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Hydrogen/Deuterium Exchange Mass Spectrometry for the Study of Methanol-induced Conformational Transitions in Ubiquitin

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HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY
FOR THE STUDY OF METHANOL-INDUCED
CONFORMATIONAL TRANSITIONS
IN UBIQUITIN

A Thesis

Presented to

The Faculty of the Department of Chemistry

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Steven Raul Rocha

December 2013

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The Designated Thesis Committee Approves the Thesis Titled

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by

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ABSTRACT

HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY FOR THE STUDY OF METHANOL-INDUCED CONFORMATIONAL TRANSITIONS IN UBIQUITIN

In aqueous solutions with high concentrations of methanol at low pH, the global conformation of ubiquitin denatures to a structure of increased helical character at 50% methanol to a highly helical denatured structure at 90% methanol. Circular dichroism analysis and hydrogen/deuterium exchange mass spectrometry experiments have been reported for the monitoring of alcohol-induced conformational transitions of ubiquitin upon exposure to increasing concentrations of methanol. A bottom-up analysis of deuterium labeled ubiquitin was used in this study to examine the local conformational transitions of ubiquitin upon exposure to solutions of varying concentrations of methanol. Analysis of ubiquitin and nine peptide fragments, produced from a ubiquitin-pepsin digest, were monitored through the use of a Varian 500-LCMS ion trap. The bottom-up approach to hydrogen/deuterium exchange mass spectrometry isolated most deuterium exchange to the β -strands of ubiquitin, suggesting the methanol-induced transitions of ubiquitin were highly characterized by the unfolding of the native-state β -sheets.

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1.0 Introduction

Protein-focused applications in mass spectrometry have become an indispensable tool for the identification and quantification of proteins since the discovery of electrospray ionization (ESI) allowed for the electrical charging of full protein structures. Researchers have long studied the three-dimensional structure of proteins as a means of understanding the specificity and functionality of specific enzymatic reactions, but only until recently have scientists begun to study protein conformation through changes in protein mass. Mass spectrometry provides a method for observing global transitions in protein conformation as well as identifying local regions on a protein that are of increased flexibility and dynamic activity. Studies focused on the mechanics of protein folding and structure have become increasingly important in the search for cures to specific human biological disorders, a number of which have been directly linked to the misfolding of specific proteins;¹ for example, some cases of Alzheimer's Disease have been directly attributed to the misfolding of protein HypF-N.² These misfolded proteins have been shown to have a higher propensity for aggregation and set the foundation for the formation of destructive amyloid fibrils that interfere in the neurological pathways of the human brain.

The complexities in studying protein conformation are derived from the interwoven relationship amongst a multitude of inherent forces originating from the protein's immediate environment and a combination of intra-molecular forces from the protein's amino acid sequence. A protein's functionality is entirely reliant on its structure and thus becomes indirectly tied to the culmination of inherent forces responsible for producing a

stable protein structure; making the study of protein conformations extremely difficult, as external molecules, such as water and metallic ions, along with other external influences, such as pH, temperature, molecular crowding and external fields (magnetic, electrical) all have the potential for dramatically altering the conformation of a protein.³

1.1 Hydrogen/Deuterium Exchange

Hydrogen/deuterium exchange (HX) has become an important technique for the study of protein conformational transitions. By introducing a substantial source of deuterium to the external environment of a protein, typically by substituting H₂O with D₂O and through the addition of [D]⁺, HX can take advantage of the secondary structure's propensity to exchange hydrogen at neutral pH. Although the rates of hydrogen exchange differ between labile hydrogens in the amino acid sequence, exchange is often complete within a few minutes for small peptides in which all labile hydrogen is accessible. This is often the case for the more slowly exchanging backbone amide hydrogen when compared to the very rapid rates of exchange for labile hydrogen on the amino acid side chains.⁴

Hydrogen exchange rates for a peptide can be divided into three groups based on the hydrogen position within a peptide: hydrogens covalently bonded to carbon with extremely slow rates of exchange, side chain hydrogens with very rapid exchange rates, and hydrogens bonded to the backbone amide which exchange at well documented rates.⁴ The rate of exchange for hydrogens on the amide backbone has been shown to deviate by only small increments resulting from minor influences presented by the amino acid side

chain. These influences have been shown to rely overwhelmingly on the steric hindrance of additional amino acid side chains rather than any dipole moment influences presented by various side chains.⁵ HX utilizes these known rates of exchange for the amide hydrogen as the focal point for determination of deuterium uptake on a protein.

At neutral pH, hydrogen exchange on the amide backbone has been shown to occur rapidly (at rates of $k_{\text{Hexchange}}=10^1\text{s}^{-1}-10^3\text{s}^{-1}$)⁵ following a base catalysis mechanism with H₂O. The rapid rates of exchange yield an extremely short half-life, in the order of milliseconds, making all deuterium exchange at neutral pH highly susceptible to rapid rates of back exchange.⁶

The average rates of peptide amide hydrogen exchange as a function of pH showing a minimum in the rate as the mechanism for exchange is altered from a base-catalysis to an acid-catalysis method.⁷ The minimum rate of exchange for the amide hydrogen resides at a pH of 2.7 with a half-life of about 10 minutes under normal laboratory conditions, further cooling of the substrate to 0°C substantially quenches the exchange of amide hydrogen to a half-life of about 2h.⁸ The kinetic isotope effects from the substitution of hydrogen to deuterium has been shown to have minimal impact on the quenching state of deuterium amide exchange.⁹ By locking deuterium into place at the amide backbone, the quenching state allows ample time for an experiment to monitor deuterium uptake on the amide backbone.

Deuterium uptake at the protein backbone alters measurable characteristics at the points of exchange, most notably the increase in mass and the alteration of magnetic spin from the substitution of hydrogen to deuterium. The presence of high concentrations of deuterium in a HX experiment, compared to those of hydrogen, ensure an extremely high probability that all exchange at the amide backbone occurs with the chemically reactive analog, deuterium.

The dependency between the rate of deuterium substitution and the position of the amide backbone in the three-dimensional structure of a protein is the fundamental basis for probing protein conformations and fluctuations within the tertiary structure. HX experiments⁷ have shown the rate of deuterium substitution to have a profound relationship to the amide's location within the tertiary structure of a protein, illustrating a higher propensity for amides located on the outer-surface of a protein to undergo higher rates of deuterium substitution when compared to amides buried within a protein. These observations have yielded a deuterium-amide exchange rate that is dependent on two main factors: (1) the degree to which the amide backbone is protected from the solvent, and (2) the extent to which it is involved in internal hydrogen bonds to surrounding residues in the secondary and tertiary structure.⁷

Higher rates of deuterium exchange for amides are not necessarily exclusive to locations on the outer surface of a protein but can result from areas of high flexibility within the protein structure.¹⁰ These regions may increase the probability of deuterium exchange by increasing the exposure of solvent-shielded amides to the external

environment or by weakening internal hydrogen bonds that could have otherwise bounded the amide and restrained it from any exchange. These areas on a protein with increased mobility and dynamics are of particular interest when studying important sites of activity in biological enzymes.¹¹ Mass shift observations derived from the mass difference in hydrogen bound proteins to those obtained in a hydrogen/deuterium exchange experiment are measured through the use of a mass spectrometer and offer a quantitative way of monitoring the degree of deuterium uptake.

1.2 Protein Conformation and Mass Spectrometry

Polar amino acids in the secondary structure of a protein facilitate most of the ionization of proteins for introduction into a mass spectrometer. Ionization techniques, such as Electrospray Ionization (ESI), ionize proteins through the implementation of high voltages and temperatures to the protein solution as it passes through a nebulizer. Positive polarities applied to proteins in solution oxidize the basic amino acids, lysine, arginine, and histidine, through the addition of a proton, resulting in a positive charge on the amino acid side chain and constituting one part of the total charge on a protein.

Ionization techniques like ESI and matrix assisted laser desorption ionization (MALDI) often use the protein solution as an intermediary in the production of a protein ion. Methods for positive ionization in ESI are often run at low pH to provide a substantial source of $[H]^+$ to protonate the basic amino acids. This dependency of $[H]^+$ in solution has been used to explain shifts in charge state population for different protein conformations.

Circular dichroism analysis^{12,13} involving β -lactoglobulin, cytochrome *c*, and ubiquitin have shown shifts to structures with greater helical content as the concentration of methanol in solution is increased. Mass spectrometry experiments^{4,14,15} on these same proteins reported shifts to higher charge states as the proteins were exposed to increasing concentrations of methanol. Positive ESI of these proteins in solutions, thought to promote a native state structure, were observed to ionize at relatively low charge states, while solutions that promote high helical structure are shown to ionize at higher charge states.¹⁵

In much the same way as hydrogen deuterium exchange is heavily reliant on the exposure of the amide backbone to the external environment for the uptake of deuterium, so too is the oxidation of the amino acid side chains dependent to the external environment. Access of $[H^+]$ for oxidation of the basic side chains from solution is a direct consequence of the position of the amino acid in the tertiary structure of the protein. This dependency has led to observations illustrating a general tendency for compacted protein structures with decreased surface area to reside at lower charge states; a spatial conformation that inherently reduces accessibility of any basic amino acids to oxidation from the electric potential of the ESI source.¹⁶ While an unfolded protein with greater surface area was viewed with a tendency to populate higher charge states, the result of greater exposure of the polar amino acids to the external solvent and additional stability gained from the dispersion of charges within the protein, limiting repulsion from multiple charge states that may have otherwise been unattainable in a more compacted protein. Experiments by Babu et al.¹⁵ reported major shifts to higher charge state for ubiquitin as

the concentration of methanol in solution was increased, suggesting that methanol will interfere with the native state structure of ubiquitin and cause it to undergo changes to its tertiary structure.

1.3 Bottom-Up Approach for Hydrogen/Deuterium Exchange Analysis

Proteolytic cleavage of proteins has been used to generate a “fingerprint” identification method for qualitative protein analysis. Typically, a protease with a high degree of specificity is used to cleave proteins at particular points within the protein sequence based on a high preference for a specific peptide sequence. The chromatographic separation and subsequent mass spectral analyses of these generated peptides are compared to calculated peptide masses or mass spectral library analysis to yield a piece-wise analysis of a full protein sequence.

The digestive protease enzyme, pepsin, present in the stomach of many organisms is a very strong digestive enzyme used to cleave many different types of proteins at many different peptide sites. The very low specificity of pepsin makes it a poor choice for fingerprinting the peptide sequence. But because its inherent function is that of a digestive enzyme, it is fully active at pH 1.9, making it an ideal enzyme for proteolysis at the quenching state conditions in a HX experiment.

The deuterium-labeled peptide fragments resulting from digestion with pepsin can be chromatographically separated for individual mass spectral analysis. Mass shifts observed in the peptide fragment mass spectra are used as a means of quantifying the deuterium uptake to more specific regions on the protein. The analysis of protein

fragment mass shifts in a hydrogen/deuterium exchange experiment offers a localized view of deuterium uptake on a protein and clues to local modifications within the protein structure that are otherwise hidden in an analysis of a fully intact protein.¹⁷

1.4 Ubiquitin

Ubiquitin is a small protein consisting of 76 amino acids and a molecular mass of about 8.5 kDa; it is present in all eukaryotes with a structure and function well studied in many scientific journals. It has been shown to have a fundamental role in human physiology, serving as a molecular label for a myriad of biological functions, including cell-cycle regulation, DNA repair, cell growth and immune function.¹⁸ The protein has been observed in all eukaryotes with a highly conserved amino acid sequence between many different organisms, differing by only 4% when comparing human ubiquitin to that of yeast. The native state structure of ubiquitin has been observed through x-ray diffraction experiments with a single 3.5 turn α -helix and four β -sheets with a few interwoven random turns (Fig. 1).¹⁹

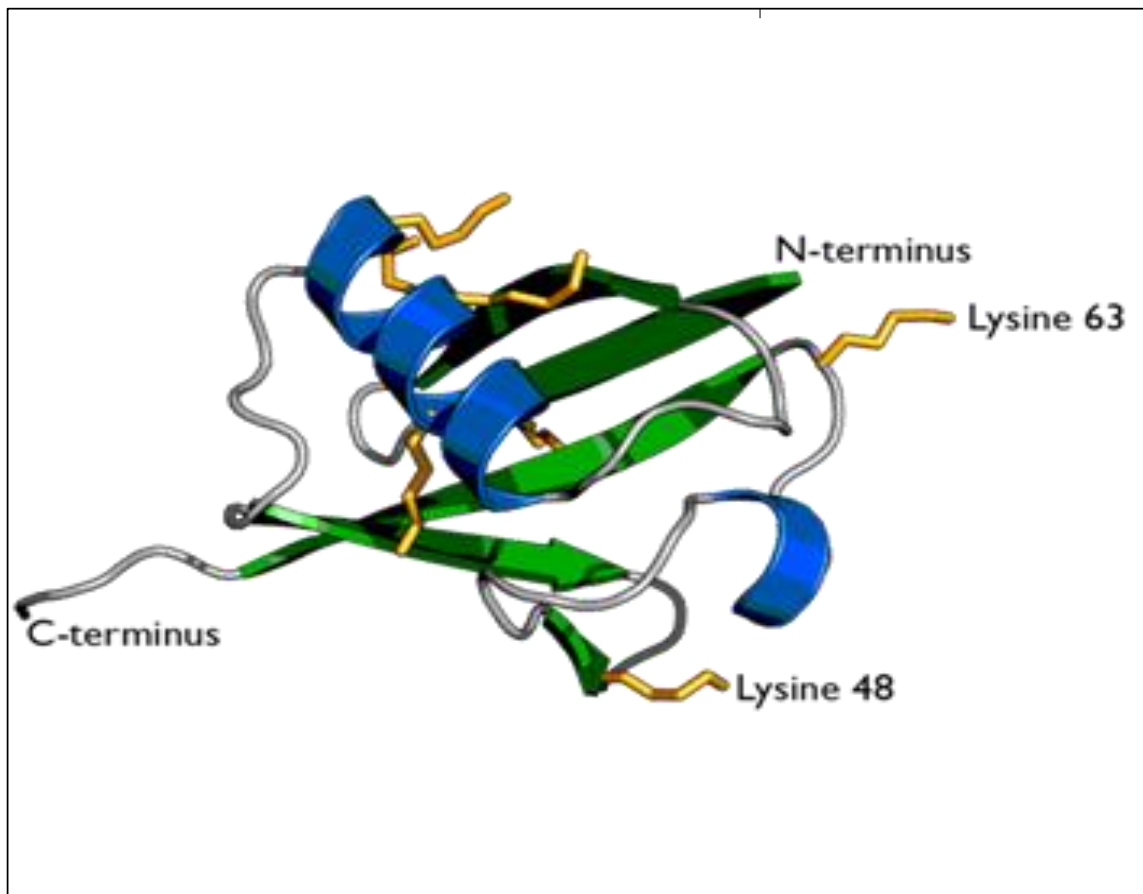


Fig. 1. Illustration of ubiquitin. Illustration of crystal structure of ubiquitin showing the tertiary structure of native state ubiquitin with helical structure in blue, beta sheet structure in green and six lysine residues.²⁰

The ubiquitin amino acid sequence from bovine erythrocytes contains 144 labile hydrogens with 72 residing on the amide backbone and 72 on the side chains. The protein has 13 basic side chains suggesting 14 points of oxidation (including the n-termini) in positive electrospray ionization (Fig.2).

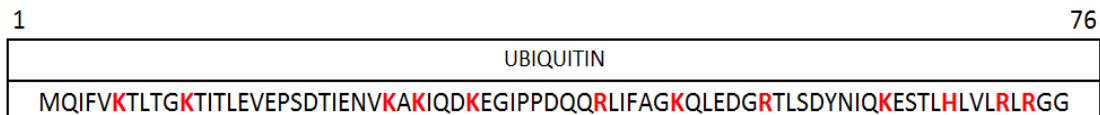


Fig. 2. The ubiquitin sequence. The polar basic amino acids, lysine (K), arginine (R), and histidine (H) are in red font.

1.5 Conformational Analysis of Ubiquitin Using Hydrogen/Deuterium Exchange Mass Spectrometry

Spectral studies¹³ using the $[\Theta]_{222}$ values of the CD spectra were able to deduce two distinct protein conformations apart from the native state of ubiquitin, in solutions of increasing concentrations of methanol. Ubiquitin was shown to increase helical structure over its native state by 26% in solutions of 35% methanol, reaching a highly helical denatured state at 90% methanol with a 62% increase in helical structure subsequently, having the same helicity in 90% methanol as a urea-induced denatured state.²¹

Mass spectral analysis¹⁵ of ubiquitin at low pH showed a progression to higher charge states as the concentration of methanol increased from 0 to 90%. A custom laboratory quadrupole mass spectrometer was able to deduce two distinct stages of charge state progression, indicating a shift in the equilibrium of native and denatured states and the presence of large-scale conformational changes. The study showed native state ubiquitin (0% methanol) to produce a bimodal charge state distribution at 5⁺ and 6⁺, retaining this distribution as the methanol concentration is increased to 25%. At 30% methanol, ubiquitin was shown to ionize at higher charge states with a bell-shaped distribution around 10⁺, continuing this trend until the bimodal charge state distributions at 5⁺ and 6⁺ are significantly decreased at 90% methanol.

Hydrogen deuterium exchange mass spectrometry experiments (HXMS)^{15,16,21,22} have been conducted on ubiquitin to provide greater resolution in monitoring the large scale conformational changes associated with the electrospray ionization mass spectrometry (ESIMS) full scan experiments on methanol-induced conformational changes to ubiquitin. The HXMS experiments on ubiquitin showed moderate uptake of deuterium as the concentration of methanol increased from 0 to 30%, reaching a maximum rate of exchange at ~50% methanol and steadily decreasing, as the concentration approaches 90%, to rates near the native state structure.

The rate of exchange is indicative of a conformational transition in ubiquitin from its native state structure at 0% methanol to a slowly evolving intermediate state in concentrations of 10-30% methanol. This slow increase in deuterium uptake on the protein is in agreement with the ESIMS full scan experiments which show a slow progression in charge state distribution up to 30% methanol before experiencing a more aggressive transition to 35%, a point at which HXMS experiments were shown to reach a more rapid increase in the rate of exchange.

Further analysis of the deuterium exchange rate shows a maximum at ~50% methanol followed by a slow decrease in exchange to 90% methanol; these findings were in accordance with the ESIMS full scan experiments which showed an almost complete transition from low to high charge states at 60% methanol.²² Further increases in methanol showed no significant changes to the charge state distribution while HD exchange experiments did show deuterium uptake to steadily decrease over this range

suggesting that the protein conformation was still undergoing a dynamic transition as the concentration of methanol increased beyond 60%. A point in which Babu et al.¹⁵ contributes a continuing transition in 60 to 90% methanol to a higher state of helicity and small changes in the overall cross section of the protein which is in agreement with the CD spectra, ESIMS full scan and HXMS experiments. Using these observations and those of β -lactoglobulin and cytochrome *c*, the transitions observed in ubiquitin as it is exposed to greater concentrations of methanol can be generalized to four transition states (Fig.3).

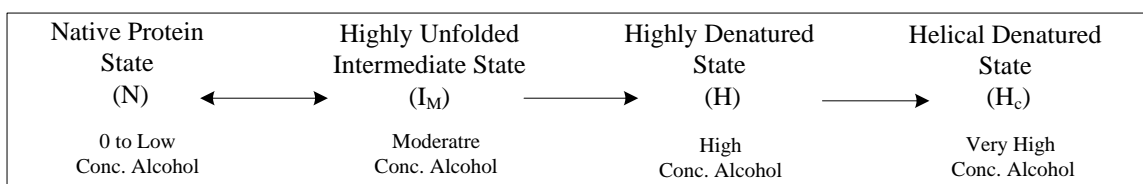


Fig. 3. General transitions of ubiquitin in solutions of increasing concentrations of methanol.

1.6 Bottom-up Hydrogen/Deuterium Exchange Analysis of Ubiquitin

This thesis relies on observations from past experiments that monitored the global methanol-induced transitions of ubiquitin as a direct consequence of methanol in solution; most notably experiments conducted by Babu et al.¹⁵ and Wright et al.^{21,22} are recreated using an alternative mass spectrometer and differences in calculating the mass shift in ubiquitin, serving as a means to test procedures employed in the bottom-up hydrogen\deuterium exchange mass spectrometry analysis of ubiquitin.

Through the use of a bottom-up approach to hydrogen/deuterium exchange mass spectrometry, this thesis intends to study local methanol-induced transitions in ubiquitin as the concentration of methanol is increased from 0 to 90%. The experiment uses methanol as a denaturant for inducing helical structure in ubiquitin. The thesis experiment aims to isolate local conformations in a way that may be used to identify regions of high dynamicity in other proteins; the expectation is that these regions are of extreme importance in the study of protein functionality and could be precursor to studying pathways and possible causes of protein misfolding or the identification of protein active sites for the production of enzymatic inhibitors.

2.0 Experimental Methods

Ubiquitin from bovine erythrocytes, U6253, pepsin from porcine gastric mucosca, P6887, D₂O (99.990, atom % D), DCl (35% in D₂O, 99.5 atom % D), CH₃COOD (98 atom % D), CH₃OH (anhydrous, 99.8%), ACN (anhydrous, 99.8%), HCl (ACS reagent, 37%) and formic acid (~98%, for mass spectroscopy) were all obtained from Sigma-Aldrich (St. Louis, MO.). All H₂O was purified to >18.0 MΩ through PURELAB[®] Option-R *Water Purification System (Siemens Pittsburgh, PA)*.

Mass spectral analysis was conducted on a Varian 500-LCMS ion trap utilizing an electrospray ionization source with a needle potential of 5000 V. All samples were injected at a flow rate of 10 μl/min with 35 psi nebulizing gas pressure. The drying gas temperature was set to 350°C, voltages to the spray shield set to 600 V, and the capillary set to 80 V; the trap was filled with He damping gas at 0.8 ml/min.²²

Ubiquitin was diluted to 1000 μM/H₂O and used as the base solution for all ubiquitin analysis. Deuterium exchange solutions of D₂O with varying percentages (0, 10, 25, 50, 75, 90%) of methanol were adjusted with DCl to pD 2.0 (pD=pH+0.4)²⁶; ubiquitin base solution was dissolved in deuterium exchange solution to yield a final concentration of 10 μM d-ubiquitin for full scan experiments and 100 μM d-ubiquitin for the peptic digest analysis.

2.1 Hydrogen/Deuterium Exchange Rate Analysis

Mass spectral full scan experiments utilizing the enhanced scan mode at 5000 μs⁻¹ scan rate over a mass range of 50-2000 m/z were used to monitor mass shifts in ubiquitin

as a function of exchange time. The 10 μM d-ubiquitin solution was manually injected using the Varian 500-LCMS syringe pump at flow rate of 10 $\mu\text{l}/\text{min}$ for 120 min; injections were commenced rapidly after combining of ubiquitin base solution and deuterium exchange solutions. The experiment focused on the mass shift of [d-ubiquitin+6D⁺]⁺⁶ ion over the span of 120 min.

2.2 Reverse Phase Chromatography for HiRes Spectral Analysis

The d-ubiquitin peptide fragments were separated using two Varian 212-LC chromatography pumps in conjunction with a Varian Pursuit XRs-C18 column (250X2.0 mm, 3 μm) using a binary gradient elution (Fig. 4), utilizing 23.5 μM formic acid/H₂O (Solvent A, pH=2.7 \pm 0.1) and a second solution consisting of 100 mL of Solvent A and 100 mL ACN + 75.5 μL formic acid (Solvent B, pH=2.7 \pm 0.1). Both mobile phase solutions were chilled to 0°C using an ice bath.

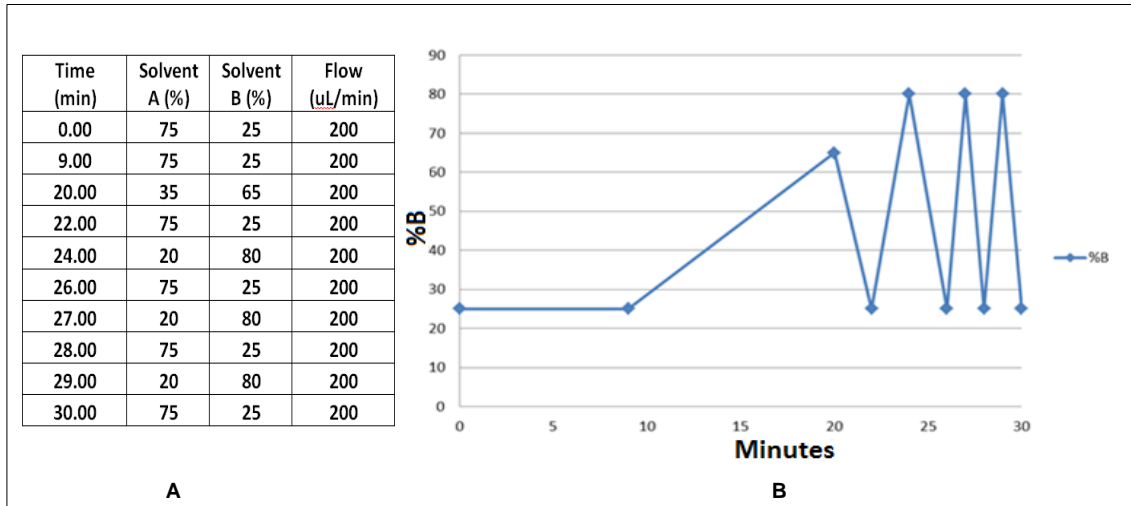


Fig. 4. A) Set HPLC parameters for the gradient elution of d-ubiquitin fragments. B) Graphical representation of HPLC Solvent B composition following the injection of d-ubiquitin peptic digest at 0 min.

Multiple components of the chromatographic setup, including the peak tubing used for solvent delivery, the LC pump heads, the 10 uL sample loop with six port valve, the 200 uL mixer, and the sample syringe, were chilled using frozen artificial ice packs filled with propylene glycol (Fig. 5-8). Particular interest in cooling was paid to the chromatographic column using large ice packs to promote uniform cooling throughout the column stationary phase.

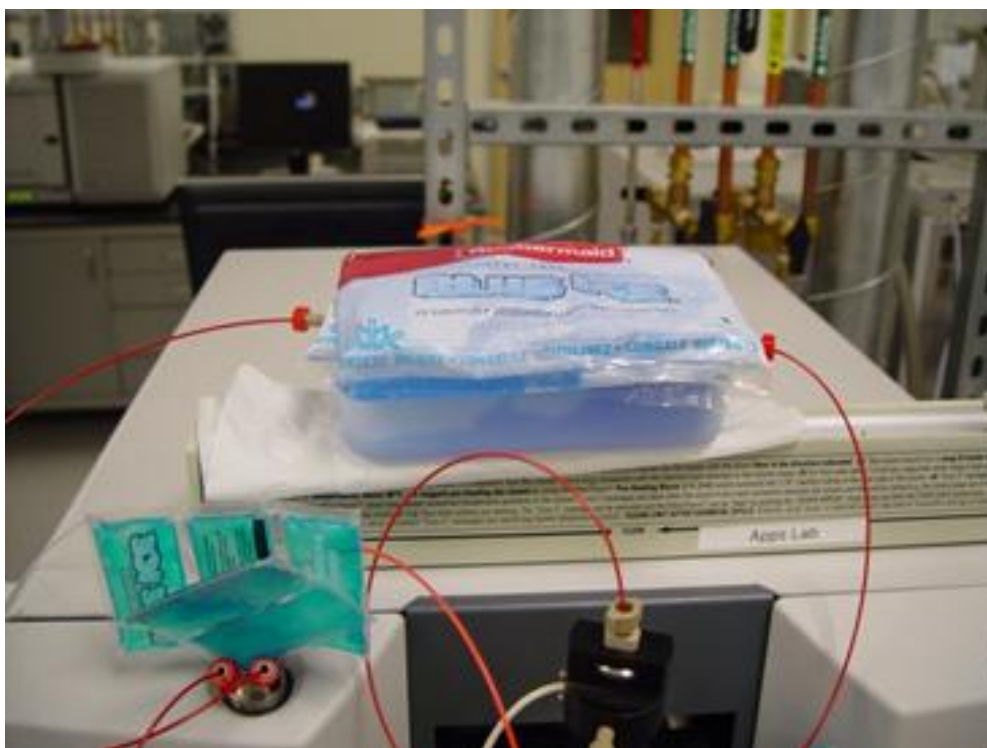


Fig. 5. HPLC column covered using frozen artificial ice packs filled with propylene glycol



Fig. 6. Solvents A and B immersed in ice bath.



Fig. 7. HPLC lines and pumpheads wrapped in propylene glycol ice packs.

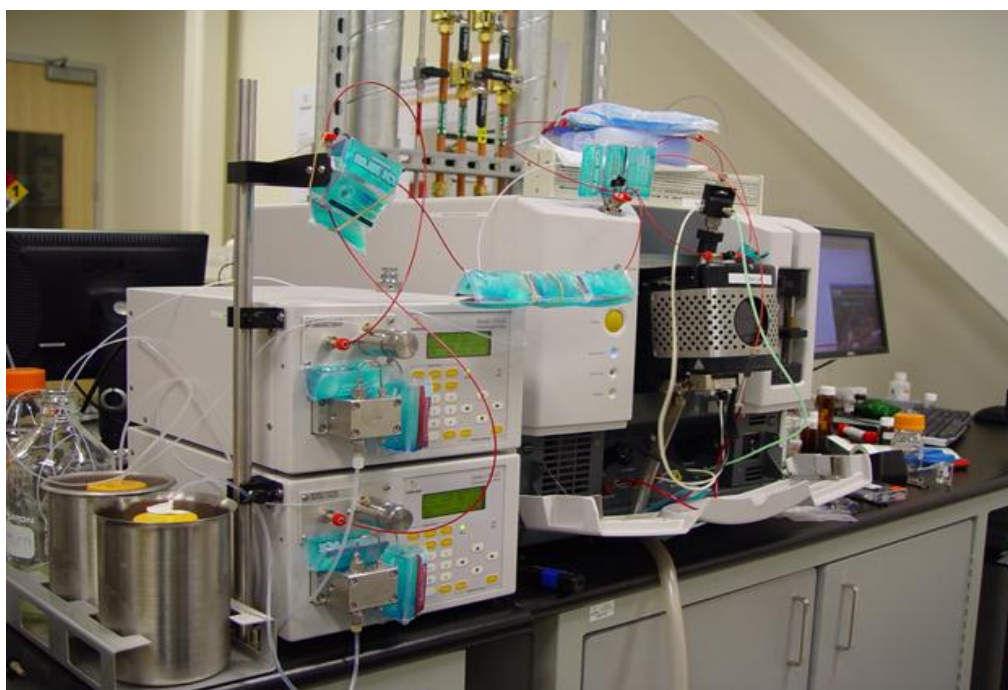


Fig. 8. Complete LCMS system in preparation for d-ubiquitin peptic digest.

2.3 Bottom-up Hydrogen/Deuterium Exchange for HiRes Spectral analysis

Pepsin was diluted to 1000 $\mu\text{M}/\text{H}_2\text{O}$ and then activated upon dilution with 23.5 μM formic acid/ H_2O (Solvent A, $\text{pH}=2.7\pm 0.1$) to yield a final concentration of 11 μM pepsin digest solution. The activated pepsin solutions were chilled to 0°C using an ice bath in 900 μL aliquots; 100 μL of 100 μM d-ubiquitin exchange solution was incubated at room temperature for 1 hr prior to immersion in an ice bath for 3 min before the commencement of digestion in 900 μL pepsin digest solutions. The digestion of ubiquitin yielded a 1:1 mixture of d-ubiquitin and pepsin to ensure a rapid rate of proteolytic cleavage. The digest was retained in the ice bath for 5 min to allow for adequate digestion. A rapid 10 μL sample loop injection of d-ubiquitin digest sample was chromatographically separated using a Varian 212-LC pump.

Mass spectral analysis of d-ubiquitin peptide fragments required a high degree of mass spectral resolution to resolve the mass shifts associated with deuterium exchange in each peptide. In order to achieve the highest possible resolution a mass analysis of five selected peptides, representing $\sim 90\%$ of the ubiquitin protein, was conducted under HiRes scan mode at a $250 \mu\text{s}^{-1}$ scan rate over a scan window of 15 m/z , set to rapidly observe two present charge states in each peptide (Fig. 9).

The narrow 15 m/z scan window associated with HiRes scan mode required predetermination of the degree of mass shift for each peptide to prohibit any mass shift of d-peptides outside the m/z analysis window. A preliminary mass analysis utilized a $5000 \mu\text{s}^{-1}$ scan rate with a scan window of $+200 \text{m/z}$ from the average mass of each peptide

(Fig. 10). The scan window was set to rapidly observe the m/z of two peptide charge states. The preliminary mass analysis for the determination of the HiRes focal m/z was conducted for all five selected peptides in all concentrations of denaturant.

The HiRes scan windows for the mass shift analysis of the five peptides were preset into individual timed segments, centered on the elution time of the individual peptides with the scan window centered on the “focal” m/z derived from the preliminary mass analysis of d-ubiquitin fragments in the various concentrations of methanol (Fig. 11).

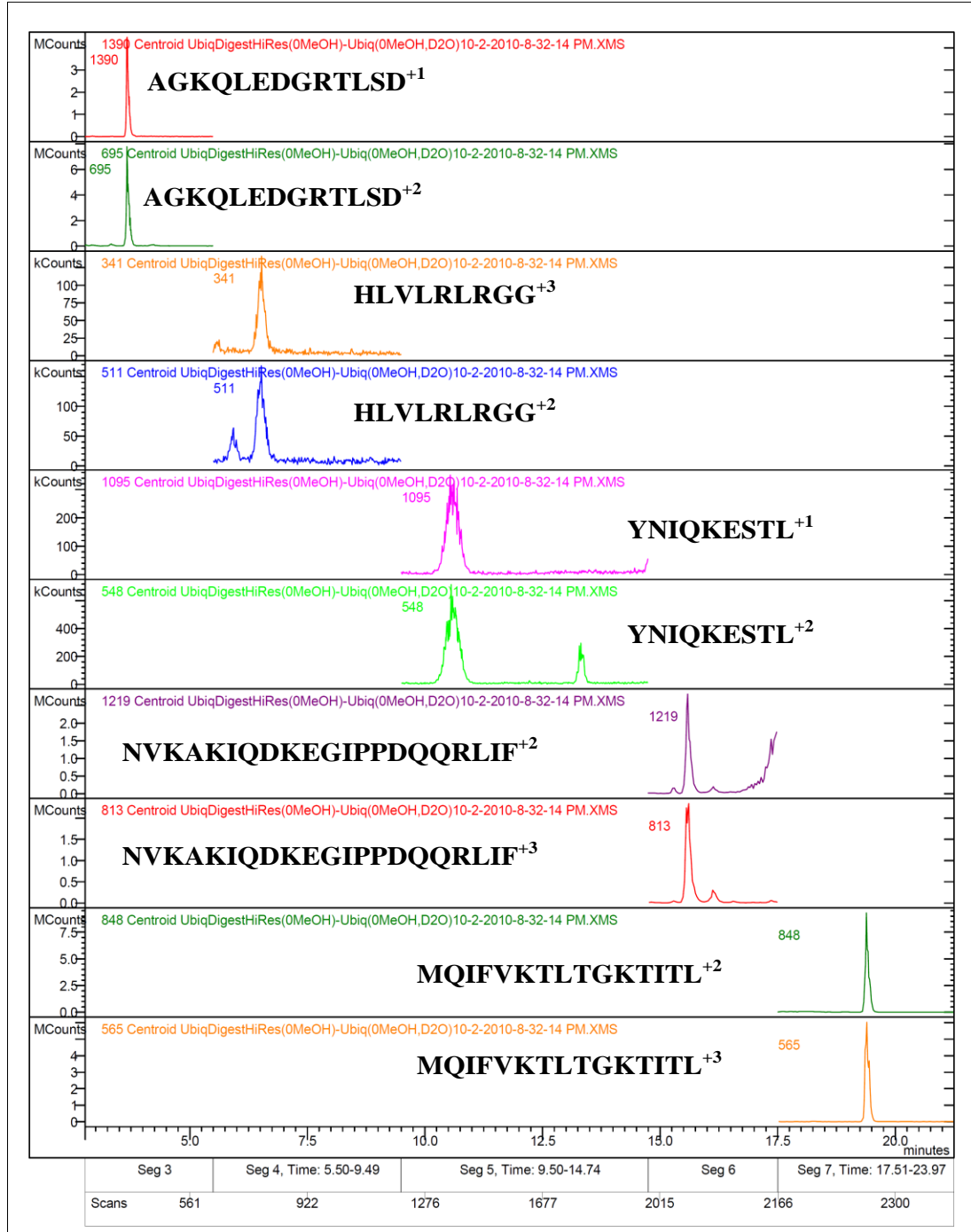


Fig. 9. HiRes analysis of five ubiquitin peptides were separated into five chronological segments utilizing a scan rate of 250 usec^{-1} in scan window of 15 m/z . The focal m/z derived from a preliminary mass analysis can be seen next to each peak.

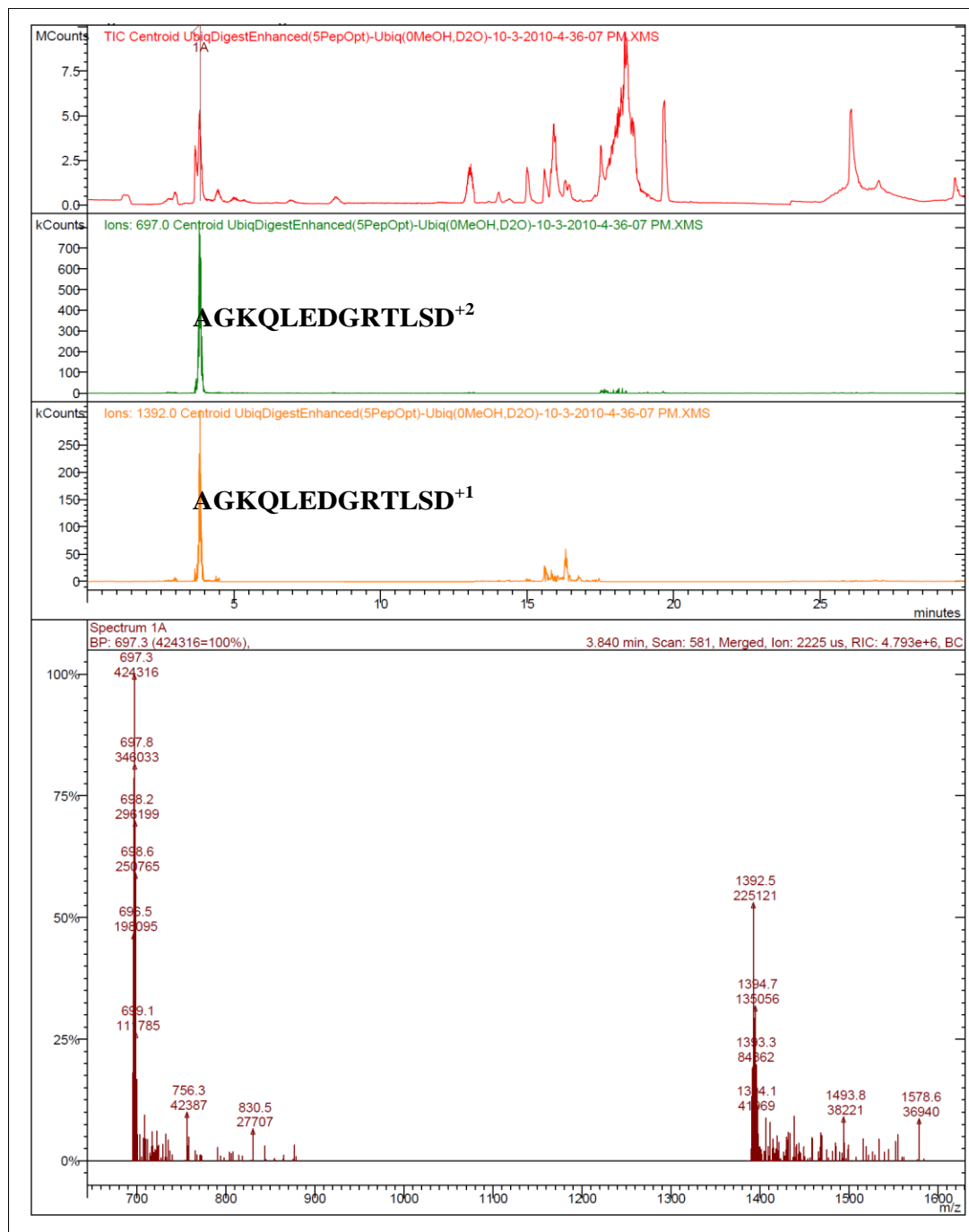


Fig. 10. Preliminary mass analysis for the determination of the HiRes “focal” m/z setpoints. Mass analysis on the selected ubiquitin peptide AGKQLEDGRTLSD in 0% MeOH, utilizing a $5000 \mu\text{sec}^{-1}$ scan rate with a scan window of $+200\text{m/z}$ from the average peptide mass. TIC in red, TIC-697.0 m/z filter in green, TIC-1392.0 m/z filter in yellow.

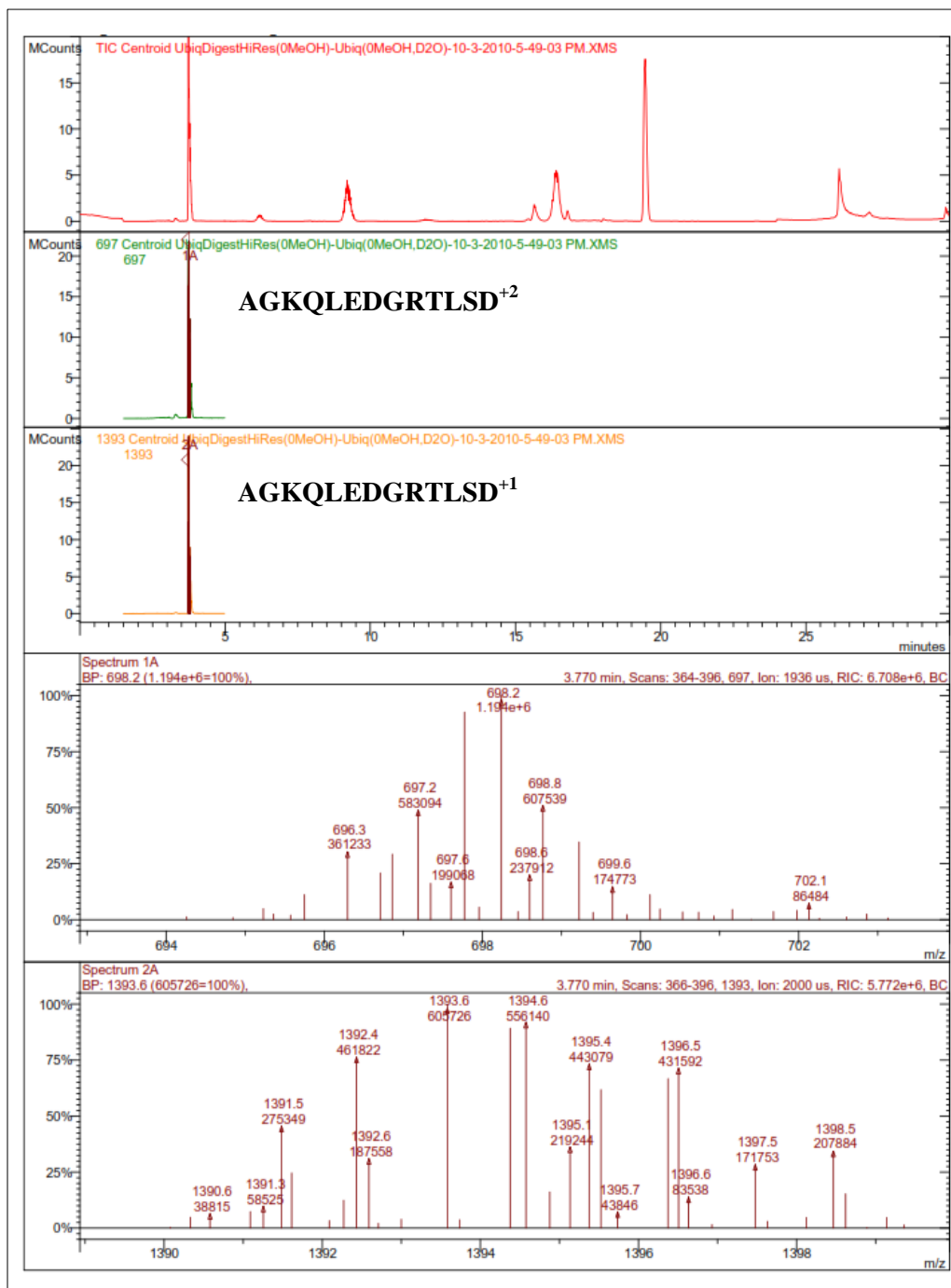


Fig. 11. HiRes analysis of ubiquitin peptide, AGKQLEDGRTLSD. Utilizing a scan rate of 250usec⁻¹ in scan window of 15m/z. The focal m/z derived from a preliminary mass analysis at 1397m/z for z=+1 and 697 for z=+2.

2.4 Circular Dichroism Experiments

Methanol-induced conformational changes in ubiquitin protein were observed with an Aviv Model 215 circular dichroism spectrometer equipped with a Peltier-type thermoelectric cell holder; 600 μL samples of 20 μM ubiquitin in D_2O with varying percentages of methanol (0, 10, 25, 50, 75, 90%) were placed in a 2 mm path quartz cuvette. Spectra were recorded at 25.0°C in the far-UV region with scans between 190 to 260nm with 1.00 nm wavelength steps and averaged over 1.00 sec.

3.0 Calculations

3.1 Calculations for a Single Point m/z Reference for the Exchange Rate Determination of $[d\text{-Ubiquitin}+6D^+]^{+6}$

Ionization of ubiquitin produces a broad range of m/z for each charge state due to the statistical distribution of isotopes within the amino acid sequence. The distribution of m/z for a single ubiquitin ion varies with distinct local maxima for ions of a specific charge (Fig. 12a); difficulty arises when trying to compare mass differences of ubiquitin ion due to the ambiguity of a single m/z reference point. The comparison of time-dependent mass shifts for ubiquitin, as it undergoes hydrogen deuterium exchange, requires a process for identifying a point of reference (single m/z) within the statistical distribution of ubiquitin ion to successfully monitor mass shifts of ubiquitin as a function of time.

In the hydrogen deuterium exchange rate analysis of ubiquitin in increasing concentrations of methanol, a single m/z was used to represent the full m/z distribution of $[d\text{-ubiquitin}+6D^+]^{+6}$ at a specific point in time. Specific criteria were defined for all calculations to narrow the m/z distribution of ubiquitin by eliminating all m/z peaks that did not meet a 60% threshold based on the intensity of the base m/z peak (Fig. 12b). Furthermore, all m/z peaks meeting the 60% threshold but existing at a range greater than 5 m/z from the base peak were deemed secondary spectral peaks existing in alternate local maxima away from the base peak and were eliminated from contention in the single point m/z calculation (Fig. 12c).

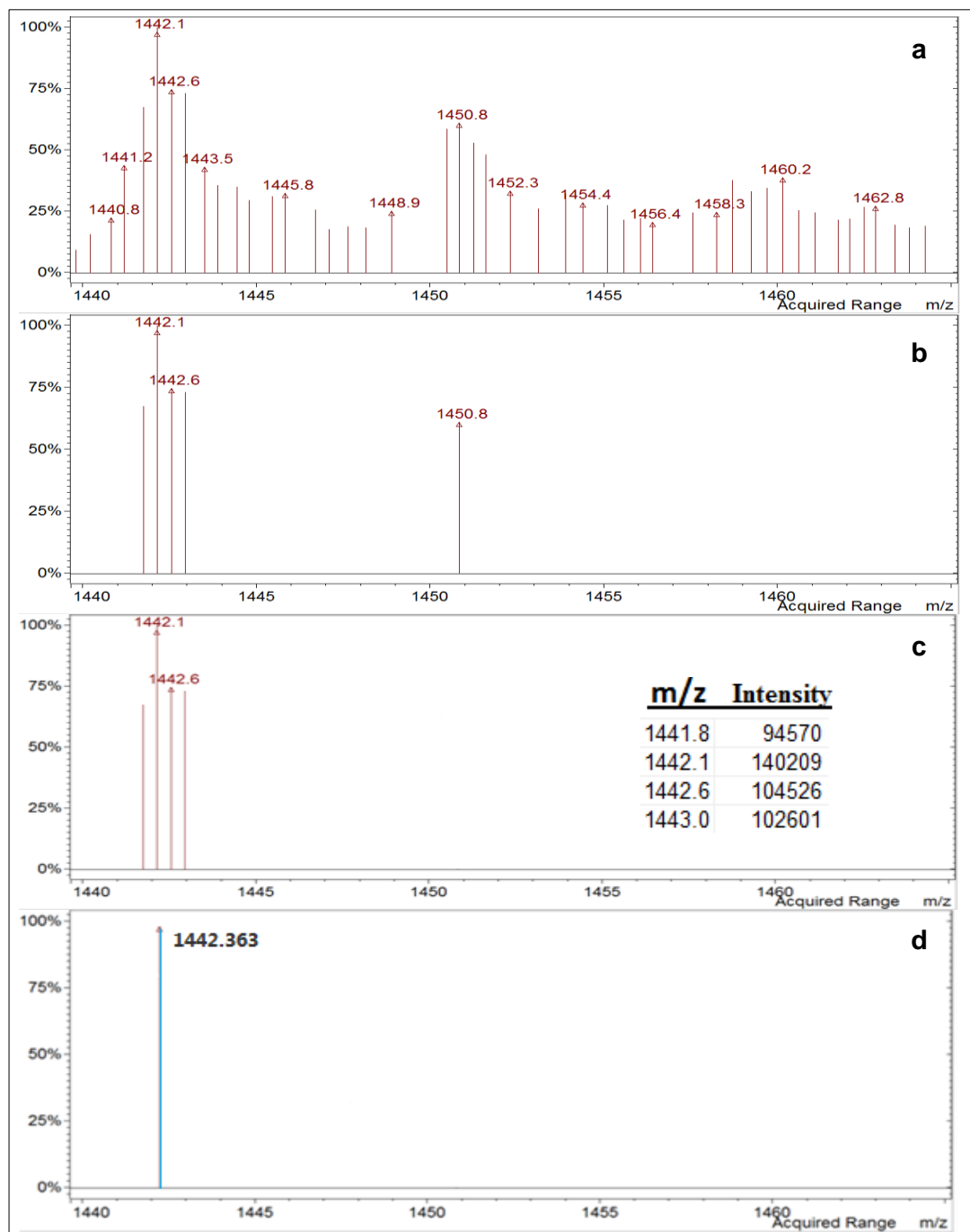


Fig. 12. Illustration of single point m/z calculation. (a) Average m/z distribution for [d-ubiquitin]⁺⁶ in 25% MeOH at 50-51 min with a base peak of 1442.1 m/z. (b) 60% intensity threshold set for for the m/z distribution of [d-ubiquitin]⁺⁶. (c) Elimination of peaks residing more than 5m/z from base peak with list of remaining ions and associated ion intensities. (d) Weighted average calculation applied to ions in Fig. 12c, resulting in single peak at 1442.363m/z used to represent entire m/z distribution in Fig.12a.

A weighted average calculation (Fig. 13) was applied to the remaining m/z peaks to produce a single m/z point based on the m/z and the associated intensity (Fig. 12d). The calculated single point m/z was used to represent the entire m/z distribution of [d-ubiquitin+6D⁺]⁺⁶ at a specific chronological point within the exchange rate analysis.

$$\text{Single m/z point} = \frac{m/z_1 \cdot \text{Intensity}_1 + m/z_2 \cdot \text{Intensity}_2 + \dots + m/z_x \cdot \text{Intensity}_x}{\text{Intensity}_1 + \text{Intensity}_2 + \dots + \text{Intensity}_x}$$

Fig. 13. Single point m/z calculation. Used to represent full mass spectra of [d-Ubiquitin+6D⁺]⁺⁶ and to serve as point of reference for observing mass shifts brought on by the absorption of deuterium.

3.2 Identification of Peptide Fragments from the Peptic Digestion of Ubiquitin

The proteolytic enzyme, pepsin, exhibits a relatively high degree of non-specificity and optimum enzymatic activity at pH 1.8 to 2.0 with some experiments reporting a slight affinity towards large bulky hydrophobic side chains.²⁷ This lack of specificity most often results in a broad range of overlapping and over-digested protein fragments, making it an extremely difficult enzyme in a bottom-up approach to protein characterization. Despite this difficulty, pepsin remains one of the most commonly used proteases in hydrogen deuterium exchange experiments due to its robustness at 0°C and pH 2.7, the optimal hydrogen/deuterium exchange quenching conditions.²⁸ The digestion of proteins following a deuterium exchange enables a more localized analysis of deuterium uptake by offering a method for the mass comparison of peptide fragments.

The experiment for the mass comparison of ubiquitin peptides following a hydrogen deuterium exchange first requires the successful identification and mapping of peptides to the fully intact ubiquitin protein. These peptides then serve as identifiers for gauging the varying degree of deuterium uptake in localized regions of the tertiary structure of a full protein.

Multiple studies^{27,29,30} have identified ubiquitin peptides resulting from peptic digestion through diverse experiments, most notably, Paloshoff et al., which reported six ubiquitin fragments in a study focused on the specificity of pepsin. The identification of ubiquitin peptide fragments used for the bottom-up hydrogen/deuterium exchange experiment, focused on a collection of identified ubiquitin fragments from other studies to form a list of 12 ubiquitin peptides known to occur as the result of pepsin proteolysis. These peptides allowed further determination of other possible peptides that may have occurred based on a process of elimination. The m/z spectra of a chromatographically separated ubiquitin-pepsin digest was filtered for 12 known and 19 determined ubiquitin peptide masses which served as the basis for identifying ubiquitin fragments.

The digestion of ubiquitin yielded numerous peptide fragments as evident in the multitude of base peaks achieved in the chromatographic separation and subsequent qualitative mass analysis (Fig. 14). The process of identifying these peaks as a particular peptide of ubiquitin required matching the known and determined peptides, average isotopic mass to masses observed in the mass spectra of the chromatograms base peaks.

The calculated m/z of known peptides was placed in a spreadsheet with charges (z) of +1 to +6 (Fig. 15).

Further credibility for a successful identification of a ubiquitin peptide was placed on base peaks which had matches to multiple charge states. Of the 31 known and determined peptides, 21 were shown to have m/z matches to calculated mass with only 12 having matches to more than one charge. Peptides selected for further research under a hydrogen exchange experiment were selected from these 12 peptides having multiple matches to m/z .

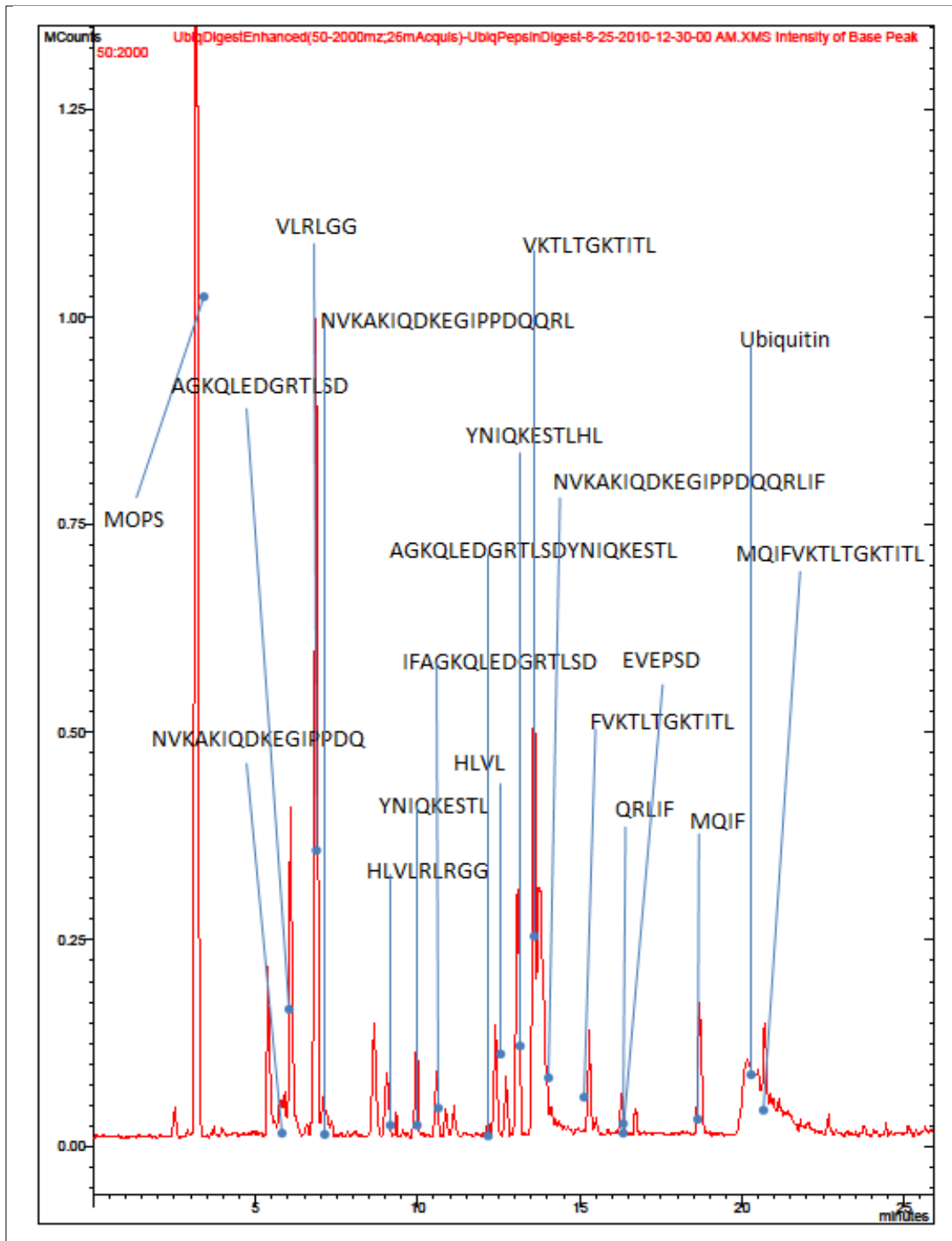


Fig. 14. The chromatographically separated m/z spectra of ubiquitin pepsin digest. Filtered to show the m/z base peaks. Identification for various points of peptide elution was identified through filtering the spectra for known masses.

Seq. #	Sequence	p.I	Avg M.W.	M+1H	M+2H	M+3H	M+4H	M+5H	M+6H
1-15	MQIFV K TLTG K TITL*	10.00	1694.1	1695.1	848.1	565.7	424.5	339.8	283.4
1-3	MQI	5.28	390.5	391.5	196.3	131.2	98.6		
1-4	MQIF	5.28	537.7	538.7	269.8	180.2	135.4		
4-15	F V KTLTG K TITL	10.00	1321.6	1322.6	661.8	441.5	331.4	265.3	221.3
5-13	V K TLTG K TI	10.00	959.6	960.6	480.8	320.9	240.9		
5-15	V K TLTG K TITL	10.00	1174.5	1175.5	588.2	392.5	294.6	235.9	196.7
12-15	V K TLTG K TITL E	8.56	1303.6	1304.6	652.8	435.5	326.9	261.7	218.3
16-21	EVEPSD	3.88	674.7	675.7	338.3	225.9	169.7	135.9	113.4
16-23	EVEPSDTIE	3.50	1018.0	1019.0	510.0	340.3	255.5	204.6	170.7
21-23	TIE	4.00	361.4	362.4	181.7	121.5			
25-45	N V KAKIQD K EGIPPD Q Q R LIF*	8.50	2437.8	2438.8	1219.9	813.6	610.5	488.6	407.3
44-45	IF	5.52	278.4	279.4	140.2	93.8	70.6	56.7	47.4
25-40	N V KAKIQD K EGIPPD Q	6.12	1780.0	1781.0	891.0	594.3	446.0	357.0	297.7
25-42	N V KAKIQD K EGIPPD Q Q R	8.50	2064.3	2065.3	1033.2	689.1	517.1	413.9	345.1
25-45	N V KAKIQD K EGIPPD Q Q R L	8.50	2177.5	2178.5	1089.7	726.8	545.4	436.5	363.9
41-45	Q R LIF	9.75	675.8	676.8	338.9	226.3	170.0	136.2	113.6
50-59	LE D G R TLSD Y	4.03	1168.2	1169.2	585.1	390.4	293.1	234.6	195.7
50-58	LE D G R TLSD	4.27	1005.1	1006.1	503.5	336.0	252.3	202.0	168.5
44-58	IF A G K QLED G R T LS D	4.78	1649.0	1650.0	825.5	530.7	413.3	330.8	275.8
52-56	D G R T L	5.84	560.6	561.6	281.3	187.9	141.2	113.1	94.4
46-52	A G K Q LED	4.67	759.8	760.8	380.9	254.3	191.0	153.0	127.6
46-53	A G K Q LED G	4.37	816.9	817.9	409.4	273.3	205.2	164.4	137.1
46-58	A G K Q LED G R T LS D *	4.78	1389.5	1390.5	695.7	464.2	348.4	278.9	232.6
46-67	A G K Q LED G R T LS D Y N I Q KE S T L	4.78	2466.7	2467.7	1234.3	823.2	617.7	494.3	412.1
59-66	Y N I Q KE S T	6.00	982.1	983.1	492.0	328.4	246.5	197.4	164.7
59-67	Y N I Q KE S T L *	6.00	1095.2	1096.2	548.6	366.1	274.8	220.0	183.5
59-69	Y N I Q KE S T L H L	6.75	1345.5	1346.5	673.8	449.5	337.4	270.1	225.3
68-69	H L	6.74	268.3	269.3	135.2	90.4	68.1	54.7	45.7
68-71	H L V L	6.74	480.6	481.6	241.3	161.2	121.2	97.1	81.1
68-76	H L V L R L R G G*	12.00	1020.3	1021.3	511.1	341.1			
70-76	V L R L R G G	12.00	770.0	771.0	386.0	257.7			

* Peptides used in HiRes analysis

- Determining the Specificity of Pepsin for Proteolytic Digestion²⁷
- Mass Spectrometry Assisted Assignment of NMR Resonances in Labeled Proteins²⁹
- The Immunosuppressive Activity and Solution Structures of Ubiquitin Fragments.³⁰
- Determined Fragments

Fig. 15. Spreadsheet for peptide identification. Showing the calculated m/z of known and determined ubiquitin peptides. Basic amino acids on each peptide segment is in red. Observed m/z in the mass spectra of chromatographically separated ubiquitin digest is filled in green. The m/z corresponding to the highest charge possible for each peptide is in bold red.

In order to create a more focused and efficient approach to studying the localization of deuterium exchange on ubiquitin, peptide fragments resulting from the peptic digest were further selected for continued study based on the intensity observed in the qualitative mass analysis and the degree of resolution achieved in the chromatographic separation. Based on these factors, five peptide fragments (Fig. 16) were selected for the continued study of deuterium exchange while still retaining the same coverage of ubiquitin offered by the 21 original peptides identified. The five peptides selected for the bottom-up approach to the study of hydrogen exchange on ubiquitin were able to account for close to 90% of the ubiquitin sequence, with 66 of the 76 ubiquitin amino acids identified in various peptide fragments. Optimization of the gradient for separation of the selected peptides was carried out to yield a timelier spaced elution (Fig. 17).

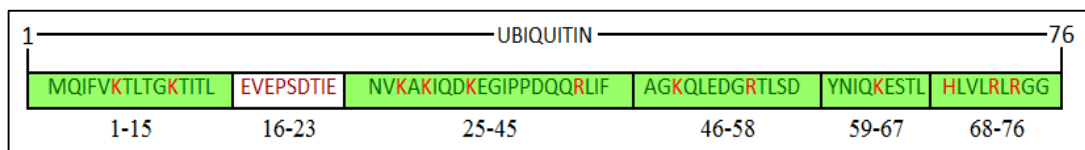


Fig. 16. The five ubiquitin peptides selected for the bottom-up hydrogen/deuterium exchange. Including 66 of the 76 ubiquitin amino acids identified in five peptide segments accounting for close to 90% coverage of ubiquitin.

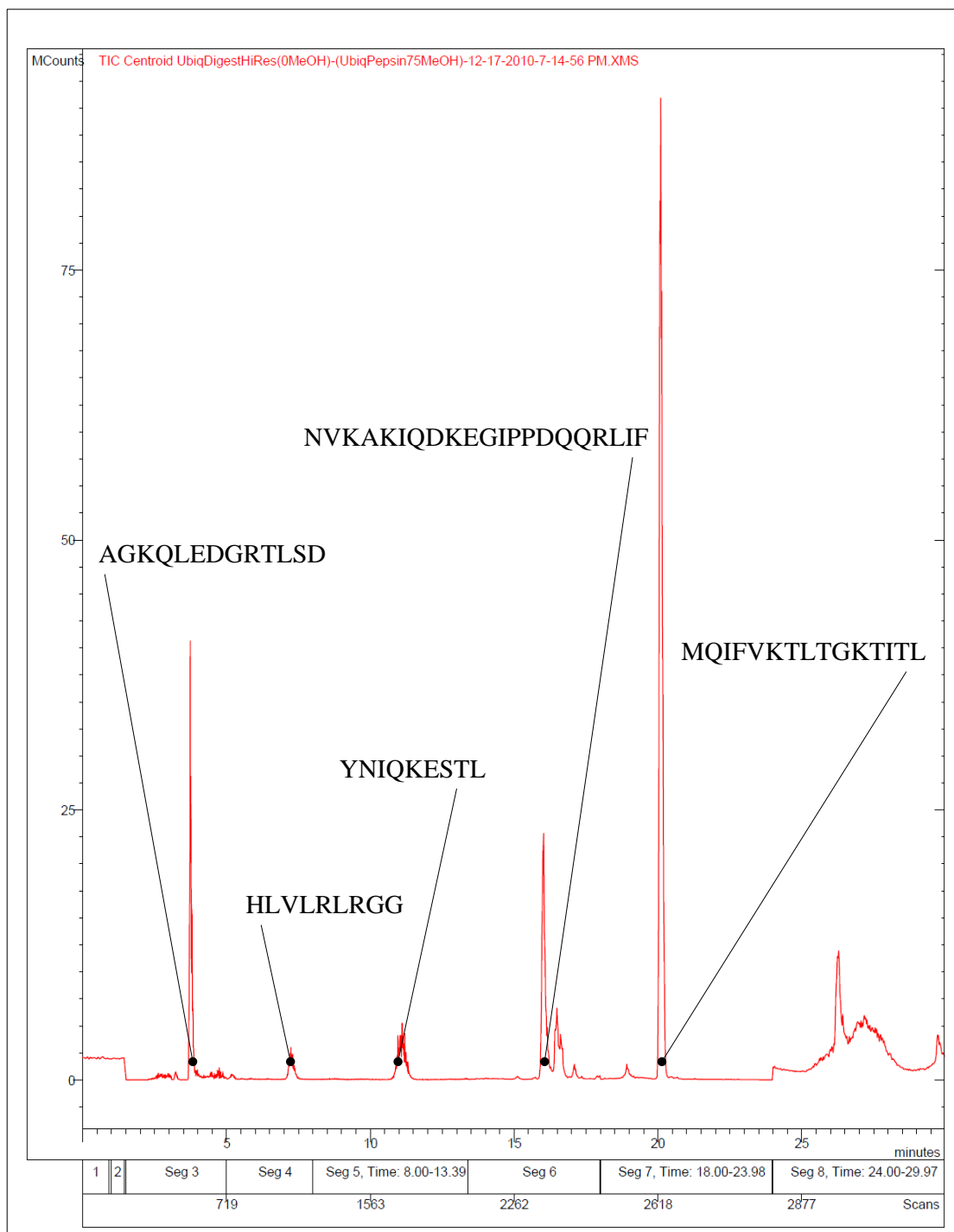


Fig. 17. The five peptides chromatographically separated in a gradient elution. The peptides were optimized to elute each peptide into a distinct Hi-Res mass spectral window. The mass window centered on masses set from preliminary full scan investigation of peptide mass shift.

3.3 Mass Shift Calculations for d-Ubiquitin Peptides

Mass determination of d-ubiquitin peptides for the bottom-up analysis of deuterium uptake on ubiquitin focused on five selected peptides representing ~90% of the ubiquitin sequence. The peptides were chromatographically optimized to elute at equidistant time intervals for mass analysis at 250s^{-1} scan rates under segments of a predetermined mass window for each peptide. The determination of peptide mass shift in various concentrations of denaturant was calculated in similar fashion to the exchange rate analysis of ubiquitin, utilizing a weighted average for the single point m/z calculation for the determination of d-peptide mass shift.

The HiRes mass analysis of each peptide was conducted in a 15 m/z scan window for each peptide (Fig. 18); the mass spectra obtained from each mass segment was averaged over the entire mass peak to yield the average mass spectral plot, converted into list form to indicate the ion m/z and the associated intensity (Fig. 18b). The ion mass assignments and their associated intensities obtained from the mass spectral analysis were populated into the same weighted average calculation used in the ubiquitin exchange analysis with a 50% abundance threshold relative to the base peak.

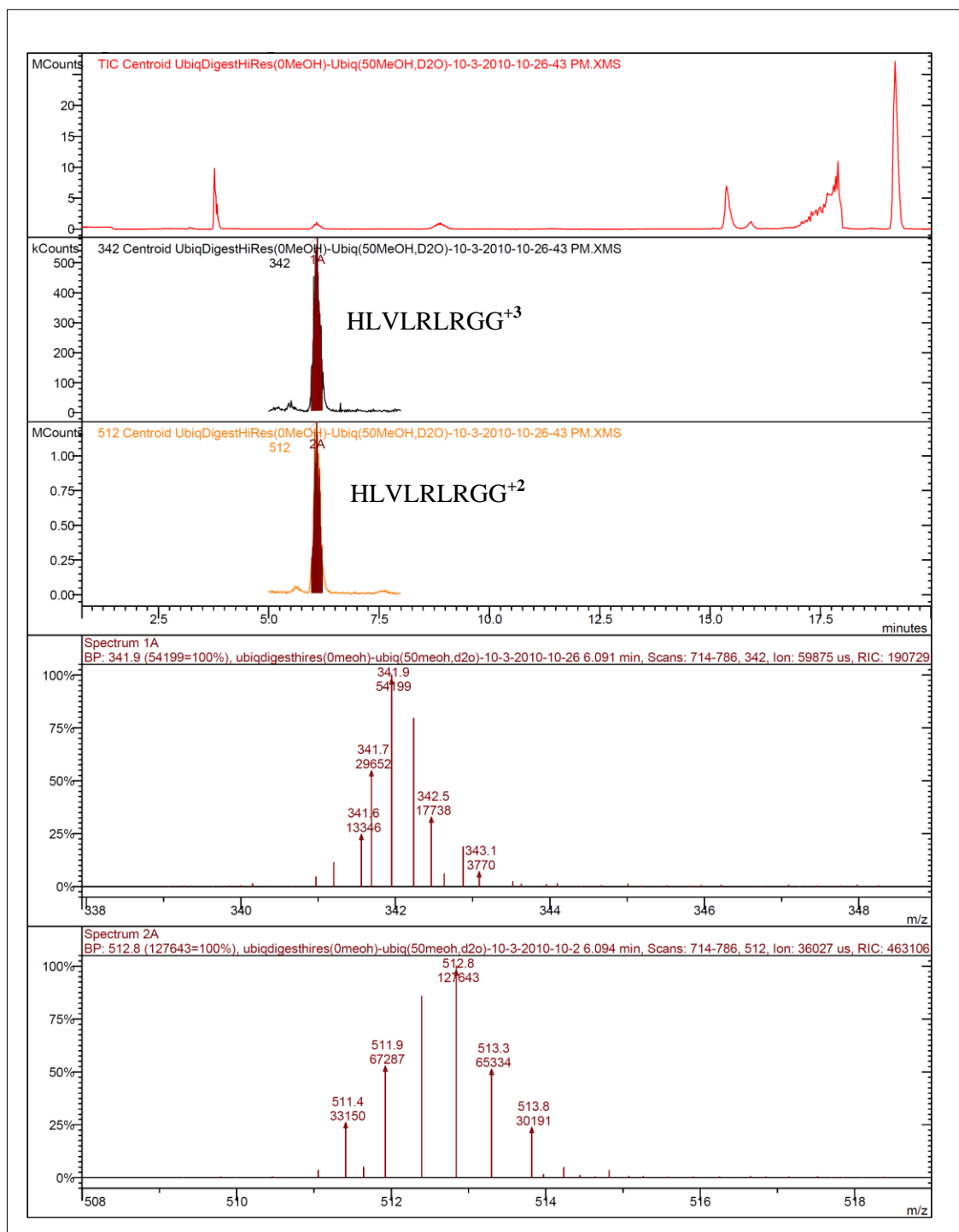


Fig. 18. HiRes scan of peptide HLVLRRLGG in 50% MeOH. The total ion chromatogram filtered for 342 and 512 m/z associated with the +2 and +3 charge states of peptide HLVLRRLGG. The average m/z spectra from scan 714-786 (full peak average) obtained at z = 2, 3.

Ion	1A Intensity	1A % Base ...	Ion	2A Intensity	2A % Base ...
341.9	54199	100.0	512.8	127643	100.0
342.2	43081	79.5	512.4	109456	85.8
341.7	29652	54.7	511.9	67287	52.7
342.5	17738	32.7	513.3	65334	51.2
341.6	13346	24.6	511.4	33150	26.0
342.9	10157	18.7	513.8	30191	23.7
341.2	6118	11.3			

Fig. 18b. List of Ions and associated intensities for the average m/z spectra of HLVLRLRGG at z=2,3, used in the single point m/z calculation for mass shift observations.

4.0 Results and Discussion

4.1 Circular Dichroism

Circular dichroism experiments for the observation of methanol-induced conformational changes in ubiquitin depicted an increase in the helicity of the secondary structure as the concentration of methanol increased from 0 to 90% (Fig. 19). The spectra showed three regions of profound progression. Solutions containing 0, 10, and 25% methanol were shown to yield structures with both β -sheet and α -helix structure indicated by the relatively tame degree of ellipticity in the 190 to 200 nm wavelength region and again in the opposite elliptical node in the 200 to 230 nm region.

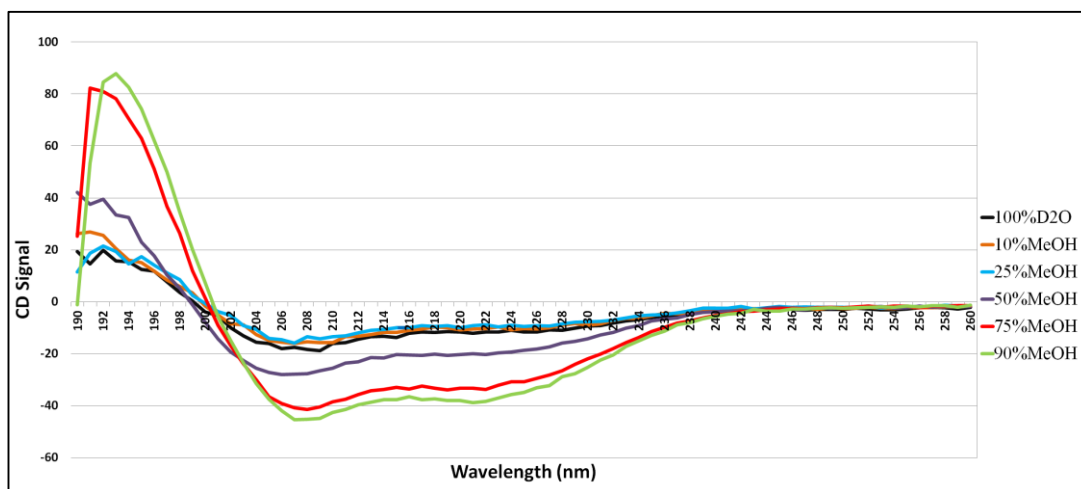


Fig. 19. CD spectra for ubiquitin at 20 μ M in various concentrations of methanol in D₂O.

Solutions of 75 and 90% methanol exhibited the most changes in ellipticity with relatively intense spectra profiles between 190 and 200 nm wavelengths and again in the 200 to 230 nm range, revealing the presence of high helicity in the protein. The absorption for ubiquitin in 90% methanol showed a slight increase in intensity consistent

with greater helical structure than the ubiquitin conformation in 75% methanol solutions. The pattern of absorption for both solutions demonstrates a penchant towards a bimodal distribution in the 200-230 nm wavelength region, consistent with a structure of high helicity, becoming more pronounced as the solution increases to 90% methanol.

The absorption response for ubiquitin in solutions of 50% methanol were observed to reside in a distinct region from the rest of the solutions with absorption rates that were close to mid-level, between the 25 and 75% methanol solutions, indicative of an increase in helical structure compared to the native state (0% methanol) but less than the highly helical structure observed in the 75 and 90% solutions. Further examination of the absorption of ubiquitin at 50% methanol shows the beginning of the bimodal pattern of response seen so prevalent in the 75 and 90% methanol solutions.

4.2 Deuterium Exchange Rate Analysis

The deuterium exchange rate analysis of ubiquitin in various concentrations of methanol focused on mass shifts to the +6 charge state of ubiquitin, [ubiquitin]⁺⁶ and were monitored in full scan mode (100-2000 m/z) for 120 minutes. The full scan analysis of ubiquitin showed a clear transition to higher charge state distributions as the concentration of methanol increased from 10 to 90% an observation that was in close agreement with Babu et al.¹⁵ and Wright et al.^{21,22} (Fig. 20)

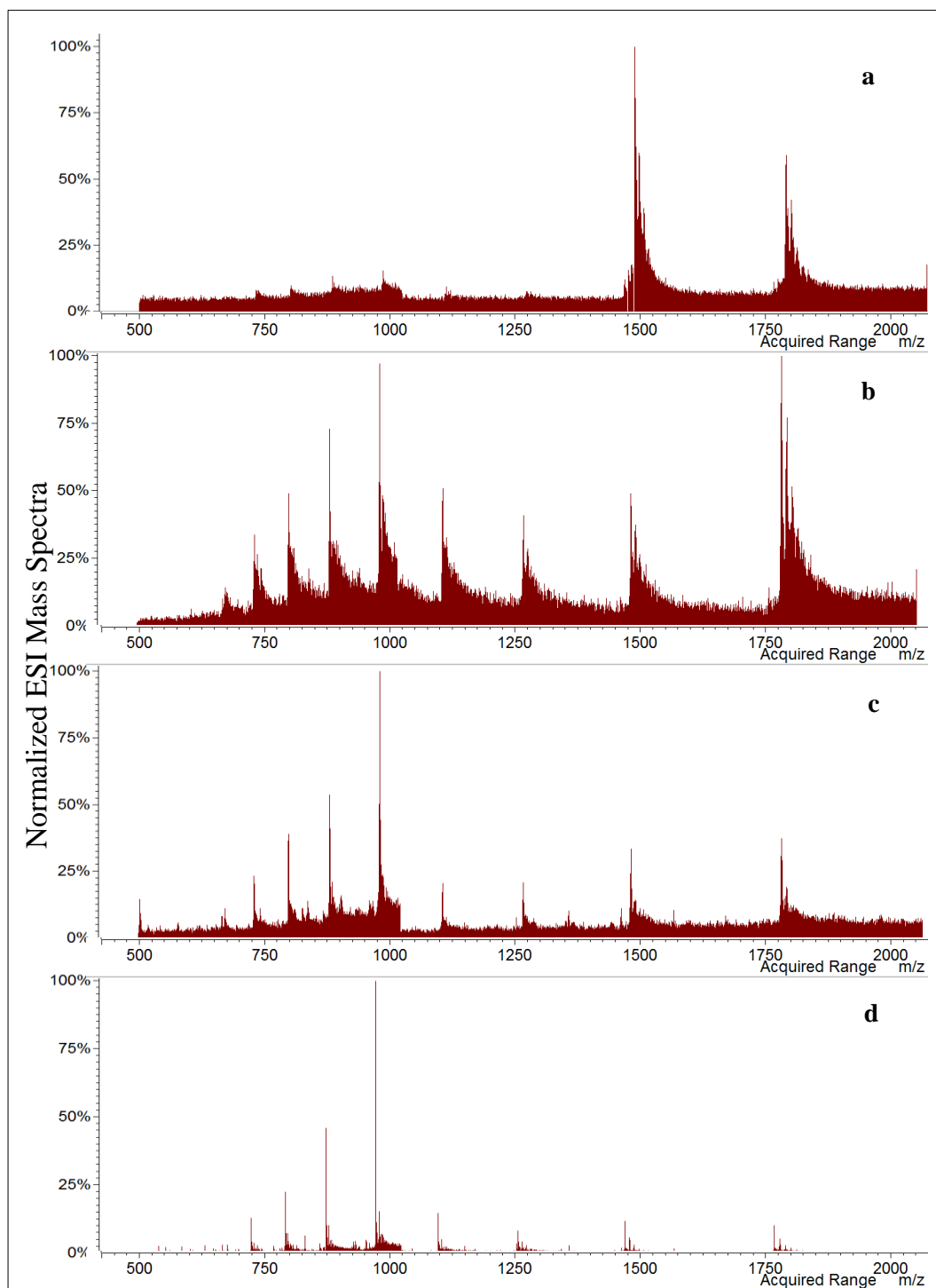


Fig. 20. ESI mass spectra of ubiquitin at pH 2.7 in various concentrations of methanol, (a) 10%, (b) 25%, (c) 50%, and (d) 90% methanol. The protein concentration was around 10 μ M.

Mass spectral analysis commenced immediately upon the introduction of ubiquitin to the various MeOH/D₂O solutions. The m/z of [ubiquitin]⁺⁶ was plotted against time using the single point m/z calculation to represent the overall m/z of [d-ubiquitin]⁺⁶. Mass analysis of [d-ubiquitin]⁺⁶ showed a rapid increase in mass upon injection of the analyte, followed by a gradual leveling off after about 60 minutes for each of the five methanol solutions (Fig. 21). The rates and degree of mass shift were clearly affected by the concentration of MeOH in solution, indicating a direct influence on the rate of deuterium exchange.

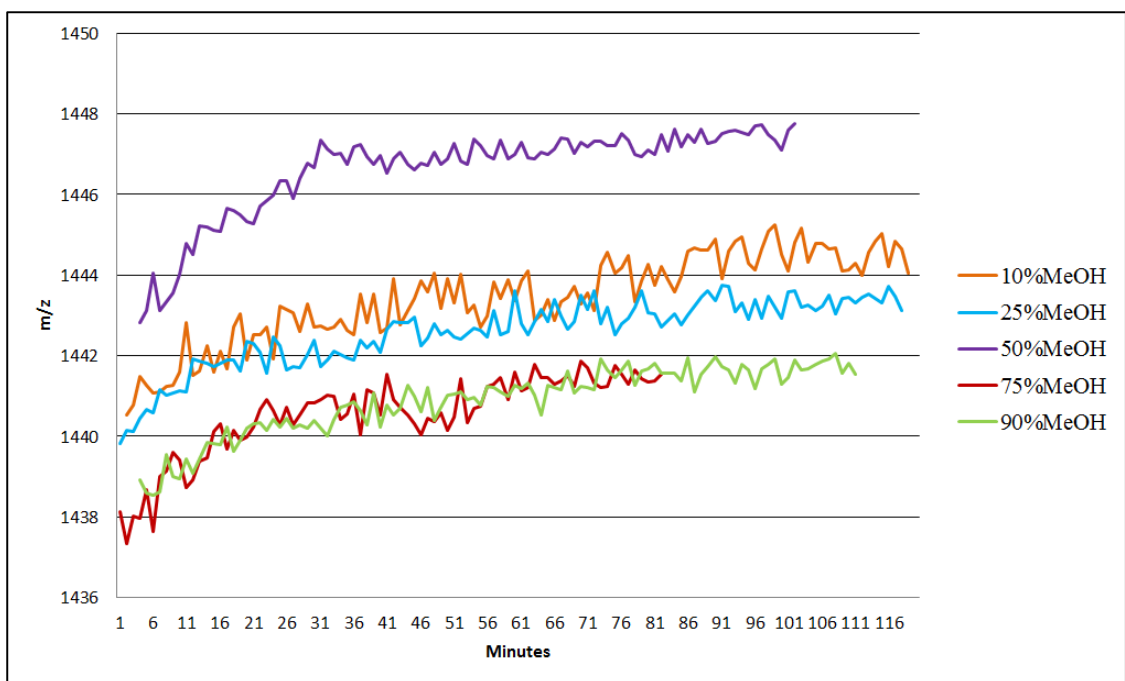


Fig. 21. Mass assignments of [d-ubiquitin]⁺⁶ upon exposure to various concentrations of MeOH/D₂O.

Comparison of the deuterium exchange rates in ubiquitin showed distinct changes to the rate and degree of deuterium exchange as the concentration of methanol increased from 10 to 90%, with the most significant changes occurring in the 25 to 75% methanol range. The degree of exchange is highest at 50% methanol with mass shifts leveling off at approximately 1448 m/z and the lowest degree of exchange residing at the 75 and 90% methanol concentrations with mass shifts leveling off at approximately 1442 m/z. Most notable are the similarities in the deuterium exchange rates and the overall mass shift of [d-ubiquitin]⁺⁶ in the 10 and 25% methanol solutions and those in the 75 and 90% methanol solutions.

4.3 High Resolution Scan of d-Ubiquitin Peptide Fragments

The high resolution mass analysis of chromatographically separated d-ubiquitin fragments focused on two charge states for each of the five preselected peptides. The high resolution scans, with a scan window of 15 m/z, were used to observe the mass shifts of peptide fragments produced from the peptic digest of deuterium labeled ubiquitin in various concentrations of methanol. The m/z for each peptide was obtained for each charge state, analyzed, and plotted using the average masses of the entire chromatographic peak (Fig. 22).

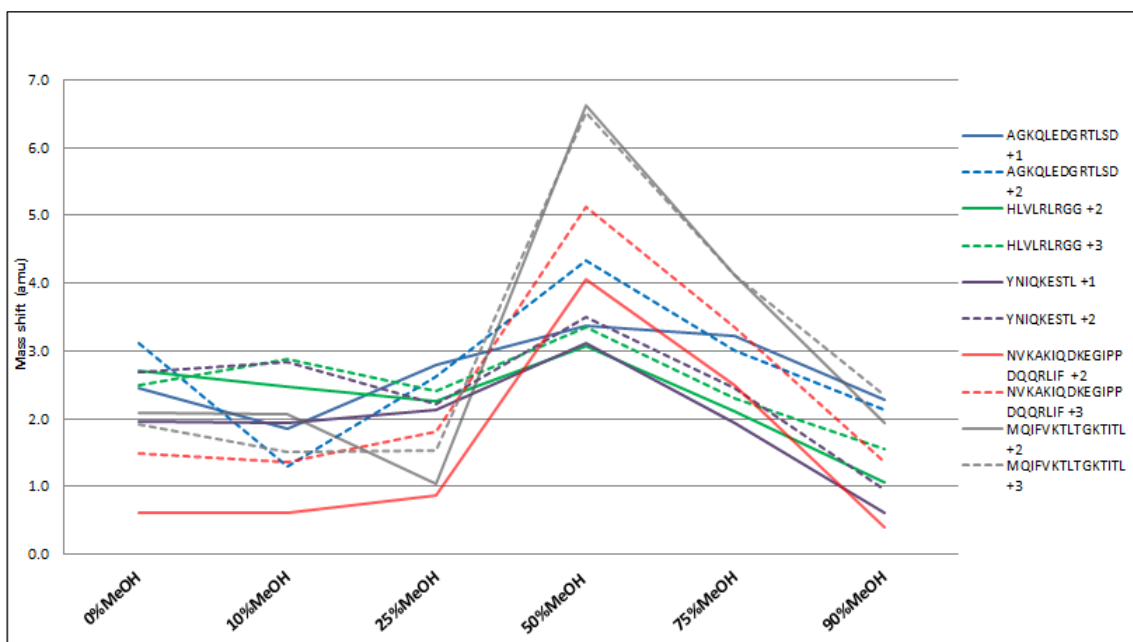


Fig. 22. Mass shifts for two charge states of the five preselected ubiquitin peptides from the peptic digestion of deuterium labeled ubiquitin in various concentrations of methanol/D₂O. The mass shifts are relative to h-ubiquitin peptide mass measurements and reported in atomic mass units (amu).

The mass spectral analysis showed increases in mass for all of the five d-ubiquitin peptide fragments across all six solutions of varying concentrations of methanol. The mass increase was most prevalent in the 25 to 75% methanol range with all peptides experiencing some degree of positive mass shift and decreasing as the concentration of methanol reaches 90%. The peptide MQIFVKTLTGKTITL exhibited the most increase, shifting more than 6 amu from its non-deuterated mass at 50% methanol; a substantial increase in mass was also observed in peptide NVKAKIQDKEGIPP DQQLIF with a shift of more than 4 amu. Minor overall increases in AGKQLEDGRTLSD, HVLRLRGG and YNIQESTL were observed with the most prevalent being in the 50% methanol solutions.

Examination of the overall increase in mass for each peptide can be shown to have close agreement with the ubiquitin conformational transitions suggested by Babu et al.¹⁵ and the deuterium exchange rates observed for ubiquitin. The collective analysis of each peptide fragment shows no overall increase in deuterium uptake until the concentration of methanol increases from 25 to 50% with a subsequent reduction as ubiquitin is exposed to 90% methanol. By examining all peptides collectively the transitions in ubiquitin conformation can be seen through the HiRes analysis of ubiquitin peptide fragments.

A clearer picture for the susceptibility of deuterium exchange in the ubiquitin peptides can be seen when averaging the mass shifts of the two peptide charge states used in the mass spectral analysis (Fig. 23). The degrees of mass shift for the d-ubiquitin peptides were clearly affected by methanol in solution showing distinct shifts in mass as the concentration of methanol changes. More evidence for the influence of methanol on ubiquitin can be seen in the relative position of each peptide when comparing the degree of mass shift (Fig. 24).

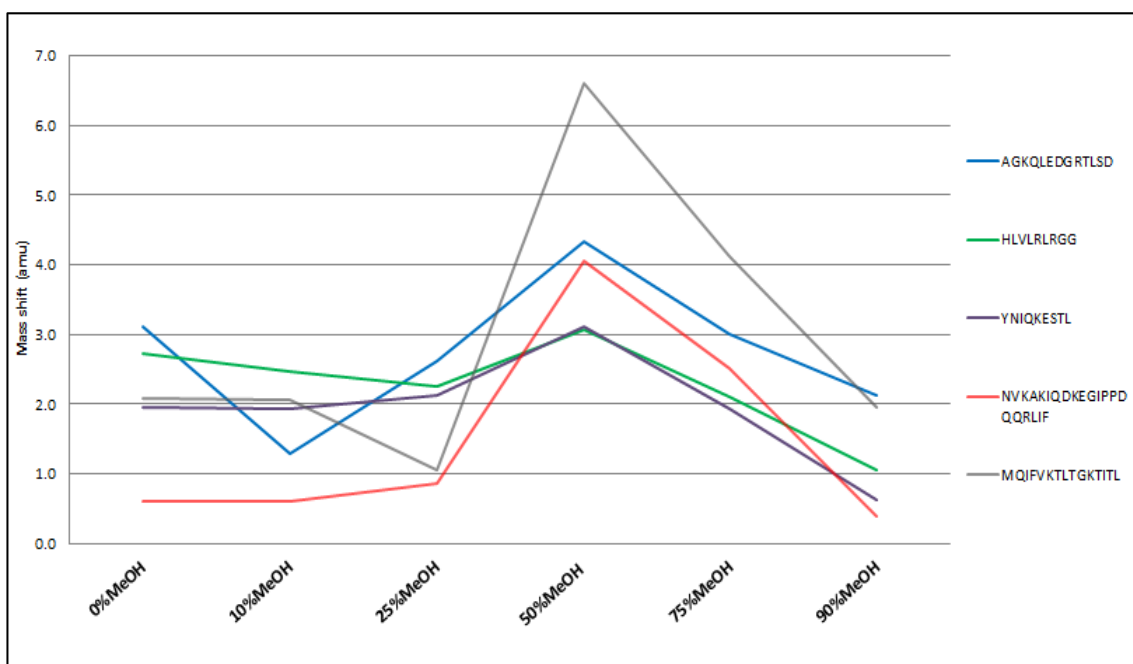


Fig. 23. The average mass shift between two observed charge states for each of the five preselected peptides from the peptic digestion of deuterium labeled ubiquitin in various concentrations of methanol/D₂O. The mass shifts are relative to h-ubiquitin peptide mass measurements. Values are reported in atomic mass unit (amu).

0% MeOH		10% MeOH		25% MeOH	
AGKQLEDGRTLSD	2.79	HLVLRRLGG	2.67	AGKQLEDGRTLSD	2.71
HLVLRRLGG	2.60	YNIQKESTL	2.38	HLVLRRLGG	2.33
YNIQKESTL	2.32	MQIFVKTLTGKTITL	1.78	YNIQKESTL	2.18
MQIFVKTLTGKTITL	2.00	AGKQLEDGRTLSD	1.70	NVKAKIQDKEGIPPDQQLIF	1.34
NVKAKIQDKEGIPPDQQLIF	1.05	NVKAKIQDKEGIPPDQQLIF	0.98	MQIFVKTLTGKTITL	1.29
50% MeOH		75% MeOH		90% MeOH	
MQIFVKTLTGKTITL	6.57	MQIFVKTLTGKTITL	4.12	AGKQLEDGRTLSD	2.20
NVKAKIQDKEGIPPDQQLIF	4.58	AGKQLEDGRTLSD	3.12	MQIFVKTLTGKTITL	2.15
AGKQLEDGRTLSD	3.85	NVKAKIQDKEGIPPDQQLIF	2.92	HLVLRRLGG	1.31
YNIQKESTL	3.31	HLVLRRLGG	2.20	NVKAKIQDKEGIPPDQQLIF	0.87
HLVLRRLGG	3.20	YNIQKESTL	2.19	YNIQKESTL	0.78

Fig. 24. The mass shift values based on the average mass shift of the two observed charge states for each of the five preselected peptides. Values are reported in atomic mass unit (amu).

The shifts in mass were greatest in 50% methanol with the lowest mass shift at 3.20 amu for HLVLRLRGG, which is still a greater shift in mass observed in almost every other solution studied. The increase of masses at this concentration was even more profound when comparing the shifts in mass for MQIFVKTLTGKTITL and NVKAKIQDKEGIPPDQQLIF in the 0-25% solutions, where the greatest shifts had been 2.0 and 1.3 amu, respectively. In comparison the shift for MQIFVKTLTGKTITL had been 5.2 amu and 4.2 amu for NVKAKIQDKEGIPPDQQLIF in the transition from 25 to 50% methanol. The 90% methanol solutions produced the lowest overall mass shift, restricting NVKAKIQDKEGIPPDQQLIF and YNIQKESTL to the lowest rate of deuterium exchange in the experiment.

In overview, each peptide followed a distinct path of deuterium exchange; with MQIFVKTLTGKTITL and NVKAKIQDKEGIPPDQQLIF demonstrating minor influences to mass from the presence of 0-25% methanol to dramatic shifts at 50% methanol, then a slow decrease in mass shift as the concentration increases to 90%, to levels below those seen in the 0-25% methanol solutions. In contrast, the deuterium uptake for peptides YNIQKESTL, HLVLRLRGG, AGKQLEDGRTLSD was shown to be the least influenced by the greater presence of methanol.

5.0 Conclusion

5.1 Circular Dichroism Experiments

Circular dichroism analysis of ubiquitin in various concentrations of methanol showed a clear increase in the helical structure of ubiquitin as the concentration of methanol reached 90%; these results were consistent with observations described by Babu et al.¹⁵ and studied by Wilkinson et al.¹³ In conducting the complimentary analysis, this experiment indicated changes in the secondary structure of ubiquitin in various concentrations of methanol, with clear transitions in ubiquitin conformation that closely matched the exchange rate analysis conducted by Babu et al.¹⁵ and Wright et al.^{21,22} In addition, the independent CD analysis suggested that β -sheet character in the native state of ubiquitin is lost as the concentration of methanol increases to 50%.

5.2 Deuterium Exchange Rate Analysis

The deuterium exchange rate analysis conducted on ubiquitin in various concentrations of methanol was in close agreement with observations reported by Babu et al.¹⁵ and Wright et al.^{21,22} The complimentary rate analysis showed three distinct regions of exchange for varying concentrations of methanol, with 10 and 25% methanol having the slowest rates of deuterium exchange, 75 and 90% methanol having the highest rates of exchange, and solutions of 50% methanol falling in-between. The three distinct groupings of ubiquitin exchange rates are consistent with the conformational transitions observed in the circular dichroism analysis.

5.3 High Resolution Scan of d-Ubiquitin Peptide Fragments

The three-dimensional structure of ubiquitin has been studied through X-ray diffraction experiments^{19,31} to determine the native structure of ubiquitin, revealing a hydrophobic core with a 3.5 turn α -helix at residues 23-34 (IENVKAKIQDKE) and four β -strands. Two of these β -strands were shown to be in parallel and form an inner sheet at residues 1-7 (MQIFVKTLT) and 64-72 (ESTLHLVLR). The other two β -strands were antiparallel and form an outer structure, incorporating residues 11-17 (KTLTLEV) and 40-44 (QQRLI).

Comparison of the five peptides used for HX experiments and the location of secondary structures in the native state of ubiquitin (Fig. 25) showed little correspondence with the sites of peptic cleavage, voiding any determination of deuterium uptake to the secondary structure. It is important to note that the five peptides used for analysis were selected based on their relative abundance, and no criteria had been set to select peptides that may have closely matched residues with specific structural elements in the native state.

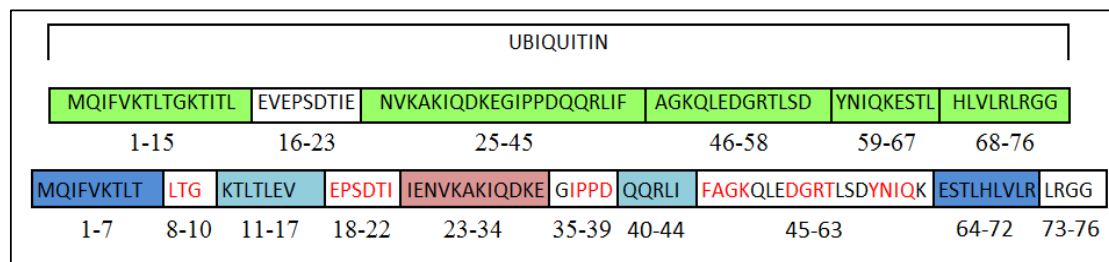


Fig. 25. Diagram for the representation of ubiquitin native state structure and the five preselected ubiquitin peptides used in the hydrogen/deuterium exchange experiments. Above) The 5 peptides selected for the bottom-up deuterium-exchange experiment in green. Below) The ubiquitin sequence showing different structures in the native state of ubiquitin and the associated residues; blue represent β -sheets in the inner structure, β -sheets in the outer structure in aqua, and α -helix in red. Residues showing reverse turns in red font.

The amounts of deuterium exchange for each peptide in the bottom-up HX experiment show the most significant uptake in peptide MQIFVKTLTGKTITL followed by peptide NVKAKIQDKEGIPPDQQLIF. In the native structure, peptide sequence MQIFVKTLTGKTITL has been shown to contain two β -strands residing in different locations of the three-dimensional structure of ubiquitin. The peptide sequence NVKAKIQDKEGIPPDQQLIF was shown to contain the sole α -helix and a portion of one of the outer residing β -strands. In the hydrogen/deuterium exchange experiments, neither of these peptides was shown to have any significant change in deuterium uptake until the methanol solution reached a concentration of 50%.

The peptide sequences AGKQLEDGRTLSD and YNIQKESTL, despite a few random turns and partial inner β -sheet structure in YNIQKESTL, were shown to have little regular structure in the ubiquitin native state. These peptides had little changes to the degree of deuterium exchange as a result of increases in the concentration of methanol. In analyzing the amount of deuterium exchange in the two peptides, it can be

stated that each peptide shows the least change from its native state conformation to the highly helical structure of ubiquitin in 90% methanol. The explanation for the relatively modest changes in deuterium uptake may be linked to the random turns and lack of overall structure of these peptide sequences in their native state. It is quite possible that as the conformation of ubiquitin changes to a more helical state these peptides, with an already ambiguous structure of random turns in the native state, require little manipulation to achieve the well-defined structured α -helix found in the 90% methanol solutions. The main argument for minimal hydrogen exchange resides in the preservation of hydrogen bonds within the secondary structure of AGKQLEDGRTLSD and YNIQKESTL, thus blocking any exchange with these hydrogen-bonded amides to deuterium.

The peptide sequence HLVLRLRGG in the native state of ubiquitin contains a partial inner β -sheet structure and is absent of structure in half of the sequence. The most important feature of this sequence is it includes the N-terminus of ubiquitin and is roughly seen to follow the same exchange levels seen in AGKQLEDGRTLSD and YNIQKESTL.

In the bottom-up hydrogen/deuterium exchange experiments the peptides MQIFVKTLTGKTITL and NVKAKIQDKEGIPPDQQLIF proved to have the most significance in using the acquired data to derive some conformational insight into local changes on the ubiquitin protein. It can be seen that these two peptides contain three of the four β -strands in the native state ubiquitin protein. Of these three β -strands, two of

them lie in the outer β -sheet in contact with the solvent. CD spectral analysis suggested elimination of β -sheet character as the concentration of methanol increased beyond 25%, ultimately ending with very prevalent helical structure in the entire protein at 90% methanol concentrations, consistent with observations made by Babu et al.¹⁵ implying that the two β -sheets residing on the outer structure of ubiquitin were converted to a structure with high helicity. The unfolding of the outer β -strands can be seen as the cause for high exchange of deuterium at the amide backbone due to the breaking of all hydrogen bonds at the amide backbone upon degradation or unfolding of the β -strands.

The unfolding of β -sheets could be seen as having the most significant effect for higher degrees of deuterium exchange; however the peptides YNIQKESTL and HLVLRLRGG have both been shown to contain an inner β -strand but do not show the same changes in deuterium uptake, possibly indicating that the β -strands located within the ubiquitin core structure are more stable and therefore experience less exchange of deuterium than those of the outer β -sheet. It is also worth noting that peptide sequence MQIFVKTLTGKTITL has inner and outer β -sheets in its native state, possibly explaining the highest degree of exchange seen in all five peptides.

5.4 Bottom-up Hydrogen/Deuterium Exchange Analysis of Ubiquitin

The bottom-up hydrogen/deuterium exchange analysis was better able to illustrate the methanol-induced transition of ubiquitin, showing a native state structure at 25% methanol and then quickly denaturing at 50% methanol, through what Babu et al.¹⁵ describes as an intermediary transition state. Data acquired in this experiment suggested

intermediary transitions were the result of denaturation at the β -sheets, and the residues involved in the most dynamic regions reside at 11-17 (KTLTLEV) and 40-44 (QQRLI).

Although this thesis first set out to study areas of high dynamicity as a way to investigate active sites of proteins, it may be possible to study areas of increased structural stability as well. In this experiment it can be said that the β -sheets in ubiquitin are doing exactly what β -sheets in a protein maybe intended to do, offer structural stability to the entire protein. In this case the β -sheets may be seen as very strong stabilizing structures in the protein by holding the native state constant in a very extreme environment, in solutions of at least 25% methanol. It can also be said that once the β -sheets structure was denatured the entire structure of the protein began to unfold.

6.0 Future Work

The project attempted to observe the conformational changes in ubiquitin by focusing on the relative mass changes of five ubiquitin peptides after undergoing a bottom-up approach to hydrogen/deuterium exchange mass spectrometry. It is important to note that there is still more research required in order to gain a clearer picture into the change in conformation of ubiquitin as it is exposed to greater concentrations of methanol.

One clear limitation of this research was the instrumentation used in obtaining the mass spectra. It is clear that mass spectral analysis of proteins requires a much greater degree of resolution than that presented in this work. The requirement for a single point m/z to track the mass shift is one of the major limitations of this work. In using a higher performing mass spectrometer, it may be possible to overcome this limitation and actively monitor mass shifts more accurately, not to mention an ability to monitor the mass shifts of a complete protein isotopic distribution rather than a weighted average indicator.

In the research presented, the concentrations of methanol used were 10, 25, 50, 75, and 90% giving a low resolution illustration of the conformational changes experienced by ubiquitin as it is exposed to greater concentrations of methanol. One issue this research overlooked was the transition state reported by Babu et al.¹⁵ which described a very dynamic change to the ubiquitin structure at the 30 to 50% methanol concentration range. This research did not look into this area and it would be appropriate for further

research to conduct greater analysis at this level possibly taking smaller incremental steps in concentration, than the broad steps in concentration used in this research.

The thesis tries to find areas of increased flexibility in the protein by denaturing it with methanol, it was hoped that places of activity may become denatured more easily and first to take up deuterium. This may not have been the case in this work as it may have been able to see areas of increased stability much easier. Employing multiple solvents in the analysis may offer different pathways of denaturation of the protein. Any future work should consider using multiple solvents for manipulating the protein structure in different ways, for example, non-polar solvents can be used to try and destabilize hydrophobic regions within the protein which have been shown to be the site of many protein active sites.

It is understood that any deuterium that has exchanged on to the amide backbone of a protein inherently becomes likely to back exchange with hydrogen and thus eliminate any mass shift in the protein. It is therefore extremely important to take any additional steps to ensure a minimal amount of deuterium back exchange. This requirement is most important in the chromatographic separation of peptides in the bottom-up analysis of hydrogen/ deuterium exchange. It is therefore recommended that any future work attempt to develop procedures that would ensure attainment of pH 2.7 and the stable retention of 0°C during the deuterium quenching state to reduce the possibility of deuterium loss at the amide backbone.

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