Protein Folding and Unfolding on the Millisecond Time Scale using Contained-
Electrospray Ionization

THESIS

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By

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Abstract

Top-down proteomics involves measuring the mass to charge ratio of an analyte protein without digestion. Unfolded proteins give better sequencing information and the workflow frequently involves a denaturing step before analysis. Manipulation of bulk solutions can be wasteful of valuable analyte solutions and can miss intermediates that describe the unfolding process. This thesis uses contained-electrospray ionization to perform online denaturing steps by mixing droplets in the electrospray plume with acidic vapors to decrease the pH of the droplet between the ESI emitter and the mass spectrometer inlet. The extent of protein denaturation can be controlled using different operational modes of the contained-electrospray ionization set-up. Operation mode Type I offers rapid protein denaturation where intermediated unfolding stages can be studied. For operation mode Type II, a cavity (5 mm) is created within the outer capillary which converts the electrospray droplets into thin liquid film and so increases the denaturation/reaction time for a more efficient unfolding. Operation modes Type I and Type II were tested using four proteins, myoglobin, ubiquitin, carbonic anhydrase, cytochrome C, with lysozyme as a control. Operation mode Type II was able to achieve higher average charge states using HCl vapor as a reagent than manipulating the bulk solution for myoglobin, ubiquitin, and cytochrome C. Lysozyme is unable to unfold without reducing disulfide bonds, and showed a minimal increase, indicating that changes...
are from proteins unfolding. In operation mode Type I myoglobin is highly charged with a heme group still attached, indicating that the reaction time is on the low millisecond to sub millisecond time scale. When the operation mode is switched to operation mode Type II, the heme completely falls off, indicating a longer reaction time.

This thesis also introduces operation mode Type III, where the outer capillary of the contained-ES apparatus is replaced with a borosilicate theta capillary. This was used to test myoglobin, cytochrome c, and carbonic anhydrase II in 1% acetic acid with 80 mM ammonium acetate and 20 mM triethylammonium acetate to look at how the proteins refold when the pH is increased. All proteins showed a decrease in their average charge state. Charge states that were lower than what was detected in pure water were observed for both myoglobin and cytochrome C.
Dedication

For my friends and family
Acknowledgments

Thank you to Dr. Abraham Badu-Tawiah for accepting me into his group and providing valuable feedback and assistance when I needed it. Also to the Badu-Tawiah group and to Jay Kim especially for helping me in the lab and to Dmytro Kulyk for assistance in working with the apparatus.
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Chapter 1 Introduction

1.1 Proteomics using Mass Spectrometry

The field of proteomics is one of the most widely researched fields in life sciences. Proteomic experiments are designed to identify and characterize proteins, protein pathways, localizations, and intracellular pathways. For studying proteins using mass spectrometry there are two major methods of analysis, the bottom up approach and top down approach. Bottom-up involves digesting the protein before analysis and top-down involves analyzing the protein without digestion and possibly fragmenting the protein in the mass spectrometer.

The bottom up approach is more used for protein identification. It begins with digesting the protein or protein mixture and reducing disulfide bonds to break the protein into peptide sequences. The peptides can be separated using liquid chromatography before analysis. The peptide sequences are ionized using ESI and then fragmented in the mass spectrometer, usually with collisional induced dissociation (CID). The fragments are then analyzed to find the amino acid sequence for each peptide fragment and software is used to compare the proteins with theoretical sequences and databases to identify the protein(s). Bottom up has the advantage that the peptide sequences are easy to analyze in low mass range mass spectrometers, and that with decent separation on the front end and statistical software, complex mixtures of proteins can be identified easily. However,
Bottom up has the disadvantage that labile post translation modifications are lost during either CID or during digestion. Any structural information or sequencing is lost during digestion however. It also can require analytical software to match the peptide mixtures with the expected protein results. For a variety of reasons however (e.g. incomplete digestion, ion suppression) some of the predicted values might not match what is observed.

In Top-down, the protein is tested without digestion. As the protein is fully intact when it enters the mass spectrometer, top-down experiments give data on PTMs, sequence, and conformational information. Molecular weight of protein are typically in range of kDa to MDa and the mass analyzer must be able to have a large mass range to detect it or the ionization source needs to increase the charges on the analyte to decrease the m/z value. For this consideration, ToF analyzers are used for their speed and practically unlimited mass range and electrospray ionization is frequently used as an ionization source as it can add multiple charges and can ionize proteins in solution.

Top-down experiments frequently use either electron capture dissociation (ECD) or electron transfer dissociation (ETD). ETD and ECD deposit the energy for fragmentation in one step as opposed to CID and provide better sequencing information. The small collisions during CID can cause a loss of PTMs, conformational change, and water loss. Top-down then has the advantage in that it can differentiate between different isoforms of the same protein. Conformation information of the protein is obtained by either using ion mobility spectrometry to compare the drift times of molecules to calculate the collisional cross section or to look at the charge
state distribution on the protein (CSD). However, the top-down method is lower throughput than bottom-up. Top-down experiments frequently require a denaturing step for the protein analysis. The denaturing step unfolds the protein and reveals more basic sites to the solution, allowing more hydrogen ions to add to the protein. The increased charge on the protein will decrease the $m/z$ value, and increases the ionization efficiency of ECD and ETD (due to increased cross-section). There are different methods to denature a protein but any manipulation of the bulk solution can waste valuable sample and adds another step to the workflow. This thesis aims to increase the throughput of top-down experiments by performing online protein denaturing and ionization in a single experimental step.

1.2 Electrospray Ionization

Electrospray ionization (ESI) mass spectrometry (MS) has become an invaluable tool for proteomic experiments. ESI is an ionization technique that allows for fast analysis, protein identification, conformational information, and can add multiple charges to large proteins, decreasing the $m/z$ value into the range of many mass analyzers. ESI has the analyte solution across from the mass spectrometer (MS) inlet with a potential applied on either side. The potential difference deforms the liquid surface into a point, which is called the Taylor cone. When the potential overcomes the surface tension of the solution, droplets containing the analyte are ejected from the tip of the Taylor cone towards the mass spectrometer. The droplets desolvate leaving the analytes in the gas phase. In positive mode ESI forms $[M+nH]^n+$ where $n>1$ compared to MALDI where $n=1$ or 2. Larger values of $n$ increase the mass of the analyte but decrease the $m/z$ value from
the increased charge on the analyte. ESI frequently adds multiple values of n H\(^+\) ions creating a distribution of peaks for the analyte creating multiple charges detected during any one scan. The charge on individual peaks can be calculated using the equation: \(^{19}\)

\[
n_1 = \frac{m/z_1 - m_H}{m/z_2 - m/z_2}
\]

where \(m/z_1\) is a peak with \(n_1\) H\(^+\) ions attached, and \(n^+\) charge, and a higher \(m/z\) value, \(m/z_2\) has \((n_1+1)H^+\) attached and a lower \(m/z\) value, and \(m_H\) is the mass of a hydrogen ion.

As solvent evaporates from the droplets, the charge to volume ratio increases. This increases the charge density and the coulombic repulsion taking place increases, with the repulsion forces being balanced out by the surface tension of the droplet. The Rayleigh limit\(^{20}\) equation give the amount of elementary charge (\(z_R\)) when the repulsion is balanced by the surface tension (\(\gamma\)):

\[
z_R = \frac{8\pi}{e} \sqrt{\varepsilon_0 R^3 \gamma}
\]

Where \(e\) is the elementary charge, \(R\) is the droplet radius, and \(\varepsilon_0\) is the vacuum permittivity. As the Rayleigh limit is approached the increase in the coulombic repulsion will increase relative to the surface tension and the droplet will undergo fission events. A larger droplet forms a Taylor cone of its own and emits smaller, analyte containing droplets. Analytes within the droplet can undergo multiple fission events before they are ionized by three proposed mechanisms.

The ion evaporation model was proposed in 1976 by Iribarne and Thompson.\(^{21}\) Small ions go through the ion evaporation model (IEM) where the droplet desolvates until the charge in the droplet overcomes the surface tension. The charged analytes are ejected from the surface of the droplet. Molecular dynamic calculations performed by
Konerman et al, indicated that this model matches smaller molecules.\textsuperscript{18} As all of the analytes in this study are on the kilodalton mass range, none will go through IEM.

Large, globular shaped molecules (e.g. native conformation proteins proteins) go through the charged residue model (CRM). When the analyte is too big to be ejected from the droplet, the droplet solvent evaporates, leaving the charge in solution to remain on the analyte.\textsuperscript{18,22,23} The third method is the chain ejection model (CEM) and is describes non-polar polymers or unfolded protein chains. A charged terminus is ejected from the surface of the droplet, pulling the next monomer unit to the surface of the droplet. This process continues until the whole molecule is ejected from the droplet.\textsuperscript{18} Molecular dynamic studies indicated that folded proteins undergo CRM while denatured proteins, which have taken on a more linear conformation, undergo CEM instead of CRM.\textsuperscript{18,24} CEM is a faster process than CRM and accounts for the higher signal intensities seen for unfolded proteins compared to native proteins. The microsecond time scale for the droplets to desolvate in CRM can lead to analytes not fully desolvating before analysis. CEM takes place on a nanosecond time scale, allowing for a higher amount of analyte to be fully desolvated and increasing the signal.\textsuperscript{18} As the denatured protein is ionized via being ejected from the droplet, there is evidence that it will take on less sodium adducts than ionization via CRM. The sodium adducts require more solvation and are therefore further down in the bulk of the droplet than H\textsuperscript{+} ions which are located on the surface. As the protein monomers are ejected from the surface of the protein, the amino acids are protonated by the hydrogen atoms at the surface of the droplet as opposed to the sodium adducts buried below.\textsuperscript{24}
Electrospray ionization is able to be coupled to liquid separation techniques such as capillary electrophoresis or reverse phase liquid chromatography.\textsuperscript{3,25,26} This makes it easier to analyze large mixtures of proteins using bottom-up proteomics and to clean up hard to read spectra. Matrix assisted laser desorption ionization (MALDI), another common ionization technique for proteins, does not directly couple to LC online, making ESI valuable for looking at protein mixtures.\textsuperscript{1,3}

1.3 Protein Unfolding and Folding

When in solution at their native conditions, proteins have a specific structure called their native form. Using mass spectrometry using aqueous or buffered aqueous solutions is called native mass spectrometry and can be used to look at the proteins structure.\textsuperscript{27} These can be based on hydrogen bonds, disulfide bonds and solvent interactions can cause large molecules to become folded in on themselves. The speed of the folding or unfolding process can range from micro to millisecond range.\textsuperscript{28,29} The protein’s native conformation is a specific structure that is required for the protein to carry out the needed function. When a protein unfolds it loses tertiary and secondary structure.\textsuperscript{30} Secondary structure is defined as how the amino acids arrange themselves in beta sheets and alpha helixes, or more local structures. Tertiary structure is defined as how the alpha helixes and beta sheets are arranged in the monomers of the proteins. As the protein transitions from the native state to the unfolded, linear state, the protein can experience stable intermediates where parts of the protein are unfolded and other parts are mostly folded.\textsuperscript{31} These intermediates can be short lived (millisecond time scale or below).\textsuperscript{29}
Current methods at looking at protein unfolding and folding are nuclear magnetic resonance (NMR) spectroscopy, circular dichroism (CD), fluorescence, ion mobility spectrometry, or shifts in a charge state distribution (CSD) in mass spectrometry. NMR, fluorescence, and CD all involve looking at the average of many analytes instead of just one molecules. Mass spectrometry based instruments however have the unique ability to look at one conformation at a time instead of seeing an average of all of the states in the solution.\textsuperscript{32}

Most globular proteins exist in their native form at a neutral pH. The native state is highly compact with little mobility. The native conformation is the form in which the proteins are found in the body and can perform the intended function. The type of protein in this paper are termed globular proteins as their native shape in solution has a globular shape to it as opposed to other proteins such as ones that are contained in the cell membrane. A native protein has a well-defined tertiary and secondary structure. As the pH decreases the protein will begin to unfold into a molten globular state. This is indicative of various sidechains beginning to unfold for the protein. These molten globular states are important to understanding the mechanisms of protein folding as they are transitional folding steps.\textsuperscript{30} The protein has begun to lose tertiary structure but the secondary structure is still mostly intact.\textsuperscript{30} The conformation has a less structured form than the native form, but the minor unfolding gives the protein a greater amount of mobility. There is not one molten globular state, as with the native state, instead it is a variety of conformations between the native and fully denatured state.
1.4 Charge State Distribution and Manipulation

As previously stated, ESI has the benefits of being able to add multiple protons to the protein. Adding larger amounts of charge will decrease the m/z value for the protein into the range of low mass range mass spectrometers such as linear ion traps or triple quadrupoles as opposed to a time-of-flight instrument. The other benefit is that the charge state distribution is indicative of the protein’s conformation. The more compact the protein is, the less basic sites are available for the hydrogen ions to attach to. When a protein unfolds, more basic sites are available to the hydrogen ions in solution. This lets mass spectrometry probe the conformation changes in proteins using the shift in charge state. The CSDs are mostly compared using the intensity weighted average charge state \( q_{\text{avg}} \) calculated using the equation:

\[
q_{\text{avg}} = \frac{\sum_{i=1}^{N} q_i w_i}{\sum_{i=1}^{N} w_i}
\]  

(1)

Where \( q_i \) is the charge on the \( i \)th charge state, \( w_i \) is the intensity of the \( i \)th charge state, and \( N \) is the number of analyte charge states.

Previous methods of manipulation CSDs having included bulk solution methods such as using solution additions, (e.g., organics, acids, bases, supercharging reagents), heating, or electro-thermal supercharging. Changing the bulk solution can require a large amount of sample for manipulation and requires long mixing times as opposed to using microliters of sample. Transitional folding or folding steps cannot be seen using bulk manipulation. It can also be difficult to look at transitional steps during the mixing process. Supercharging reagents (e.g. m-NBA) work by having low vapor pressures. During the droplet desolvating process, the droplets with
supercharging reagents undergo less evaporative cooling. The droplets are then much warmer than droplets without the supercharging reagent. The longer droplet lifetime and hotter conditions cause the droplet to unfold in the droplet.\textsuperscript{38} Supercharging reagents do require bulk solution manipulation, and therefore a large enough sample solution to manipulate.

Bulk solution modifications have the disadvantage of requiring a large amount of sample to manipulate and can create a larger amount of waste. To get around that requirement there has been a growth in research in recent years to create methods that are focused on developing methods that avoid reactions with the bulk phase. This includes reactions using theta capillaries,\textsuperscript{45,46} leaking in vapors between the emitter and the mass spectrometer,\textsuperscript{36,37} and rapid laminar flow mixers.\textsuperscript{47} These methods have the benefit of using small amounts of samples (µL of samples) of being able to see folding intermediates.

The effect of vapor on the ESI droplets have been previously studied, primarily by the Mcluckey group, though there have been others as well. Kharlamova et al. studied the effect of acidic vapors on droplets by having acidic vapors leaked in through a curtain gas placed between the emitter and the mass spectrometer inlet. They showed that the proteins did have a significant shift in charge state distribution. Holo-myoglobin in water showed an increase of $q_{\text{avg}}$ from 11.1 to 20.2 when mixed with HCl vapors and the % apo detected went from 10% to over 50%. They saw that the apo form of the protein had a $q_{\text{avg}}$ higher than when using 1% acetic acid to denature the protein suggesting that using HCl vapor was a way to decrease the pH less than 3. The also saw that $q_{\text{avg}}$ would shift
base on the pKa of the acid, where the lowest pKa caused a greater shift.\textsuperscript{36} Kharlamova et al. would later show similar results in negative mode when the basic vapors of ammonia, triethylammonia (TEA) and piperidine, where the more basic reagents resulted in a larger shift in CSD in negative mode.\textsuperscript{37} The reaction time for this is limited as the process happens between the emitter and the mass spectrometer. Myoglobin did not fully denature during the process as evidenced by the amount of holo myoglobin still present.

Other methods to avoid bulk manipulation have included Ziare’s fused droplet electrospray technique. Based on, extractive electrospray, this technique has two ESI emitters aimed at 90\(^\circ\) of each other. The droplets collide with each other between the emitter and inlet of the mass spectrometer. Any droplets that do not collide with another droplet will keep going and not enter the mass spectrometer. They were able to change the reaction time by moving the apparatus closer to or further away from the mass spectrometer inlet. This fused droplet method has the benefit of being able to use reagent with low vapor pressure as opposed to the vapor leak-in experiments and by have the benefit of being an easy method to measure the kinetics of the reaction.

Theta capillaries have also been used for rapid mixing. A theta capillary is a borosilicate capillary that has been pulled to a point for nano-electrospray, but with a divider down the center of the capillary to form two chambers for reagents. During the nano-spray process, the reagents in either chamber begin to mix in the Taylor cone. The Williams group looked at the mixing rates of theta capillaries and calculated that there should be a reaction time maximum of 274 ± 60 \(\mu\)s. They also found that most reactions seemed to only last up to one tenth of that, between 27 \(\mu\)s and 270 ns.\textsuperscript{48} Further studies
were able to view unfolding processes that were on the 1 µs time scale. The McLuckey and the Williams group have looked at protein folding and unfolding using rapid mixing using theta capillaries. Both groups were able to observe protein folding intermediates during the process but neither were able to fully denature proteins, which would still require bulk solution manipulations. Fisher et al. used theta capillaries to mix various proteins with acetic acid to denature proteins. They had myoglobin mixed with various concentrations of acetic to shift the $q_{\text{avg}}$ of the protein. The $q_{\text{avg}}$ and the % apo increased as the concentration of acetic acid increased when tested using theta capillaries for rapid mixing. However, in all cases, there was a mixed amount of apo and holo myoglobin. The limited reaction time gives proteins a limited amount of time on for any folding or unfolding process. Theta capillaries would then be unable to be a replacement for solution phase denaturing. Like the droplet fusion theta capillaries are capable of using non-volatile reagents. However, it has the drawback that reagents such as HCl, which are corrosive are then sprayed directly into the mass spectrometer, while vapor leak does not. The Williams group calculated the flow rate with in the theta capillaries to be on the nL/min to pL/min scale, making coupling to an LC system difficult.

McLuckey’s group pioneered ion/ion reactions as a way of shifting the CSD of the protein and even being able to increase the signal of a specific charge state by consolidating the distribution into one charge state. This is accomplished using collisions an ion trap to remove/add hydrogens. The reaction can be controlled by using RF pulses to excite ions in a specific $m/z$ value. The excited ions have an increased velocity that decreases the amount of collisions. And allows for all of the ions to be ‘parked’ at one
charge state. Ion/Ion reactions can be coupled to HPLC but an ion trap is required to perform the experiments.

1.5 Micro-reactors and Charged Droplet Reactions

During a chemical reaction in the bulk phase, the mixing time can become a significant factor in the overall reaction time. In order to minimize this, micro-reactors react microliter amounts of sample with microliter amounts of reagent. The goal of any micro-reactor is to decrease the amount of mixing time to zero, where the observed reaction time is only based on how fast the reactions themselves can occur. Previously described continuous flow mixers have been able to get their dead time down to the microsecond time scale. However, the best rapid mixing attached to a MS so far has had a dead time of 200 µs. However, any folding or unfolding events that happen faster than 200 µs would be missed.

Previous work has been done on Droplets and thin films have been shown to increase the reaction time of reagents by decreasing the time required to mix the solutions. When the droplets are desolvating, the concentration of the reagents within the droplets increase, which then decreases the mixing time compared to the bulk solution. Badu-Tawiah et al. looked at using the aza-michael reaction. The reaction was done under thin film condition where 2 µL of the reagents was pipetted onto a surface and allowed to dry. The samples were then dissolved and analyzed using nano-electrospray and compared to the results of the comparable bulk solution. Of the four systems tested, three had 99% reagent consumed and one had 87% consumed compared to the bulk phase which had 16%, 5%, 92%, and 5%. The increased reagent consumed
can be attributed to the increase in concentration as the film dries, and the decrease in mixing time.

Mortensen and Williams showed that mixing nL amounts of sample during nanospray using theta capillaries was on the range of microseconds, with an upper reaction time of 274±60 µs. They monitored the reduction of 2,6-dichloroindophenol (DCIP) using L-ascorbic acid (L-AA). They estimated that the droplet lifetime is between the 27 µs and 270 µs with some of the reaction taking place in the Taylor cone of the nano-spray emitter. Recently have been able to view protein folding events on a 1 µs time scale. The mixing of the theta capillary begins in the Taylor cone where the solutions on either side of a borosilicate theta capillary meet and ends when the droplet finally desolvates.

1.6 Contained-Electrospray Ionization

The contained-electrospray apparatus (Figure 1) has been previously described by Kulyk et al, in which two separate operation modes (Figure 1B, (i and ii) were developed to enable online droplet modification and used to decrease matrix affects and increase the limit of detection of analytes that are difficult to ionize.
Figure 1.1 Scheme of the contained-electrospray apparatus. (A) The contained-electrospray apparatus (B) The three different mode of operation (i) Type I has the ES-emitter pulled outside of the outer capillary for a short reaction time with volatile reagents, (ii) Type II has the ES-emitter pulled 5 mm with in the outer capillary for an increase in reaction time and more extended droplet modification, and (iii) Type III has the outer capillary replaced with a borosilicate theta capillary for using non-volatile reagents. Modified with permission from Kulyk, D. S.; Miller, C. F.; Badu-Tawiah, A. K. Anal. Chem. 2015, 87 (21), 10988–10994. Copyright 2016 American Chemical Society.

Operation modes Type I and Type II are for use with volatile reagents. The volatile reagent is placed below the nebulizing gas (in these experiments N₂ is the only nebulizing gas tested). At higher nebulizing gas pressures the volatile reagent is suppressed causing a decrease in the affect. At lower pressures there is an increase in the amount of volatile reagent present in the vapor that allows for extended mixing times and greater droplet modification.

Operation modes Type I has the emitter pulled slightly outside of an outer capillary. The outer capillary nebulizing gas carries the volatile reagent through the outer capillary. The reagent then mixes with the analyte in Taylor cone and in the droplets between the emitter and the mass spectrometer. This gives a limited amount of rection
time for the proteins to undergo folding. These charged droplets desolvate and increase the concentration of the acid and lowering the pH of the droplet. The amount of acid actually needed to see an affect is minimal. Operation mode Type I only consumes 6.4 nL/min at 30 psi and operation mode Type II only consumes 5.8 nL/min. When the pressure is increased to 140 psi, operation mode Type I used 3.5 nL/min and operation mode Type II used 3.1 nL/min.\textsuperscript{59}

Operation mode Type II has the inner capillary pulled 5 mm with in the outer capillary. This provide a reaction chamber for an increased mixing and reaction time. It is hypothesized that the analyte solution sprays onto the walls of the outer capillary forming a thin film. The volatile reagents then dissolve into the thin film providing faster mixing than bulk solutions but also longer reaction times than operation mode Type I. Previous data indicates that the droplets formed from operation mode Type II are smaller and more reactive than the droplets in operation mode Type I.\textsuperscript{59}

Operation modes Type I and Type II have already been used to increase the limit of detection of hard to ionize analytes such as steroids and certain drugs of abuse by increasing the amount of protons present and decreasing the competition for protons among analytes during the ESI process.\textsuperscript{59} In this thesis, a new operation mode Type III operational mode (Figure 1B, iii) was developed that has the outer capillary replaced with a borosilicate theta capillary. The emitter is placed in one chamber and a non-volatile reagent is placed in the other chamber. When a backpressure (10 psi) the reagent is sprayed along with the analyte. The reagents mix in the Taylor cone and in the droplets, giving a finite amount of time for the reaction to occur.
The chemical systems of interest in this thesis are proteins, and the contained-ES apparatus is used to perform online charge state manipulation using all three operation modes of the contained-ESI ion source. Operation mode Type I is used to look at various intermediates of the protein unfolding process by decreasing the pH of the droplets. Operation mode Type II uses the cavity for extended droplet modification, obtaining results similar to solution phase denaturing without any bulk manipulation. Operation mode Type III mixes proteins in 1% acetics acid with a triethylammonium acetate/ammonium acetate solution. Triethylammonium acetate and ammonium acetate are volatile enough to remove protons from the droplet and taylor cone. The loss of protons is reflected in a decrease in the $q_{avg}$ for the proteins.
Chapter 2 Methods

2.1 The Apparatus

This thesis concerns itself with using the contained electrospray ionization source (Figure 1). This source is made from a 1/16 swag-lock cross element with three inputs and one output. The top input is the nebulizer gas, in this case N₂. The input 90° to it is the electrospray emitter, a fused silica capillary with an inner diameter (ID) of 100 µm. The bottom inlet is the container for the volatile reagent and it is 180° from the nebulizing gas input. The outlet of the apparatus depends on which of the three modes of operation that is being used. For operation modes Type I and Type II, the outlet is the ES-emitter placed inside of a fused silica outer capillary with an ID of 250 µm. The nebulizer gas carries the reagent fumes through the outer capillary to the ES-emitter. Operation mode Type I has the tip of the emitter placed slightly outside outer capillary while operation mode Type II has the emitter placed 5 mm with in the outer capillary for increased interaction with the volatile reagent.

This thesis introduces operation mode Type III, where the outer capillary is replaced with a borosilicate theta capillary and the emitter is placed with in one of the chambers. The opposite chamber is filled with a non-volatile reagent. When a backpressure of 10 psi is applied both the reagent and the analyte are sprayed and mix in the Taylor cone and the droplet plume for a limited time. The distance between the
emitter and the mass spectrometer is 2-5 mm. The speed of droplets has previously been reported as \( \sim 100 \text{ m/s} \)\(^{60}\) to give a reaction time on the microsecond time scale for operation mode Type I. Some mixing may begin in the Taylor cone that can increase the unfolding time to the low milli-seconds.

### 2.2 The Mass Spectrometer

The mass spectrometer used in this experiment is a Thermo Fisher Scientific Velos Pro LTQ (San Jose, CA, USA). The commercial ESI source has been removed and been replaced with the contained-ES ionization source. The mass spectrometer is otherwise unmodified. The linear ion trap mass spectrometer is a commonly used mass spectrometer for proteomic experiments as they are fast, sensitive and capable of MS\(^n\) experiments.\(^{2,3}\) The ionization of the analytes is done at atmospheric pressure, allowing for easy coupling to the contained-electrospray apparatus or to LC systems for future experiments. The detectors are electron multipliers.

![Figure 2.1 Scheme of the Thermo Fisher Scientific Velos Pro LTQ mass spectrometer used in these experiments. Retrieved from Thermo Scientific, website: http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_51541.pdf](http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_51541.pdf)
The MS parameters used were: 5 µL/min solvent flow rate; 5 kV spray voltage; N₂ nebulizer gas; 0.5 mL of HCl; 150°C capillary temperature; 3 microscans; 100 ms ion injection time. Thermo Fisher Scientific Xcalibur 2.2 SPI software was used for data analysis. Spectra are averaged for 30 to 60 seconds.

2.3 Reagents

Myoglobin (Holo form MW=17.6 kDa, Apo form MW 17.0 kDa, pI=7.4), Ubiquitin (MW=8.57, pI=5.2), Carbonic Anhydrase II (MW=29.1 kDa, pI=5.9), Cytochrome C (MW=12.4 kDa, pI=10.4), Lysozyme (MW=14.3 kDa pI=11.4) and triethylammonium acetate were all purchased from Sigma Aldrich (St Louis, MO, USA) and used without further purification. Ammonium acetate, hydrochloric acid, acetic acid, and formic acid, were all purchased from Fischer Scientific (San Jose, CA, USA). Borosilicate theta capillaries were bought from Sutter Industries (Novato, CA, USA). All proteins samples were prepared in 100% 18.2 MΩ water from a Milli-Q water purification system (Millipore, Billerica, MA) for protein unfolding experiments (Ch. 3.1) and 50 µM in 1% acetic acid for protein folding experiments (Ch. 3.2).
Chapter 3 Results

3.1 Protein Unfolding

Five model proteins are tested in operation modes Type I and Type II of contained-ESI with HCl as the source for acidic vapors. Myoglobin is tested using formic acid and acetic acid as well to determine how changing the pKa and vapor pressure changes the affect the $q_{\text{avg}}$ of the protein compared to HCl. The flowrate is varied in both operation modes Type I and Type II for testing myoglobin with HCl. An increase of flow showed little effect on the $q_{\text{avg}}$ for myoglobin but a decrease in the amount of myoglobin that has fully denatured. Lysozyme is constrained with four disulfide bonds that prevent any sort of large scale unfolding. As it is the unfolding process that increases the charge on the protein, the $q_{\text{avg}}$ does not show any significant increase when tested. This indicates that the significant increase in $q_{\text{avg}}$ seen by other proteins are from the proteins unfolding.

3.1.1 Myoglobin

Myoglobin is a protein with a mass of 17.6 kDa and a heme cofactor non-covalently attached when the protein is folded. When the heme is attached to the protein it is in its holo form. When the protein is denatured and in a more linear conformation, the heme group detaches from the protein, giving a mass of 17.0 kDa and it is in its apo form. Konerman et al. used time resolved mass spectrometry to show that the fully
denatured protein happens on the millisecond time scale and goes through a two-step process of:

\[(\text{holo-myoglobin})_{\text{native}} \rightarrow (\text{Holo-myoglobin})_{\text{unfolded}} \rightarrow \text{Heme} + (\text{apo-myoglobin})_{\text{unfolded}}.\]  

The multiple steps in the unfolding/folding process make it easy to look at myoglobin’s unfolding process. The native holo-myoglobin will form a narrow CSD with relatively high \( m/z \) values. The unfolded holo-myoglobin should form a wide CSD at lower \( m/z \) values. As the protein transitions from \((\text{Holo-myoglobin})_{\text{unfolded}}\) to \(\text{Heme} + (\text{apo-myoglobin})_{\text{unfolded}}\), the CSD will stay in a similar place but shift to account for the loss of the heme group.
Figure 3.1 Myoglobin tested in various modes. (A) ESI, no reagent. (B) Operation mode Type II with HCl. (C) Operation mode Type I with HCl (D) ESI, in water with 1% acetic acid

Using conventional ESI with no reagent vapor, myoglobin gave a $q_{\text{avg}}$ of 8.7 with a unimodal charge state and 0% of the myoglobin detected was in the apo form. Adding HCl and using a low N$_2$ pressure (20 psi) the $q_{\text{avg}}$ increased to 20.8, the % apo increased to 31%, and the CSD became bimodal with one distribution centered on the +22 charge state and a third centered on the +13 charge state. The pH of the droplets in operation mode Type I has been determined to be 1.2 at low N$_2$ pressures using Micro Essential Laboratory ultrasensitive pH paper (Hydron Ultrafine; Brooklyn NY, USA). This pH is low enough to denature myoglobin and could cause some refolding. The intermediate holo-myoglobin$_{\text{unfolded}}$ is detected, indicating that the reaction is on the milli-second time scale Myoglobin tested in operation mode Type II gave a similar $q_{\text{avg}}$ to operation mode.
Type I (+20.4 compared to +20.8). However, the percent of the apo conformation of myoglobin dramatically increased from 31% to 100%. There was no holo conformation of myoglobin detected. The CSD detected was monomodal, with the distribution centered on the +13 charge state gone completely. The pH of the droplets using low pressures of N2 in operation mode Type II is 1.2 as well.$^{59}$

![Myoglobin tested using (A) Operation mode Type I and (B) Operation mode Type II with HCl vapors as the reagent. Red labels the apo form detected and blue labels the holo peaks detected. The greater amount of apo peaks present is from the increased mixing time present from operation mode Type II compared to operation mode Type I.](image)

The increase in the % heme loss is from the amount of time mixing time taking place when using operation mode Type II. Operation modes Type I and Type II were then
compared to performing a bulk denaturation of myoglobin using acetic acid. Myoglobin denatured in 1% acetic acid and sprayed using conventional ESI. The $q_{avg}$ was +19.2 with a unimodal distribution and the 100% detected myoglobin in its apo form. The pH of a solution of 1% acetic acid is 2.8, over twice the pH of the droplets using HCl in both operation modes Type I and Type II. The lower pH achieved using contained-ESI are able to achieve higher $q_{avg}$ than using acetic acid in solution. Myoglobin is at its most unfolded at a pH of 2. At pH lower than it can begin to refold again as the present in solution stabilize a more non-linear structure.\textsuperscript{31} The increase in $q_{avg}$ over the acetic acid solution suggests that the protein is still more unfolded than at a pH of 2.8.

**Figure 3.3** Myoglobin tested with contained-ESI using operation mode Type I with various reagents at a $N_2$ pressure of 20 psi. The $q_{avg}$ and the percent of myoglobin detected with no heme group (\% Apo) is listed for each spectrum. (A) ESI, no vapor (B) Operation mode Type I, Acetic Acid (C) HCl vapors (D) Formic acid
Myoglobin was then tested using different acids in operation mode Type I. The size of the shift in CSD is determined by a number of factors, but the ones that are most concerning the reagent are vapor pressure and the pKa of the acid. The vapor pressure determines how much acid will be able to interact with the droplets and the pKa determines the amount of H+ available to protonate the protein.

Table 3.1: The volatile reagents tested using Types I and Types II using myoglobin as a analyte. The reagents are listed in ascending vapor pressure and descending pKa.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Vapor Pressure (mm Hg)</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid (CH₃COOH)</td>
<td>11.7</td>
<td>4.76</td>
</tr>
<tr>
<td>Formic Acid (HCOOH)</td>
<td>44.8</td>
<td>3.75</td>
</tr>
<tr>
<td>Hydrochlorid Acid (HCl)</td>
<td>114.8</td>
<td>-7</td>
</tr>
</tbody>
</table>

When tested using no reagent in water, myoglobin was 100% in its holo form with an average charge of 8.7. When tested in operation mode Type I with acetic acid, myoglobin showed no shift in qavg and was effectively identical to being tested using conventional ESI. While bulk additions of acetic acid can denature myoglobin, when using contained-ESI, the low vapor pressure and relatively high pKa prevent a detectible drop in the droplets’ pH to achieve the microsecond unfolding steps. Operation mode Type I with HCl gave a significant shift in both qavg (8.7 to 20.8) and the % apo detected (0% to 31%). HCl has a higher vapor pressure and a lower pKa than acetic acid. The vapor pressure of HCl is ten times that of acetic acid. The distribution presented is bimodal with multiple CSD present in the spectrum. The first is the CSD centered on the +13 peak and 100% of the detected myoglobin was in its holo form. The second is a
distribution is centered on the +22 charge state and includes both holo-myoglobin and apo-myoglobin. Highly charged holo is a transitional state, existing on the millisecond to low second time scale in bulk reactions. The % apo detected is not as high as was reported for the vapor leak in studies mentioned above or mixing with the 100% acetic acid using theta capillaries. Formic acid was tested as an intermediate pKa and vapor pressure. The shift in formic acid was slight, $q_{avg}=11.0$. The distribution became wider as well, from two peaks to five peaks, another indication of protein unfolding. This indicates that the protein is no longer in its native form and is now in a molten globular state as tertiary structure is lost. Contained-ESI can the probe different steps in the unfolding process using multiple reagents as long as the vapor pressure is high enough and the pKa is low enough.
3.1.2 Lysozyme

Lysozyme is a protein with four disulfide bonds that give it structure. These disulfide bonds prevent any sort of large scale unfolding processes. The inability for the protein to fully unfold prevents it from ever having a significant increase in charge state.\textsuperscript{36,61} In this way it acts as a control for the rest of the proteins tested. If the increase observed in $q_{\text{avg}}$ is from the protein unfolded, than lysozyme should show a small or no increase in $q_{\text{avg}}$. When tested using operation mode Type II there was only a minor shift in charge state, ($q_{\text{avg}}=9.5$ in conventional ESI, $q_{\text{avg}}=10.5$ in operation mode Type II with HCl). Operation mode Type II showed a slightly larger shift than using 1% acetic acid in

Figure 3.4 Lysozyme tested using A) conventional ESI in water, B) Operation mode Type II contained-ESI with HCL, and C) conventional-ESI in 1% acetic acid.
the bulk phase, \( q_{av}=9.9 \). The minor increase in \( q_{av} \) is from the protein’s inability to unfold. The constrained structure prevents any new protonation sites to be exposed to the protein, even under denaturing conditions. The disulfide bonds require a reducing agent to break the bonds. The significant shifts in \( q_{av} \) are then from the proteins denaturing in the droplet or thin film instead of the highly acidic reagents adding protons without any unfolding taking place.

3.1.3 Ubiquitin

Ubiquitin is a small protein (8.6 kDa) that is highly resistant to pH changes.\(^{62}\) There is a hydrophobic core Katta and Chait found that to fully denature it, the pH had to be 2.3 in a 1:1 (v/v) methanol: HCl solution. Contained-ESI can form droplets with lower pHs in both modes using HCl vapors, (operation mode Type I at low pressures and operation mode Type II for all pressures).\(^{59}\)

![Figure 3.5 Ubiquitin tested using (A) ESI, (B) operation mode Type I with room temperature HCl, (C) operation mode Type II with room temperature HCl, and (D) operation mode Type II with 40°C HCl.](image)

28
Using conventional ESI with only water as a solvent, the solution had a high amount of noise, with extraneous peaks. This could be explained as the pI of ubiquitin is close to the pH of pure water and the protein was mostly neutral. There was a visible distribution that looked to center on the +8 charge. Using operation mode Type I of the contained ESI apparatus the S/N rose dramatically to 26 and there was a slight shift in the CSD as the as the +6 charge state was no longer detected and the +9 and +10 peaks now detected. The decrease in pH Testing in operation mode Type II increased the $q_{avg}$ to +8.7 The slight shift in pH from conventional ESI to contained-ESI in both operation modes Type I and Type II are indicative of the difficulty in unfolding the protein. The HCl reagent was heated to increase the vapor pressure and the $q_{avg}$ increased to +9.9. The +13 charge state was now detected as well. Ubiquitin has 13 basic amino acids and 10 acidic amino acids, a charge state of +13 would indicate that all of the side chains are protonated.

### Table 3.2 Signal to noise Ratios for the +8 charge state in ubiquitin.

<table>
<thead>
<tr>
<th></th>
<th>ESI</th>
<th>Type I Room Temp. HCl</th>
<th>Type II Room Temp. HCl</th>
<th>Type II 40°C HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>$q_{avg}$</td>
<td>N/A</td>
<td>8.1</td>
<td>8.7</td>
<td>9.9</td>
</tr>
<tr>
<td>S/N</td>
<td>2.1</td>
<td>26.8</td>
<td>11.8</td>
<td>7.9</td>
</tr>
</tbody>
</table>

3.1.4 Cytochrome C
Cytochrome C is a commonly tested protein in mass spectrometry as a standard with a mass of 12.4 kDa and a pI 10.4. It has been previously studied and shown through H/D exchange experiments that cytochrome C goes through a two-step folding phase with transitional states. Cytochrome C is resistant to change in pH. At pH of 2 in 50% methanol cytochrome C shows a loss of tertiary structure but significant secondary structure remains. In solutions of 2% acetic acid, a bimodal charge state remains, one centered on the +9 charge state and another centered on the +15 or +16 charge state. The two distributions were tested using H/D exchange experiments and were found to have similar exchange rates. If the two distributions were from protein unfolding the higher charge distribution should have had a higher exchange rate. The similar exchange rate
came from the different conformations rapidly converting from one to the other; the exchange rate was an average of exchange rate of the folded and unfolded forms. The conversion process was blocked by heating the protein to oxidize it and the distribution centered on the +9 state was shown to have slower H/D exchange rate. This is evidence that the structure of the distribution centered on the +9 charge state is more compact with less sites available for hydrogen deuterium exchanges to occur. Studies using ion mobility showed that each charge state from +9 to +20 have a specific collisional cross section, increasing with the increase in charge. Multiple CCS were detected for the +7 and +8 charge states. Like myoglobin, cytochrome C has a heme group, however it is covalently bonded to the protein and therefore does not fall off when the protein is denatured, therefore no apo forms can be detected.

When tested using operation mode Type I with HCl vapors, the There was a shift in the $q_{avg}$, (8.5 for ESI, 9.7 for Type I with HCl) and the CSD became bimodal. The initial distribution shifted to a slightly higher charge state (centered on the +9 charge state for operation mode Type I and on the +8 charge state for ESI). The +11 and +12 charge states were detected as well while absent in conventional ESI. The second distribution (+20 to +13 charge states) was a lower intensity than the initial distribution at lower charge states (+7 to +11). Higher pressures were used to try and suppress the reagent as cytochrome C was so responsive to a lower pH. The increase in $q_{avg}$ could indicate an initial, unfolding step is occurring for the high m/z CSD or that when the protein is in pure water, there are unprotonated basic amino acids on the surface. Previous studies using ion mobility show that the collisional cross section of the +11 charge state is
slightly higher than the collisional cross section of the +10 charge state.\textsuperscript{66} The small intensity of the second distribution could be from the short reaction time, where only a small amount of cytochrome c is able to denature fully.

When tested with operation mode Type II of contained-ESI there was another slight shift in $q_{\text{avg}}$ (from 8.5 to 12.1). The distribution was still bimodal with a distribution centered on the +9 charge state and another centered on the +13 or +14 charge states. The different conformations have been shown to be at equilibrium with each other, even at a low pH. The increased mixing for operation mode Type II is able to increase the intensity of the higher CSD but it is unable to shift it to a monomodal distribution. The $q_{\text{avg}}$ is higher than cytochrome C in 1% acetic acid however ($q_{\text{avg}}=11.7$). When the N\textsubscript{2} pressure is increased to 150 psi there is a slight shift in $q_{\text{avg}}$ from 12.1 to 11.6. The distribution centered on the +15 charge state decreased in intensity but was still detected.
3.1.5 Carbonic Anhydrase II

![Graphs of Carbonic Anhydrase II tested using different operation modes and conditions.]

Figure 3.7 Carbonic Anhydrase II tested using (A) ESI, (B) operation mode Type I with HCl, 20 psi N₂, (C) operation mode Type I with HCl, 100 psi N₂, (D) operation mode Type II with HCl, 20 psi N₂.

Carbonic Anhydrase II is the largest protein tested in this study. The mass is 29.1 kDa, over seven times the maximum of the instrument. The pI is 5.9. CA2 is has a zinc atom that is non-covalently bonded to the protein. Like myoglobin, when CA2 is denatured under acidic conditions, the zinc cofactor separates from the protein and is lost into the solution. This provides a minor mass shift compared to myoglobin but it is still measurable. Testing in operation mode Type I gave a shift in $q_{\text{avg}}$ to +12.4 from +10.1 in conventional ESI. All of the detected peaks had the zinc cofactor attached. There was a bimodal CSD with one centered on the +16 charge state and the other distribution remaining centered on the +10 charge state. When the N₂ pressure is increased to 100 psi, the CSD centered on +16 disappears entirely, and the $q_{\text{avg}}$ returns to +10.1, the same as conventional ESI. By switching to operation mode Type II, the $q_{\text{avg}}$ was increased to +27. This was the only protein where the $q_{\text{avg}}$ after testing in operation mode Type II was below the $q_{\text{avg}}$ when denatured in 1% acetic acid.
3.1.6 The Effect of Flow Rate

Figure 3.8 The flow rate was varied in both operation mode Types I and Type II while testing myoglobin. The N\textsubscript{2} pressure was 50 psi and myoglobin was tested with 40\textdegree\textsubscript{C} HCl and with no reagent.

The effect of flow rate was tested using myoglobin. The flow rate was varied and the effect on $q_{\text{avg}}$ and % heme loss, measured in percent of myoglobin detected in its apo form. This is used as an indicator for the amount of myoglobin that is fully unfolded. The HCl was heated to 40\textdegree\textsubscript{C} to increase the amount of acid present to interact with the droplets and the N\textsubscript{2} pressure was at 50 psi.

For operation mode Type I, increasing the flow rate from 0.5 \(\mu\text{L/min}\) to 20 \(\mu\text{L/min}\) showed no real change in $q_{\text{avg}}$, with or without HCl. The % apo-myoglobin did decrease as the flow rate increased. At 0.5 \(\mu\text{L/min}\) the % Apo detected was 59\%. As the flow rate increased, the % apo decreased until 5 \(\mu\text{L/min}\), where it leveled off to \(\sim 23\%\).
The smaller amount of liquid present during low flow rates will create smaller droplets that are slower moving than the droplets at high flow rates creating more efficient mixing. At high flow rates, the increase in the droplet size and the added velocity prevent a significant unfolding. No signal was detected for 0.5 µL/min when using no reagent, while it was detected when using HCl, allowing for smaller amounts of sample to be used at low flow rates.

Operation mode Type II was tested up to 50 µL/min. At low flow rates (3 and 5 µL/min) the % apo started out at almost 100% and then began to decrease. At 10 µL/min the % Apo dropped to 89% and then continued to drop to 53% at 50 µL/min. The increased amount of liquid could prevent significant pH drops on the time scale. The amount of unfolded myoglobin was still high at over 50% with a flow rate of 50 µL/min indicating that even at high flow rates, operation mode Type II of contained ESI can still cause an extensive shift in $q_{avg}$ and would be amenable to a LC system. At very low flow rates (0.5-2 µL/min) the signal was not stable enough to be detected even when using HCl. At 3 µL/min. At low flow rates for operation mode Type II, the protein began to unfold with in the cavity, even with no HCl gas. At low flow rates, the turbulent mixing going on with in the cavity is denaturing the protein without any reagent.

3.2 Protein Folding

To experiment with protein folding operation mode Type III of the apparatus was used. The reagents used were the commonly used salts ammonium acetate (AA) and triethylammonium acetate (TEAA). The two buffers form a solution of pH 7 but differ in triethylamine has a greater gas-phase basicity (951 kJ/mol) than ammonia (819 kJ/mol).
The reagents are basic and remove protons from the Taylor cone and droplets to remove the charges attached to the protein. Previous studies have shown that an increase in gas-phase basicity (GB) will decrease the charge on the protein and decrease the CSD.\textsuperscript{67} TEAA/AA solutions have already been shown to increase the stability of non-covalent complexes and can help detect charge states lower than what is detected using ammonium acetate alone.\textsuperscript{68,69} Charged-reduced proteins can be beneficial in preventing unfolding during CID and SID.\textsuperscript{68–70} While the reagents are volatile, they are not volatile enough to affect a change in $q_{\text{avg}}$ using operation mode Type I or Type II.

For experiments in in operation mode Type III, myoglobin, cytochrome C, and carbonic anhydrase II were denatured in 1% acetic acid. When the N$_2$ gas is turned on (10 psi), the reagent mixture is sprayed along with the analyte solution and mixes in the Taylor cone and the droplets. As protons are removed from solution and the protein, the $q_{\text{avg}}$ increases, indicating that the protein is refolding.
3.2.1 Myoglobin

Figure 3.9 Myoglobin tested using operation mode Type III of contained-ESI. (A) Myoglobin in 1% acetic acid. (B) Myoglobin reacted with 80 mM ammonium acetate and 20 mM triethylammonium acetate.

Myoglobin was the tested using ESI in 1% acetic acid and had a $q_{avg}$ of +19.2. 100% of the detected myoglobin was in its apo form. When sprayed with the reagent solution in the opposite chamber, the $q_{avg}$ decreased from +19.2 to +11.2. This is still higher than the protein in water (+8.7). This is a significant shift in $q_{avg}$ with the charge states that were detected when spraying using pure water (+8 and +9) being detected. The +7 charge state was detected which was not detected when myoglobin sprayed in pure
water. No holo form of myoglobin was detected. The folding step to reincorporate the heme group is on the milli-second time scale, too long to be seen using this technique.\textsuperscript{71}

3.2.2 Carbonic Anhydrase II

Figure 3.10 MS spectra of Carbonic Anhydrase II tested in operation mode Type III of Contained-ESI. (A) No reagent. (B) The reagent is 80 mM ammonium acetate and 20 mM triethylammonium acetate.

CA2 showed a significant shift in $q_{\text{avg}}$ when mixed using operation mode Type III. It has previously been shown that larger proteins have more complex structures that can take a longer amount of time to refold.\textsuperscript{72} The folding time for CA2 could take too long for CA2 to fully refold. The zinc cofactor is not detected in the spectrum, again indicating that the protein is not able to fully refold. CA2 is the only protein where charge states lower than
the charge states in water were not detected and where the native charge states were not detected either. As with myoglobin, CA2 remains monomodal. There overall distribution is not a symmetric Gaussian peak, a possible indicator of uneven mixing caused by dissimilar flow rates of the reagent and analyte solutions. The significant shift in $q_{\text{avg}}$ does indicate that contained-ESI is a useful technique to look at intermediate steps during the folding process and at reducing charge on proteins.

3.2.3 Cytochrome C

![Graph showing charge states of Cytochrome C in different conditions](image)

Figure 3.11 Cytochrome C in 1% acetic acid tested using operation mode Type III contained-ESI with: (A) no reagent, (B) 80 mM ammonium acetate, 20 mM triethylammonium acetate.

Cytochrome C was tested using operation mode Type III to mix the analyte solution (cytochrome C in 1% acetic acid) with the reagent solution (80 mM ammonium acetate/20 mM triethylammonium acetate). The shift in $q_{\text{avg}}$ was only 0.8 (11.7 to 10.1) compared to 8.0 for myoglobin or 6.0 for CA2. Even though the protein is smaller than both of them. When denatured in 1% acetic acid, cytochrome C gave a strong bimodal
CSD. One is centered on the +16 charge state and the other is centered on the +9 charge state. These distributions was observed when testing cytochrome C in operation mode Type II. Previous studies have shown that the protein exists in an equilibrium between these two distributions, alternating between the unfolded state (low m/z distribution) and the more folded state (high m/z distribution). The +6 charge state did become detectable when during testing with operation mode Type III though. The +6 charge state was not detected in conventional ESI of the protein in pure water. It is not clear if the +6 charge state is more compact than the +7 charge state. TEAA might be able to remove a proton on the +7 charge state without inducing further folding on the part of the protein. Previous studies on protein complexes using TEAA suggest that using TEAA over AA might reduce the charge but not show significant structural changes.68
Chapter 4 Conclusion and Future Work

4.1 Conclusion

Contained-electrospray ionization can probe the folding and unfolding processes of proteins without modifying the bulk solution using three operation modes. Operation mode Type I can be used to induce initial folding steps by having the droplets act as a short-lived reaction vessels for monitoring protein unfolding. As the droplet only exists on the sub-millisecond time scale the unfolding process cannot finish, giving a CSD indicative of transitional unfolding steps. To increase the reaction time, the emitter is pulled 5 mm with in the outer capillary for operation mode Type II. This forms a reaction cavity with an increase in mixing time that allows for a greater amount of protein unfolding to take place. The \( q_{\text{avg}} \) was similar too or higher than the \( q_{\text{avg}} \) detected using bulk denaturing of 1% acetic acid. Operation mode Type III was used to mix denatured proteins with basic reagents to refold the proteins. By changing the pressure of the nebulizing gas, the user can shift the CSD online in real time. The contained-ESI apparatus is easily reorganized for online CSD manipulation.

4.2 Future Work

For future work the goal is to show that contained-ESI can be coupled directly to liquid chromatography and is capable of protein modification without any bulk solution modification. There are three steps to accomplish this larger goal. The first is to separate
a simple protein mixture using an LC system with the contained-ES apparatus set up between the LC and the mass spectrometer detector. The second step is to look at performing online protein modifications such as disulfide bond reduction and crosslinking using a continuous spray Type-III mode or coaxial spray Type-II mode during an LC/MS experiment. H/D exchange experiments will be performed in operation mode Type III to determine the kinetics of the experiments and an upper and lower time limit of the reactions.
References

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