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Analysis of Protein Conformation and Dynamics by Hydrogen/Deuterium Exchange MS

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Abstract

synopsis—Recent technological advances hydrogen exchange MS have led to improvements in the technique's ability to analyze the shape and movements of proteins. John Engen of Northeastern University gives a much needed update on the field. The cover, created by Engen, shows proteins "swimming" in an H_2O/D_2O solution with a sample mass spectrum in the background.

Eight years ago, the first Feature on HX MS appeared in *Analytical Chemistry* (1). A lot has happened in the field since then, and this analytical method now seems even more useful and important than many thought at the time. Most of the challenges that were posed at the end of the 2001 article have been met and far exceeded. A continuously growing following for HX MS includes a well-populated interest group in the American Society for Mass Spectrometry (group website: www.hxms.com/asms), and the number of publications and citations on HX MS continues to climb steeply (Figure 1). This Feature will reintroduce the topic of HX MS for the benefit of newcomers, give an update of progress in the field, and attempt to forecast what the future holds.

The analyte: proteins

Proteins, the analytes in HX MS, are complicated. In addition to the chemical composition (amino acid sequence) of the molecule, factors such as shape (structure/conformation) and protein movements in solution (dynamics) must be considered. Structure and dynamics contribute significantly to the function of proteins, and therefore, to fully understand a protein, the interplay of structure, function, and dynamics must be investigated. Historically, functional studies have largely been in the domain of biologists and biochemists, whereas structural and dynamics studies have been dominated by physical and analytical chemists. Structure and dynamics are closely intertwined, and today, no one analytical method or focused area of expertise can provide the amount and diversity of information that is required for complete protein analysis. Combinations of skills and techniques—including HX MS—usually yield the most comprehensive picture.

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Understanding as much as possible about proteins in the shortest amount of time has long been a goal of hydrogen exchange (HX) MS. Recent technological advances have led to improvements in the technique, but has this goal yet been achieved? (To listen to a podcast about this Feature, please go to the *Analytical Chemistry* website at pubs.acs.org/ac.)

Understanding the folded conformation of proteins is essential. Unlike small molecules, the properties of a protein might change depending on its folded conformation. For example, an enzyme that is improperly folded (perhaps as a result of denaturation, mistakes in synthesis, post-translational modifications, and so forth) may not be able catalyze a reaction, whereas one that is properly folded will. In another example, a protein that functions by binding to target proteins or molecules might be unable to bind if its conformation is wrong. Further, some proteins might assume different functions depending on their conformation (2). Understanding those conformations is essential when developing small molecule drugs that target different forms of the protein. Producing and managing recombinant proteins for use as therapeutics also require rigorous control of the properly folded conformation because misfolded molecules may lose efficacy and/or produce undesirable effects (3,4). Thorough knowledge of the structure and dynamics can help us understand protein function as well as provide diagnostic markers for properly folded proteins.

Many proteins are highly dynamic and may populate higher energy states according to the Boltzmann distribution. Examples of protein dynamics include natural protein “breathing” or flexing in solution, structural changes in response to interactions either within the protein itself or with substrates, binding to ligands, and changing solution conditions. Determining which regions of a protein change structure, rate constants for these changes, and free energies of different structures are some of the most important and fundamental goals of protein dynamics studies. Whereas methods for determining protein structure such as X-ray crystallography and high-resolution NMR have yielded a very large number of extremely valuable structures, information on protein dynamics, as was pointed out in the previous feature (1), still lags behind because of a lack of robust methods and analytical tools.

Detecting HX by MS is an approach for characterizing protein dynamics and changes to protein conformation (5,6). Because protein conformation affects the rate of exchange of deuterium for hydrogen in proteins, measuring the deuteration of proteins over time can reveal aspects of conformation as well as changes to conformation when a protein structure is perturbed by any number of factors (drugs, protein interactions, modifications, denaturation, pH, and so forth). Other methods exist for such protein characterization, but many of them sample global conformational properties and do not provide the level of detail desired. CD, for example, only reports on a protein’s content of β-sheet and α-helix. If those properties remain constant, even during an important structural rearrangement, CD will not detect conformational differences. Other methods such as fluorescence, differential scanning calorimetry, analytical ultracentrifugation, side-chain reactivity, binding assays, and various chromatographic methods have the same limitation. Also, because all proteins and/or protein systems are not compatible with crystallography or NMR, alternatives such as HX MS become more and more attractive. Again, the complementarity of these methods should be emphasized: one method alone cannot provide all the information usually desired about a protein. As an example, unlike crystallography, HX MS cannot provide the position of atoms; on the other hand, crystallography might not be possible for very dynamic proteins that could be probed by HX MS.

The HX reaction

The HX phenomenon was first described by Kaj Ulrik Linderstrøm-Lang in the 1950s (for a review, see Englander et al., ref. 7). Linderstrøm-Lang exposed proteins to D_2O and made detailed measurements of protein deuteration using density gradient tubes (8) long before the development of more selective detection methods such as NMR or MS. Radioactivity measurements have been used to measure exchange for tritium, and several kinds of spectroscopy, including IR and UV, have been used to follow the exchange of deuterium for hydrogen. Once NMR became available, researchers employed it extensively for the analysis

of HX in proteins (9,10). Because the mass of deuterium is roughly double the mass of hydrogen, MS is also appropriate for detecting HX. However, HX MS is a relative newcomer to HX analysis because MS for biological molecules did not become commonplace until the early 1990s. The first protein HX MS work appeared in 1991 (11).

Many excellent reviews have been published regarding the details and mechanisms of the exchange of hydrogens between solvent and protein (for examples, see refs. 6,10,12,13). The reader is referred to these other works for details beyond the scope of this Feature. The hydrogens that are most easily measured by HX MS are those located at the peptide backbone amide linkages (NHs) (Figure 2). Some hydrogens in proteins, such as those bonded to carbon, almost never exchange; others, such as many side chain hydrogens, exchange so quickly that measuring them by LC/MS is difficult.

The ability to measure exchange of the backbone amide hydrogens has other advantages: these hydrogens help hold secondary structure elements (β-sheets, α-helices) together, and every amino acid except proline has a backbone amide hydrogen (Figure 3). Folded proteins can have exchange rates at NHs that are eight orders of magnitude slower than those in an unfolded version of the same sequence. Exchange is a function of two intertwined protein properties: solvent exposure and hydrogen bonding. Slow exchange is indicative of solvent protection and/or hydrogen bonding, whereas fast exchange indicates solvent exposure and/or no hydrogen bonding. For example, in Figure 2, the regions exposed to the solvent generally become deuterated rapidly, but those parts of the protein buried in the core are generally labeled much more slowly. However, sometimes structural elements that are highly solvent exposed are hydrogen bonded (as are several solvent-exposed α-helices in Figure 2) and exchange slowly. The linked nature of solvent exposure and hydrogen bonding must always be considered when interpreting data: whereas rapid deuteration is usually indicative of high solvent exposure, slow deuteration does not necessarily indicate low solvent exposure and could be instead caused by high hydrogen bonding. Changes to solvent exposure and hydrogen bonding can be sensed over the entire length of the backbone by measuring the amount and rate of deuterium incorporation at NHs. Unfortunately, interactions that involve primarily side chains may not alter exchange at the backbone, and HX MS data on those interactions should be interpreted cautiously. Figure 3 provides another example of the backbone amide hydrogens in a typical protein, which are color coded according to the type of secondary structure. Notice that there are a number of places in this example protein (indicated by arrows) where interactions might occur that would not necessarily alter the exchange at NHs.

HX is both an acid- and base-catalyzed reaction. The general mechanism for isotope exchange in folded proteins at physiological pH (therefore, predominantly base-catalyzed) requires abstraction of the amide proton by base (OD^-) followed by reprotonation/deuteration of the amide nitrogen with a proton/deuteron from the solvent (recently reviewed in ref. 14).Hydrogens that exchange slowly do so most readily following structural changes that free these hydrogens from intramolecular hydrogen bonding and provide access to the aqueous solvent and OD^- (10,15). Exchange at a single peptide NH can be facilitated by low amplitude structural changes—that is, changes involving only a few atoms. This form of HX occurs while most of the protein remains folded. Alternatively, structural changes may involve movements of large segments of the polypeptide backbone or even complete unfolding of the protein. This form of HX can be viewed as exchange from unfolded (or partially unfolded) forms of a protein (16).

Determining HX by MS

A general scheme for the analysis of proteins with HX MS is shown in Figure 4. The most simple implementation of HX MS is the continuous labeling experiment (for a recent review,

see Yan and Maier, ref. 17). In this procedure, protein in 100% H_2O buffer is diluted with a buffer in which all components are the same except that the H_2O is replaced with D_2O. The dilution is typically 10-20× such that the kinetics favor unidirectional exchange (deuteration). The labeling is carried out in physiological buffers typically with pH ~7.0. The exchange reaction proceeds for defined periods of time (for example, 10 seconds, 1 minute, 1 hour, 8 hours) and is quenched at each time point by lowering the pH to 2.5 and the temperature to 0 °C. These simple changes decrease the intrinsic rate of HX by approximately five orders of magnitude. Under quench conditions, the half-life for exchange in unfolded polypeptides is ~1 hour (meaning that if the deuterated protein were suddenly exposed to a 100% H_2O environment, it would take ~1 hour for half of the deuterium to exchange back to hydrogen). The mass of the quenched sample is determined by injection into an LC system coupled to a mass spectrometer with ESI (note that MALDI MS without LC has also been used; see ref 18). A digestion step may be introduced just before the LC, but the digestion must be done under quench conditions, necessitating the use of acid proteases. Digestion is intended to break the protein into peptides so that the location of deuteration can be determined. This increase in spatial resolution, which was first conceived (19,20) before peptides were analyzed by MS, is one of the great advantages of HX MS: it reveals the location of deuteration—or more importantly, the *location of changes* in deuteration (see also below). Note that in all HX MS experiments—and especially in experiments that include digestion—the conformational information of the protein under native conditions and physiological pH has been captured by the deuterium labeling. As long as the quench conditions are maintained and the samples are analyzed rapidly, deuteration in peptides is a reflection of the solution conformation at the labeling pH.

There are many advantages to detecting HX by MS. One of the main advantages is the miniscule amount of protein that is required: as little as 500-1000 picomoles for an entire experiment including the analysis of 10 time points of exchange. In addition, the concentration of the protein can be as low as 0.1 μM. These requirements are orders of magnitude smaller than those of many other biophysical methods for studying protein conformation and dynamics. Thus, proteins that precipitate at higher concentrations, proteins that are hard or costly to obtain, and proteins that are otherwise incompatible with other methods can be analyzed by HX MS. Another great advantage is that HX MS can analyze samples containing multiple proteins and ligands because of the ability of LC and MS to deal with mixtures. And because the readout is ultimately based on mass, mixtures are not as problematic for HX MS as for other methods in which signals from one species might overlap and obscure signals from the species of interest.

The breadth and power of HX MS have been illustrated many times over the last 8 years. Analyses cover all manner of proteins and protein systems, including the analysis of protein constructs to assist with crystallographic and NMR structural determination (21,22), the analysis of whole viruses (23,24), the investigation of biopharmaceuticals (25,26) and the delineation of antibody epitopes (27,28), and the analysis of drug binding in important disease targets (29-31). The next 8 years of HX MS research will no doubt be even more revealing and insightful.

Progress in HX MS

The aforementioned 2001 article presented a list of challenges for HX MS (1), which included automated sample prep, automated data processing, scaling down the LC to take advantage of sensitive MS, and validating gas-phase fragmentation methods that determine deuterium levels at specific peptide linkages. All of these challenges have been addressed, and some of them have been met. This section will describe these recent developments and how they make the application of HX MS more and more mainstream.

One major challenge to HX MS is the ability to exactly define the location of deuteration. This issue has been the subject of many papers over the past decade, and significant progress has been made. There are two ways to increase the spatial resolution (the ability to localize the deuterium to individual amino acids). The first method, digestion modification, is not a new development and has been in use since the earliest analyses by HX MS. If the conditions of the digestion step are slightly changed, different peptides can be generated; hopefully, sequences of many of them will overlap with each other. When peptides overlap, deuterium can be pinpointed to smaller and smaller segments of the backbone. The results of the digestion can be changed by altering the time, varying the quantity of enzyme, adding denaturants, or using enzymes with different specificities. In the last few years, there has been a rise in the use of aspergillopepsin (EC 3.4.23.18; also known as Factor XIII) as an alternative to porcine pepsin because the digestion pattern is slightly different. Multiple groups (32-34) have used tandem digestion with both pepsin and aspergillopepsin to increase and change digestion even further.

Deuterium can also be localized with the mass spectrometer itself using MS/MS experiments. The dream is to fragment each deuterium-labeled peptide in the gas phase and determine the location of each deuterium atom by looking at the fragment ions; unfortunately, it has taken much longer to resolve this issue than many had anticipated. At first, collision induced fragmentation in a cloud of argon or helium gas without hydrogen/deuterium migration (also referred to as scrambling) seemed possible for certain product ions. However, other groups provided evidence that hydrogen/deuterium migration was actually a major problem and that the location(s) of deuterium in product ions did not represent its pre-fragmentation location in the precursor peptide ions. The idea of using MS/MS for deuterium localization was in limbo until the scrambling issue could be solved. When alternative fragmentation methods such as electron capture dissociation and electron transfer dissociation (ETD) appeared, it was shown that scrambling could be minimized (35-37). Because fragmentation by ETD occurs very rapidly and a low amount of internal energy is imparted to the ions during the fragmentation process, deuterium does not have a chance to scramble during the actual electron transfer and bond cleavage. If, however, ions have sufficient internal energy or receive vibrational excitation at stages prior to the actual electron transfer event, one can induce scrambling even in MS/MS by ETD (36,37).Therefore, when properly performed with the right fragmentation method, it appears that MS/MS can be used to reliably determine the location of every deuterium in every peptide. Hopefully, HX MS/MS will become commonplace in the next 3-5 years and provide the reliable, specific, single-amino-acid deuteration information that researchers have been craving since the method’s inception.

Another challenge for HX MS in 2001 was automation: automation of the labeling and automation of the data processing. Much progress has been made in these areas as well (see Figure 4). The deuterium labeling reaction itself has become automated with robotics (38-40), which not only make the sample preparation more reproducible but less labor intensive as well. The other area that has seen automation is data processing, a surprisingly difficult aspect of HX MS for a number of reasons. Data processing automation is essential because a major problem area for HX MS has been the perception that the data processing steps are so labor intensive that only the foolhardy would dare undertake HX MS experiments. Many users that want and/or need HX MS have shied away from the whole notion, particularly those in environments in which data analysis needs to happen rapidly. For them, the luxury of having a single person sit and pour over thousands of mass spectra looking for deuterium isn’t possible. These problems are diminishing, and the landscape is changing. A number of software packages have been developed (for example, see Pascal et al., ref. 41) that reliably reduce the burden of data processing from a time frame of weeks to only hours. Yet software development and automation in the data analysis portion of an HX MS experiment remain great challenges of this method that until solved, will not allow HX MS to enter the routine and mainstream

position that it really should occupy. Thankfully, a validated, unified software platform is on the horizon.

A final challenge from 2001 is the analysis of larger and more complex proteins/systems with ever smaller amounts of material. Realizing this challenge requires improvements in all stages of the analytical measurement portion of HX MS experiments. Mass spectrometers themselves have continued to become more and more sensitive, and the LODs have steadily dropped. Whereas 10 years ago it might have required ≥200 pmol of material for a single high-quality MS analysis, with the current state-of-the-art mass spectrometers, these requirements are routinely 10-50× less. Smaller consumption opens the door to analysis of proteins that cannot be obtained in sufficient quantity or that do not behave at higher concentrations (for example, highly disordered proteins, low-copy-number proteins, or proteins that are hard to overexpress and purify).

In addition, improvements in the chromatographic separation prior to mass analysis are also important. Because separation must occur at 0 °C to maintain quench conditions, chromatographic performance in HPLC is compromised. Using smaller particles significantly improves the chromatographic performance of HX MS experiments (29,42,43) and simultaneously reduces the amount of sample required because the molecules that are analyzed are highly concentrated into very narrow chromatographic peaks. With such improvements in separation, more and more peptides can be analyzed within the confines of short separation at 0 °C (43). Expanded use of improved chromatographic approaches will hopefully occur in future years, driven by creation of commercial solutions that implement this superior separation methodology. In addition, orthogonal separation within the mass spectrometer itself in the form of ion mobility (44) has already been demonstrated to further increase the ability of HX MS to deal with increasingly complex analyses.

Challenges for HX MS as an analytical technique

Despite the great strides HX MS has made and its impressive ability to examine and describe the exchange of hydrogen in proteins in ways that Linderstrøm-Lang would be both amazed at and proud of, more remains to be done. Improvements in HX MS, driven by the goal of understanding as much about all proteins as possible in the shortest amount of time, will continue in the coming years. Above all, HX MS needs to become a “turnkey” method with ultrafast turnaround in an integrated platform. When this happens, the limiting step will be obtaining the protein to be studied, and goals such as true high-throughput screening of conformational changes in the presence of binding inhibitors or interactions could be realized.

One particular application of “turnkey” HX MS is likely to be the characterization of biopharmaceuticals. Integration of HX MS into the standard platform for interrogating the physical properties of protein molecules, particularly those of pharmaceutical interest, would significantly aid in the understanding of structure-function relationships. HX MS could help answer many questions. For example, how does structure change during various storage conditions? What might certain additives or contaminants do to conformation? And, (during development stages) how can molecules be altered to deliver the desired effects yet be stabilized to prevent degradation or aggregation?

We are still in the early stages of making HX MS analysis of biopharmaceuticals a reality. As an example, consider Figure 5. Here, the HX properties of a recombinant, therapeutic monoclonal antibody were measured (25). The baseline exchange, or the exchange of the molecule in its native state as used in the current drug formulation, is shown after 10 minutes in deuterium buffer (this is one time-point of an entire time-course taken in these experiments). These are simple, continuous labeling experiments that include rapid identification of all the peptic peptides (>250) and high-resolution separation with UPLC. The data processing is

semiautomated, and an entire experiment can be completed and analyzed fairly rapidly. If one were to measure the HX of the same molecule under various conditions and compare the results to the HX for the baseline state, any conformational changes observed would be indicative of structural alterations. For many purposes, the best case would be to find no changes in HX, therefore confirming that nothing detrimental had happened to the molecule when changing to the new condition/state. In a therapeutic molecule, the signature HX of the protein in the desired functional state (the active form, for example) becomes the baseline for what would be considered a "good" conformation. Functional assays would be necessary to validate the functional state, again bringing us back to the need for multiple methods of analysis described earlier. Biological and biochemical assays are essential; when combined with the HX MS experiments, connections between the HX MS readout of higher-order structural information can be made to the functional status indicated by the biological assays.

Many more interesting days lie ahead for HX MS. By the next time this topic is featured in *Analytical Chemistry*, perhaps many of the current methodological issues of HX MS will be nonexistent, and HX MS will just be a routine method churning out exciting information about proteins and protein conformation.

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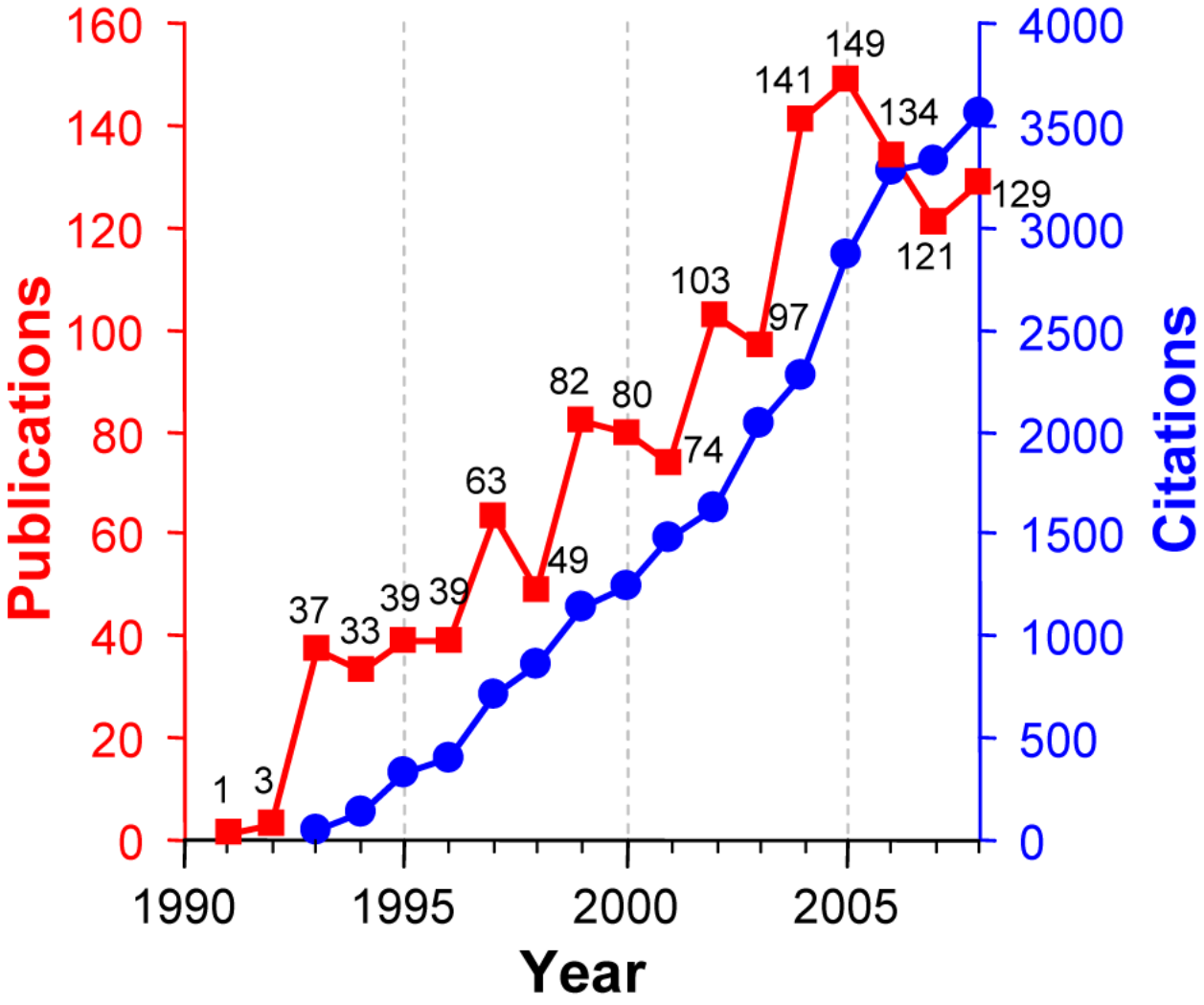

Figure 1.

The growth of HX MS since 1990. The number of publications (red squares) and citations (blue circles) was determined using ISI Web of Science (Thompson Reuters) with a keyword search for “hydrogen exchange mass spectrometry” over the period 1991-2008. The totals for 1991-2008 were 1374 publications and 25,249 citations. During that same period, 16 laboratories accounted for 25% of the papers in the field.

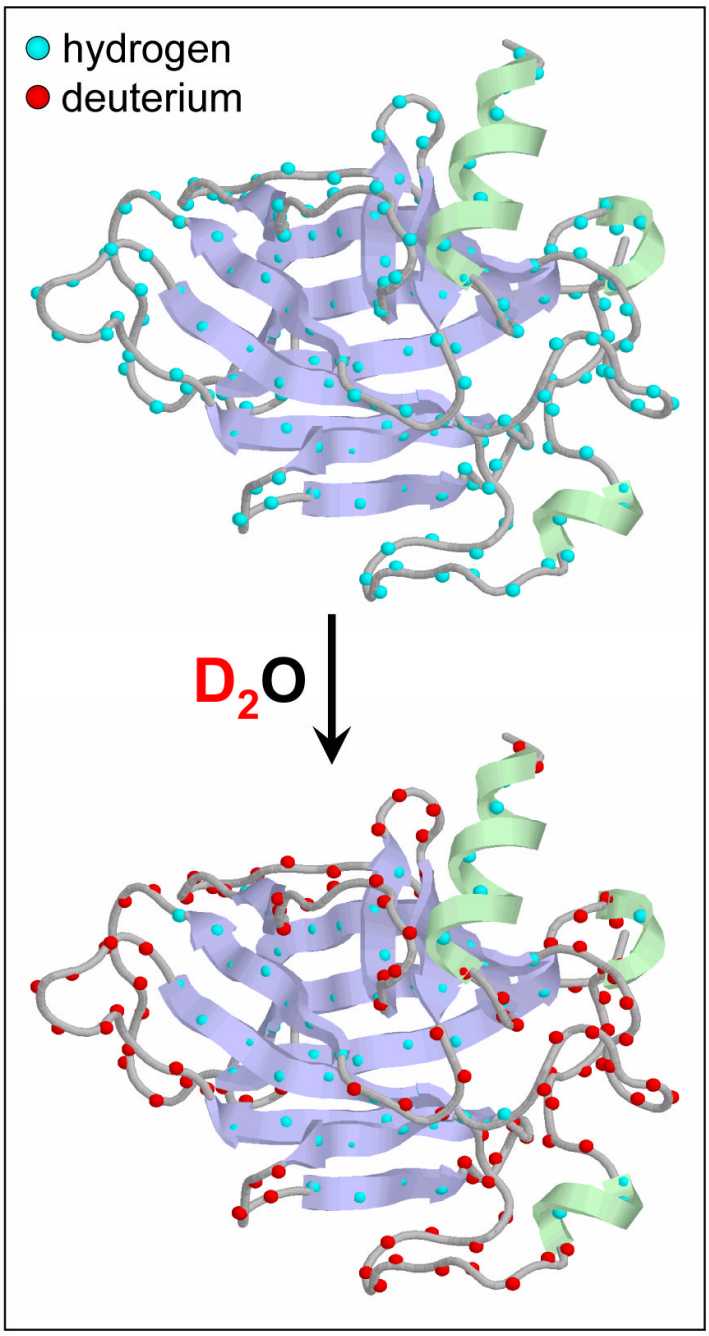

Figure 2.

HX into proteins. Labile NHs, rendered here as blue balls, can become deuterated when a protein is placed in a D_2O solution. Other hydrogens that exchange too slowly or too quickly to be measured are not shown. The rate of deuteration depends on factors including solvent accessibility, hydrogen bonding, pH, and temperature. In this figure, for example, NHs that are not hydrogen bonded and reside near the surface of the protein become deuterated rapidly, but highly solvent-exposed NHs in structured elements at the surface (several α-helices in this case) are protected from exchange by hydrogen bonding.

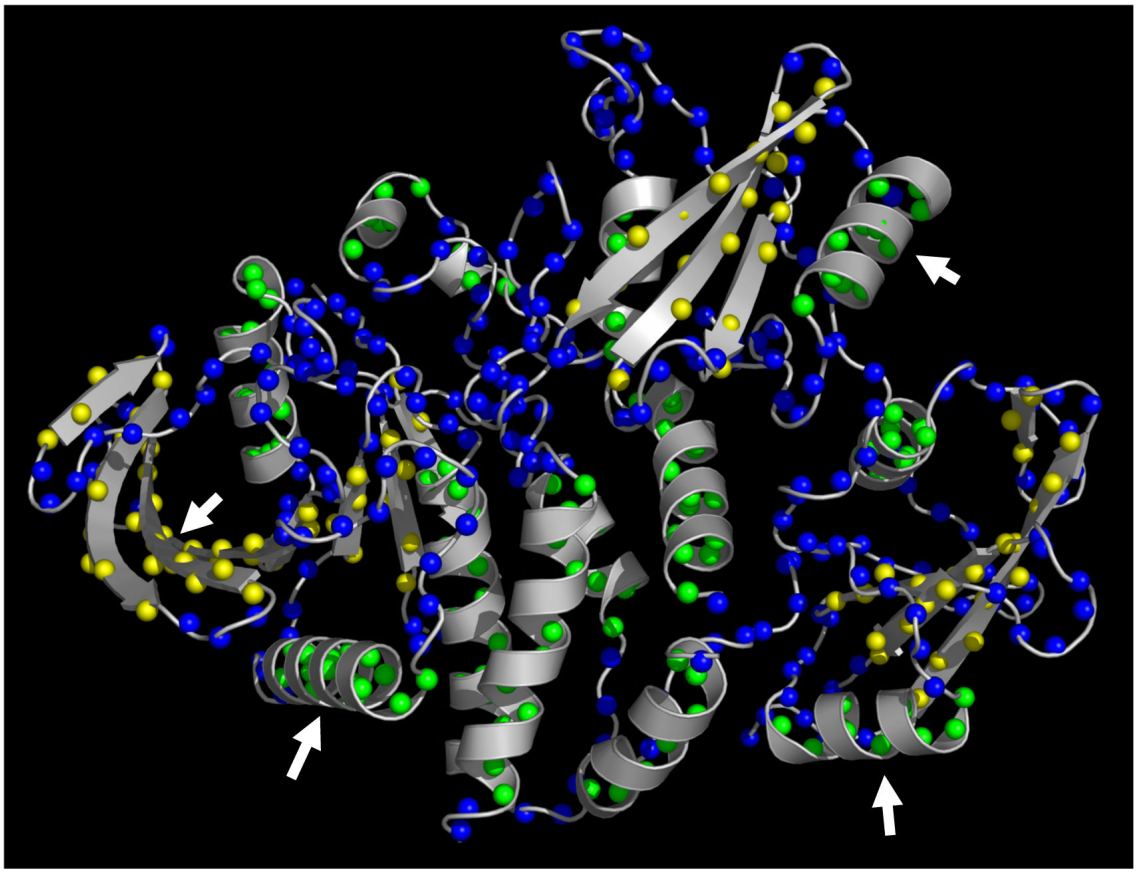

Figure 3.
Backbone amide hydrogens sorted according to secondary structure: those in α-helices are shown in green, those in β-sheets are shown in yellow, and the remainder are shown in blue. If structural elements such as helices or multistrand beta sheets (such as those indicated by white arrows) remain highly stable during protein-ligand interactions, there may be no change to deuteration at NHs. Interactions driven by side chains may not perturb backbone amide HX.

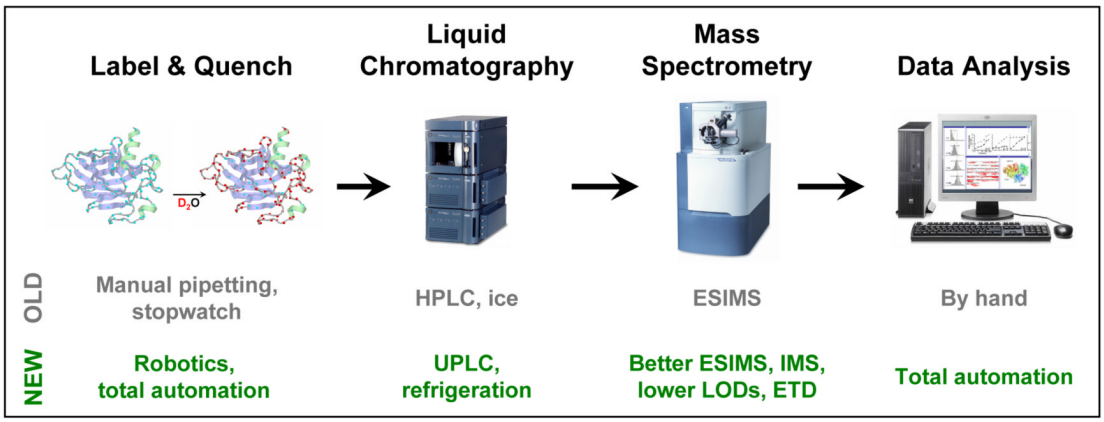

Figure 4.
The general steps that make up a typical HX MS experiment. The old techniques shown are being replaced by the newer, better methods at each step during the overall experiment.

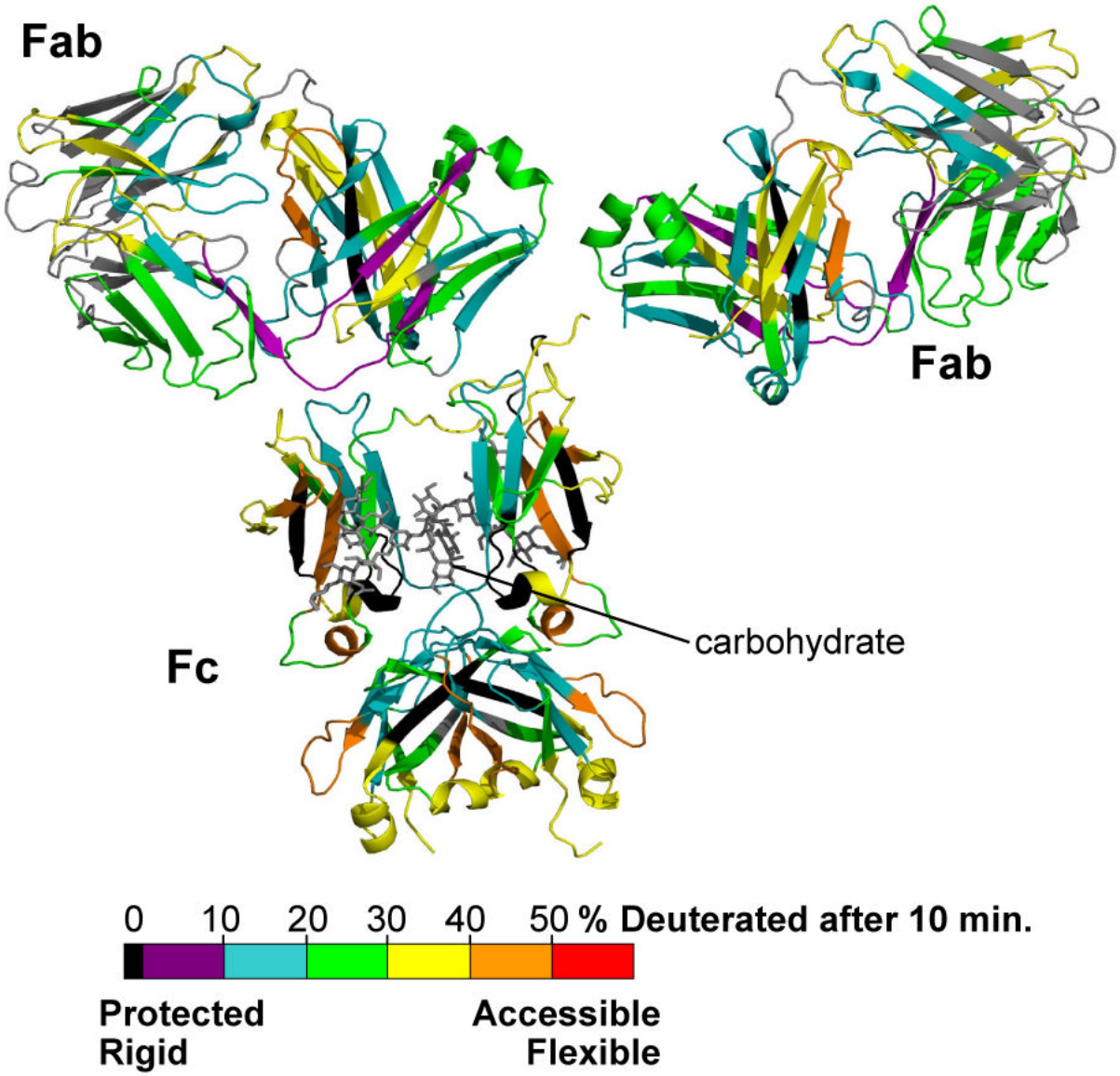

Figure 5.
Deuteration of a recombinant biopharmaceutical monoclonal antibody (adapted from Houde et al., ref. 25). The relative deuterium levels for this antibody are shown after 10 minutes of labeling. Some regions are much more deuterated than others. Data such as these form the baseline against which comparisons can be made to determine if the conformation of the molecule is altered (for example, by post-translational modifications or storage conditions).