Spectrometry. *Anal. Chem.* 1999; 71:4154–4159. [PubMed: 10517140]
50. Simmons DA, Konermann L. Characterization of Transient Protein Folding Intermediates during Myoglobin Reconstitution by Time-Resolved Electrospray Mass Spectrometry with OnLine Isotopic Pulse Labeling. *Biochemistry.* 2002; 41:1906–1914. [PubMed: 11827537]
51. Gillespie B, Plaxco KW. Nonglassy Kinetics in the Folding of a Simple Single-Domain Protein. *Proc. Nat. Acad. Sci. USA.* 2000; 97:12014–12019. [PubMed: 11050233]
52. Bágel'ová J, Antalík M, Tomori Z. Effect of Polyglutamate on the Thermal Stability of Ferricytochrome c. *Biochem. Mol. Biol. Int.* 1997; 43:891–900. [PubMed: 9385449]
53. Milne J, Xu Y, Mayne L, Englander SW. Experimental Study of the Protein Folding Landscape: Unfolding Reactions in Cytochrome c. *J. Mol. Biol.* 1999; 290:811–822. [PubMed: 10395831]
54. Heck AJR. Native Mass Spectrometry: a Bridge between Interactomics and Structural Biology. *Nature Methods.* 2008; 5:927–933. [PubMed: 18974734]
55. Hernández H, Robinson CV. Determining the Stoichiometry and Interactions of Macromolecular Assemblies from Mass Spectrometry. *Nat. Protoc.* 2007; 2:715–726. [PubMed: 17406634]
56. Cacace MG, Landau EM, Ramsden JJ. The Hofmeister Series: Salt and Solvent Effects on Interfacial Phenomena. *Q. Rev. Biophys.* 1997; 30:241–277. [PubMed: 9394422]

57. Salis A, Ninham BW. Models and Mechanisms of Hofmeister Effects in Electrolyte Solutions, and Colloid and Protein Systems Revisited. *Chem. Soc. Rev.* 2014; 43:7358–7377. [PubMed: 25099516]
58. Gavriilidou AFM, Gülbakan B, Zenobi R. Influence of Ammonium Acetate Concentration on Receptor-Ligand Binding Affinities Measured by Native Nano ESI-MS: A Systematic Study. *Anal. Chem.* 2015; 87:10378–10384. [PubMed: 26399292]
59. Hargrove MS, Olson JS. The Stability of Holomyoglobin Is Determined by Heme Affinity. *Biochemistry.* 1996; 35:11310–11318. [PubMed: 8784185]
60. Benesch JL, Sobott F, Robinson CV. Thermal Dissociation of Multimeric Protein Complexes by Using Nanoelectrospray Mass Spectrometry. *Anal. Chem.* 2003; 75:2208–2214. [PubMed: 12918957]
61. Geels RBJ, Calmat S, Heck AJR, van der Vies SM, Heeren RMA. Thermal Activation of the Cochaperonins GroES and gp31 Probed by Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 2008; 22:3633–3641.
62. Wang G, Abzalimov RR, Kaltashov I. Direct Monitoring of Heat-Stressed Biopolymers with Temperature-Controlled Electrospray Ionization Mass Spectrometry. *Anal. Chem.* 2011; 83:2870–2876. [PubMed: 21417416]
63. Yang F, Zhou B, Zhang P, Zhao Y, Chen J, Liang Y. Binding of Ferulic Acid to Cytochrome *c* Enhances Stability of the Protein at Physiological pH and Inhibits Cytochrome *c*-Induced Apoptosis. *Chem.-Biol. Interact.* 2007; 170:231–243. [PubMed: 17875304]
64. Makhatadze G, Lopez M, Richardson J, Thmos S. Anion Binding to the Ubiquitin Molecule. *Protein Sci.* 1998; 7:689–697. [PubMed: 9541401]

Figure 1.

(a) Positive nESI of a solution of bovine CA II in 5 mM NH₄ OAc (AA) solution, sprayed out of a theta tip with no electroosmosis. Mass spectra of the same CA II solution after electroosmosis via 100 ms of a 10 Hz square wave at (b) +/-200 V and (c) +/-230 V. The circles at the right of the spectra indicate the theta tip schematic; an aliquot of the same sample was loaded in each channel, and the lightning bolts depict the voltage applied to each side

Figure 2.

A plot of the percentage of aMb ions relative to all myoglobin ions (aMb+hMb) as a function of square wave voltage. Insert (a) - Positive nESI mass spectrum of a solution of hMb in 5 mM NH₄OAc (AA) solution, sprayed out of a theta tip with no electroosmosis. Insert (b) - Mass spectrum of the same hMb solution after electroosmosis via 100 ms of a 10 Hz square wave at +/-230 V. Insert (c) - Mass spectrum obtained with a square wave voltage of +/-300 V. Green open circles represent charge states of aMb, red closed circles represent charge states of hMb, blue triangles represent hMb with an additional heme group, filled gold square represents heme ion

Figure 3.

A plot of the abundance weighted average charge state of eCyt *c* ions as a function of square wave voltage after 100 ms of a 10 Hz square wave applied to a solution of eCyt *c* in 5 mM NH₄ OAc (AA) solution. Insert (a) – Positive nESI mass spectrum obtained using ± 290 V. Insert (b) – Mass spectrum obtained using ± 310 V. Insert (c) – Mass spectrum obtained using ± 340 V

Figure 4.

Electroosmosis of myoglobin at ± 230 V for 100 ms in (a) de-ionized water; (b) 10 mM NH_4OAc (AA) solution. The circles at the right of the spectra indicate the theta tip schematic; an aliquot of the same sample was loaded in each channel, and the lightning bolts depict the voltage applied to each side

Figure 5.

Raman thermometry measurements of 5 mM NH_4OAc solution in a theta tip. Maximum temperature reached during the electroosmosis step is plotted with respect to the applied square wave peak amplitude. The dotted black line included to guide the eye is a quadratic fit to the data points. Insert (a) shows the temperature profile during the electroosmosis-spray-MS detection process with +/-100 V (violet), +/-300 V (green) and +/-500 V (red). Insert (b) shows representative training spectra (lines) for two temperature values and shows experimental spectra taken during electroosmosis (dots) to illustrate the sensitivity of the Raman measurements to temperature

Figure 6.

Electroosmosis of a solution mixture of ubiquitin, bCyt *c* and myoglobin in 5 mM NH_4OAc solution in a theta tip at (a) ± 200 V; (b) ± 230 V; (c) ± 250 V; (d) ± 300 V and (e) ± 500 V. The circles at the right of the spectra indicate the theta tip schematic; an aliquot of the same sample was loaded in each channel, and the lightning bolts depict the voltage applied to each side