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Tracking Higher Order Protein Structure by Hydrogen-Deuterium Exchange Mass Spectrometry

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Abstract

Structural biology has provided a fundamental understanding of protein structure and mechanistic insight into their function. However, high-resolution structures alone are insufficient for a complete understanding of protein behavior. Higher energy conformations, conformational changes, and subtle structural fluctuations that underlie the proper function of proteins are often difficult to probe using traditional structural approaches. Solution state structural techniques are needed to interrogate the dynamic nature of proteins under native conditions. Hydrogen/deuterium exchange with mass spectrometry (HDX-MS) provides a means to probe the accessibility of backbone amide protons under native conditions, which reports on local structural dynamics of solution protein structure. HDX-MS also provides a tool for tracking complex structural rearrangements that occur in the course of a protein's function. Here we review recent advances in HDX methodology and highlight some novel applications where HDX has shed light on particularly challenging systems.

Keywords

Hydrogen deuterium exchange; footprinting; protein structure

1. INTRODUCTION

The three-dimensional arrangement of the amino acids in proteins governs their function and regulates nearly every fundamental biological process. Much of our understanding of protein structure is derived from high resolution structural studies, predominantly obtained using X-ray crystallography. Such studies reveal the secondary, tertiary, and quaternary structure that is necessary for the activity of the protein, but this is only part of the story. Crystal packing forces can introduce local structure that may not recapitulate the solution structure. More importantly, proteins often sample an ensemble of higher energy states and the details on the structural fluctuations (i.e. "dynamics") that proteins undergo in solution is not directly evident from crystal structures. Without such breathing motions, even the simplest of enzymes would be unable to rearrange chemical bonds to mediate catalytic steps.

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CONFLICT OF INTEREST

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Hydrogen/deuterium exchange coupled to mass spectrometry (HDX-MS) has become a widespread tool for structural analysis of proteins. HDX-MS probes the accessibility of amide hydrogens on a protein backbone by monitoring the exchange of amide protons with deuterium. The exchange reaction is highly dependent on pH and temperature and it is critical that both are carefully controlled so that the exchange reaction directly reports on protein structural dynamics. After the labeling step, the exchange is subsequently slowed ("quenched") by lowering the pH and temperature to freeze deuterium into place, and mass spectrometry is used to quantify deuterium uptake. The kinetics of amide exchange provide a means to track structural dynamics [1]. Additionally, HDX-MS data has been used to assess conformational heterogeneity [2], map protein-protein interactions [3–4], screen libraries for ligand binding [5–6] screen protein constructs for crystallization [7–8], and determine binding affinities [9].

In a typical labeling experiment, a protein in an aqueous buffer is rapidly diluted into a high percentage deuterium buffer for varying amounts of time ("continuous labeling" Figure 1A). The exchange reaction is then slowed nearly to cessation by diluting the reaction into quench conditions (pH 2.5 and 0°C) effectively locking the deuterium label on the protein backbone. The labeled proteins are then rapidly proteolyzed into peptide fragments using an aspartic protease under quench conditions and the peptides are resolved by reverse phase liquid chromatography coupled to mass spectrometry (LC-MS). The resulting HDX profiles of all the observable peptides report the local structural dynamics of the protein. Alternatively, HDX-MS has been employed to obtain snapshots to monitor protein structural changes using a "pulse labeling" scheme (Figure 1B). Here, the protein is perturbed and labeled with a consistent short pulse of deuterium exchange to monitor protein transitions along with the corresponding kinetics. This provides a powerful way to track the local changes that, for example, occur during protein folding and refolding events. This review will cover recent advances in HDX-MS methodology and applications to interrogate protein structure and function in biological systems that are frequently refractory to structural characterization by other methods. For the fundamental theory and methodology of HDX the reader is referred to these publications [1, 4, 10].

1.1. Advances in sample processing and analysis

One of the advantages of HDX-MS is that it is applicable to nearly any protein system of interest. With the high sensitivity of modern mass spectrometers, the limits of detection are sufficient to enable comprehensive studies with very little sample requirements; nowadays, a few hundred picomoles of protein is sufficient. HDX is now routinely used to study large protein assemblies, heavily glycosylated proteins, and membrane proteins, all of which can be challenging targets for most structural techniques. In practice, the size or complexity of systems to be interrogated by HDX-MS is mainly limited by the peak capacity of the LC-MS system, which is a metric of how well it can resolve and track all relevant peptides. The peak capacity is hindered by the additional measures that are necessary to minimize loss of the deuterium during sample handling. Even at quench conditions (pH 2.5 and 0°C), a moderate level of back-exchange still occurs. The goal is to digest the protein(s), chromatographically resolve the peptide mixture, and detect and track the deuteration of the peptides by MS, all while minimizing deuterium back-exchange. This also proven useful for

HDX-MS by providing a new dimension for the separation complex peptide mixtures [12]. The increasing sensitivity of mass spectrometers enables observation and tracking of more of the peptides thereby increasing the data quality and information content for HDX-MS. Modern systems are capable of obtaining high quality data for large multi-protein complexes > 100 kDa [13], such as the recent report on the inner workings of the base complex of the yeast 19S proteasome, one of the most complex systems investigated by HDX-MS to date [14].

HDX-MS has also benefited from several advances in LC technology. Ultra-high pressure chromatography (UPLC) provides increased chromatographic resolution even at the low temperatures required for minimizing back-exchange, boosting peak capacity and the limits of detection [15]. LC separation times are still generally kept short, but by using sub-zero temperatures and aprotic polar solvents, the separation times can be extended to obtain better chromatographic resolution and increased peak capacity constraint severely limits options for sample processing such as denaturation and proteolysis, resulting in suboptimal digestion efficiency. Minimizing deuterium back-exchange also necessitates short LC-MS analyses that are typically carried out at lower temperatures, which is detrimental to chromatographic peak shape and overall resolution.

Over the past decade there have been numerous advances in MS techniques for sample processing and analysis that have greatly expanded the complexity of systems amenable to HDX-MS. Mass analyzers with very high resolving power have greatly expanded peak capacity [11] by resolving nearly-isobaric peptides that previously could not be resolved by their m/z. Ion mobility spectrometry which is increasingly incorporated on high-end MS instruments, has without increased back-exchange [16–17]. In 2015, Black *et al.* described the first application of HDX using microchip capillary electrophoresis coupled to mass spectrometry (CE-MS) as an alternative means to rapidly resolve a complex peptide mixture [18]. They achieved a threefold increase in peak capacity with significantly shorter analysis times, illustrating the potential of CE-MS for HDX-MS.

Many proteins of interest contain disulfide bonds that need to be reduced for optimal digestion and peptide coverage. This has traditionally been accomplished chemically by the addition of high concentrations of tris(2-carboxyethyl)phosphine (TCEP), which can reduce disulfide bonds even under quench conditions [19]. However, reduction can be inefficient with highly disulfide bonded proteins [20–21]. In 2014, Mysling *et al.* described an electrochemical cell for rapid online disulfide reduction that is compatible with HDX-MS [22]. With this system, the same group was able to expand the sequence coverage of the highly disulfide-bonded nerve growth factor β from 44% to 96% [23]. The electrochemical cell is now commercially available and is likely to be incorporated into the next generation of HDX-MS platforms.

1.2. Application to membrane proteins

Analysis of integral and peripheral membrane proteins is one of the applications where HDX-MS has gained much attention in recent years [24]. Membrane proteins present one of the most challenging systems for structural studies as they generally require either detergent solubilization or, more preferably, reconstitution into artificial lipid environments to

maintain a native conformation. A significant effort is being made to advance synthetic lipid environments to more closely recapitulate that of a native biological membrane including: anionic detergents, lipid nanodiscs, synthetic liposomes, and lipid bicelles. For HDX-MS, depending on the level of detergent or lipid added there may be a need for additional clean-up steps for their removal, as introduction of detergents can lead to a rapid decline in performance [25]. For many systems, detergent solubilization does not appropriately mimic the native lipid environment. To this end researchers have focused on studying integral membrane proteins in more native like lipid environments using detergent-lipid bicelles, lipid nanodiscs, synthetic liposomes, and intact native membranes. These platforms enable tight control of the lipid composition as well as greater interrogation of peripheral and integral membrane protein activity.

G protein-coupled receptors (GPCRs) are perhaps the most widely studied group of integral membrane proteins due to their pharmacological significance, yet there remains a significant gap in our understanding of the structural mechanisms of GPCR signaling and ligand induced structural changes. Zhang *et al.* demonstrated the application of HDX-MS to study of the conformational dynamics of the β2-Adrenergic GPCR (β2AR) [26]. Using detergent solubilized β2AR bound to the inverse agonist carazol, they presented an optimized HDX-MS method for the study of the conformational dynamics of β2AR and its interactions with pharmacologically relevant ligands [27]. Around the same time, Chung *et al.* characterized the structural differences between active and inactive β2AR [28]. The HDX-MS characterization of β2AR revealed flexible regions of the protein that were not picked up from the available structural information [29]. Duc *et al.* compared the conformational dynamics of three GPCRs, including the β2AR, reconstituted in dodecyl maltoside (DDM) and lipid bicelles [30]. While the overall conformational dynamics of β2AR were largely similar in DDM and bicelles, intracellular loop 3 was significantly more dynamic in DDM compared to bicelles. Solubilization in bicelles also yielded dramatically improved sequence coverage of β2AR following pepsin digestion by nearly 20% for all regions of the protein.

These results highlight just some of the advantages of using reconstitution into synthetic lipid environments over detergent solubilization. HDX-MS was used to track the extensive light-induced changes in the photo-receptor bacteriorhodopsin, an integral membrane protein, reconstituted in bicelles [31]. However, lipid bicelles have drawbacks that prevent their widespread use including their size range and limitations on lipid composition. Synthetic liposomes have been used to study integral and peripheral membrane proteins by HDX-MS and provide some advantages compared to bicelles, nanodiscs, and detergents. Using HIV envelope glycoprotein gp41 segments displayed on liposomes Kim *et al.* were able to characterize the interactions between the envelope glycoprotein and neutralizing antibodies [32]. Liposomes were also used to the track the structural transitions of the various subdomains of ATP synthase during its catalytic cycle [33].

Lipid nanodiscs are an attractive platform for the study of integral membrane protein structure and function. Integral membrane proteins can be inserted into lipid nanodiscs of varying size and lipid composition with relative ease after detergent solubilization. HDX-MS with lipid nanodiscs requires additional steps for removal of the lipids prior to LC-MS. Hebling *et al.* pioneered a nanodisc disassembly followed by phospholipid depletion to

enable HDX-MS analysis of an integral membrane proteins [34]. Their approach was used to examine the conformational dynamics of the membrane scaffold protein and the effects of ligand binding to gamma glutamyl carboxylase (GGCX) embedded in nanodiscs [35–36]. Nanodiscs have also been used for studying peripheral membrane proteins such as cytochrome P450 3A4 (CYP3A4) to interrogate the structural dynamics of the apo and ligand bound conformations [37].

HDX-MS of integral membrane proteins in lipid nanodiscs has most recently been applied to study the conformational dynamics of LeuT, a neurotransmitter-sodium symporter (NSS) [38]. All NSSs exist in a dynamic conformational equilibrium between two states; however, attempts at characterizing the structural transition between these two states, and the structural effects of clinically relevant drugs and polymorphisms, have met limited success. Adhikary *et al*. were able to demonstrate clear HDX signatures corresponding to each state, thus enabling future interrogation of the structure and functional dynamics involved in this conformational equilibrium as well as the effects of clinically relevant drugs and polymorphisms in human homologs [38].

These artificial membrane systems all aim to accurately recapitulate the native lipid environment of integral and peripheral membrane proteins to better interrogate their structure and function by HDX-MS. However, the best option to observe how these proteins behave under native conditions is to study them as they exist unperturbed in their native lipid environment. Rey *et al*. presented a method for studying the conformational dynamics of the mitochondrial ADP/ATP carrier (bAnc1p) and the structural basis for inhibition by two known inhibitors using HDX-MS in intact mitochondria [39]. The data obtained with bAnc1p in intact mitochondria was markedly different than previous HDX-MS data using detergent solubilized bAnc1p [40]. The authors suggested this difference may be attributed to a partial disruption of the native conformation that occurred with detergent solubilization in the earlier study.

These studies illustrate the extension of HDX-MS to monitoring membrane protein structure and conformational dynamics under true native conditions. Most exciting is the feasibility of performing HDX-MS on proteins *in situ.* Garcia *et al*. investigated the conformational dynamics of the influenza hemagglutinin (HA) fusion glycoprotein on intact influenza virions, demonstrating that the soluble HA ectodomain recapitulates the structure and dynamics of the full-length HA on the viral surface [41]. More recently Lim *et al*. characterized the temperature-dependent dynamic changes that play a role in the activation of the fusion glycoprotein on intact dengue virus particles [42]. With further advancements in HDX-MS techniques we anticipate that complex membrane-bound systems can be routinely examined in their complete native environment.

1.3. Ligand binding site and epitope mapping

One of the applications where HDX-MS has shined for decades is for mapping protein-protein interactions [3], particularly epitope mapping [43]. The past 5 years have seen a surge in HDX for epitope mapping of complex antigens including: Malaria P vivax Duffy binding protein [44], HIV envelope glycoprotein: [32, 45], various surface proteins on *Neisseria meningitides* Factor H binding protein [46], Neisseria heparin binding antigen

[47], Neisseria adhesin A [48], *Staph aureus* antigens [[49]], Influenza: [50], Dengue Virus [42], and Ricin Toxin [51]. While epitope mapping with HDX-MS focuses on monoclonal antibodies, Yang *et al.* demonstrated the ability to map the general epitopes of affinity enriched polyclonal sera [52]. Though this application is at an early stage, it demonstrates the feasibility of HDX-MS to qualitatively track the predominant structural targets of the immune response on an antigen.

Sometimes the differences in conformational dynamics upon ligand binding may be invisible by HDX-MS because they manifest as differences on either very long or very short time scales that is outside the typical temporal window that is sampled (seconds to hours). Since the intrinsic exchange rate is dependent on pH and temperature it is possible to manipulate both variables to expand the observed temporal range for better coverage of the ~10 orders of magnitude that amide protons can exchange within proteins [53–54] Seeing the full kinetic profile can help alleviate false negatives in epitope mapping studies.

1.4. Glycoproteins and biotherapeutics

Highly glycosylated proteins often present a challenge for structural studies. For HDX-MS, high levels of glycosylation can limit proteolytic efficiency, and the glycan structural variants present at the glycosylation sites creates a level of heterogeneity that can impede the observation and assignment of many glycopeptides. Furthermore, glycans themselves incorporate deuterium under HDX conditions, and complicate the analysis of glycopeptide data [55–56]. In 2016, Jensen *et al.* reported that the glycosidase PNGase A, a commonly used reagent for glycobiology, retains sufficient activity at low pH that it can be used to remove asparagine-linked glycan chains from glycopeptides under quench conditions [57]. This additional glycosidase step provides a convenient way to decrease the complexity of the sample and simplify the analysis of glycopeptides by separating the glycan and peptide contributions to the overall HDX profile.

HDX-MS is becoming increasingly incorporated into biotherapeutic development as it provides a fast structural evaluation of intact therapeutic antibodies [58] and a structural tool for ensuring biocomparability [59]. The structural consequences due to altered glycosylation on IgG molecules has now been extensively investigated by HDX-MS [60–63]. HDX-MS is now being applied for formulation screening, construct stability, characterization of aggregates and chemical modifications [64–68], and will likely be adopted for further applications within the biopharmaceutic industry.

1.5. Obtaining higher sequence resolution

The sequence resolution and coverage obtained from a typical HDX-MS platform is limited by the extent of peptides generated through rapid proteolysis. Pepsin is by far the most commonly used protease for HDX-MS, with rhizopuspepsin and aspergillopepsin used to a lesser degree, often combined in tandem with pepsin [69–71]. More recently additional proteases including the stomach protease from the rice field eel [72] and *Nepenthes* aspartic protease [73–74] have been used with HDX-MS experiments. Both show promise for HDX as their sequence specificity is considerably different from the earlier set of available proteases. While buffer conditions cannot deviate far from the quench conditions, it has been

shown that elevated pressures can enhance proteolytic digestion without affecting back-exchange [75]. By immobilizing the proteases both the digestion efficiency and the resulting background signal from the autolysis fragments can be minimized [76]. Proteases for HDX-MS are now available immobilized on high-strength beads to enable high-pressure online digestion [77].

A shared characteristic of all currently available proteases for HDX is that they digest proteins in a relatively non-specific, but reproducible manner. As a consequence, proteolysis generally yields multiple overlapping peptides that can be used to provide higher sequence resolution [78–79]. The extent of sequence resolution enhancement through this approach was nicely demonstrated by Mayne *et al.* By combining in-line proteolysis using immobilized pepsin and/or aspergillopepsin, they were able to generate an average of 10 unique peptides covering each amino acid position for four unique proteins of varying size and structure [78]. This approach has been incorporated into more recent HDX analysis software [80–81]. However, some recent observations reported by Sheff *et al.* have highlighted some caveats of this analysis; namely that each individual peptide from a series of overlapping fragments can have drastically different back-exchange kinetics [82]. This can significantly confound the interpretation of exchange on a residue-specific level and higher-resolution information obtained from overlapping peptides should be interpreted carefully. Another drawback to relying on a diverse set of peptides is that the selection of proteases and digestion conditions must be empirically determined for each protein to generate an appropriate set of overlapping peptides, which is not always obtainable.

A different approach for obtaining higher sequence resolution has been through tandem mass spectrometry. In the early days of HDX-MS several groups attempted to use collision-induced dissociation (CID) for fragmentation of peptide ions with the goal of obtaining amino acid specific exchange information. Unfortunately, CID is ineffective for localizing deuterium on amides because the conditions for CID results in gas-phase scrambling of amide hydrogens and deuterons, preventing definitive assignments for the deuterium labels [83]. The more recent electron capture dissociation (ECD) or electron transfer dissociation (ETD) methods have been far more promising for obtaining higher resolution with HDX-MS. Unlike CID, these are soft fragmentation techniques where labile post-translational modifications are unperturbed [84–85]. More importantly, ECD and ETD largely alleviate gas phase deuterium scrambling, which enables the analysis of HDX data with amino acid level resolution [86–88].

ETD is being increasingly incorporated into HDX-MS to provide a higher level of resolution for localizing structural changes [62, 89–90]. In the case of glycopeptides, ETD is particularly useful as it can be used to distinguish the contribution of deuterium exchange of the carbohydrate as compared to the underlying peptide backbone [56]. This offers a new tool for tracking any changes to the accessibility of the glycan groups within glycoproteins, for which very few tools exist. ECD/ETD also presents a method for fragmentation of intact proteins to provide residue-specific information on deuterium incorporation in a “top-down” approach for HDX-MS analysis [91–93]. The top-down approach has been a useful tool for examining systems refractory to typical bottom-up HDX-MS, for example the conformational changes leading to amyloid formation of Aβ 1–42 [94]. The combination of

traditional peptide-based HDX-MS (“bottom-up”) and intact protein MS with ECD/ETD (“top down”) is now providing increased sequence coverage of challenging targets, such as intact antibodies [58]. There are, however, some experimental limitations that limit the broader implementation of ETD for HDX. It is only effective for multiply charged ions, and generally best suited for at least triply charged ions, which are not always present for the array of peptides throughout the protein sequence. Controls are still necessary to ensure that the source and fragmentation conditions are adequately avoiding deuterium scrambling [95]. The levels of scrambling can greatly depend on the peptide sequence and further studies are warranted to understand the full process and primary sequence effects on deuterium scrambling [95]. Despite these limitations, ETD continues to become more prevalent with HDX-MS paving the road to probing amide exchange at residue-level resolution making HDX-MS even more powerful and widely applicable for studying complex protein interactions and behavior.

1.6. Monitoring transient structures or folding intermediates

Many proteins undergo large refolding events through the course of their function. High resolution structures can often reveal the initial and final states, but are not always informative about the sequence of structural rearrangements during the transitions. Often the intermediates are short-lived conformational states that can be difficult to capture by most structural methods. Pulse-labeled HDX-MS is a powerful approach that can monitor protein motions during such dynamic events. Unlike typical continuous-labeling, where a protein is incubated in deuterated buffer to probe the exchange kinetics, pulse labeling HDX-MS captures snapshots of proteins on rapid time scales as they undergo conformational transitions. In a typical pulse labeling HDX-MS experiment, a protein is mixed with a perturbant that triggers a structural change. In the case of protein folding this perturbing condition would be removal of a denaturant, initiating the protein to unfold. After select amounts of time the perturbed protein is then rapidly diluted into a high percentage deuterium buffer for a short amount of time (milliseconds to seconds), and the labeled protein is then rapidly quenched, digested, and analyzed by LC-MS as described above (Figure 1B). The resulting data provides a series of snapshots of the protein through the course of its structural changes, which can resolve the kinetics of multiphasic protein folding/unfolding events on a local level.

A millisecond pulse-labeling HDX-MS platform using a quench-flow system was applied to study the kinetic folding of ribonuclease H (RNase H), maltose binding protein (MBP), and PI3K SH3 domain [96–98]. In all cases a fully deuterated protein was diluted into folding conditions for various amounts of time and then rapidly pulsed with H_2O for tens of milliseconds to seconds. The rapid H_2O pulse was optimized to label only unstructured (non-hydrogen bonded) amides. The labeled samples were then immediately quenched and proteolyzed using in-line digestion, and analyzed by LC-MS. Fine temporal control of the folding times enabled the researchers to track amide sites through the course of protein folding. Analysis of the kinetic transitions of RNase H and PI3K SH3 demonstrated the formation of individual secondary structure elements that are retained during folding to the native structure, highlighting the stepwise process of protein folding. Folding of MBP, which is considerably larger than RNase H, was found to form an obligate folding intermediate

consistent with a dominant folding pathway (Figure 2). This work, along with other HDX-MS studies, indicate protein folding occurs through distinct folding intermediates in a sequential manner thereby reducing conformational entropy and accelerating folding. The high sequence and temporal resolution of pulse labeled HDX-MS in these studies enabled the researchers to identify folding intermediates and chart the pathways traversed by proteins during folding.

Pulse labeling approaches have also benefitted from advancements in ETD, and can now track refolding motions at even higher resolution [99]. This was well illustrated in examining the aggregation pathways of amyloid beta with residue specific information [100]. Overall, HDX-MS offers one of few techniques for tracking refolding events and transient conformational changes on a local level, and the advancements to the field will increase the resolution and data quality in the future.

1.7. HDX for informed modelling and structural elucidation

HDX-MS can also provide valuable information to help constrain or guide predictive structural modeling approaches when no high-resolution structures exist [101–105]. Structural modeling platforms, such as the Integrated Modelling Platform (IMP), rely on homology modeling in combination with structural restraints obtained from various techniques [106]. These hybrid modeling approaches incorporate information from a myriad of techniques such as electron microscopy, small angle X-ray scattering, crosslinking, chemical labeling, and hydroxyl footprinting [67, 107–109]. Amide accessibility obtained from HDX provides an additional level of information for structural modeling as it can differentiate regions of stable secondary structure from unfolded regions. Computational tools are available to predict the HDX behavior of model structures to enable HDX-MS data for model evaluation [110], and this approach is now being incorporated into established modeling platforms [111].

1.8. Localizing order in intrinsically disordered proteins

Throughout this review, we have reinforced the idea that by better understanding a protein's structural dynamics we can better appreciate its function. It is well known, however, that not all proteins are wholly structured. Many proteins feature intrinsically disordered domains that are integral to the protein's function *in vivo*. The flexibility inherent to specific domains is often highly relevant to the regulation of protein systems. For example, the inhibitor of nuclear factor kappaB, IKBα, was shown by HDX-MS to be relatively flexible at three of its ankyrin repeat domain, and this local flexibility is critical for its proper regulation through proteosomal degradation [112]. Disorder to order transitions are hallmarks of many cell signaling systems and protein interactions. Such transitions pose a major challenge to most high resolution structural methods due to the inherent flexibility in the system.

The receptor tyrosine kinases EGFR, HER2, and HER3 are integral membrane proteins featuring C-terminal domains involved in autophosphorylation and signal transduction. HDX-MS showed that the C-terminal domains for all three proteins are largely disordered compared to the well-structured kinase domain [113]. It was suggested that the disorder and extended conformation of the C-terminal domains of these receptors aid in increasing the

capture radius for signaling partners [114]. The disordered nature of the domains was also suggested to lower the thermodynamic barrier for binding of signaling partners, enabling interactions with multiple diverse partners through a lack of encoded structured binding specificity [113]. A similar approach revealed that the intracellular domain of the low-density lipoprotein related receptor (LRP-1) adopts local secondary structure that is disrupted by activation through tyrosine phosphorylation to increase accessibility to its many binding partners [115].

Many promiscuous proteins use disorder and structural plasticity to increase their capacity to interact with multiple diverse partners; these interactions often feature disorder to order transitions that can be effectively monitored by HDX-MS. The peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) interacts with the estrogen-related nuclear receptor ERRγ, and many others, to regulate transcription of metabolically important genes. HDX-MS revealed stabilization of the ERRγ ligand binding domain upon binding PGC-1α [116]. A similar approach monitored the binding of human La (hLa) protein to dissimilar RNAs. When binding unstructured RNAs previously structured regions of hLa became unstructured, whereas binding a structured RNA induced structure in hLa [117].

Protein disorder is not always functionally relevant and in some instances, can have undesirable consequences. The cellular prion protein (PrP) can spontaneously misfold under certain conditions and induce misfolding of native PrP leading to a cascade of aggregation and amyloid formation. Native PrP has a defined structured, but is highly flexible in solution, possibly correlating with its misfolding and aggregation potential. Native state HDX-MS identified potential misfolding intermediates and precursor conformations that initiate misfolding of other native PrP [118–119]. These intermediate or precursor conformations were shown to be partially unfolded forms of PrP and lacked global structure. In a separate study, Singh *et* al identified key residues involved in the pH dependent misfolding of PrP by HDX-MS [120]. These data provide a more complete understanding of the PrP propensity to misfold and the mechanism of prion formation.

The amyloid beta (Aβ) peptide is well known for its propensity to aggregate into β-sheet rich amyloid fibrils and is implicated in the progression of Alzheimers disease. Researchers have employed a combination of biophysical and structural tools in an effort to understand both the free solution structure and amyloid structure of Aβ. Previous efforts at interrogating the structure of Aβ amyloid fibrils have not been widely successful owing to the large heterogeneity of soluble Aβ aggregates. Recently pulse-labeling HDX-MS was used to monitor conformational changes in Aβ during aggregation [100]. The time-resolved monitoring of Aβ aggregation yielded valuable information on the kinetics and mechanism of amyloid formation and the environmental factors contributing to fibril formation.

To better understand the link between protein disorder, function, and dysfunction researchers search for the presence of transiently sampled structural motifs. Keppel et al presented a method for monitoring transiently preferred structural motifs in intrinsically disordered proteins using millisecond HDX-MS [79]. By comparison of the various overlapping peptides, the authors could narrow down the regions of secondary structure with near amino acid level resolution (Figure 3). Their work identified secondary structure elements in the

p300 domain of the activator for thyroid hormone and retinoic acid receptors (ACTR) consistent with previous NMR data [79].

The studies highlighted here, along with many others, demonstrate the ability of HDX-MS to monitor weak or transient protein structure [121–122]. With recent and continuing advances in HDX-MS methodology enabling higher spatial and temporal resolution the study of disordered proteins will become increasingly accessible. We anticipate the range, and environmental complexity, of disordered systems studied by this method to greatly expand. The ability to extract kinetic and mechanistic information about protein aggregation from single experiments across a range on environmental conditions will further our understanding of protein misfolding and disorder as it relates to protein function and dysfunction.

CONCLUSION

HDX-MS continues to expand as a general tool for structural biology. This versatile approach can be used to track the local structural dynamics of known structures, track folding intermediates, or provide a glimpse of structure where none is available. Thanks to the benefits of the new generation of LC-MS systems HDX-MS is achieving ever expanding lower limits of detection, improvements in resolution, better sample handling, and online integration. Commercialization options and automated software tools are now available through multiple instrument vendors, making HDX-MS increasingly accessible to the greater structural biology community. Tackling complex biological targets such as membrane proteins and large glycoprotein complexes, which was once a daunting and at times impossible task, can now be performed on a nearly routine basis. HDX-MS is now entering a phase where analyzing structures of complex membrane proteins *in situ* is not only feasible but underway.

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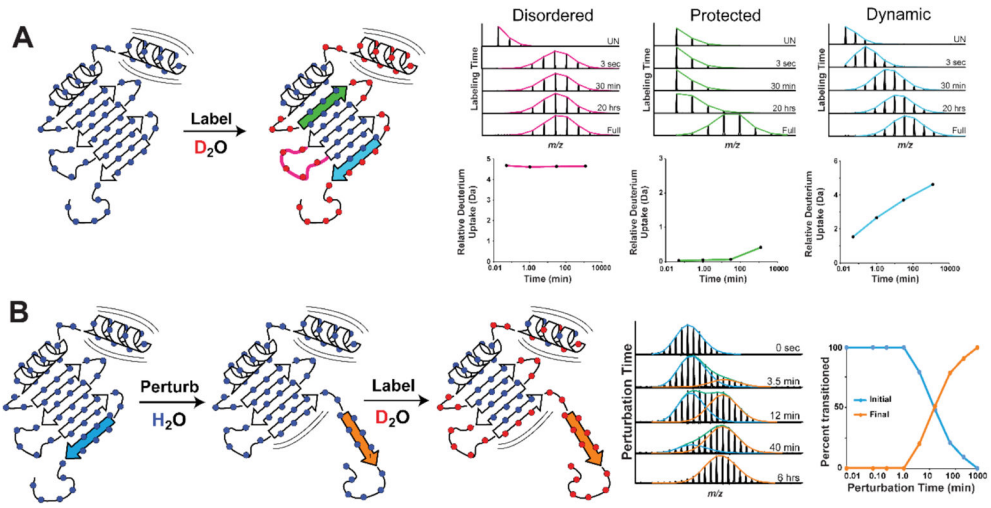

Figure 1.

Continuous labeling (A) and pulse labeling (B) of proteins by HDX-MS. **A)** A protein composed of secondary structural elements (beta sheets and alpha helices) in an aqueous solvent contains a proton at each protein backbone amide (blue circles). In a deuterated solvent the amide protons exchange for deuterium (red circles). Regions of secondary structure are involved in hydrogen bonds and exchange much slower than accessible amides, or regions of high flexibility (e.g. helix on the top right). The exchange kinetics for three example peptides are shown on the right showing a disordered region (pink), a highly protected region (green), and a dynamic region (blue). **B)** With pulse labeling HDX-MS a protein is perturb to trigger a conformational change, in this case unfolding. After a specific time, the sample is rapidly exposed to deuterium to label all accessible sites. By tracking the populations of the protected (folded, blue) and unprotected (unfolded, orange) populations the kinetics of unfolding can be precisely monitored.

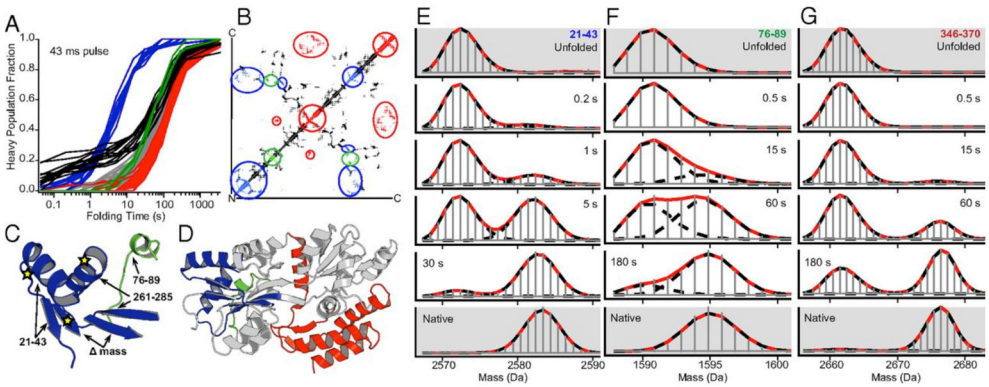

Figure 2.

Tracking the sequence of protein folding by pulse labeled HDX-MS. Maltose binding protein was denatured, fully deuterated, and refolding triggered by dilution. At specific times through the refolding process samples were rapidly labeled with H_2O to exchange deuterium at all accessible (unfolded) amides. By clustering the kinetics of the various regions it becomes clear that the secondary structural elements in blue form an obligate intermediate in the folding pathway, and must be present before the other regions (green, red and black) are able to fold **(A, B).** Individual peptides with population shifts that change with folding time reveal the local kinetics of folding throughout the protein **(C, D)**. Example spectra are shown in **E–G**. Figure modified from reference (Walters, PNAS 2013) with permission.

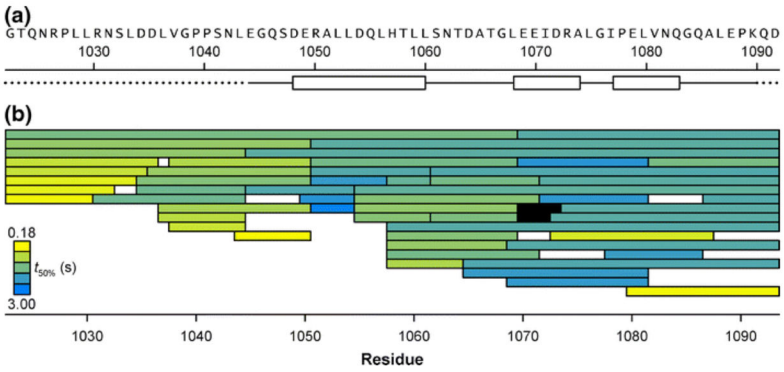

Figure 3.

a) Residual secondary structure in ACTR. **b)** HDX-MS was used map residual secondary structure in intrinsically disordered domains. The set of peptide from ATRK were analyzed for subtle protection by assessing their exchange half-life ($t_{50\%}$). Comparison of the kinetics on all observable peptides can qualitatively localize the positions of residual secondary structure within intrinsically disordered domains. Figure modified from reference (Keppel JASMS 2015) with permission.