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Isotope Labeling of Biomolecules: Structural Analysis of Viruses by HDX-MS

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Abstract

The structural analysis of viruses is often a complex task. In many cases, the details of the viral architecture, especially for enveloped viruses, are limited to low-resolution techniques such as electron microscopy. These structural proteins and assemblies of viruses often populate multiple conformational states and undergo dramatic structural changes, making them difficult to study by most structural methods. They also frequently include highly dynamic regions that are of key functional importance. Many viruses present large surface glycoproteins, which have also proved to be challenging for structural biology due to the intrinsic flexibility and heterogeneity of the glycan decorations. Over the past two decades, hydrogen deuterium exchange coupled to mass spectrometry (HDX-MS) has provided a wealth of information on many diverse viral proteins, glycoproteins, and complexes, in many cases, in multiple conformational states. Here, we describe the methodology for using HDX-MS to investigate the rich structural dynamics of viral systems, and we briefly review the type of systems that have been examined through this type of approach. Though the technique is relatively simple, several potential pitfalls exist at both the sample preparation and the data analysis stage that investigators should be aware of for obtaining reliable data.

1. INTRODUCTION

Hydrogen deuterium exchange utilizes the naturally occurring proton exchange that occurs at amides on proteins in solution. By incubating a protein in its native solution state in a deuterated buffer, the amide protons exchange for deuterium. The rate of this exchange is governed by solution conditions such as temperature and pH and also is highly dependent on the local environment of the amide (Englander, 2006; Marcisin & Engen, 2010). Amides involved in stable hydrogen bonds, such as in alpha helices and beta-sheet secondary structure, exchange several orders of magnitude slower than exposed amides. The conformational dynamics of the structural elements will also influence the amide exchange rate, as breathing and dynamic fluctuations expose hydrogen-bonded amides for brief periods, allowing their protons to exchange with solvent. The kinetics of amide exchange thus can reveal sequence-specific information about secondary structure as well as the local conformational dynamics throughout a protein.

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As a technique hydrogen deuterium exchange has been around since the 1950s, but only in the early 1990s was mass spectrometry applied for analyzing deuterium uptake in intact proteins (Englander, 2006; Katta & Chait, 1991). After exchange under native conditions, the deuterium at backbone amides is locked in place by lowering the temperature and the pH to a regime where amide exchange is minimized or “quenched” (~0 °C and pH 2.5), thus enabling analysis by mass spectrometry. Acid-active proteases, such as pepsin, can be used under such quench conditions to digest the protein while leaving the deuterium label in place, thus providing local information on the exchange throughout the protein (Zhang & Smith, 1993; Fig. 1).

The real limitation on the size or complexity of the protein/complexes that can be analyzed by HDX-MS is the ability to resolve and detect the large number of peptides generated. Over the past two decades, these limits have continually expanded thanks to developments in liquid chromatography and mass spectrometry instrumentation. Ultra-high-pressure liquid chromatography (UPLC) offers increased chromatographic resolution that greatly increases the complexity of samples that can be analyzed, more effectively resolving the hundreds of peptides in a typical digest reaction (Wales, Fadgen, Gerhardt, & Engen, 2008). The continual improvement in the sensitivity and resolution of mass spectrometers also expands the limits on sample complexity while also helping to lower the limit of detection. With modern mass spectrometers, complete HDX analysis can be accomplished with as little as ~100 pmol, or around 5 µg of a 50-kDa protein. Software for HDX-MS data processing has also greatly facilitated the analysis of experimental data, though manual validation remains a critical part of generating reliable, complete datasets (Iacob & Engen, 2012).

Because HDX-MS provides a sensitive method for probing local structural dynamics, it has become widely used for mapping protein–protein or protein–ligand interactions, monitoring conformational changes induced by ligand binding or changes in solution conditions, and comparing variants of proteins such as mutants that may differ in stability (Chalmers et al., 2011; Englander, 2006; Marcisin & Engen, 2010). The method has also been adopted by the biopharmaceutical industry for assessing conformational purity and comparing biosimilars (Houde, Berkowitz, & Engen, 2011). While HDX-MS has primarily been employed to study soluble proteins, with additional steps for detergent or lipid removal prior to LC–MS, it has become possible to investigate the structure of membrane proteins as well (Hebling et al., 2010; Kim et al., 2011; Rey et al., 2010; Zhang, Chien, et al., 2010).

A major focus of structural virology is aimed at obtaining an understanding of construction and structural basis for stability of the protein capsids that encapsulate the viral genomic information. While the structures of many intact capsids have been determined by crystallography and electron microscopy, due to their large size, monitoring the structural or dynamic changes during assembly and subsequent maturation can be challenging. Large-scale changes also take place during receptor binding, entry, and uncoating or delivery of the viral genome. HDX-MS provides unique insights into structural changes in viral capsids and can be used to compare capsids that are in different conformational states, helping to pinpoint the regions that undergo significant local reorganization (Tuma, Coward, Kirk, Barnes, & Prevelige, 2001). HDX-MS has been used to measure local conformational dynamics and gain insight into the mechanism of assembly and capsid maturation for

various bacteriophages (Domitrovic et al., 2013; Fu & Prevelige, 2006; Gertsman, Fu, Huang, Komives, & Johnson, 2010; Gertsman et al., 2009; Kang, Hawkrig, Johnson, Muddiman, & Prevelige, 2006; Morton et al., 2010; Veesler et al., 2014), human immunodeficiency virus (HIV) (Lanman et al., 2003, 2004; Monroe, Kang, Kyere, Li, & Prevelige, 2010), Rous sarcoma virus (Bush et al., 2014), Adenovirus (Snijder et al., 2014), Hepatitis B (Bereszczak, Watts, Wingfield, Steven, & Heck, 2014), Brome mosaic virus (Wang, Lane, & Smith, 2001), and Rhinovirus (Wang & Smith, 2005).

One strength of HDX-MS is the ability to detect bimodal spectra, reflecting distinct conformations that a given region of a protein may populate in a sample. Under most exchange conditions, amides are only exposed for short times through fast local structural fluctuations and take up deuterium gradually ("EX2" kinetics shown in Fig. 2A). However, in some cases, proteins can undergo large motions that expose several amides for long periods leading to the complete exchange of this region ("EX1" kinetics shown in Fig. 2B). The result is a bimodal mass spectrum with two populations whose relative intensities shift over the time course of deuteration (Zhang, Post, & Smith, 1996). Such EX1 behavior has been observed in Hepatitis B capsids providing a more detailed level of information about their conformational dynamics (Bereszczak et al., 2014). Alternatively, a bimodal will also result if there are two distinct noninterchanging conformations present within an ensemble (Fig. 2C). This type of behavior was observed in HK-97 bacteriophage capsids, where HDX-MS revealed two conformational states that correlated well with the known quasiequivalent states within the capsid assembly (Gertsman, Komives, & Johnson, 2010). This type of spectra can also reveal conformational heterogeneity in a sample, for example, the presence of some misfolded or aggregated protein (Garcia, Guttman, Ebner, & Lee, 2015; Kaltashov, Bobst, Abzalimov, Berkowitz, & Houde, 2010).

Many viruses contain proteins that interact with host proteins during infection and may be in part or largely unstructured on their own. Unlike many structural techniques, HDX-MS can provide information on even intrinsically disordered proteins in their solutions state (Balasubramaniam & Komives, 2013). Using short timescale deuterium exchange, even weak residual secondary structure can be mapped (Keppel & Weis, 2015). HDX-MS has been used to elucidate the residual structure present in HIV Nef (Hochrein et al., 2006) and nonstructural proteins from Dengue virus (Zhao et al., 2015).

An important question for virology and vaccinology is how the viral surface proteins mediate viral infectivity while at the same time protecting themselves from immune surveillance. In many cases, the surface proteins are highly glycosylated as a means to protect the protein from the immune system (Scanlan, Offer, Zitzmann, & Dwek, 2007). Even for HDX-MS, viral glycoproteins present a challenge not only because material is often only available in very limited quantities, but also because the glycosylation on particular N-linked or O-linked sites tends to exhibit varying degrees of heterogeneity. This results in a multiplicity of ions that all may share the same underlying peptide sequence but have different glycan decorations (microheterogeneity) (Varki et al., 2009). The signal for glycopeptides thus can be relatively low, especially for glycopeptides that have multiple glycan chains. Furthermore, glycopeptides may be too hydrophilic to be retained by the

reverse phase columns commonly used for HDX-MS. Together these factors can dramatically limit the detection and sequence coverage of glycosylated segments.

Despite these challenges, in recent years, HDX-MS has been used to examine Hepatitis C E2 glycoprotein (Khan et al., 2014), influenza hemagglutinin (Garcia et al., 2015), HIV envelope glycoprotein (Davenport et al., 2013; Guttman et al., 2014; Guttman et al., 2015; Kim et al., 2011; Kong et al., 2010), and Ebola GP1/GP2 (Bale et al., 2011), revealing their conformational dynamics in solution and providing insight into mechanisms of viral glycoprotein recognition and virus neutralization by antibodies and small molecule inhibitors. The continued progress in the advancement of LC and MS technologies should eventually enable examination of viral proteins on their intact, infectious virions. The first steps have been taken toward applying HDX-MS to analyze surface glycoproteins *in situ* on whole enveloped viruses (Garcia et al., 2015).

2. METHODS

2.1 Initial Considerations for HDX-MS and Sample Requirements

For an HDX experiment, it is important to get suitable deuteration of the protein resulting in a final volume amenable to LC–MS analysis and minimizing deuterium loss (back-exchange) once the exchange is quenched. All samples within a series must be treated identically to ensure there are no deviations in percent deuterium content, buffer conditions, or extent of back-exchange between samples. The buffers should be selected so that the protein retains its native conformation throughout the deuterium exchange, and it is useful to perform native and SDS-PAGE before and after HDX to ensure that longer incubations in deuterium do not result in aggregation or degradation, both of which would confound the analysis.

The starting protein solution should be made to allow a 5- to 20-fold dilution into deuteration buffer, followed by at least a two-fold dilution into the quenching buffer. The final quenched sample should yield around 10–100 pmol of protein per injection on LC–MS, depending instrument sensitivity. The final volume is often limited by the size of the injection loop, typically 100 or 200 μ L. It is generally preferable to inject on the higher side to get better data for peptides of weaker intensity, thereby gaining more complete sequence coverage. However, it is important to be aware that too much signal in the MS may cause detector saturation, which can distort the mass envelope, leading to inaccurate deuteration measurements. For aggregation-prone proteins, the starting concentration may need to be lower, and final volumes of up to 1 mL can be used, but will require longer injection times.

Buffers for HDX, especially the quench solution, should be made with LC–MS grade water. Using MilliQ water is not advised as it often contains polymeric species that can interfere with MS analysis. For studies that aim to examine binding of small molecules, including a cosolvent such as 1–2% dimethyl sulfoxide may be required for compound solubility. In this case, it is also important to include the cosolvent in the unliganded protein exchanges, as the solvent may subtly perturb the structure of the protein and thereby alter the deuterium exchange kinetics. It is also important to note that when the ligand is in large excess, the

compound may give rise to a large peak in the LC gradient that can affect the detection of the peptides.

2.2 Deuterium Exchange Reaction

The exchange reaction is initiated by diluting the protein stock into deuterated buffer. Ideally, the deuteration times should be selected to get an adequate measure of the exchange rate for all amides. With many proteins, unprotected amides will be exchanged within milliseconds, while very highly ordered amides may require weeks. It is generally best to sample a broad timescale from seconds to several hours to maximize the temporal sampling. It is also important to consider that both pH and temperature have a large effect on the intrinsic exchange rate (the rate that an unprotected, completely accessible amide exchanges with solvent deuterium). Increasing the pH by 1 or the temperature by ~24 °C will increase the exchange rate 10-fold (Bai, Milne, Mayne, & Englander, 1993). One approach for expanding the temporal range covered by HDX-MS has been to run parallel experiments under different conditions and combine the exchange data (Coales et al., 2010).

Whenever working with D₂O (>99.9%), use freshly opened containers and keep samples well sealed as the D₂O will exchange with moisture in the air. If the HDX data are to be used for thermodynamic measurements, then it is important measure the pD during deuterium exchange (Bai et al., 1993; Hamuro et al., 2003). The pH* (the pH measured in D₂O) should be recorded for the buffers, which is used to calculate the pD (Krezel & Bal, 2004). It is also important to record the exact temperature during the deuterium exchanges. The downstream LC-MS should be compatible with nearly all buffer systems commonly used for proteins (with the exception detergents). For neutral pH samples, phosphate buffer is preferable as it also contributes to buffering capacity at the pH used for the subsequent quench step, pH 2.5.

For experiments that aim to compare two or more sets of data by HDX, it is advisable to include in an internal standard to provide an exact measure of the exchange conditions. This is best accomplished by spiking the starting protein stock solution with the tetrapeptide HN-Pro-Pro-Pro-Ile-OH (PPPI), which contains only a single, slow-exchanging amide (Zhang, Zhang, & Xiao, 2012) (~10 pmol per LC-MS injection works well in our hands). This provides a means to assess whether the exchange conditions were identical in all samples under comparison. Alternatively, the standard's exchange profile can be used to normalize data sets performed under different buffer conditions. Small peptides can also be spiked and preequilibrated in the buffered D₂O solution prior to the addition of the protein, to be used to test and correct for any deviations in back-exchange between samples (Zhang et al., 2012). When performing studies with protein-ligand complexes, it is necessary to use a sufficient excess of ligand and preequilibrate the complex to ensure that the complex is fully formed and remains intact during deuterium exchange.

2.2.1 Quenching the Exchange—After deuteration for a specified time, the amide exchange is slowed (or “quenched”) by lowering the temperature to ~0 °C and the pH to 2.5. This decreases the exchange rate by an order of about 10,000 where the average half-life of the deuterium label is ~1 h. The protein can then be either left intact for global deuterium

examination or rapidly digested under quench conditions with an acid-active protease to generate peptides that retain the deuterium labels. In either case, there will still be a small amount of deuterium loss (back-exchange) that should be kept as consistent as possible between samples. Quenching is typically performed by mixing deuteration reactions 1:1 into ice-cold acidification buffer (e.g., 0.1% trifluoroacetic acid [TFA]). Mock samples (containing buffers but not protein) are used to empirically adjust the pH of the quench solution to obtain a final pH of 2.5 after mixing. It is best to use thin walled PCR tubes for more efficient heat transfer during the quench step.

2.2.2 Digestion Optimization and Peptide Identification—Unless the deuteration is to be monitored on the intact protein level, a major consideration is whether enough peptides can be observed throughout the protein by LC–MS to comprehensively monitor the entire sequence (a metric referred to as the “sequence coverage”). An early step in HDX-MS studies is optimizing quench and digest conditions to provide the highest possible observable peptides to ensure maximal sequence coverage. Since the sample needs to be maintained at low pH, the availability of proteases is limited. Most commonly pepsin, which is very robust and works under quench conditions, is used. Alternative acid proteases such as protease XVII from *Rhizopus*, protease type XIII from *Aspergillus Saitoi*, and *Nepenthesin* protease have also been used with HDX-MS to provide complementary sequence coverage due to their differences in sequence specificity (Cravello, Lascoux, & Forest, 2003; Kadek et al., 2014). Due to the nonspecific nature of the proteases used, multiple partially overlapping peptides are often observed. This is beneficial as it provides multiple data points for a given region of the protein providing a higher level of redundancy.

Many proteins are sufficiently destabilized under such acidic conditions to enable subsequent digestion, though some may require additional denaturants to increase accessibility of the peptide backbone to the protease. In this case, the quench solution can be supplemented with urea or guanidine to help unfold the protein. For disulfide bond-containing proteins, tris-2-carboxy-ethylphosphine (TCEP) is included in the quench to reduce disulfides during protease digestion (Zhang, McLoughlin, et al., 2010). The amount of denaturant/TCEP, digestion time, and the protein:enzyme ratio should be varied when determining optimal quench and digest conditions. Pepsin will still retain activity in 8 *M* urea, up to 2 *M* GndHCl, or up to 1 *M* TCEP. Some hyper-stable proteins may be so protease resistant that even with high concentrations of denaturants under quench conditions the digestion will produce very few peptides, severely limiting the sequence coverage.

Some proteins may require detergent for solubility or stability that can be included during deuterium exchange. It is possible to supplement buffers with low concentrations of certain nonionic detergents (e.g., 0.1% *n*-dodecyl maltoside or β -octyl glucoside) that are compatible with LC–MS. For higher concentrations of detergents, an additional organic extraction step can be used for detergent removal after quenching (Rey et al., 2010). For membrane-embedded proteins, zirconium affinity has been shown to be an effective approach for phospholipid depletion under quench conditions (Hebling et al., 2010).

Digestion can be carried out manually with soluble or immobilized protease. Alternatively, in many cases, an online protease column is incorporated into the LC–MS system. With in-

solution digestion, a method of rapidly mixing the protease into the quenched solution is to add the small volume of protease solution into the cap of the microcentrifuge tube. This way after the protein is added to the quench it is possible to immediately vortex and efficiently combine the protease with the quenched sample. Immobilized proteases or protease columns have the advantage of generating a digest with a lower background (no autolysis fragments, or other impurities from the protease). Immobilized pepsin on high-pressure resistant particles is now also commercially available (Enzymate column, Waters). This enables digestions at pressures up to 10,000 psi, which can greatly aid in digestion efficiency by disrupting the protein's residual structure, without contributing to back-exchange (Ahn, Jung, Wyndham, Yu, & Engen, 2012). One disadvantage of using online digestion columns is that they add another source of carryover (discussed below) during LC, which can confound HDX-MS data.

Another choice for HDX sample preparation is whether to freeze the sample or proceed directly to LC-MS. Since it is best to match all conditions as closely as possible for all deuteration time points, many labs perform all deuterium reactions side by side and flash freeze in liquid nitrogen. Deuterated samples can be stored at -80°C for months with no detectable loss of the deuteration. Modern automated HDX systems that utilize robots to perform exchanges immediately inject the quenched samples onto LC-MS. This approach has automated the mixing, quenching, and injection steps and has enabled HDX as a tool for high-throughput analysis and screening (Chalmers et al., 2011; Englander et al., 2003). Only pepsin columns compatible with HDX-MS should be used, as some pepsin columns have been observed to cause severe deuterium loss (Wu, Kaveti, & Engen, 2006).

2.2.3 Additional HDX Controls—An undeuterated control is made by identical dilution and subsequent digestion of the protein in purely aqueous buffer. This sample is used for peptide identification and also serves as a reference for deuterium shift analysis. A fully or maximally deuterated control is helpful to include as they serve as a reference for whether deuterium exchange went to completion at the longest time points. For simple comparative studies (i.e., free protein vs. ligand bound), the quantitative differences at each time point will already highlight the locations where changes occur, and the fully deuterated control may not be necessary.

Preparation of a fully deuterated sample may not always be trivial. It has to achieve full deuteration, but the buffer should match the other time points to ensure consistent back-exchange conditions. With many proteins unfolding a starting protein stock with denaturant (e.g., heating in 3–6 M GndHCl), diluting into the buffered D_2O and extensive heating (65°C for 1 h) should provide a fully deuterated sample that approximately matches the buffer composition of the other samples. Performing the deuteration step in high concentrations of denaturant (i.e., GndHCl) may significantly offset the intrinsic exchange rate (Zhang et al., 2012), and thereby lead to altered back-exchange. Since the fully deuterated sample is fully denatured (and reduced), it can also have a different digestion profile (due to increased digestion efficiency). Using a lower concentration of protease can help generate digestion profiles to match closer to the other deuterated samples while keeping the same level of back-exchange. In some cases, it may be necessary to digest the

protein first and deuterate the peptides. For this approach, the exchange and quench buffers must be carefully matched to the other time points to ensure identical back-exchange.

During the digestion step, the sample is exposed to the residual deuterium content after the quench mixing. This can cause artifactual “in-exchange,” especially with longer digestion times, where the unfolded and digested protein slowly takes up deuterium in the quenched solution (Hoofnagle, Resing, & Ahn, 2003). Some peptides will appear to have become partially deuterated when in fact they did not exchange at all during the deuterium incubation step. For this reason, many labs perform a zero time point correction by premixing the quench with the D₂O buffer, and subsequently adding the stock protein, thereby only sampling the in-exchange.

2.2.4 Liquid Chromatography–Mass Spectrometry—Once the sample is digested, it is then analyzed by mass spectrometry to measure incorporation of deuterium. It is critical that quench conditions be maintained throughout the analysis to avoid further back-exchange. Both matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have been used for HDX-MS studies (Mandell, Falick, & Komives, 1998; Zhang & Smith, 1993); however, the coupling of ESI to reverse-phase UPLC has made it the dominant method for analysis, in general providing greater sequence coverage and more complete structural analysis. In a typical setup, the quenched sample is desalted and preconcentrated on a trapping column, and subsequently the peptides are resolved with a relatively short (10 min) gradient, though for highly complex samples longer gradients can be used (Walters, Ricciuti, Mayne, & Englander, 2012). LC gradients should be empirically optimized for each sample, but a good starting condition is 5–40% B over 10 min (A: 0.1% formic acid (FA), 5% acetonitrile with ~0.02% TFA for pH 2.5; B: 0.1% FA, 100% acetonitrile). The syringe, injection loop, lines, and columns are kept at or near 0 °C to minimize back-exchange. Systems for maintaining cold temperature LC runs can be constructed rather inexpensively (Keppel, Jacques, Young, Ratzlaff, & Weis, 2011). Alternatively, commercial systems are now also available that maintain low temperatures and automate the injection process (HDX Manager, Waters).

To avoid any deviations in the data that could arise from day-to-day variation, it is always preferable to collect data on all samples on the same day with identical conditions (Houde et al., 2011). A major concern during LC–MS data collection is residual sample from a previous injection appearing in the subsequent run (sample “carryover”). Significant carryover will result in a convoluted mass envelope leading to an inaccurate measure of deuterium incorporation (Fig. 2E). Therefore, minimizing such carryover during a sequence of injections is critical for reliable HDX-MS data. Elaborate washing protocols have been established to minimize this (Fang, Rand, Beuning, & Engen, 2011); however, it is still advisable to run periodic blanks to ensure that carryover is not significant (<5%). Incorporation of an online protease column adds another source of carryover, which requires an additional set of wash cycles (Majumdar et al., 2012). In this case, it is also important to keep in mind that some of the harsher wash solvents for cleaning the trapping column should not be passed over the protease column.

High-resolution quadrupole time-of-flight mass (Q-TOF) analyzers have been popular for HDX-MS as they provide the necessary resolution for observing each isotopic peak. More recent FT-based instruments offer even higher resolution, but suffer from potential destructive interference effects that can result in inaccurate deuteration measurements (Burns, Rey, Baker, & Schriemer, 2013). Another consideration with the mass spectrometer is that the heating during ESI will also result in some back-exchange (Katta & Chait, 1993). For this reason, HDX-MS on most instruments is conducted using a lower source temperature. MS instrumentation with an ion mobility stage adds an additional dimension for the gas-phase separation of peptides, thereby expanding the complexity of samples that can be analyzed (Iacob, Murphy, & Engen, 2008). The limits on sample size and complexity for HDX-MS are expected to expand in parallel with the continual advancement of LC and MS technologies.

2.2.5 Peptide Identification—Due to the characteristic proteolytic activity of pepsin, the peptides observed in HDX-MS vary greatly in length, ranging from 3 to 30 residues, and identification is accomplished using exact mass and fragmentation data from an undeuterated sample. Due to the inherent hydrogen scrambling by collision-induced dissociation (CID), MS/MS of deuterated peptides does not yield additional information regarding deuterium localization (Bache, Rand, Roepstorff, Ploug, & Jorgensen, 2008; Ferguson & Konermann, 2008). However, MS/MS by electron capture (Abzalimov, Kaplan, Easterling, & Kaltashov, 2009) and electron transfer dissociation (Rand, Pringle, Morris, Engen, & Brown, 2011) provide gas-phase fragmentation without inducing hydrogen scrambling, thus enabling deuterium exchange measurements with single-residue resolution (Landgraf, Chalmers, & Griffin, 2012).

Identification of glycosylated peptides, which are common on viral surface proteins, is more challenging as conventional CID MS/MS yields little fragmentation of the peptide backbone (Medzihradszky, 2005). Electron transfer dissociation is often used as an effective alternative MS/MS tool for identification of glycopeptides (Syka, Coon, Schroeder, Shabanowitz, & Hunt, 2004). Samples can also be reneutralized after digestion, treated with endoglycosidases to remove glycans, and then used to obtain CID fragmentation data for the deglycosylated peptides. With regard to glycopeptides, it is also important to note that amido groups within the glycan chains also take on and retain deuterium during HDX-MS analysis (Guttman, Scian, & Lee, 2011).

2.2.6 HDX Data Analysis—Once the datasets have been collected, the deuterium incorporation needs to be extracted from the mass spectra. Most often, determination of deuterium uptake for a given peptide involves measuring the geometric center (centroid) of each mass envelope for each time point for each observable peptide, which can be hundreds to thousands of spectra. To this end, countless software tools have been developed to provide automation and turn weeks of manual analysis into days. Today, there are both commercial and academic software packages including: DynamX (Waters); HD-Examiner (Sierra Analytics), Mass Spec Studio (Rey et al., 2014), HD-Workbench (Pascal et al., 2012), EX-MS (Kan, Mayne, Chetty, & Englander, 2011), and HX-Express (Weis, Engen, & Kass, 2006), to name only a few.

Techniques have also been developed for utilizing overlapping peptides, commonly generated by the nonspecific proteases used for HDX-MS, for obtaining higher resolution exchange information (Fajer, Bou-Assaf, & Marshall, 2012; Zhang et al., 2012). However, unpredictable back-exchange due to residual secondary structure or column interactions adds a complication that can confound this approach to gaining higher resolution information from the data (Sheff, Rey, & Schriemer, 2013). It should be appreciated that a simple centroid analysis is insufficient for interpreting the full information content within HDX data (Zhang, Ramachandran, Kumar, & Gross, 2013). Slow correlated protein motions, or mixtures of conformational states in a sample, can give rise to bimodal mass spectra, which can sometimes be subtle and not readily evident (Fig. 2; Weis, Wales, Engen, Hotchkko, & Ten Eyck, 2006). Recent software developments have begun to address detection and even deconvolution of such bimodal mass spectra (Chik, Vande Graaf, & Schriemer, 2006; Guttman, Weis, Engen, & Lee, 2013; Kan et al., 2011).

HDX-MS is often used for mapping protein–protein and protein–ligand interactions (Coales, Tuske, Tomasso, & Hamuro, 2009; Mandell, Baerga-Ortiz, Falick, & Komives, 2005). However, ligand binding can cause long-range allosteric effects, which are also readily observed by HDX-MS (Chalmers et al., 2011). When comparing the effects of ligand binding, it is also important to consider the pathway of deuterium exchange. For some protein–ligand complexes, the predominant exchange pathway actually occurs through the unliganded state during transient ligand dissociations (Wildes & Marqusee, 2005). These caveats should be considered during the interpretation of HDX-MS comparisons, especially in the absence of other structural information.

3. EXAMPLE HDX-MS EXPERIMENT WITH BOVINE RIBONUCLEASE A

Prepare buffers¹: wherever possible use LC–MS grade water for all solutions;

20 × phosphate-buffered saline (PBS): 400 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 1% sodium azide, 20 mM ethylenediamine-tetraacetic acid (EDTA);

Ribonuclease A (RNaseA) stock: 0.1 mg/mL RNaseA in 1 × PBS;

Pepsin solution: 1 mg/mL pepsin (3 × recrystallized, Worthington Biochemicals) in 100 mM sodium phosphate, pH 4.0;

Quench buffer: 0.2% FA, 100 mM TCEP (Sigma-Aldrich) in LC–MS grade water;

Buffered D₂O²: Freshly opened D₂O (>99.9%, Cambridge isotope labs), mixed with 20 × PBS for 1 ×.

Prepare mock samples for optimizing the quench pH:

90 μL buffered D₂O,

10 μL 1 × PBS,

¹Optional: include 0.5 μg/mL of the tetrapeptide: Pro-Pro-Pro-Ile (PPPI).

²Keep this solution sealed as the D₂O will exchange with ambient moisture.

100 μ L quench buffer,

10 μ L pepsin buffer (100 mM sodium phosphate, pH 4.0).

Vortex and test the pH. Make small additions of 1 M hydrochloric acid or 1 M sodium hydroxide to the master quench solution and continue to prepare mock samples until the final pH is 2.5. Approximately 2 μ L of 10 M sodium hydroxide per milliliters of quench solution will be needed to achieve this final pH. Have a container of liquid nitrogen ready for freezing-quenched samples.

3.1 Deuteration and Quenching

To initiate deuterium exchanges add 10 μ L of the RNaseA stock into 90 μ L of buffered D₂O, pipette up and down to mix, seal the tube. For the sake of consistency, it is best to use the same pipettor and identical pipette tips for all deuterium exchanges. For incubation times longer than 1 h, it is advisable to wrap the tube cap with parafilm to avoid exchange with ambient moisture. About 1 min before the desired deuteration time, place the quench tube with 100 μ L of quench solution on ice. Open the tube and place 10 μ L of pepsin solution in the cap, leave on ice with the cap open. At the desired deuteration time, pipette the entirety of the deuteration mixture and inject into the quench tube. Quickly close and vortex the tube to mix and initiate digestion. Leave the tube on ice for 5 min to reduce and digest, and flash freeze in liquid nitrogen. For incubations less than 15 s, place 10 μ L RNaseA in new tube. Using a 100 μ L pipette transfer 90 μ L of buffered D₂O to the protein, mix rapidly and with the same pipette tip, transfer the full volume into the quench tube. For incubations greater than 8 h, it is best to initiate the exchange the evening before and leave sealed overnight, so it can be quenched side by side with the shorter incubations. Samples can be stored at -80°C for months.

Undeuterated sample—Prepared as all deuterium incubations but with buffered H₂O instead of buffered D₂O.

Fully deuterated sample—Prepare a denatured solution of RNaseA at 0.1 mg/mL:

0.1 mg/mL RNaseA,

1 $\times$ PBS,

4 M guanidine hydrochloride,

10 mM dithiothreitol (DTT),

Heat at 90°C for 30 min.

Mix 10 μ L denatured RNaseA into 90 μ L buffered D₂O, seal the cap with parafilm, and heat to 65°C for 1 h. Allow the sample to cool and quench, digest, and freeze as described above.

Optional: zero time point: Prepare a quench tube with quench buffer and pepsin as described above.

In quick succession, add 90 μ L of buffered D₂O and then 10 μ L of RNaseA stock. Digest and freeze as described above.

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ABBREVIATIONS

ESI	electrospray ionization
FA	formic acid
HDX-MS	hydrogen deuterium exchange coupled to mass spectrometry
TFA	trifluoroacetic acid
UPLC	ultra-high-pressure liquid chromatography

REFERENCES

- Abzalimov RR, Kaplan DA, Easterling ML, Kaltashov IA. Protein conformations can be probed in top-down HDX MS experiments utilizing electron transfer dissociation of protein ions without hydrogen scrambling. *Journal of the American Society for Mass Spectrometry*. 2009; 20(8):1514–1517. [PubMed: 19467606]
- Ahn J, Jung MC, Wyndham K, Yu YQ, Engen JR. Pepsin immobilized on high-strength hybrid particles for continuous flow online digestion at 10,000 psi. *Analytical Chemistry*. 2012; 84(16):7256–7262. [PubMed: 22856522]
- Bache N, Rand KD, Roepstorff P, Ploug M, Jorgensen TJ. Hydrogen atom scrambling in selectively labeled anionic peptides upon collisional activation by MALDI tandem time-of-flight mass spectrometry. *Journal of the American Society for Mass Spectrometry*. 2008; 19(12):1719–1725. [PubMed: 18640053]
- Bai Y, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. *Proteins*. 1993; 17(1):75–86. [PubMed: 8234246]
- Balasubramaniam D, Komives EA. Hydrogen-exchange mass spectrometry for the study of intrinsic disorder in proteins. *Biochimica et Biophysica Acta*. 2013; 1834(6):1202–1209. [PubMed: 23099262]
- Bale S, Liu T, Li S, Wang Y, Abelson D, Fusco M, et al. Ebola virus glycoprotein needs an additional trigger, beyond proteolytic priming for membrane fusion. *PLoS Neglected Tropical Diseases*. 2011; 5(11):e1395. [PubMed: 22102923]
- Bereszczak JZ, Watts NR, Wingfield PT, Steven AC, Heck AJ. Assessment of differences in the conformational flexibility of hepatitis B virus core-antigen and e-antigen by hydrogen deuterium exchange-mass spectrometry. *Protein Science*. 2014; 23(7):884–896. [PubMed: 24715628]
- Burns KM, Rey M, Baker CA, Schriemer DC. Platform dependencies in bottom-up hydrogen/deuterium exchange mass spectrometry. *Molecular & Cellular Proteomics*. 2013; 12(2):539–548. [PubMed: 23197788]
- Bush DL, Monroe EB, Bedwell GJ, Prevelige PE Jr, Phillips JM, Vogt VM. Higher-order structure of the Rous sarcoma virus SP assembly domain. *Journal of Virology*. 2014; 88(10):5617–5629. [PubMed: 24599998]
- Chalmers MJ, Pascal BD, Willis S, Zhang J, Iturria SJ, Dodge JA, et al. Methods for the analysis of high precision differential hydrogen deuterium exchange data. *International Journal of Mass Spectrometry*. 2011; 302(1–3):59–68. [PubMed: 21528013]
- Chik JK, Vande Graaf JL, Schriemer DC. Quantitating the statistical distribution of deuterium incorporation to extend the utility of H/D exchange MS data. *Analytical Chemistry*. 2006; 78(1):207–214. [PubMed: 16383329]

- Coales SJ, E SY, Lee JE, Ma A, Morrow JA, Hamuro Y. Expansion of time window for mass spectrometric measurement of amide hydrogen/deuterium exchange reactions. *Rapid Communications in Mass Spectrometry*. 2010; 24(24):3585–3592. [PubMed: 21108306]
- Coales SJ, Tuske SJ, Tomasso JC, Hamuro Y. Epitope mapping by amide hydrogen/deuterium exchange coupled with immobilization of antibody, on-line proteolysis, liquid chromatography and mass spectrometry. *Rapid Communications in Mass Spectrometry*. 2009; 23(5):639–647. [PubMed: 19170039]
- Cravello L, Lascoux D, Forest E. Use of different proteases working in acidic conditions to improve sequence coverage and resolution in hydrogen/deuterium exchange of large proteins. *Rapid Communications in Mass Spectrometry*. 2003; 17(21):2387–2393. [PubMed: 14587084]
- Davenport TM, Guttman M, Guo W, Cleveland B, Kahn M, Hu SL, et al. Isolate-specific differences in the conformational dynamics and antigenicity of HIV-1 gp120. *Journal of Virology*. 2013; 87(19):10855–10873. [PubMed: 23903848]
- Domitrovic T, Movahed N, Bothner B, Matsui T, Wang Q, Doerschuk PC, et al. Virus assembly and maturation: Auto-regulation through allosteric molecular switches. *Journal of Molecular Biology*. 2013; 425(9):1488–1496. [PubMed: 23485419]
- Englander SW. Hydrogen exchange and mass spectrometry: A historical perspective. *Journal of the American Society for Mass Spectrometry*. 2006; 17(11):1481–1489. [PubMed: 16876429]
- Englander JJ, Del Mar C, Li W, Englander SW, Kim JS, Stranz DD, et al. Protein structure change studied by hydrogen-deuterium exchange, functional labeling, and mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(12):7057–7062. [PubMed: 12773622]
- Fajer PG, Bou-Assaf GM, Marshall AG. Improved sequence resolution by global analysis of overlapped peptides in hydrogen/deuterium exchange mass spectrometry. *Journal of the American Society for Mass Spectrometry*. 2012; 23(7):1202–1208. [PubMed: 22528203]
- Fang J, Rand KD, Beuning PJ, Engen JR. False EX1 signatures caused by sample carryover during HX MS analyses. *International Journal of Mass Spectrometry*. 2011; 302(1–3):19–25. [PubMed: 21643454]
- Ferguson PL, Konermann L. Nonuniform isotope patterns produced by collision-induced dissociation of homogeneously labeled ubiquitin: Implications for spatially resolved hydrogen/deuterium exchange ESI-MS studies. *Analytical Chemistry*. 2008; 80(11):4078–4086. [PubMed: 18459737]
- Fu CY, Prevelige PE Jr. Dynamic motions of free and bound O29 scaffolding protein identified by hydrogen deuterium exchange mass spectrometry. *Protein Science*. 2006; 15(4):731–743. [PubMed: 16522798]
- Garcia NK, Guttman M, Ebner JL, Lee KK. Dynamic changes during acid-induced activation of influenza hemagglutinin. *Structure*. 2015; 23(4):665–676. [PubMed: 25773144]
- Gertsman I, Fu CY, Huang R, Komives EA, Johnson JE. Critical salt bridges guide capsid assembly, stability, and maturation behavior in bacteriophage HK97. *Molecular & Cellular Proteomics*. 2010; 9(8):1752–1763. [PubMed: 20332083]
- Gertsman I, Gan L, Guttman M, Lee K, Speir JA, Duda RL, et al. An unexpected twist in viral capsid maturation. *Nature*. 2009; 458(7238):646–650. [PubMed: 19204733]
- Gertsman I, Komives EA, Johnson JE. HK97 maturation studied by crystallography and H/2H exchange reveals the structural basis for exothermic particle transitions. *Journal of Molecular Biology*. 2010; 397(2):560–574. [PubMed: 20093122]
- Guttman M, Cupo A, Julien JP, Sanders RW, Wilson IA, Moore JP, et al. Antibody potency relates to the ability to recognize the closed, pre-fusion form of HIV Env. *Nature Communications*. 2015; 6:6144.
- Guttman M, Garcia NK, Cupo A, Matsui T, Julien JP, Sanders RW, et al. CD4-induced activation in a soluble HIV-1 Env trimer. *Structure*. 2014; 22:974–984. [PubMed: 24931470]
- Guttman M, Scian M, Lee KK. Tracking hydrogen/deuterium exchange at glycan sites in glycoproteins by mass spectrometry. *Analytical Chemistry*. 2011; 83(19):7492–7499. [PubMed: 21863800]
- Guttman M, Weis DD, Engen JR, Lee KK. Analysis of overlapped and noisy hydrogen/deuterium exchange mass spectra. *Journal of the American Society for Mass Spectrometry*. 2013; 24(12):1906–1912. [PubMed: 24018862]

- Hamuro Y, Coales SJ, Southern MR, Nemeth-Cawley JF, Stranz DD, Griffin PR. Rapid analysis of protein structure and dynamics by hydrogen/deuterium exchange mass spectrometry. *Journal of Biomolecular Techniques*. 2003; 14(3):171–182. [PubMed: 13678147]
- Hebling CM, Morgan CR, Stafford DW, Jorgenson JW, Rand KD, Engen JR. Conformational analysis of membrane proteins in phospholipid bilayer nanodiscs by hydrogen exchange mass spectrometry. *Analytical Chemistry*. 2010; 82(13):5415–5419. [PubMed: 20518534]
- Hochrein JM, Wales TE, Lerner EC, Schiavone AP, Smithgall TE, Engen JR. Conformational features of the full-length HIV and SIV Nef proteins determined by mass spectrometry. *Biochemistry*. 2006; 45(25):7733–7739. [PubMed: 16784224]
- Hoofnagle AN, Resing KA, Ahn NG. Protein analysis by hydrogen exchange mass spectrometry. *Annual Review of Biophysics and Biomolecular Structure*. 2003; 32:1–25.
- Houde D, Berkowitz SA, Engen JR. The utility of hydrogen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. *Journal of Pharmaceutical Sciences*. 2011; 100(6):2071–2086. [PubMed: 21491437]
- Iacob RE, Engen JR. Hydrogen exchange mass spectrometry: Are we out of the quicksand? *Journal of the American Society for Mass Spectrometry*. 2012; 23(6):1003–1010. [PubMed: 22476891]
- Iacob RE, Murphy JP 3rd, Engen JR. Ion mobility adds an additional dimension to mass spectrometric analysis of solution-phase hydrogen/deuterium exchange. *Rapid Communications in Mass Spectrometry*. 2008; 22(18):2898–2904. [PubMed: 18727141]
- Kadek A, Mrazek H, Halada P, Rey M, Schriemer DC, Man P. Aspartic protease nepenthesin-1 as a tool for digestion in hydrogen/deuterium exchange mass spectrometry. *Analytical Chemistry*. 2014; 86(9):4287–4294. [PubMed: 24661217]
- Kaltashov IA, Bobst CE, Abzalimov RR, Berkowitz SA, Houde D. Conformation and dynamics of biopharmaceuticals: Transition of mass spectrometry-based tools from academe to industry. *Journal of the American Society for Mass Spectrometry*. 2010; 21(3):323–337. [PubMed: 19963397]
- Kan ZY, Mayne L, Chetty PS, Englander SW. ExMS: Data analysis for HX-MS experiments. *Journal of the American Society for Mass Spectrometry*. 2011; 22(11):1906–1915. [PubMed: 21952778]
- Kang S, Hawkrigde AM, Johnson KL, Muddiman DC, Prevelige PE Jr. Identification of subunit-subunit interactions in bacteriophage P22 procapsids by chemical cross-linking and mass spectrometry. *Journal of Proteome Research*. 2006; 5(2):370–377. [PubMed: 16457603]
- Katta V, Chait BT. Conformational changes in proteins probed by hydrogen-exchange electrospray-ionization mass spectrometry. *Rapid Communications in Mass Spectrometry*. 1991; 5(4):214–217. [PubMed: 1666528]
- Katta V, Chait BT. Hydrogen/deuterium exchange electrospray ionization mass spectrometry: A method for probing protein conformational changes in solution. *Journal of the American Chemical Society*. 1993; 115(14):6317–6321.
- Keppel TR, Jacques ME, Young RW, Ratzlaff KL, Weis DD. An efficient and inexpensive refrigerated LC system for H/D exchange mass spectrometry. *Journal of the American Society for Mass Spectrometry*. 2011; 22(8):1472–1476. [PubMed: 21953202]
- Keppel TR, Weis DD. Mapping residual structure in intrinsically disordered proteins at residue resolution using millisecond hydrogen/deuterium exchange and residue averaging. *Journal of the American Society for Mass Spectrometry*. 2015; 26(4):547–554. [PubMed: 25481641]
- Khan AG, Whidby J, Miller MT, Scarborough H, Zatorski AV, Cygan A, et al. Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. *Nature*. 2014; 509(7500):381–384. [PubMed: 24553139]
- Kim M, Sun ZY, Rand KD, Shi X, Song L, Cheng Y, et al. Antibody mechanics on a membrane-bound HIV segment essential for GP41-targeted viral neutralization. *Nature Structural & Molecular Biology*. 2011; 18(11):1235–1243.
- Kong L, Huang CC, Coales SJ, Molnar KS, Skinner J, Hamuro Y, et al. Local conformational stability of HIV-1 gp120 in unliganded and CD4-bound states as defined by amide hydrogen/deuterium exchange. *Journal of Virology*. 2010; 84(19):10311–10321. [PubMed: 20660185]
- Krezel A, Bal W. A formula for correlating pKa values determined in D2O and H2O. *Journal of Inorganic Biochemistry*. 2004; 98(1):161–166. [PubMed: 14659645]

- Landgraf RR, Chalmers MJ, Griffin PR. Automated hydrogen/deuterium exchange electron transfer dissociation high resolution mass spectrometry measured at single-amide resolution. *Journal of the American Society for Mass Spectrometry*. 2012; 23(2):301–309. [PubMed: 22131230]
- Lanman J, Lam TT, Barnes S, Sakalian M, Emmett MR, Marshall AG, et al. Identification of novel interactions in HIV-1 capsid protein assembly by high-resolution mass spectrometry. *Journal of Molecular Biology*. 2003; 325(4):759–772. [PubMed: 12507478]
- Lanman J, Lam TT, Emmett MR, Marshall AG, Sakalian M, Prevelige PE Jr. Key interactions in HIV-1 maturation identified by hydrogen-deuterium exchange. *Nature Structural & Molecular Biology*. 2004; 11(7):676–677.
- Majumdar R, Manikwar P, Hickey JM, Arora J, Middaugh CR, Volkin DB, et al. Minimizing carry-over in an online pepsin digestion system used for the H/D exchange mass spectrometric analysis of an IgG1 monoclonal antibody. *Journal of the American Society for Mass Spectrometry*. 2012; 23(12):2140–2148. [PubMed: 22993047]
- Mandell JG, Baerga-Ortiz A, Falick AM, Komives EA. Measurement of solvent accessibility at protein-protein interfaces. *Methods in Molecular Biology*. 2005; 305:65–80. [PubMed: 15939994]
- Mandell JG, Falick AM, Komives EA. Measurement of amide hydrogen exchange by MALDI-TOF mass spectrometry. *Analytical Chemistry*. 1998; 70(19):3987–3995. [PubMed: 9784743]
- Marcisin SR, Engen JR. Hydrogen exchange mass spectrometry: What is it and what can it tell us? *Analytical and Bioanalytical Chemistry*. 2010; 397(3):967–972. [PubMed: 20195578]
- Medzihradsky KF. Characterization of protein N-glycosylation. *Methods in Enzymology*. 2005; 405:116–138. [PubMed: 16413313]
- Monroe EB, Kang S, Kyere SK, Li R, Prevelige PE Jr. Hydrogen/deuterium exchange analysis of HIV-1 capsid assembly and maturation. *Structure*. 2010; 18(11):1483–1491. [PubMed: 21070947]
- Morton VL, Burkitt W, O'Connor G, Stonehouse NJ, Stockley PG, Ashcroft AE. RNA-induced conformational changes in a viral coat protein studied by hydrogen/deuterium exchange mass spectrometry. *Physical Chemistry Chemical Physics*. 2010; 12(41):13468–13475. [PubMed: 20877857]
- Pascal BD, Willis S, Lauer JL, Landgraf RR, West GM, Marciano D, et al. HDX workbench: Software for the analysis of H/D exchange MS data. *Journal of the American Society for Mass Spectrometry*. 2012; 23(9):1512–1521. [PubMed: 22692830]
- Rand KD, Pringle SD, Morris M, Engen JR, Brown JM. ETD in a traveling wave ion guide at tuned Z-spray ion source conditions allows for site-specific hydrogen/deuterium exchange measurements. *Journal of the American Society for Mass Spectrometry*. 2011; 22(10):1784–1793. [PubMed: 21952892]
- Rey M, Mrazek H, Pompach P, Novak P, Pelosi L, Brandolin G, et al. Effective removal of nonionic detergents in protein mass spectrometry, hydrogen/deuterium exchange, and proteomics. *Analytical Chemistry*. 2010; 82(12):5107–5116. [PubMed: 20507168]
- Rey M, Sarpe V, Burns KM, Buse J, Baker CA, van Dijk M, et al. Mass spec studio for integrative structural biology. *Structure*. 2014; 22(10):1538–1548. [PubMed: 25242457]
- Scanlan CN, Offer J, Zitzmann N, Dwek RA. Exploiting the defensive sugars of HIV-1 for drug and vaccine design. *Nature*. 2007; 446(7139):1038–1045. [PubMed: 17460665]
- Sheff JG, Rey M, Schriemer DC. Peptide-column interactions and their influence on back exchange rates in hydrogen/deuterium exchange-MS. *Journal of the American Society for Mass Spectrometry*. 2013; 24(7):1006–1015. [PubMed: 23649779]
- Snijder J, Benevento M, Moyer CL, Reddy V, Nemerow GR, Heck AJ. The cleaved N-terminus of pVI binds peripentonal hexons in mature adenovirus. *Journal of Molecular Biology*. 2014; 426(9):1971–1979. [PubMed: 24613303]
- Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(26):9528–9533. [PubMed: 15210983]
- Tuma R, Coward LU, Kirk MC, Barnes S, Prevelige PE Jr. Hydrogen-deuterium exchange as a probe of folding and assembly in viral capsids. *Journal of Molecular Biology*. 2001; 306(3):389–396. [PubMed: 11178899]

- Varki, A.; Cummings, R.; Esko, J.; Freeze, H.; Stanley, P.; Bertozzi, C., et al. Essentials of glycobiology. Plainview, NY: Cold Spring Harbor Laboratory Press; 2009.
- Veesler D, Khayat R, Krishnamurthy S, Snijder J, Huang RK, Heck AJ, et al. Architecture of a dsDNA viral capsid in complex with its maturation protease. *Structure*. 2014; 22(2):230–237. [PubMed: 24361271]
- Wales TE, Fadgen KE, Gerhardt GC, Engen JR. High-speed and high-resolution UPLC separation at zero degrees celsius. *Analytical Chemistry*. 2008; 80(17):6815–6820. [PubMed: 18672890]
- Walters BT, Ricciuti A, Mayne L, Englander SW. Minimizing back exchange in the hydrogen exchange-mass spectrometry experiment. *Journal of the American Society for Mass Spectrometry*. 2012; 23(12):2132–2139. [PubMed: 22965280]
- Wang L, Lane LC, Smith DL. Detecting structural changes in viral capsids by hydrogen exchange and mass spectrometry. *Protein Science*. 2001; 10(6):1234–1243. [PubMed: 11369862]
- Wang L, Smith DL. Capsid structure and dynamics of a human rhinovirus probed by hydrogen exchange mass spectrometry. *Protein Science*. 2005; 14(6):1661–1672. [PubMed: 15883190]
- Weis DD, Engen JR, Kass IJ. Semi-automated data processing of hydrogen exchange mass spectra using HX-Express. *Journal of the American Society for Mass Spectrometry*. 2006; 17(12):1700–1703. [PubMed: 16931036]
- Weis DD, Wales TE, Engen JR, Hotchko M, Ten Eyck LF. Identification and characterization of EX1 kinetics in H/D exchange mass spectrometry by peak width analysis. *Journal of the American Society for Mass Spectrometry*. 2006; 17(11):1498–1509. [PubMed: 16875839]
- Wildes D, Marqusee S. Hydrogen exchange and ligand binding: Ligand-dependent and ligand-independent protection in the Src SH3 domain. *Protein Science*. 2005; 14(1):81–88. [PubMed: 15576569]
- Wu Y, Kaveti S, Engen JR. Extensive deuterium back-exchange in certain immobilized pepsin columns used for H/D exchange mass spectrometry. *Analytical Chemistry*. 2006; 78(5):1719–1723. [PubMed: 16503628]
- Zhang X, Chien EY, Chalmers MJ, Pascal BD, Gatchalian J, Stevens RC, et al. Dynamics of the beta2-adrenergic G-protein coupled receptor revealed by hydrogen-deuterium exchange. *Analytical Chemistry*. 2010; 82(3):1100–1108. [PubMed: 20058880]
- Zhang HM, McLoughlin SM, Frausto SD, Tang H, Emmett MR, Marshall AG. Simultaneous reduction and digestion of proteins with disulfide bonds for hydrogen/deuterium exchange monitored by mass spectrometry. *Analytical Chemistry*. 2010; 82(4):1450–1454. [PubMed: 20099838]
- Zhang Z, Post CB, Smith DL. Amide hydrogen exchange determined by mass spectrometry: Application to rabbit muscle aldolase. *Biochemistry*. 1996; 35(3):779–791. [PubMed: 8547258]
- Zhang J, Ramachandran P, Kumar R, Gross ML. H/D exchange centroid monitoring is insufficient to show differences in the behavior of protein states. *Journal of the American Society for Mass Spectrometry*. 2013; 24(3):450–453. [PubMed: 23397137]
- Zhang Z, Smith DL. Determination of amide hydrogen exchange by mass spectrometry: A new tool for protein structure elucidation. *Protein Science*. 1993; 2(4):522–531. [PubMed: 8390883]
- Zhang Z, Zhang A, Xiao G. Improved protein hydrogen/deuterium exchange mass spectrometry platform with fully automated data processing. *Analytical Chemistry*. 2012; 84(11):4942–4949. [PubMed: 22571272]
- Zhao Y, Soh TS, Zheng J, Chan KW, Phoo WW, Lee CC, et al. A crystal structure of the Dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication. *PLoS Pathogens*. 2015; 11(3):e1004682. [PubMed: 25775415]

Figure 1.

Overview of HDX-MS. (A) The protein is incubated in deuterated buffer under native conditions to allow the amide protons to exchange for deuterium. (B) The exchange is then slowed to lock the deuterium in place and an acid protease is used to rapidly digest the protein into peptides. (C) The peptides are resolved by fast reverse phase liquid chromatography, which is directly infused into the mass spectrometer. (D) The mass spectra are recorded for each eluting peptide where the mass shift due to deuterium incorporation for each deuteration time is quantified (E).

Figure 2.

Examples of different exchange behaviors during HDX-MS. (A) Most commonly peptides uptake deuterium gradually by exposing amides through fast local structural fluctuations (EX2). (B) Large protein motions occurring on relatively slow timescales will expose large segments for long periods of time, whereby all amides become fully exchanged in a correlated manner (EX1). This is evidenced by a bimodal distribution where the population shifts to the exchanged species over the course of deuterium exchange. (C) Samples may contain populations of alternate conformational states. This is evidenced by a bimodal where the relative intensities of the two species remain constant over the course of deuteration. In this case, there is approximately 20% contribution to the signal from an unfolded population that exchanges fully by the earliest time point (denoted by *). (D) Proteins can undergo motions on various timescales resulting in mixed EX1/EX2 kinetics. In this case, the peptide is gradually deuterated (EX2) while also shifting to the fully deuterated state via EX1. (E) Sample carryover during LC-MS analysis can cause artifactual bimodals. In this case, the 1 min and 1 h time points show an additional component corresponding to ~15% carryover from a previous injection. The fact that it is also evident in the fully deuterated spectrum (depicted by an arrow), which should be uniformly and completely deuterated, is strong evidence that carryover is responsible for the bimodal behavior.