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Review

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Applications of Hydrogen/Deuterium Exchange MS from 2012 to 2014

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Hydrogen/deuterium exchange (HDX) detected by mass spectrometry (MS) is extraordinarily useful in the study of many aspects of proteins, especially the analysis of protein conformation and dynamics. While once a challenging and therefore sparingly used method, modern HDX MS is more straightforward, rapid, and routine than in the past. As a result, the breadth of applications of the method has expanded. This Review catalogs applications of HDX MS that have appeared in the literature during the 30 months from January 2012 to June 2014. As penetration of the method into nonacademic sectors where confidentiality is necessary is also at an all-time high, many more applications of this method likely exist that have not been reported in the literature.

A synopsis of the recent applications of HDX MS is shown in Figure 1 where classifications have been made in terms of sector in which the work was performed, geography, and general topic. We elected to categorize the publications in these ways to emphasize that the method is used not only in all sectors but also on nearly every continent. Many different possibilities existed for characterizing the applications by topic, and this was not a perfect task. Some papers belong in multiple topics or could arguably be placed in different categories than we finally decided. An Excel database and an Endnote library of the 234 articles we surveyed, both of which contain the topic groupings, are available from the authors.

Academia is the largest sector contributing to published HDX MS applications (Figure 1). Approximately 25% of the papers surveyed included multiple sectors, which we have classified as mixed. As mentioned above, such categorization is biased against work that has not been published. Industrial research and research from governmental laboratories must/may remain confidential. Therefore, the results in Figure 1A are to be interpreted with this in mind. The United States was the primary source of publications in HDX MS during the January 2012 to June 2014 time period (Figure 1B,C), although significant and important work originated from 22 other countries (Figure 1D). To illustrate the HDX MS applications in much greater detail, we have divided the remainder of the article into six sections that each discuss one of the topical classifications shown in Figure 1E.

PROTEIN FOLDING

The study of protein folding is a small (14% of papers surveyed) but nonetheless an important application of HDX MS. The creation of structure during folding slows exchange, particularly in positions that become hydrogen bonded or solvent protected. Monitoring changes in deuteration during folding can reveal not only what parts fold and when but also what factors may affect folding and various folded states. In addition to HDX MS studies of individual proteins folding and unfolding, HDX MS can also be used to understand how other proteins participate in folding or maintaining folded states.

Folding Mechanisms. Work by Tsutsui et al.[1] provides a classic example of the utility of HDX MS for studying protein folding (Figure 2A). The protein α_1-antitrypsin was placed in denaturant; the denaturant was diluted, and the refolding was allowed to proceed for various amounts of time. A short pulse of deuterium (10 s) labeled parts of the protein that were not yet folded at each folding time and the results were summarized in light of the known crystal structure of the protein. HDX MS can access rates, energies, and pathways of protein folding in vitro, as described for a number of other systems including ubiquitin, staphylococcal nuclease, ribonuclease H, and maltose binding protein.[2−4] Folding and/or unfolding as a result of pressure,[5] chromatography,[6] carboxyl-group modification,[7] mutation,[8] or binding[9−11] were also explored in recent publications.

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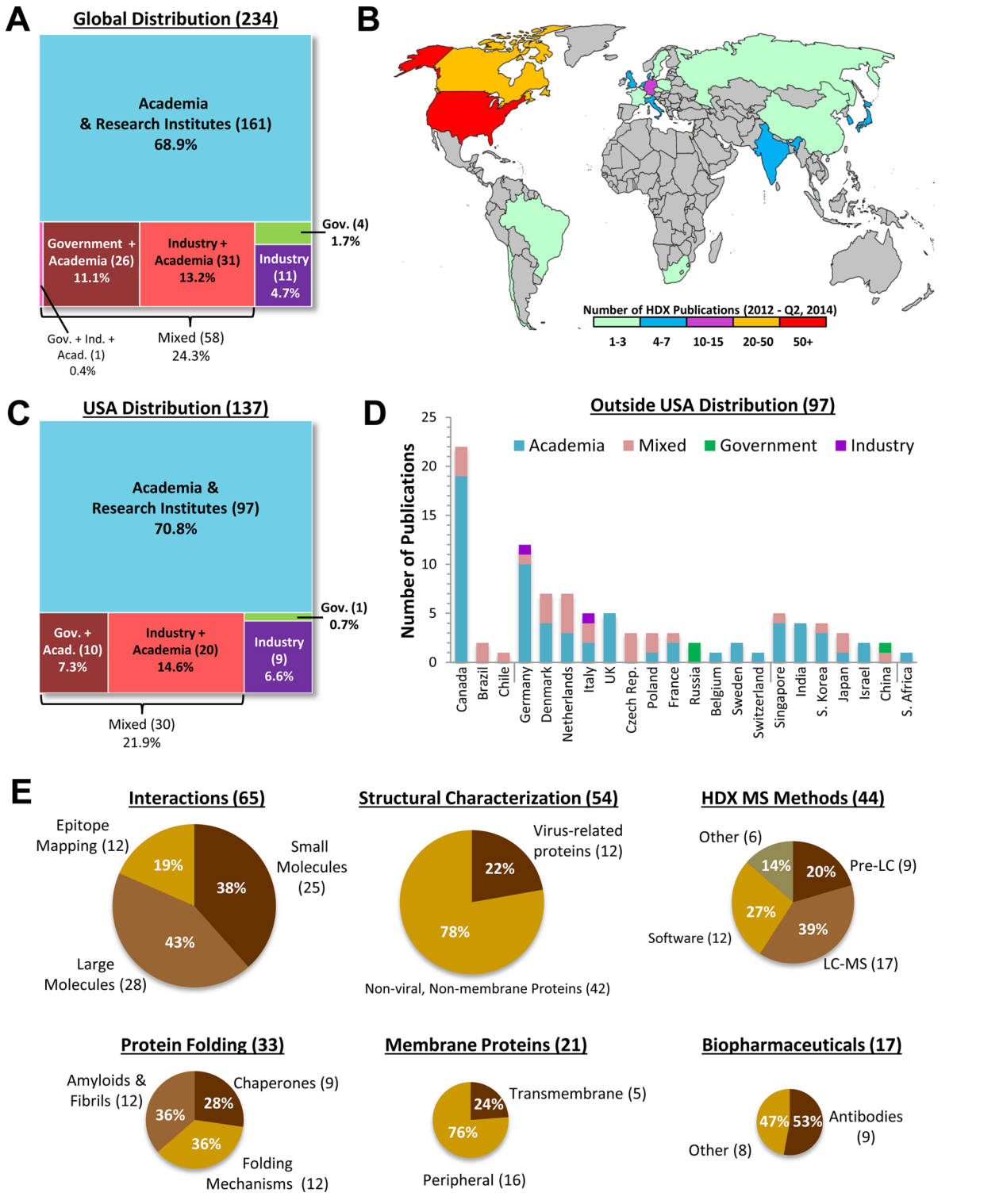

Figure 1. Synopsis of the published applications of HDX MS from January 2012 to June 2014. (A) A total of two-hundred thirty-four (234) articles were published and classified according to the scheme shown, based on author affiliations. The number of articles in each category is shown in parentheses; review articles were not included. (B) The global distribution of the articles in panel A, based on the home institution of the communicating author of each article. (C) Similar to panel A, but for the largest source of articles, the United States of America. (D) Breakdown of the non-USA publications according to country and to sector. The data are divided roughly by continent going from left to right. (E) The six topical classifications chosen for the Review are shown as pie graphs, with the size of each pie equivalent to the number of articles (shown in parentheses) in each topic. Further subdivision within topics follows the order of the main text of the article.

Amyloids and Fibrils. Failures in protein folding or other disruptions in protein structure can lead to aggregation and/or fibril formation. HDX MS can probe what parts of proteins associate in fibrils, what regions participate in aggregation, and how various conditions affect conversion of soluble proteins into insoluble forms. β_2-Microglobulin (β_2m) forms amyloid fibrils in dialysis-related amyloidosis when degradation in the kidney is compromised. The intrinsic stability of β_2m was studied by HDX

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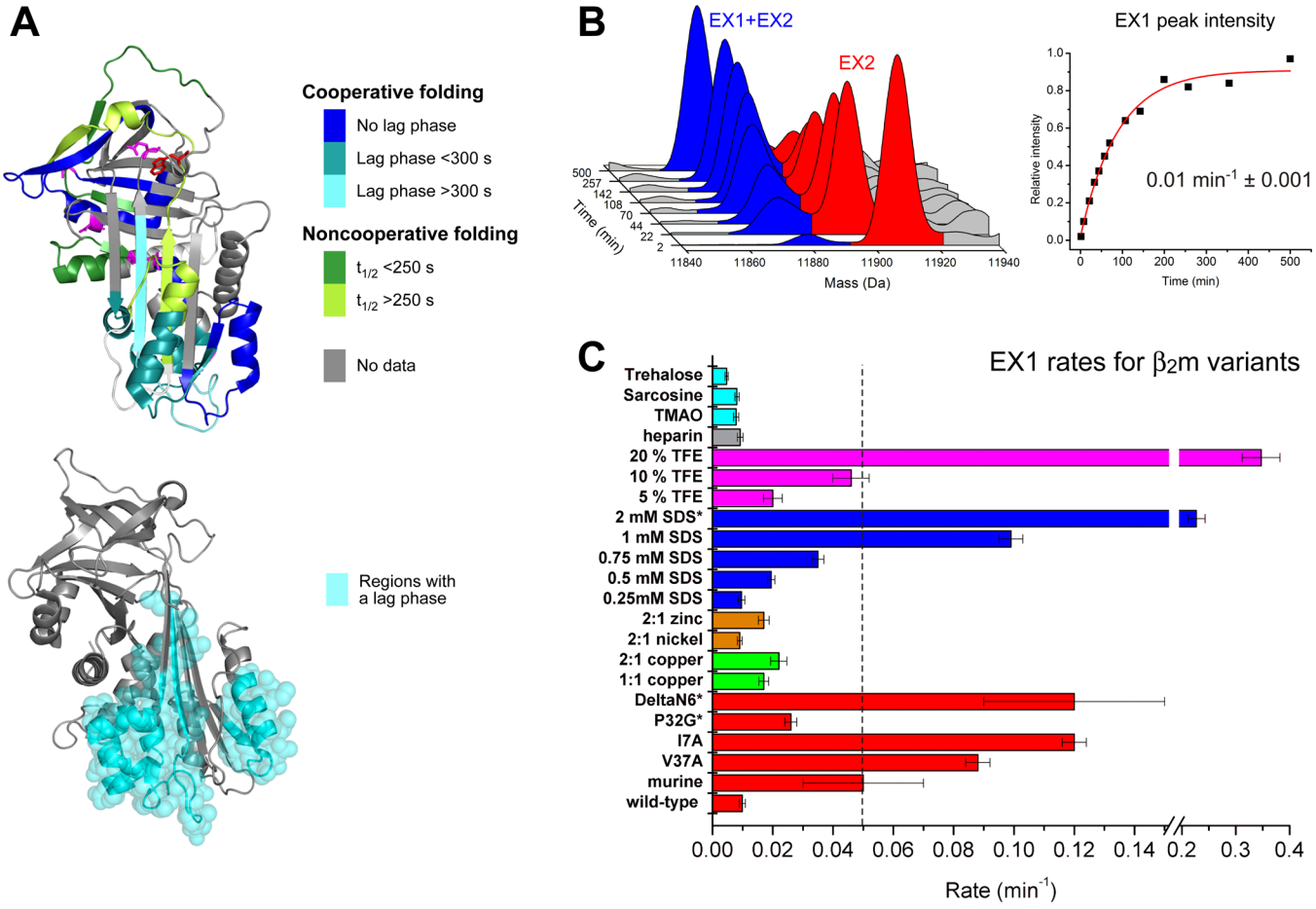

Figure 2. Application to the study of protein folding and unfolding. (A) Example of determining the folding order of a protein with HDX MS, here the order of α1-antitrypsin. Modified with permission from ref 1. Copyright 2012 National Academy of Sciences of the United States of America. (B) Example of monitoring protein unfolding via conformational dynamics. Totally deuterated β2-microglobin (β2m) was incubated in H_2O buffer and intact mass spectra acquired at the times shown. Exchange through EX1+EX2 kinetics (blue peaks), representing a very unfolded unprotected form, was observed from which a rate of EX1 unfolding could be extracted (right graph). (C) The EX1 rate of β2m unfolding was measured for a host of other conditions, including mutation and solution additives. Panels B and C modified and used with permission from ref 12. Copyright 2012 John Wiley and Sons.

MS,[12] and it was found that the protein visited a highly unprotected, globally unfolded conformational state (Figure 2B). Various conditions were tested, and the rate of unfolding was remeasured in each set of circumstances (Figure 2C) with the goal of determining if global unfolding dictated β_2m aggregation and fibril formation. While the dynamics of β_2m were easily measured by HDX MS, there was poor correlation between the rate of EX1 unfolding and fibril formation, interpreted as meaning that other fibril nucleation mechanisms besides global unfolding are at play. Fibril formation was studied for other proteins/peptides known to aggregate including amyloid beta (Aβ) peptide,[13,14] α-synuclein,[15] prions,[16−19] tau,[20] insulin,[21] and the prostatic acidic phosphatase fragment of 39 residues (PAPf39).[22] The regions and rates of protection during fibril formation were interrogated in each case.

Chaperones. Cells are preprogrammed to deal with many types of protein folding and misfolding problems through the use of molecular chaperones. HDX MS is highly useful for studying not only how protein folding is altered by chaperones but also how molecular chaperones function. Many of the HDX MS reports on chaperones included in our analysis were aimed at understanding how the chaperones themselves function, e.g., in response to binding or ATP. A number of elegant studies were reported, including those on small heat shock proteins,[23] Hsp90,[24−26] the Hsp90 cochaperone Sti1,[27] Hsp70,[26] Hsp104/ClpB,[28,29] and GroEL/ES.[30] Following the actual folding of a protein substrate in the presence of a chaperone is a much more technically challenging HDX MS experiment. A study of the effects of GroEL/ES on a TIM-barrel substrate was reported by the laboratory of Hartl and colleagues.[31] The largely 2-state, cooperative folding of the substrate in the absence of GroEL/ES (Figure 3A) was changed by the chaperonin to be more than 30-fold faster and stepwise (Figure 3B). The spatial resolution from HDX MS allowed delineation of what regions of the substrate protein folded at what rates. Comparison of folding for a related substrate from a species with no GroEL/ES demonstrated that the pathway GroEL/ES enforced is correlated to folding in conditions of chaperonin independence. More HDX MS studies of protein folding and its machinery are certain to come in the future.

■ PROTEIN STRUCTURAL CHARACTERIZATION

Understanding the structure of a protein is an important contributor in understanding its function. HDX MS has an important role to play in this endeavor, as illustrated by this section with nearly 23% of the articles published during the 30

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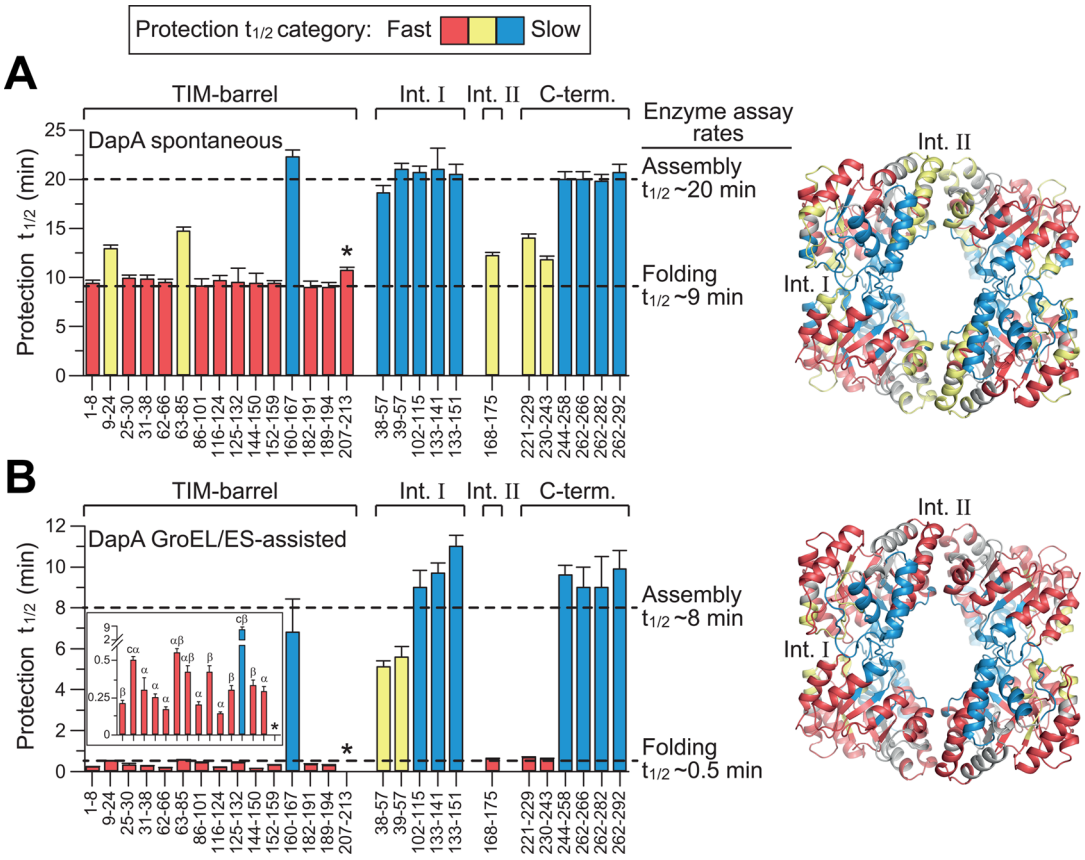

Figure 3. HDX MS in the study of chaperone-assisted protein folding. The TIM-barrel protein DapA was unfolded with denaturant and, upon dilution of the denaturant, allowed to fold and assemble into its native tetramer spontaneously (A) or in the presence of GroEL/ES (B). After various periods of refolding, pulse labeling, pepsin digestion, and mass analysis were performed. The protection half-times (colored by the categories shown: red, yellow, blue) for segments of the protein (left) were greatly accelerated by the chaperones compared to the spontaneous folding. The chaperones also changed the order of folding. The locations of each protection category are shown at the right on the crystal structure of the assembled tetramer. Reprinted with permission from ref 31. Copyright 2014 Elsevier.

month period in question. Most experiments of this type compare at least two conformational forms and look for differences between the forms, interpreting the data on the crystal structure of one (or both) of the forms. The perturbations that may create multiple forms (not including interactions, discussed in the next section) include things such as outside forces (e.g., light, temperature, pressure), mutation, post-translational modifications (e.g., phosphorylation), multimerization, and intramolecular interactions. Experiments involving HDX MS data combined with other structural information from X-ray crystallography, NMR, cryo-EM, small-angle X-ray scattering (SAXS), and molecular dynamics simulations can draw the most informed conclusions about the structure of the protein(s). To explore the many examples of how HDX MS can be used for structural characterization, this section has been divided into two broad categories of proteins that are related to viruses and those that are not. Structural characterization is also found in articles that were classified in other sections because they contain multiple topics or because structural characterization was performed during protein interaction(s), performed on biopharmaceuticals, or performed on membrane proteins.

Nonviral, Nonmembrane Proteins. Protein structural characterization has been an increased focus of HDX MS in the past few years. An excellent example of HDX MS analysis of protein conformation was provided by Lee et al.[32] wherein they describe the conformational changes of the LOV (light–oxygen–voltage) domain protein VIVID (VVD) in the absence and presence of the light. VVD adopts a more protected conformation in the light (Figure 4). The mass spectra (Figure 4A) of an intact protein continuous labeling experiment of the dark state (VVD_D) and the light state (VVD_L) revealed very different time scales of unfolding between the two states. Comparisons of deuteration at the peptide level (Figure 4B) showed the regions that incorporated more deuterium upon dimer formation in the dark state (Figure 4C). Light-induced conformational changes are only one variable that could be studied: reports of HDX MS analysis of the effects of temperature, wherein a study of the effects of cold on structure were examined[33] or the effects of being in the solid state[34] also appeared.

HDX MS is most informative when used in conjunction with information generated by other methods such as functional data, binding information, modeling, activity assays, or other structural tools. The combination of HDX MS with structural analysis using other tools is incredibly valuable, as shown by a number of recent examples. Noble et al.[35] studied COPII proteins, which participate in transporting proteins from the ER to the Golgi apparatus, with cryo-EM, HDX MS, and modeling. Residues that mediate the COPII cage assembly were identified: HDX data identified four unique contact regions that interface two proteins Sec13 and Sec31, each of which is involved in the assembly and flexibility of the COPII cage. One of the peptides in the hinge

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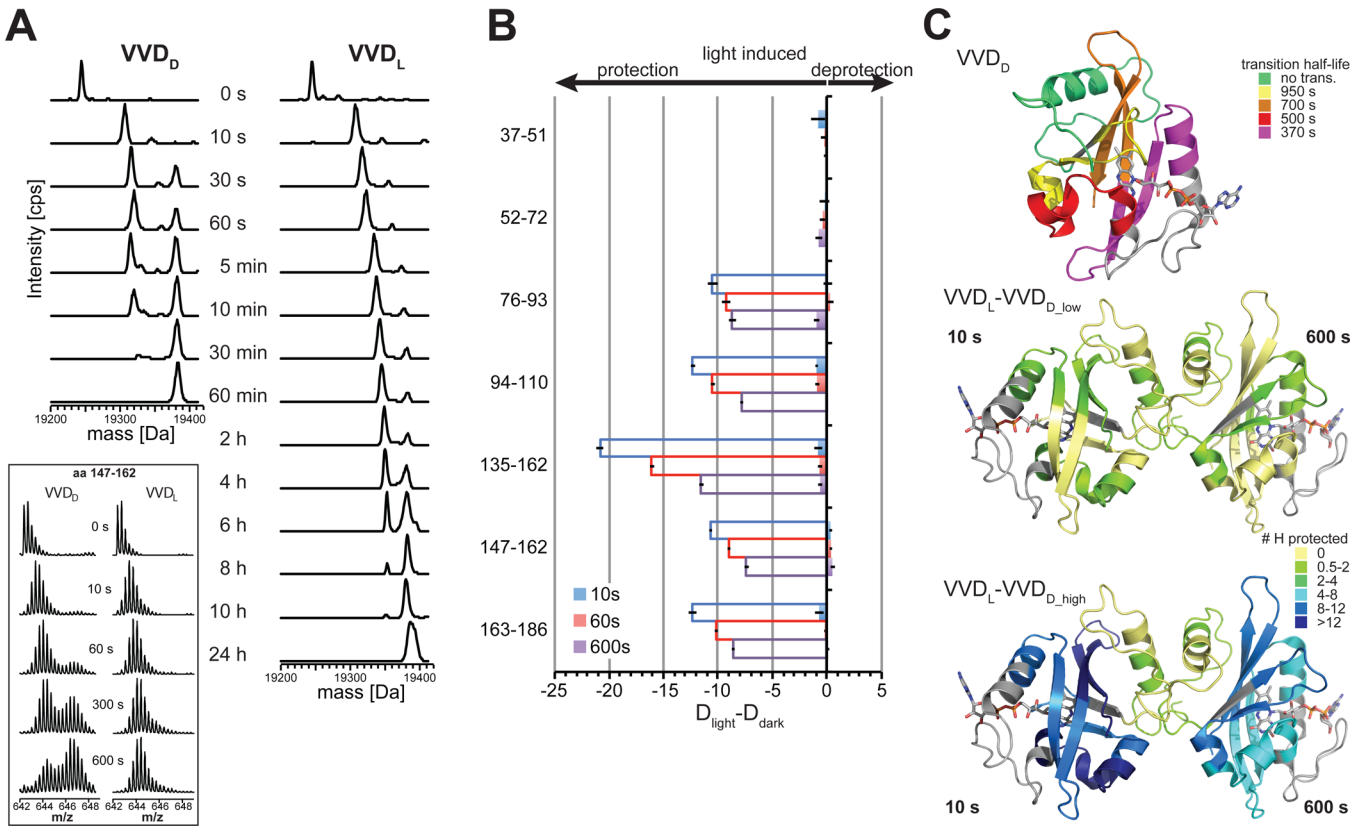

Figure 4. Observing structural changes during function. It was proposed that the LOV (light−oxygen−voltage) domain protein VIVID (VVD) underwent a structural change upon illumination and HDX MS was used to show that this was the case. (A) Continuous labeling of the dark state (VVDD) and light state (VVDL) revealed unfolding with EX1 kinetics and very different time scales of unfolding for the two states. The location of the unfolding was determined with analysis of pepsin fragments (example spectra shown in panel A inset). (B) Comparison of deuterium levels in peptides and (C) interpretation of the data on the structure of the protein and its dimer indicated that all structural elements of VVDD incorporate more deuterium in nearly all regions except for the N-cap. See ref 32 for full details. Modified with permission from ref 32. Copyright 2013 Elsevier.

region was found to exhibit bimodal isotope distributions. It was shown that cage assembly leads to a large conformational change at the interface between Sec13 and Sec31 and that Sec13 plays an important part in rigidifying the hinge by providing structural integrity. In another study,[36] a combination of cross-linking and HDX MS was used to map the homodimer interface of the human 14−3−3 protein. Willander et al.[37] used a crystal structure, molecular dynamics, and HDX MS on the BRICHOS domain from lung surfactant protein C to study the conformation of the wild-type and a BRICHOS mutant D105N. The dynamics of Family 1 glycoside transferase were studied by HDX MS in combination with molecular dynamics simulations, and the data revealed which parts of the enzyme become more solvent exposed despite the fact that most regions are buried in the crystal structure.[38] Using HDX MS, X-ray structures, and SAXS modeling, detailed studies of heterodimers of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR) were completed.[39] Solution NMR and HDX MS were combined to study the Cyclin-depdent kinase 2-associated protein 1 (CDK2AP1) and showed a region of intrinsic disorder and a 4-helix bundle.[40]

HDX MS has seen multiple uses for analysis of multidomain proteins, quaternary interactions, and evolutionary relationships in proteins. For multidomain proteins, two studies we wish to highlight exemplify the possibilities. In the first study, Tsukamoto et al.[41] used HDX MS of the four domain protein PDZK1, a regulator of an HDL receptor, to show that the PDZ4 domain does not exert its regulatory influence by modulating the conformation of the other PDZ domains. In the second study,[42] a detailed analysis of the interactions that bridge the nitric oxide receptor and the catalytic domains of soluble guanylate cyclase was made to reveal the interdomain interactions that communicate nitric oxide occupancy from one region of the complex to the active site. Several other studies of interest include analysis of a two-domain protein with HDX MS to characterize the domain interface[43] and analysis of ATP-induced dimerization.[44]

The subdomains within a large prodomain protein, pro-enkephalin, were probed.[45] Oligomerization of the RAGE receptor, which has been difficult to investigate with crystallography, was studied with HDX MS, molecular modeling, and molecular dynamics.[46] The conformation of fibrinogen was probed with HDX MS to gain insights into regions that were not resolved in the crystal structure, such as the αC region which belongs to the Aα chain.[47] In addition, the fibrinogen variant Bβ 235 Pro/Leu, which is responsible for abnormal fibrin structure, was investigated by HDX MS. This variant showed enhanced deuteration in one region of the protein, immediately preceding the Bβ 235 mutation.

There were several reports of the HDX MS analysis of orthologs, proteins with the same function but from different species. Fang et al.[48] studied the conformation of sliding clamps which function as DNA polymerase processivity factors. HDX MS data for a series of clamps from eight different species indicated that, despite their highly similar three-dimensional

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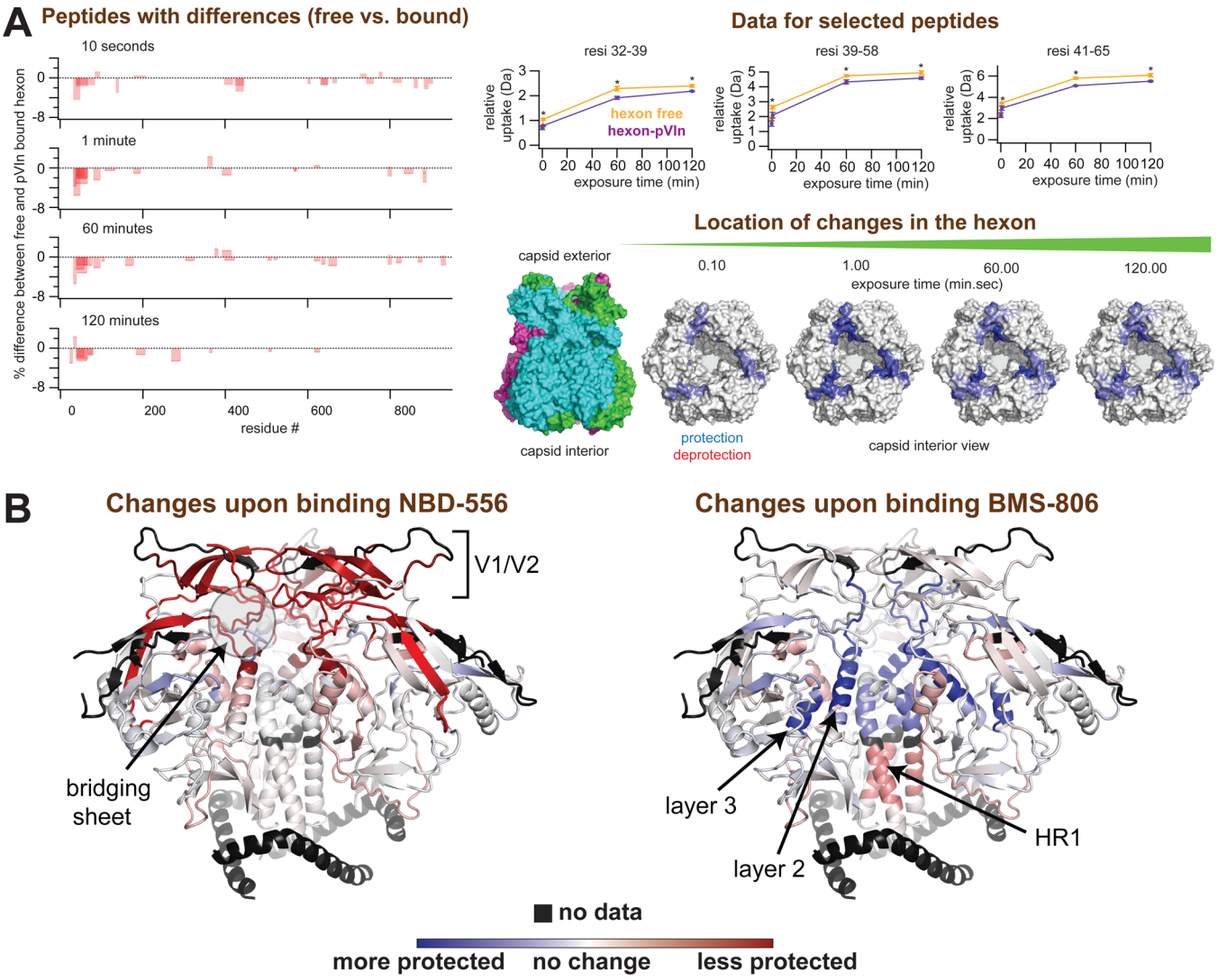

Figure 5. Application of HDX MS to the study of viruses and virus-related proteins. (A) Analysis of the binding to peripentonal hexons of the 33-residue N-terminal fragment of the precursor VI protein (pVIn) from human adenovirus. Exchange into purified hexons was compared with and without pVIn and the affected regions were localized. Modified with permission from ref 72. Copyright 2014 Elsevier. (B) Binding-induced changes in HIV Env trimers were determined when complexed with two different small molecule HIV entry inhibitors (NBD-556 or BMS-806) that block the CD4 binding site. Changes in exchange were compared to those seen when Env trimmers were bound to CD4 alone (not shown here). Modified with permission from ref 81. Copyright 2014 Elsevier.

structures, clamp proteins show a large range of dynamic behavior. A conserved pattern of alternating dynamics was observed in specific regions of the proteins. In a study of the orthologs of the receptor for activated kinase (RACK1) from human, yeast, and plant,[49] rigid and dynamic regions in the orthologs were identified and compared to conclusions based on the crystal structure.

Recent studies of tRNA-synthetases exemplify how HDX MS analysis of the effects of mutation is powerful. Analysis of the Y341A mutant of human tyrosyl-tRNA synthetase, a functional mutant that would not crystallize, with HDX MS and SAXS revealed conformational changes essential for activation via uncovering of an internal tripeptide sequence.[50] In the seryl-tRNA synthetase, HDX MS was used[51] to understand why a mutation (F383V) located ~100 amino acids upstream of the nuclear localization signal (NLS) somehow abolished nuclear localization. The NLS was not resolved in a crystal of the protein, but HDX MS showed that the NLS was protected from exchange in the F383 V mutant versus the wild-type. In other mutagenesis analyses, HDX MS was used to show that mutation E208Q in the SescA translocase, a mutation that alters the active site, changes the global conformational state.[52] Further HDX MS analysis of SecA focused on dimerization and how mutation would disrupt formation of the dimer.[53] Mutagenesis in exchange of a protein activated by cAMP (EPAC) showed that a single mutation can shift the conformational dynamics toward the extended active conformation.[54]

Changes to protein conformation as a result of post-translational modifications are quite tractable by HDX MS, including analysis of the effects of S-nitrosation,[55] oxidation,[56] addition of glutathione,[57] modification to metal atoms such as in aquomet-hemoglobin,[58] or binding to metal atoms (e.g., Mg^{2+}, Mn^{2+}, or Ca^{2+}).[59] Phosphorylation, one of the more recognizable post-translational modifications, is catalyzed by kinases while the reverse reaction is carried out by phosphatases. The effects of phosphorylation/dephosphorylation were studied in many proteins by HDX MS. Examples included interrogation of structural changes as a result of mutations that mimic

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phosphorylation (phosphomimetics S770D/S771D in the plasma membrane pH regulatory protein NHE1),[60] the effects of serine phosphorylation in phenylalanine hydroxylase,[61] mutation that alters the activity of the phosphatase SHP2,[62] and conformational changes in an enzyme due to dephosphorylation.[63] In addition, a number of studies that included HDX MS was performed on the enzymes that carry out phosphorylation, the kinases.

Knowledge of kinase structure and kinase regulation is important because kinases are tied closely to diseases such as cancer through signaling pathways. Therefore, many groups have studied kinases in an effort to understand how their conformation might affect their function. Both receptor and nonreceptor kinases have been studied by HDX MS. Itk and Btk are nonreceptor tyrosine kinases that belong to the Tec family kinases. HDX MS analysis of Itk and Btk[64] showed that these two kinases have different dynamic behavior and that the activation loop of Btk is more solvent accessible than that of Itk thereby affecting activity. Panjarian et al.[65] studied the Abl kinase in the context of SH3-linker mutations and showed that the there is a remarkable allosteric network linking the SH3 domain, the myristic acid binding pocket, and the active site of c-Abl core. Activation of ERK2 occurs by enhancing hinge flexibility, shown by HDX MS analysis revealing that hinge mutations increase the flexibility and induce changes in the nucleotide binding mode.[66] For the receptor-tyrosine kinases category, the kinase domain of epidermal growth factor receptor (EGFR) was recently studied by HDX.[67] Wild-type EGFR kinase was compared to an L834R mutation that causes abnormally high activity by promoting EGFR dimerization. The HDX data indicated that the monomeric EGFR kinase has higher HDX rates and is dynamic especially in the C-terminal portion of the αC helix. In the L834R mutant, HDX was not significantly altered showing that the disordered state remains predominant. West et al.[68] studied Snf1-related kinases (SnRKs) and regulation via abscisic acid (ABA), a plant hormone important for controlling growth and stress responses. The structural mechanism of proteins in the ABA signaling pathway (the ABA receptor PYL2, HAB1 phosphatase, and two kinases, SnRK2.3 and 2.6) were investigated with HDX MS.

Among other methods, HDX MS has proven very effective in probing allostery. An allosteric effect may result from any type of ligand binding, post-translational modifications, or other conformational changes. Some of the articles already discussed above, and others not in this section but covered below, include an allosteric component in addition to their other findings. In a recent book chapter,[69] Beckett describes the application of HDX to investigate an allosteric system focusing on the *E. coli* biotin repressor/BirA. The effects of phosphorylation and mutagenesis on the RegA response regulator were studied,[70] and an allosteric coupling between the site of phosphorylation and the activating mutation was described. Winkler et al.[71] studied the elements involved in allosteric regulation of phosphodiesterase activity by comparing different states of the blue-light-regulated phosphodiesterase 1.

Virus-Related Proteins. The structural characterization of viruses is an ongoing topic of interest for the HDX MS community, with 12 papers appearing during January 2012 through June 2014. HDX MS can be used to understand whole viruses, assembled capsids, or just a single protein encoded by a viral genome. In an excellent example of the analysis of viral capsids, Snijder et al.[72] characterized the N-terminal fragment of human adenovirus pVI protein (termed pVIn). As shown in Figure 5A, when pVIn was incubated with purified hexons, there was protection from the exchange in several regions suggesting how and where pVIn associated. In another study,[73] the procapsid (prohead-1) of bacteriophage HK97 was analyzed by cryo-EM and HDX MS to understand how the protease cleavage events of maturation occur. Given the recent outbreaks of Ebola in Africa, two very timely papers described the role of HDX MS in studying Ebola virus (a negative-strand RNA virus). The conformation of the Ebola virus matrix protein VP40 and the role of the N- and C-terminal tails in assembly were investigated.[74] Earlier HDX MS results localizing conformational differences between the only protein on the surface of the Ebola virus, the membrane-attached glycoprotein $GP_{1,2}$ protein, and $GP_{1,2}$ from the related Sudan virus Gulu variant (SUDV-Gul) were discussed in light of structural data for complexes of $GR_{1,2}$ with neutralizing antibodies.[75] HDX MS was applied to another negative-strand RNA virus, Toscana virus which causes pediatric meningitis, and revealed which regions of the hexameric form of nucleoprotein N were dynamic.[76] Hepatitis viruses were studied with HDX MS: hepatitis B capsid protein conformation was probed as were complexes with antibodies;[77,78] a crystal structure of the hepatitis C envelope glycoprotein 2 was obtained with the aid of HDX MS identification of disordered regions.[79]

There were many HDX MS analyses of HIV proteins, including the very detailed analyses of the envelope glycoprotein (Env, various portions of the entire protein, gp 160, are called gp41 or gp120) by the Lee laboratory at the University of Washington[80−83] as well as analysis of HIV viral accessory factors such as Nef.[84] One example of the analysis of the Env protein is shown in Figure 5B where, in the presence of HIV entry inhibitors (NBD-556 or BMS-806), exchange into trimeric gp120 was altered in the variable regions V1 and V2 for NBD-556 but in quite different regions for BMS-806.[81] Interactions between gp41 and gp120 were observed for monomers but not for trimers,[82] and key differences in conformational stability between the gp120 proteins from different viral isolates were revealed.[80]

INTERACTIONS

The activity and function of proteins is often regulated via binding to other molecules; understanding these events, therefore, can give critical insight into protein function and disease related states. In this section, we will focus on how HDX MS has been utilized (28% of the articles published during the 30 month period in question) to study protein interactions. We note that protein interaction studies are also present in articles that have fallen into other categories, again because classification is inevitably an imperfect process when articles cover multiple topics. The applications we will highlight in this section include antigen/antibody interactions (i.e., epitope mapping) and will include work on vaccine design, clotting factors, and allergens. Studies involving proteins in complex with large molecules, which we define as greater than molecular weight of 800 Da, will be discussed and include examples involving the effects of binding to protein subdomains and nucleic acids. Lastly, protein interactions with small molecules, defined here as smaller than molecular weight of 800 Da, will be reviewed and will cover topics involving nucleotide binding proteins and receptor/drug binding as well as the effects of anticancer drugs on protein targets.

Epitope Mapping. Epitope mapping is a crucial step in designing therapeutic antibodies and vaccines. HDX MS has been a useful, some would argue indispensable, technique for this

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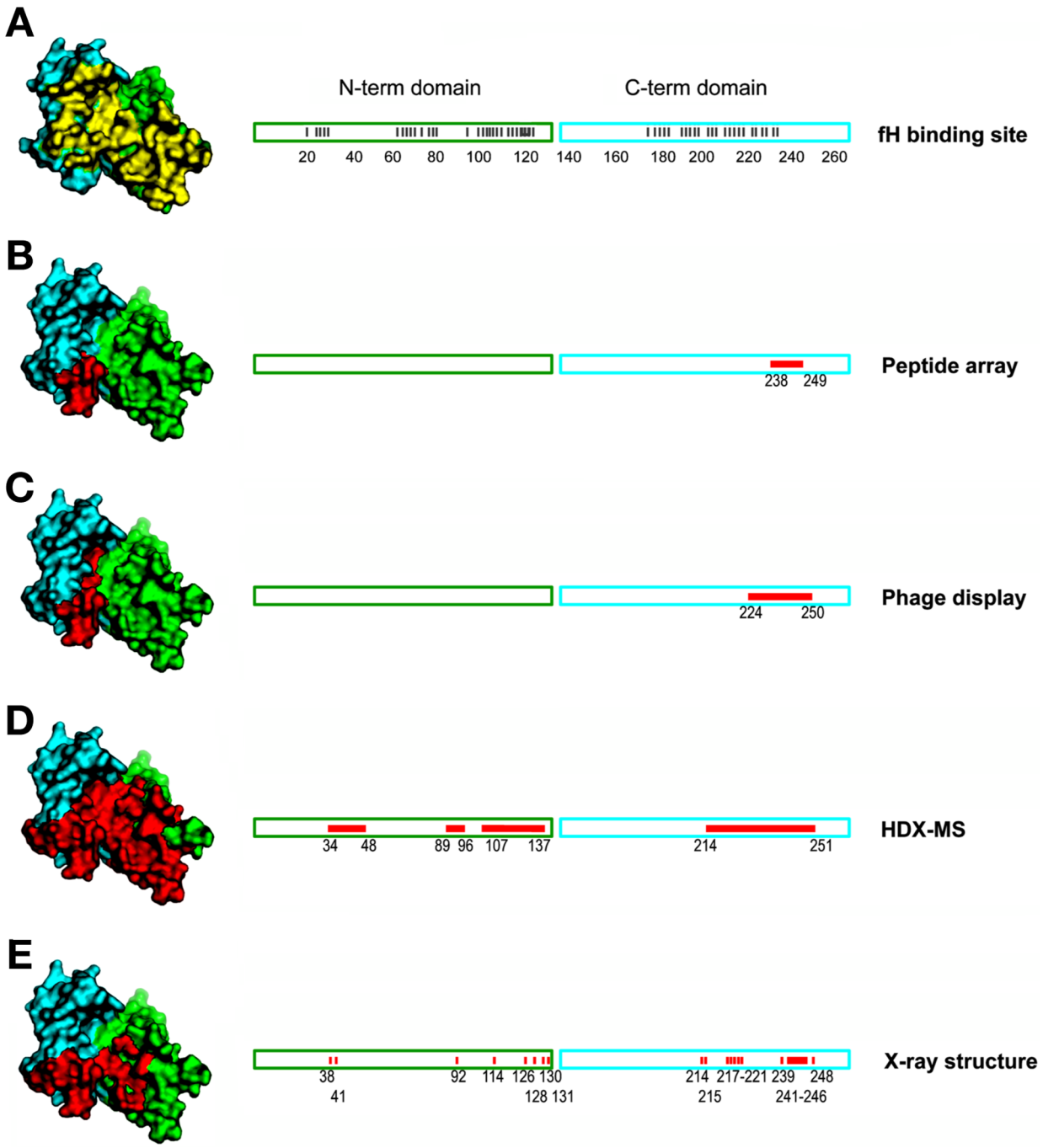

Figure 6. HDX MS for epitope mapping of a monoclonal antibody (mAb) against factor H binding protein (fHbp), a virulence factor, and vaccine antigen of the causative agent of bacterial meningitis, *Neisseria meningitidis*. Comparisons were made against (A) the known interface between factor H (fH) and fHbp, interface residues colored yellow in the structure at the left, and (B–E) epitope mapping data, colored red on the structure and linear representation, for mAb 12C1 and fHbp by various methods. HDX MS mapping (panel D) identified all regions but not with the resolution of the cocrystal structure. Reprinted with permission from ref 86. Copyright 2013 National Academy of Sciences of the United States of America.

purpose.[85] Malito et al.[86] probed the binding between a monoclonal antibody against factor H binding protein (fHbp), the vaccine antigen of *Neisseira meningitidis*. The epitope was probed with peptide arrays, phage display, X-ray crystallography, and HDX-MS; the agreement of the methods is shown in Figure 6. As the authors point out, HDX MS was *"the most effective method to rapidly supply near-complete information about epitope structure"*. Some other methods for epitope mapping, while suitable for linear epitopes, struggle with providing full pictures of interactions that cover discontinuous conformational epitopes,[87] a deficiency apparent in the missing regions in panels B and C of Figure 6. A second study[88] by the same group analyzed two broadly cross reactive antibodies to fHbp with HDX MS to very rapidly characterize the complex, identify the epitopes, and compare the antibodies. These studies highlight how HDX MS is becoming an essential methodology for epitope characterization. While crystal structures still remain the gold-standard for complex characterization, HDX MS provides a rapid means, either in the absence of or in conjunction with an X-ray

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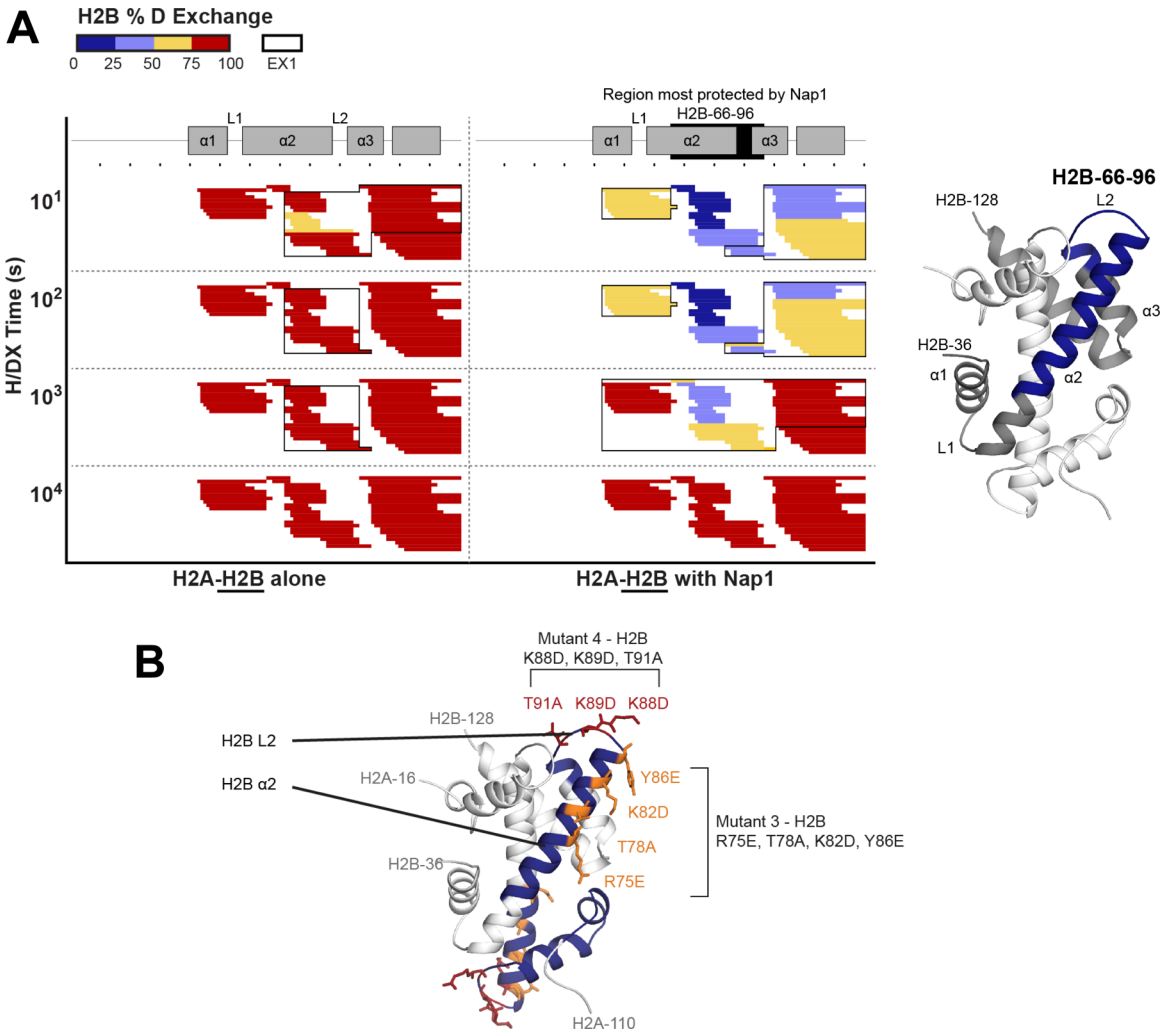

Figure 7. Example of mapping protein/protein interactions by HDX MS. (A) The effects of binding the nucleosome assembly protein 1 (Nap1) to the histone H2A−H2B heterodimer were shown by comparing exchange into the H2B portion of H2A−H2B alone (left) to exchange into H2B when bound to Nap1 (middle). The regions most affected by binding were mapped to the crystal structure of H2B (colored blue, right panel). (B) Analysis by HDX MS suggested regions where mutations might be made and tested in other assays; mutants 3 and 4 (residues changed indicated in yellow and red, respectively) later showed reduced binding to Nap1 in FRET assays. Modified with permission from ref 97. Copyright 2013 Elsevier.

structure, to focus attention on regions that are involved in interactions and inform other experiments that test such regions.

HDX MS can be used not only to characterize therapeutic antibodies destined to combat diseases but also to study antibodies that appear as the result of intervention with other therapeutic proteins. As an example, consider hemophilia A, a disorder characterized by the functional absence of a critical blood clotting protein, factor VIII (FVIII). Treatment with recombinant FVIII is hampered by the patient's immune system whereupon intravenous infusion of recombinant FVIII causes many patients to develop antibodies against the clotting factor. HDX MS was used to characterize antibody binding to FVIII. Bloem et al.[89] used HDX to identify epitopes in two C1 domain spikes on FVIII when in complex with a human mAb, KM33, while Sevy et al.[90] identified epitopes of four anti-FVIII mAbs using HDX MS. Other interesting examples of HDX MS in epitope mapping studies include: analysis of allergens from almond nuts (pru du 6)[91] or cashew nuts (ana o 2);[92] studies of innate immunity where binding to IgG to ficolin complexes were characterized by HDX MS;[93] generation of an algorithm[94] for predicting B cell epitopes using as a model system the D8 protein of the vaccinia virus, a major target of the small pox vaccine. The prediction algorithm was validated using a combination of X-ray crystallography, ELISA, site-directed mutagenesis, and HDX MS. Lu et al.[95] generated and characterized 13 mAbs against GroEL found in *Francisella tularensis* (FtGroEL) and used HDX to map the target epitopes and compare the antibodies. The HDX data suggested that the protective effects of the mAbs are due to stabilization of a structural rearrangement in FtGroEL. In the final example covered in this section, protein stabilization by antibody binding was described by Tiyanont et al.[96] who monitored protein dynamics during activating- or inactivating-antibody binding to the regulatory region of human Notch3. Notch3 in complex with either EDTA or an activating antibody resulted in increased deuteration, signifying increased dynamics whereas binding of an inhibitory antibody reduced deuteration. This study points out that, although binding of an antibody to an antigen can be strong, the biological effects are not always inhibition of the function of the antigen.

Protein/Large Molecule Interactions. The previous section on epitope mapping covered the special case of protein/protein interactions where one of the partners is an antibody. Many other protein/protein interactions exist where no antibodies are involved. A multitude of proteins realize their

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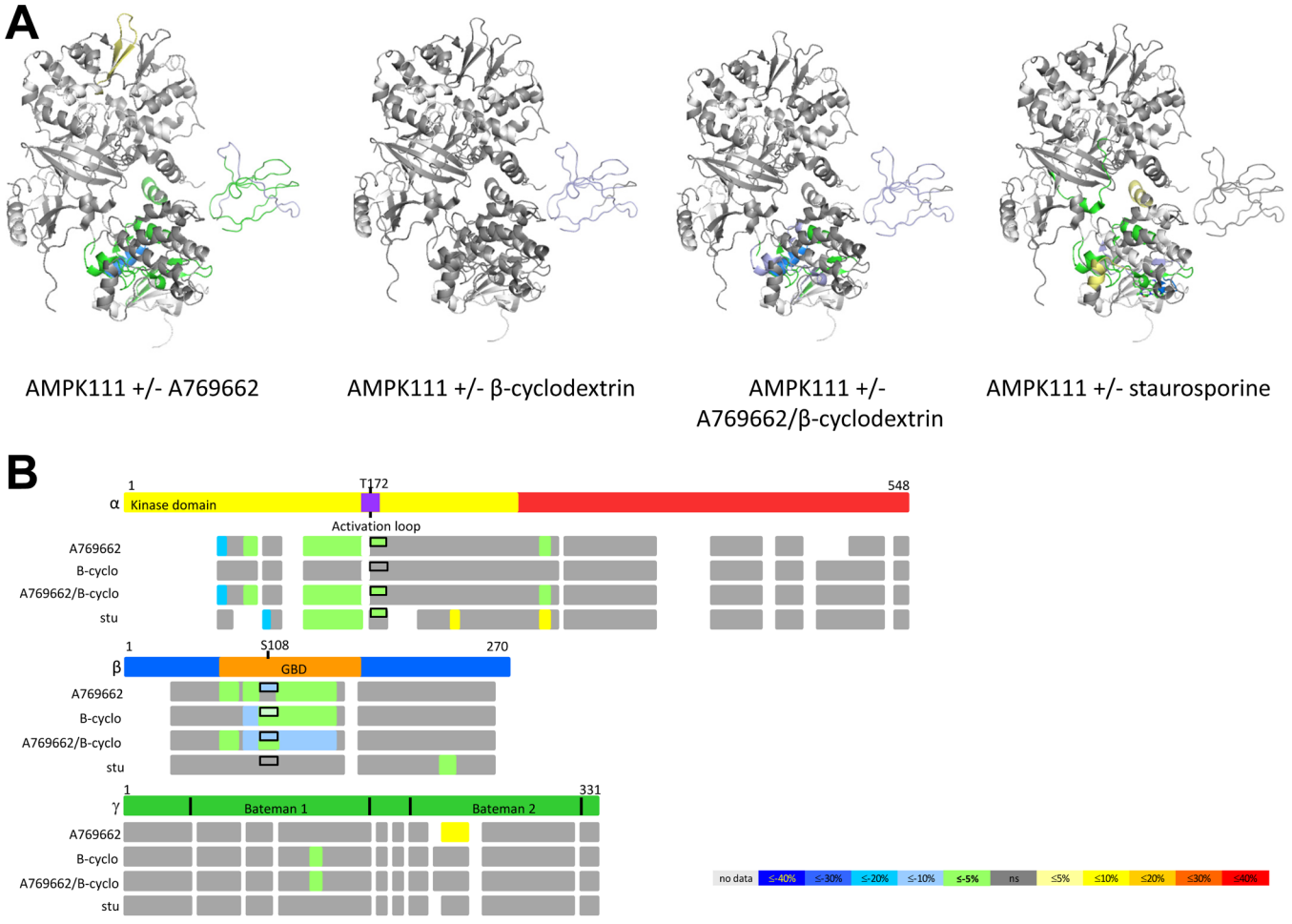

Figure 8. Small molecule interactions with a protein. The AMP-activated protein kinase (AMPK) was incubated with various small molecules (A769662, beta cyclodextrin, and staurosporine), and HDX MS was performed. Changes in deuteration relative to unbound AMPK were both (A) mapped to the crystal structure of AMPK and (B) displayed schematically. The percentages of deuterium differences were mapped according to the key shown. Reprinted with permission from ref 124. Copyright 2013 Elsevier.

functional roles through binding with other large biomolecules, including nucleic acids, peptides, or other proteins. In recent years, HDX MS has been applied to investigate protein/large molecule complexes, and here, we define a large molecule as something with molecular weight in excess of 800 Da, in order to further understand many biological systems. In general, the dissociation constant of the complex is known, and this simplifies the experiment because the mixing ratio of the component proteins can be calculated to maximize the amount of proteins that are bound. In cases where the dissociation constant is not known, 5-, 10-, or 20-fold excess of one protein can be added and the results compared, for example. Complications can arise in these types of HDX MS experiments when the binding constant is weak because signals from peptides of a protein that is not of interest can interfere with the signals of peptides from proteins that are of interest.

A good example that characterizes a protein/large molecule HDX MS is shown in Figure 7. Here, D'Arcy et al.[97] investigated interactions between the histone H2A−H2B heterodimer and the nucleosome assembly protein Nap1. The authors report that the helices in the H2A-H2B sample partially disordered conformations at low ionic strength. Binding of Nap1 reduces these sampling events and competes for histone−DNA and interhistone interactions within the nucleosome. On the basis of the results of HDX MS, the authors proposed other mutants that should block interactions, which were then verified by non-MS methods. Such experiments highlight the importance of combining HDX MS measurements of protein/protein interactions with other types of assays.[98]

There were many examples of how HDX MS can be used to study protein/protein interactions,[84,99−113] ranging in application from analysis of identifying contact surfaces to monitoring structural changes in one or more members of the complex upon binding. Studies were performed on complexes with only two members as well as larger systems with multiple proteins; the proteins studied came from all types of organisms including human, yeast, *E. coli*, cyanobacteria, or other microorganisms. HDX MS data were often combined with other techniques, including methods such as circular dichroism, homology modeling, X-ray crystallography, and NMR.

Several reports focused on protein binding to peptides or small polypeptides including: analysis of how the conformation of the $\alpha\beta$ T cell receptor (TCR) is influenced by binding to peptides presented by the class I major histocompatibility complex (MHC-I);[114,115] a study of the protection of the plasminogen activator inhibitor-I (PAI-I) by binding to the small 39-residue somatomedin B domain of the plasma glycoprotein vitronec-

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tin;[116] and analysis of the effects of various peptide ligands binding to thrombin.[117]

The analysis of protein binding to nucleic acids, or binding between proteins involved with nucleic acids, was described in a number of articles. Choi et al.[118] used HDX MS to study the Notch transcription complex formed between the Notch intracellular domain (NCID), the DNA-binding factor CSL, and Mastermind Family 1 protein (MAML1). The role of each member of cooperative assembly of the complex was ascertained. Winkler et al.[119] studied the interaction between the light-sensing antirepressor AppA and the transcriptional repressor PpsR. The HDX data for the complex showed how light activation of AppA–$PpsR_2$ influences the PpsR effector region, altering how the complex would interact with DNA. A study[120] of the TATA box-binding protein (TBP) and its interactions with domains of the progesterone receptor (PR) revealed conformational changes in both AF1 and AF2 domains of PR upon binding, as well as changes in flexibility within TBP. Binding between the processivity factor proliferating cell nuclear antigen (PCNA) in complex with an inhibitory protein called TIP[121] identified regions involved in binding between TIP and PCNA, and the authors concluded that TIP binding disrupts the PCNA trimer formation and decreases its activity. In a study focusing solely on the effects of DNA binding to a protein, Roberts et al.[122] utilized HDX MS and computational docking to investigate uracil–DNA glycosylase UNG, which is responsible for identifying and cleaving uracil from DNA, as it binds to a 30 bp DNA sequence. They reported that the UNG–DNA complex showed increased protection at the enzyme active site and two areas surrounding the active site. The effects of protein binding to sequences of RNA, in the form of aptamers, were investigated for the serpin plasminogen activator inhibitor 1 (PAI-1).[123] RNA aptamer binding resulted in major reduced deuterium incorporation within PAI-1, and the areas of reduction were mapped.

Protein/Small Molecule Interactions. We have just discussed where HDX MS has been used to characterize protein interactions with large biomolecules. Small molecule (defined here as smaller than a molecular weight of 800 Da) binding to proteins has also been investigated extensively with HDX MS. The small molecules in question are often therapeutic drugs but can also be natural substrates, regulatory molecules, metal ions, or antibiotics. Other possibilities include lipids, glycans, aromatic pollutants, metabolites, and the list goes on. One surprising finding from many HDX MS studies of small molecule binding is how influential these interactions are on proteins. Given the size differences between most small molecules and peptic peptides, even high concentrations of small molecules (i.e., weak dissociation constants) do not generally interfere with the LC/MS steps or lead to suppression of peptide signals of interest.

Publications from Patrick Griffin's laboratory epitomize analyses of protein/small molecule binding by HDX MS. An example is shown in Figure 8 where structural changes in AMP-activated protein kinase (AMPK) were monitored upon binding of nucleotides, cyclodextrin, and a small molecule activator.[124] AMP binding resulted in conformational changes principally in the γ subunit of AMPK with other small changes in the α and β subunits. The synthetic inhibitor exhibited large changes in the β subunit and the kinase domain of the α subunit. Other Griffin publications further demonstrate analyses of this type for the estrogen receptor α (ERα),[125] the vitamin D receptor,[126,127] peroxisome proliferator-activated receptor gamma (PPARγ),[128] and the retinoid X receptor α (RXRα).[129]

The effects of cyclic AMP, a regulatory molecule for a number of proteins, on protein conformation and dynamics were explored in several publications.[130,131] Underbakke et al.[132] showed that, in the GMP binding protein guanylate cyclase, nitric oxide binding produced profound changes in domains responsible for heterodimerization and signal transmission. HDX MS was used to study oxygen binding to myoglobin and hemoglobin,[133] to understand the binding of several aza-sugar molecules to a glycanase,[134] and to probe progesterone and propranolol binding to α1-acid glycoprotein (AGP), a protein in plasma that can bind to a variety of molecules.[135] Hernychova et al.[136] studied the interactions between a small molecule, Nutlin-3, and the protein MDM2 and showed that Nutlin-3 binding caused reduced HDX kinetics in regions surrounding the binding site and the hydrophobic pocket of the protein. Brier et al.[137] investigated the regulatory effects of 4-hydrophenylacetic acid (4-HPA) on the DNA-binding protein neisserial adhesion (NadR). The HDX data show that 4-HPA binds between the two DNA-binding lobes of NadR and stabilizes this state with little conformational perturbation. These data suggest that 4-HPA regulates NadR by stabilizing a conformation unable to bind to DNA. Ghose et al. studied spore photoproduct lyase (SPL), which repairs UV legion spore photoproducts in a *S*-adenosyl-L-methionine (SAM) dependent manner.[138] SPL in complex with SAM and a synthetic nucleotide results in a significant decrease in deuterium incorporation, suggesting stabilization. However, SPL in complex with only the nucleotide produces no change in deuterium exchange, indicating that SAM is a required binding partner.

Many small molecule therapeutics that combat cancer have been discovered. HDX MS has been applied to better characterize the effects of such molecules on the target protein. The effects of Taxol on microtubule associated protein 4 (MAP4) were studied.[139] Yu et al.[140] investigated the antileukemia agents that target the SAM binding site in the methyltransferase DOT1L using a combination of techniques, including HDX MS. It was shown that a base excision repair enzyme (APE1) tied to cancer can be inhibited with a small compound, E3330, and in doing so, HDX is modified in a few key regions.[141] A small molecule compound that suppresses metastasis in several mouse models was shown by HDX MS to alter the conformational properties of lysyl-tRNA synthetase (KRS).[142] Liang et al.[143] investigated the binding effects of inhibitor ML323 on the human deubiquitinase complex USP1-UAF1 using HDX MS and showed four moderately protected and two strongly protected peptides located outside the USP1 catalytic site, supporting the idea that ML323 is an allosteric inhibitor of USP1-UAF1.

Cisplatin is used to treat several types of cancer, and its main chemotherapeutic effects involve cross-linking DNA leading to apoptosis. Side effects of cisplatin include platination of a number of proteins, including cytochrome c. The effects of cisplatin on cytochrome c conformation were recently studied by HDX MS.[144] Other metal-binding events in proteins were investigated by HDX MS including zinc binding to β-2-microglobulin[145] and the related analyses of porphyrin binding to human ferrochelatase.[146]

In the last example of small molecule binding to proteins, HDX MS was utilized to understand the mechanism of a class of antibiotics called acyldepsipeptides (ADEPs). ClpP protease in *E. coli* is a tetradecamer regulated by binding of ATP that opens an axial pore and allows substrates to enter the degradation chamber. HDX MS analysis[147] of the ADEP1 interaction with

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ClpP revealed, among other things, that ADEP1 destabilizes the N-terminal regions of ClpP subunits while stabilizing the equatorial belt, highlighting the allosteric nature of inhibition.

BIOPHARMACEUTICALS

Biopharmaceuticals are proteins that are used as drugs.[148] A very important part of the development of such drugs is characterization of the protein, which occurs at many stages (e.g., research, development, processing, formulation, etc.). There are tremendous challenges to manufacturers during characterization due to the size of proteins, their complexity, and the necessity of analyzing higher-order structure. This section reviews the published work in the period that involved biopharmaceutical characterization by HDX MS. As mentioned near the beginning of this article, it is almost certain that a large body of HDX MS work on biopharmaceuticals goes unpublished; as a result, in this category, we are only discussing a fraction of the applications in this field. We will cover 17 articles in this section, half of which are related to antibodies. Several papers related to biopharmaceuticals have been covered in other sections of the review (e.g., epitope mapping) although we have tried to consolidate known biopharmaceutical products to the following section. Three subcategories addressed here are (1) general antibody characterization with a focus on the effects of post-translational modifications on antibody conformation and dynamics; (2) antibody aggregation; (3) studies that describe the conformation and dynamic behavior of nonantibody biopharmaceuticals.

Antibodies. Monoclonal antibodies primarily of the IgG isotype make up the majority of biopharmaceutical proteins currently employed for the treatment of diseases. Due to their large size and complexity (e.g., disulfide bonding, glycosylation, etc.), their structural characterization has been challenging. HDX MS is playing an important role in understanding the conformation and dynamics of such complex molecules.[149] Work has been done to understand the effects of post-translational modifications, such as oxidation and glycosylation, on antibody structure.[150−152] For example, a recent paper[150] described how a single mutation in the C_H3-Fc domain of an antibody dramatically altered the conformation of the antibody by altering its glycosylation pattern. Antibody−drug conjugates have also been characterized by HDX MS,[153] and the data indicate that, for the IgG in question, no major structural changes occurred in the molecule upon addition of the drug conjugates. A comparison between an originator antibody (Rituximab) and a biosimilar was published,[154] highlighting how HDX MS can be used in comparability analyses of antibodies.

Proteins *in vitro* may be susceptible to aggregation induced by the environment. Biopharmaceuticals undergo many events in their lifetime, from expression and purification to formulation and storage. There can be a tendency to form aggregates which are obviously detrimental to the manufacturing process and most importantly can have a toxic effect on the patients. There are ongoing efforts to catalog, characterize, and ultimately understand aggregation, especially what might trigger it and what parts of the molecule are more prone to aggregation. HDX MS is a valuable method in the quest to understand aggregation, and several articles on aggregation recently appeared.[155−157] Zhang et al.[155] investigated the effects of thermal stress on bevacizumab (Avastin), a monoclonal antibody that targets the vascular endothelial growth factor A (VEGF-A) and is used as an anticancer drug. The HDX MS data indicated that some peptides in the Fab and the C_H2-Fc regions incorporate more deuterium upon aggregation, and others, in the variable region, incorporate less deuterium upon thermally induced aggregation. Manikwar et al.[157] explored how excipients influence aggregation, using HDX MS as the readout. Iacob et al.[156] detailed how HDX MS and other methods in the biopharmaceutical industry can be used in combination to gain a more complete picture of aggregation for three monoclonal antibodies that have a propensity for forming dimers.

Other/Nonantibody Biopharm Proteins. In addition to antibodies, there were other biopharmaceuticals characterized by HDX. In an excellent example of the use of HDX MS for comparability studies, Houde and Berkowitz[158] characterized recombinant coagulation factor IX (rFIX), a fusion protein version in which an Fc homodimer domain was connected C-terminally. Figure 9 shows the difference index plot for rFIX in the absence and presence of Ca^{2+} (calcium binding is required for activation). Conformational changes are apparent upon Ca^{2+} binding but expected due to the influence of calcium on the structure of rFIX. The same conformational changes were observed for the rFIX-Fc fusion protein, indicating that it too responds to activation in the same way as the nonfusion version. Other HDX MS data in the same study also show that, by all measures, the conformation of rFIX is not altered by the presence of the Fc domain. Experiments of this type clearly have implications not only for creation of new entities but also for demonstrating comparability during process change, upon modification, or for biosimilars.

The conformation and dynamics of insulin were characterized by HDX MS,[159,160] including analyses of various forms/versions of insulin with different therapeutic properties. The effects of Endo H trimming of high mannose glycans from α-amylase were studied.[161] Several forms of recombinant glucocerebrosidase, sold as Cerezyme, a treatment for Gaucher's disease, were investigated by HDX MS,[162] including mutants and ligand-bound forms. The effects of pegylation on the conformation and dynamics of granulocyte colony stimulating factor (G-CSF) were studied.[163] Exchange into a new class of therapeutic agents, "stapled peptides", was characterized by HDX MS,[164] and the authors found that the location of the staple is important and is linked to both the deuteration kinetics and the rates of proteolysis of the stapled peptide. Finally, HDX of lyophilized protein was studied[165] with the goal of understanding what may happen to protein structure during dehydration. The results of this work have important implications in formulation of biopharmaceuticals, including on the conditions during the freeze-drying process and for reconstitution. HDX MS can be used to probe all such events and inform each level of processing.

MEMBRANE PROTEINS

The application of HDX MS to the investigation of membrane proteins is a growing area of application. Membrane proteins are inherently difficult to deal with by many biophysical methods. HDX MS has the advantage of only needing small amounts of material and the ability to differentiate protein from lipid. Exchange can be performed in detergent or in membrane mimetics, provided the lipid component of the membrane mimetic can be removed sufficiently so as to not interfere with peptide ionization and detection. A great deal of the methodological challenges of analyzing membrane proteins by HDX MS have been met, leading to a number of publications in this area and increased understanding of these proteins. Topics in this section are divided into peripheral membrane proteins, which are less challenging to study, and transmembrane proteins.

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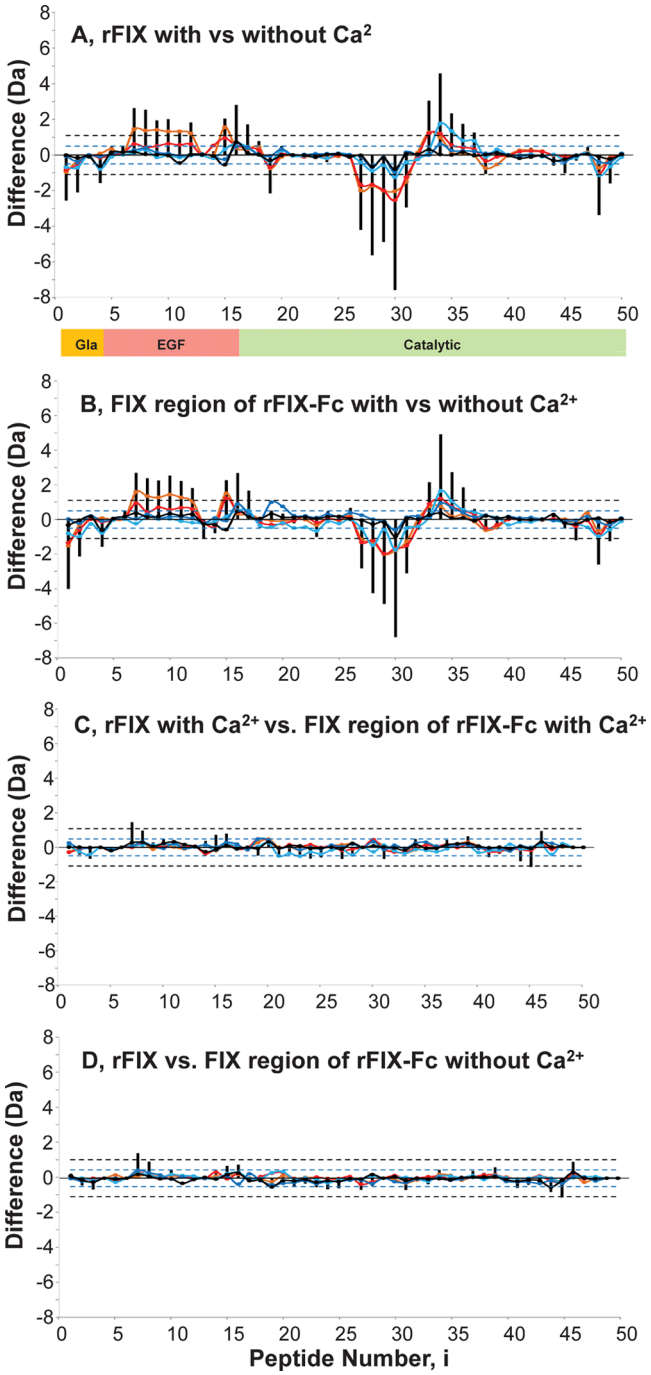

Figure 9. Characterization of Fc fusion proteins and how their parts relate to naturally occurring versions. HDX MS of recombinant factor IX (rFIX) was compared to a fusion of rFIX with an Fc of an antibody (termed rFIX-Fc). The pattern of differences in deuterium levels of rFIX as a result of calcium binding (A) was the same as that observed when the fusion form, rFIX-Fc, bound to calcium (B) meaning that the FIX portion was not impaired in its calcium binding activity by being attached to the Fc. There were essentially no differences (C, D) between exchange into rFIX and rFIX-Fc with calcium or without calcium. Reprinted with permission from ref 158. Copyright 2012 Wiley.

Peripheral Membrane Proteins. Peripheral membrane proteins are not completely embedded but rather anchored to the membrane in some way. Protein conformation and dynamics can be influenced by the membrane itself, through electrostatic and hydrophobic interactions. It is therefore important to study peripheral membrane proteins in the presence of an actual membrane in order to obtain meaningful data. Several excellent, recent examples of the application of HDX MS to the analysis of peripheral membrane proteins come from Roger Williams' lab. They have been investigating phosphoinositide 3-kinases (PI3K) which act just downstream of membrane receptors and are in close proximity to the membrane. There are two classes of PI3Ks: Class 1A includes isoforms p110α, p110β, and p110δ, which are regulated primarily by p85 and are activated by receptor tyrosine kinases; Class 1B contains isoform p110γ, is regulated by p101, and is activated by G-protein coupled receptors (GPCRs). HDX MS was used to study p110α[166] and showed that p110α in complex with a regulatory subunit (p85α) constitutes a complex catalytic cycle characterized by distinct conformational steps. There was disruption of the SH2 domain of p85α with interfacial regions of p110α, movement of the adapter binding domain in p101α, and interaction of the kinase domain with lipids. Oncogenic mutants of p110α were then probed. All mutations changed the way the protein interacted with the lipid membrane, and selected mutants induced a variety of conformational changes in both p110α and its interaction p85α. Some mutants mimicked conformational changes observed in natural activation. The other isoforms (p110β and p110δ) of Class 1A PI3K were also investigated[113] by HDX MS, and results for all isoforms were compared. The binding of the regulatory subunit p85 to each isoform was mapped, and it was shown that each isoform had unique relationships with p85. Figure 10A summarizes some of the most recent findings wherein HDX MS probed the class 1B isoform PI3Kγ catalytic subunit (p110γ) as it interacts with its regulatory subunit (p101), lipid membranes, and G-protein G$\beta\gamma$ heterodimers. HDX MS analyses, which involved multiple proteins and liposomes and are a technical challenge in themselves, showed that the helical domain of p110γ is substantially protected when in complex with p101, exposed when bound to lipids, and protected once more when binding to G-protein G$\beta\gamma$ heterodimers. Then, Walser et al.[167] used HDX MS to investigate how the p110γ catalytic subunit interacted with the adaptor subunit p84. Not only do all these studies with PI3K provide essential new information that is very difficult to obtain by other methods, but also they highlight the extreme utility of HDX MS for studying membrane proteins in lipid environments.

There were a number of papers in the last 30 months that described investigations of human apolipoprotein A-1 (ApoA1), a protein essential for solubilizing and transporting cholesterol in the blood. ApoA1 is the primary component of high density lipoproteins (HDLs) and plays a major role in the structure of most HDL particles. HDL particles start out as discs of lipid molecules with two copies of ApoA1 circling the hydrophobic lipid tails at the equator. Rearrangements of the ApoA1 structure and addition of cholesterol can then transform the disc into spheres. HDX MS was used to obtain conformational information for ApoA1 in discoidal HDL particles of two sizes,[168] to probe the effects of point mutations in ApoA1 that lead to reduced HDL levels,[169] and to compare the conformation of ApoA1 in discs versus in spherical HDL particles.[170] The HDX MS data indicated that several regions of lipid-free ApoA1 become significantly protected when incorporated into HDL particles with a 9.6 nm diameter whereas, for particles with a diameter of 7.6 nm, approximately 20% more ApoA1 residues are forced out of contact with lipids. EX1 kinetics were observed for ApoA1 indicating coexisting helical and nonhelical populations. The Iowa (G26R) and Milano (R173C) mutations of ApoA1 change helix packing, albeit in different ways and magnitudes,

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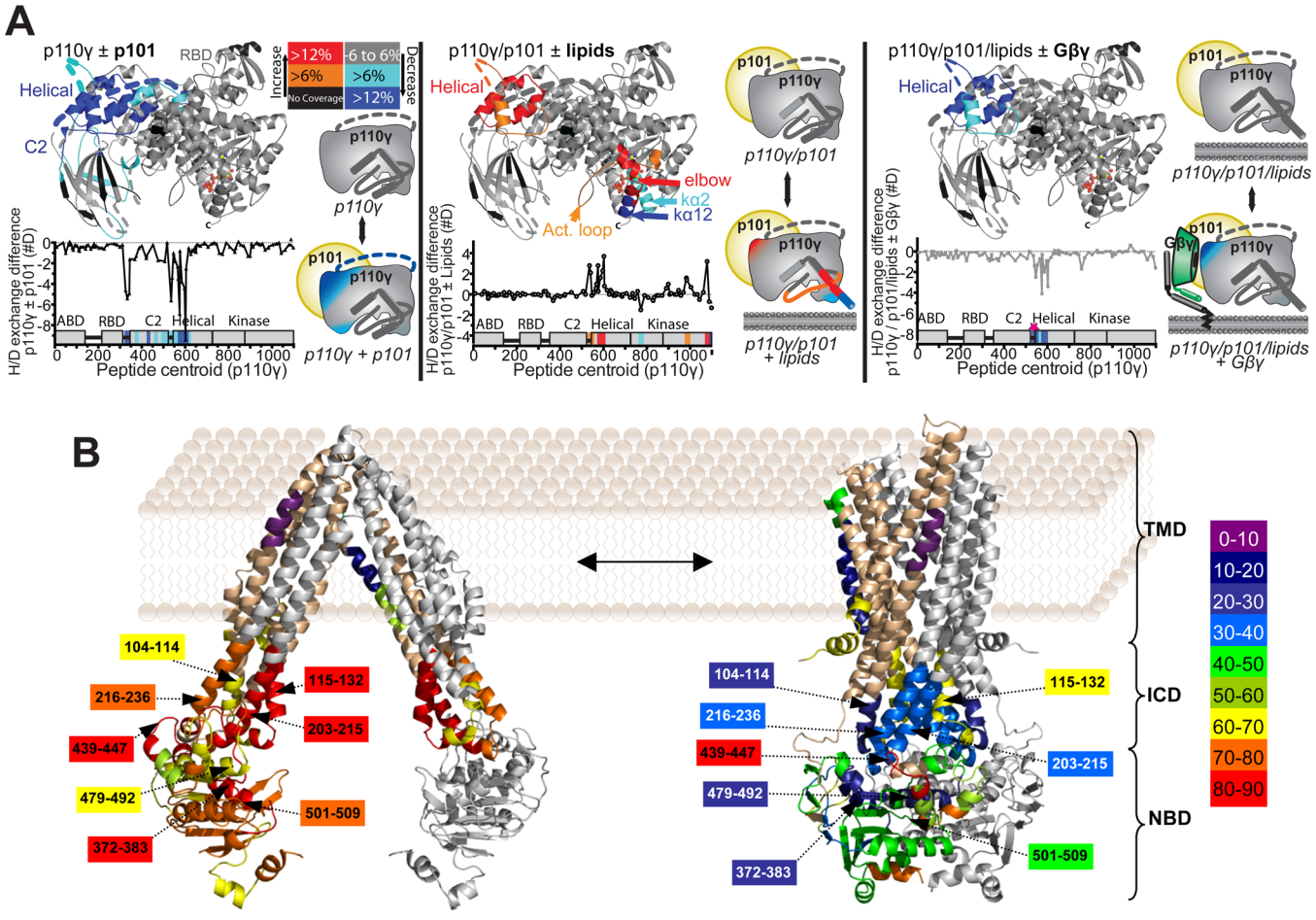

Figure 10. Membrane protein investigations with HDX MS. (A) Exchange into phosphoinositide 3-kinase γ (also termed p110γ) was compared to exchange during interactions with its regulatory/adaptor subunit p101 (left). The complex of p110γ/p101 was then labeled with and without empty liposomes (middle) or liposomes containing G-proteins (G$\beta\gamma$) (right) to ascertain the role of the membrane and effects of binding. See ref 229 for full details. Reprinted with permission from ref 229. Copyright 2013 National Academy of Sciences of the United States of America. (B) HDX MS of BmrA, a bacterial multidrug ABC transporter, was performed while the protein was in *n*-dodecyl-β-D-maltoside (DDM) detergent. HDX results for analysis of an apo form were superimposed on the 3D model of the open conformation (left), and analysis of a closed form ATP-Mg-bound mutant were superimposed on a 3D model of the closed conformation (right). Coloring is based on the percentage of deuterium after 1 h of labeling, according to the color scale at the right. Reprinted with permission from ref 181. Copyright 2012 National Academy of Sciences of the United States of America.

which contributes to proteolysis and the potential to form amyloids. A combination of methods including small angle neutron scattering, cross-linking MS, and HDX MS were used to construct a model of HDLs, including full length ApoA1.[171] Both HDX MS and lysine acetylation MS were implemented to study conformational changes in ApoA1 caused by Apo A-II in discoidal HDL.[172] When both ApoA1 and ApoA-II were incorporated in the same HDL particle, there were differences in deuterium incorporation in ApoA1 helices 3−4 and 7−9.

Phospholipase A_2 (PLA_2) was also a topic of interest. PLA_2 is a large protein superfamily, members of which catalyze the hydrolysis of phospholipids to produce fatty acids. They are involved in signaling, inflammation, and lipid membrane maintenance. Group VIIA lipoprotein associated (Lp-PLA_2) and its interactions with ApoA1 and HDLs were studied by HDX MS,[173] and the identity of three specific regions in PLA_2 with decreased deuteration were found. The authors note that the same regions in PLA_2 do not exhibit the same protection when associated with ApoA-II. Group VI Ca^{2+} independent (iPLA_2) was also studied[174] as was an inhibitor binding to iPLA_2[175] using a combination of MD simulations and HDX MS. It was shown that inhibitor binding resulted in protection in loop regions surrounding the active site, with some minor changes in regions distant from the active site, indicating that the whole enzyme is affected.

Monoacylglycerol lipase (Mgl) was studied by HDX MS.[176,177] Mgl is a serine hydrolase that associates with lipid membranes and deactivates cannabinergic signaling in the central nervous system by hydrolyzing the lipid signaling molecule 2-arachidoylglycerol. Karageorgos et al.[176] used HDX MS to monitor changes to Mgl when bound to small several molecule inhibitors. Reaction with a covalent inhibitor AM6580 that carbamylates Ser122 in Mgl resulted in HDX protection of helices 6α and 8α. Decreases in deuterium incorporation were also noted for another inhibitor AM6701 that was reversible; however, the decreased protection was not as pronounced. In a second publication, Nasr et al.[177] used HDX MS to compare the conformation of Mgl free in solution versus bound to phospholipid nanodiscs to delineate the region of lipid association. Membrane anchoring was found to proceed through helix α4 in the lid domain and neighboring helix α6, and this positions Mgl into an open conformation to facilitate ligand binding. Using the AM6580 inhibitor, it was shown that Mgl remains membrane bound, even when inactivated.

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Other peripheral membrane proteins were investigated. HDX MS aided in understanding how an enzyme required for synthesis of phosphatidylcholine is activated via a lipid-induced amphipathic helix.[178] Koshy et al.[179] studied the cytoplasmic fragment of CheW and CheA proteins, which are bacterial chemotaxis receptors. Global HDX data show that CF exhibits much slower exchange when in complex with CheW and CheA compared to when in solution. Lee et al.[180] monitored the structural transitions of α-synuclein as it bound to phospholipid vesicles.

Transmembrane Proteins. Transmembrane proteins, or those that span the membrane, are even more challenging than peripheral membrane proteins. The highly hydrophobic regions that span the membrane can be difficult to digest and separate from the lipid component. Nevertheless, some success has been realized, including the example of this type of HDX MS shown in Figure 10B. In the work shown in Figure 10B,[181] the bacterial ABC transporter, BmrA, was investigated. BmrA is a multidrug transporter that normally protects healthy cells by binding and expelling foreign organic compounds but must shift between open and closed conformations for this function. HDX MS revealed regions located in the intercellular domain with very different exchange kinetics depending on the conformational state. Another carrier, the bovine mitochondrial ADP/ATP carrier, has also been studied by HDX MS.[182] Similar to BmrA, mitochondrial ADP/ATP shifts between two distinct conformations to function and HDX revealed differences between these states were localized to the loop regions within the mitochondrial matrix. The glycerol facilitator (GF), a transmembrane protein responsible for transporting water and glycerol, was probed with HDX MS.[183] One of the transmembrane helices, TM7, displayed much faster exchange kinetics relative to the other helices suggesting that TM7 is less stable than the other helices in order to aid transport of molecules across the membrane.

Methodological developments are key to the analysis of transmembrane proteins. The proteins must be in the lipid to be in their native state for deuteration, but then the lipid must be removed. Various methods are described in these articles for accomplishing this. The folding of membrane proteins and ways to study it are also interesting. Bacteriorhodopsin, the prototypical GPCR, was studied by pulse-labeling HDX MS[184] to monitor refolding induced by dilution from sodium dodecyl sulfate (SDS). The protein obtained structure rather slowly as it refolded from SDS. The methodology explored could be applied to other membrane proteins. The transmembrane enzyme gamma-glutamyl carboxylase (GGCX), for which there is no solved structure, was studied with HDX MS.[185] GGCX converts select glutamic acid to γ-carboxy glutamic acid in vitamin K-dependent (VKD) proteins, and GGCX mutations have been linked to clotting disorders. GGCX in nanodiscs was labeled when free or bound to an 18 residue consensus propeptide from VKD substrates. Major differences in hydrogen exchange were observed for sites involved in propeptide and glutamate binding. The authors also noted other regions within GGCX exhibiting minor differences when in complex with the propeptide. Overall, HDX MS work on transmembrane proteins is progressing slowly but is expected to increase once the methods become more tractable.

METHODS

In the course of applying a method to problems, better ways of addressing the problems invariably appear. It is through a wide variety of applications that methods refinement and development can truly become efficient. During the past 30 months of applications, there have been a number of developments in the methodology that have made the applications possible. Nineteen percent of the articles surveyed had something to do with methods development. We have divided their classification into pre-LC, LC-MS, software, and other.

Improvements to the Pre-LC steps were reported and include work describing the enzymatic digestion step,[186−188] disulfilde bond reduction using electrochemical cells,[189,190] affinity capture,[191] methodological consideration for reducing back-exchange in MALDI[192] and electrospray,[193] and offline fast mixing for HDX labeling.[194] Reports of methods improvements in the LC-MS portions of the experiment included subzero cooling to minimize back exchange during LC/MS,[195,196] descriptions of top-down HDX MS studies using ECD or ETD fragmentation[197−203] where the goal is the elimination of proteolytic digestion and/or improvement to the spatial resolution of the exchange information, microfluidic systems for exchange, LC and MS,[204,205] work related to improving separation and understanding LC column parameters,[206−209] comparison of various platforms for HDX MS,[210] and description of fine isotope structure in HDX MS.[211] A number of software improvements was made including new software packages or updates to existing software,[83,212−215] studies of how to process data,[216,217] software approaches to improve spatial resolution,[218,219] and other work on computational and processing improvements.[220−222] Several other interesting studies, including methods descriptions, histidine exchange, and useful tools, were published.[223−228]

CONCLUDING REMARKS

Methods developments and refinements in HDX MS technology have placed this tool in the hands of nonspecialists. There will no doubt be additional future improvements in the methodology, but even at the current stage, a great deal of studies are now possible by more people than ever before. The applications of the method are therefore expected to continue to be strong and even increase in coming years. When one considers the vast number of possible forms of proteins, some known but many unknown, perhaps HDX MS will never run out of proteins to study. It is imperative that those active in the field continue to expand the reach of the method, apply the method to more and more types of proteins, systems, and purposes, train new investigators, and push forward with more improvements. There are still many things that remain to be studied.

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Notes

The authors declare the following competing financial interest(s): J.R.E. is a consultant of the Waters Corporation.

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