TABLE OF CONTENTS

ABSTRACT

" Protein Stability Measurements Using Hydrogen/Deuterium Exchange, Mass Spectrometry, and NMR Spectroscopy"

Protein stability is one of the most important properties of proteins and is directly related to their function. The way proteins interact with each other depends highly on their conformation. Measuring the stability of the protein structure assesses the extent to which the native conformation of the protein has changed in a binding assay or as a result of a denaturant present.

In my research project, I am working with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in combination with nuclear magnetic resonance spectrometry (NMR) to measure the rate of Hydrogen/Deuterium exchange of proteins in solution. The rates of Hydrogen/Deuterium exchange depend on solvent accessibility. For sheltered exchangeable groups (e.g. backbone amide protons), the accessibility to the solvent is determined by the overall stability of the protein structure. In denaturing conditions, the more the protein structure is destabilized, the more it unfolds, and the more hydrogen atoms are exposed to the deuterated solvent, producing a faster rate of H/D exchange. On the other hand, when binding to a ligand, the protein sometimes undergoes a structural conformational change and more amide hydrogens become sheltered either by the conformational change that increases stability or by the ligand itself. Thus, the rate of H/D exchange is slower. Both NMR Spectroscopy and Mass Spectrometry can be used to monitor rates of hydrogen/deuterium exchange. Through NMR Spectroscopy, the H/D exchange of specific residues can be measured. Mass Spectrometry measures the overall gain in mass of the protein, although it is capable of providing peptide-specific information through pepsin digestion. Although NMR gives specific residue information about the protein, mass spectrometry is advantageous in that it requires picomole quantities of sample protein, can analyze large proteins, can handle unpurified samples (1), is much easier to use than NMR, requires less time for data collection, is open to variety of methods in acquiring data, and can be done in a high-throughput fashion.

I have cloned, expressed and purified the maltose-binding protein (MBP) and lambda CI. Using these samples, NMR was utilized to generate HSQC spectra and Mass Spectrometry was utilized to monitor the change in mass of the protein throughout the time of the reaction. From the collected data I was able to calculate the rates of exchange. Different buffers for the matrix, standards and temperature were experimented with, as well as the effects of Guanidine on the rate of exchange.

In this work, we have worked out a method to perform H/D exchange experiments in Dr. Montelione's laboratory. The method is yet to be optimized for high-throughput screening and ligand binding.

1. Ghaemmaghami, S., Fitzgerald, M.C. & Oas, T.G. (2000) *PNAS* **97,** 8296-8301.

INTRODUCTION

Hydrogen/Deuterium Exchange and Protein Stability

Stability is one of the most important properties of proteins and is directly linked to their function. It is of an utmost interest to those in the protein field and the pharmaceutical industry to be able to assess the structural stability or the conformational changes of the protein in question or of many proteins in a high throughput fashion. From that information, the function of the protein or the effect of a chemical or other molecules on the protein's structure, and hence the function can be deduced. The ultimate goal of the project is to use Hydrogen/Deuterium exchange as a method for studying the effects of protein-ligand interactions on the conformational stability of the protein structure in a high-throughput fashion. When a protein's structure is stable, the protein is said to be properly folded, which can be indicative of a native conformation. When a protein is in a denaturing environment and is not in its native conformation, its peptide chain(s) unfold and more of its structure is exposed to the solvent. Conformational changes or instability can be monitored by hydrogen/deuterium exchange. When placed in a deuterated buffer, a protein undergoes hydrogen-deuterium exchange of the amide or hydroxy hydrogen atoms, and that results in gain in mass over the course of time of exchange because deuterium is a heavy isotope of hydrogen. Overall, there are three categories into which amide hydrogen atoms can be grouped. The fast- and intermediate-exchanging hydrogens are unprotected amide hydrogen atoms that are solvent-accessible and

exchange rapidly. The slow-exchanging hydrogens are those that are buried inside the protein can exchange

only if they are exposed to the solvent, even if for a short time through the dynamic opening and closing of the side-chains (Hoofnagle, A.N. et.al, 2003). In a protein, amide hydrogen atoms are protected through hydrogen bonding and by being buried in the core of the protein. The more stable the protein structure, the more time the protein spends in its native conformation and the smaller the H/D exchange rate for those hydrogen atoms. There are many factors, other than solvent accessibility that affect the H/D exchange rate. These include temperature and pH. It has been discovered that the slowest rate of exchange occurs at pH~3 and is increased tenfold with every pH increment and threefold with every 10° increase in temperature (see Fig. 5). If a protein is introduced to a denaturing environment and unfolds, it exposes more of its core amides to the solvent, allowing the buried hydrogen atoms to be exchanged more readily with the deuterium of the solvent. These buried and/or hydrogen-bonded amide hydrogens are able to exchange through fluctuations in the protein that allow transient solvent penetration. These local unfolding events can take anywhere from milliseconds to months or longer (Hoofnagle, A.N., et.al, 2003). Measuring the different rates of exchange can give information about general protein stability and can be used to calculate the desired rate values and change in Gibbs Free Energy of the opening conformation (ΔG_{op}) that assesses the thermodynamic stability of the open structure, hence providing information on how favorable are the fluctuations in the protein structure. H/D exchange occurs through a catalyzed reaction of proton abstraction and deuterium transfer, see Fig. 3 (a). When the protein

is highly stable, its structure rarely unfolds and exchange occurs usually through global unfolding of the protein. These proteins follow the EX2 equilibrium exchange mechanisms, where the rate of structural closing (k_{cl}) is much greater than the

chemical rate of exchange (k_{ch}). On the other hand, more dynamic proteins have a k_{cl} that is much smaller than k_{ch} and have the amide hydrogens exposed to the solvent and follow the EX1 mechanism, where the observed rate of exchange (k_{obs}) is equal to the rate of structural opening (k_{op}), see Fig. 3 (b),(c). From Fig. 3 (b), the equilibrium constant between the open and closed states (K_{op}) in EX2 mechanism can be obtained by dividing the two rate constants.

$$K_{op}=k_{op}/k_{cl}$$

The observed rate of exchange for EX2 is given in Fig. 3 (c).

$k_{obs}=(k_{op}/k_{cl})\cdot k_{ch}$ Therefore,

$K_{op}=k_{obs}/k_{ch}$ where $k_{ch}=k_{int}[D_2O]$

$K_{op}=k_{obs}/(k_{int}[D_2O])$ where k_{obs} can be measured in the laboratory and $k_{int}[D_2O]$ is calculated from the amino acid sequence.

pD= pH(measured)+0.4

k_{ch}=k(acid)+k(base)+k(water)

this equation is given in Bai,Y. et.al. (1993) paper. Calculate k_{ch} by adjusting k(acid), k(base) and using the values log k_A=1.62, log k_B=10.05, log k_W=-1.5 for

PDLA (known ribonuclease sequence), and log $[OD^-]$= pD-15.65 as recommended and described in the Bai,Y. et.al. (1993) paper. See "Results" for calculations.

For temperature adjustments:

$k_{ch}(T)= k_{ch}(293)\cdot\exp(-Ea[1/T-1/293]/R)$

where Ea is the activation energy and the recommended values in Bai,Y. et.al. (1993) paper are 14, 17 and 19 kcal/mol for k_A, k_B, and k_W, respectively. $R=1.9847\ cal\cdot mol^{-1}\cdot K^{-1}$ and T is the temperature in degrees Kelvin.

$\Delta G_{op}= -RTln(K_{op})$ for EX2 mechanism

$\Delta G_{op}= -RTln(k_{obs}/(k_{int}[D_2O])$

where $R=1.9847\ cal\cdot mol^{-1}\cdot K^{-1}$ and T is the temperature in degrees Kelvin.

Joules are converted to calories when multiplied by 0.2397

From Mass Spectroscopy data, k_{obs} can be obtained by plotting $ln(\Delta Mass_{max}-\Delta Mass)$ vs. time. The slope of that line is the $-k_{obs}$. From NMR Spectroscopy data, $-k_{obs}$ is calculated as the slope of the graph ln(Peak Heights) vs. time.

The change in Gibbs free energy (ΔG_{op}) is a mathematical way to measure to what extent is the forward reaction of opening in the protein structure is favorable and can be obtained through rate of H/D exchange measurements. Therefore, H/D

exchange rate can be a very useful tool for assessing the stability of the protein structure.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

The general increase in mass of the protein, as it incorporates the heavier deuterium, can be measured by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). In these experiments, MALDI-MS/TOF (Time-Of-Flight) instrument was used, see Fig. 4. The instrument used in these experiments has a straight vacuum tube for ion acceleration. When analyzed in MALDI-MS/TOF, the protein is ionized by the matrix, evaporated from the matrix by a laser beam and accelerated through a tube until it reaches the analyzer. The time from acceleration to detection is the Time-Of-Flight and is a direct measurement of the mass of the species. Since in these experiments the protein is ionized only once, it is singly charged and the m/z ratio can therefore be considered to be its mass. As the protein gains mass due to the H/D exchange, the total change in mass can be monitored with MALDI-MS/TOF by calculating the difference between protein m/z ratios at different time points of the exchange. Increased number of laser shots reduces the noise level and increases the peak height. Mass spectrometer has a very high sensitivity for very tiny (picogram) quantity of protein. However, one drawback to this system is the back exchange that can occur when the partially or fully deuterated protein in mixed with a hydrogenated matrix. This back exchange can be quite significant, ranging anywhere from 10-50%, depending on the measures taken to minimize it (Kipping, M. et.al). H/D exchange is pH- and temperature- dependent. It has been observed that the slowest rate of exchange occurs at a pH~3, see Fig. 5. Furthermore, it has been

observed that lowering the temperature also decreases the H/D exchange rate (Kipping, M. et.al). Under the H/D exchange reducing conditions, Mass Spectrometry can be an extremely useful tool for monitoring the H/D exchange. If the protein studied is undigested, the rate of exchange is given for the whole protein. Specificity can be achieved by digesting the protein with pepsin, which has a low specificity and thus generates peptides of varying sizes. By sequencing and overlapping the fragments, the exchange rates can be monitored for different regions of the protein (Hoofnagle, A.N., et.al). Furthermore, Mass Spectrometry can be performed in a high-throughput manner, using a 100-well or higher Mass Spectrometry plate for spotting the samples.

Nuclear Magnetic Resonance Spectroscopy

Another way to monitor the H/D exchange rate is by Nuclear Magnetic Resonance (NMR) Spectroscopy. In an NMR experiment, application of a magnetic field to the sample, causes the magnetic fields of the nuclei to interact with it, giving rise to increased energy levels and flipping of spin states of the nuclei. Each nucleus normally aligns either with or against the applied magnetic field. Since a greater population aligns with the magnetic field, the net magnetization vector of the sample is aligned along the direction of the applied magnetic field. Through a coil around the sample, pulses of radiofrequency waves are applied, tilting the magnetization vector of the nuclei. As the magnetization vector relaxes, due to solvent interactions, back to its alignment with the field, the resulting time domain data is transformed to frequency domain data by the Fourier Transform mathematical method. A spectrum of resonance frequency of a signal expressed as a chemical shift, the difference

between resonance frequencies of the sample nucleus and a standard, is generated. Through NMR, each specific residue can be monitored for the H/D exchange rate. NMR data can generate a two-dimensional plot of peaks, each corresponding to a specific nitrogen-hydrogen (^{15}N-^{1}H) pair. In these experiments, NMR cannot detect deuterium because it has a net spin of 1 and is considered spin-inactive for these experiments. Since NMR is blind to deuterium, the peaks gradually disappear as the time of exchange goes by. When natural log of peak intensities is plotted versus time and the resulting graph is line-fitted, the absolute value of the slope is calculated to give the H/D exchange rate constant (k_{obs}), which can in turn be used to calculate the ΔG_{op}. While NMR is advantageous in that it can give residue-specific information about the protein, it requires a large amount of protein. Moreover, it can monitor only one reaction at a time and requires significant NMR instrument time. Also, the data is complex and arduous to process and to analyze. Mass Spectrometry, on the other hand, is more suited for high-throughput stability screens because it uses very small (picogram) quantities of protein, demands less time for analysis, is much easier to use than NMR, is capable of analysis of many targets in one experiment, and has a high sensitivity of resolution even with impure protein extracts (Ghaemmaghami, et. al, 2000). Even though it still needs to be optimized to be more efficient for high-throughput screens, Mass Spectrometry is the preferred method for protein structure stability determination by H/D exchange today. Our goal was to use the complementary methods of NMR and MALDI-MS to probe protein stability by H/D exchange. Further, we were interested in using this technology to possibly screen for protein-ligand interactions by measuring the increased protein stability associated

with ligand binding. To do this and work out the conditions, we used two proteins as model systems.

Maltose-Binding Protein

The maltose-binding protein (MBP) segment that was described in Ghaemmaghami et. al paper was cloned through steps described in "Materials and Methods". The protein, residues 1-357, was tagged with a His tag on the C-terminus of the clone and was ^{15}N-labeled. Together with the tag, the molecular weight of MBP is 40,803.8 Da, the theoretical isoelectric point (pI) is 5.57 and the theoretical number of exchangeable amide hydrogen atoms is 344. MBP, expressed from the MalE gene, is involved in transport and regulatory maltose/maltodextrin system that functions to efficiently uptake and catabolize maltodextrins in *E.coli* (http://www.che.utoledo.edu/nadarajah/ webpages/mbp.htm). Besides maltose and maltodextrin, it is also known to bind β-cyclodextrin. As can be seen in Fig.1, the conformation of MBP changes dramatically to a more compact form when a ligand is bound. This conformational change makes the protein structure more rigid, and hence more stabilized from internal fluctuation (Doring, K. et.al). It is highly soluble and is sometimes used as a fusion protein to increase solubility of otherwise insoluble proteins. Furthermore, its binding affinity to amylose is exploited by using MBP as a fusion protein to a protein of interest, which is then purified by passing it through amylose column (http://www.bio.mtu.edu/campbell/ purifica.htm). It is a periplasmic membrane protein that helps transport maltose through cell membranes and there is evidence that its association with a maltose pore leads to increased selectivity for

maltose and its dissociation leads to transmembrane diffusion through the maltose pore of

proteins other than maltose (Heuzenroeder, M.W. et. al).

Lambda Repressor

Another protein that I was working with and that has also been used in the Ghaemmaghami et. al paper is Lambda repressor (called Lambda in this paper). This protein was also cloned according to the protocol in "Materials ad Methods". It is composed of residues 6-85, has the molecular weight of 10,029.3 Da, theoretical pI of 7.02 and 87 theoretical number of exchangeable amide hydrogen atoms. Lambda repressor is a DNA-binding protein in Lambda phage, which regulates the life cycle of the lambda phage. It is involved in transcriptional activation of both itself and another protein Cro through interactions with RNA polymerase (http://www.web-books.com/MoBio/Free/Ch4H2.htm). Its C-terminal domain is the dimerization domain and its N-terminal domain is its DNA-binding domain (Leeds, et.al). The N-terminus that is responsible for DNA binding is composed of amino acids 1-92 of the full protein and has a characteristic DNA-binding helix-turn-helix motif, see Fig 2. If the first three amino acids are removed, the affinity for DNA decreases 30-fold, and removing the first six amino acids results in decrease of binding affinity in three orders of magnitude

(http://www.mun.ca/biochem/courses/4103/topics/Lambda/Lambda_Repressor.html). By having cloned a C-terminal Lambda, I mean that the 6xHistidine tag is on the C-terminus of my clone, which is residues 6-85, and is therefore a helix-turn-helix DNA-binding region. It is important to note that Lambda repressor binds to the

operator region of DNA in a dimerized form. It has been shown by Leeds, et.al that disrupting dimerization

abolished transcription and non-dimerized proteins were present in very low concentrations due to proteolysis. Therefore, my protein is theoretically incapable of either DNA binding or dimerizing.

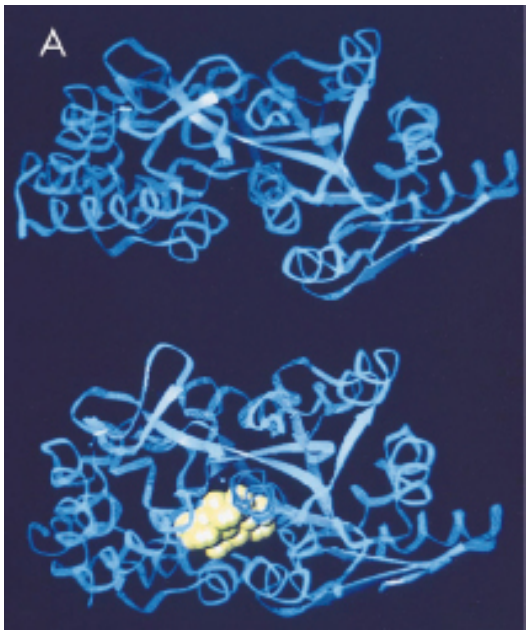


Figure 1: Crystal structures of the open (top) and closed (bottom) forms of MBP. The yellow structure represents maltose bound within the binding site. The figure is from the recent review of the maltose/maltodextrin system in *E. coli* by W. Boos & H. Shuman, *Microbiology and Molecular Biology Reviews*, **62**, 204-229 (1998) (originally from B.H. Shilton, M.M. Flocco, M. Nilsson & S.L. Mowbray, *Journal of Molecular Biology*, **264**, 350-363 (1996)).

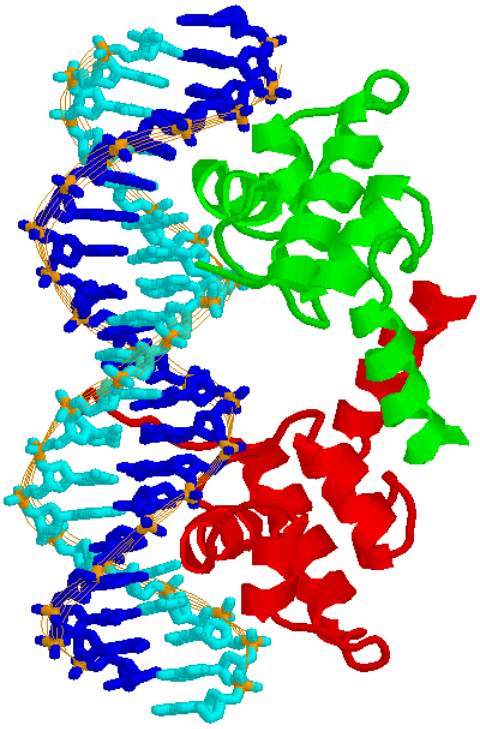


Figure 2: Lambda repressor/operator complex.

http://chemistry.gsu.edu/glactone/PDB/Proteins/NucProt/1lmb.html

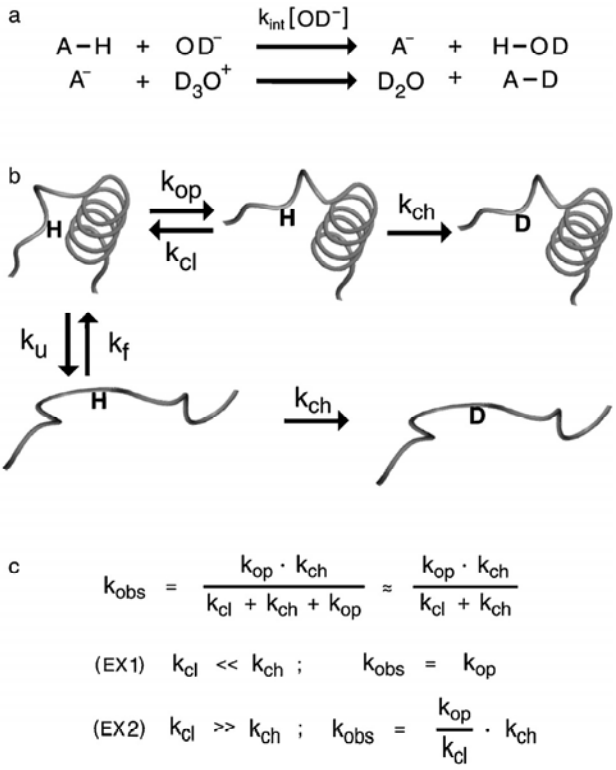


Figure 3: Mechanism of hydrogen/deuterium exchange. (a) Amide exchange at neutral pH involves a base catalyzed proton abstraction and acid catalyzed transfer of deuterium from solvent. Proton abstraction is rate limiting. (b) Hydrogen exchange occurs through different mechanisms, including local structural fluctuations (upper pathway) or complete unfolding (lower pathway). (c) The observed rate of exchange (k_{obs}) for small-amplitude fluctuations is a function of the rate of structural opening (k_{op}), the rate of structural closing (k_{cl}), and the chemical rate of exchange ($k_{ch}=k_{int}$[catalyst]), where catalyst is OH- or buffer. The native proteins follow the EX2 exchange mechanism, while denatured proteins or areas of high fluctuation follow the EX1 mechanism. (Koofnagle, A.N. et.al)

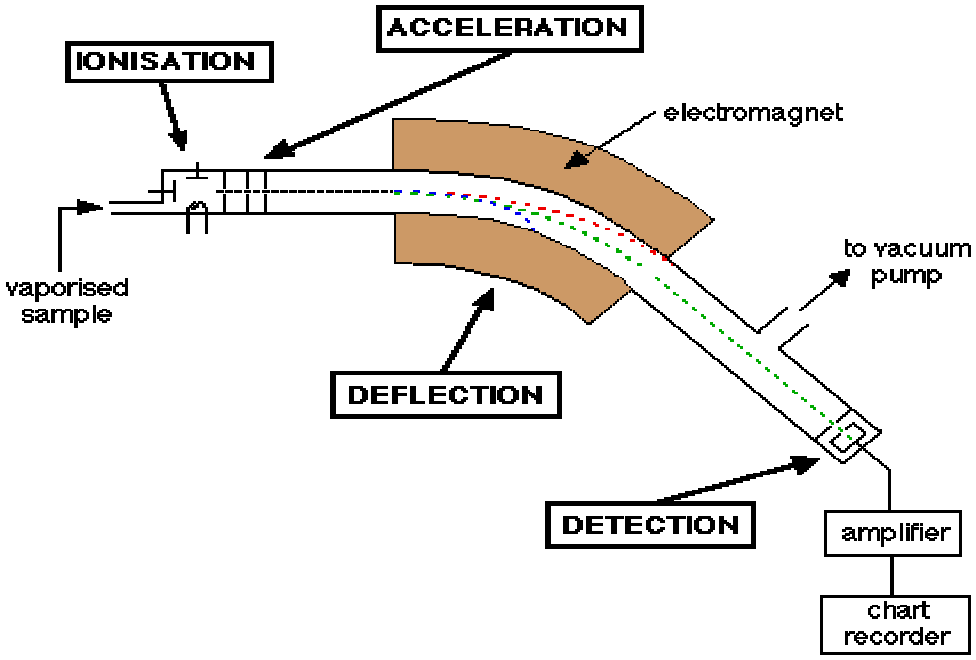


Figure 4: Basic diagram of a MALDI-MS/TOF instrument.
http://masspec.scripps.edu/information/history/

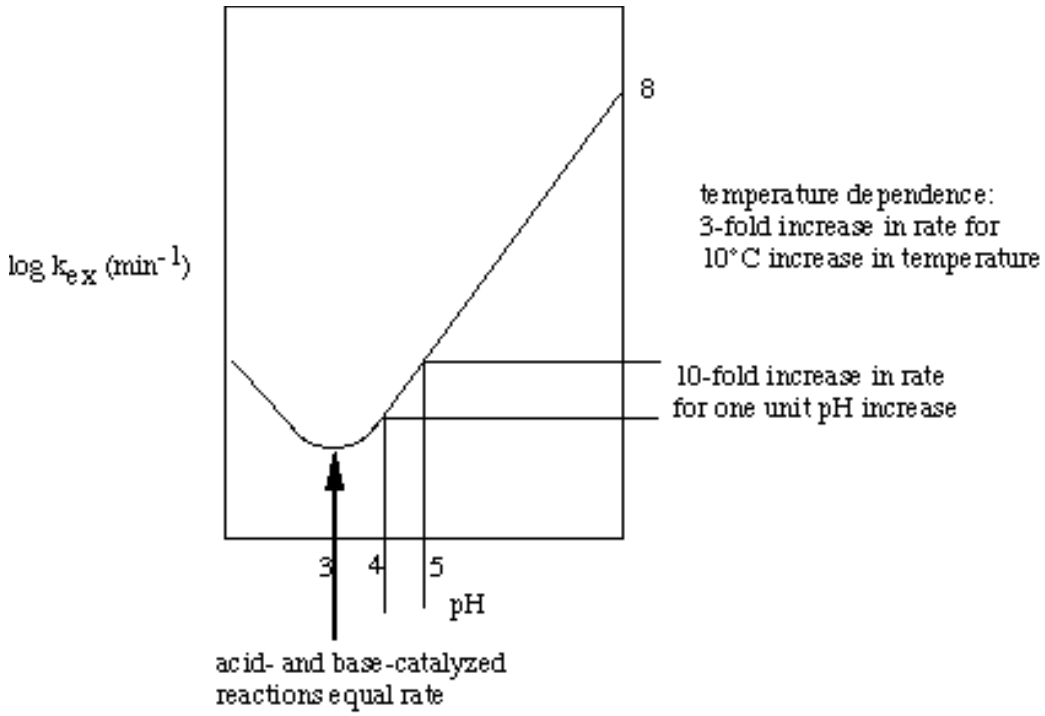


Figure 5: H/D exchange rate dependence on pH. In this figure, k_{ex} is equivalent to k_{obs} in this paper.
http://www.mbg.cornell.edu/nicholson/biobm631/folding/folding.html

RESULTS

Protein Production

The two proteins, MBP and Lambda repressor, were cloned in *E.coli* according to the techniques described in "Materials and Methods." In the beginning, several clones were tested for solubility based on where the 6xHis tag was attached. The proteins used in the latter experiments were MBP 1-357 tagged at the C-terminus and Lambda repressor 6-85 also tagged at the C-terminus. Interestingly, Lambda repressor 6-85 was not soluble when the tag was attached at the N-terminus. As the SDS-PAGE gels showed, the concentration of the protein was always very high (See Fig.6). The protein was purified through Ni-NTA column and Gel Filtration, resulting in >95% purity and yield of 1.81mg/ml for MBP and 2.95mg/ml for Lambda. (see Fig. 7). Total volume of MBP after purification was 37mls and OD 3.03 at A280. Total volume of Lambda after purification was 23mls and OD 0.87 at A280. The proteins were concentrated by centrifugation through concentrator columns to reach the final volume of 1.5mls and concentration of 24.72 mg/ml for MBP and 0.5mls and concentration of 13.98 mg/ml for Lambda.

Hydrogen/Deuterium Exchange by Nuclear Magnetic Resonance

For the initial protein assessment, protein samples were prepared by adding 15ul of D_2O to 300ul of MBP or Lambda repressor protein sample for final concentrations of 5% D_2O, 1.52mM of Lambda repressor and 0.60mM of MBP. The HSQC spectrum for Lambda repressor and the TROSY spectrum for MBP did not show aggregations of distorted peaks and therefore showed that both proteins were in

their stable native conformations (see Fig.8). For Lambda repressor H/D exchange experiment #1, the low salt buffer (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN3) was lyophilized, resuspended in D_2O, and the pH was adjusted to pH 6.4 with HCl (note that $pD=pH_{read}+0.4$). Then, 60ul of the protein was resuspended in 440ul of the deuterated buffer for NMR analysis at 10°C (see Fig.9). Analysis of the NMR data indicated that the peaks were initially extremely weak, indicating that the exchange was very rapid and almost all the residues exchanged within the first 10 minutes of the start of the exchange. Therefore, the peak height data obtained was not useful in that the protein has already exchanged and the peak heights were oscillating in the noise (see Fig. 10). It is important to note that distinct peaks were labeled with numbers and letters for identification purposes. The experiment was repeated with Lambda repressor as the NMR experiment #2. Since the peaks were extremely weak in the previous experiment, it was hypothesized the H/D exchange needed to be slowed down in order to be able to analyze the data. Therefore, 10mM ND_4Ac deuterated buffer, pH 4.0 was used for NMR analysis at 20°C (see Fig.11). The peaks were strong in the beginning of the experiment and even though only five peaks were visible after the 4th hour, they showed an exponential pattern of disappearance (see Fig. 12). Not only was the experimental data useful for further analysis but it also demonstrated the impact of lowering the pH from 6.3 to 4.0 on lowering the H/D exchange rate. The exchange rate was dramatically slower, even including the increase in temperature from 10°C to 20°C. Using Microsoft Excel program and the above data, natural logarithm of the peak heights was taken and the ln(peak height)

vs. time plot was generated (see Fig. 13). From that data, the following calculations were made:

Lambda repressor using NMR

k_{ex}(average)=0.7682/hr =0.0128/min

ln(Peak Height at t_0)=12.6796

Peak Height (average) at t_0=321,129

Disregarding the peak DZN-H:

k_{ex}(average)=0.8412/hr =0.01402/min

ln(Peak Height at t_0)=13.105

Peak Height (average) at t_0=491,393

The chemical rate of exchange was calculated using equations from Bai,Y. et.al (1993) paper as follows:

pD= 4.0+0.4=4.4

k_{ch}= $\log^{-1}$(1.62-4.4)+ $\log^{-1}$(10.05-11.25)+ $\log^{-1}$(-1.5)=9.64x10^{-2} min^{-1}

The ΔG_{op} was calculated as follows:

ΔG_{op}= -RTlnK_{op}= -(1.987cal·mol^{-1}·K^{-1})(293K)ln(0.0128/0.0964)=1,175.48cal/mol= =1.18kcal/mol

For MBP H/D exchange experiment, the protein was buffer exchanged with 20mM NH_4Ac, pH 6.3. Then, it was lyophilized in speed vacuum and resuspended in low salt (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN_3), pH 6.3 deuterated buffer for TROSY NMR analysis at 24°C (see Fig. 14). TROSY was used instead of HSQC because of the large size of the protein. The resulting spectra showed some peaks that never disappeared throughout the experiment (see Fig. 15). There were

other peaks that showed a gradual decrease (see Fig. 16) but it was not in a smooth fashion like was seen with Lambda repressor (Fig. 12). Instead, these peaks were decreasing in general but were decreasing and increasing in particular. It is important to note that the peak height was initially low and even though it was stronger than in Lambda repressor H/D experiment #1 (see Fig. 10), the peaks were still close to noise. This kind of pattern might indicate that some peaks exchanged quickly and showed a pattern of disappearance and some residues were hidden deep inside the protein where there would be little solvent accessibility and therefore did not show any indication of exchange in the time span of the experiment. This pattern might be indicative of the structural complexity of MBP. From those peaks that did exchange, using Microsoft Excel program and the above data, natural logarithm of the peak heights was taken and the ln(peak height) vs. time plot was generated (see Fig. 17). From that data, the following calculations were made in the following manner:

<u>MBP with NMR</u>

k_{ex}(average)=0.1114/hr =0.00186/min

ln(Peak Height at t_0)=11.973

Peak Height(average) at t_0=158,351

The chemical rate of exchange:

pD= 6.3+0.4=6.7

$k_{ch}= \log^{-1}(1.62-6.7)+ \log^{-1}(10.05-8.95)+ \log^{-1}(-1.5)= 12.62\ min^{-1}$ at 293K

Temperature correction:

$k_{ch}(297)= 12.62\cdot\exp(-17{,}000[-4.60\times10^{-5}]/1.987)= 31.21\ min^{-1}$

The ΔG_{op} was calculated as follows:

$\Delta G_{op}= -RTlnK_{op}= -(1.987 cal\cdot mol^{-1}\cdot K^{-1})(297K)ln(0.00186/31.21)=5{,}740.82 cal/mol=$

=5.74kcal/mol

Hydrogen/Deuterium Exchange by Mass Spectrometry

In the beginning of the experiments, was no protocol to operate by so, in trying to reproduce the experiments in Ghaemmaghami, et.al (2000) paper and in trying to obtain as accurate results as possible, many preliminary trials were made. First, to find out what was the largest optimal dilution, serial dilution experiments were performed. As a result of those experiments, the proteins were diluted in the subsequent experiments in the following ratios: 1:50 in the H/D exchange buffer and then 1:20 with the matrix, for a total dilution of 1:1000. Different buffers in matrix were also experimented with in order to bring the matrix to pH 3.0 and quench the back-exchange reaction (see "Discussion"). The homogeneity of the matrix was compromised and where Acetonitrile gave very low peaks, Citrate and Succinate often produced peaks that were just a little above the noise level. An H/D experiment #1 was performed with MBP in deuterated buffer (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN_3), pH 6.3 at 24°C (see Fig. 18). BSA was used as internal standard, which was not a good idea because of its heterogeneity and its large size, BSA had a higher standard deviation within the same well as the protein itself. As Fig. 19 shows, there was an increase in mass but that increase did not follow a smooth pattern and so could no be used for k_{ex} and ΔG calculations. The inaccurate data obtained was probably due to a large amount of back exchange, heterogeneity of the standard, as well as a possible handling error (see "Sources of Error"). The MBP H/D

exchange experiment #2 was done using the low salt deuterated buffer mentioned above as the exchange buffer at 24°C with Apomyoglobin and Aldolase as external standards. No buffer was used to calibrate the matrix to pH 3, so the pH of the matrix was 1.8, and the plate was previously frozen at -20°C and then put on ice under the hood for spotting(see Fig.20). The time it took for the samples to dry was about 4 minutes. The results demonstrate a gradual increase in mass (see Fig. 21). The success might be attributed to the better quenching of H/D back exchange reaction when the plate is on ice, and to the less heterogeneous external double standard used for calibration, but further research is needed to prove this. The rate of exchange and other calculations were made from the plot of ln (ΔM_{max}-$\Delta M_{t=0}$) vs. time (see Fig.22) in the following manner:

MBP with Mass Spectrometry

k_{ex}=0.246/hr =0.0041/min

$\ln(\Delta M_{max}-\Delta M_{t=0})$=5.5673

$\Delta M_{max}-\Delta M_{t=0}$=261.73

$\Delta M_{t=0}$=82.2735

The ΔG_{op} was calculated as follows:

k_{ch}= 31.21 min^{-1}

ΔG_{op}= $-RT\ln K_{op}$= $-(1.987 cal \cdot mol^{-1} \cdot K^{-1})(297K)\ln(0.0041/31.21)$=5,274.37cal/mol=

=5.27kcal/mol

Another experiment was done to monitor H/D exchange with different Guanidine concentrations (see Fig. 23). The results did not show any effect of Guanidine on the exchange reaction, perhaps because of inefficiency of the method in quenching the back-exchange reaction. An H/D exchange experiment was performed with Lambda

repressor using a low-salt phosphate buffer at pH 7.0 with Apomyoglobin as an internal standard (see Fig. 24). The experiment at pH 7.0 showed a very fast initial H/D exchange rate at the first minute and a very slow exchange rate after that (see Fig. 25). These results were consistent with the NMR data that showed peaks of very low intensity in the beginning of the experiment, suggesting that Lambda repressor exchanges very quickly. The fluctuations of the mass can be due to back exchange and experimental error. Because there was no gradual exchange curve observed, the experimental data could not be used for calculation analysis. Lambda repressor Mass Spectrometry H/D exchange experiment #2 was performed with modifications to the protocol. The exchange buffer used was 10mM ND_4Ac, pH 4.0 to reduce the H/D exchange rate and to reproduce the conditions in the NMR experiment (see Fig.26). The temperature was 22°C, which was the room temperature that could be monitored in the water bath. As can be seen in Fig. 27, the resulting curve showed a gradual exchange. That exchange could be measured by plotting $\ln(\Delta M_{max}-\Delta M_{t=})$ vs. time (see Fig. 28 and Fig. 29) and used for the rate of exchange and other calculations in the following manner:

<u>Lambda repressor with Mass Spectrometry</u>

k_{ex}=0.624/hr =0.0104/min

$\ln(\Delta M_{max}-\Delta M_{t=0})$=3.6015

$\Delta M_{max}-\Delta M_{t=0}$=36.653

$\Delta M_{t=0}$=50.347

Disregarding time points t=10 and t=60, the calculations are as follows:

k_{ex}=0.666/hr =0.0111/min

$\ln(\Delta M_{max}-\Delta M_{t=0})$=3.6015

ΔM_{max}-$\Delta M_{t=0}$=36.653

$\Delta M_{t=0}$=50.347

Temperature correction:

k_{ch}= 9.64x10^{-2} min^{-1} at 293K

$k_{ch}(295)$= 0.0964·exp(-14,000[-2.314x10^{-5}]/1.987)= 0.14 min^{-1}

The ΔG_{op} was calculated as follows:

ΔG_{op}= -RTlnK_{op}= -(1.987cal·mol^{-1}·K^{-1})(295K)ln(0.0111/0.14)=1,485.75cal/mol=

=1.49kcal/mol

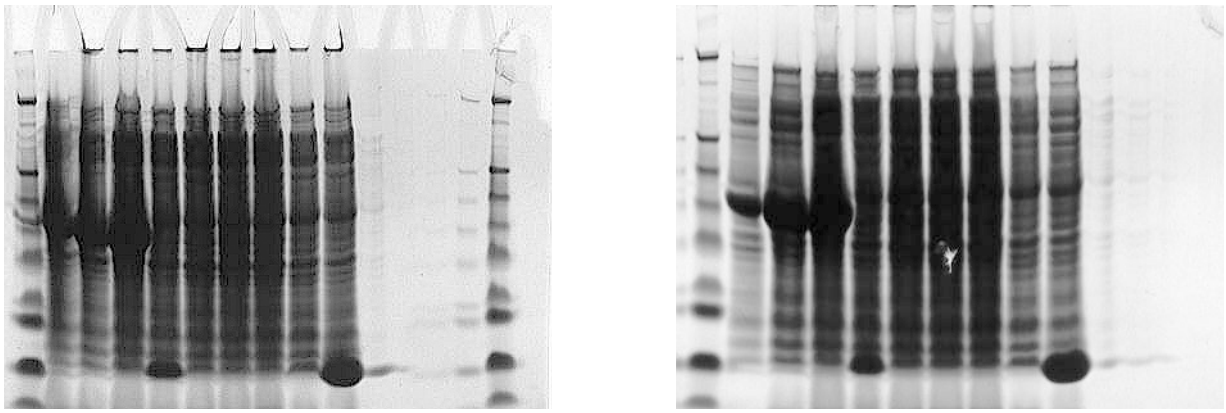
The first two time points (t=1 and t=10) could be disregarded for the calculations because they describe the fast-exchanging unprotected amide hydrogens. The last time point (t=60) was disregarded for the Lambda repressor Mass Spectrometry experiment #2 because the protein showed a decrease in mass, which is indicative of an error that could probably be attributed to a greater back exchange for that particular time point due to a handling mishap. It is also important to note that the plate used in the experiment was previously frozen at -20°C and then transferred to ice. Towards the end of the exchange experiment, the temperature in the previously frozen plate might have increased substantially to increase the rate of back-exchange.

As a result, the ΔG_{op} values were obtained but did not compare to the values in the literature. The results can be observed in the following table:

ΔGop values for Lambda and MBP in kcal/mol		
Lambda (literature)****	Lambda (NMR)**	Lambda (MS)***
5±0.4	1.18	1.49
MBP (literature)****	MBP (NMR)*	MBP (MS)*
16±3	5.74	5.27

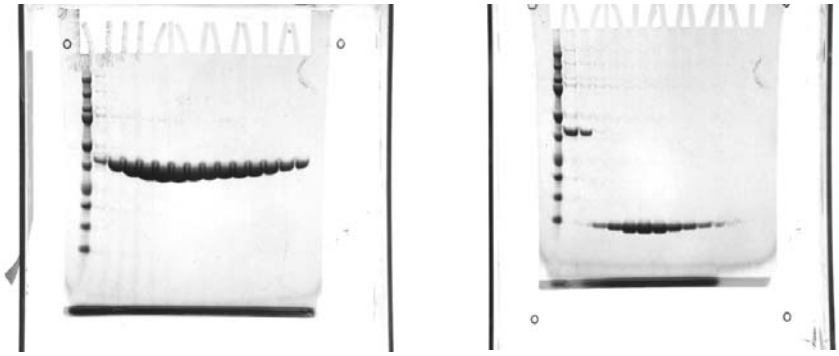
* Stabilities measured at 24°C and pH 6.3
** Stability measured at 20°C and pH 4.0
*** Stability measured at 22°C and pH 4.0
**** Stabilities measured using SUPREX technique from Ghaemmaghami, S. et.al. (2000) paper

The stability measurements for MBP could be disregarded because of the low quality of the data collected by both NMR and MS. For Lambda repressor, the k_{obs} might have been too high because some intermediate-exchanging amide hydrogens were most likely included in the rate determinations, see "Discussion."



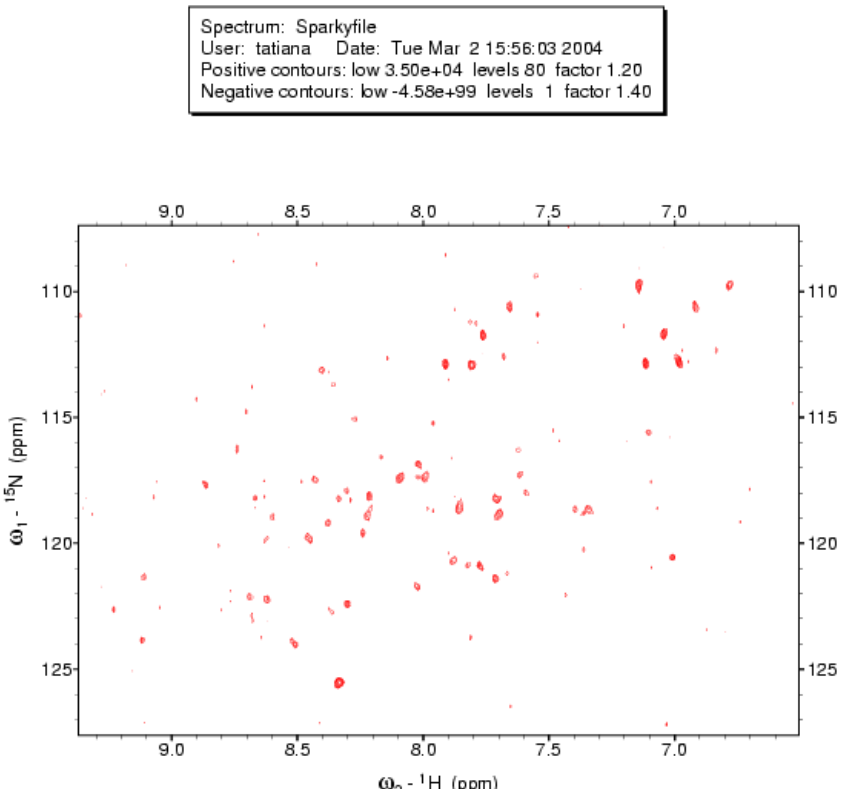
a.) b.)

Figure 6: Gel pictures of production. Lanes 1-3 are MBP. Lanes 4-9 are Lambda repressor. The proteins picked for experiment are MBP from lane 3 and Lambda repressor from lane 4. a) Expression. b.) Solubility.



a.) b.)

Figure 7: SDS-PAGE gels of Gel Filtration-purified proteins. a.) MBP in lanes 15-28 b.) MBP in lanes 29-30, Lambda repressor in lanes 34-41.



a.)

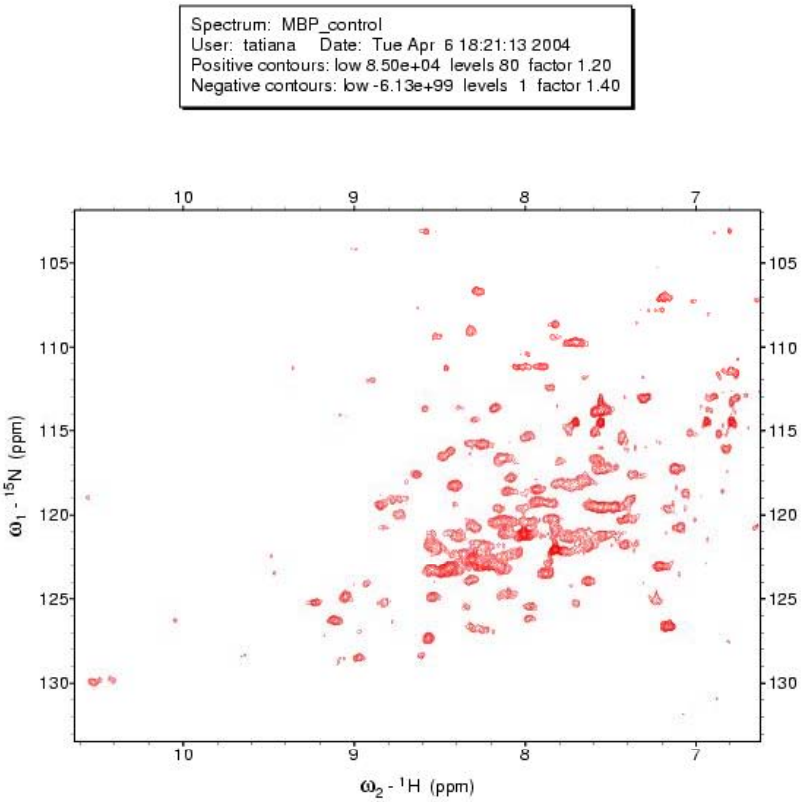


Figure 8: a.) HSQC spectrum of hydrogenated Lambda repressor b.) TROSY spectrum of hydrogenated MBP.

b.)

NMR Protocol #1 for Lambda repressor

- Use Varian 600
- 10°C, pH 6.3
- Mix 60ul of protein with 440ul of D_2O buffer in NMR tube for 1.68 mg/mL concentration
- Buffer: lyophilize low salt phosphate buffer (20mM NaPi, 20mM NaAc, 100mM NaCl, 0.02% NaN_3, pH 6.3), re-suspend in D_2O, adjust pH with HCl
- HSQC data collected for 14 hours

Figure 9: NMR protocol for Lambda repressor NMR H/D exchange experiment #1.

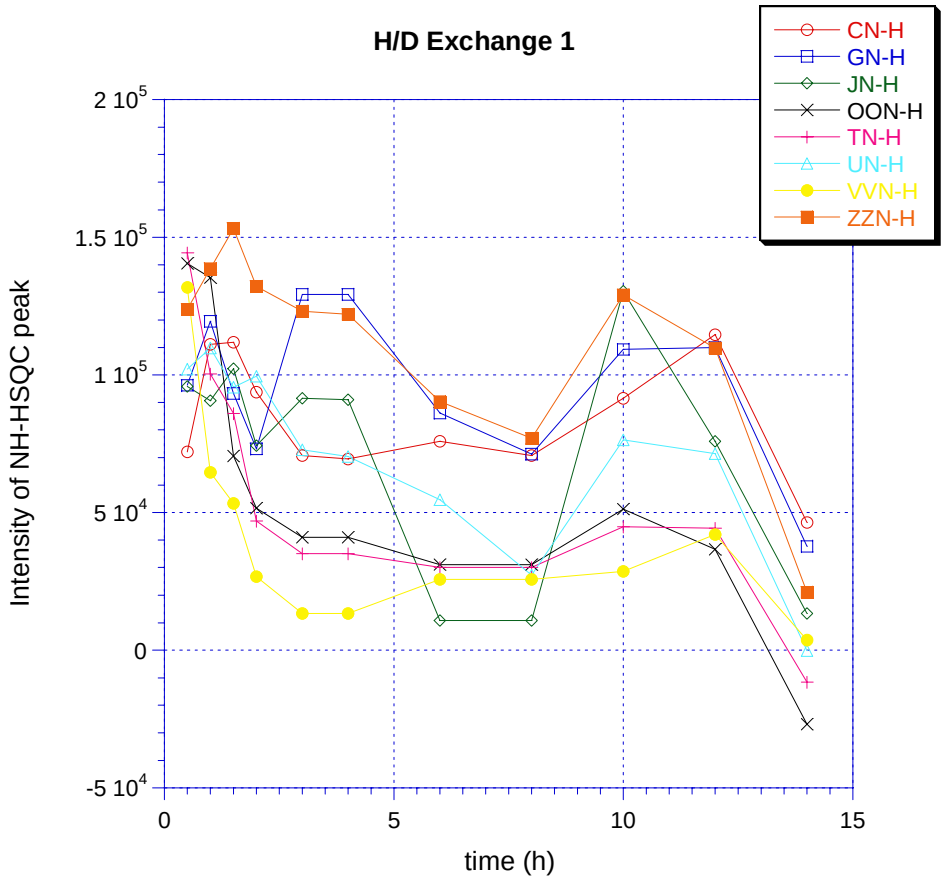


Figure 10: Peak heights vs. time for Lambda repressor NMR H/D exchange experiment #1. The peaks show no pattern of disappearance.

NMR Protocol #2 for Lambda repressor

- Use Varian 500
- 20°C, pH 4.0
- Mix 1.52mg of lyophilized protein with 500ul of D_2O buffer in NMR tube for 3.04mg/mL concentration
- Buffer: 10mM ND_4Ac
- HSQC data collected for 14 hours

Figure 11: Protocol for Lambda repressor NMR H/D exchange experiment #2.

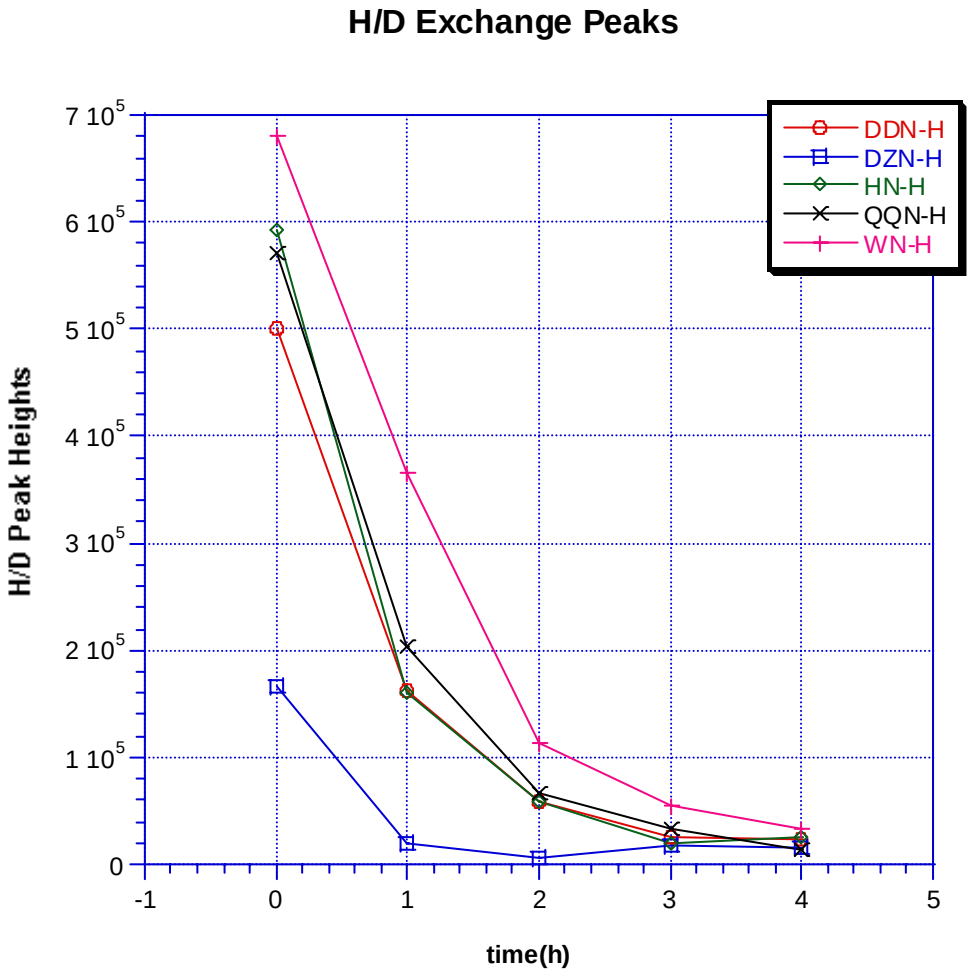
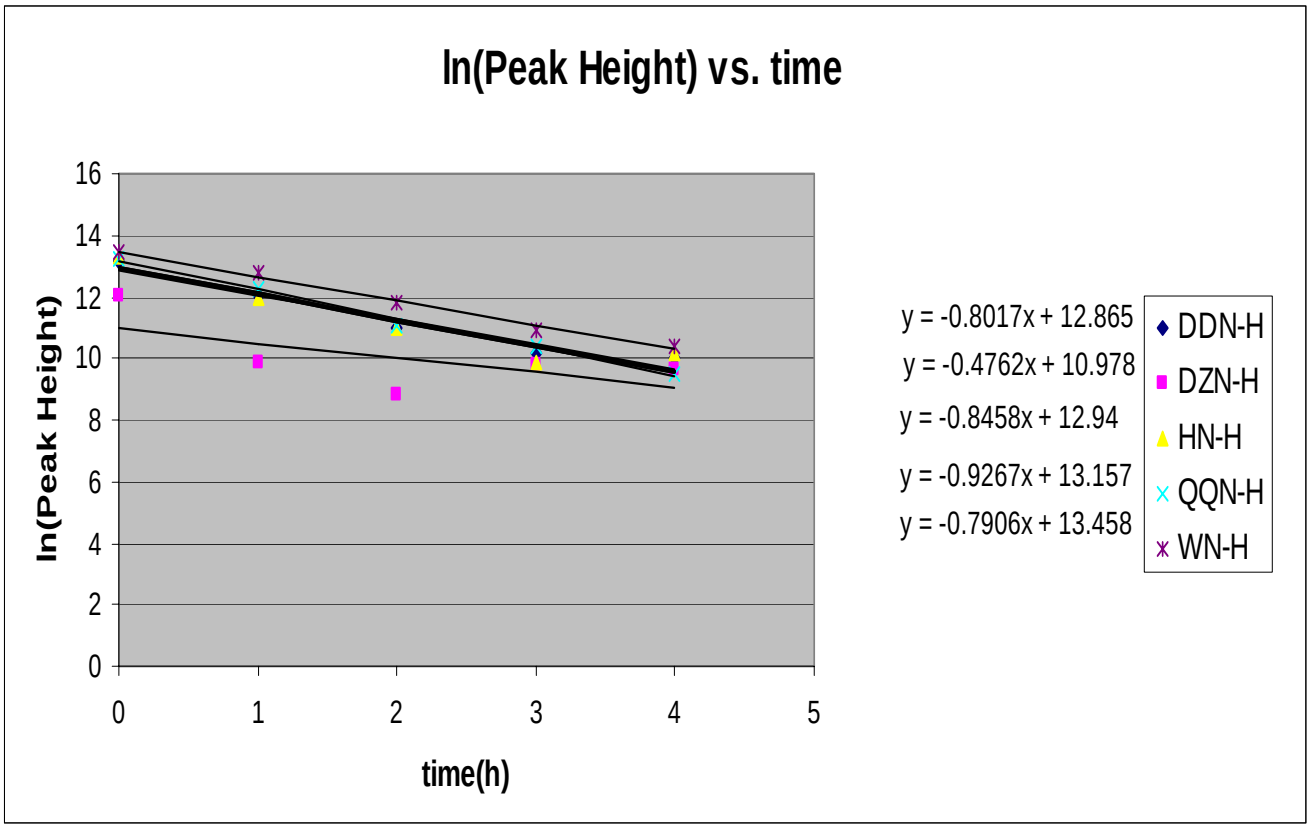


Figure 12: Peak heights vs. time for Lambda repressor NMR H/D exchange experiment #2. The peaks show an exponential pattern of disappearance.



		ln (Peak Heights)			
	DDN-H	DZN-H	HN-H	QQN-H	WN-H
0	13.12396	12.01829	13.29304	13.25576	13.42968
1	12.00049	9.859692	11.98165	12.22685	12.80804
2	10.97893	8.786609	10.99264	11.09548	11.80399
3	10.18029	9.793282	9.843631	10.41012	10.92111
4	10.02553	9.670546	10.13293	9.530683	10.42008

Figure 13: ln(peak height) vs. time plot for Lambda repressor NMR H/D experiment #2.

NMR Protocol for MBP

- Use Varian 600
- 24°C, pH 6.3
- Mix 2.47mg of protein (buffer exchanged with 20mM NH_4Ac, lyophilized) with 300ul of buffer in NMR tube
- Buffer: deuterated low salt phosphate buffer (20mM NaPi, 20mM NaAc, 100mM NaCl, 0.02% NaN_3, pH 6.3)
- TROSY data collected for 15 hours

Figure 14: Protocol for MBP NMR H/D exchange experiment.

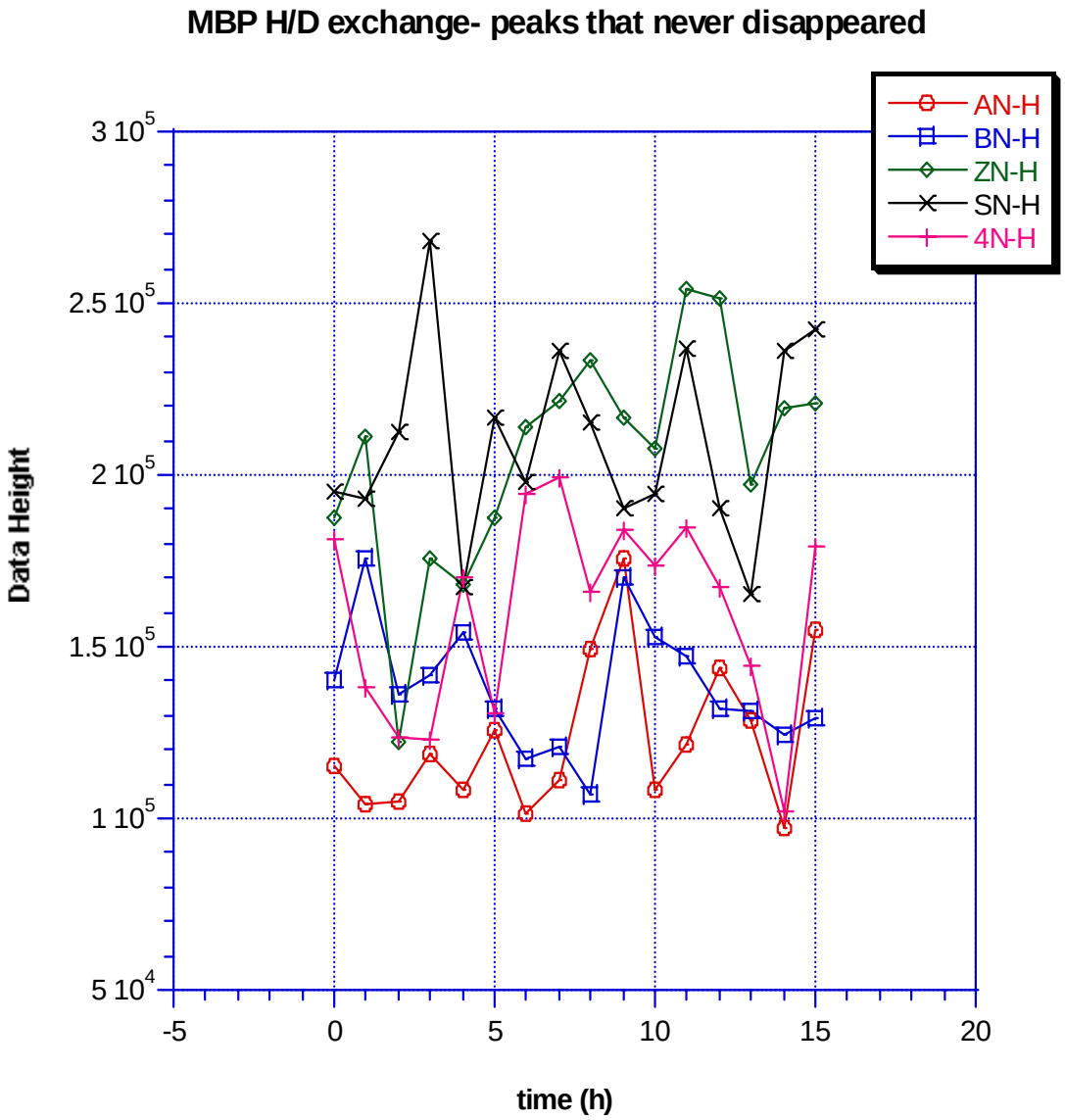


Figure 15: Peak Height vs. time for MBP NMR H/D exchange experiment. These are the peaks that remained throughout the 16 hours of the exchange reaction.

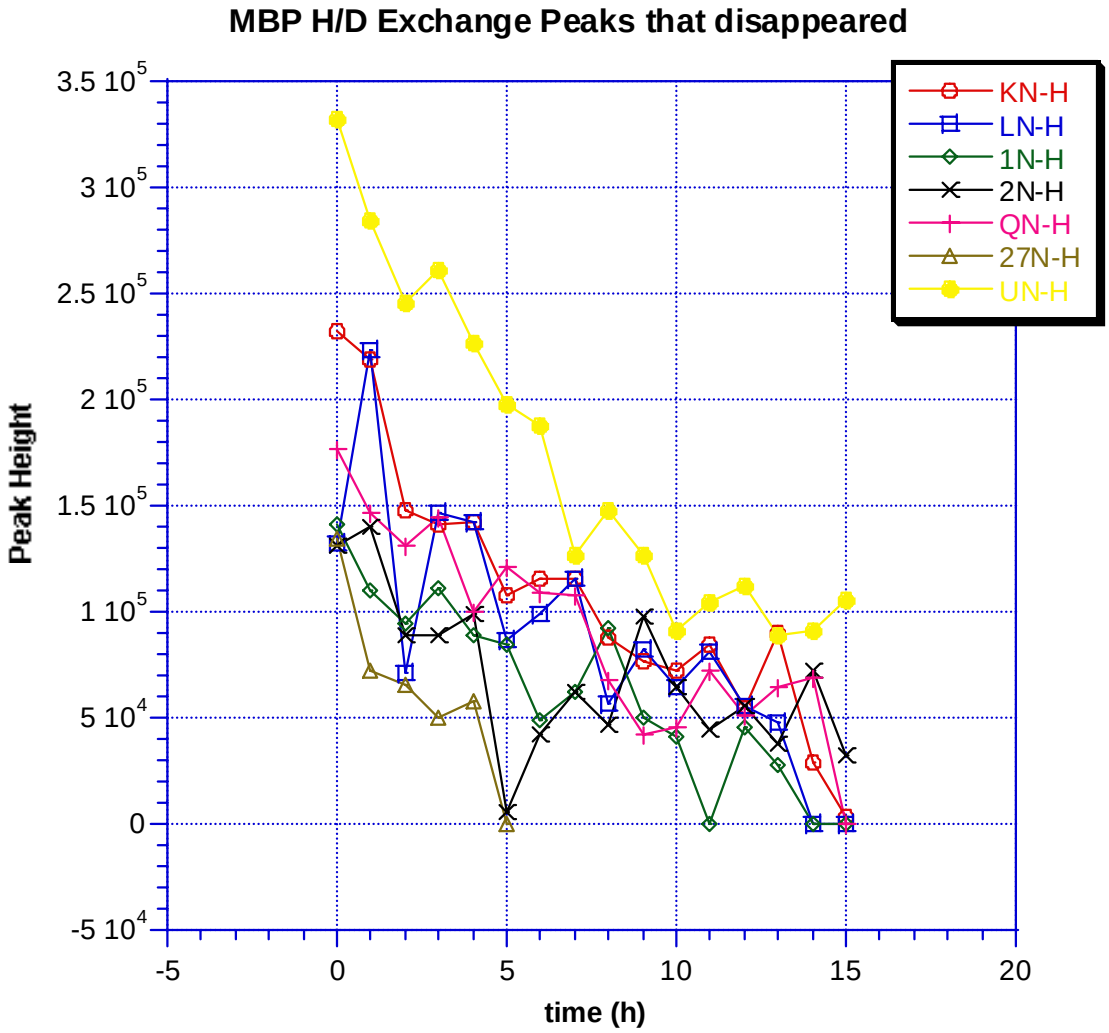
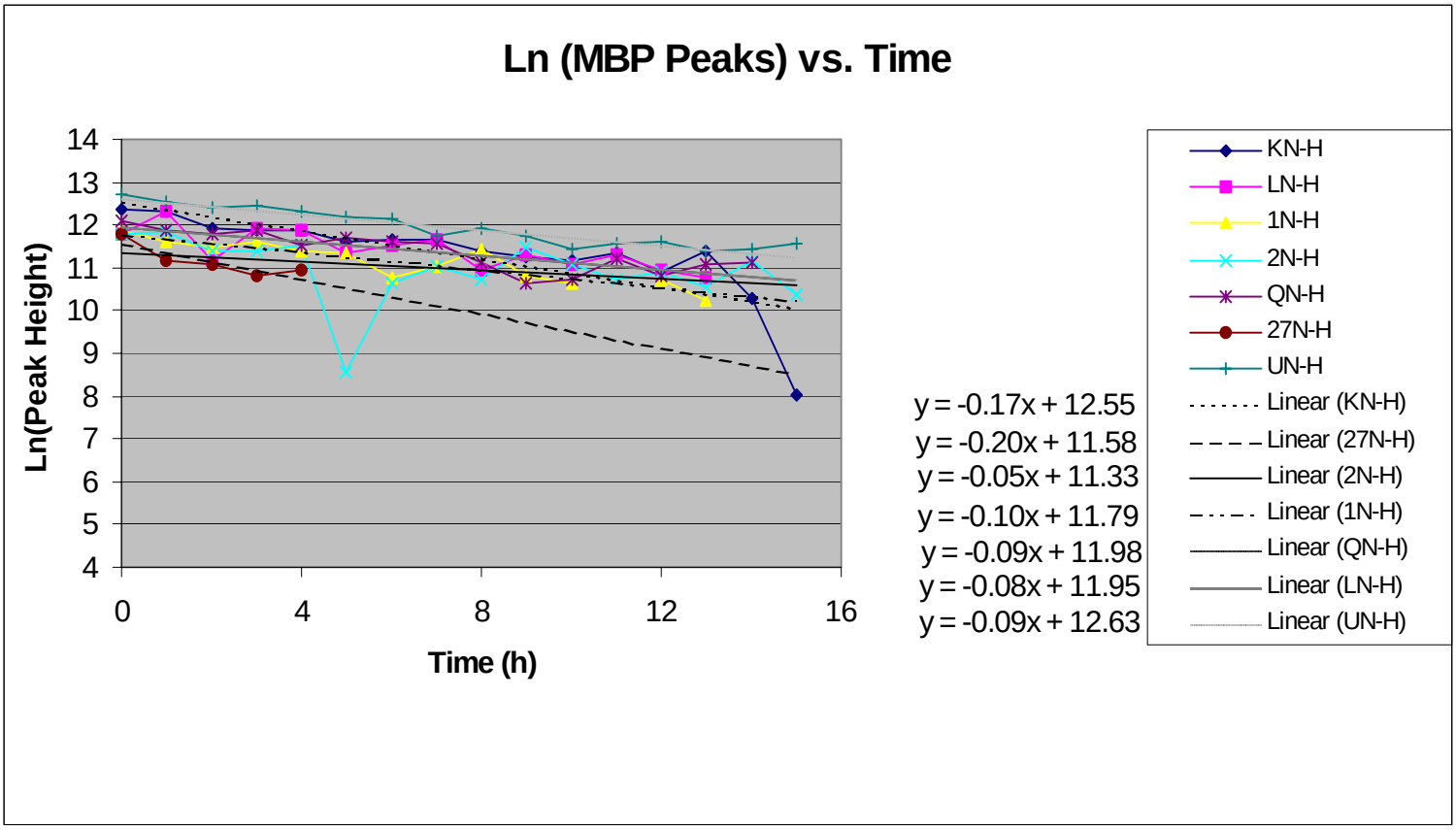


Figure 16: Peak Height vs. time for MBP NMR H/D exchange experiment. These are the peaks that demonstrated a pattern of disappearance through the course of the exchange reaction.



	ln (MBP H/D Exchange Peaks That Disappeared)						
Time (h)	KN-H	LN-H	1N-H	2N-H	QN-H	27N-H	UN-H
0	12.35	11.79	11.86	11.78	12.08	11.81	12.71
1	12.30	12.32	11.61	11.85	11.90	11.18	12.56
2	11.90	11.18	11.46	11.40	11.78	11.09	12.41
3	11.86	11.90	11.62	11.39	11.88	10.83	12.47
4	11.87	11.87	11.40	11.51	11.51	10.96	12.33
5	11.59	11.37	11.34	8.54	11.71		12.20
6	11.66	11.50	10.79	10.65	11.59		12.14
7	11.66	11.66	11.03	11.04	11.59		11.75
8	11.38	10.95	11.43	10.75	11.12		11.90
9	11.24	11.32	10.82	11.49	10.65		11.75
10	11.19	11.07	10.63	11.08	10.74		11.42
11	11.34	11.31		10.71	11.19		11.55
12	10.90	10.94	10.72	10.92	10.83		11.63
13	11.41	10.77	10.24	10.54	11.08		11.40
14	10.29			11.18	11.14		11.42
15	8.02			10.38			11.57

Figure 17: ln(peak height) vs. time plot for MBP H/D experiment.

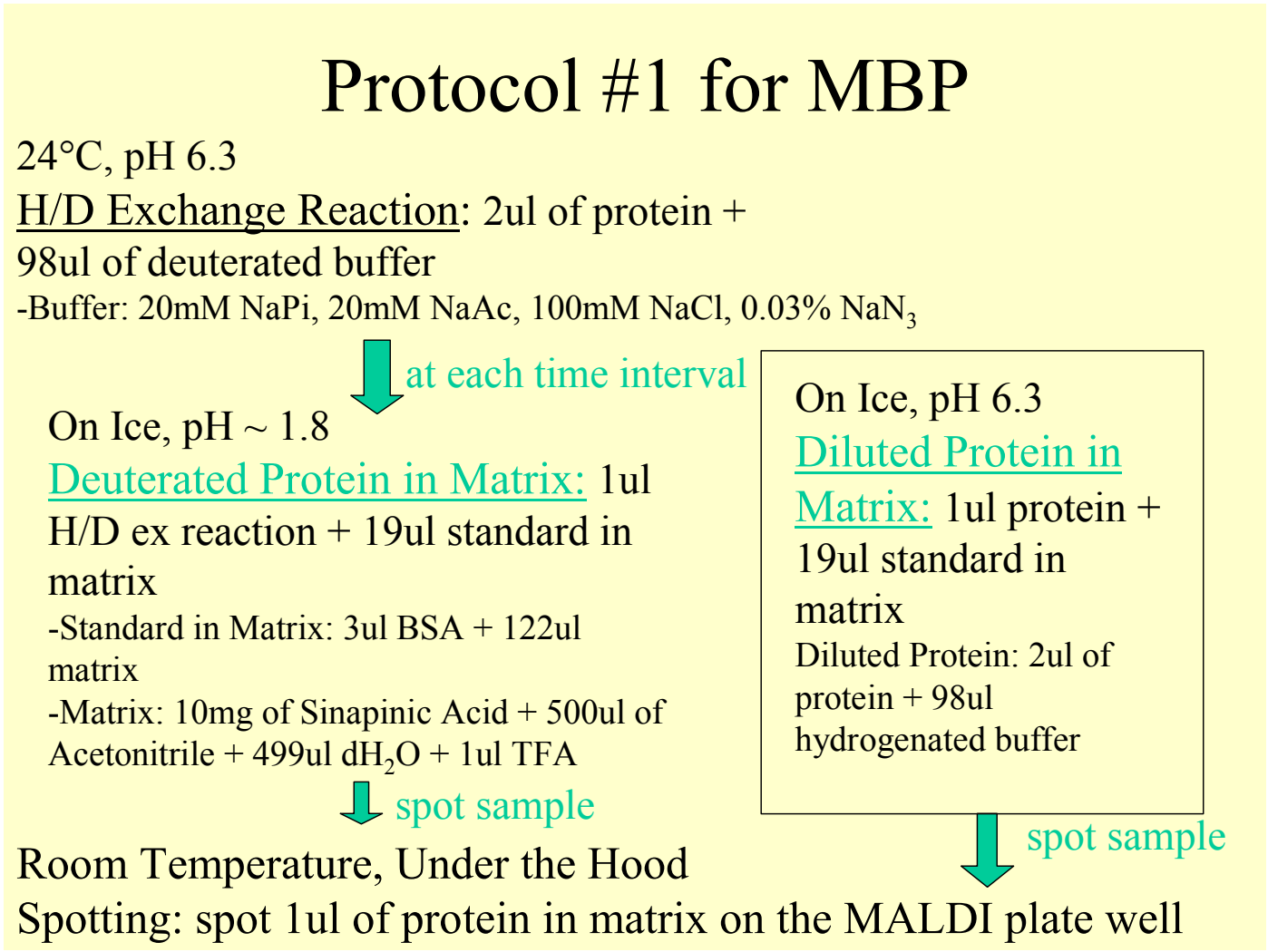
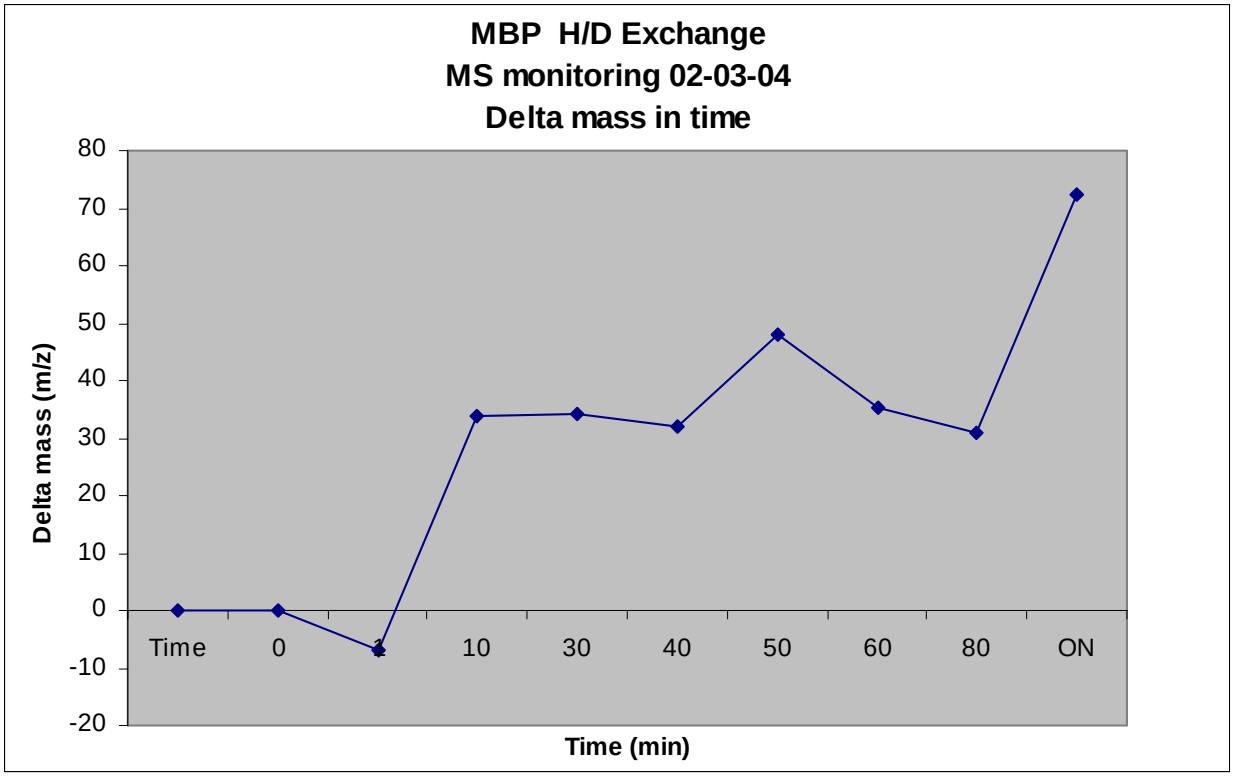


Figure 18: Protocol for MBP Mass Spectrometry H/D exchange experiment #1



	40817.45		
	MBP		
Time	Average m/z	Delta mass	SD
0	40817.45	0.00	4.56
1	40810.50	-6.95	6.48
10	40851.34	33.89	13.56
30	40851.64	34.19	13.15
40	40849.29	31.84	8.39
50	40865.40	47.95	22.79
60	40852.60	35.15	9.36
80	40848.24	30.79	6.48
ON	40889.77	72.32	11.99

Figure 19: Change in mass vs. time of MBP H/D exchange #1using BSA as an internal standard in matrix. The table provides the original masses of the protein, as well as the standard deviation of the data in each well.

Protocol #2 for MBP

24°C, pH 6.3

H/D Exchange Reaction: 2ul of protein + 98ul of deuterated buffer

-Buffer: 20mM NaPi, 20mM NaAc, 100mM NaCl, 0.03% NaN_3

↓ at each time interval

On Ice, pH ~ 1.8
Deuterated Protein in Matrix: 1ul H/D ex reaction + 19ul matrix
-Matrix: 10mg Sinapinic Acid + 500ul Acetonitrile + 499ul dH_2O + 1ul TFA

On Ice, pH 6.3
Diluted Protein in Matrix: 1ul protein + 19ul matrix
-Diluted Protein: 2ul of protein + 98ul hydrogenated buffer

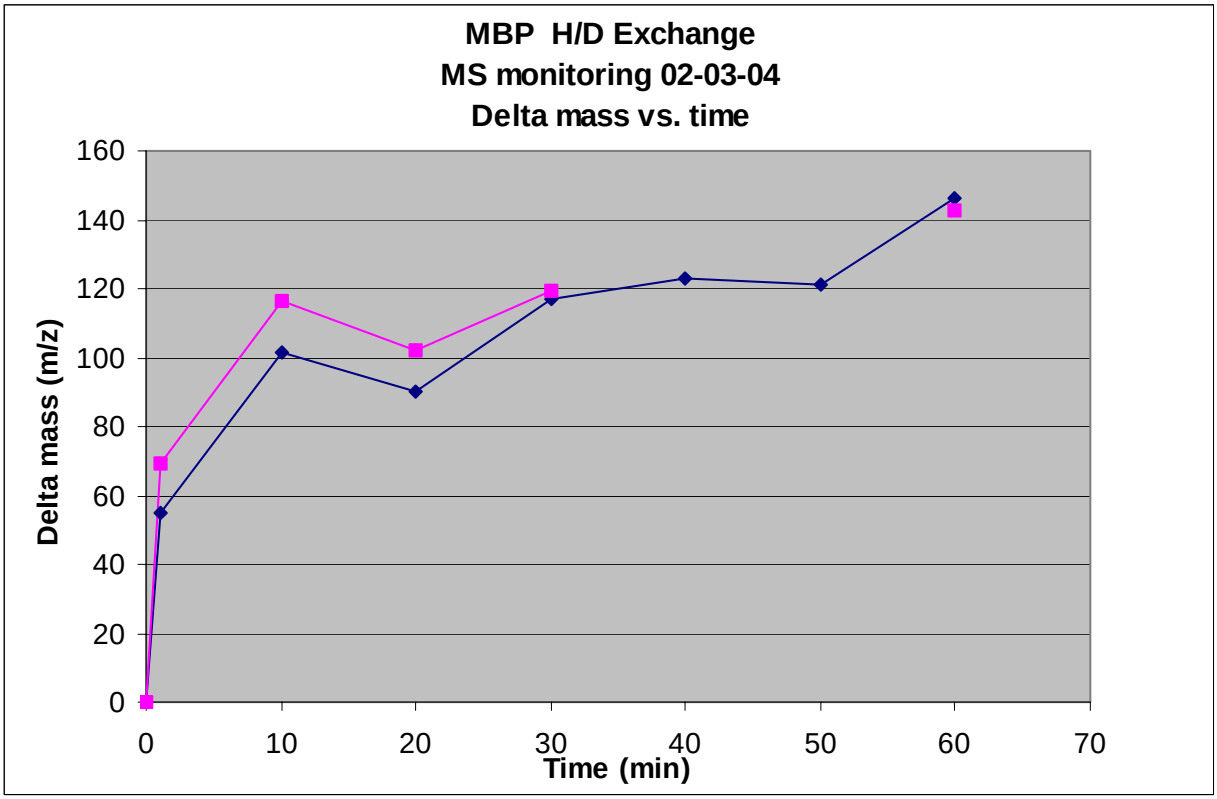
On Ice, pH ~ 1.8
External Standard in Matrix I: 1ul Aldolase + 1ul Apomyoglobin + 38ul matrix
External Standard in Matrix II: 20ul ESM I + 20ul matrix

↓ spot sample ↓ spot sample ↓ spot sample

Previously at -20°C, kept On Ice under the hood

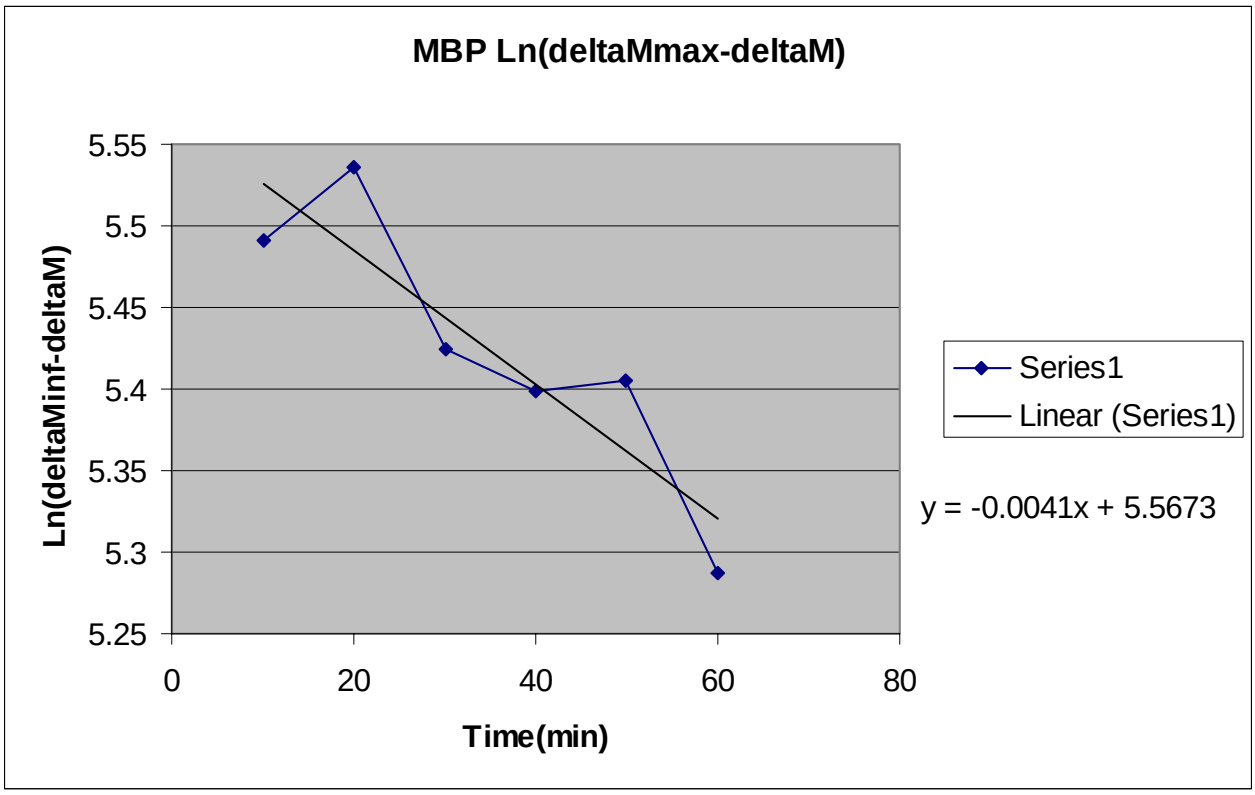
Spotting: spot 1ul of protein in matrix in each well on the MALDI plate

Figure 20: Protocol for MBP Mass Spectrometry H/D exchange experiment #2.



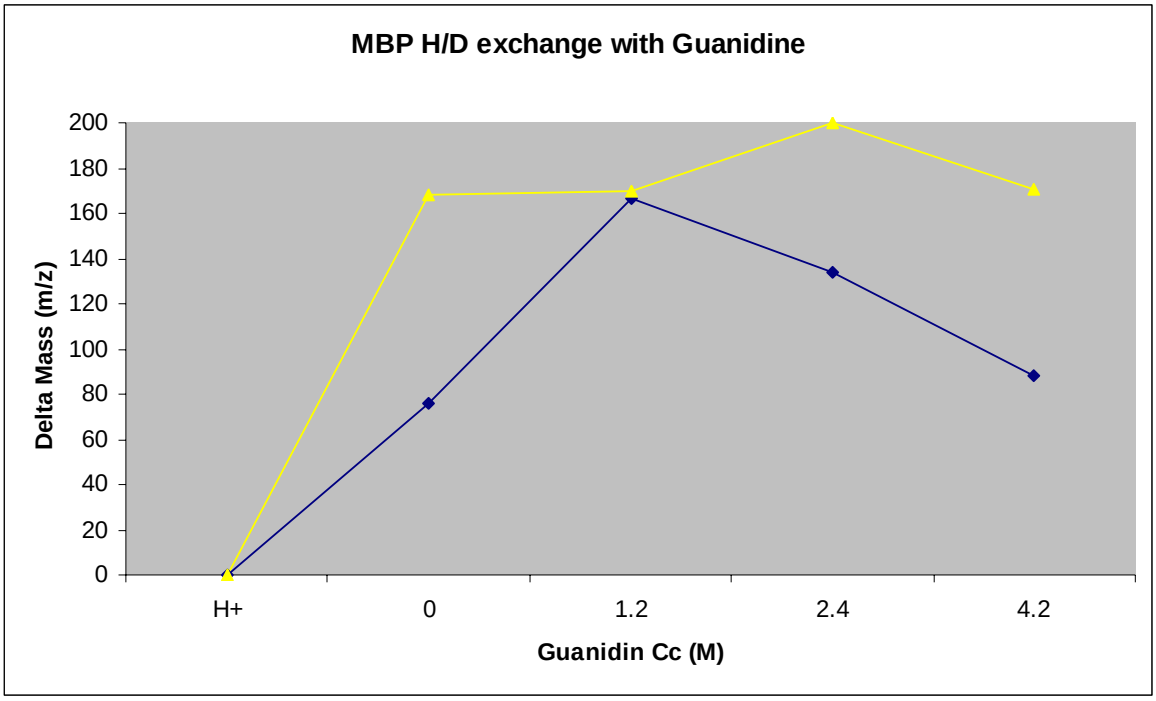
	MBP			MBP		
Time	Average m/z	Delta mass	SD	Average m/z	Delta mass	SD
0	40709.96	0.00	3.55	40710.20	0.00	5.40
1	40765.16	55.20	2.42	40779.21	69.01	6.88
10	40811.50	101.54	4.95	40826.46	116.26	4.23
20	40800.36	90.40	6.17	40812.02	101.81	2.97
30	40827.19	117.23	4.91	40829.48	119.28	2.92
40	40832.82	122.86	1.90			
50	40831.43	121.47	4.93			
60	40856.27	146.31	6.80	40853.02	142.82	7.19
ON						

Figure 21: Change in mass vs. time plot of MBP H/D exchange #2 using Aldolase and Apomyoglobin as external standards. Duplicates were done in parallel from the same reaction tube but in different matrix tubes to show the reproducibility of the method. The table provides the original masses of the protein, as well as the standard deviation of the data in each well.



	dMmax=	344	
	MBP		
Time=x	delta M	dMmax-dM	ln(dMmax-dM)
10	101.54	242.46	5.49
20	90.40	253.60	5.54
30	117.23	226.77	5.42
40	122.86	221.14	5.40
50	121.47	222.53	5.41
60	146.31	197.69	5.29

Figure 22: $\ln(\Delta Mass_{max} - \Delta Mass)$ vs. time for MBP Mass Spectrometry H/D exchange #2.



	MBP			MBP Calibrated		
Guanidin CC (M)	Average m/z	SD	Delta mass	Calibrated Average m/z	SD	Delta mass
H+	40768.33	5.96	0	40664.73	32.66	0.00
0	40843.98	5.5	75.648	40833.19	28.56	168.46
1.2	40935.14	5.06	166.808	40834.16	50.12	169.44
2.4	40902.3	4.83	133.972	40865.03	35.13	200.30
4.2	40856.33	4.91	88	40835.13	7.4	170.40

Figure 23: Change in mass of MBP calibrated with BSA as an internal standard. The blue line represents MBP without calibration to BSA and the yellow line represents MBP with calibration to BSA.

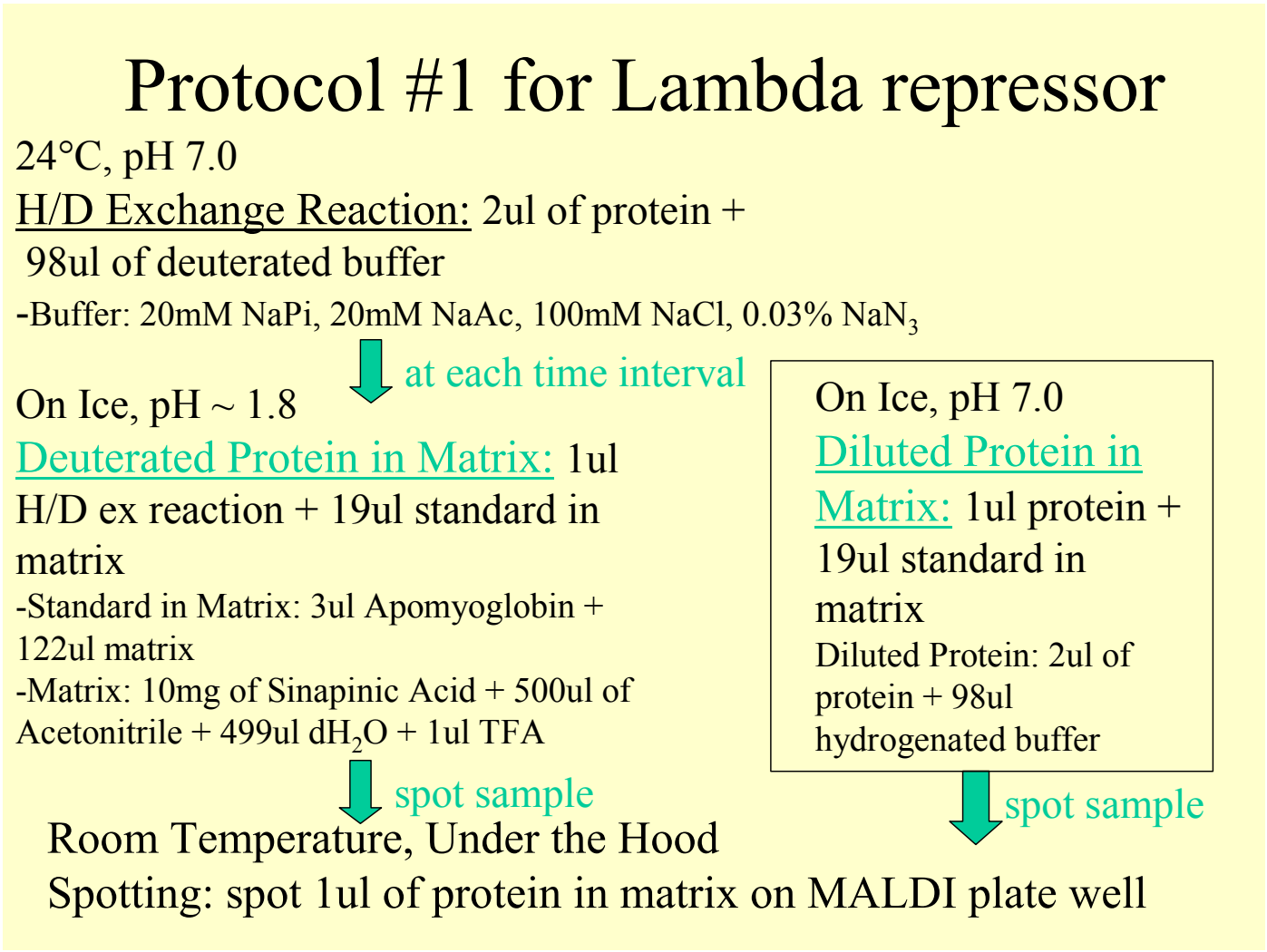
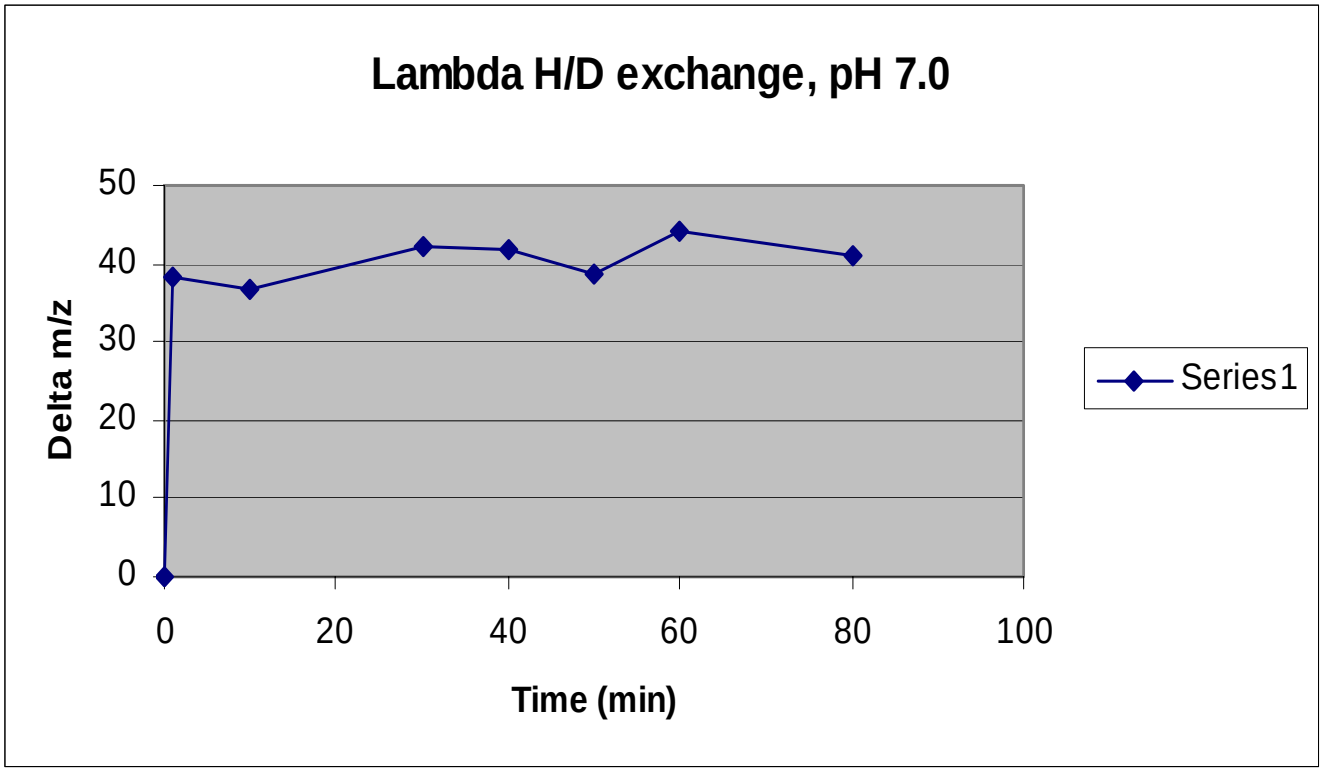


Figure 24: Protocol for Lambda repressor Mass Spectrometry H/D exchange experiment #1



	LAMBDA		
Time	m/z	Delta m/z	SD
0	10046.1	0.00	0.67
1	10084.56	38.46	3.37
10	10082.63	36.54	2.25
30	10088.33	42.24	3.06
40	10087.98	41.89	3.82
50	10084.58	38.48	5.72
60	10090.09	43.99	2.12
80	10087.08	40.98	4.84
ON	10087.26	41.16	6.49

Figure 25: Change in mass vs. time for Lambda repressor H/D exchange #1 at pH 7.0 in the phosphate low salt buffer and with Apomyoglobin as internal standard. The table shows the original m/z values and the standard deviation within each well.

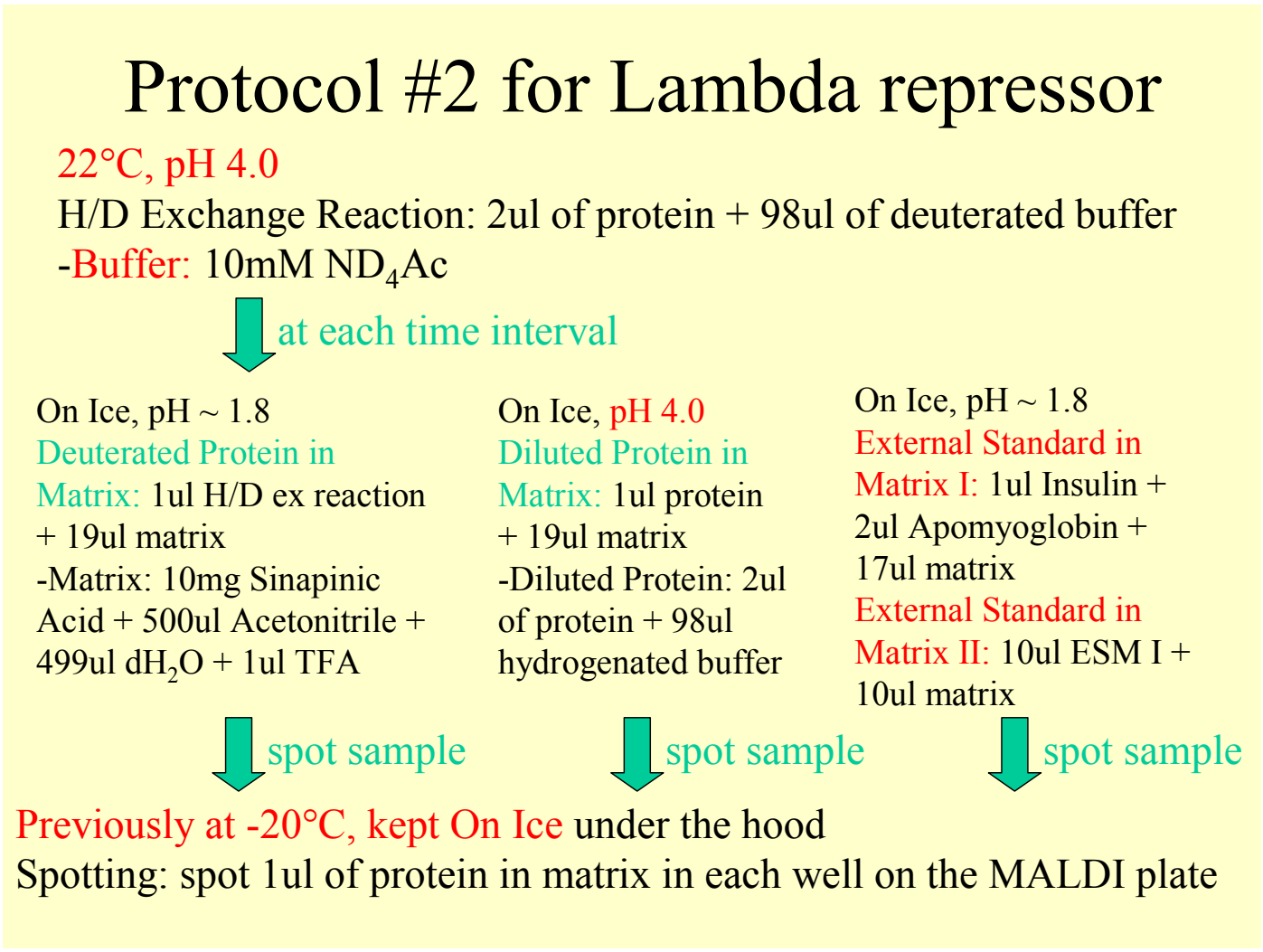
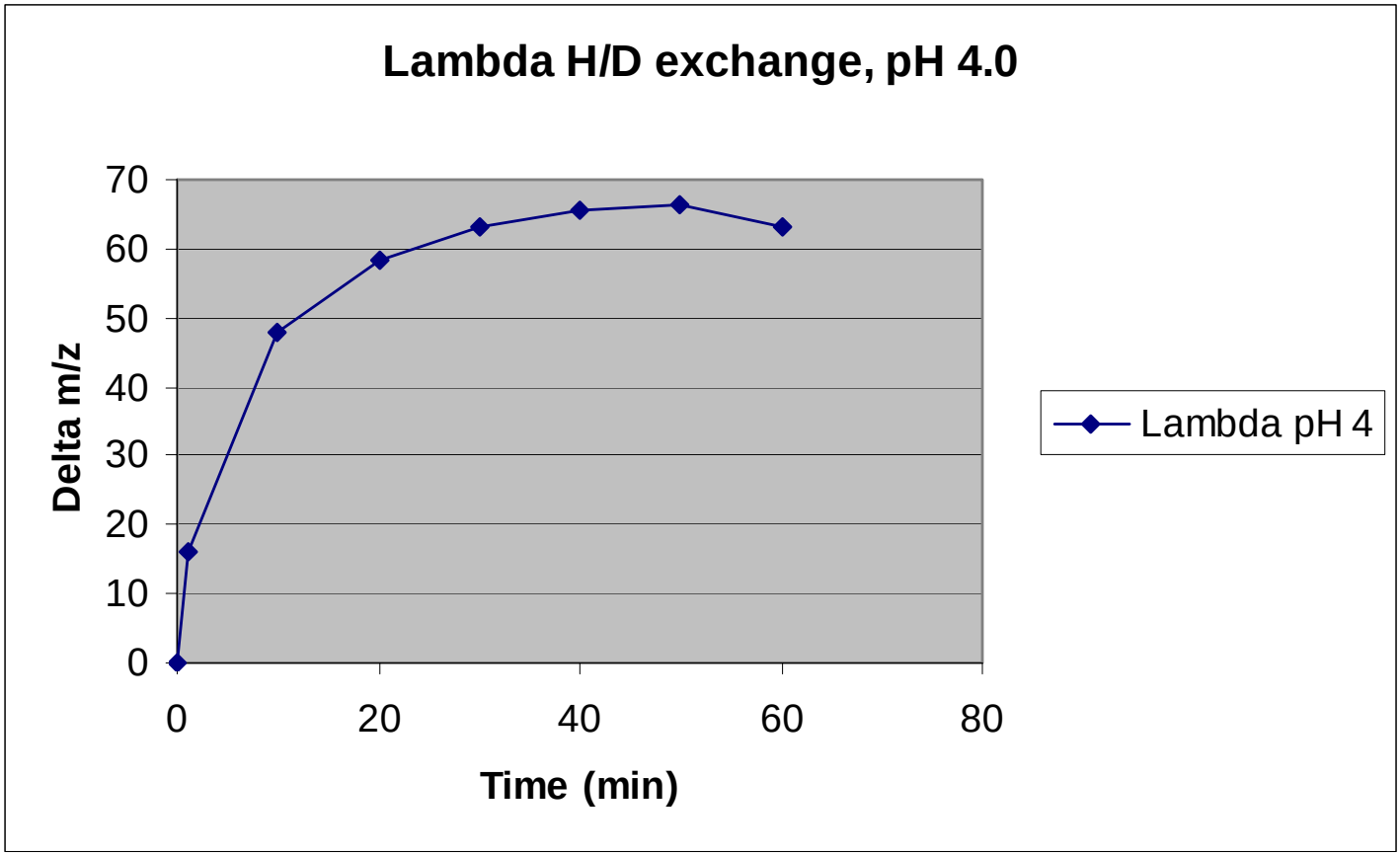
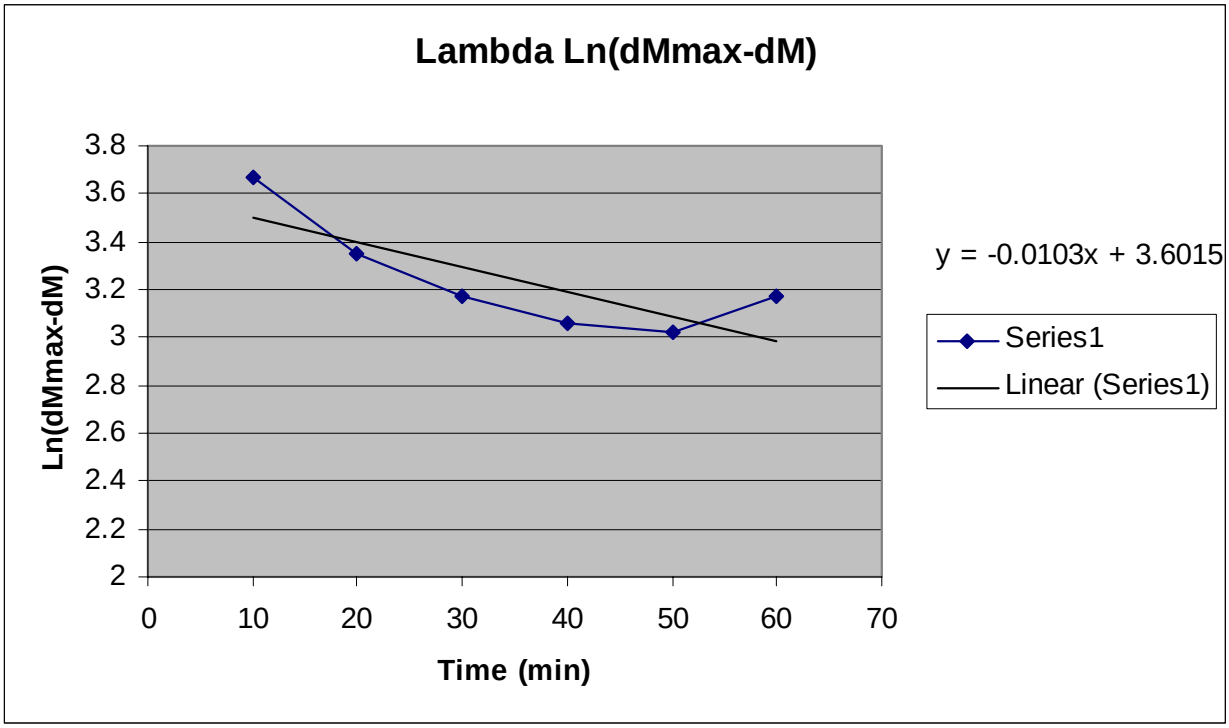


Figure 26: Protocol for Lambda repressor Mass Spectrometry H/D exchange experiment
#2.



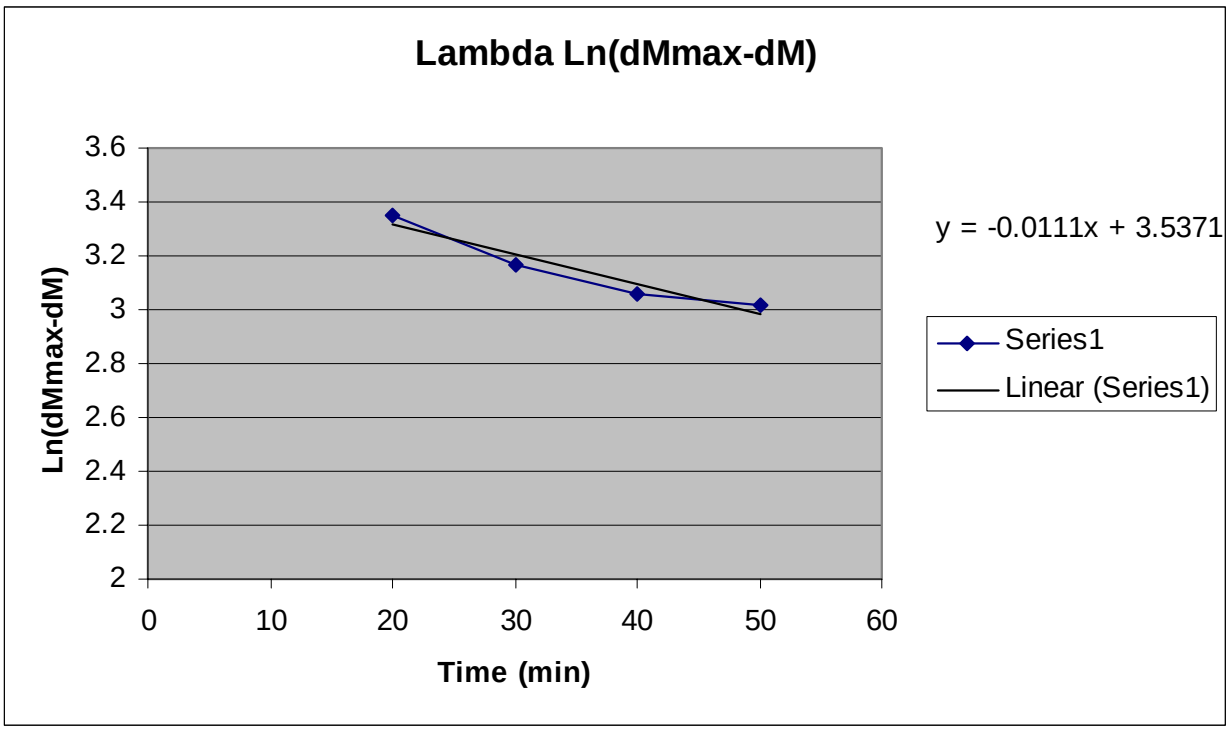
Time	Lambda Average m/z	TFA Delta mass	SD
0	10055.64	0.00	3.73
1	10071.57	15.92	0.86
10	10103.34	47.69	1.49
20	10114.09	58.45	1.01
30	10118.89	63.25	2.56
40	10121.37	65.73	0.70
50	10122.18	66.54	0.64
60	10118.86	63.22	3.74

Figure 27: Change in mass vs. time for Lambda repressor H/D exchange #2 at pH 4.0 in the Ammonium Acetate buffer and with Apomyoglobin and Insulin as external standards.



	dMmax=	87	
	Lambda		
Time=x	delta M	dMmax-dM	ln(dMmax-dM)
10	47.69	39.31	3.67
20	58.45	28.55	3.35
30	63.25	23.75	3.17
40	65.73	21.27	3.06
50	66.54	20.46	3.02
60	63.22	23.78	3.17

Figure 28: $\ln(\Delta Mass_{max} - \Delta Mass)$ vs. time for Lambda repressor Mass Spectrometry H/D exchange #2.



	dMmax=	87	
	Lambda		
Time=x	delta M	dMmax-dM	ln(dMmax-dM)
20	58.45	28.55	3.35
30	63.25	23.75	3.17
40	65.73	21.27	3.06
50	66.54	20.46	3.02

Figure 29: $\ln(\Delta Mass_{max} - \Delta Mass)$ vs. time for Lambda repressor Mass Spectrometry H/D exchange #2 disregarding time points t=10 and t=60.

DISCUSSION

H/D exchange is a very useful tool in assessing protein stability and behavior. As deuterium gets incorporated into the protein, the protein gains in mass and that gain can be observed with Mass Spectrometry. This is a technique with a high-throughput potential that measures the mass of the protein in very tiny quantities, does not involve lengthy and intricate data collection and is tolerant to impurities in the sample. Measuring the deuterium incorporation into the protein over time, the rate of exchange is obtained from which protein structure stability measurements can be calculated. This technique can be very useful, especially for the industry, if it can be automized and performed in a high-throughput fashion, saving a lot of time, money, manpower and resources.

In this experiment, I have assessed the protein stability through monitoring the rates of hydrogen/deuterium exchange. As the protein's hydrogen atoms exchange with the solution's deuterium atoms, the protein gains mass and that can be measured either by watching the disappearance of the peaks through NMR Spectroscopy or directly measuring the mass of the protein at different times of exchange by Mass Spectrometry. My goal for the project was to obtain the exchange measurements through Mass Spectrometry and to use NMR Spectroscopy as a technique for comparison. Although the process might sound simple, there is a lot of work involved in improving the techniques and optimizing the methods. In Mass Spectrometry, the main preoccupation was quenching the H/D exchange at the time the protein was

mixed into the matrix, which is hydrogenated. Because of the artificial denaturing conditions of the matrix, it cannot be

deuterated because then there will be an artificial incorporation of deuterium atoms into the protein, or as it is called, the in-exchange. On the other hand, when the protein is placed into a hydrogenated matrix under denaturing conditions of pH 1.8, the protein then undergoes a back exchange, which can be pretty significant. It is known that H/D exchange is slowest at pH of about 3. Adjusting the pH of the matrix to 3 could theoretically decrease the H/D exchange by more that 20-fold (Kipping, M. et.al). We have tried several buffers to achieve that pH 3. The first set of buffers was as follows: 1.) 50% Acetonitrile, 0.1% TFA, and 25mM Ammonium Acetate, pH 3.02, 2.) 50% Acetonitrile, 24mM Ammonium Acetate, pH 2.95, 3.) 50% Acetonitrile, 0.1% TFA, 25mM Succinate, 25mM Citrate, pH 3.01, 4.) 50% Acetonitrile, 25mM Succinate, 25mM Citrate, pH 3.03. When analyzed in the MALDI-MS/TOF, the peaks for MBP in the above buffers were very weak and fragmented. Especially when using buffers 3 and 4 with Citrate and Succinate, there were no peaks observed for MBP. However, when the buffers were used together with the standards (Aldolase and Apomyoglobin), the analysis gave very nice peaks. For some reason, MBP does not behave well in those buffers. Another observation for the above buffers is that the matrix was flaky and therefore it was difficult to obtain the signals. While Citrate buffers gave no or extremely low peaks, Acetate buffers gave a signal but it was very low compared with that of just TFA alone However, we decided to use Acetonitrile because it buffered the matrix to pH 3 and at the same time the peaks were decent to work with, even though they were very weak. Later on

in the experiment, we concluded that the peaks were more likely to be heterogeneous and with more standard deviation among them than when using matrix with just TFA.

Decreasing the temperature by 10°C results in a three-fold reduction of the H/D exchange rate (Kipping, M. et.al.), so we decided to experiment with different techniques of manipulating the temperature. For the first try, we froze the MALDI 96-well plate in -20°C overnight and then transferred it to -80°C for 3hrs. After the plate was taken out from -80°C, it was transferred onto dry ice to maintain the temperature. From the very beginning, the results did not look promising because the plate was covered in white powdery flakes and the sublimation vapors from the dry ice were accumulating on the plate. We were wiping the flakes off with tissue paper, which was not very scientific. Nevertheless, we tried to spot some samples. The first time we tried, the sample froze solid in the pipette tip before even having the chance to be dispensed. The second time, we managed to quickly spot the sample, but it froze in a solid 3-D ball on the plate and would therefore be impossible to analyze. In the subsequent trials, we have opted for freezing the plate in -20°C and then placing it on ice under the hood for spotting. The hood was essential to minimize the drying time, which would prevent further back-exchange.

In the future, it is recommendable to increase the air flow under the hood in order to minimize drying time, which is increased with colder temperature. At room temperature, it took about 2.5 minutes for the samples to dry, but on ice and previously frozen at -20°C, it took 4-5 minutes to dry. Furthermore, the time it took from mixing the sample with matrix, vortexing it, and spotting it onto the plate could be minimized if the water bath where the H/D exchange reaction was kept, the vortex,

and the hood were in the same place. It took us time to place the tubes on ice, pick up the pipette and the tips, walk over to the hood, place the ice bucket and open the tube. All that could be avoided if were working in the same location.

Overall, both the Mass Spectrometry and NMR results for Lambda repressor demonstrated a clear dependence of pH on H/D exchange rate (see Figures 24, 25, 26 and 27). For MBP, the H/D exchange rates are not dependable since the data collected demonstrated many fluctuations in the pattern (see Figures 15, 16 and 21).

As noted in the "Results," NMR experiments #1 for Lambda repressor and #2 for MBP did not work well because the peaks were low to begin with and therefore were lost in the noise and did not show the real pattern of disappearance. Both experiments were done at pH 6.3, although Lambda experiment was done at 10°C and MBP experiment was performed at 24°C. After completing the experiment, precipitation of the protein was noticed in the NMR tubes of both proteins. It is known that H/D exchange is faster at high pH, so one problem is that the amide hydrogens exchanged really fast, leaving the peaks to be observed at low intensities. Furthermore, as the protein might have precipitated early in the experiment, the concentration of the protein detected might not have been sufficient, resulting in lower peak intensities.

Overall, Lambda repressor generated data that could be used for stability determinations and the rates of exchange of the core amides were very similar: 0.666/hr according to Mass Spectrometry and 0.7682/hr according to the NMR Spectroscopy and including the slowest exchanging peak (see "Results" for calculations). This is a surprising result because the back exchange is predicted to be

high because we have not yet optimized the method to quench it. A factor that might have lowered the H/D exchange rate for Lambda repressor in the Mass Spectrometry could be the back exchange that had not been quenched in the matrix, something that is not an issue in the NMR experiment.

Even though the data for MBP is inconclusive, it nevertheless demonstrates a slower H/D exchange rate than Lambda repressor, even when MBP was exchanged at a higher pH (6.3 for MBP vs. 4.0 for Lambda). We did not want to perform MBP H/D exchange experiments at pH 4.0 because at this pH MBP would probably exchange even slower due to the effect of pH on H/D exchange rates (see Fig. 5). Its large mass and more intricate structure allow more amide hydrogen atoms to be buried inside, thus being solvent-inaccessible. Furthermore, we did not want to change the environment of MBP to pH 4.0 because we were concerned about the precipitating effects that might have on the protein. MBP has a pI of 5.57 and it was purified at the pH 6.3. If the MBP would be introduced into pH 4.0, it would have to cross its pI and it might precipitate or otherwise behave differently. It was safe to introduce Lambda repressor into pH 4.0 because since its pI is 7.02, it will not have to cross that point when going from pH 6.3 to pH 4.0.

As it is, the rate of exchange was very slow and measurable in the time span of the experiment only for the intermediate-exchanging amide hydrogens. To be able to measure the exchange rate of the core amide hydrogen atoms, denaturation curves have to be plotted with mass vs. concentration of the denaturant.

After the ΔG_{op} values were calculated, they did not match the literature values, see "Results." The ΔG_{op} values that were calculated were much smaller than

the literature values, and that might be due to the calculation of k_{obs} that was larger due to incorporation of intermediate-exchanging amide hydrogen atoms. For Lambda repressor it would have to be advisable to perform the Mass Spectrometry H/D exchange experiments for a longer time period to monitor the rate of exchange of the more protected amide hydrogen atoms. For both proteins, it would be more accurate to calculate the stability measurements from the denaturation curves. In the literature, (Ghaemmaghami, S. et.al, 2000), the stability measurements were obtained using different concentrations of the denaturant to monitor the H/D exchange rates. In these experiments, no denaturant was used and therefore, for MBP experiments, the rates of exchange of core amide hydrogens were not measured either with NMR or Mass Spec and it is possible that not all the core amide hydrogens were included in the rate calculations for the Lambda repressor since only ~ 66 of the theoretical 87 amide hydrogens were exchanged according to the Mass Spec experiments.

CONCLUSIONS

Protein stability has been studied for many years, but probing the structural stability by Hydrogen/Deuterium exchange and Mass Spectrometry is fairly recent. Although a lot of studies have been done on this topic, there are still plenty of opportunities for improving and optimizing the methods and the applications. One major problem is quenching the back exchange in the matrix. Although it is possible to buffer the matrix to pH 3, the quality of the data is greatly compromised. The temperature can be adjusted to even lower levels with the right equipment, and drying times can be reduced by introducing air flow. Another problem that has not been solved yet is accomplishing high-throughput method for plating the samples and acquiring data. That will require careful selection of targets, grouping them in different mass and pI categories and using several H/D exchange reactions at various pH levels.

In this paper, I have measured rates of H/D exchange for two proteins. Lambda repressor was the one that demonstrated a smooth H/D exchange curve from which the rate of exchange was obtained. It was very comparable with the rate of exchange obtained from NMR analysis and gave a promising result that even though the method were not optimized, it was giving promising results.

To obtain the stability measurements of the proteins through total unfolding mechanism, studies can be done with the denaturant-dependent change in mass by H/D exchange. From those denaturation curves, the concentration of denaturant that is sufficient to completely unfold the protein can be obtained and stability

measurements can be calculated. Also, ligand-binding experiments can be performed to measure the effects of a ligand on the structural conformation of the protein.

MATERIALS AND METHODS

PCR

PCR mixture included DNA template, 10xPCR buffer, 10xDNTPs, Taq polymerase enzyme, sterile dH_20, forward primer or reverse primer. The primers used were C-terminal forward 'ATCGATCGCATATGAAAATCGAAGAAGGTAAACTC' and reverse 'ATCGACTCGAGAGTCTGACGACCGCTGGCG' for MBP; C-term forward 'ATCGATCGCATATGAAACCATTACACAGAGCAGC' and reverse 'ATCGACTCGAGGATTTCTCTGGCGTTGAAGGG' for Lambda repressor. These were the proteins used for the experiments. The PCR reaction cycle was 94˚-52˚-68˚-94˚-57˚-68˚. The resulting bands were thicker for MBP than for Lambda.

GEL EXTRACTION OF PCR FRAGMENTS

2% agarose gel were run at 120V and bands of correct size were excised. A Qiagen Gel Extraction kit was used to purify the cut PCR bands.

RESTRICTION DIGESTS

A two-step reaction was performed; first the vector and insert were cleaved with NdeI enzyme. Following overnight incubation, it was further diluted and adjusted for XhoI buffer conditions. In each case, 40ul of enzyme were added.

LIGATIONS

The NdeI/XhoI digestions were purified using a Qiagen MinElute kit and following the instructions on the kit manual. To 150ul of DNA in each tube, 450ul of QG buffer, 10ul of Sodium Actate, 150ul of Isopropanol were added and mixed. Then the

tubes were transferred into elution columns and centrifuged for 1min. The flow through was disposed and the column was washed with 750ul of PE buffer + ethanol, centrifuged and the column placed in a new tube. The process was repeated and the spin column dried after spinning in a fresh tube. Each spin column was placed in 1.5ml eppendorf tube and to each spin column, 15ul of EB buffer was added, the column was left to stand for 1 min, centrifuged, another 10ul of EB buffer was added and the column was centrifuged, saving the eluate.1ul of DNA from the eluate was mixed with 1ul of vector, 1ul of Ligase, 2ul 10xT4 DNA ligase buffer and 15ul dH_2O. Ligations were carried out overnight at 16˚C. The ligations worked well for both proteins.

RESTRICTION DIGEST OF LIGATIONS

Restriction digest of the ligations was used to linearize the plasmids that do not contain the DNA inserts. In this way, the linear plasmid gets digested inside the cell by nucleases and circular plasmid with the insert is retained. Ligations were incubated in a 74˚C water bath to kill Ligase. Since none of the primers or ORFs contain EcoRI or BamHI internal restriction sites and are present between the cloning sites used, these enzymes are used for restriction digest. An EcoRI digest mix for each 20ul of ligation sample uses 3ul of EcoRI buffer, 1ul of EcoRI, and 6ul of dH_2O for a total volume of 30ul. BamHI digest for each 20ul of ligation sample uses 3ul of BamHI buffer, 0.5ul of 100x BSA, 1ul of BamHI and 5.5ul of dH_2O for a total volume of 30ul. Each digestion reaction is incubated at 37˚C for 1 hour.

TRANSFORMATIONS INTO XL-10 CELLS

2.4ul of DNA was added to 20ul XL-10 Gold *E.coli* cells, which were previously thawed on ice. The mixture was then incubated for 30 minutes. The cells were heat shocked at 42˚C for 1 minute and incubated on ice for 5 minutes. Then, 100ul of SOC was added to each tube and incubated at 37˚C for 1 hour. The LB/Amp plates were labeled and sterile glass beads were added following the incubation. The entire transformation was plated. The number of colonies varied from 1 to 10, per construct.

COLONY PCR

Colony PCR is done to screen for constructs with the correct size insert. Each XL-10 Gold colony is resuspended in 50ul of dH_2O. From that resuspension, 10ul is transferred into a PCR tube. For each reaction, the PCR mix contains 2.5ul of 10xdNTPs, 2.5ul of 10xPCR buffer, 1ul of T7 Forward primer, 1ul of T7 Reverse primer, 1ul of Taq polymerase and 7ul of dH_2O. 15ul of PCR mix was added to each of the PCR tubes. Colony PCR is run using a set program in the PCR machine which uses a 94˚C-45˚C -68˚C cycle. The reactions are then run on a 2% Agarose gel. It is noteworthy that the constructs appear 200bp longer in the gel because of the added length of the primers. Each colony with the correct construct was inoculated in 2ml Superbroth/Amp.

GLYCEROL STOCKS

700ul of cells in Superbroth/Amp were added to 300ul of 80% glycerol in a glycerol stock tube. The cells were vortexed and put on dry ice for 30 minutes and transferred for storage to -80˚C.

MINIPREPS

Overnight cultures in Superbroth/Amp mixture were grown for 6 hours after addition of 2ul of Chloramphenicol. The cells were spun for 10 minutes at 3000 XG. The minipreps were done using a Qiagen Miniprep kit protocol, buffers and tubes. The only deviation was that in the elution step, elution buffer was added twice (50ul and 20ul). The miniprep DNA was stored at -20˚C.

TRANSFORMATIONS INTO BL21 CELLS

New transformations were done using BL21(DE3)+Magic cell line following the same procedure as the transformations into XL-10 Gold cells, except no SOC was added and the LB/Amp/Kan/Glucose plates were used. The BL21 cells are protein expression cell line and usually give a lot of healthy colonies. It is important to note that the amount of cells and DNA added can vary, but the ratio of DNA to cells is usually greater for the XL-10 Gold cell line transformations.

EXPRESSIONS

The colonies then were inoculated with 500ul of LB/Amp/Kan and grown throughout the day at 37˚C and then 200ul of the culture was transferred into 500ul of MJ9 media (for a 10ml mix, add 250ul of glucose, 50ul of $MgSO_4$-$7H_2O$, 10ul of Ampicyllin, 10ul of Kanamycin, 10ul of Trace Elements and 10ul of Vitamins) and grown overnight at 37˚C. Then, 300ul of the cells were grown for 3hrs at 37˚C in 2mls of MJ9 mixture. OD was taken at A280 at mid-log phase and IPTG was added to induce protein production and put on shaker at 17˚C overnight. The cells were centrifuged, supernatant aspirated and cells resuspended with cold lysis buffer+ 2-Mercaptanol. The cells were then broken open by sonication and 20ul of the sonicated cells were transferred into PCR tubes. The rest of the cells were spun and 20ul of the supernatant

was transferred into another set of PCR tubes. Each sample was stained with 8ul of NuPage buffer + DTT, heated at 72˚C for 10 minutes and run on an 12% agarose gel at 200V to test for solubility and expression. The gels were washed with water 3 times for 15 minutes and then stained overnight. The most soluble clones were expressed in large scale.

Large scale is analogous to small scale and was done by inoculating cells in LB-Amp/Kan and then inoculating cells into 40mls of N15-labeled MJ9 and incubated overnight. The cells were transferred into 1L of MJ9(^{15}N) for 3 hrs. After induction with IPTG overnight in 17˚C, the final OD was usually ~3.0. Centrifugation was done at 7000 rpm for 25 min and the pellets were stored in –20˚C.

Ni-NTA COLUMN PURIFICATION

Cells were resuspended in lysis buffer (50mM NaPi, 300mM NaCl, 5mM Imidazole, 5mM 2-ME, pH 8.0), sonicated on ice, centrifuged for 20min at 15000rpm. Supernatant was loaded into Ni-NTA column, washed in buffer (50mM NaPi, 300mM NaCl, 20mM Imidazole, 5mM 2-ME, pH 8.0). The protein was then eluted from the Ni-NTA column by 10mls of elution buffer (50mM NaPi, 300mM NaCl, 250mM Imidazole, 5mM 2-ME, pH 8.0) and fractions were collected in eppendorff tubes. SDS-PAGE gels of the fractions were used to estimate the concentrations of the pooled fractions..

GEL FILTRATION

A low salt buffer at pH 6.3 (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN3) was used to equilibrate GF column. Concentrations were estimated by SDS-PAGE gels and OD was measured of samples that were 10X diluted with

Guanidine/Tris pH7.0. Total volume of MBP after purification was 37mls and OD 3.03 at A280 for a concentration of 1.81mg/ml. Total volume of Lambda after purification was 23mls and OD 0.87 at A280 for a concentration of 2.95mg/ml. The proteins were concentrated by centrifugation through concentrator columns to reach the final volume of 1.5mls and concentration of 24.72mg/ml for MBP and 0.5mls and concentration of 13.98mg/ml for Lambda.

MALDI/TOF/MS MASS SPECTROMETRY

For initial mass of the protein before exchange, the protein was analyzed by Mass Spectormetry. Matrix used for Mass Spectrometry was Sinapinic acid. 24ul of matrix mix (for 1 eppendorf tube, 10mg of SA, 0.5mls of Acetonitrile, 1ul of TFA and 499ul of dH_2O) and 1ul of diluted protein sample were mixed by vortexing. The dilution of the protein with the protonated buffer was the same as the dilution of the protein with deuterated buffer in the H/D experiments. In later experiments, 19ul of matrix mix was mixed with 1ul of protein solution. To make the pH of the matrix solution 3, which most efficiently quenches the H/D exchange, the solution was buffered with Acetonitrile instead of TFA, but it was unfavorable because it gave a very weak signal. Then, 1ul of that mixture was dotted on the Mass Spectrometer 96-well plate well, position of the protein sample was recorded and other wells were filled the same way. The samples were dried under the hood and then analyzed in the Voyager MALDI-MS/TOF Mass Spectrometer. The laser intensity was 2551, 25000V and the delay time for each laser shot is 750 nsec. The number of laser shots was 100 per sample. The spectra was analyzed in the Data Explorer program, using the following parameters: 1000 mass resolution in Peak Detection, Noise Filtering in

Noise Filter/Smooth, Default in Noise Filter/Smooth and Centroiding. The peak mass m/z values were then recorded in Excel spreadsheet and the data was plotted in Excel.

For Hydrogen/Deuterium exchange experiments, the protein was suspended in a deuterated buffer (20mM sodium phosphate, 20mM sodium acetate, 100mM NaCl, pH6.3 for MBP and 7.0 for Lambda) and then mixed with matrix at certain time increments (0min, 10min, 20min, etc.) and crystallized by drying under the hood. It is important to note that 0min increment was not possible to achieve by human means because the proteins exchanged their outer unprotected hydrogen atoms as soon as they were immersed into a deuterated buffer, as there is a time lapse between mixing the protein and the buffer, mixing the protein solution and the matrix, vortexing, and then crystallizing the mixture on the plate. Therefore, time 0min might as well be regarded as the mass of fully hydrogenated protein spotted from a dilution of the protein in a protonated buffer. Initially, internal standards were used as follows: BSA for MBP and Apomyoglobin for Lambda. Later on, it was discovered through literature that BSA is inaccurate standard to use because of its heterogeneity. As a result, a double standard of Apomyoglobin and Aldolase was used for MBP. Also, to avoid any possible protein-protein interactions in the matrix, external standard was used in the later experiments.

As our final method of acquisition, the 2ul of protein sample was mixed with deuterated buffer (20mM sodium phosphate, 20mM sodium acetate, 100mM NaCl, 0.02% NaN_3, pH6.3 for MBP and 10mM ND_4Ac, pH 4.0 for Lambda repressor). Then, 1ul of the H/D exchange reaction was pipetted into 19ul of matrix (50% Acetonitrile, 49.9% dH_2O, 0.1% TFA). The mixture was vortexed and spotted onto

Mass Spectrometry plate (pre-chilled at -20°C, kept on ice under the hood). Exchange reaction samples are taken at the time of mixing and every 10 minutes after mixing with the deuterated buffer. The fully protonated protein sample is also spotted as a reference. Two external standards were made in two concentrations for each. For MBP, standard in matrix was prepared in the following way: mix 1ul of Aldolase, 1ul of Apomyoglobin with 38ul of matrix (50% Acetonitrile, 49.9% dH_2O, 0.1% TFA). A dilution was achieved by mixing 20ul of the above standard in matrix with 20ul of matrix. For Lambda repressor, the standard in matrix was prepared by mixing 1ul of Insulin, 2ul of Apomyoglobin with 17ul of matrix (50% Acetonitrile, 49.9% dH_2O, 0.1% TFA). A dilution of standard in matrix was achieved by mixing 10ul of the above standard in matrix with 10ul of matrix. The dilution of standard in matrix served as a backup for the more concentrated standard in matrix, a the dilution usually produced smoother peaks. The external standards were spotted on the same plate as the other samples from the experiment. External standard is achieved by shooting a well with the standard in matrix, calibrating the values to the known mass m/z values, processing the spectrum and storing it as the external standard calibration file. The spectra of the wells with the protein of interest are then acquired with automatic calibration to the external standard. Several spectra were generated for each well of the protein sample to obtain an average value and a standard deviation. Variations were made on laser intensity, buffers in matrix, temperature of the plate and the standards to optimize the method. For the data and discussion of Mass Spectrometry experiments, see Results and Discussion.

NMR SPECTROSCOPY

The machine used was Varian Inova 600. For the initial protein assessment, protein samples were prepared by adding 15ul of D_2O to 300ul of MBP or Lambda repressor protein sample for a final concentration of 5% D_2O. Each sample was placed into a Shighammy tube and run at 20°C. See Results for discussion and data.

For the H/D exchange experiments, the deuterated buffer was obtained by lyophilizing the low salt buffer (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN3), suspending it in D_2O, and adjusting to pH 6.4 by adding 5ul HCl to 1ml of deuterated buffer. Then, 60ul of protein were added to 440ul of D_2O buffer and the mixture was placed into NMR tube. NMR data was generated on Varian Inova 600 at 10°C at the following time intervals: 0 minutes, 15 minutes, 60 minutes, 90 minutes, 120 minutes, 3 hours, 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, and 14 hours. The data was processed in SPINS, generating HSQC plot and a peak list. It is important to note that the 0 minutes and 15 minutes data points were generated using nt value of 4 by mistake. The rest of the data points were generated using nt value of 8 and so only those could be compared with each other. The 30 minute spectra peaks were assigned in alphabetical order (AN-H, AAN-H, BN-H, etc.) and the labeled peak locations were superimposed onto the other spectra, which then generated a list of peak heights corresponding to the same peaks in all spectra. The data was then processed in KelidaGraph and a H/D exchange plot was generated. The data did not fit the expected pattern. See "Results" for discussion and data. An H/D exchange experiment #2 was done with Lambda repressor using 10mM ND_4Ac as the deuterated exchange buffer at pH 4.0 on Varian 500. To lyophilize the protein, 109ul of protein was frozen

at -20°C and then in -80°C. The protein was then lyophilized in a speed vacuum for 3.5hrs and resuspended in 500ul of the deuterated buffer for a total concentration of 0.349mM or 3.04mg/mL. The nt value was 16 and each experiment lasted 60 minutes, consecutively and the experiments were run at 20°C. The experiments were started 10 minutes after mixing. The peaks were very strong in the beginning and demonstrated an exponential pattern of decay. See "Results" for discussion and data. An H/D exchange experiment was performed with MBP protein on Varian 600, but instead of HSQC, TROSY application was utilized because of the large size of the protein. For this experiment, 100ul of MBP protein (0.63mM, in low salt buffer (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN_3), pH 6.4) was buffer exchanged through dialysis in 20mM NH_4Ac buffer pH 6.3. The sample was recovered, frozen in -20°C and then in -80°C. Afterwards, the sample was lyophilized by speed vacuum and reconstituted in 300ul of low salt deuterated buffer (20mM NaPi, 20mM NaOAc, 100mM NaCl, 0.02% NaN_3), pH 6.3 for a total concentration of 0.21mM or 8.24mg/mL. The H/D exchange experiment was then performed at 24°C with nt value of 16 and the experiments were run consecutively for 15 hours, each experiment lasting for 60 minutes. The experiments were started 20 minutes after mixing. The peaks showed initially low intensity, but were able to give a pattern of disappearance. See "Results" and "Discussion" for discussion and data.

DNA ARCHIVAL

For C-Lambda 6-85 DNA, construct CL#1st21.1, 2ug of plasmid DNA was archived in plate 5-1-A-8-2 well # E7

For C-Lambda 6-85 DNA, construct CL#1st21.2, 2ug of plasmid DNA was archived in plate 5-1-A-8-2 well # F7

For C-Lambda 6-85 DNA, construct CL#1st21.3, 2ug of plasmid DNA was archived in plate 5-1-A-8-2 well # G7

For MBP 1-357 DNA, construct MBP21.1, 2ug of plasmid DNA was archived in plate 5-1-A-8-2 well # H7

INDEX OF ABBREVIATIONS

ΔG_{op}:	change in Gibbs free energy of unfolding
H/D exchange:	hydrogen-deuterium exchange
k_{ch}:	chemical rate of exchange
k_{cl}:	rate of structural closing
k_{obs}:	observed rate of exchange
k_{op}:	rate of structural opening
K_{op}:	equilibrium constant of [open]/[closed] states of the protein
Lambda:	Lambda repressor
ΔM:	change in mass of the protein at a time point during an H/D exchange reaction expressed as the difference between M_{max} and the mass of the protein at that time point
ΔM_{max}:	theoretical change in mass of the protein if all of its available hydrogen atoms exchanged with deuterium
$\Delta M_{t=0}$:	change of the protein due to fast- and intermediate- exchanging hydrogens at time t=0, extrapolated from $\ln(\Delta M_{max}-\Delta M_{t=})$ vs. time plot of slowly exchanging hydrogen atoms
MALDI-MS/TOF:	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry/ Time-of-Flight
MBP:	Maltose-Binding Protein
m/z ratio:	mass-to-charge ratio
N-H:	nitrogen-hydrogen pair
NMR:	Nuclear Magnetic Resonance
pI:	isoelectric point
TFA:	Triflouroacetic acid

ACKNOWLEDGEMENTS

This project has been a wonderful experience thanks to the people in Dr. Montelione's lab. First and foremost, I would like to thank Cecilia della Valle for Mass Spectrometry protocol development, help and discussion. Also, thanks Dr. Montelione, Dr. Tom Acton, and Dr. Rong Xiao for their counseling on this project; Dr. Jim Aramini for indispensable NMR help; Ritu Shastry and Chi Kent Ho for help and advice with protein production; Yiwen Chiang and Rong Xiao for help in protein purification, as well as the rest of the Montelione lab for advice, discussion and a friendly atmosphere.

REFERENCES

Arrington, C.B., and Robertson, A.D. (2000). "Correlated motions in native proteins from MS analysis of NH exchange: evidence for a manifold of unfolding reactions in ovomucoid third domain." *J. Mol. Biol.* **300**: 221-232.

Arrington, C.B., Teesch, L.M., and Robertson, A.D. (1999). "Defining Protein Ensembles with Native-state NH Exchange: Kinetics of Interconversion and Cooperative Units from Combind NMR and MS Analysis." *J. Mol. Biol.* **285**: 1265-1275.

Baerga-Ortiz, A., Hughes, C.A., Mandell, J.G., and Komives, E.A. (2002). "Epitope mapping of a monoclonal antibody against human thrombin by H/D-exchange mass spectrometry reveals selection of a diverse sequence in a highly conserved protein." *Protein Science* **11**: 1300-1308.

Bai, Y., Englander, J.J., Mayne, L., Milne, J.S., and Englander, S.W. (1995). "Thermodynamic parameters from hydrogen exchange measurements." *Methods Enzymol.* **259**: 344-356.

Bai, Y., Milne, J.S., Mayne, L., and Englander, S.W. (1993). "Primary structure effects on peptide group hydrogen exchange." *Proteins: Struct. Funct. Genet.* **17**: 75-86.

Bell, C.E., and Lewis, M. (2001). "Crystal structure of the λ repressor C-terminal domain octamer." *J. Mol. Biol.* **314(5)**: 1127-1136.

Chowdhury, S.K., Katta, V., and Chait, B.T. (1990). "Probing conformational changes in proteins by mass-spectometry." *J. Am. Chem. Soc.* **112**: 9012-9013.

Chernushevich, I.V., Loboda, A.V., and Thomson, B.A. (2001). "An introduction to quadrupole-time-of-flight mass spectrometry." *J. Mass Spectrom.* **36**: 849-865.

Deng, Y.H., and Smith, D.L. (1998). "Identification of unfolding domains in large proteins by their unfolding rates." *Biochemistry* **37**: 6256-6262.

Dharmasiri, K., and Smith, D.L. (1996). "Mass spectrometric determination of isotopic exchange rates of amide hydrogens located on te surfaces of proteins." *Anal. Chem.* **68**: 2340-2344.

Doring, K., Surrey, T., Nollert, P., Jahing, F. (1999). "Effects of ligand binding on the internal dynamics of maltose-binding protein." *Eur. J. Biochem.* **266(2)**: 477-483.

Duan, X., Hall, J.A., Nikaido, H., and Quiocho, F.A. (2001). "Crystal structures of the maltodextrin/maltose-binding protein complexed with reduced oligosaccharides: flexibility of tertiary structure and ligand binding." *J. Mol. Biol.* **306**: 1115-1126.

Ehring, H. (1999). "Hydrogen exchange electrospray ionization mass spectrometry studies of structural features of proteins and protein/protein interactions." *Anal. Biochem.* **267**:252-259.

Englander, S.W., Sosnick, T.R., Englander, J.J., and Mayne, L. (1996). "Mechanisms and uses of hydrogen exchange." *Curr. Opin. Struct. Biol.* **6**: 18-23.

Gardner, K.H., Zhang, X., Gehring, K., and Kay, L.E. (1998). "Solution NMR studies of a 42 KDa *Escherichia coli* maltose binding protein/β-cyclodextrin complex: chemical shift assignments and analysis." *J. Am. Chem. Soc.* **120**: 11738-11748.

Ghaemmaghami, S., Fitzgerald, M.C., and Oas, T.G. (2000). "A quantitative, high-throughput screen for protein stability." *PNAS* **97(15)**: 8296-8301.

Ghaemmaghami, S., and Oas, T.G. (2001). "Quantitative protein stability measurement *in vivo*." *Nat. Struct. Biol.* **8(10)**: 879-882.

Heuzenroeder, M.W., Reeves, P. (1980). "Periplasmic maltose-binding protein confers specificity on the outer membrane maltose pore of Escherichia coli." *J Bacteriol.* **141(2)**: 431–435.

Hoofnagle, A.N., Resing, K.A., Ahn, N.G. (2003). "Protein analysis by hydrogen exchange mass spectrometry." *Annu. Rev. Biophys. Biomol. Struct.* **32**: 1-25.

Huang, G.S., and Oas, T.G. (1995). "Submillisecond folding of monomeric λ repressor." *Proc. Natl. Acad. Sci. USA* **92**: 6878-6882.

Kaltashov, I.A., and Eyles, S.J. (2002). "Crossing the phase boundary to study protein dynamics and function: combination of amide hydrogen exchange in solution and ion fragmentation in the gas phase." *J. Mass Spectrom.* **37**: 557-565.

Kapron, J.T., Pace, E., Van Pelt, C.K., Henion, J. (2003). "Quantitation of midazolam in human plasma by automated chip-based infusion nanoelectrospray tandem mass spectrometry." *Rapid Commun. Mass Spectrom.* **17**: 2019-2026.

Kipping, M., Schierhorn, A. (2003). "Improving hydrogen/deuterium exchange mss spectrometry by reduction of the back-exchange effect." *J. Mass Spectrom.* **38**: 271-276.

Lamosa, P., Turner, D.L., Ventura, R., Maycock, C., and Santos, H. (2003). "Protein stabilization by compatible solutes." *Eur. J. Biochem*. **270(17)**: 4606.

Leeds, J.A., Beckwith, J. (1998). "Lambda repressor N-terminal DNA-binding domain as an assay for protein transmembrane segment interactions in vivo." *J. Mol. Biol.* **280(5)**:799-810.

Li, R., and Woodward, C. (1999). "The hydrogen exchange core and protein folding." *Protein Sci.* **8**: 1571-1590.

Mandell, J.G., Falick, A.M., and Komives, E.A. (1998). "Measurement of amide hydrogen exchange by MALDI-TOF mass spectrometry." *Anal. Chem.* **70(19)**: 3987-3995.

Millet, O., Hudson, R.P., and Kay, L.E. (2003). "The energetic cost of domain reorientation in maltose-binding protein as studied by NMR and fluorescence spectroscopy." *PNAS* **100(22)**: 12700-12705.

Molday, R.S., Englander, S.W., and Kallen, R.G. (1972). "Primary Structure Effects on Peptide Group Hydrogen Exchange." *Biochemistry*. **11**: 150-158.

Mossing, Michael C. (1998). "Solution structure and dynamics of a designed monomeric variant of the lambda Cro repressor." *Protein Science* 7: 983-993.

Powell, K.D., Ghaemmaghami, S., Wang, M.Z., Ma, L., Oas, T.G., and Fitzgerald, M.C. (2002). "A general mass spectrometry-based assay for the quantitation of protein-ligand binding interactions in solution." *J. Am. Chem. Soc.* **124**: 10256-10257.

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

Spurlino, J.C., L, G-Y., and Quiocho, F.A. (1991). "The 2.3-Å resolution structure of the maltose- or maltodextrin-binding protein, a primary receptor of bacterial active transport and chemotaxis." *The J. of Biol. Chem.* **226(8)**: 5202-5219.

Zhang, Z., Smith, D.L. (1993). "Determination of amide hydrogen exchange by mass spectrometry: A new tool for protein structure elucidation." *Protein Science*. **2**: 522-531.

Online References:

1. http://chemistry.gsu.edu/glactone/PDB/Proteins/NucProt/1lmb.html

2. http://masspec.scripps.edu/information/history/

3. http://www.bio.mtu.edu/campbell/ purifica.htm

4. http://www.che.utoledo.edu/nadarajah/ webpages/mbp.htm

5. http://www.mbg.cornell.edu/nicholson/biobm631/folding/folding.html

6. http://www.mun.ca/biochem/courses/4103/topics/Lambda/Lambda_Repressor.html

7. http://www.web-books.com/MoBio/Free/Ch4H2.htm