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Article

Efficacy of mass spectrometry versus 1H nuclear magnetic resonance with respect to denaturant dependent hydrogen-deuterium exchange in protein studies

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SUMMARY

Understanding how proteins fold holds the key to many crucial advancements in the medical field, such as the treatment of chronic diseases. Measuring the rate of hydrogen-to-deuterium exchange for labile hydrogens within proteins exposed to deuterium oxide (D_2O) can help elucidate the folding pathway of a protein. Mass spectrometry can be used to determine the extent of deuterium exchange by calculating ΔG_{HX} (mass-to-charge ratio) values. These values were then compared the mass spectrometry results to previous exchange measurements taken by 1H NMR to investigate if mass spectrometry can be a viable method for measuring hydrogen-deuterium exchange in proteins. Once all of the reactions at different time points (10 seconds to 172,800 seconds) were conducted in the mass spectrometer in a 1.5M guanidine hydrochloride and D_2O solution, the plotted data resulted in sigmoidal curves which showed dissimilar exchange rates to the previously archived 1H NMR data. After completion of the experiment, we concluded that further studies are necessary to draw a sound conclusion, as the field of study in which we tested solely 1.5M guanidine hydrochloride was too narrow. If reactions at other concentrations of guanidine hydrochloride yield the same exchange rates, denaturant-dependent hydrogen-deuterium exchange techniques can be applied in novel protein studies.

INTRODUCTION

In protein folding research, what is the efficacy of mass spectrometry versus 1H nuclear magnetic resonance spectroscopy (1H NMR) in the study of protein folding pathways?

Researching protein folding will lead to a better understanding of degenerative diseases caused by protein misfolding and improper aggregation such as Alzheimer's, Parkinson's, and Type II Diabetes (1). Even after many years of extensive research in protein folding, many laboratories around the world are still striving to find a plausible explanation as to how proteins fold. Linderstrøm-Lang's hydrogen exchange methods now enable us to define the structure of protein folding in various situations (2). In essence, a protein stock is diluted in a solution of deuterium oxide (D_2O). Then, the labile hydrogen atoms can be observed exchanging with deuterium atoms due to the increase in mass from the extra neutron of the deuterium isotope. The denaturant induces the loss of the quaternary structure, tertiary structure, and secondary structure present in a protein's native state, causing more and more of the labile hydrogens in the amide backbone to be exposed, increasing the ΔG_{HX} (mass-to-charge ratio). ΔG_{HX} helps define the change in mass that a peptide experiences after hydrogen-deuterium exchange has occurred for a set time interval. The longer the protein stays dissolved in the denaturant deuterium oxide solution, the more the protein will unfold, gradually increasing the overall mass of each peptide. Through the results of the hydrogen-deuterium exchange, we can note the degree of protection (P_{struc}) which indicates the degree to which a protein has unfolded at that moment. In viewing the patterns of weight increases, we can observe which areas of the protein exchange faster and which exchange slower compared to other areas to determine the overall hydrogen-deuterium exchange rate.

Hydrogen-deuterium exchange can be monitored either using nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry, two methods widely used to observe proteins (3). Mass spectrometry is an analytical technique that ionizes chemical species and then sorts the respective ions based on their ΔG_{HX} (mass-to-charge ratio); 1H Nuclear Magnetic Resonance or Proton Nuclear Magnetic Resonance (1H NMR) is the application of nuclear magnetic resonance to target hydrogen-1 nuclei in the molecules of an organic substance to determine their respective structures (4). One prominent difference between the 1H NMR and mass spectrometry is that 1H NMR helps to find the structure of a molecule whereas mass spectrometry helps distinguish the constituents of a molecule. The main area where 1H NMR is more capable than mass spectrometry is in the observation of denaturant-dependent reactions, when the D_2O solution contains the denaturant guanidine hydrochloride (5). Also, NMR spectroscopy is quantitative and does not require extra steps for sample preparation (6). In this study, deuterium was used instead of tritium, hydrogen-1 hydrogen-4, hydrogen-6, and hydrogen-7 as they are all unstable, making them unfit for hydrogen exchange. However, 1H NMR is more expensive due to uniform isotopic labelling of ^{13}C and ^{15}N, which enhances the NMR sensitivity but also allows for site-specific interrogation of structures and intermolecular contacts (7);

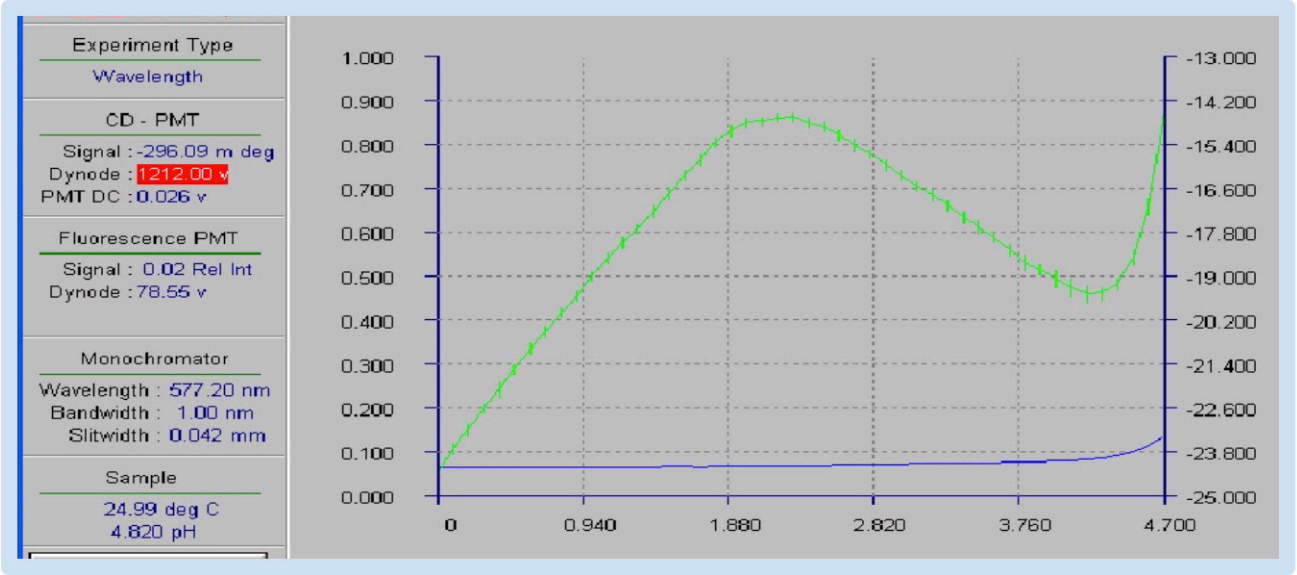

Figure 1: Circular Dichroism Readings. A Circular Dichroism (CD) experiment was performed to deduce the structure of equine Cytochrome *c* and the concentration of guanidine hydrochloride needed as denaturant in the deuterium oxide solution. The CD uses multiple automated titration mechanisms to induce changes in the concentration of guanidine hydrochloride and equine Cytochrome *c* to collect fluorescence readings. Green is the CD reading. x-axis numbering represents the concentration of guanidine hydrochloride. The y-axis numbering represents the fluorescence reading.

1H NMR requires more expertise to operate whereas a mass spectrometer can be used with minimal training;there are limits on the size of the observable protein with 1H NMR but a mass spectrometer can be used to view almost any protein. Due to their convenience, mass spectrometers are much more commonly used in laboratories around the world than 1H NMR. Furthermore, measuring hydrogen-deuterium exchange by mass spectrometry can make the EX1 (bimodal isotopic distribution) and EX2 (unimodal isotopic distribution) behavioral difference clear, illustrating a significant advantage of using mass spectrometry (5). In order to draw on the vast benefits of mass spectrometry, we conducted research on denaturant-dependent hydrogen-deuterium exchange reactions via mass spectrometry. Then, we drew a comparison between archived data of cytochrome c denaturant-dependent hydrogen-deuterium exchange via nuclear magnetic resonance and the current collected data of Cytochrome *c* denaturant dependent hydrogen-deuterium exchange via mass spectrometry.

Cytochrome *c* was used in this study because of its crucial role in the body; it is vital for oxidative phosphorylation in mitochondria, and it plays an important role in the production of life-sustaining ATP by participating in electron transport (8). Furthermore, due to their structural similarity, both human Cytochrome *c* and equine Cytochrome *c* can be studied using the same techniques (9). Also, the ubiquitous nature and sequence of Cytochrome *c* as a 12 kDa protein with a 104 amino acid peptide chain and a single heme group, allow for it to be used as a model protein for molecular evolution (8).

When all of the hydrogen-deuterium exchange runs at different time points are conducted in the mass spectrometer in a 1.5 M guanidine hydrochloride deuterium oxide solution, the ΔG_{HX} values will be the same as those obtained from the archived 1H NMR data prior to this study.

RESULTS

We determined the variables used in this study using information from both background research and pre-study trials. The independent variable was the timepoints for the various mass spectrometry hydrogen-deuterium exchange runs that ranged from 10 seconds to 172,800 seconds, or 48 hours. The rationale for the 10-second time point came from the notion that completing the process of hydrogen-deuterium exchange occurs with increasingly less precision under that time frame. By this point, it becomes necessary to use a stopped-flow kinetics machine, which was not used in this study. The rationale for the 48-hour time frame is derived from the notion that after dissolving Cytochrome *c* in the D_2O solution, most of the protein's peptides should have fully exchanged after 48 hours, reaching a point of saturated deuterium occupancy. The dependent variable was determined to be deuterium occupancy as a function of time. This value was then used to calculate ΔG_{HX} (mass-to-charge ratio) values. The scientific controls were all-hydrogen runs in a 1.5 M guanidine hydrochloride deionized water solution and all-deuterium runs (48 hours of exchange to reach saturated deuterium occupancy) in a 1.5 M guanidine hydrochloride deuterium oxide solution.

Initially, to determine the structure of the equine Cytochrome *c* and the concentration of guanidine hydrochloride needed as denaturant in the deuterium oxide solution, a titration plot was produced, which displayed the fluorescence readings from

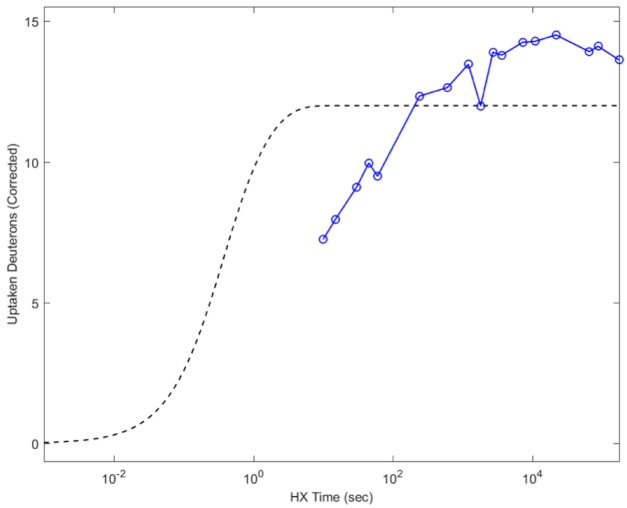

Figure 2: Centroid Average Maps. Example of centroid average map MS-HX data of Peptide 67-82 +2 (+2 is the overall charge) corrected for back-exchange using an all-deuterium run. This means that the protein was in solution for 48 hours for full H-D exchange. The solution was kept in a dry bath at a constant temperature of 45°C. This serves as a control for the all-hydrogen runs and the all-deuterium runs to compare the data. The dotted black line is the estimated amount of deuterium isotopes that were taken up over a given HX time.

the circular dichroism (**Figure 1**). As shown by the low values in the circular dichroism graph which then rise to a peak and then drop off, it is evident that Cytochrome *c* is dominated by alpha-helical secondary structure as opposed to beta-pleated sheet secondary structure. From the CD study, 1.5 M guanidine hydrochloride was selected as the denaturant concentration for the D_2O solution.

As the study was conducted solely at 1.5 M guanidine hydrochloride, there was one main obstacle that prevented us from reaching the objective of the study. There was still not a lot of separation between the exchange rates of different larger scale openings caused by unfolding in the protein, Cytochrome *c*.

The sigmoidal curves are different for the mass spectrometry data and the 1H NMR data suggesting different hydrogen-deuterium exchange rates and disproving the hypothesis. The sigmoidal curves were compared by looking at the slope of the sigmoidal curve and the overall general shape. If the hydrogen-deuterium exchange rates for the 1H NMR and mass spectrometry were the same, then they would reflect the same shape. For the collected data, there are significant differences in the hydrogen-deuterium exchange rates. **Figure 4** shows the comparison between the mass spectrometry data and 1H NMR data for three randomly selected peptides. If the data collected from the MS-HX were similar to the data of the 1H NMR, further protein folding studies could use similar methods. However, as the size of the protein changes, components of the experiment such as the concentration of the denaturant need to be adapted

DISCUSSION

From the results of the circular dichroism, the structure of the studied protein can be identified. As shown by the low values in the circular dichroism graph which rise to a peak and then drop off, it is evident that Cytochrome *c* is dominated by alpha-helical secondary structure as opposed to beta-pleated sheet secondary structure.

The peptide coverage of the MS-HX was highly complete. This was due to the multiple trials done at each time point to ensure a set of data was available for a large number of peptides. Ensuring proper protein coverage was an important step, as collecting data for each peptide would allow for a reasonable comparison between the MS and 1H NMR data.

A wide range of time points were conducted for the MS-HX runs in order to gain an idea of the full spectrum of the protein unfolding pathway. The lower end of the time point range was 10 seconds. This was the smallest time point because at time points lower than 10 seconds, a stopped-flow kinetics is required. The largest time point was 48 hours which was selected because there is almost complete hydrogen to deuterium exchange at that time.

The denaturant dependent hydrogen-deuterium exchange mass spectrometry method was finalized from pre-study trials and background research in order to prevent error in the collected data that was used to calculate the ΔG_{HX} values and to ensure accuracy. From the calculations of the ΔG_{HX} by the lab proprietary software ExMS, the data was shown in hundreds of ΔG_{HX} by time (seconds) graphs. To prevent error, the graphed data was cancelled for back-exchange using an all deuterium run as shown in centroid average maps (**Figure 2**). This data was compared to the NMR data, as the NMR data was previously collected and cross-checked through various other experiments.

From the observations, the folding pathway of the protein can be deduced, including - which segments of the protein fold first, last, and which segments dominate the hydrogen-deuterium exchange rate. For example, the slow rate of hydrogen exchange in a protein, relative to the exchange rate observed with simple peptides under the same experimental conditions is closely related to the conformation of the protein molecule in aqueous solution of denaturant and D_2O(10).

At 1.5 M guanidine hydrochloride, larger scale openings caused by unfolding in Cytochrome c dominate the hydrogen-exchange rates of residues included in each opening segment, making it difficult to study smaller local openings in the protein. As the larger scale openings from the unfolding of the protein dominate the exchange rates, the hydrogen-deuterium exchange rate was not similar to that of the 1H NMR. The exchange rate contributed to the EX2 behavior shown by the unimodal isotopic distribution slowly moving over to the right (**Figure 3**).

Through the results of the denaturant-dependent hydrogen-exchange reaction in the mass spectrometer, the degree of protection (P_{struc}) was noted, which helped draw conclusions regarding the folding pathway of the equine Cytochrome *c* (5). The results also showed that in EX2 exchange (**Figure**

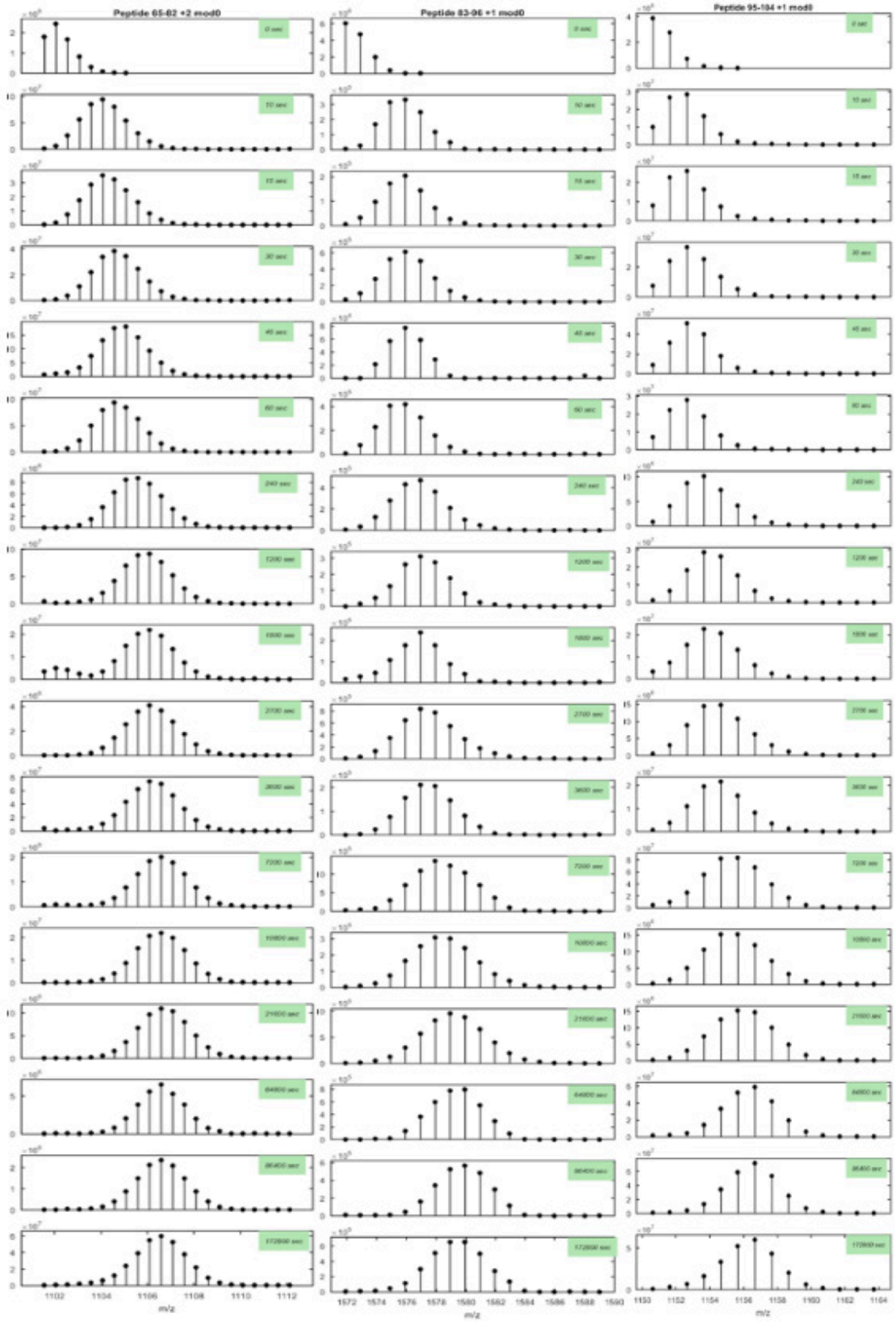

Figure 3: MS-HX data. These samples of mass spectrometry hydrogen-deuterium exchange (MS-HX) data were collected from the time point runs done with Peptide 83-96 +1, Peptide 65-82 +2, and Peptide 95-104 +1 (+1 and +2 are the overall charges). The unimodal isotopic distributions represented at each timepoint for each peptide are shifting due to a positive increase in the ΔG_{HX} (mass-to-charge ratio) as the time points also increase in duration, representing EX2 behavior. Y-axis numbering represents the mass-to-charge ratio.

3), individual amino acids are not correlated. Thus, they will always show a unimodal isotopic distribution that moves from starting to ending conditions (hydrogen to deuterium or deuterium to hydrogen) with longer exchange times.

The data collected using the mass spectrometer during the time point runs indicated that the hydrogen-to-deuterium exchange rates were not similar at 1.5 M guanidine hydrochloride when compared to the archived 1H NMR data disproving the hypothesis. Further testing with the same hypothesis but at a different guanidine hydrochloride concentration may provide more promising results.

In order to elucidate further conclusions regarding the efficacy of hydrogen-deuterium exchange mass spectrometry in conjunction with denaturant-dependent reactions, further experiments are necessary. The spectrum of the study can be increased by gathering data at more time points to check for a better concentration of guanidine where the denaturant-dependent hydrogen exchange can occur with similar exchange rates as 1H NMR. This can be done by simply changing the concentration of guanidine hydrochloride in the deuterium oxide solution and keeping the rest of the procedure the same. If further experimentation in denaturant-dependent hydrogen-deuterium exchange mass spectrometry methods yields the same ΔG_{HX} values, this novel mass spectrometry technique can then be applied to other protein folding studies aiding in the research on chronic diseases.

METHODS

An AVIV Circular Dichroism Spectrometer Model 202 was used to deduce the structure of equine Cytochrome *c*. The CD used multiple automated titration mechanisms to induce changes in the concentration of guanidine hydrochloride and Cytochrome *c* to collect fluorescence readings (**Figure 1**). The protein stock sample in the cuvette was placed in the CD and 1.5 M guanidine hydrochloride solution was titrated in by the CD. This data enabled the visualization of the protein's secondary structure, which allowed us to determine the approximate order in which each segment of the protein unfolds.

The variables were determined using information from both background research and pre-runs. The independent variables consisted of the various guanidine hydrochloride concentrations in the pre-runs and timepoints for the various actual runs ranging from 10 seconds to 172,800 seconds, or 48 hours.

The mass spectrometer used was a Mass Spectrometer OrbiTrap XL. 1H nuclear magnetic resonance ΔG_{HX} values were previously calculated from prior lab study data and the data from the mass spectrometry hydrogen-deuterium exchange were both calculated using the lab proprietary ExMS software. Then, the 1H NMR ΔG_{HX} values and the mass spectrometry ΔG_{HX} values were graphed on the same plot to compare the resulting approximate sigmoidal curves (**Figure 4**). Using the protection factor (P_{struc}) derived from the deuterium occupancy, the ExMS program calculates ΔG_{HX}

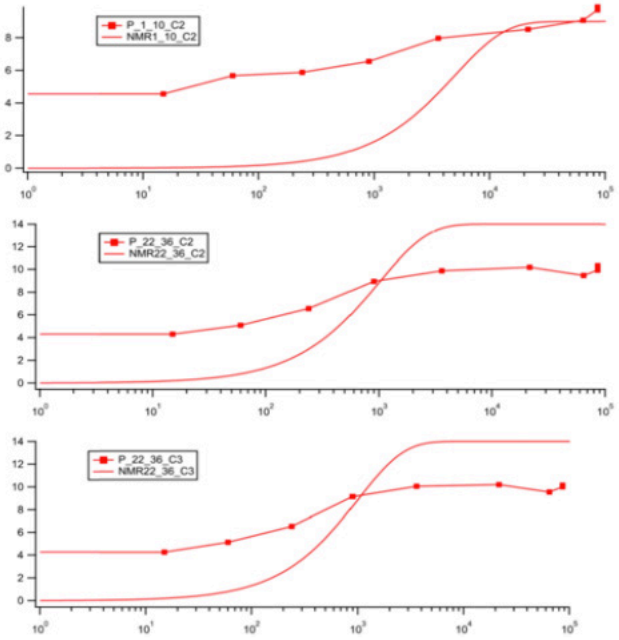

Figure 4: 1H NMR and MS-HX data comparison . A sample of the data from the mass spectrometer and the 1H NMR on the same graph for each peptide. The x-axis shows the time in seconds and the y-axis shows the mass/charge values. While the curves for the MS-HX data are relatively smooth, they do not align with the perfectly sigmoidal 1H NMR-HX data. The x-axis shows the time in seconds. The y-axis shows the mass-to-charge ratio. Legend: MS-HX data is shown by 'P' and the charge is shown by 'C'; 1H NMR data is shown by 'NMR' and the charge is shown by 'C'. (Peptide 1-10 +2, Peptide 22-36 +2, Peptide 22-36 +3).

which is also often reported in free energy units as $\Delta G_{HX} = -RT\ ln\ (K_{op})$: Where T is the temperature, R is the gas constant, and $1/K_{op}$ is the protection factor of the protein segment, with K_{op} as the structural opening rate that exposes the amide.

For the mass spectrometer hydrogen-deuterium exchange (MS-HX) runs, two main 10 mL solutions were made. One consisted of Cytochrome *c* (pH 7) and the other was a D_2O solution (pH 7) prepared with 1. 5 M guanidine hydrochloride as a denaturant. The concentration of guanidine hydrochloride deuterium oxide solution was checked with a refractometer. Before the actual run in the mass spectrometer, the protein solution samples consisting of 10 µL protein stock and 90 µL D_2O solution were mixed, and the hydrogen-deuterium exchange was allowed to occur for time intervals of 0 (all hydrogen run), 10, 15, 30, 45, 60, 240, 1200, 1800, 2700, 3600, 7200, 10800, 21600, 64800, 86400, and 172800 seconds. Multiple trials were conducted for each time interval in order to ensure full peptide coverage. The reaction was then quenched using 25 µL of diluted phosphoric acid, which lowered the pH to 2.5 in order to slow the hydrogen-deuterium exchange reaction. The solution was injected into the lab-proprietary HPLC (High-Performance Liquid Chromatography) apparatus, wherein proteins were cut into peptides by a pepsin trap column and then sprayed into the mass spectrometer. The experiment was replicated three

times for each time point.

The resulting data from the mass spectrometer, which was collected in ExMS, was then cancelled for back-exchange (error) to ensure accuracy. If the reacting sample solution is kept at room temperature too long after mixing the deuterium solution with the protein solution or if the ambient temperature is below 0°C, there may be back exchange of deuterium atoms to hydrogen atoms.

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