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REVIEW ARTICLE

HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY AND ITS UTILIZATION

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Summary

Hydrogen/deuterium exchange connected with mass spectrometry is increasingly applied for the interrogation of protein conformation, mapping protein dynamics, identification of protein-ligand interaction sites, and allosteric conformation changes. The dynamics of protein changes is determined with the m/z value of deuterated and non-deuterated protein or percentage of deuterium incorporation for the digested peptides. Hydrogen/deuterium exchange data are processed with selected software and finally quaternary structure of protein is visualized showing how different protein and ligand chains hook up with each other. Obtained results can help understand how protein interacts with its ligand and elucidate the role of this complex in a living organism. Utilization of this method is demonstrated by the amyloid-beta peptide aggregation associated with Alzheimer´s disease; determination of the structure toxin-co-regulated pili *Vibrio cholerae* in connection with its pathogenesis or revelation of binding sites on Mouse double minute 2 homolog complex with small molecule Nutlin-3 which is important for elucidation of drug effects in cancer research.

Key words: mass spectrometry; protein-protein interaction

INTRODUCTION

Many biophysical methods such as circular dichroism, differential scanning calorimetry, and ultra centrifugation, measure characteristic properties of a protein and provide rather global information about protein behavior. Only X-ray crystallography (crystallography) and nuclear magnetic resonance (NMR) give localized and high-resolution information about protein structure, however, in spite of substantial effort to develop these technologies they still have limitations in applicability and throughput. NMR is limited by protein size and crystallography offers only static snapshots. Besides that, both techniques require special conditions for preparation of protein samples, such as solid state for crystallography or high protein concentrations for NMR. The solid state is limiting for many membrane proteins and intrinsically disordered proteins inherently non-crystallizable and therefore they are not measurable by crystallography. Moreover, in many cases the static protein structures alone are not sufficient to describe their function. These difficulties can be overcome by hydrogen/deuterium (H/D) exchange approach monitored by mass spectrometry (MS). This method is based on a chemical reaction when covalently bonded amide hydrogen in the peptide bonds

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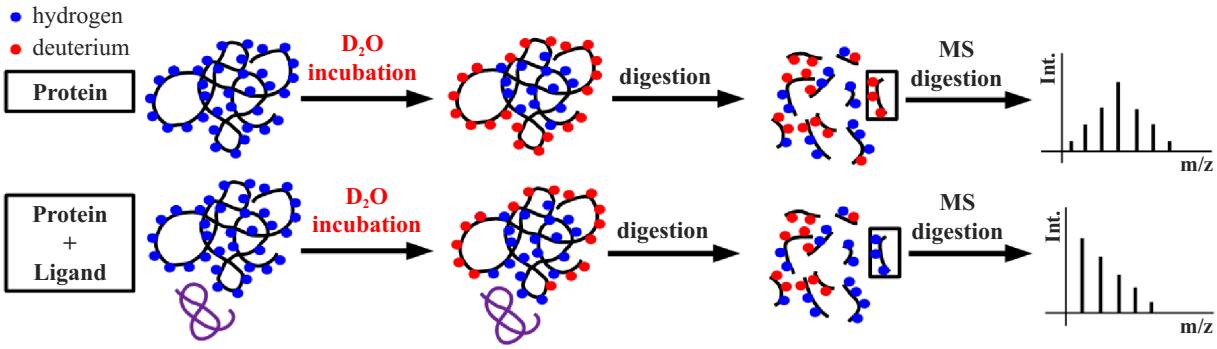

Figure 1. Monitoring of hydrogen/deuterium exchange. Protein free or with ligand is cultivated in buffer with H_2O under physiological pH and room temperature. Hydrogen/deuterium exchange starts with the dilution of protein with the same buffer with D_2O. Hydrogens buried inside of protein structure or shielded by ligand are exchanged slower than hydrogens on protein surface. Differences in deuterium uptake in digested proteins are detected by mass spectrometry.

of the proteins is replaced by deuterium atom. The exchange reactions are based on the comparison of solvent accessibility of various parts of the proteins in solutions with either water (H_2O) or deuterium oxide (D_2O). This powerful technique enables to study protein structure [1], protein dynamics, protein-protein interactions [2], and conformational flexibility related to function properly. Main advantages of this method are requirement of low protein concentration (500 - 1000 picomols), virtually no limitation of protein size or protein complex, as well as high speed, dynamics and flexibility of analysis.

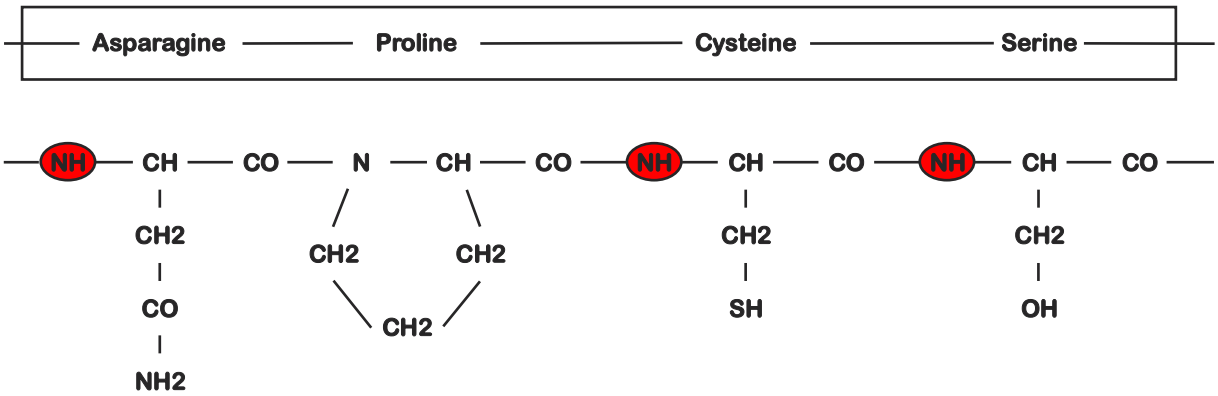

Figure 2. Polypeptide with highlighted hydrogens at the backbone amide positions (red) exchanges for deuterium atom at rates that can be measured by mass spectrometry.

OVERVIEW OF H/D EXCHANGE METHOD

Sample preparation

H/D exchange followed by MS analysis derives the protein structure dynamics based on the mass increase of a protein when the backbone protons are exchanged with solvent deuterium (Figure 1) [3]. The hydrogens located within the peptide backbone amide linkages are highly exchangeable and therefore most easily measured by H/D exchange MS. The location of these hydrogens is displayed in the structural formula of polypeptide (Figure 2) when each amino acid has one amide hydrogen except proline. H/D exchange analysis usually starts with the addition of deuterated buffer to the lyophilized or concentrated samples. The H/D exchange rates are determined at various incubation time points ranging from seconds to days for each protein state. After a defined incubation period the H/D exchange reaction is quenched, directly by addition of acidic solution into sample resulting in the pH shift to ~ 2.5 and followed by rapid freezing. Quench conditions minimize the back H/D exchange during subsequent procedures.

Moreover, low pH conditions allow using acid-stable proteases like pepsin or alternative acid proteases with different cleavage preferences (protease type XIII, rhizopuspepsin, nepenthesin) [4]. Digestion times usually range from 10 to 180 sec at temperature 0°C. Generally, H/D exchange uses two approaches for analysis of proteins. The first procedure, global analysis, is focused on global reshaping of intact protein cultivated in different biological states. Commonly at least two different states of the proteins are compared by this method. Chosen states depend on the type of study, for example structure changes caused due to protein mutation [1, 5] posttranslational modification; interaction of proteins with ligand [6] or environmental changes like temperature or pH. The overview of global analysis workflow is showed in Figure 3. The more detailed protein analysis able to reveal structure changes in the peptide level is named local analysis. The main steps of analysis are described in Figure 4. Related approach to H/D exchange is PLIMSTEX (protein ligand interaction by mass spectrometry, titration, and H/D exchange), the strategy which determines the conformational change, binding stoichiometry and affinity in a variety of protein-ligand interactions including those involving small molecules, metal ions, and peptides [7].

The peptide mapping

The peptide mapping of protein incubated in H_2O buffer is performed before each H/D exchange analysis to obtain experimental data used for evaluation of H/D results. The goal of peptide mapping consists of measurement and identification of all peptides which cover whole amino acid protein sequence, their charge and retention time (workflow is described in Figure 4). Tens or hundreds of peptides (depending on molecular weight of proteins) are typically generated when protein is digested by acid protease. Prior to mass spectrometry analysis, the peptides are separated by High Performance Liquid Chromatography (HPLC) to minimize mass overlap and suppression of peptide ionization. The HPLC eluent is introduced directly into ion source and the peptide ions are measured in mass spectrometer in data dependent mode. The spectra created by fragmentation of selected peptide peaks are evaluated and verified to identify the amino acid sequence for each parent peptide ion.

H/D exchange measurement

Samples for **global analyses** are measured directly without digestion. Quenched sample of intact protein at the temperature near 0°C is eluted from HPLC to mass spectrometer and m/z of protein is measured. This measurement monitors the mass of the protein which increases during the time-course of the deuteration as more backbone amide hydrogens are being exchanged for deuterons. When this protein interacts with a ligand and/or undergoes structural changes accompanied by changes in hydrogen bonding then the deuteration kinetics is altered.

Samples for **local analyses** are digested before measurement. In order to localize regions of protein involved in binding or structure changes, the deuterated protein is subjected to enzymatic digestion followed by mass spectrometric analysis. Quenched freezed protein sample is thawed immediately before analysis to temperature near 0°C and is injected into column with immobilized protease. Then the peptides are trapped and desalted on-line on peptide microtrap column, eluted onto an analytical column and separated by a linear gradient. Subsequently, peptides are eluted directly to mass spectrometer and their m/z is measured. Time of HPLC separation should be as short as possible and columns are kept at 0°C to minimize the loss deuterium through back exchange with the solvent.

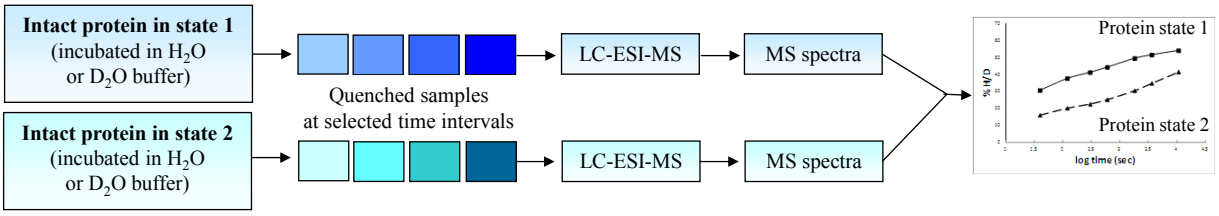

Figure 3. H/D exchange global analysis
H/D exchange reaction starts by the dilution of intact proteins in deuterated buffer. For every protein state, one undeuterated sample as control is performed. At selected time intervals the exchange reaction is quenched and samples are directly measured within the global analysis without digestion. Differences in the incorporation of deuterium between protein states are visualized in deuterium uptake plots.

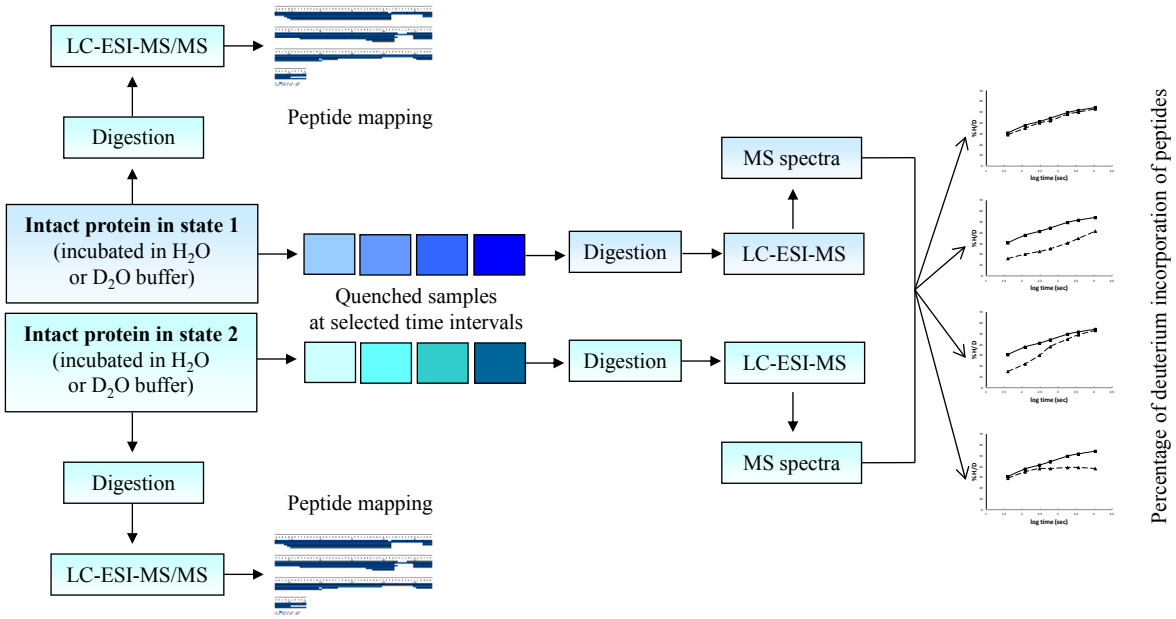

Figure 4. H/D exchange local analysis
In local analysis the peptide mapping is performed before each H/D exchange experiment to detect all digested peptides which are identified by tandem mass spectrometry. H/D exchange reaction is initiated by the dilution of proteins in deuterated buffer. The reaction is quenched at selected time intervals and proteins are digested before measurement. Peptides obtained by mapping are used for its detection in H/D exchange data. Results are visualized by deuterium uptake plots for every detected peptide.

H/D exchange data evaluation

Data processing from H/D exchange experiment always represents a challenging task and various software platforms have been developed. Most of them focus on calculating the m/z value from raw mass spectrometry data for the deuterated peptide, and then evaluate the m/z value increase on the basis of time intervals.

Manual evaluation involving peptide sequence assignment, retention time alignment, and centroid mass extraction from the large number of data points is cumbersome and time-consuming. In addition, the data-mining of H/D datasets require data displaying and statistical cross-comparison which is still lacking in the most of the currently available software. Nevertheless, recently some computational methods and tools for data processing and visualization with reasonable accuracy and limited human intervention were developed. Free-available for non-commercial purposes HDX Workbench [8] is a standalone application that combines a complete analysis workflow with a full graphical user interface and therefore offers rich data visualization with a possibility of manual data correction. A commercial program HDExaminer [9] offers faster data analysis and comparison of more than two experiments.

Structure modeling and molecular docking

The knowledge of three-dimensional protein structures (or their homology with related group of proteins) is critical for structure modeling and brings benefits to the interpretation of H/D exchange results. The structure changes with molecular docking help create the quaternary structure of protein showing how different protein and ligands chains hook up with each other. Moreover, H/D exchange data and known tertiary protein structure are also valuable for the animation of folding and unfolding processes. For visualization of H/D exchange results on protein spatial structures, molecular graphic packages PyMOL (Schrödinger, LLC) [10] and Visual molecular dynamics [11] are used.

UTILIZATION OF H/D EXCHANGE METHOD

Many biological methods and predictive studies followed by experimental confirmation have tried to reveal behavior of protein complexes, their structure changes, binding sites, and functions. This knowledge is necessary for understanding the cellular functions

and biological processes as a whole unit in a macroorganism. On the other hand, the failure of protein interactions causes many diseases. Therefore, several reports are concerned with studying the interface properties of proteins and elucidating their role in the interaction networks.

H/D exchange approach was applied for the characterization of Alzheimer´s disease that causes dementia among old people. The investigation was focused on probing into the conformation changes of the amyloid-beta (Aβ) peptide aggregation that correlates with the amount of neuritic plaque pathology in the human brain. The pulsed H/D exchange method based on the incubation of aggregated Aβ peptides in the presence or absence of Cu^{2+} ions for 1 min at 0°C was used for this study. The presence of Cu^{2+} slowed down the Aβ42 aggregation and stabilized the soluble Aβ42 species [12].

Another study used H/D exchange and computational modeling for the characterization of structure of toxin-coregulated pili (TCP) exposed on the surface of the bacterial pathogen *Vibrio cholerae*. The authors compared the solvent accessibility of the intact TCP filament with the monomeric TcpA subunit. They precisely identified regions of the pilin protein that are uncovered in the monomeric TcpA subunit. These regions were buried in the intact pilus filament and were involved in the subunit-subunit interactions. Subsequently, these data were used for generating a new molecular computational TCP filament model tested by mutating key residues at predicted subunit-subunit interfaces which would disrupt pilus assembly. This knowledge about TCP filament model elucidates its role in the pathogenesis and reveals a new potential therapeutic target for *Vibrio cholerae*. [13].

H/D exchange has been plentifully used in cancer research namely for studying interactions of proteins with small-molecules which change their structure and function. Nutlins belong among these candidates. These small molecules (cis-imidazol analogs) were discovered by screening a chemical library. Nutlin-3 binds the Mouse double minute 2 homolog (MDM2) protein which inhibits creation the MDM2-p53 complex and blocks the catalytic role of the MDM2 in tagging p53 for proteasomal degradation [14]. Data from H/D exchange study demonstrated two distinct stages to Nutlin-3 interaction sites on the MDM2 N-terminal domain [15].

CONCLUSION

H/D exchange is envisioned as a general tool to support future efforts for the study of protein conformation and dynamics within different fields like medicine, pharmacology, toxicology, microbiology, protein engineering etc. This approach offers diverse conditions for the cultivation of protein and its complexes (temperature, pH, addition of stimulants or inhibitors etc.) at various time intervals. Further advantages of this method are the requirement of low concentration of protein for analysis and possibility to analyze large protein-ligand complexes. Moreover progression of mass spectrometry instruments and bioinformatics involve into detection of structure changes at the amino acid level and single amino acid resolution is possible.

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