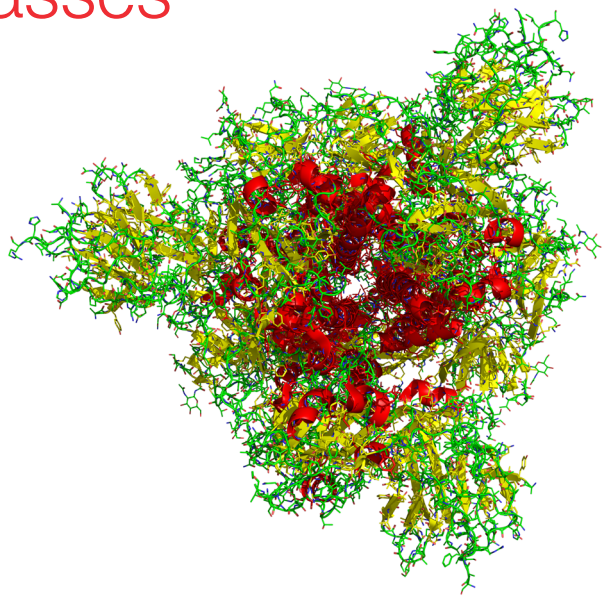
Hydrogen deuterium exchange mass spectrometry for the masses

Executive summary

Determining the shape and dynamics of proteins and protein complexes is crucial to understanding protein function and the roles proteins play in biological systems. Traditional techniques employed to examine protein structures have a number of limitations, including the large sample amounts required, the size of the complex under interrogation and the availability of the sample in the condition needed for analysis. In recent years, innovations in hardware, software and automated workflows have pushed mass spectrometry (MS) to the forefront of the range of techniques used for structural analysis. Hydrogen deuterium exchange mass spectrometry (HDX-MS) has become a powerful tool in the MS toolbox. This white paper focuses on the information provided by HDX-MS experiments, and how new tools developed for processes from sample preparation to data analysis have made this workflow more informative and streamlined.



Introduction

The characterization of protein structures and protein complexes is essential for understanding protein function and the mechanisms of action in a biological system. These proteins, the complexes and networks they form, the various interactions that occur during complex formation and the folding they undertake all govern biological activities.

Traditional techniques for studying protein complex structures, folding and protein-protein interactions include X-ray crystallography, cryo-electron microscopy (cryo-EM) and nuclear magnetic resonance (NMR). However, these techniques may not allow for the analysis of proteins in their native conditions and are limited to examining structures in their static states. These traditional techniques are also limited by the size of the biomolecule that is being examined, and the fact that many proteins are simply not amenable to these types of analysis limits their applicability. NMR is one such example where the technique is limited to proteins that include less than 200 amino acids and are soluble for analysis and amenable to isotopic labeling. In X-ray crystallography it is often very difficult to produce the well-diffracting crystals needed for analysis. MS techniques have been used for many years to study protein complexes, protein structures and protein-protein interactions; however, they were limited to highly specialized and instrument-savvy research laboratories. Additionally, due to their complexity and requirements for specialized sample preparation, advanced MS features and fit-for-purpose data analysis, MS-based structural proteomics workflows have lagged behind traditional proteomic analysis.

This white paper introduces HDX-MS, a powerful tool for studying protein structures, dynamics, folding, complexes and interactions. This paper will address the advantages of HDX-MS, the information it provides and the complementary role it plays with traditional techniques, as well as the latest tools and workflows that have been developed to simplify HDX-MS.

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Protein and protein complex studies

Proteins are pivotal to virtually every biological process that occurs within a cell, from gene expression to cell growth and proliferation, intercellular communication and apoptosis. Examining the role proteins play in biological processes can be challenging due to their dynamic nature. Proteins within a cell are continuously stimulated by external factors that change their conformation and properties. Since proteins are expressed in a cell-dependent fashion, they can vary in type and structure from one cell to another. Structural conformational changes play a significant role in protein function. These protein characteristics suggest a complexity that can be difficult to investigate when trying to understand protein function in a biological context. The complexity is further compounded by the fact that the majority of proteins interact with one another via non-covalent interactions, forming larger complexes. The structure of a protein influences its interactions with other proteins, as well as with lipids, glycans, small molecules and nucleic acids. These interactions, in turn, can affect protein function. Characterizing the three-dimensional structure of a protein or protein complex helps explain the role of proteins in biological functions.

HDX-MS

A number of MS tools are available for elucidating protein or protein complex structures (Figure 1). Each approach provides complementary information about the structures of proteins and protein complexes. HDX-MS is one such tool in the MS toolbox for structural biology, and it takes advantage of the labile nature of protons present on protein backbone amides. When dissolved in solution, proteins exchange these protons with hydrogen groups present in the buffer. In a deuterated buffer, protons from the protein are exchanged with deuterium. Only the protons present on backbone amides are measured, as protons found on the functional groups of amino acid side chains exchange too rapidly to measure, and the ones on the carbons are too slow to exchange.[1] The rate of hydrogen to deuterium exchange provides solvent accessibility data, which can be used to infer information on protein structure and conformation. MS can be used to measure the rate of deuterium uptake. For single proteins and protein complexes, HDX-MS can be used to obtain information on structure, protein-protein or protein-ligand interaction sites, allosteric effects, intrinsic disorder, and conformational changes induced by posttranslational modifications (PTMs). HDX-MS has the added advantage of not being limited by the size of proteins or protein complexes, compared to traditional structural approaches. It also has the advantage of being highly sensitive and detects coexisting protein conformations.

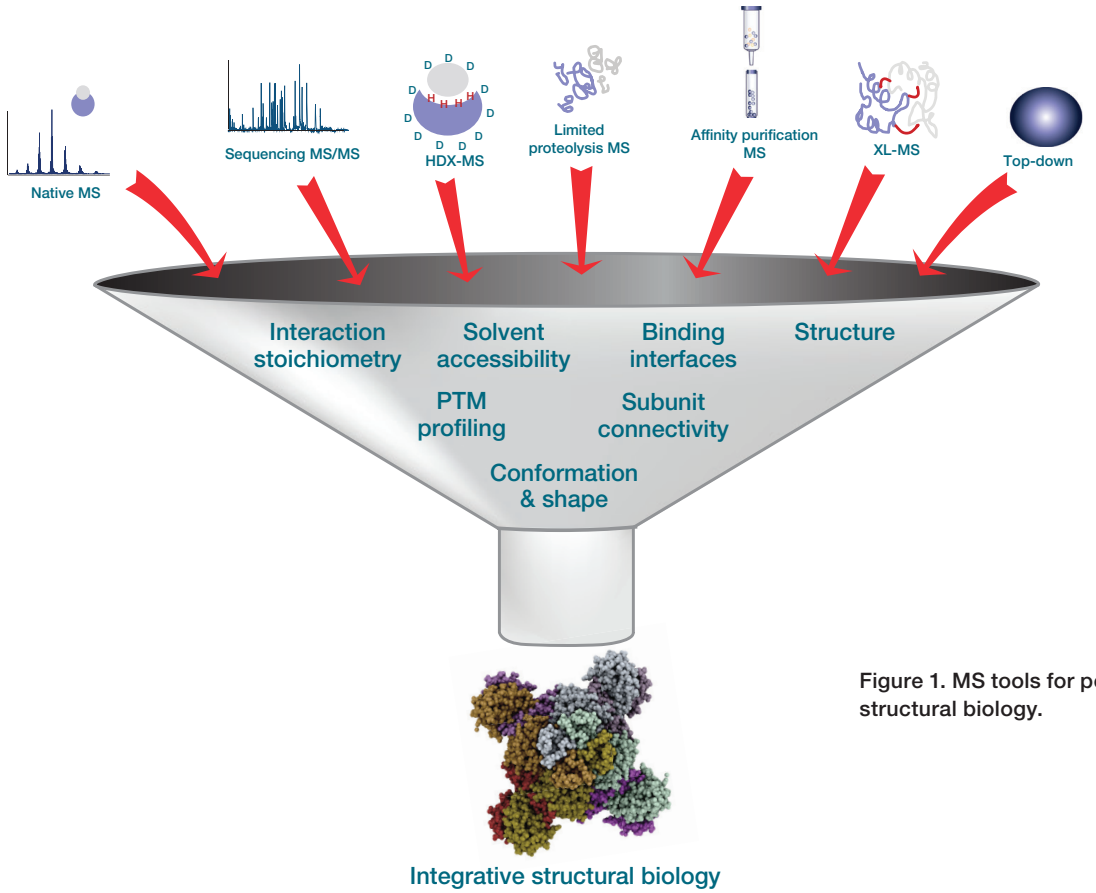

Figure 1. MS tools for performing integrative structural biology.

HDX-MS workflow

All HDX-MS experiments involve deuterium labeling prior to MS analysis. Specifically, the protein is incubated in a deuterium buffer, which allows for the amide hydrogens present on the protein backbone to exchange with the deuterium buffer. As mentioned previously, the most commonly used labeling approach is continuous labeling, in which a protein in its steady state is incubated in deuterium buffer continuously over different time periods and the exchange of hydrogen to deuterium is measured as a function of time. The time period can span from seconds to several hours or days. After labeling, the samples are quenched by lowering the temperature of the experiment to 0 °C and the pH of the reaction to 2.5. HDX-MS experiments can be performed in either a bottom-up or a intact/top-down fashion (Figure 2).

Bottom-up HDX-MS

Identification

The most commonly used strategy for HDX-MS is to digest the proteins into peptides and analyze them using MS (Figure 2). This ensures complete sequence coverage and captures region-specific information from the protein. Before hydrogen-deuterium exchange is performed, the protein is digested and analyzed in a data-dependent fashion using multiple fragmentation techniques (collision-induced dissociation [CID], higher-energy collisional dissociation [HCD] and electron transfer dissociation [ETD]). The goal is to identify as many overlapping peptides as possible. This is done to maximize sequence coverage of the protein for identification. This process is followed by the HDX-MS experiment. Since low pH is used in HDX-MS experiments to minimize deuterium back-exchange, acidic enzymes such as pepsin are the preferred tools for digestion. The digestion can be performed in solution or on immobilized pepsin columns, the latter being the preferred approach. Currently available commercial platforms, such as the H/D-X PAL™ Hydrogen Deuterium Exchange sampler system (LEAP Technologies), enable automated labeling and digestion. Upon digestion, the samples are desalted on a trap column and separated using reversed-phase chromatography prior to analysis by MS.

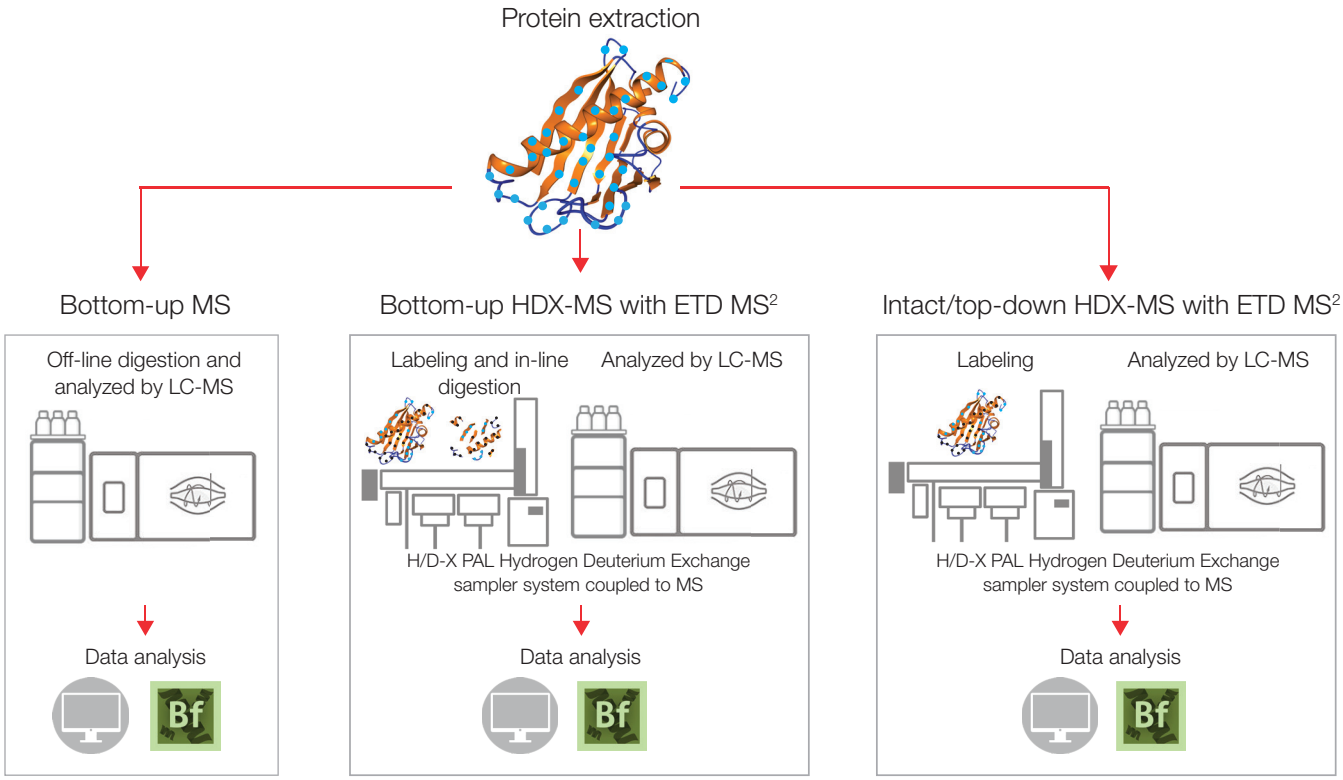

Figure 2. Schematic representation of HDX-MS workflows.

Deuterium uptake information

The HDX-MS experiment can be performed in two ways. The first method is to collect full-scan MS to get deuterium uptake information on peptide levels to probe the protein conformation. The second method is to access higher-resolution amino acid level information and thus requires acquiring peptide fragment level deuterium uptake data. Here a full-scan MS with data-dependent ETD MS^2 is acquired for both unlabeled and deuterated samples. With this design, the full-scan MS experiment establishes the peptide-level deuterium incorporation value, while the higher resolution, single amino acid level deuterium incorporation value is obtained by ETD MS^2. In this case, ETD is preferred over CID or HCD to avoid deuterium scrambling, a known phenomenon when energy-driven fragmentation methods are employed.[2-5] During scrambling, the protons on the peptide backbone migrate and don't reflect the state of the peptides in solution. Studies have shown that ETD, a non-ergodic fragmentation technique, is far better suited as an activation choice due to the very low levels of hydrogen scrambling that occur during this process; therefore ETD allows for an accurate localization of incorporated deuterium at the single residue resolution level.[6,7]

Intact/top-down HDX-MS

The alternative to bottom-up HDX-MS is intact/top-down analysis. In intact/top-down HDX-MS, proteins are introduced into the mass spectrometer after deuterium exchange without any digestion. For complex mixtures, some level of separation is performed before introducing proteins into the mass spectrometer, typically using a C4 column. Deuterium uptake may be measured at the intact level or ETD may be employed to sequence the proteins. The primary types of information obtained by HDX-MS are briefly explained below:

- **Protein or protein complex conformations:** A protein or protein complex can have multiple three-dimensional shapes, known as conformations. HDX-MS can provide information on the conformational differences between different states of a protein or protein complex, and can help elucidate a protein's structured versus unstructured regions.[8]
- **Protein dynamics:** A protein or protein complex can undergo conformational changes to form new three-dimensional structures, known as protein dynamics. HDX-MS can provide information on the various short-lived intermediate structures and the series of events that led from one state to another.[9-11]
- **Biomolecule binding:** This process provides information on the interaction interface between different subunits or ligands. The interactions may occur between a protein and another protein, or between a protein and ligands such as nucleic acids, lipids, glycans and small molecules. The locations of the sites on the protein involved in the binding can be ascertained, as well as how ligand binding affects protein conformation.
- **Allosteric effects:** HDX-MS provides information on the effects of ligand binding on protein sites other than the binding site, or throughout the whole protein.
- **Intrinsic disorder:** The process provides information on proteins that lack a three-dimensional structure. This can be the entire protein or part of a protein that exists as flexible polypeptides or loops.
- **Protein aggregation:** HDX-MS provides information on the regions involved in protein aggregation, conformational changes and the intermediate structures that form during aggregation.

Protein or protein complex conformations

The three-dimensional structure of a protein is not static. As a protein performs its functions, it can rearrange itself to form new three-dimensional structures referred to as its conformations. HDX-MS can provide information on the conformational differences. HDX-MS experiments are conducted over multiple states or time points, and the amount of deuterium uptake can vary depending on the protein's structure. Flexible regions (e.g., loops) will exchange faster, and more structured regions and those in the core of the protein will exchange slower (Figure 3).

In order to obtain finer details on specific regions within a protein, the protein can be digested into peptides. The deuterium uptake can then be measured and plotted onto the three-dimensional structure of the protein.

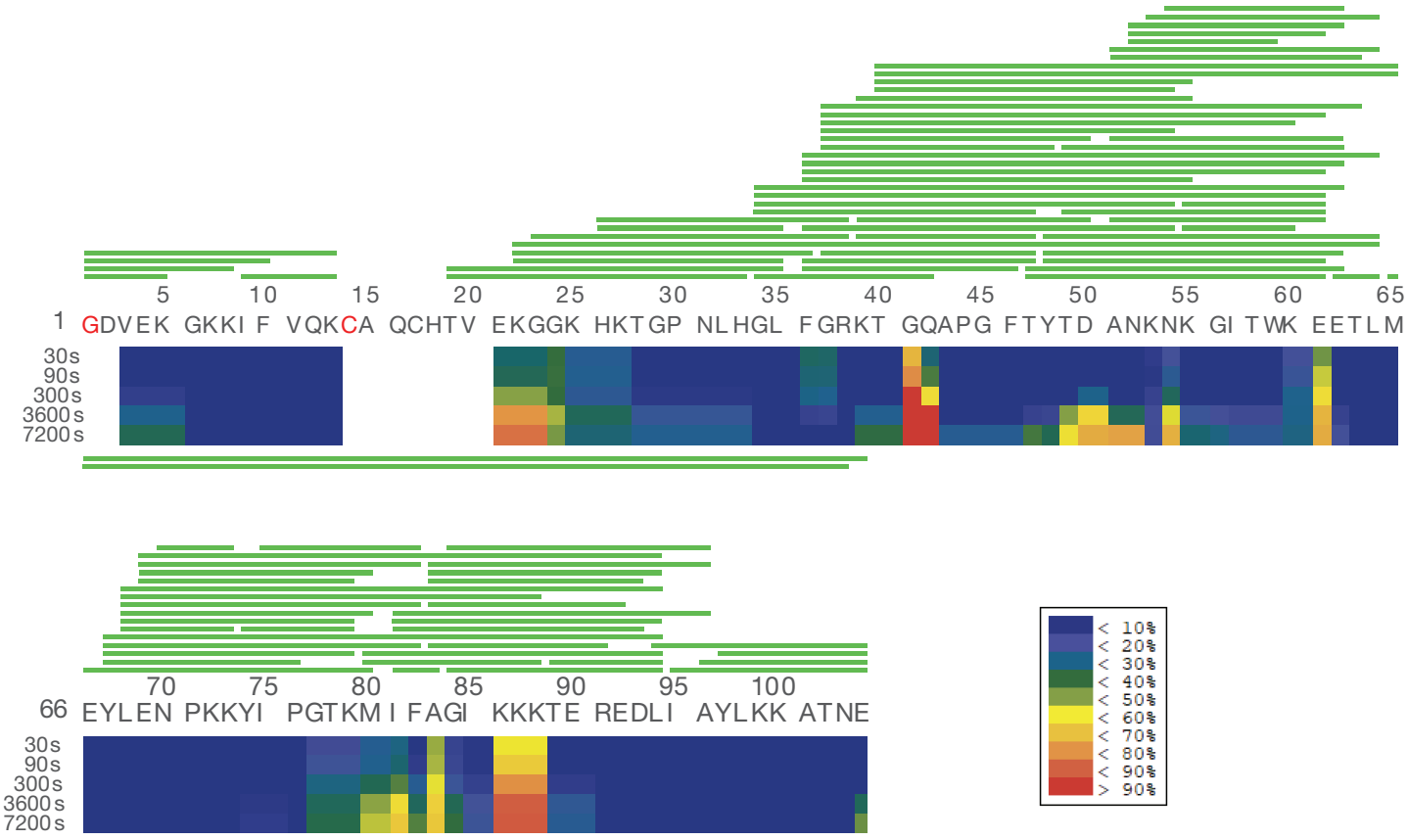

Figure 3. Cytochrome c deuterium uptake heat map. Deuterium uptake ranges from low (blue) to high (red) depending on the protein's conformation.

Protein dynamics

A protein's function is not determined by its fixed structure but rather by the various changes the structure undergoes. In order to understand protein function, researchers need to characterize the changes between the static states and the events that lead from one state to another. Therefore, researchers undertake differential HDX-MS experiments to monitor the sequence of events. The experiments performed for protein dynamics are very similar to examining protein structure, as there are overlaps in the information obtained from them. However, the big difference is that far more information is needed about the various intermediates that form as a protein transitions from one state to another.

There are several different approaches taken by researchers in these experiments, which are referred to as pulsed HDX-MS[9-11] or continuous-labeling HDX-MS. The naming comes from the length of the hydrogen-deuterium exchange windows. Continuous-labeling is the more widely used approach, in which protein conformations are continuously exposed to deuterated solvent. Multiple measurements are taken varying the time of exposure. The samples are then digested to their peptide components and analyzed by MS. Using dedicated software, differential analysis can be performed at both the peptide level and the individual amino acid level.[12] Figure 4A compares the individual amino acid level protection plots of two samples. Finally, the data can be mapped to three-dimensional structures generated from complementary approaches to understand the protein dynamics that are occurring (Figure 4B).[13]

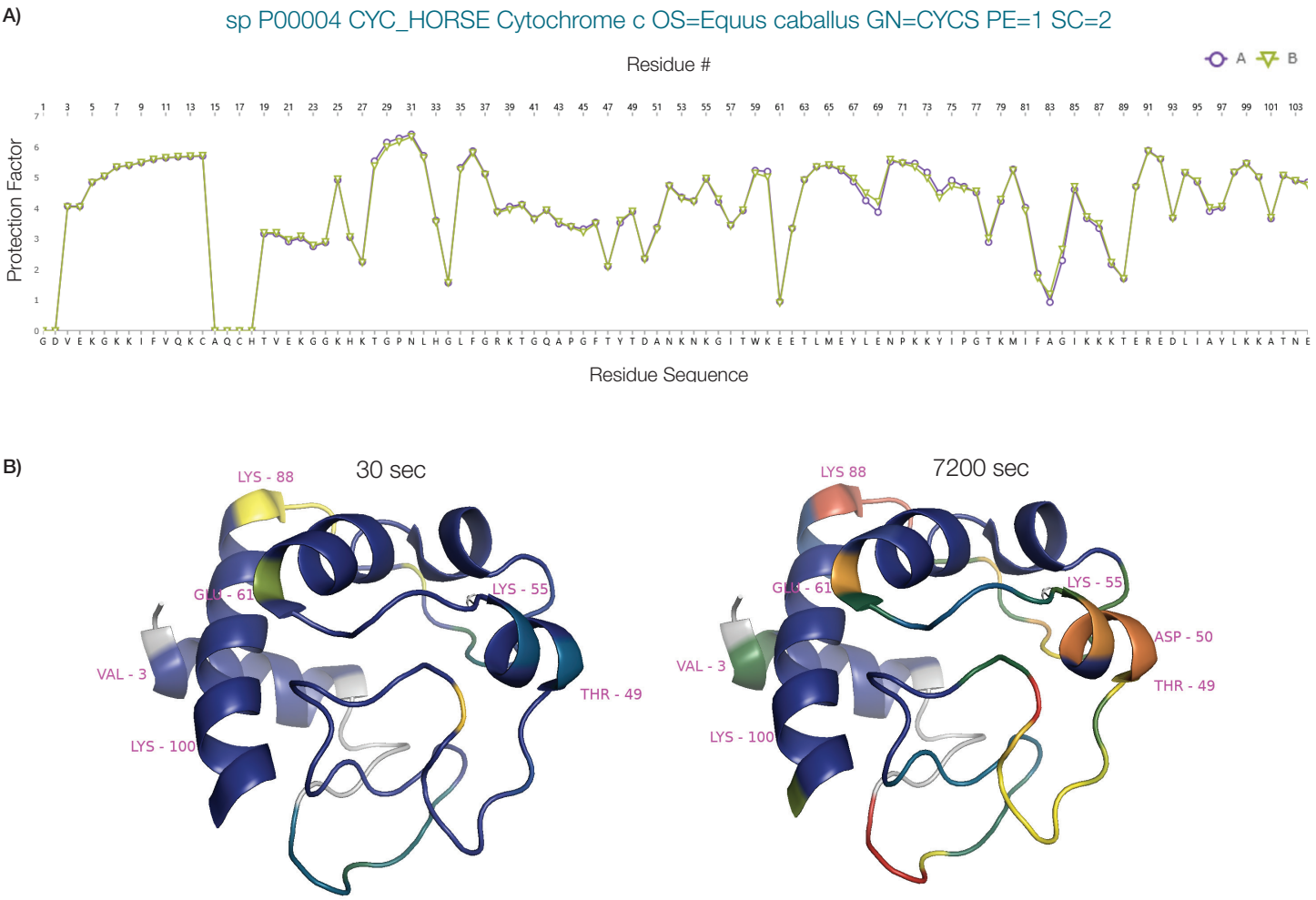

Figure 4. Example of continuous labeling experiment. (A) Cytochrome c protection factor plots of different experiments: purple (sample A) and green (sample B). (B) The deuterium uptake mapped onto cytochrome c crystal structure (PDB: 1HRC). The deuterium uptake increases over time.

Protein-ligand interactions

HDX-MS can aid in the localization of protein-ligand interaction sites. The experiments consist of analyzing a protein with and without ligands in the presence of deuterium, enabling the measurement of differences in deuterium uptake. The areas where protein-ligand interaction occurs exchange at a much slower rate than the regions that do not participate in the interaction, as the former tend to be shielded while the latter are much more exposed to deuterium. MS data is then used to determine proton exchange rates, helping to localize interaction sites and elucidate their structural information.

Further, ligand binding can also affect protein conformation and dynamics. Similar differential HDX-MS experiments can be performed. Samples are analyzed by bottom-up approaches, and higher resolution can be obtained when many overlapping peptides are detected.[14] Deuterium uptake can be used to pinpoint conformational changes

The differential HDX-MS data obtained here can reveal the stabilization or destabilization of a protein's region upon ligand binding. Regions that show slower deuterium exchange indicate stabilization, while increases in deuterium exchange indicate destabilization (Figure 5).

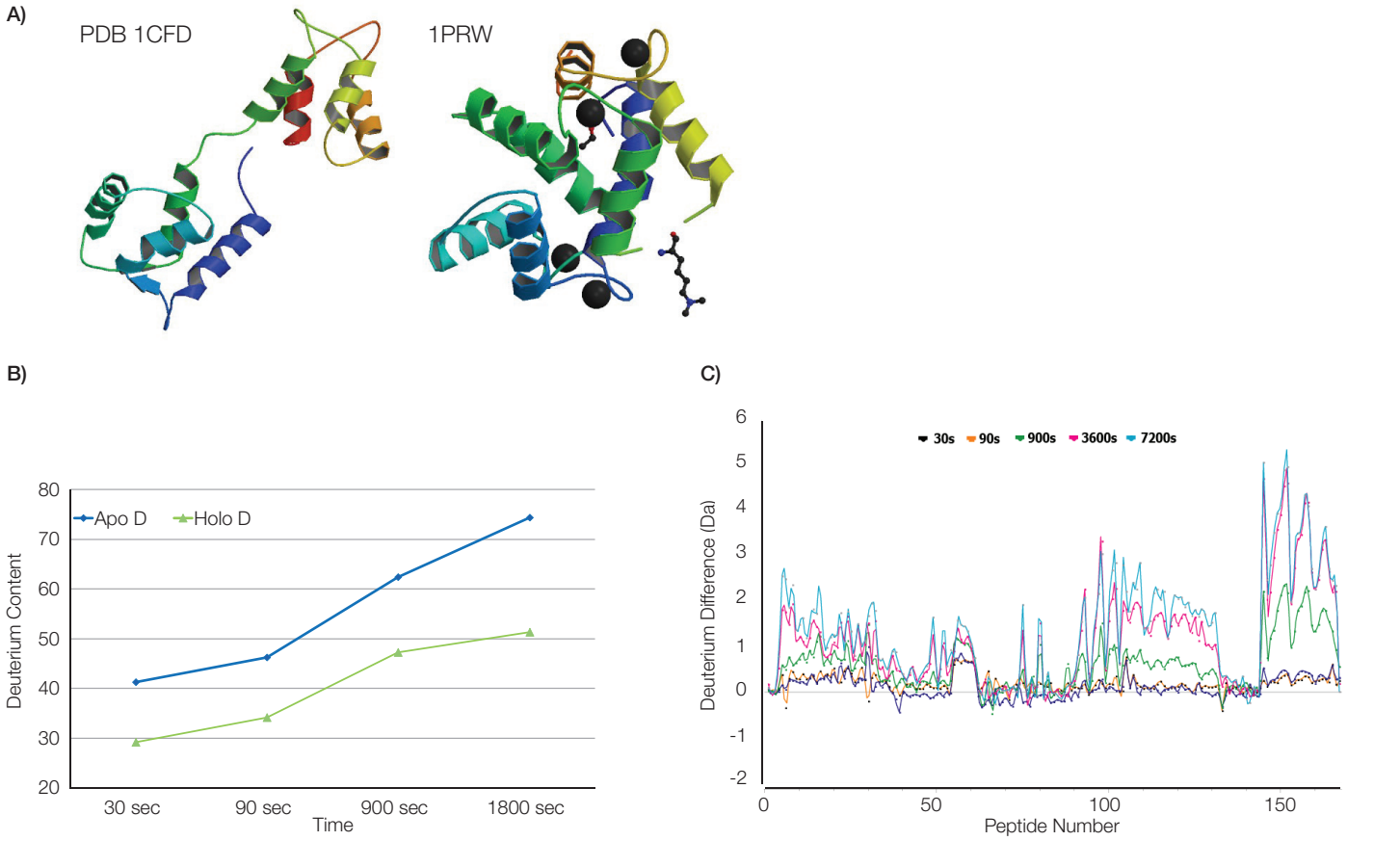

Figure 5. (A) Crystal structure of calcium-binding protein calmodulin with and without calcium (apo and holo, respectively). (B) Deuterium uptake plots for intact calmodulin apo and holo forms. The holo form has a more rigid structure than the apo form, with less deuterium uptake. (C) Apo vs holo calmodulin peptide deuterium incorporation residual plot. All the peptides from the holo form show less deuterium incorporation than those from the apo form. C-terminal peptides show more differences than N-terminal peptides.

Allosteric effects

The conformational changes arising from ligand binding are not restricted to the binding site but may extend to other regions of the protein and will be reflected in the deuterium uptake measurements in these regions. By comparing the protection factors from HDX-MS data, the allosteric changes occurring throughout the protein can be determined (Figure 6).

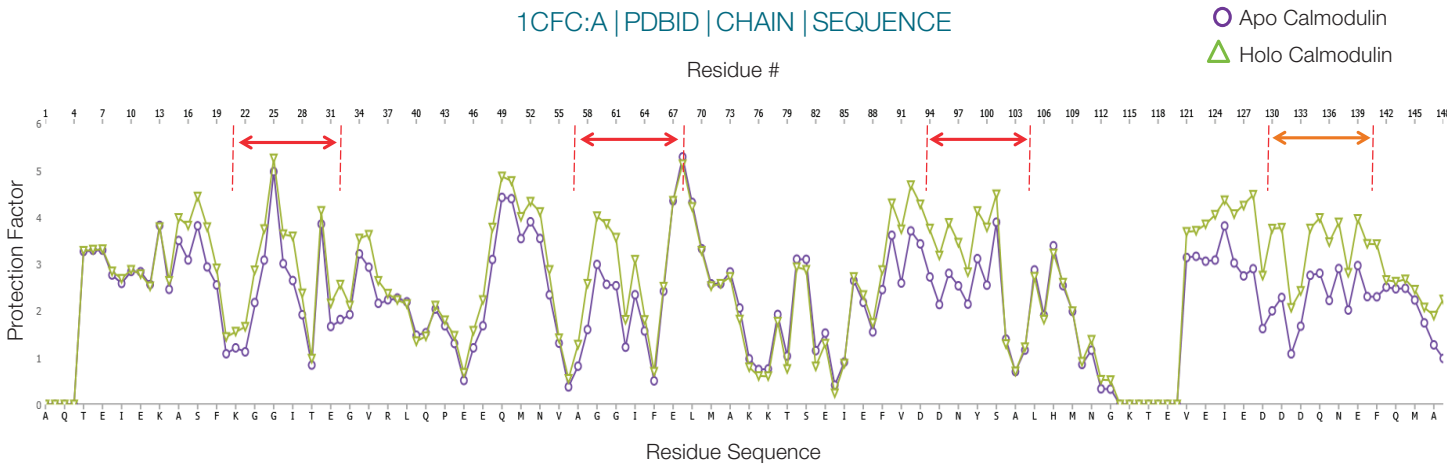

Figure 6. Allosteric effects of calcium binding for calmodulin structure. Protection factor plot for apo vs holo calmodulin is shown. Calmodulin has four calcium binding sites, indicated in orange. However, differences are observed in regions outside of the calcium binding sites.

Intrinsic disorder

HDX-MS can also provide information on intrinsically disordered proteins or protein regions.[15] These are proteins or parts of proteins that exist as flexible polypeptides and lack a three-dimensional structure. HDX-MS can provide information on the dynamics of these proteins and can be used to study ligand binding. The primary challenge with using HDX-MS experiments to study intrinsically disordered proteins is their rapid hydrogen-deuterium exchange rate, which is faster (millisecond) than the time period used in typical HDX-MS experiments. In order to circumvent this issue, experiments may be executed at a lower-than-physiological pH, where the exchange rate is slower. This process expands the time required (from micro to millisecond range) to measure the HDX kinetics of the intrinsically disordered region.

Protein aggregation

It is important to understand the mechanism of protein aggregation and the conformational changes of the aggregates when dealing with therapeutic proteins. The aggregation of therapeutic proteins can result in an incorrect drug dosage, or an undesired and potentially fatal immune response. Consequently, monitoring protein aggregates is crucial for safety and quality assurance. HDX-MS can also be used to probe protein aggregation. The experiments involve looking at aggregated and non-aggregated forms of the protein at multiple time points. Upon deuterium uptake, samples are quenched and analyzed by peptide mapping using MS^1 spectra. Peptide-level deuterium uptake plots are constructed to pinpoint regional-level conformation changes. Figure 7 is an example of aggregation study of monoclonal antibody drug Trastuzumab using HDX-MS. The results showed that the degree of deuterium uptake increased with the degree of aggregation, implying that aggregation would impact the three-dimensional structure of the protein.

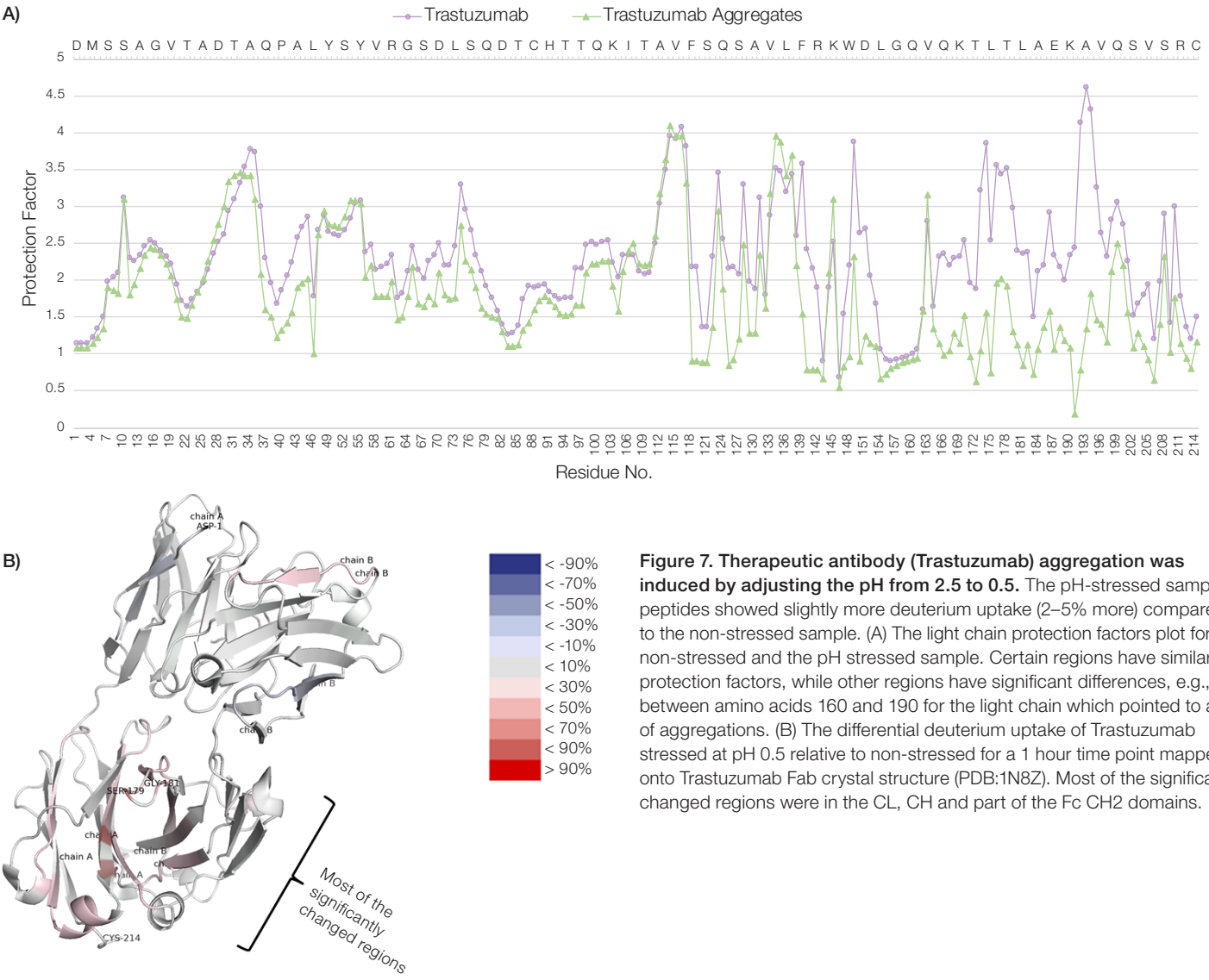

Figure 7. Therapeutic antibody (Trastuzumab) aggregation was induced by adjusting the pH from 2.5 to 0.5. The pH-stressed sample peptides showed slightly more deuterium uptake (2–5% more) compared to the non-stressed sample. (A) The light chain protection factors plot for the non-stressed and the pH stressed sample. Certain regions have similar protection factors, while other regions have significant differences, e.g., between amino acids 160 and 190 for the light chain which pointed to area of aggregations. (B) The differential deuterium uptake of Trastuzumab stressed at pH 0.5 relative to non-stressed for a 1 hour time point mapped onto Trastuzumab Fab crystal structure (PDB:1N8Z). Most of the significantly changed regions were in the CL, CH and part of the Fc CH2 domains.

Technical advances in MS

MS has made it possible to study protein and protein complex structures, making these experiments accessible to a wider community of researchers. These days, mass spectrometers are available in research institutes, providing services and resources to scientists engaged in proteomics experiments. The same instruments can be used for structural protein work, making it possible to elucidate proteins and protein complex structures. By tapping into these resource laboratories, structural biologists can obtain a much more complete picture of protein structures.

However, there are challenges associated with HDX-MS. Primarily, researchers are dealing with samples that are heavily modified by deuterium, resulting in very wide isotopic envelopes in the mass spectrum. This issue, along with large number of peptides eluting in a short liquid chromatography (LC) time scale, can result in overlapping peaks in the mass spectrum making it tough to resolve them. In HDX-MS, the mass spectrum acquisition requires mass spectrometers with ultra-high resolution to resolve these peaks and assign accurate charge states and masses to the peptide precursors. Also, the resolution needs to be high enough to distinguish between small mass differences that can aid in revealing ligand binding sites, PTMs and interactions. Top-down HDX-MS experiments also benefit from mass spectrometers that have high-resolution and accurate-mass measurements. Such instruments can help mitigate overlapping charge states and minimize mass interferences for proteins, making it easier to assign correct charge states and masses. The emergence of Thermo Scientific™ Orbitrap™ mass spectrometers has benefited the field of HDX-MS. These instruments provide ultra-high resolution, high mass accuracy, high sensitivity and high acquisition speeds, all of which are mandatory requirements for performing both bottom-up and intact/top-down HDX-MS experiments.

HDX-MS has benefited immensely from advancements in fragmentation, as well as the availability of multiple fragmentation techniques (CID, HCD, ETD, electron-transfer and higher-energy collision dissociation [EThcD], ultraviolet photodissociation [UVPD]) in a single mass spectrometer. The use of multiple fragmentations enables confident identification and sequence coverage of peptides and proteins. ETD has made a big difference in HDX-MS experiments due to its low HDX scrambling effect and its ability to provide information at the single amino acid level.[6,7] UVPD fragmentation is a recent implementation on commercial mass spectrometers. Studies have shown that when used in parallel with ETD, UVPD can provide complementary sequence information which enables amino acid level resolution for top-down HDX-MS experiments.

Data analysis

One of the biggest bottlenecks in HDX-MS experiments is data processing. HDX-MS data analysis involves identification of the peptides and/or proteins as well as the calculation of deuterium content for each identified peptide. The large number of data points in a conventional HDX-MS experiment makes this task cumbersome and time-consuming. In addition, many HDX-MS projects require the statistical cross-comparison of many data sets. In recent years, a number of software tools have emerged for HDX-MS. However, they tend to be developed for in-house use in research laboratories and are not available to the wider community. Those that are available to broader community have issues with ease of use, developer support and software stability.

Thermo Scientific™ BioPharma Finder™ software's HDX-MS support was developed to address these shortcomings. It is a comprehensive software package that can perform both peptide identification and HDX-MS data analysis. The HDX model simulates the hydrogen-deuterium exchange and back-exchange process at the single-residue level. The protection factor plot can be used to evaluate the whole protein's conformational properties.

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Conclusion

HDX-MS can be used to obtain protein structure and conformation information. Additionally, for protein complexes, HDX-MS can provide information on protein-protein or protein-ligand interaction sites and conformational changes induced by PTMs. It is also complementary to traditional techniques such as cryo-EM, X-ray crystallography, NMR and MS structural biology techniques, such as crosslinking mass spectrometry (XL-MS). For example, HDX-MS experiments can help guide decisions about which complementary techniques to use to acquire additional information.

Recent innovations in hardware—such as improvements in mass resolution, sensitivity, acquisition speed, the introduction of multiple non-ergodic fragmentation techniques such as ETD, UVPD and PTCR, and the ability to perform multiple fragmentations in a single experiment—have allowed researchers to study structures that were previously inaccessible. Advancements in software and overall workflow for HDX-MS are making it possible for the workflow to leave specialized research laboratories and finally enter the mainstream.

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