UNRAVELING MOLECULAR RECOGNITION IN PROTEINS: PROTOCOL DEVELOPMENT FOR THE MALDI-TOF MS

Melissa A. Fullner
Mount Marty College
Yankton, SD 57078

William D. Price
Department of Chemistry
Marshall University
Huntington, WV

ABSTRACT

With the established link of protein misfolding in such diseases as Alzheimer's disease, Mad cow disease, Creutzfeldt-Jakob disease, cystic fibrosis, and several forms of cancer (Thomasson, N.D.), protein folding research has recently moved to the forefront in the areas of biology and chemistry. This experiment addressed the issue of protein folding using Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS) to monitor hydrogen/deuterium (H/D) exchange. Methods to investigate binding sites of protein folding as well as thermodynamic properties of protein unfolding were created for both chemically and thermally denatured proteins. The protein Myoglobin from horse heart was unfolded by varying amounts of the chemical denaturant guanidine hydrochloride (GuHCl), ranging in concentration from 0 to 6.0M, as well as by varying the temperature, ranging from 27 to 90 degrees Celsius. Mass spectrometry was then used to monitor the molecular weight shift of the protein, with a standard protein, Cytochrome C, used for calibration purposes. The findings for both methods showed proper unfolding of the protein, following the two state thermodynamic model for protein unfolding. Although more investigation needs to be performed to increase the precision and accuracy of each protocol, solid groundwork is established and presumed to be useful as another tool for uncovering the mysteries behind protein folding and its related diseases.

INTRODUCTION

Proteins are essential for all living organisms (McKee & McKee, 2003). In fact, they have the most diverse functions of all the classes of molecules encountered in living systems (McKee & McKee, 2003). A few examples of these numerous functions include catalysis of biological reactions (enzymes), serving as defense mechanisms (immunoproteins), providing structure and support, aiding movement, and regulating the binding of hormones and growth factors (Lodish

et al., 2004; McKee & McKee, 2003; Weaver & Hedrick, 1997). The function of a protein is highly dependent upon the structure (McKee & McKee, 2003; Lodish et al., 2004); in fact, the two are inextricably entwined. To truly understand the functions of proteins, and by extension the complexities of biological systems, one must have a battery of methods to characterize protein structure under various conditions.

Proteins are actually composed of twenty different amino acids (Lodish et al., 2004; McKee & McKee, 2003; Rupp, 2000; Weaver & Hedrick, 1997). These amino acids are linked together by peptide bonds to create an overall linear structure known as the primary structure (Lodish et al., 2004; McKee & McKee, 2003; Rupp, 2000; Weaver & Hedrick, 1997). Figure 1 shown below depicts how a peptide bond is created between two amino acids (Rupp, 2000).

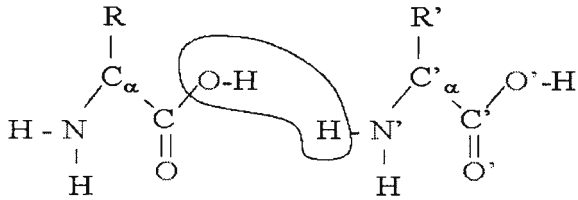

Figure 1. Primary structure of a protein (Rupp, 2000). The circled portion indicates where a peptide bond forms to link two amino acids.

This chain of amino acids then interacts with nearby regions of itself to cause local folding of the protein and the creation of several repeating arrangements or patterns (Lodish et al., 2004; McKee & McKee, 2003; Rupp, 2000; Weaver & Hedrick, 1997). This level of folding is known as the secondary structure of the protein (Lodish et al., 2004; McKee & McKee, 2003; Rupp, 2000; Weaver & Hedrick, 1997). The secondary structure of the protein then folds again and creates what is known as the tertiary structure or overall three-dimensional shape (Lodish et al., 2004; McKee & McKee, 2003; Rupp, 2000; Weaver & Hedrick, 1997; McClure, 2004), which is shown below in Figure 2 (McClure, 2004). This unique shape is what allows the protein to carry out its specific function in the biological system (Lodish et al., 2004; McKee & McKee, 2003).

Figure 2. Three-dimensional structure of a protein, depicting the numerous folding patterns that occur and condense the structure from a linear chain to a globular structure (McClure, 2004).

Since the function of proteins is so highly dependent upon their structure, misfolding can lead to many diseases and pathologies. It is speculated to be the cause of some neurodegenerative diseases such as Huntington's disease (Rupp, 2000). In addition, protein misfoldings have been linked to Alzheimer's disease, Mad cow disease, Creutzfeldt-Jakob disease, scrapie, cystic fibrosis, several different types of cancer, and an inherited form of emphysema (Thomasson, N.D.). Thus, understanding the folding mechanism of proteins as well as specific binding sites is of utmost importance in treating these disorders.

Fortunately, there is a relatively simple method for investigating protein folding known as hydrogen/deuterium (H/D) exchange. In H/D exchange, some sites of hydrogen from the protein exchange with molecules of deuterium when incubation occurs in deuterium oxide, D_2O (HXMS.COM, 2001). This exchange causes a subsequent shift in mass, which can then be used to determine the number of sites of exchange, since each deuterium added to the protein increases the overall mass of the protein by one mass unit (HXMS.COM, 2001), which is measured in Daltons, with one Dalton equal to one mass unit (Lodish et al., 2004).

Specifically, the backbone amide hydrogens are the ones that undergo this H/D exchange (Engen & Smith, 2001; HXMS.COM, 2001; Sivaraman & Robertson, 2001). An example of where these hydrogen atoms are located in relation to the rest of the protein is shown below in Figure 3 (Rupp, 2000).

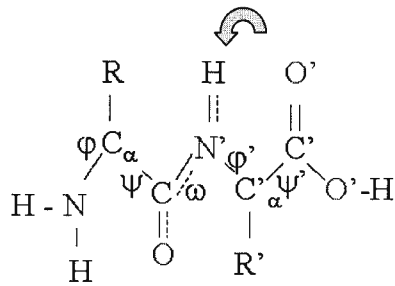

Figure 3. Primary structure of a protein, with the arrow indicating an amide hydrogen (Rupp, 2000).

These amide hydrogens are easy to monitor because each amino acid, except for proline, has one amide hydrogen (HXMS.COM, 2001). They are also useful to investigate because these sites are highly involved in protein folding and forming the overall folded protein structure (HXMS.COM, 2001; Sivaraman & Robertson, 2001).

For these reasons, H/D exchange has become a widely used technique for recent research in the field of protein investigation. It has been used to investigate protein structure (Raza, Dharmasiri, & Smith, 2000; Tobler & Fernandez, 2002), identify areas of ligand-binding (Zhu, Rempel, & Gross, 2004), determine conformational changes in protein structure (Figueroa & Russell, 1999), investigate protein dynamics in solution (Deng, Zhang, & Smith, 1999), and

identify various rates of exchange and unfolding (Mandell, Falick, & Komives, 1998; Deng & Smith, 1999). Although several different techniques can be used with H/D exchange, mass spectrometry (MS) is one of the most popular choices of today (Deng & Smith, 1999; Deng, Zhang, & Smith, 1999; Figueroa & Russell, 1999; Mandell, Falick, & Komives, 1998; Tobler & Fernandez, 2002; Zhu, Rempel, & Gross, 2004).

Mass spectrometry is a method used to measure the mass of a molecule, or in this case a protein, by ionizing the sample into the gas phase and separating the created ions by their mass to charge ratios, m/z (Mass spectrometry, 2004). The mass spectrometer uses an ion source to convert the solid molecules to the gas phase and excite them to their ionic forms (Mass spectrometry, 2004). The mass spectrometer then separates these formed ions by means of a mass analyzer into their m/z ratios and finally utilizes an electron multiplier to detect these ions (Mass spectrometry, 2004). This information is then recorded onto a mass spectrum, displaying the intensity of the various ions versus their mass to charge ratios (Mass spectrometry, 2004).

There are various types of mass spectrometers, but one of the most useful with H/D exchange in protein analysis is the Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometer, commonly referred to as the MALDI-TOF MS. It is oftentimes preferred because of its ability to analyze extremely large molecules that may have a molecular mass in excess of 200,000 Daltons (Young, N.D.), thus allowing for a wide range of proteins to be utilized and investigated. Figure 5 below shows the typical set-up and components of a MALDI-TOF MS (Young, N.D.).

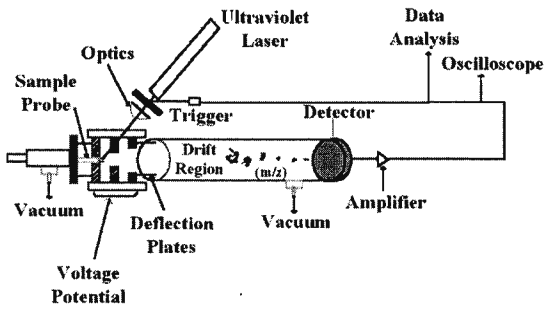

Figure 5. Schematic of a typical MALDI-TOF mass spectrometer (Young, N.D.).

Like the name indicates, this technique incorporates the basic ideas of mass spectrometry, but adds a matrix to the sample in order to prevent degradation with the ionization process (Young, N.D.). The matrix/sample solution is spotted onto a steel plate and placed into the ion source where it is under high vacuum (Mass spectrometry, 2004). The ionization process then occurs by firing a pulsed nitrogen laser at 337 nm onto the sample, causing an absorbance of energy, which is later transferred to heat, causing the molecules to move into the gas phase and leave the plate (Mass spectrometry, 2004; Young, N.D.). This is displayed in Figure 6 shown below (Young, N.D.).

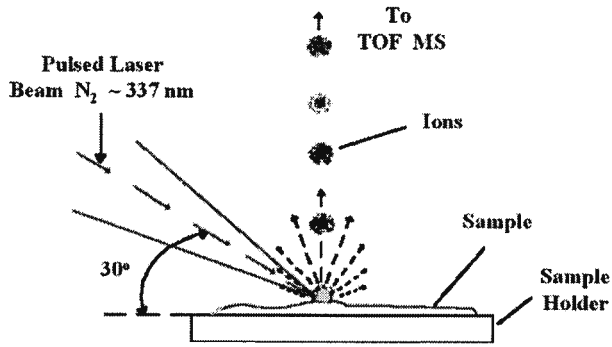

Figure 6. Schematic of the sample ionization process (Young, N.D.).

The ions formed then travel down a field-free flight tube, known as the drift tube or TOF tube (Mass spectrometry, 2004; Young, N.D.). All the ions have the same translational kinetic energy, due to receiving the same potential energy (Mass spectrometry, 2004; Young, N.D.). Thus, the differences in velocity as these ions travel to the detector can be used to determine the mass, since the time of flight is proportional to $(m/z)^{1/2}$ (Mass spectrometry, 2004; Young, N.D.). This is based on the equation $K.E.=1/2mv^2$, where K.E. is the kinetic energy, m is the mass of the ion and v is the velocity of the ion (Mass spectrometry, 2004; Lennon, 1997). The ions are then separated based on mass to charge (m/z) ratios (Mass spectrometry, 2004; Young, N.D.). A graphic of this procedure is provided in Figure 7 (Young, N.D.).

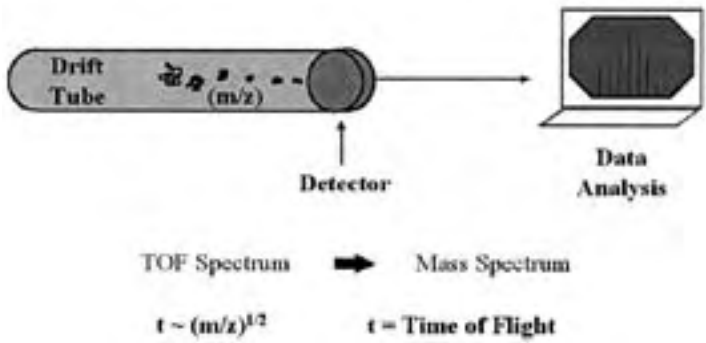

Figure 7. Conversion from the time of flight to the mass spectrum (Young, N.D.).

This technique of MALDI-TOF MS combined with the procedure of H/D exchange is providing an ever-expanding field of knowledge. Dr. Michael C. Fitzgerald of Duke University is one of the primary investigators in this procedure. He and his colleagues have published numerous articles dealing with method development and protein investigation in this area. Specifically, he and his colleagues have created a method known as SUPREX (Stability of Unpurified

Proteins from Rates of H/D Exchange) which allows for picomole quantities of proteins to be studied, unpurified protein samples to be utilized, and many readings to be obtained by means of automation and fast analysis (Ghaemmaghami, Fitzgerald, & Oas, 2000). This technique has been used to investigate thermodynamic properties and stability of various proteins (Powell & Fitzgerald, 2003; Powell, Wales, & Fitzgerald, 2002; Powell et al., 2003) as well as to investigate protein-ligand binding interactions (Powell et al., 2002). Furthermore, investigations have been performed to modify and improve this protocol, including efforts to increasingly minimize the concentration of protein necessary to perform the reaction (Powell & Fitzgerald, 2001).

The procedures from this SUPREX method (Powell & Fitzgerald, 2001) were used as guidelines for protocol development in this experiment. Previous studies performed at Marshall University were also used (Adams, 2004; Harris & Kingery, 2003). The purpose of this study was to determine methods to investigate binding sites of protein folding as well as thermodynamic properties of protein unfolding for the MALDI-TOF MS using H/D exchange. In these procedures, unfolding was done with the addition of a chemical denaturant, guanidine hydrochloride (GuHCl), as well as by heating. Both of these methods are proven effective at denaturing proteins (McKee & McKee, 2003). With protein denaturation, H/D exchange would lead the unfolded protein to a larger mass shift as more amide sites have access to the D2O solvent and can undergo exchange. A schematic representation of this is outlined below in Figure 4.

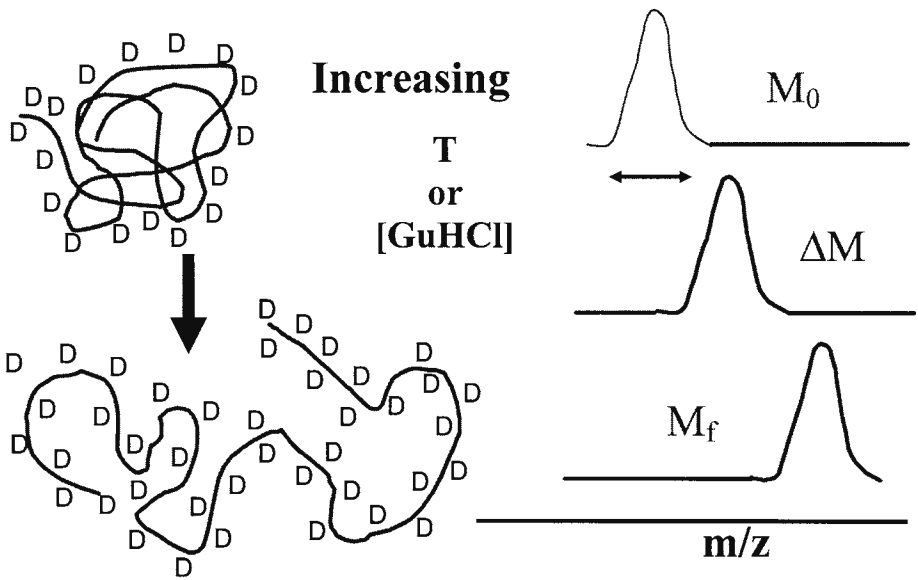

Figure 4. Schematic of the unfolding (thermally or chemically induced) of proteins and noting the subsequent mass shift that occurs as more amide sites become accessible to the solvent and undergo H/D exchange.

MATERIALS

The various peptides and proteins used, including Insulin from Bovine Pancreas, Ubiquitin from Bovine Red Blood Cells, Cytochrome C from Bovine Heart, and Myoglobin from Equine Heart were purchased from the Sigma Chemical Company (St. Louis, Missouri). The chemical denaturant guanidine hydrochloride (GuHCl) was also purchased from the Sigma Chemical Company (St. Louis, Missouri). 99.9% deuterium oxide (D2O), trifluoroacetic acid (TFA), methanol, acetone, sodium phosphate, and ammonium bicarbonate were all purchased from the Aldrich Chemical Company (Milwaukee, Wisconsin). Acetonitrile was purchased from Akros. Nanopure water (H_2O) was obtained from a purifier in the laboratory. Sinapinic acid (SA) used in the MALDI matrix was purchased from Bruker Daltonics. The Bruker Company was also the supplier of the Bruker Biflex™ III MALDI-TOF instrument, which was operated to obtain and analyze data from the reactions. 0.5mL and 1.5mL flip-top plastic sample tubes, a Micro7™ Centrifuge, and a Genie 2™ Vortex were purchased from Fisher and utilized throughout experimentation. Finally, various sizes of automatic pipetters were purchased from BioRad to perform volumetric transfers.

Methods

There were three distinct methods identified and utilized in this experiment. The first was an initial method focused on determining the correct spotting technique and incubation period necessary to obtain consistent mass readings of various peptides and proteins with the Bruker Biflex™ III MALDI-TOF. The second method utilized the findings from the first method and focused on examining chemically induced protein unfolding by means of the chemical denaturant guanidine hydrochloride (GuHCl). Finally, the third method explored thermally induced protein unfolding and also utilized the information obtained from the initial method. These methods are listed in further detail below in sections A through C.

Experimental A: H/D Exchange with Folded Proteins

An approximately 200 µM protein solution of Insulin and Ubiquitin was prepared, with nanopure water serving as the solvent. The solution was thoroughly mixed by using the Genie 2™ Vortex. A total of 1mL of the protein solution was created and placed in a 1.5mL tube. It was then divided into 100µL increments in 10, 0.5mL flip-top sample tubes. These vials were placed in a freezer when not being used to ensure that the samples remained viable and in excellent condition for analysis.

The MALDI matrix solution was also made prior to the reaction. First, a 0.5% trifluoroacetic acid solution was created by mixing trifluoroacetic acid (TFA) and nanopure water (H2O) in a 10mL plastic vile. Mixing of the solution was done by placing the tube on the vortex for several seconds. Following this, 333µL of this solution was combined with 666µL of acetonitrile in a 1.5mL

tube using appropriate micropippeters. Approximately 5mg of sinapinic acid (SA) was then added to this solution and vortexed to fully dissolve the SA and thoroughly mix the solution. The final pH of this solution was very acidic, being around 2.5.

For the experiment, 1µL of the protein solution was removed from a sample vial and placed in a 0.5mL flip-top tube. This was continued several times, depending upon the number of incubation periods that were investigated. Then, 100µL of solvent was added to each tube. One tube received nanopure water (H2O) and was used as the mass standard in the experiment. All other tubes were given 100µL of the 99.9% deuterium oxide (D2O) to allow for the H/D exchange.

Special care was taken when using the D_2O to prevent exchange with hydrogen from the atmosphere. Lids and rims of the tubes and D_2O bottle were sprayed with nitrogen gas and quickly closed. The bottle of D_2O was also stored under nitrogen in a dessicator to ensure that minimal exchange occurred between uses.

The exchange time for each protein sample containing D_2O solvent was then monitored. Each vial was given a different duration of time for D_2O incubation, ranging from 0.1 minutes, or as little time as possible, to 60 minutes. Following this span of time, each reaction was quenched in a small amount of ice cold, acidic MALDI matrix solution. The matrix solution was kept at this low temperature by placing the tubes in an ice bath. The protein sample in H_2O solvent was also quenched in this matrix solution.

In the case of the one layer spotting method, quenching was done by placing 1µL of the protein solution/solvent mixture into 9µL of the ice cold matrix solution, which was placed in a 0.5mL plastic tube. The solution was then mixed by swirling with the pipet tip (Price, 2002). Following this, 1uL of the protein/matrix solution was pippetted and spotted onto the MALDI-TOF plate.

In using the two layer spotting method, a base layer was first established. According to findings by Dai, Whittal, & Li (1999), a mixture of 600µL methanol, 400µL acetone and 6mg SA was the established protocol. 0.3µL of this mixture was first placed on each spot of the MALDI-TOF plate needed for sample analysis. This layer quickly evaporated due to being highly organic and created a fine layer of matrix crystals on the plate. In addition, for quenching the reaction, 10µL of each protein solution/solvent mixture was placed into 10µL of ice cold matrix located in a 0.5mL plastic vial and swirling was again performed with the pipet tip (Price, 2002). 0.5µL of each sample was then pippetted onto the established base layer of a spot on the MALDI-TOF plate.

The plate was dried under vacuum to minimize the amount of back-exchange that might occur with water from the environment as well as to improve crystal growth (Mandell, Falick, & Komives, 1998). The samples were then placed in the MALDI-TOF and analyzed by using the 66klin parameter of the instrument (Price, 2002). The number of shots was adjusted to at least 500 (Price, 2002) and a minimum of 5 readings were obtained from each sample spot, spanning all different areas.

An average and standard deviation of the mass of both Insulin and Ubiquitin were then obtained for each solvent and period of incubation in both the

one layer and two layer spotting methods. The obtained average masses were graphically plotted versus the period of D_2O incubation for each technique. These graphs were then interpreted to determine the length of time necessary for maximal H/D exchange, as well as the most accurate method of spotting. The findings from investigating both Insulin and Ubiquitin were a five minute incubation period in the D_2O along with using the two layer method for spotting. These findings were used in the future experimental procedures B and C.

Experimental B: H/D Exchange with a Chemically Denatured Protein

For this protocol, a method published by Powell and Fitzgerald was used as a guide (2001). Approximately 10mL of a 20mM sodium phosphate buffer (Powell & Fitzgerald, 2001) in nanopure H_2O was created as a stock solution. This was then used as the solvent in creating 1mL of a 1.5mM solution of Myoglobin for the protein solution. An approximately 7M guanidine hydrochloride (GuHCl) stock solution, in 50mM ammonium bicarbonate solution prepared with nanopure H_2O, was created for the basic chemical denaturant. This was then diluted with various amounts of the 20mM sodium phosphate buffer from before to give a range of 0 to 6.0M GuHCl, in 0.5M increments. A 10µL sample of the protein solution was then added to a 90µL amount of each appropriately concentrated GuHCl solution/sodium phosphate buffer. Each sample mixture was placed in a 0.5mL tube, mixed thoroughly by placing the tube on the vortex, adequately labeled for later identification, and allowed to incubate for a period of roughly 2 hours.

A 20mM sodium phosphate buffer was also created using D_2O. 100uL volumes of this solution were placed in separate 0.5mL tubes. Care was again taken to minimize the amount of exchange with the air. Following incubation, 10µL of each protein/GuHCl/sodium phosphate buffer solution was added to a D_2O tube. These solutions were allowed to react for approximately 5 minutes, the length of time indicated to be ideal from the initial experimental method (Experimental A).

Quenching was again performed by using the MALDI matrix solution, as in the initial method (Experimental A). It was performed by placing 1µL of the reaction mixture with 19µL of the highly acidic, ice cold matrix. However, slight alterations were made to the matrix. The solution was saturated with sinapinic acid (SA) followed by centrifugation for 5 minutes at 14000 rpm, as directed by the Bruker Company in their MALDI-TOF matrix protocol. Only the supernatant of the solution was used for the matrix solution, in order to eliminate excess SA in the sample. Additionally, the matrix solution contained Cytochrome C for mapping purposes (Powell & Fitzgerald, 2001). The concentration of this protein was approximately 0.75µM, being close in value to the final concentration of the Myoglobin. This protein was necessary to accurately track the shift of Myoglobin's unfolding by serving as a calibration point for analysis.

The same two layer method (Dai, Whittal, & Li,1999) that was used in the first procedure (Experimental A) was utilized, as were the same means of drying and MALDI-TOF analysis. Cytochrome C was calibrated for each sample and helped to identify a more accurate Myoglobin mass reading for each. Five

sample readings across each spot were recorded and averaged to obtain the mass reading for the subsequent GuHCl concentrations.

From these mass readings, the approximate concentration of GuHCl necessary to unfold the protein was determined. This was found to be a concentration of about 2.0M GuHCl. This concentration was then further investigated by performing the same procedure with a range of 1.5 to 2.5M, in 0.1M increments. The same methods of analysis were used and the average mass readings were obtained. The mass readings for all trials were then converted to a fraction of unfolding. In this case, the highest mass was considered to be the completely unfolded form and thus was assigned a value of one; the lowest mass reading was considered to be the completely folded protein and thus was given a value of zero. All other readings fell somewhere within this range. The fractions of unfolding were then plotted versus the concentration of GuHCl.

Experimental C: H/D Exchange with a Thermally Denatured Protein

This protocol also utilized the published method by Powell and Fitzgerald (2001). A 150μM protein solution of Myoglobin was created by using the 20mM sodium phosphate buffer (Powell & Fitzgerald, 2001) in nanopure H_2O from the chemical unfolding experiment (Experimental B). 2mL of this solution was prepared by mixing 2, 1mL aliquots in 2, 1.5mL flip-top tubes. Each tube was well mixed using a vortex. The solution was then divided into 8 different 0.5mL flip-top tubes. Smaller amounts (~ 100μL) of the protein solution were placed in the vials that would be exposed to lower temperatures, while larger volumes (~300μL) were placed in tubes which would undergo high amounts of heat. This was performed to ensure that evaporation during the heating period would not eliminate the protein sample.

Temperatures studied ranged from 30 to 90 degrees Celsius, in approximately 10 degree intervals. A solution was also kept at room temperature, which for the trial was recorded at 27 degrees Celsius. The ~ 100μL protein solutions were used for temperatures ranging from 27 to 50 degrees Celsius, and the ~300μL protein solutions were used for the 60 to 90 degree range. Heating to these specific temperatures was performed by numerous water baths, with the temperature for each sample being maintained for a period of 1 hour.

Following the heating, 10μL of each protein solution was transferred from the heated vial and placed in 100μL of the 20mM sodium phosphate D_2O buffer. This buffer was the same as that prepared from Experimental B. Incubation in the buffer was then allowed for the 5 minute increment for each sample, as determined from the initial experimental results (Experimental A).

Quenching of the reaction then occurred by placing 1μL of the mixture into 19μL of the highly acidic, ice cold matrix. The matrix was prepared identical to the Experimental B procedure and the two layer spotting method (Dai, Whittal, & Li, 1999) was the same as well. Analysis with the MALDI-TOF was performed as in the two other trials (Experimental A and B) and calibration of the Cytochrome C was again used to obtain accurate Myoglobin mass readings.

The optimal temperature for unfolding was determined by following the same data analysis steps as in the chemical denaturing process (Experimental

B). The fraction of unfolding was graphed versus the temperature, which was converted from Celsius to Kelvin in order to simplify further calculations.

EXPERIMENTAL RESULTS

The charts below were created using Microsoft Excel and show the results from the H/D exchange with folded Insulin and Ubiquitin (Experimental A). Charts 1 and 3 represent the findings from performing the one layer spotting technique, whereas Charts 2 and 4 graphically represent data collected using the two layer spotting method. All charts contain the calculated range of standard deviations observed from the various recorded mass readings. These standard deviations were used to determine the best method of spotting, which was found to be the two layer method in both Insulin and Ubiquitin. The graphs also revealed the length of time necessary for maximal H/D exchange, indicated by the earliest high point in the curve. For all graphs, this was found to be approximately five minutes of incubation. These results were used in further experimentation with the unfolding of proteins (Experimental B and C)

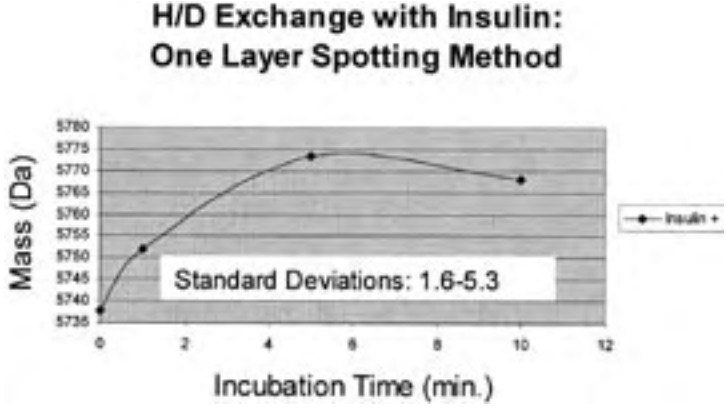

Chart 1. Results from D_2O exchange with Insulin, using the one layer spotting method. The graph shows the averages of points taken from the MALDI-TOF plate, as well as the calculated range of standard deviations for these points.

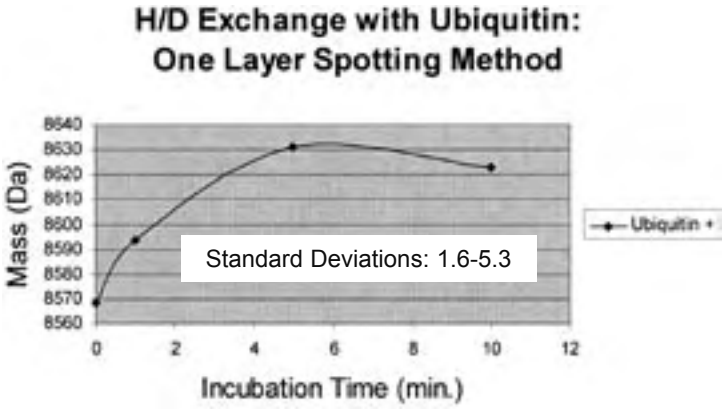

Chart 2. Results from D_2O exchange with Ubiquitin, using the one layer spotting method. The graph shows the averages of points taken from the MALDI-TOF plate, as well as the calculated range of standard deviations for these points.

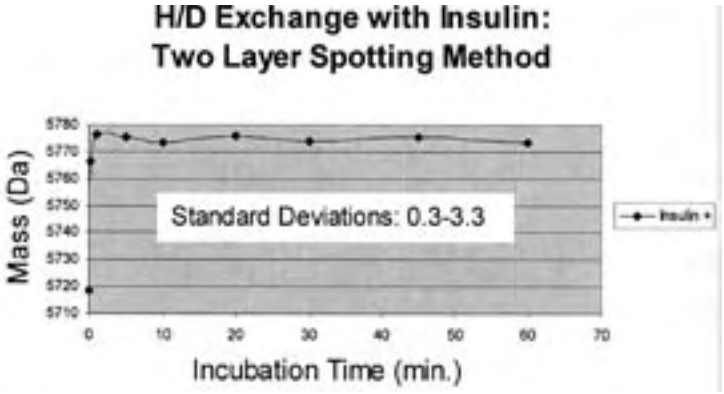

Chart 3. Results from D_2O exchange with Insulin, using the two layer spotting method. The graph shows the averages of points taken from the MALDI-TOF plate, as well as the calculated range of standard deviations for these points.

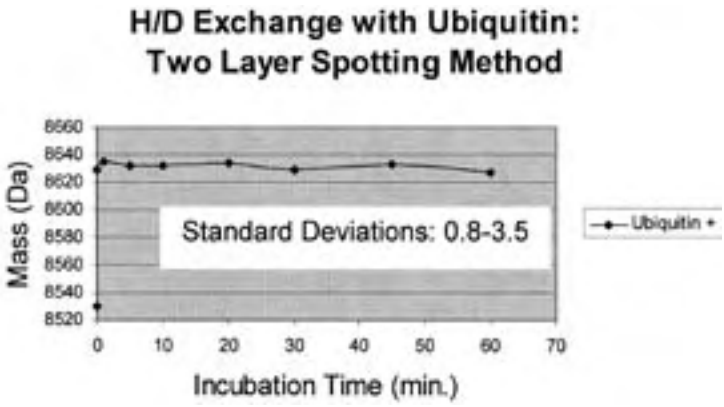

Chart 4. Results from D_2O exchange with Ubiquitin, using the two layer spotting method. The graph shows the averages of points taken from the MALDI-TOF plate, as well as the calculated range of standard deviations for these points.

The results from H/D exchange with a chemically denatured protein, in this case Myoglobin, are shown below in Chart 5. The mass readings of the Myoglobin were converted to fractions unfolded to simplify interpretation of the chart as well as to manipulate the data for further calculations. The graph was created by using IGOR Pro by WaveMetrics, Inc. and a least-squares fit using Genfit. The two state thermodynamic model for protein folding was used as the model. This model states that proteins can exist in only two forms, folded and unfolded (Pace, 1986 and Finn et al., 1992; as cited in Price, 1999). A comparison was also made with results from similar studies that had been performed using the technique of Fluorescence Spectroscopy. These investigations were done previously by students attending Marshall University and enrolled in Chemistry 307 (Price, 1999).

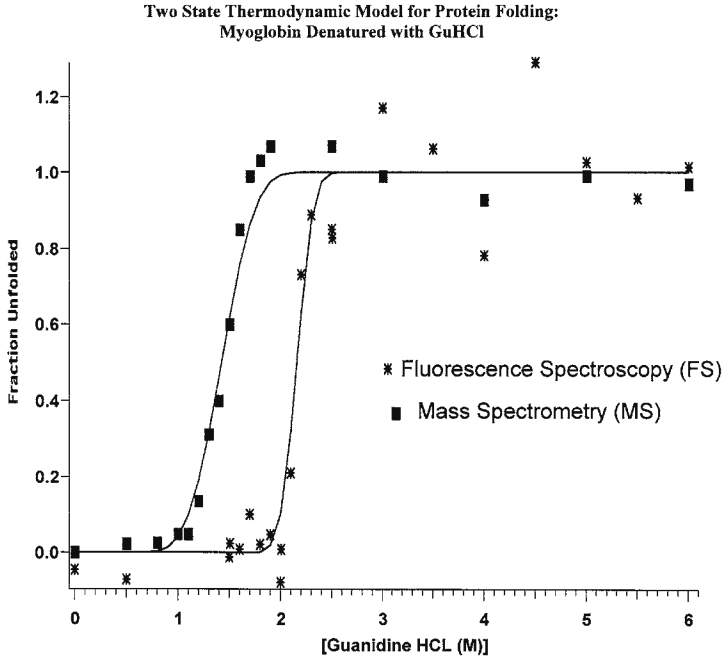

Chart 5. Two state thermodynamic model for protein folding using the chemical denaturant GuHCl on the protein Myoglobin. The squares indicate the data observed using mass spectrometry. The asterisks show the typical results obtained from Marshall University students who perform a similar experiment with fluorescence spectroscopy in Chemistry 307 (Price, 1999).

Following the established two state thermodynamic model, various equations listed below were utilized to determine the concentration of GuHCl necessary to unfold Myoglobin as well as the associated free energy of the unfolding reaction. Calculations were done for both methods of analysis, mass spectrometry and fluorescence spectroscopy, so comparisons could be made. These findings are listed in Table 1.

$$f = \frac{\exp\left[-m\frac{(C_m - C)}{RT}\right]}{1 + \exp\left[-m\frac{(C_m - C)}{RT}\right]}$$

Equation 1. Two state thermodynamic model equation derived by Dr. Price (Price, 1999) which was used to fit the data with only the two fit parameters, m and Cm. m is a parameter related to the surface area of the protein that measures the rate of change of the free energy of denaturation with respect to the denaturant concentration. Cm is the GuHCl concentration at the midpoint of the denaturation curve. These are then used to find ΔG, the free energy of unfolding.

$$\Delta G°_u = \Delta G°_{H2O} - mC$$

Equation 2. The free energy equation (Dautrevaux, Boulanger, Han, & Biserte, 1969; as cited in Price, 1999) which assumes that the denaturant gives rise to a linear perturbation of the free energy that continues to zero denaturant concentration. $\Delta G°_u$ is the free energy of unfolding; $\Delta G°_{H2O}$ is the free energy of unfolding in the absence of denaturant; m is the parameter found in Equation 1; and C is the molar concentration of GuHCl.

Table 1. The calculated Gibbs free energy for the unfolding of Myoglobin by means of the chemical denaturant GuHCl for each technique used. This was calculated by using the standard Gibbs free energy equation: ΔG=-RTlnK. In this case, R is the gas constant 8.314 J/(mol*K), T is the room temperature of 293 Kelvin (K), and K is the equilibrium constant equal to the concentration of the native, folded protein [N] divided by the concentration of the unfolded protein [U] (Price, 1999).

Technique	C_m(M)	*m* (J L/mol^2)	ΔG (kJ/mol)
Fluorescence	1.424	21540	34.4
Mass Spectrometry	2.159	60210	130.0

The results from H/D exchange with the thermally denatured protein Myoglobin are shown on the following page in Chart 6. The same analysis technique was applied as used in the chemical denaturing of Myoglobin found in the previous chart (Chart 5), with the temperature in Kelvin replacing the GuHCl concentration. The IGOR Pro by WaveMetrics, Inc. and a least-squares fit using Genfit were again utilized to create the graph. The two state thermodynamic model for protein folding was once more used as the model (Pace, 1986 and Finn et al., 1992; as cited in Price, 1999). The results of this procedure were compared to a similar study of Myoglobin using the technique of Circular Dichroism (Hicks & Price, 2000).

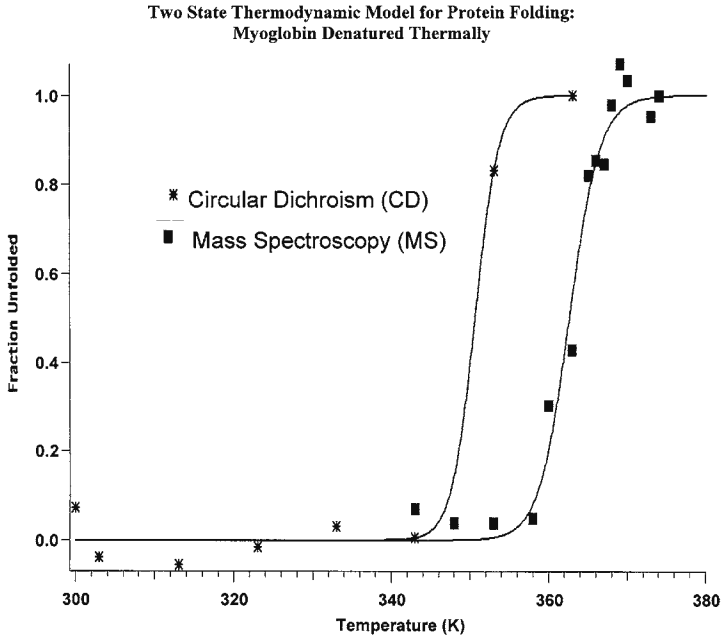

Chart 6. Two state thermodynamic model for protein folding using the protein Myoglobin which was thermally denatured. The squares indicate the data observed using mass spectrometry. The asterisks show results obtained from a previous study by Dr. Price using the method of Circular Dichroism (Hick & Price, 2000).

Equation 3 listed below was utilized to determine the melting temperature of Myoglobin as well as enthalpy of the reaction. These values were then manipulated to determine the entropy along with the Gibbs free energy of the unfolding. Calculations were performed for both methods of analysis, mass spectrometry and circular dichroism, allowing for comparisons to be made between the two techniques. Table 2 contains the findings of these calculations.

$$Fu = \frac{e^{\frac{\Delta H}{R}\left(\frac{1}{Tm} - \frac{1}{T}\right)}}{e^{\frac{\Delta H}{R}\left(\frac{1}{Tm} - \frac{1}{T}\right)} + 1}$$

Equation 3. The equation used to fit the data with only the two fit parameters, ΔH and Tm (Hicks & Price, 2000). ΔH represents the enthalpy of the reaction, whereas the Tm is the melting temperature of Myoglobin. As before, these parameters are used to find ΔG, the free energy of unfolding.

Table 2. The calculated values for enthalpy of the reaction (ΔH), melting temperature of Myoglobin (Tm), entropy of the unfolding process (ΔS), and the Gibbs free energy (ΔG) of the denaturing for each method used. The ΔH and Tm were obtained from Equation 3 using the MathCad program. These values were then used to find the ΔS, by taking ΔH and dividing by the Tm. From this information, each ΔG could be obtained from the equation: ΔG= ΔH-TΔS. In this case, T was used to represent the normal body temperature or approximately 309 K.

Technique	ΔH (kJ)	Tm (K)	Entropy (ΔS) kJ/K	ΔG (kJ/mol)
CD	568.3	362.6	1.567	84.1
MS	689.6	350.6	1.967	81.8

DISCUSSION

The first experiments performed with folded proteins, specifically Insulin and Ubiquitin, showed that H/D exchange did occur in the native, three-dimensional state for both. Charts 1-4 all displayed an immediate increase in the protein's mass as soon as the D_2O solvent was applied. The amount of exchange then continued to escalate with increased incubation in the D_2O, reflected by the subsequent rise in mass values with time. This pattern persisted until every amide hydrogen in the studied protein had been exchanged with deuterium. Following this, the mass of each protein stabilized and a plateau in readings was obtained for all lengthier incubation periods. From these plotted curves, the length of time necessary for maximum H/D exchange in both Insulin and Ubiquitin was determined to be approximately five minutes. A similar length of incubation had been identified in previous research performed at Marshall University (Adams, 2004), and further legitimated the validity of these findings. This period of incubation was then used for future investigations involving H/D exchange.

Furthermore, this procedure minimized the variation in mass readings from the observed spectrums. One layer and two layer spotting methods were performed to determine which technique was capable of producing more consistent readings across the samples. For both Insulin and Ubiquitin, the two layer spotting method was found to minimize mass fluctuation. Charts 2 and 4 respectively, showed much smaller standard deviation values and ranges in comparison to the one layer spotting method seen in Charts 1 and 3. Thus, this technique was used for all subsequent studies investigating protein unfolding, as it allowed for a more precise determination of the number of exchange sites in the given protein.

For both methods of protein unfolding, thermally and chemically induced, the two state thermodynamic model was used as the focal point. This model is based on the principle that proteins can only exist in two states, folded and unfolded (Pace, 1986 and Finn et al., 1992 as cited in Price, 1999). Hence, according to this model, when a protein undergoes H/D exchange upon denaturation, the graph of the mass versus the amount of denaturant (GuHCl or temperature) produces a trend similar in shape to that of a titration curve (Price,

1999). A sharp increase in mass occurs over a very small change in the means of denaturation. However, for easier analysis and interpretation purposes, the mass readings of the protein, Myoglobin, were converted into the fractions unfolded and these were plotted versus the amount of denaturant.

Chart 5 produced this titration-like curve for the unfolding of Myoglobin by means of the chemical denaturant GuHCl. Thus, the established method of analysis utilizing mass spectrometry was successful, as it followed the protein unfolding process of the two state thermodynamic model. However, this method did produce some areas of concern. In comparison with results obtained from Marshall University students using fluorescence spectroscopy (Price, 1999), notable differences were found between the two methods of analysis.

As shown in Table 1, the experimental results from using mass spectrometry found that unfolding occurred with an approximately 2.2M concentration of GuHCl, whereas fluorescence spectroscopy results indicated a concentration of around 1.4M (Price, 1999). Therefore, although these concentration values were relatively close, there was still a great amount of variation present, especially when considering the concentrations of GuHCl were investigated at 0.1M increments for both techniques (Price, 1999). Likewise, the calculated Gibbs free energy displayed in this table showed a significant difference between CD and MS, producing 34.4 and 130.0 kJ/mol respectively. The higher concentration of GuHCl determined by the MS study increased the energy by a factor of nearly four, in comparison to that obtained by means of Fluorescence.

The overall conclusion from these results was that the method did work as far as performing the H/D exchange on a chemically denatured protein. However, more research needs to be done to precisely identify what is the exact concentration of GuHCl that unfolds Myoglobin and the related energy associated with this reaction. From this information, further modifications can be made to the methodology in order to obtain more accurate measurements in future protein investigations. With a few alterations, a simple, straight-forward protocol for investigating protein unfolding by means of chemical denaturant has been created.

The results from the thermally denatured Myoglobin experiment showed similar findings to those obtained with the chemical denaturation. The developed methodology for mass spectrometry produced a titration curve shape, as shown in Chart 6, indicating that the H/D exchange with unfolding had occurred and followed the expected pattern of the two state thermodynamic model. Nevertheless, when comparison was made with another study of thermal denaturation performed by the method of circular dichroism (Hicks & Price, 2000), discrepancies were again present.

As shown in Table 2, the two studies were slightly different as far as the determined melting temperature of Myoglobin. The study performed with CD found a melting temperature that was about twelve degrees Kelvin higher than the one observed using MS. Furthermore, a comparison of the energies associated with the unfolding reaction, also located in this table, indicated some variation. The CD data produced a higher enthalpy than that found by using MS, 1.967 and 1.567 kJ/K respectively. However, this was counterbalanced by the entropy, which had the opposite trend, being higher in MS and lower in CD,

at 689.6 and 568.3 kJ. Subsequently, this made the overall free energy of the two techniques about the same, with CD being 84.1 kJ/mol and MS finding the value to be 81.8 kJ/mol.

Once more, the created methodology was proven successful, but not complete. Further investigations need to be done on this reaction with MS, in an attempt to obtain a more accurate melting temperature closer to that determined by the published CD investigation (Hicks & Price, 2000). Heating the protein solution to higher temperatures must be performed in future procedures to obtain the best curve and line of fit for the unfolding reaction. However, the framework is firmly established for a highly effective, relatively simple method of analyzing thermally unfolded proteins.

REFERENCES

Adams, P. R. (2004, April 27). Analytical monitoring of H/D exchange in proteins and peptides via MALDI-TOF mass spectrometry: Curriculum development for physical chemistry. Unpublished manuscript.

Dai, Y., Whittal, R. M., & Li, L. (1999). Two-layer sample preparation: A method for MALDI-MS analysis of complex peptide and protein mixtures. Analytical Chemistry, 71, (5), 1087-1091.

Deng, Y., & Smith, D. L. (1999). Rate and equilibrium constants for protein unfolding and refolding determined by hydrogen exchange-mass spectrometry. Analytical Biochemistry, 276, 150-160.

Deng, Y., Zhang, Z., & Smith, D. L. (1999). Comparison of continuous and pulsed labeling amide hydrogen exchange/mass spectrometry for studies of protein dynamics. Journal of the American Society for Mass Spectrometry, 10, 675-684.

Engen, J. R., & Smith, D. L. (2001). Investigating protein structure and dynamics of hydrogen exchange- mass spectrometry. Analytical Chemistry, 73, (9), 256A-265A.

Figueroa, I. D., & Russell, D. H. (1999). Matrix-assisted laser desorption ionization hydrogen/deuterium exchange studies to probe peptide conformational changes. Journal of the American Society for Mass Spectrometry, 10, 719-731.

Ghaemmaghami, S., Fitzgerald, M. C., & Oas, T. G. (2000). A quantitative, high-throughput screen for protein stability. Proceedings of the National Academy of Sciences of the United States of America, 97, (15), 8296-8301.

Harris, J. K., & Kingery, J. R. (2003, May 1). Analysis of myoglobin protein unfolding intermediates via MALDI-TOF mass spectrometry and H/D exchange: A physical chemistry curriculum development. Unpublished manuscript.

Hicks, & Price, W. D. (2000). Questions of life and death explored in the physical chemistry lab: How and why proteins fold. 219th National Meeting of the American Chemical Society. San Francisco, CA.

HXMS.COM a site devoted to the study of proteins with hydrogen exchange and mass spectrometry [On-line]. (2001, April 7). Available: http://www.hxms.com/intro.htm

Lennon, J. J. (1997, June 13). Matrix assisted laser desorption ionization time-of-flight mass spectrometry [On-line]. Available: http://www.abrf.org/ABRFNews/1997/June1997/jun97lennon.html

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, S. L., & Darnell, J. (2004). Protein structure and function. In Molecular Cell Biology (5th ed.) (pp. 59-96). New York: W. H. Freeman and Company.

Mandell, J. G., Falick, A. M., & Komives, E. A. (1998). Measurement of amide hydrogen exchange by MALDI-TOF mass spectrometry. Analytical Chemistry, 70, (19), 3987-3995.

Mass spectrometry: an introduction [On-line]. (2004, May 25). Available: http://www.vib.be/Research/EN/Service+-+Facilities/Proteomics+-+Facility/MALDI-TOF/

McClure, W. (2004, January 19). Protein architecture [On-line]. Available: http://info.bio.cmu.edu/courses/BiochemMols/ProtG/ProtGMain.htm

McKee, T., & McKee, J. R. (2003). Amino acids, peptides, and proteins. In Biochemistry: The Molecular Basis of Life (3rd ed.) (pp.108-158). New York: McGraw Hill.

Powell, K. D., & Fitzgerald, M. C. (2003). Accuracy and precision of a new H/D exchange- and mass spectrometry-based technique for measuring the thermodynamic properties of protein-peptide complexes. Biochemistry, 42, 4962-4970.

Powell, K. D., & Fitzgerald, M. C. (2001). Measurements of protein stability by H/D exchange and matrix-assisted laser desorption/ionization mass spectrometry using picomoles of material. Analytical Chemistry, 73, (14), 3300-3304.

Powell, K. D., Ghaemmaghami, S., Wang, M. Z., Ma, L., Oas, T. G., & Fitzgerald, M. C. (2002). A general mass spectrometry-based assay for the quantitation of protein-ligand binding interactions in solution. Journal of the American Chemical Society, 124, (35), 10256-10257.

Powell, K. D., Wales, T. E., & Fitzgerald, M. C. (2002). Thermodynamic stability measurements on multimeric proteins using a new H/D exchange- and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry-based method. Protein Science, 11, 841-851.

Powell, K. D., Wang, M. Z., Silinski, P., Ma, L., Wales, T. E., Dai, S. Y., Warner, A. H., Yang, X., & Fitzgerald, M. C. (2003). The accuracy and precision of a new H/D exchange- and mass spectrometry-based technique for measuring the thermodynamic stability of proteins. Analytica Chimica Acta, 496, 225-232.

Price, W. D. (2002, June 28). H/D exchange protocol. Unpublished manuscript.

Price, W. D. (1999). Two state thermodynamic model for protein folding. Unpublished manuscript.

Raza, A. S., Dharmasiri, K., & Smith, D. L. (2000). Identification of non-covalent structure in apocytochrome c by hydrogen exchange and mass spectrometry. Journal of Mass Spectrometry, 35, 612-617.

Rupp, B. (2000, August 9). Protein structure basics [On-line]. Available: http://www-structure.llnl.gov/Xray/tutorial/protein_structure.htm

Sivaraman, T., & Robertson, A. D. (2001). Kinetics of conformational fluctuations by EX1 hydrogen exchange in native proteins. In Methods in Molecular Biology, vol. 168: Protein Structure, Stability, and Folding (pp. 193-210). Totowa, NJ: Humana Press.

Thomasson, W.A. (N.D.). Unraveling the mystery of protein folding [On-line]. Available: http://www.faseb.org/opar/protfold/protein.html

Tobler, S. A., & Fernandez, E. J. (2002). Structural features of interferon-? aggregation revealed by hydrogen exchange. Protein Science, 11, 1340-1352.

Weaver, R. F., & Hedrick, P. W. (1997). Translation. In Genetics (3rd ed.) (pp. 255-292). Dubuque, IA: Wm. C. Brown Publishers.

Young, S. (N.D.). MALDI-TOF mass spectrometry [On-line]. Available: http://www.psrc.usm.edu/mauritz/maldi.html

Zhu, M. M., Rempel, D. L., & Gross, M. L. (2004). Modeling data from titration, amide H/D exchange, and mass spectrometry to obtain protein-ligand binding constraints. Journal of the American Society for Mass Spectrometry, 15, 388-397.