Mapping Protein–Ligand Interactions with Proteolytic Fragmentation, Hydrogen/Deuterium Exchange-Mass Spectrometry

Elyssia S. Gallagher*,†,1, Jeffrey W. Hudgens*,†,1

*Institute for Bioscience and Biotechnology Research, Rockville, Maryland, USA
†Biomolecular Measurement Division, National Institute of Standards and Technology, Rockville, Maryland, USA
1Corresponding authors: e-mail address: Elyssia_Gallagher@baylor.edu; jeffrey.hudgens@nist.gov

Contents

1. Introduction 2
2. H/D Exchange Theory 3
   2.1 H/D Exchange 3
   2.2 Acid and Base Catalysis 5
   2.3 Temperature 6
   2.4 Protein Structure 6
   2.5 Effects of Ligand-Binding Interactions upon H/D Exchange Rates 8
3. The HDX-MS Experiment 12
   3.1 Synopsis 12
   3.2 Protein Preparation 13
   3.3 Exchange Reaction 15
   3.4 Automated Versus Manual HDX-MS 16
   3.5 Reaction Quenching 17
   3.6 Enzymatic Digestion 18
   3.7 Chromatography 19
   3.8 Mass Spectrometry 20
   3.9 Generating a Proteomic Map 27
   3.10 Data Analysis 28
   3.11 Uncertainty Evaluations: Which Deuterium-Uptake Differences Are Meaningful? 30
   3.12 Data Display 31

1 Current address: Department of Chemistry and Biochemistry, Baylor University, Waco, Texas, USA.
Biological processes are the result of noncovalent, protein–ligand interactions, where the ligands range from small organic and inorganic molecules to lipids, nucleic acids, peptides, and proteins. Amide groups within proteins constantly exchange protons with water. When immersed in heavy water (D₂O), mass spectrometry (MS) can measure the change of mass associated with the hydrogen to deuterium exchange (HDX). Protein–ligand interactions modify the hydrogen exchange rates of amide protons, and the measurement of the amide exchange rates can provide rich information regarding the dynamical structure of the protein–ligand complex. This chapter describes a protocol for conducting bottom-up, continuous uptake, proteolytic fragmentation HDX-MS experiments that can help identify and map the interacting peptides of a protein–ligand interface. This tutorial outlines the fundamental theory governing hydrogen exchange; provides practical information regarding the preparation of protein samples and solutions; and describes the exchange reaction, reaction quenching, enzymatic digestion, chromatographic separation, and peptide analysis by MS. Tables list representative combinations of fluidic components used by HDX-MS researchers and summarize the available HDX-MS analysis software packages. Additionally, two HDX-MS case studies are used to illustrate protein–ligand interactions involving: (1) a continuous sequence of interacting residues and (2) a set of discontinuously numbered residues, residing spatially near each other.
transduction across the plasma membrane and within living cells (Chen, Chattopadhyay, Bergen, Gadd, & Tannery, 2007).

Hydrogen/deuterium exchange-mass spectrometry (HDX-MS) is an important tool for characterizing protein–ligand interactions (Katta & Chait, 1991; Zhang & Smith, 1993). HDX-MS measures deuterium uptake of amide N–H groups along the protein backbone. This information is useful for identifying portions of the protein engaged in binding and the dynamics of the protein–ligand interface. While once quite challenging and labor intensive, advances in instrumentation and software have enabled research groups to rapidly complete insightful, conclusive HDX-MS studies. As a result, more laboratories are conducting analyses that utilize HDX-MS methods (Pirrone, Iacob, & Engen, 2015).

This chapter describes protocols for conducting studies with continuous uptake, proteolytic fragmentation HDX-MS, which has demonstrated utility for identifying the interacting peptides in protein–ligand interactions. The power of this HDX-MS protocol for mapping protein–protein interactions is demonstrated in a study by Malito et al. (2013), who used multiple techniques to map the epitope of complement factor H–binding protein (fHbp) against a monoclonal antibody (mAb 12C1). Malito et al. studied the interaction of fHbp with bactericidal mAb 12C1 with diverse mapping techniques, including peptide arrays, phage display, X-ray crystallography, and HDX-MS (Fig. 1A–D). The map obtained with each technique was compared to the epitope of fHbp (Fig. 1E), determined from modeling the fHbp:fH crystal structure (Schneider et al., 2009). Their HDX-MS study accounted for nearly the entire $\approx 1000 \, \text{Å}^2$ contact interface between mAb 12C1 and fHbp. They concluded that “…hydrogen/deuterium exchange-mass spectrometry was the most effective method to rapidly supply near-complete information about epitope structure” (Malito et al., 2013).

2. H/D EXCHANGE THEORY

2.1 H/D Exchange

Within proteins, hydrogen atoms manifesting ionic character exchange with the labile protons of the surrounding solvent. When the solvent contains an isotope of hydrogen (deuterium or tritium), exchange can be used to characterize protein structure and dynamics via an analytical method that detects a difference between the isotopes. Since hydrogen and deuterium have masses of 1.008 and 2.014 Da, respectively, MS can be utilized to monitor exchange by detecting the mass increase of the protein.
Each hydrogen bond type has a distinct range of H/D exchange rates (Fig. 2). For instance, covalently bound C–H groups do not undergo solvent-mediated exchange. Covalently bound hydrogen atoms in amine, indole, and hydroxyl functional groups of amino acid side chains and the N-terminal amide group of the protein backbone undergo rapid H/D exchange with half-lives on the order of $t_{1/2} \approx 0.01–1$ ms (Bai, Milne, Mayne, & Englander, 1993; Liepinsh & Otting, 1996). The other amides in the protein backbone exhibit exchange rates with half-lives ranging from $t_{1/2} = 5$ s to 60 days. These rates can be measured using proteolytic fragmentation HDX-MS. Since a backbone amide hydrogen is present at every amino acid (except proline) throughout the protein, monitoring the rates of exchange associated with these functional groups allows the entire protein structure to be sampled. In addition to the functional group, H/D exchange rates vary with solution pH, temperature, solvent accessibility, and protein structure (Balasubramaniam & Komives, 2013; Englander & Kallenbach, 1984; Mandell, Falick, & Komives, 1998).
2.2 Acid and Base Catalysis

A completely unfolded protein has essentially no protection against proton exchange between the solvent and each amide group of the protein backbone, that is, each amide is in the exchange-competent state, N–H open. Proton exchange between an unprotected amide group and aqueous solvent is a chemical reaction catalyzed by acid, base, or water. Accordingly, the exchange rate ($k_{\text{ch}}$) is written (Bai et al., 1993; Englander, 2006; Englander & Kallenbach, 1984):

$$k_{\text{ch}} = k_{\text{acid}}^{\text{int}}[\text{H}_3\text{O}^+] + k_{\text{base}}^{\text{int}}[\text{OH}^-] + k_{\text{water}}^{\text{int}}[\text{H}_2\text{O}]$$  \hspace{1cm} (1)

The $k_{\text{acid}}^{\text{int}}$ and $k_{\text{base}}^{\text{int}}$ are rate coefficients for the acid- and base-catalyzed exchange, and $k_{\text{water}}^{\text{int}}$ is the intrinsic rate coefficient for the water-catalyzed exchange reaction (Englander, 2006). At physiological pH (7.0–8.0) the base-catalyzed mechanism is dominant. The acid-catalyzed mechanism becomes dominant as pH decreases below 3. Between pH 2.0 and 3.0, the exchange rate, $k_{\text{ch}}$, attains a minimum with the acid- and base-catalyzed mechanisms contributing equally to exchange, and the water-catalyzed mechanism being dominant. At the pH corresponding to minimum $k_{\text{ch}}$, the exchange rate for backbone amides is two orders of magnitude less than any other functional group (except for the guanidinium group of arginine).
The small contribution associated with $k_{\text{water}}$ is generally ignored, as it accounts for $\approx 38\%$ of $k_{\text{ch}}$ at the pH minimum and becomes negligible as the acid or the base rates increase (Bai et al., 1993; Gregory, Crabo, Percy, & Rosenberg, 1983). Of great consequence to the HDX-MS experiment, the exchange rate for an average backbone amide is reduced by 10,000-fold at pH 2.5 compared to pH 7.0 (Dempsey, 2001).

### 2.3 Temperature

The chemical exchange rate, $k_{\text{ch}}$, shows strong temperature dependence as described by the Arrhenius equation. A rate decrease of $\approx 10$-fold is observed when the temperature is reduced from 20 to 0 °C (Englander, 2006). Decreasing the temperature lowers the kinetic energy of the molecules in solution, reducing the rate of diffusion and leading to fewer collisions and reactions between the protein, catalyst, and protium or deuterium donor. When the protein is under quench conditions (pH 2.5 and 0 °C), the average half-life for H/D exchange of a backbone amide hydrogen is between 30 and 120 min (Engen & Smith, 2000; Morgan & Engen, 2009; Thevenon-Emeric, Kozlowski, Zhang, & Smith, 1992), providing sufficient time for rapid digestion and separation of the resulting peptides prior to MS. To ensure highly reproducible analyses, it is necessary to precisely control solution pH and temperature at every step in the HDX-MS experiment, including protein equilibration, exchange, quench, and processing prior to MS.

### 2.4 Protein Structure

The exchange rate of a backbone amide N–H is dependent on the primary, secondary, tertiary, and quaternary protein structure. For an unfolded protein or peptide, the identities of the neighboring amino acids affect the magnitude of $k_{\text{ch}}$. Englander (Bai et al., 1993; Connelly, Bai, Jeng, & Englander, 1993; Englander, 2015) provides tables that can predict $k_{\text{ch}}$ for unfolded peptides based on pH, temperature, and neighboring amino acids.

The Linderstrøm-Lang model of hydrogen exchange describes exchange by localized unfolding. Amides can reside in either exchange-incompetent ($\text{N-H}_{\text{closed}}$) or exchange-competent ($\text{N-H}_{\text{open}}$) states (Hvidt & Linderstrøm-Lang, 1954). Any hydrogen-bonded proton is exchange-incompetent because it is sterically inaccessible to exchange (England, Mayne, Bai, & Sosnick, 1997). Generally, secondary structural features (e.g., $\alpha$-helices, $\beta$-sheets) in a folded protein are comprised of amides residing in
exchange-incompetent states since they are stabilized through intramolecular hydrogen bonds. The Linderstrøm-Lang model posits that an amide N–H group becomes exchange-competent through unspecified transient events. The Linderstrøm-Lang model is defined kinetically by the structural opening and reclosing rates, \( k_{\text{op}} \) and \( k_{\text{cl}} \), of an amide:

\[
\text{N–H}_{\text{closed}} \xrightarrow{k_{\text{op}}} \text{N–H}_{\text{open}} \xrightarrow{k_{\text{ch}}} \text{D}_2\text{O} \text{ exchanged}
\] (2)

The exchange process proceeds during transient, exchange-competent intervals when the hydrogen bond donor and acceptor are not bound (N–H_{open}) and are solvent accessible. The Linderstrøm-Lang model attributes the variation of \( k_{\text{op}} \) and \( k_{\text{cl}} \) to the presence and absence of secondary structure, and these dynamical variables connect protein structure to thermodynamic properties (Englander et al., 1997; Hvidt & Linderstrøm-Lang, 1954; Hvidt & Nielsen, 1966). Protection against amide N–H exchange, conferred by secondary structure through variation of \( k_{\text{op}} \) and \( k_{\text{cl}} \), can reduce the H/D exchange rate at amide groups by as much as \( 10^8 \) (Fleming & Rose, 2005; Skinner, Lim, Bédard, Black, & Englander, 2012).

When a hydrogen bond is broken, a kinetic competition ensues between the rates of chemical exchange, \( k_{\text{ch}} \), and structural reclosing, \( k_{\text{cl}} \) (Baldwin, 2011; Englander & Kallenbach, 1984; Hvidt & Nielsen, 1966). Under most physiological conditions, reclosing is faster than chemical exchange (\( k_{\text{cl}} \gg k_{\text{ch}} \)), requiring the same hydrogen bond to break multiple times before a successful exchange event. The hydrogen exchange rate then depends on the fraction of time the exchange-competent (open) form exists and therefore the structural opening equilibrium constant (\( K_{\text{op}} \)). The kinetic expression for this EX2 condition is written with a preequilibrium step:

\[
k_{\text{HDX}} = \frac{k_{\text{op}}k_{\text{ch}}}{k_{\text{op}} + k_{\text{ch}} + k_{\text{cl}}} \approx K_{\text{op}}k_{\text{ch}}
\] (3)

where \( k_{\text{HDX}} \) is the observed exchange rate. The equilibrium term, \( K_{\text{op}} \), represents the fraction of time that the amide is exchange-competent. The approximation shown in Eq. (3) holds for any significant level of structural protection (\( k_{\text{op}}/k_{\text{cl}} \approx K_{\text{op}} < 1 \)). The Gibb’s energy change, \( \Delta_{\text{op}} G^\circ \), is the opening energy for exchange at amide N–H:

\[
\Delta_{\text{op}} G^\circ = -RT \ln K_{\text{op}} = -RT \ln \left( \frac{k_{\text{op}}}{k_{\text{cl}}} \right)
\] (4)
As the chemical rate is made progressively faster, for example, by raising the pH, the amide exchange rate will increase linearly with the solvent catalyst concentration in the EX2 range. Ultimately, when \( k_{ch} > k_{cl} \), the HX exchange rate will reach the asymptotic EX1 limit at which the deuteration rate coefficient, \( k_{HDX} \), becomes

\[
k_{HDX} = k_{op}
\]  

(5)

EX1 behavior can be documented by its insensitivity to pH or by observation of correlated D-uptake behavior, where multiple amides simultaneously exchange and exhibit the same EX1 exchange rate (England, 2006).

Many studies have been devoted to determining how protein structure governs amide hydrogen exchange patterns. These studies have explored solvent and catalyst penetration models. Analysis of model-based expectations for hydrogen exchange indicate that local unfolding pathways manifest activation energies of \( E_a = 70–145 \text{ kJ/mol} \) and penetration mechanisms manifest \( E_a > 165 \text{ kJ/mol} \) (England & Kallenbach, 1984). Studies of protein amide N–H exchange dynamics have successfully accounted for HDX-MS and HDX-NMR data with local unfolding and foldon models (England, Mayne, & Krishna, 2007; Hu et al., 2013; Krishna, Hoang, Lin, & Englander, 2004; Krishna, Maity, Rumbley, Lin, & Englander, 2006).

2.5 Effects of Ligand-Binding Interactions upon H/D Exchange Rates

Most commonly, protein–ligand-binding interactions reduce the observed deuterium uptake at the binding site; however, such binding is sometimes accompanied by allosteric effects. Sowole and Konermann offered scenarios where H/D exchange rates are reduced (type 1), unaffected (type 0), or enhanced (type 2) by ligand binding (Sowole & Konermann, 2014). Accounting for these scenarios requires no modification of the Linderstrøm-Lang model.

The complex NL formed by the binding of protein N and ligand L is characterized by its dissociation constant, \( K_d \), defined as:

\[
K_d = \frac{\{N\}\{L\}}{\{NL\}}
\]  

(6)

where curly braces denote the chemical potential of each species in solution, as referenced to a standard state (e.g., 1 mol/L) (Gilson, Given, Bush, & McCammon, 1997). (Formally, \( K_d \) is unitless; however, since activity
coefficients $\approx 1$ in dilute solution, the effects of reference states are nil, allowing direct use of species concentrations in Eq. (6). Hence, $K_d$ values are customarily listed in concentration units.) Formation of complex NL is accompanied by its stabilization energy:

$$\Delta_d G^\circ = -RT \ln K_d$$

(7)

The magnitude of $\Delta_d G^\circ$ is governed by an interplay of entropic and enthalpic factors originating from the protein, ligand, and solvent (Bissantz, Kuhn, & Stahl, 2010; Tzeng & Kalodimos, 2009). Contributions to $\Delta_d G^\circ$ may originate from any structural element of the protein. Consequently, structural reorganization in the protein arising from complexation can affect N–H groups local to the binding interface, and it can allosterically affect remote N–H groups. With knowledge of the stabilization energy of NL, $\Delta_d G^\circ$, statistical mechanics can be used to reveal the spectrum of effects that complexation may have on amide hydrogen exchange rate coefficients.

In the statistical mechanics terminology of Sowole and Konermann a backbone amide N-H exchanging under EX2 conditions has the H/D exchange rate coefficient (Sowole & Konermann, 2014):

$$k_{\text{HDX}} = p_{\text{op}} k_{\text{ch}}$$

(8)

where $p_{\text{op}}$ (as with $K_{\text{op}}$) is the fraction of time that the N–H bond spends in the exchange-competent (N–H\text{open}) state (ref. Eq. 2) (England et al., 2007; Wooll, Wrabl, & Hilser, 2000). From Boltzmann statistics, the $p_{\text{op}}$ coefficient is computed by

$$p_{\text{op}} = \frac{e^{-\Delta_{\text{op}} G^\circ / RT}}{Z}$$

(9)

where $\Delta_{\text{op}} G^\circ$ is the opening energy and $Z$ is the partition function, defined as $Z = 1 + \exp(-\Delta_{\text{op}} G^\circ / RT)$, describing the manifold of all states (ground and excited) for the protein or protein complex. Stable proteins conform to the condition, $k_{\text{cl}} \gg k_{\text{op}}$, such that $\Delta_{\text{op}} G^\circ \gg 0$ and $Z \approx 1$. For convenience of illustration, Gibbs energy in the following text is expressed in $RT$ units, $\Delta j = RT \Delta_{\text{op}} G^\circ$, so that

$$k_{\text{HDX}} = e^{-\Delta j / k_{\text{ch}}}$$

(10)

Each complex NL has an energy state manifold, comprising an exchange-incompetent ground state (i.e., N–H\text{closed}) and a manifold of sparsely populated, higher energy states (Fig. 3). The higher energy states
Figure 3  Free energy level diagram of protein N and protein–ligand complex NL, where energy is plotted in \(RT\) units. All amide N–H’s of the lowest energy (ground) state are exchange-incompetent (i.e., N–H\(_{\text{closed}}\)). This simplified example depicts only three of a large ensemble of excited states. Higher energy states, denoted by asterisks, are partially unfolded states manifesting one or more exchange-competent amide N–H’s (i.e., N–H\(_{\text{open}}\)). Adjacent to each asterisk, the Boltzmann occupancy is noted, and this occupancy determines \(k_{\text{HDX}}\) of N. (A) The uncomplexed protein molecule. Excited states are presumed to be \(\Delta j = 5RT, 7RT, \text{ and } 9RT\) above the ground state. (B) Type 1 scenario, where ligand binding lowers the free energy of the ground state by \(\Delta G^\circ(\Xi - 4RT)\), and the excited-state energies are lowered (arbitrarily) by \(-2RT\). The net stabilization increases the energy gap between the ground and excited states, which reduces populations of the excited states relative to those in (A); thus, \(k_{\text{HDX}}\) decreases. (C) Type 0 scenario, where the ground state and excited-state energies are lowered equally by \(\Delta G^\circ(\Xi - 4RT)\). Formation of the protein–ligand complex NL does not alter the Boltzmann occupancies among the manifold of excited states; thus, \(k_{\text{HDX}}\) is unchanged. (D) Type 2 scenario, where the intrinsic protein–ligand binding affinity, \(\Delta G^\circ_{\text{intr}}\), is countered by the expense of a conformational change in structure, \(\Delta G^\circ_{\text{switch}}\). The energy gap between the ground and excited states is diminished, which increases the Boltzmann occupancies among the excited-states relative to those in (A); thus, \(k_{\text{HDX}}\) increases. The overall binding affinity, \(K_d\), is determined by two competing contributions (Eqs. 7 and 11). Adapted figure used by permission from Sowole and Konermann (2014).

represent the multitude of subglobal states experiencing partial or full unfolding through foldon fluctuations and local dynamics (Englander et al., 2007; Gu, Zitzewitz, & Matthews, 2007; Kaltashov, Bobst, & Abzalimov, 2013; Weinkam, Zimmermann, Ronesberg, & Wolynes, 2010). Each higher energy state has one or more amide N–H’s that is exchange-competent. In Fig. 3, each higher energy level is labeled with
its Boltzmann occupancy, $e^{-\Delta_i}$. The stable uncomplexed protein molecule $N$ (Fig. 3A) has excited states, which are arbitrarily set to $\Delta_j = 5RT, 7RT, \text{ and } 9RT$ above the ground state for this discussion. The formation of the protein–ligand complex $NL$ can result in three scenarios.

In the type 1 scenario (Fig. 3B), formation of the $NL$ complex lowers the ground state energy by $\Delta_d G^o$. This lowering is expressed as $\Delta_d G^o$ (which is arbitrarily set to $-4RT$). Formation of the $NL$ complex may also lower the energy of the excited-state manifold, which is shown (arbitrarily) by a change of $-2RT$. As a result, the net energy separation between the exchange-incompetent ground state and lowest exchange-competent higher state is greater for $NL$ than for $N$ (ref. Fig. 3A). Consequently, the thermal populations of the excited states in $NL$ are diminished relative to those of the uncomplexed protein $N$; thus, the $k_{HDX}$ observed in $NL$ is also diminished. Predominately, the HDX–MS literature comprises observations of $NL$ complexes exhibiting type 1 behavior.

The type 0 scenario (Fig. 3C) is characterized by energy lowering of the ground and excited states by the binding affinity, $\Delta_d G^o$. Since the relative energies among the ground and excited states are unchanged, excited-state fractional occupancies are also unchanged; thus, $k_{HDX}$ remains unchanged. Such behavior is difficult to detect. Sowole and Konermann (2014) suggest that the type 0 scenario is implicated in the study of some mAb complexes (Pan, Salas-Solano, & Valliere-Douglass, 2014).

The noncanonical type 2 scenario results in enhanced $k_{HDX}$. Here, the protein possesses a binding site that has a large intrinsic affinity, $\Delta_d G^o_{\text{intr}}$ $(\equiv -4RT$ arbitrarily). However, the ligand is accommodated in the $NL$ ground state only after an unfavorable structural change has taken place. The energy penalty of this conformational switching event is termed $\Delta G^o_{\text{switch}}$. The overall binding affinity in this scenario is the sum of two opposing contributions

$$\Delta_d G^o = |\Delta_d G^o_{\text{intr}}| - |\Delta G^o_{\text{switch}}|$$

The $NL$ ground state remains thermodynamically favored for $\Delta_d G^o > 0$. Ligand-induced distortion of the protein implies that $NL$ conformers with exchange-competent, open N–H sites are more readily accessible than in the uncomplexed $N$. Thus, ligand binding lowers $\Delta_j$ values, resulting in increased HDX rates throughout the protein (Eq. 4, Fig. 3D). Sowole and Konermann have demonstrated that oxygenation of myoglobin results in a $NL$ complex that exhibits type 2 behavior at residues displaced from the binding site (Sowole & Konermann, 2014). The observed behavior is
consistent with cooperative T → R switching, where part of the intrinsic O$_2$ binding energy ($\Delta$$_G^\circ$$_{\text{intr}}$) is reinvested for destabilization of the ground state ($\Delta$$G^\circ$$_{\text{switch}}$). This destabilization increases the Boltzmann occupancy of unfolded conformers, thereby enhancing HDX rates.

3. THE HDX-MS EXPERIMENT

3.1 Synopsis

As documented above, H/D exchange is dependent on any property affecting the molecular Gibb’s energy, which most commonly spans pH, temperature, solvent accessibility, and structure. Thus, the pH and temperature of HDX experiments are optimized for measuring the forward exchange rates with minimal perturbing back exchange. Further, since these variables are controlled, the collected data can be used to infer the structural dynamics and solvent accessibility of each backbone amide within the protein.

The continuous-labeling, proteolytic fragmentation HDX-MS experiment, as depicted in Fig. 4, begins when a protein sample is diluted in D$_2$O, initiating exchange at exposed amides and side chains. After exposure in D$_2$O for selected time intervals ($t_{\text{HDX}}$), aliquots of deuterated protein are extracted and diluted into a quench buffer, which is maintained at pH 2.5 (H$_2$O) and 0 °C. These conditions minimize amide exchange rates, preventing loss of deuterium at labeled backbone amides. After the solution is quenched, the protein is digested into peptides by an acidic protease, and the resulting peptides are trapped on a C18 guard column where buffer exchange removes salt. Using reverse-phase (RP) chromatography, the peptides elute from an analytical column and are analyzed by electrospray ionization mass spectrometry (ESI-MS). The quench conditions (pH 2.5, ≈1 °C) are maintained throughout the digestion, buffer exchange, and chromatographic stages to minimize back exchange loss of deuterium labels, which proceeds in H$_2$O solvent via Eq. (2). From the MS data, the average mass change of each peptide is calculated and correlated to the presence of deuterium. Since side chains have more rapid exchange rates than backbone amides at pH 2.5 and 0 °C, the deuterium labels at these positions are equilibrated back to the natural protic isotope abundance during digestion and separation. This simplifies data analyses since each amino acid (except proline and arginine) can have a maximum of one deuterium label.

Studies of protein–ligand interactions are conducted with protein in the presence and absence of ligand. The protein–ligand interaction is characterized
by examining the differential deuterium uptake of the peptides observed from the holo-protein (protein–ligand complex) and the respective apo-protein (protein alone).

3.2 Protein Preparation

To accurately map protein structure and binding interactions, the native protein conformation must be maintained in solution; however, these native conditions cannot interfere with the RP peptide separation or ionization. Most current HDX-MS work flows utilize online enzymatic digestion after which the peptides are trapped on a C18 column (Fig. 4). This set-up allows for physiological, high salt buffers to be used for protein preparation, equilibration, and exchange since the salts are washed away prior to gradient elution and ESI-MS. Compounds that interact with the stationary phase, such
as surfactant or phospholipids, should be removed prior to online analyses. Surfactants can be retained through interactions with the C18 stationary phase of the trap column, causing these molecules to accumulate and elute during the peptide separation at high concentrations of acetonitrile (CH₃CN), leading to fouling of the analytical column and ion suppression. Though detergents can affect the separation and ionization of peptides, HDX-MS analyses have been performed in the presence of 10% glycerol or low concentrations of dodecyl maltoside, a nonionic detergent.

The ideal protein concentration for an HDX experiment is determined by the sensitivity of the mass spectrometer. During an experiment, the protein is diluted into exchange buffer (D₂O, pD 7.4) and quench buffer (H₂O, pH 2.5). However, following enzymatic digestion, the peptides are concentrated on a trap column. This step allows for low concentrations of protein to be utilized in the exchange reaction since they can be detected following concentration and peptide separation. Alternatively, highly concentrated proteins can be problematic if they are not fully eluted from the proteolytic, trap, or RP column within a single run. Peptides retained on the columns and eluted during later runs experience greater back exchange, which can complicate data analysis. For current instrumentation, 10–25 pmol of protein per time point is sufficient to detect the peptides following labeling, digestion, and separation without significant peptide carry over between runs.

To study protein–ligand binding, the protein and ligand should be present at concentrations that promote interaction after the protein–ligand mixture is diluted into exchange buffer (D₂O). A comparison of the holo-complex to the apo-protein is best achieved when ≈100% of the protein is bound to ligand in the holo-state and a single protein population is present. To estimate the optimum ratio and concentrations of protein and ligand, the dissociation constant (ref. Eq. 6) for the interaction must be known. For computational convenience all activity coefficients are assumed equal to 1; thus, \( K_d \) has units of concentration. A convenient equation for estimating the optimum ratio is given by Mandell et al.:

\[
\frac{\text{%bound}}{P} = \frac{L + P + K_d + \sqrt{(L + P + K_d)^2 - 4LP}}{2P}
\]

where \( L \) and \( P \) are the total quantities of the protein and ligand, respectively (Mandell et al., 1998). For tightly binding interactions (\( K_d \approx 1 \) nmol/L), the protein and ligand can be mixed at a molar ratio near 1:1. When
$K_d = 10–100\text{ nmol/L}$, the ligand must be present at a molar excess to approach 100% binding to the protein. When the ligand is a peptide or protein, there may be a maximum amount of ligand that can be used in the experiment. A protein or peptide ligand will be digested, trapped, separated, ionized, and detected like the other protein. If the ligand is at a high molar excess, the peptides of the ligand may coelute with those of the other protein and suppress ion signal from the less concentrated protein.

### 3.3 Exchange Reaction

The buffer used for exchange should match the solution conditions (buffering agent, additives, acidity) of the equilibration buffer and be prepared with $D_2O$ solvent. The measured $pD$ of this buffer must be corrected for an artifact associated with monitoring the deuterium isotope with a glass electrode. Equation (13) describes the correction for $pD$ when $pD_{\text{read}}$ is the $pD$ read directly from the pH meter \cite{Glasoe1960}.

$$pD_{\text{corrected}} = pD_{\text{read}} + 0.4$$

To accurately measure the exchange rate, the amount of hydrogen and deuterium present in the exchange mixture should be known. HDX is often initiated by diluting protein, prepared in equilibration buffer (H$_2$O), into exchange buffer ($D_2$O) with $D_2$O at 80–95% of the final solution volume. This $D_2$O excess decreases the probability that labeled sites will be replaced by hydrogen if a second exchange event occurs at the same position during the exchange time. Alternatively, if diluting the protein sample will decrease the protein concentration below that which can be effectively detected by MS, protein can be introduced to $D_2$O using a small gel filtration spin column; however, this method requires that the shortest exchange time point be no less than 1–2 min \cite{Engen2000}.

The exchange buffer should be prepared using high purity $D_2$O (99%) and the $pD$ should be adjusted with deuterated acid (deuterium chloride solution, DCl) or base (sodium deuteroxide solution, NaOD). Alternatively, a solution of known $pD$ can be made using an acid and conjugate base pair. The dissociation of a monoprotic acid is shown in Eq. (14) where HA and $A^-$ are the acid and conjugate base, respectively.

$$HA + H_2O \rightleftharpoons A^- + H_3O^+$$

The Henderson–Hasselbalch equation (Eq. 15) can be used to calculate the ratio of acid and conjugate base to combine to make a buffer with a
particular pH or pD (Harris, 2003). In the equation below, \([HA]\) and \([A^-]\) are the molar (mol/L) concentrations of the acid and conjugate base, respectively.

\[
\text{pH} = pK_a + \log \frac{[A^-]}{[HA]} \tag{15}
\]

Since the volume of the acid and conjugate base are the same in the buffer, the volume component of the concentrations cancel and the equation can be rewritten to determine the mole ratio of the conjugate base and acid needed to generate a buffer with a particular pH or pD (Eq. 16).

\[
\frac{\text{mol A^-}}{\text{mol HA}} = 10^{(\text{pH} - pK_a)} \tag{16}
\]

If a diprotic or polyprotic acid is needed to make the buffer, the same general equation can be used, but the \(pK_a\) should be replaced by the appropriate dissociation constant (\(pK_{a1}, pK_{a2}\), etc.) and the acid and conjugate base should represent the species associated with that equilibrium.

### 3.4 Automated Versus Manual HDX-MS

During the mid-2000s, the laborious task of manually conducting continuous-labeling HDX-MS experiments was automated (Chalmers et al., 2006). By 2010, automated commercial instruments became available from the Waters Corp. and ThermoFisher Scientific. The emergence of these instruments has increased the use of HDX-MS methods within the biopharmaceutical industry for studying the effect of process changes on biotherapeutic properties and for characterizing protein–ligand interactions (Chow & Ju, 2013; Houde, Arndt, Domeier, Berkowitz, & Engen, 2009; Houde, Berkowitz, & Engen, 2011; Iacob et al., 2013; Kaltashov, Bobst, Abzalimov, Berkowitz, & Houde, 2010). The automated instruments comprise a liquid handling robotic platform that immerses a sample in D_2O, waits for the exchange reaction time to expire, injects the labeled sample into a quench buffer, and then injects the quenched sample into a series of valves for digestion, solvent exchange, and reverse-phase, ultra-performance liquid chromatography (RP-UPLC). Due to the precise timing of the exchange, quench, digestion, and peptide separation steps within temperature-controlled environments, the results obtained with automated sample handing platforms can exhibit excellent repeatability and intermediate measurement precision (Chalmers, Busby, Pascal, Southern, & Griffin, 2007;
Hudgens, Huang, & D’Ambro, 2016). Robots can interweave experiments of varying length ($t_{\text{HDX}}$), which shortens the total duration of an experiment containing multiple exchange time points. Each HDX-MS apparatus comes with specialized processing and analysis software.

Though automated platforms do exist, it is too early to declare dead the age of manual measurements. When armed with appropriate temperature and timing controls, investigators can manually perform HDX-MS experiments with highly reproducible results. For “one-off” investigations designed to augment a larger project, manual HDX-MS may be the most effective, economical strategy for adding compelling results to a biochemical study.

3.5 Reaction Quenching

After the exposure time of the sample to D$_2$O has expired, the exchange reaction is quenched by decreasing the solution to 0 °C and pH 2.5. From this step forward, the labeled protein sample is exposed to a high concentration of H$_2$O; thus, the quench conditions are necessary to minimize back exchange of labeled backbone amides.

Solution pH is best controlled using a buffer. An acid and conjugate base pair have a high buffering capacity when the solution pH is ±1 pH unit from the $pK_a$. Phosphate is a triprotic acid with three dissociation constants of $pK_a=2.15$, 7.20, and 12.15 (Harris, 2003). Phosphate is regularly used for buffering the quench buffer at pH 2.5 and preparing the equilibration and exchange buffers at pH 7.4 and pD 7.4, respectively. The buffering capacity of the quench buffer must be sufficient to decrease the pH of the exchange reaction to pH 2.5, often requiring the concentration of the buffering species to be 10 times higher in the quench solution than in the equilibration and exchange solutions. Quenching can also be accomplished by adding strong acid, e.g., formic acid, to the exchange reaction. However, when the equilibration/exchange buffer has a single dissociation constant with a $pK_a$ near physiological pH, following quenching, the solution will have a pH significantly below the $pK_a$ and may not have sufficient buffering capacity to maintain a reproducible pH $\approx$ 2.5, resulting in increased or variable back exchange in replicate analyses.

After quenching, samples can be analyzed immediately or rapidly frozen in liquid nitrogen and stored at $-80$ °C for a day. Sample storage is more common when using a manual HDX-MS platform. To minimize loss of deuterium label, samples should be frozen quickly after quenching and thawed rapidly ($<30$ s) prior to digestion.
3.6 Enzymatic Digestion

Following quenching of the exchange reaction, the protein sample is rapidly digested into peptides. To achieve higher digestion efficiencies at this rapid time scale, chaotropes and reducing agents are often added to the quench solution to aid in protein denaturation. The concentrations of these additives are limited since they must not degrade the immobilized-protease columns commonly used for digestion. Chaotropes, such as urea or guanidinium hydrochloride (Gdn–HCl), are often included at a molar concentration as high as 3 mol/L. High concentrations of these additives accelerate denaturation, minimizing the amount of time that labeled protein undergoes back exchange in the aqueous analysis solution. The optimum concentration for each reagent is protein dependent and should be determined prior to HDX–MS experiments.

For proteins containing disulfide bonds, reducing agents, such as 3,3',3"-phosphatanetriyltripropanoic acid (i.e., tris(2-carboxyethyl)phosphine hydrochloride or TCEP), are added at high millimolar concentrations (>200 mmol/L). The reducing agent breaks the disulfide bonds, which helps to unfold the protein and improve digestion efficiency. Since TCEP solutions oxidize with time (Burns, Butler, Moran, & Whitesides, 1991), solutions (pH < 5) are best prepared immediately before initiating a series of measurements. Prepared solutions are stored at ≈0 °C and used the same day. Alternately, an electrochemical cell that is designed to reduce disulfide bonds can be added to the fluidic path prior to the proteolytic column (Mysling, Salbo, Ploug, & Jørgensen, 2014).

Denaturation unfolds the protein, and the amide N–H’s along the protein backbone become exchange-competent, resulting in back-exchange rates equal to $k_{ch}$ (Reaction 2). It becomes imperative to maintain the protein sample in cold, acidic conditions. For this reason, online digestions utilize a mobile phase of water with 0.1% formic acid to maintain acidic pH ≈ 2.5, and temperature is kept at ≈0 °C by submerging all valves and tubing in an ice bath (manual experiment) or by refrigerating these components in a compartment (automated system).

Most HDX–MS workflows utilize online columns containing immobilized protease, which can digest samples within one to three minutes (Chalmers et al., 2006), due to the large proteolytic enzyme to protein sample molar ratio. Alternatively, some HDX–MS experiments have employed in-solution digestion. In-solution digestion typically employs a 1:1 ratio of protein to solution-phase enzyme and reaches completion after a digestion time of $\leq$5 min at 0 °C (Brier et al., 2012; Sarkar & Wintrode, 2011).
In-solution methods may combine enzymatic digestion with additional sample preparation steps, such as removal of additives that are incompatible with UPLC-ESI-MS.

The spatial resolution of deuterium uptake is determined by peptide length. Porcine pepsin is an acidic protease that is commonly used for HDX-MS applications due to the high activity of the enzyme in the pH range between 2 and 4. This enzyme has also been successful at digesting proteins at reduced temperatures. Pepsin digestion generally results in peptides comprising (3–30) amino acids (Zhang & Smith, 1993) with an average length of 14 amino acids (Ahn, Cao, Yu, & Engen, 2013). The sequence of these peptides often overlap, which can improve amide resolution. Pepsin is nonspecific in the sense that cleavage positions cannot be predicted from a protein sequence. However, pepsin shows strong cleavage trends, such as pepsin preferentially cleaves after phenylalanine or leucine, but rarely cleaves after a charged residue (arginine, lysine, or histidine) or proline (Hamuro, Coales, Molnar, Tuske, & Morrow, 2008).

Although pepsin is the most common acidic protease used in HDX-MS experiments, other acidic proteases have been utilized. Each enzyme exhibits a unique cleavage trend (Ahn et al., 2013). Because each protease exhibits distinct site cleavage propensity, the use of two or more proteases during an HDX-MS measurement campaign can increase the sequence coverage of the protein sample and improve amide resolution (Hu et al., 2013; Mayne et al., 2011). Alternative proteases include protease type XIII from Aspergillus saitoi, protease type XVIII from Rhizhopus species (Cravello, Lascoux, & Forest, 2003; Rey, Man, Brandolin, Forest, & Pelosi, 2009; Zhang et al., 2008), and plant aspartic proteases, nepenthesin I (Rey et al., 2013) and nepenthesin II (Yang et al., 2015) from Nepenthes gracilis. Pepsin, protease type XIII, and protease type XVIII are commercially available and can be used to prepare enzyme-immobilized columns following the protocol described by Wang, Pan, and Smith (2002) or Rey et al. (2009). Additionally, because of the prevalence of pepsin in HDX-MS, enzyme-immobilized particles and packed columns can be purchased. Nepenthesin I and nepenthesin II require expression and purification prior to enzyme immobilization and use in packed columns.

3.7 Chromatography

After the sample protein is digested, the peptide effluent passes through a C18 guard column that immobilizes the peptides. Peptides trapped on the guard
column are washed with additional mobile phase (usually water with 0.1% formic acid) for 30–60 s, which removes salts and additives (e.g., chaotropes, reducing agents, etc.) present in the equilibration, exchange, or quench buffers. Subsequently, the peptides are eluted and separated on a RP-UPLC column. Table 1 lists column configurations and solution gradients that investigators have used to accomplish HDX-MS measurements.

Following buffer exchange, the peptides are eluted using a gradient of water with 0.1% formic acid (solvent A) and a mixture of 80/20/0.1 (v/v/v) acetonitrile/water/formic acid (solvent B). To minimize back exchange at backbone amides, the RP separation is completed within 10 min. Although this rapid separation does not fully resolve the peptides, it does decrease the number of coeluting peptides, which increases the probability that more peptides will be ionized and detected. Furthermore, the mass spectrometer performs a second dimension separation based on \( m/z \). The UPLC separation also ensures that the side chain residues, with more rapidly exchanging hydrogen sites, back exchange to the protic natural abundance because the mobile phase contains 100% H\(_2\)O and 0% D\(_2\)O. This loss of deuterium label on the side chains (except for arginine) decreases the complexity of the MS data since each residue within the peptide can have a maximum of one exchange site.

### 3.8 Mass Spectrometry

The peptides eluting from the upstream UPLC apparatus pass directly into the electrospray source of an MS, which measures ion intensity versus \( m/z \) as a function of retention time. Typical fluidic flow rates of the UPLC match the required input flow rate into the ESI source. However, traditional HPLC apparatus operating at higher flow rates may need a splitter that shunts off excess effluent, though this may diminish overall sensitivity.

Numerous HDX-MS laboratories have reported results obtained using ESI tandem mass spectrometers of architectures spanning quadrupole-reflection-time-of-flight (qTOF/qRTOF), ion trap-Fourier transform (ion cyclotron resonance (ICR) or orbitrap), and quadrupole-orbitrap configurations. These systems feature resolutions \( \left[ (m/z)/\Delta m/z \right] z \) from 40,000 to 750,000 at 400 \( m/z \), where \( m/z \) is the ion mass-to-charge ratio and \( z=\) ion charge. To avoid corrupted measurements of deuterium content within peptide ions, Fourier transform instruments must be set to an appropriate resolution, which is usually somewhat lower than the advertised maximum performance (Burns, Rey, Baker, & Schriemer, 2013).
Table 1  HDX-MS Fluidic Configurations used to Conduct UPLC Separations of Peptides Prior to Their Injection into a Mass Spectrometer

<table>
<thead>
<tr>
<th>Guard Column [Column Description (Particle Size) and Vendor, Bore (mm): Dia. × Length], Desalting Procedure</th>
<th>Analytical Column [Model (Particle Size), Vendor, Bore (mm): Dia. × Length]</th>
<th>Solvent Gradients (Flow Rate, Gradient Cycle Duration, Solvents)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18 (5 μm) Grace Discovery Sciences, Carnforth, UK <strong>Bore (mm):</strong> 1.0 × 50, <strong>Desalt:</strong> 50 μL/min, 100% solvent A; 3 min</td>
<td>C18 Hypersil GOLD (1.9 μm), ThermoFisher Scientific, Waltham, MA <strong>Bore (mm):</strong> 1.0 × 50</td>
<td><strong>Flow rate:</strong> 50 μL/min  <strong>Gradient sequence:</strong> 5–35% solvent B, 3 min; 35–70% solvent B; 5 min; 70–100% solvent B, 0.5 min; 100% solvent B, 0.5 min; 5% solvent B, 0.5 min  <strong>Solvent A:</strong> H₂O, 0.1% formic acid  <strong>Solvent B:</strong> 80% CH₃CN, 20% H₂O, 0.1% formic acid</td>
<td>Li et al. (2014)</td>
</tr>
<tr>
<td>ACQUITY UPLC BEH C18 VanGuard Pre-column (1.7 μm); Waters Corp., Milford, MA <strong>Bore (mm):</strong> 2.1 × 5, <strong>Desalt:</strong> 200 μL/min, 100% solvent A; 2.5 min</td>
<td>ACQUITY UPLC BEH C18 (1.7 μm); Waters Corp., Milford, MA <strong>Bore (mm):</strong> 1.0 × 100</td>
<td><strong>Flow rate:</strong> 40 μL/min  <strong>Gradient sequence:</strong> linear gradient 15–45% solvent B; 6.8 min  <strong>Solvent A:</strong> H₂O, 0.1% formic acid  <strong>Solvent B:</strong> 90% CH₃CN, 10% H₂O, 0.1% formic acid</td>
<td>Malito et al. (2013)</td>
</tr>
<tr>
<td>Protein Micro Trap column; Michrom BioResources, Inc. <strong>Desalt:</strong> 220 μL/min; 5% buffer A, 2 min</td>
<td>C12 Jupiter Proteo C12 (4 μm, 90 Å); Phenomenex, Torrance, CA <strong>Bore (mm):</strong> 1.0 × 50</td>
<td><strong>Flow rate:</strong> 50 μL/min  <strong>Gradient:</strong> Linear gradient of 5–60% solvent B; 12 min  <strong>Solvent A:</strong> H₂O, 0.1% formic acid  <strong>Solvent B:</strong> 90% CH₃CN, 10% H₂O, 0.1% formic acid</td>
<td>Brier et al. (2012)</td>
</tr>
</tbody>
</table>
For HDX–MS, the linearity of the ion detector of the mass spectrometer is also of key importance, as it can limit the accuracy of the intensity measurement. During the analysis of HDX data, scientists should manually search for peculiar ion intensity envelopes that may evidence ion suppression induced by the coincidence of one or more abundant coeluting peptide ions.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>HDX-MS Fluidic Configurations used to Conduct UPLC Separations of Peptides Prior to Their Injection into a Mass Spectrometer—cont’d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Guard Column</strong> (Column Description (Particle Size) and Vendor, Bore (mm): Dia. x Length), Desalting Procedure</td>
<td><strong>Analytical Column</strong> (Model (Particle Size), Vendor, Bore (mm): Dia. x Length)</td>
</tr>
<tr>
<td>C8, Microm Bioresources, Auburn, CA</td>
<td>C18 Hypersil GOLD (3 μm), ThermoFisher Scientific, Waltham, MA</td>
</tr>
<tr>
<td>ACQUITY UPLC BEH C18 VanGuard Pre-column trap (1.7 μm); Waters Corp., Milford, MA Bore (mm): 2.1 x 5 Desalt solution: 100 μL/min; 100% H2O, 0.05% formic acid; 2 min</td>
<td>ACQUITY UPLC BEH C18 (1.7 μm), Waters Corp., Milford, MA Bore (mm): 1 x 100</td>
</tr>
<tr>
<td>Peptide concentration and desalting Microtrap, Bruker-Michrom, Auburn, CA Desalt: 100% solvent A; 5 min</td>
<td>ZORBAX 300SB-C18 (3.5 μm), Agilent Tech., Santa Clara, CA Bore (mm): 1 x 50</td>
</tr>
</tbody>
</table>
For HDX-MS experiments m/z accuracy of the MS is important, particularly, for measurement campaigns spanning days or weeks. HDX-MS experimentalists should know the maximum deviation drift rate of their instrument and develop a calibration schedule that maintains the instrument accuracy within the instrument specification over each anticipated instrument run.

To reduce proton–deuteron scrambling in the ESI source, the electrospray capillary temperature must be carefully controlled. It must be high enough to promote desolvation of the peptide ions, but low enough that the deuterium label is not corrupted by back exchange or H/D scrambling via intramolecular migration of protons and deuterons. Experiments have found that H/D scrambling is minimized when the desolvation temperature in the ESI source is set to 100 °C (Coales, Tomasso, & Hamuro, 2008; Walters, Ricciuti, Mayne, & Englander, 2012), rather than the 200–300 °C that is common for proteolytic studies.

Tandem ESI-MS instruments containing an electron transfer dissociation (ETD) or electron capture dissociation (ECD) stage can resolve proteolytic fragmentation HDX-MS data at the unit amide limit. In the middle-down version of this experiment, selected peptide ions are isolated and fragmented by ETD or ECD, which produces $c_k$ and $z_k$ ion fragments. The ion intensity versus m/z spectra of the secondary ion fragments are recorded. Each $c_k$ and $z_k$ ion fragment reports the nascent deuterium content of the associated fraction of the parent peptide ion sequence. Simultaneous equations describing data observed for the $c_k$ and $z_k$ ion sets can be solved to determine the D occupancy of the peptide ion sequence with resolution approaching and equal to the unit amide limit. Examples of top-down and middle-down HDX-MS of proteins using ETD (Abzalimov, Kaplan, Easterling, & Kaltashov, 2009; Huang & Hudgens, 2013; Rand, 2013; Rand, Zehl, Jensen, & Jørgensen, 2009; Sterling & Williams, 2010) and ECD (Pan & Borchers, 2014; Pan, Han, & Borchers, 2012; Pan, Han, Borchers, & Konermann, 2009) have appeared in the literature. The emergence of software tools (Table 2) designed for data analysis of ETD/ECD data will only accelerate the use of these techniques that can resolve the HDX of protein structures with single amino acid resolution.

The ETD/ECD produces fragment ions by transferring a low energy electron to the parent peptide cation, typically of charge $z = +2$ or +3. Fragment ions resulting from ETD and ECD processes are accompanied by little or no H/D scrambling and no structural rearrangements. However, as parent ion charge is increased, collisions induced by the ion optics increasingly
<table>
<thead>
<tr>
<th>Software</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUTOHD</td>
<td>Public software: Computer program that takes in a list of mass spectra, defines isotopic clusters,</td>
<td>Palmblad, Buijs, and Håkansson (2001)</td>
</tr>
<tr>
<td></td>
<td>and identifies them using a list of candidate peptides generated from enzymatic fragmentation or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dissociation URL: <a href="http://www.ms-utils.org/autohd.html">http://www.ms-utils.org/autohd.html</a></td>
<td></td>
</tr>
<tr>
<td>DynamX</td>
<td>Commercial product: Allows simultaneous visualization of raw MS data, identified peptides,</td>
<td>Waters Corp. (2015)</td>
</tr>
<tr>
<td></td>
<td>deuterium uptake graphs, and comparability plots. Multifunctional user interface. Integrates ion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mobility separation and ETD fragmentation data URL: <a href="http://www.waters.com/">http://www.waters.com/</a></td>
<td></td>
</tr>
<tr>
<td>ExMS</td>
<td>Public software: Scans through high-resolution MS data to find the individual isotopic peaks and</td>
<td>Kan, Mayne, Chetty, and Englander (2011)</td>
</tr>
<tr>
<td></td>
<td>isotopic envelopes, verifies peptide assignment, extracts deuterium incorporation information</td>
<td></td>
</tr>
<tr>
<td></td>
<td>URL: <a href="http://HX2.med.upenn.edu/download.html">http://HX2.med.upenn.edu/download.html</a></td>
<td></td>
</tr>
<tr>
<td>HDExaminer</td>
<td>Commercial product: Supports various MS data formats including high-resolution MS data and ETD</td>
<td>HDExaminer, Sierra Analytics, Inc. (2009)</td>
</tr>
<tr>
<td></td>
<td>data URL: <a href="http://www.massspec.com/HD">http://www.massspec.com/HD</a> Examiner.html</td>
<td></td>
</tr>
<tr>
<td>HDsite</td>
<td>Public software: An iterative optimization program that integrates multiple peptide acquisitions</td>
<td>Kan, Walters, Mayne, and Englander (2013)</td>
</tr>
<tr>
<td></td>
<td>together with isotopic envelope-shape information and a site-resolved back-exchange correction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>URL: <a href="http://HX2.med.upenn.edu/download.html">http://HX2.med.upenn.edu/download.html</a></td>
<td></td>
</tr>
<tr>
<td>Software</td>
<td>Commercial Status</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HeXicon 2</td>
<td>Noncommercial software</td>
<td>An automated pipeline suite for analysis and visualization of HDX-MS data. Improvements in this version include a peak detection algorithm, chromatogram alignment, and a robust peptide sequence assignment routine</td>
</tr>
<tr>
<td>HXExpress2</td>
<td>Public software</td>
<td>Semi-automatic data analysis software that generates deuterium uptake and peak width plots. Improves the speed of analyzing HDX-MS data</td>
</tr>
<tr>
<td>HDX Workbench</td>
<td>Noncommercial software</td>
<td>An integrated desktop program that facilitates automation, management, visualization, and statistical cross-comparison of large HDX data sets. This is a significant upgrade of the previous programs, The Deuterator and HD Desktop (Pascal, Chalmers, Busby, &amp; Griffin, 2009; Pascal et al., 2007)</td>
</tr>
<tr>
<td>HDXAnalyzer</td>
<td>Public software</td>
<td>HDXAnalyzer is a software package that supports statistical analyses of HDX mass spectrometry data. Program examines raw LC-MS hydrogen exchange data and finishes with complete data visualization. All information for usage and installation is available in the package</td>
</tr>
</tbody>
</table>
| HDXFinder | Public software | This Web tool application (previously named HDXAnalyzer) relies on high resolution of mass spectrometry to | Miller, Prasannan, Villar, Fenton, and Artigues (2012) | }
<table>
<thead>
<tr>
<th>Software</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>extract all isotopic envelopes before correlating these envelopes for individual peptides. The software includes a variety of graphical tools for verifying correlations and rankings of the isotopic peptide envelopes URL: <a href="http://www.kumc.edu/mspc/links.html">http://www.kumc.edu/mspc/links.html</a></td>
<td>Slysz et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>Hydra</td>
<td><em>Public software:</em> Software that processes H/D exchange data from MS or tandem MS analyses URL: <a href="http://omictools.com/hydra-hydrogen-deuterium-exchange-s7731.html">http://omictools.com/hydra-hydrogen-deuterium-exchange-s7731.html</a></td>
<td></td>
</tr>
<tr>
<td>MagTran</td>
<td><em>Public software:</em> This application calculates centroids from a user-selected $m/z$ window. This utility program uses the ZSCORE algorithm URL: <a href="http://magtran.software.informer.com/">http://magtran.software.informer.com/</a></td>
<td>Zhang and Marshall (1998)</td>
</tr>
<tr>
<td>Mass Spec Studio</td>
<td><em>Public software:</em> High-capacity HXD-MS and HDX-MS$^2$ mode of operation; supports high structural resolution of labeling chemistries; supports analyses involving ETD and CID fragmentation; can investigate peptides modified by covalent labeling; integrates structural mass spectrometry data with the High Ambiguity Driven bimolecular DOCKing (HADDOCK) program URL: <a href="https://github.com/MassSpecStudio/MassSpecStudio">https://github.com/MassSpecStudio/MassSpecStudio</a></td>
<td>Rey et al. (2014)</td>
</tr>
</tbody>
</table>
heat the parent peptide ions, which can result in H/D scrambling across the amide sequence. When adjusting ion optics prior to a measurement campaign, control measurements must be performed with defined model peptides to verify that scrambling is not occurring at the conditions used for the HDX experiment (Zehl, Rand, Jensen, & Jørgensen, 2008).

Attempts to enhance the amide resolution of HDX-MS by employing collision-induced dissociation (CID) to produce secondary fragments have proved disappointing. CID is unsuitable for HDX-MS studies because this fragmentation method heats the ions, increasing the vibrational energy and leading to significant H/D scrambling (Ferguson et al., 2007; Jørgensen, Gårdsvoll, Ploug, & Roepstorff, 2005; Rand & Jørgensen, 2007).

### 3.9 Generating a Proteomic Map

Prior to conducting any HDX-MS experiments, a map comprising the curated list of proteolytic peptide ions must be generated from the protein sample. The peptide map is the filter through which mass spectral data are selected for HDX-MS analyses. The curated list of proteolytic peptide ions includes the verified peptide sequence, ion score, charge (z), and retention time for the UPLC apparatus. Although pepsin and the other acidic proteases described above are nonspecific in that cleavage positions cannot be predicted from a protein sequence, these cleavage trends are highly reproducible when the same conditions (pH, temperature, time, and protein concentration) are used. Under controlled conditions each unique peptide elutes from the analytical chromatography column at a consistent retention time, regardless of its deuterium content.

During generation of the peptide map, the digestion and running parameters are optimized, including the temperature of the enzymatic column and the flow rate, which in combination with the column dimensions, determines the digestion time. Proteases function most effectively at physiological temperatures; however, this efficiency must be balanced against the need to reduce back exchange of the labeled backbone amide N–D within the protein, which is decreased at reduced temperature. Additionally, the faster the flow rate, the less time the protein will be in the presence of water, minimizing back exchange; yet, decreasing the flow rate increases the time the protein is in contact with the protease, potentially increasing digestion efficiency. Interestingly, pepsin more effectively digests model proteins when the protease column is maintained at high pressures (Ahn, Jung, Wyndham, Yu, & Engen, 2012; Jones, Zhang, Vidavsky, & Gross, 2010;
Digestion efficiency is determined by observing the UPLC effluent using a tandem MS (MS/MS) instrument that employs CID to fragment the parent peptide ions. CID is an acceptable fragmentation method since these studies are performed prior to exchange, and H/D scrambling is not a factor. These experiments allow digestion conditions (temperature and flow rate), solution conditions (chaotropic and reducing agent concentrations), and the UPLC gradient to be optimized. By searching a database, such as MASCOT, the \( m/z \) values of the detected peptides and their fragments can be correlated to specific peptides within the protein sequence. Good digestions yield peptides covering 70–100% of the protein sequence and a large number of overlapping peptides. The elution time and exact mass for each peptide from the database search are then utilized by the HDX-MS software to find labeled peptides and measure the mass increase associated with deuterium uptake.

As described in the chromatography section, sample carry over complicates HDX-MS data analyses. One way to test for carry over is to repeat the tandem MS analysis immediately following peptide mapping using only water as the “sample.” Since no protein is present, peptides that are detected in a database search must have eluted from the digestion, RP-trap, or RP-analytical column. To minimize carry over, the peptide mapping can be repeated with a lower protein concentration (protein concentration affects acidic protease digestion efficiency) or additional column washes can be incorporated into the HDX-MS experiment between protein analyses (Majumdar et al., 2012).

3.10 Data Analysis

Manual analysis of an HDX-MS experiment is very time consuming; however, software programs that calculate deuterium uptake for each detected peptide greatly decrease analysis times (Table 2). This reduction of analysis time has encouraged more groups to use HDX-MS for studies of protein structure, dynamics, and binding interactions. Most tools determine average deuterium uptake using the peptide centroid of the isotopic envelope, \( \langle m \rangle_{\text{centroid}} \), which is defined as:

\[
\langle m \rangle_{\text{centroid}} = z \cdot \left( \sum_{i=1}^{n} \left( \frac{m}{z} \right)_i \cdot I_i \right) - m_{H^+} \]

(17)
where \( z \) is the ion charge, \( n \) is the number of isotopic \( m/z \) features in the mass spectrum of the ion, \( (m/z)_i \), and \( I_i \) are, respectively, the measured mass-to-charge ratio and intensity of the \( i \)th ion feature, and \( m_{H^+} \) is the mass of the charge carrier (usually a proton). The centroid of the isotopic envelope is a derived measurand that reflects the average mass of an isotopic envelope originating from the same peptide. It contains no information about the measurement system or about the properties of the analyzed ion. The centroid calculation applies well for peptides that uptake deuterium under EX2 conditions. However, centroid calculations do not apply for peptides that exchange under EX1 conditions, nor can automated analysis programs detect EX1 signatures in centroid data (Zhang, Ramachandran, Kumar, & Gross, 2013). Thus, it is incumbent upon the experimentalist to verify manually the actuality of EX2 kinetics for each peptide ion. Verification is accomplished by inspecting the isotopic peak intensity distribution envelope of each peptide ion. In practice, peptide ions exhibiting EX1 kinetics are infrequently observed. Often, EX1-like signatures indicate practical problems in the data acquisition process, e.g., carryover due to incomplete column washes (Fang, Rand, Beuning, & Engen, 2011).

The isotopic envelope for each detected peptide may indicate the type of exchange occurring. Peptides that exchange with EX1 kinetics can have bimodal distributions (Fang et al., 2011; Guttman et al., 2013; Kreshuk et al., 2011; Weis, Wales, Engen, Hotchko, & Ten Eyck, 2006). Under EX1 conditions \( k_{ch} \gg k_{cl} \); therefore, multiple exchange events occur simultaneously in the unfolded protein. The two populations represent the exchanged and nonexchanged forms of the protein. If the two populations are well separated on the m/z axis, then two distinct peaks will be visible, though if the peaks are not well separated, then a broad isotopic distribution will be apparent.

For peptides that exchange with EX2 kinetics, deuterium uptake results in a binomial distribution. Following quenching, each backbone amide experiences deuterium to hydrogen back exchange with a rate, \( k'_{ch} \) (ref. Eq. 1; superscript \( i \) denotes the sequence index). Peptides exiting the UPLC column have a distribution of masses with the average mass of this population representing the average number of deuterium that were incorporated and maintained in the peptide. For most backbone amides, the loss of deuterium due to back exchange is between 20% and 30% (Walters et al., 2012). However, just as the exchange rate is dependent on the primary structure of the protein or peptide, the back-exchange rate is sequence dependent and can vary by up to 30-fold depending on the neighboring side chains. At a specific
pH and temperature, the back-exchange rate can be readily estimated for each amide hydrogen within an unfolded peptide sequence (Bai et al., 1993; Connelly et al., 1993; Englander, 2015).

For specific peptides, the amount of back exchange may deviate from its estimate due to interactions with the chromatographic columns (Sheff, Rey, & Schriemer, 2013; Walters et al., 2012). If an accurate value of the exchange rate is needed, then additional control measurements must be performed to determine the amount of deuterium that is lost due to back exchange. The corrections may be applied during data analysis. However, for most ligand-binding experiments, a relative analysis of deuterium uptake is measured between the apo- and holo-protein states with identical experimental conditions used for both proteins. This comparison is sufficient to determine regions of the protein that have different amounts of deuterium uptake in the presence and absence of ligand if the experiments are performed within a short time frame.

Data analysis for deuterium uptake can be complicated in arginine-rich peptides (Morgan & Engen, 2009). Though the backbone amide has the lowest rate of exchange at pH 2.5 and that rate is less than any other functional group, the guanidinium group of arginine has the next slowest exchange rate at this pH (Brier & Engen, 2008). During solvent exchange and UPLC, the presence of H2O ensures that the side chain residues exchange back to H prior to detection by MS. Since the exchange rate of the guanidinium group is also slow at this pH, with short wash times and UPLC gradients, some deuterium label may be retained in arginine side chains upon ionization.

3.11 Uncertainty Evaluations: Which Deuterium-Uptake Differences Are Meaningful?

The determination of dataset quality requires an uncertainty evaluation for each peptide ion that is computed from two or more replicate measurements at each t_HDX. For a few peptides, the determination of deuterium content and uncertainty can be improved by including centroid data from two or three charge states. For HDX-MS data analysis, most software packages (Table 2) automatically evaluate measurement uncertainties for the centroids. Alternately, measurement uncertainties can be evaluated manually.

Pairwise comparisons of peptides common to the apo- and holo-protein data sets can signal differences arising from a protein–ligand interaction. However, knowledge of the uncertainties of these measurements is required to identify those peptides exhibiting a significant difference. A powerful comparison method uses pairwise t-tests of each peptide across the t_HDX
time points. The t-tests presume that apo- and holo-peptide ion data follow a Student’s t-distribution (Press, Teukolsky, Vetterling, & Flannery, 2007). The threshold for statistical significance between pairs is usually set at $P \leq 0.05$. To conduct simultaneous statistical comparisons of the same peptide across multiple HDX-MS data sets, Tukey’s range test can be used (Tukey, 1949). An assortment of t-tests is available from numerous commercial and freeware statistics packages (e.g., R-language, TR Foundation, 2015).

3.12 Data Display

Deuterium uptake can be visualized in multiple ways. First, data for each peptide that is common in the apo- and holo–species can be plotted together with their measurement uncertainties on the same semi-logarithmic graph. The y-axis can show average mass increase in Daltons or it can show normalized mass increase, where the normalization is for the number of exchangeable amide hydrogens within the peptide, excluding the N-terminal amide hydrogens (Huang, Wen, Blankenship, & Gross, 2012). The x-axis is plotted as the logarithm of immersion time of the protein in D$_2$O. Peptide ions exhibiting high levels of deuterium uptake at all-time points indicate regions that are solvent-exposed, unstructured (loops) in the protein. Peptides that pass from low to high deuterium uptake over the time course of the experiment indicate that the peptides are present in dynamic regions of the protein. Peptides that have low levels of deuterium uptake at all-time points represent portions of the protein that are protected from exchange due to secondary structures, such as within $\alpha$-helices and $\beta$-sheets.

Second, a difference map of the deuterium uptake observed in the apo- and holo-protein forms can be presented in a one dimensional, linear array with the detected peptides represented by bars under the protein sequence. Customarily regions absent statistically different deuterium uptake are shown in gray. Regions showing statistical differences are exhibited as a colored heat map and referenced to a separately displayed color scale.

Third, when a crystal structure is known for the protein sample, the differential deuterium uptake can be plotted on the Protein Data Bank (PDB) image (Berman et al., 2000) to give a better indication of the sites where structural and dynamic changes are present. Plotting these data on a three-dimensional structure allow visualization of the binding interaction. This visualization can help distinguish whether the binding site is in a linear sequence or associated with multiple discontinuous regions of the protein.
Plotting the data onto the 3D structure can give significant insight into the protein–ligand interaction; however, it should be realized that the crystal structure is one model of the protein, and the HDX-MS data may represent a different solution-based equilibrium structure of the protein. Additionally, experiments should also be performed to verify that the site is the protein–ligand interface rather than a portion of the protein sequence affected by an allosteric effect.

4. INTERPRETING HDX-MS DATA TO DETERMINE PROTEIN–LIGAND INTERACTION MAPS

Proteins can interact by engaging either continuous or discontinuous sequences of amino acids (Barlow, Edwards, & Thornton, 1986; Thornton, Edwards, Taylor, & Barlow, 1986). Continuous, or linear, epitopes, such as short linear motifs (SLiMs), are short lengths of the protein sequence that mediate a protein–protein interaction. The majority of annotated SLiMs consist of 3–11 contiguous amino acids, with an average of just over 6 residues (Diella et al., 2008; Dinkel et al., 2013; Neduva & Russell, 2006). On the other hand, discontinuous or conformational protein–ligand interactions may involve several amino acid sequences that are distant in primary structure; however, conformational folding has placed the active residues proximal in tertiary structure. For example, antigen–antibody interactions often involve interactions of discontinuous epitopes (Pandit et al., 2012; Zhang et al., 2011).

This section outlines procedures that have successfully characterized a linear epitope and a discontinuous motif. For brevity, the presentation focuses on the HDX-MS portion of each study; however, these studies used auxiliary measurements, e.g., sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography, assays, etc., to assure purity, structural invariance, and biochemical activity of the protein–ligand complex under HDX-MS conditions. These auxiliary data carry additional value, as they help define properties of the protein–ligand complex.

4.1 Example: Protein–Ligand Interactions Involving Continuous Amide Contacts

In the archaeon, *Thermococcus kodakarensis*, three proliferating cell nuclear antigen (PCNA) monomers can organize into a homotrimeric ring that
encircles duplex DNA. The PCNA homotrimer tethers other enzymes in its role as the processivity factor for DNA polymerases. During laboratory investigations of *T. kodakarensis*, Li et al. (2014), discovered a small, ≈8.5 kDa protein that binds to *T. kodakarensis* PCNA1 and PCNA2 (29.3 kDa) *in vitro* (Li, Santangelo, Čuboňová, Reeve & Kelman, 2010). SDS-PAGE data showed that the 8.5 kDa protein inhibits PCNA-dependent activities, likely by preventing the formation of the homotrimer. Hence, this small protein is designated as *Thermococcales* inhibitor of PCNA (TIP). Tests demonstrated that the PCNA-TIP interaction was novel, as mutagenesis of the PCNA protein sequence in locations known to induce unclamping by other ligands did not suppress PCNA activity.

An HDX-MS study was initiated with the objective of determining the interacting regions of PCNA2 and TIP that drive the dissociation of the PCNA homotrimer (Li et al., 2014). Protein stock solution was diluted in phosphate buffer (20 mmol/L sodium phosphate, 500 mmol/L NaCl, pH 7.6) to prepare a 15 μmol/L final analytical concentration and equilibrated at 4 °C for 2 h. The PCNA-TIP complex was prepared by mixing PCNA and TIP at a molar ratio of 1:1 (PCNA monomer:TIP monomer) and equilibrated at 4 °C for 16 h.

Prior to the conduct of HDX-MS investigations, separate proteomic studies were conducted on the native PCNA2 and TIP, comprising pepsin digestion, separation of the peptic peptides by UPLC, and ESI-MS/(CID) MS of the UPLC effluent. Application of MASCOT (Matrix Science, Oxford, UK) and MassMatrix database searches (Xu, 2010) of ion signals observed by standard and tandem mass spectrometry (ESI-MS/(CID)MS) yielded a master list of identified peptide ions. Subsequently, each identification was verified manually. The ion lists provided coverage for 98% of the PCNA2 backbone (85 peptide (+1, +2, +3) ions) and 99% of the TIP backbone (13 peptide (+1, +2, +3) ions).

Robotic HDX-MS measurements were conducted by immersing each protein in buffered D₂O (pD 7.6; 3 °C) for \( t_{\text{HDX}} = 0.5, 5, 20, 40, 60 \) min. The robot dispensed protein solution (5 μL) into 21 μL D₂O buffer (20 mmol/L sodium phosphate, 500 mmol/L NaCl, pD 7.6) at 3 °C. This temperature was chosen to slow the H/D exchange rates so that the observed rates better fit the dynamic range of the instrument.

The HDX-MS data were analyzed using HDX Workbench (Pascal et al., 2012). HDX-MS data for the apo-PCNA2 (PCNA2 homotrimer alone) exhibited a range of deuterium uptake rates. After an hour of exposure to
D<sub>2</sub>O, the interior-helical regions near the trimeric interfaces exhibited little deuterium content. Exterior to the homotrimer ring, long-loop regions of each PCNA2 also required hours to achieve significant D<sub>2</sub>O exchange. On the other hand, backbone amides in some outer helices and loop regions were more labile, as these amides exhibited substantial deuterium uptake within 20 min. In contrast to the structured PCNA2 protein, HDX-MS experiments on apo–TIP (TIP alone) found no evidence of stable secondary structure. After immersion in D<sub>2</sub>O solution, amide groups of apo–TIP, especially in the C-terminal region, rapidly exchanged to contain over 80% deuterium within 30 s.

Variations in deuterium uptake rates were observed for various peptides in apo–PCNA2 (PCNA2 homotrimer alone) and holo–PCNA2 (i.e., PCNA2 peptides from the PCNA2–TIP complex). Deuterium uptake plots for peptide ions 243–259 and 187–204 (Fig. 5B and C) of apo–PCNA2 and holo–PCNA2 indicate that the holo–PCNA gains deuterium more slowly. (This conclusion is also supported by pairwise t-tests on the D-uptake versus time data, which yielded P = 0.03 and P = 0.01, respectively, inferring that the apo and holo data differ.) Since peptide 243–259 contains mostly artificial histidine residues, this decreased deuterium uptake likely arose from nonspecific interactions. Alternatively, peptides 104–110 and 34–38 of holo–PCNA2 exhibit greater deuterium uptake than apo–PCNA2 (Fig. 5D and E). These peptides reside in the trimer interfaces. The observations suggested that binding with TIP facilitates the dissociation of PCNA2 homotrimer. Such dissociation may occur by altering the Δ<sub>dG</sub>°<sub>i</sub> of these residues, so that the local amide hydrogen bonding becomes labile (K<sub>op</sub> is increased), which enables greater protic exchange with water. Such behavior is in accord with the type 2 Sowole–Konermann scenario (Sowole & Konermann, 2014).

To confirm that region 187–204 of PCNA2 binds TIP and accounts for the diminished deuterium uptake, three residues, K197, Y200, and Y204, were mutated to alanine and glutamine (ref. Fig. 5F) to make PCNA2-A and PCNA2-E, respectively. Relative to the native PCNA2–TIP complex, the PCNA2-A was expected to bind strongly with TIP, and the PCNA2-E was expected to bind weakly with TIP. Importantly, as determined by functional assays, the mutations forming PCNA2-A or PCNA2-E did not alter the PCNA activity.

Prior to conducting HDX-MS studies with PCNA2-A and PCNA2-E, ion lists providing nearly complete sequence coverage were measured and manually verified. HDX-MS experiments involving PCNA2-A,
Figure 5 HDX-MS of the protein–ligand system, PCNA2-TIP. (A) Difference D-uptake map between native apo- and holo-PCNA2 plotted on the crystal structure of homotrimeric PCNA2 (PDB: 3LX2) (Ladner, Pan, Hurwitz, & Kelman, 2011). Blue (light gray in the print version) signifies no statistical difference, red (dark gray in the print version) denotes a decreased D-uptake rate in native holo-PCNA2, and yellow (light gray in the print version) denotes increased D-uptake rate in native holo-PCNA2. (B) Temporal D-uptake of native PCNA2 peptide 243–259. (C) Temporal D-uptake of native PCNA2 peptide 187–204. (D) Temporal D-uptake of native PCNA2 peptide 104–110. (E) Temporal D-uptake of native PCNA2 peptide 34–38. (F) Partial sequence of PCNA2 showing the mutations of PCNA2-A and PCNA2-E. (G) Effects of mutations on selected peptides of PCNA2. Each colored bar plots the difference ratio (%) between the deuterium uptake by the PCNA2-TIP complex relative to its uncomplexed PCNA2. Colors correspond to the native PCNA2 (N, red (dark gray in the print version)), PCNA2-A mutant (A, yellow (light gray in the print version)), and PCNA2-E mutant (E, blue (light gray in the print version)). Each bar was computed from the average deuterium uptake found at five HDX times points, and a bracket (thin lines) denotes its standard deviation (1σ). Asterisks on the colored bars of PCNA2-E indicate data originating from slightly shorter peptides. The 187–204 peptide (red (dark gray in the print version)) contains sites subjected to mutagenesis. Each plotted peptide group exhibits statistically significant differential peptide data, as inferred by paired t-tests. Panels (B)–(G) are used with permission of Li et al. (2014).
PCNA2-E, and TIP were conducted. Relative to apo-PCNA2-A, holo-PCNA2-A mutant showed significantly decreased deuterium uptake, not only in region 187–204, but also in regions 218–229 and 39–48 (Fig. 5G). Interestingly, these regions are composed of short loops and are geometrically close to each other, suggesting potential binding interfaces. As expected for a dissociative interface, holo-PCNA2-A shows enhanced deuterium uptake for region 104–110, but the effect is less than observed for the native holo-PCNA2. Overall, the HDX-MS results for holo-PCNA2-A were consistent with the prediction that alanine mutations strengthen PCNA2-TIP interactions. In accord with an expectation for weaker binding, the holo-PCNA2-E mutant responded weakly to the presence of TIP. Decreased deuterium uptake was observed only in region 39–48 (Fig. 5G) and essentially no increase in deuterium uptake was observed in region 101–110. The absence of a deuterium uptake difference in region 187–204 of native holo-PCNA-E indicated the importance of this region in modulating the PCNA2-TIP interactions.

Examination of the differential deuterium uptake of apo- and holo-TIP found that peptides 22–28 and 55–58 of holo-TIP (Fig. 6A) show reduced deuterium uptake rates in the native PCNA2-TIP complex, evidencing a Sowole–Konermann type 1 scenario. These diminished D-uptake rates

Figure 6  Mutations in PCNA2 affect the interactions with TIP. Panels display the deuterium uptake of peptides 22–28 and 55–58 in apo-TIP (Solid red (dark gray in the print version) lines; TIP alone) and holo-TIP (dashed black lines; TIP in solution with PCNA) for (A) native PCNA2, (B) PCNA2-A mutant, and (C) PCNA2-E mutant. Figures used with permission of Li et al. (2014).
suggested that TIP engages with native PCNA2, likely through interactions with peptide region 187–204 of PCNA2. Moreover, the HDX kinetics data show that the deuterium uptake of peptide regions 22–28 and 55–58 of TIP in the presence of native PCNA2 eventually reaches the same levels as that observed in the apo form (TIP alone), thus, evidencing the flexible nature of the TIP structure and its weaker binding with PCNA2.

The PCNA2-A mutant–TIP complex exhibited nearly the same trend of HDX kinetics in regions 22–28 and 55–58 of TIP (Fig. 6B) as compared to the native PCNA2-TIP complex. This similarity is in accord with the formation of the stable PCNA2-A-TIP complex. In contrast, apo-TIP and holo-TIP for the PCNA2-E mutant–TIP complex exhibit no significant difference in deuterium uptake behaviors (Fig. 6C), which is in accord with the weaker binding expected in the PCNA2-E-TIP complex. These data taken together with the behaviors of holo-PCNA2-A and holo-PCNA2-E support assignment of the TIP binding region as residing within residues 187–204 of PCNA2.

4.2 Example: Mapping a Discontinuous Protein–Protein Interaction

Mapping of discontinuous epitopes recognized by functional mAbs is essential for understanding the nature of immune responses and designing improved vaccines, therapeutics, and diagnostics. B-cell paratopes have complex, 3D structures often composed of residues that are discontinuous in the primary structure. Therefore, immunization with an isolated synthetic peptide that mimics a single linear antigen is unlikely to elicit high titers of neutralizing antibodies (Malito et al., 2013).

Important epitopes include those of bacterial meningitis (Neisseria meningitidis) that target human complement factor H (fH) protein. Human fH protein is a 155 kDa, 20 domain, complement control protein (CCP) that binds to the glycosaminoglycans (GAGs) that are present on the surface of host cells (Józsi & Zipfel, 2008). The resulting fH glycoprotein decoration marks human host cells as “self,” protecting them from lysis by complement. Pathogenic cells and viruses not decorated with fH are effectively “unself” cells that are vulnerable to complement-mediated lysis. To evade complement-mediated killing, bacterial meningitis recruits fH by sequestering it to fHbp, a 27 kDa lipoprotein that resides on the pathogenic cell surface (Schneider et al., 2009). A crystal structure model of the fHbp:fH complex reveals that fHbp folds to form two intermingled N- and
C-terminal β-barrel domains that span amino acids 1–137 and 138–255, respectively (Malito et al., 2013; Schneider et al., 2009).

The fHbp protein on the pathogenic cell surface can serve as a vaccine antigen of bacterial meningitis. Malito et al. developed mAb 12C1, which specifically recognized fHbp (variant 1), inhibited fHbp binding to human fH, and exhibited bactericidal activity (Malito et al., 2013).

Malito et al. (2013) used HDX-MS to characterize the interaction between fHbp and mAb 12C1. During the HDX-MS study separate sets of deuterium labeling experiments were conducted on solutions of fHbp alone (apo-fHbp) and mAb 12C1 with fHbp (holo-fHbp). The antibody–antigen complex was formed by adding 225 pmol fHbp to mAb 12C1 using a molar ratio of 1:1.5, followed by incubation for 30 min at \(20^\circ C\). During the experiment, samples were immersed in deuterated phosphate-buffered saline (PBS) solution (pD 7.4, 25°C) for specific exposure times \(t_{\text{HDX}} = 0.5, 2, 10, 20, 30\) min. After each immersion period expired, a 30 μL sample was removed and the H/D exchange reaction was quenched with 30 μL ice-cold 200 mmol/L sodium phosphate (pH 2.4) buffer. The quenched aliquots were immediately frozen in liquid nitrogen and stored at \(-80^\circ C\) for less than 24 h.

When analyzing frozen aliquots, labeled samples were thawed rapidly to \(0^\circ C\) and injected into a Waters nanoACQUITY UPLC. The UPLC injector, switching valve, columns, solvents, and all associated tubing were maintained \(0^\circ C\) (ref. Table 1). The front end of this apparatus digested protein samples into peptides with a Porosyme Immobilized Pepsin Cartridge. This digestion step required 2.5 min at 20°C with a solvent flow rate \((H_2O, 0.1% \text{ formic acid})\) of 200 μL/min and resulted in desalted peptides that were trapped on a guard column. Subsequently, the peptides were eluted off the guard column, chromatographically separated, and electro-sprayed into a Waters tandem SynaptG2 mass spectrometer operating in resolution mode \(m/z 100–2000\). Mass accuracy was ensured by continuously infusing a Glu-1-Fibrinopeptide B solution \([600 \text{ fmol/μL in 50% (vol/vol) CH}_3\text{CN, 0.1% formic acid})\] or Leu-Enk \((2 \text{ ng/μL in 50% CH}_3\text{CN, 50% H}_2\text{O, 0.1% formic acid;}\) Waters Corp.) through the reference probe of the electrospray ionization source. DynamX software (Waters Corp., 2015) determined the centroid mass of each peptide as a function of labeling time. Only the peptic peptides present in at least three repeated digestions of the unlabeled proteins were considered for the analysis.

Proteomics measurements and application of the ProteinLynx Global Server proteomics search program to apo-fHbp mass spectrum data identified 19 peptide ion fragments that covered 97% of the fHbp sequence.
Similar HDX-MS measurements of holo-fHbp solutions revealed that the presence of mAb 12C1 suppressed deuterium uptake for seven of the nineteen peptide ion fragments of fHbp (Fig. 7). The diminished D-uptake evidences a Sowole–Konermann type 1 interaction scenario induced by binding between 12C1 and fHbp. The peptides define four discontinuous segments (L34-L48, I89-F96, T107-137, and Y214-L251) encompassing 92 residues distributed in the N- and C-terminal domains of fHbp. These four discontinuous segments are clustered in a distinct region (Fig. 7) and comprise the discontinuous antigenic epitope of fHbp.

The HDX-MS results are in accord with other epitope mapping techniques. Most importantly, all four peptides identified by HDX-MS are also present in the fHbp:12C1 epitope determined from crystal structure analysis. Scanning with a library of synthetic fHbp peptides suggested that the C-terminal residues, A238-I249, comprise the primary target recognized by mAb 12C1. Screening a phage-display library of fHbp peptides identified that C-terminal peptide, L224-G250, is also a target of mAb 12C1.

Although both peptides reside in the epitope identified with HDX-MS, neither peptide accounted for the binding affinity found by surface plasmon resonance (SPR). However, the HDX-MS approach not only confirmed the involvement of peptide A238-I249 in binding 12C1 but also revealed additional fHbp N-terminal regions that mediate the binding interaction. These results accounted for the strong binding constant derived from SPR data, and they also supported evidence of a conformational epitope spanning the N- and C-termini of fHbp as the target of mAb 12C1.

Malito et al. concluded “...our results highlight the importance of using sensitive epitope mapping techniques rather than relying on the identification of linear peptides when seeking to fully understand the details of B-cell epitopes mediating antigen–antibody interactions” (Malito et al., 2013). The technique of proteolytic fragmentation HDX-MS is a sensitive platform that can rapidly provide these nuanced maps of protein–ligand interactions.

**DISCLAIMER**

Certain commercial materials and equipment are identified in order to adequately specify the experimental procedure. Such identification neither implies recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material or equipment identified is the best available for the purpose.
Figure 7 Map of the antigenic epitope of fHbp during interaction with mAb 12C1, as determined by HDX-MS. The peptides protected by mAb 12C1 are highlighted in red (dark gray in the print version) on the fHbp structure. Numbered spheres label the start and end of each affected fragment. Subpanels show the deuterium uptake observed in peptides of apo-fHbp (red (dark gray in the print version), always upper line) and holo-fHbp (blue (dark gray in the print version), always lower line), showing discordant behavior during 30 min immersion in D₂O. The relative arrangement of the lines is characteristic of Sowole–Konermann type 1 behavior. Used with permission of Malito et al. (2013).
REFERENCES


Chen, Y.-B., Chattopadhyay, A., Bergen, P., Gadd, C., & Tannery, N. (2007). The Online Bioinformatics Resources Collection at the University of Pittsburgh Health Sciences


