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REVIEW

Hydrogen–deuterium exchange mass spectrometry for determining protein structural changes in drug discovery

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Abstract Protein structures are dynamically changed in response to post-translational modifications, ligand or chemical binding, or protein–protein interactions. Understanding the structural changes that occur in proteins in response to potential candidate drugs is important for predicting the modes of action of drugs and their functions and regulations. Recent advances in hydrogen/deuterium exchange mass spectrometry (HDX-MS) have the potential to offer a tool for obtaining such understanding similarly to other biophysical techniques, such as X-ray crystallography and high resolution NMR. We present here, a review of basic concept and methodology of HDX-MS, how it is being applied for identifying the sites and structural changes in proteins following their interactions with other proteins and small molecules, and the potential of this tool to help in drug discovery.

Keywords Hydrogen–deuterium exchange (HDX) · Mass spectrometry (MS) · Protein structure change · Protein–protein and -chemical interaction · Nm23-H1 · UCH-L1

Introduction

Proteins are indispensable players in all biological processes, including enzyme catalysis, metabolism, energy conversion, cell signaling, cell communication, cell structural support, cell growth and death, among others. They perform their diverse functions, via their specific three dimensional arrangement of amino acids. Three dimensional tertiary and quaternary structures are determined by their primary sequences (Anfinsen 1973). A well-defined structure of protein represents the specificity of conformation, while native protein maintains a sufficient degree of dynamics to keep functionality (Frauenfelder et al 1991; Bai et al. 1995; Henzler-Wildman et al. 2007). This is most evident for enzymes that undergo conformational changes during catalysis (Stock et al. 2000; Eisenmesser et al. 2005; Liu and Konermann 2008).

Under physiological conditions, proteins perform their biochemical functions through interacting with target proteins, or post-translational modifications (PTMs) including phosphorylation, acetylation, glycosylation and oxidation, at both global and local level (Xin and Radivojac 2012). Tertiary and quaternary protein structural changes can also occur by protein–protein and protein–ligand interactions, and PTMs. These structural changes regulate the biological functions of proteins. Therefore, progress in small compound drug discovery and protein therapeutics depends on structural analysis of higher order protein complexes (Chen et al. 2011).

Many analytical tools for characterization of protein structures and conformational transitions are currently available: Capillary electrophoresis (CE); surface plasmon resonance-based assays (SPR); spectroscopic methodologies such as UV–visible spectrophotometry; Foster resonance energy transfer (FRET); Fourier-transform infrared spectroscopy (FTIR); nuclear magnetic resonance (NMR); small angle X-ray scattering (SAXS); circular dichroism (CD); calorimetric techniques such as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) (Vuignier et al. 2010; Huang and Chen 2014). CD spectroscopy is useful in observing changes in the secondary and tertiary structures of proteins and monitoring protein–protein interactions, although not for getting information that

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helps in quantitative structural interpretations (Greenfield 2004). FRET analysis has been used to study time-resolved enzyme catalysis and protein–ligand interactions. It covers a large sensitivity range but its ability to provide biologically meaningful information diminished because of the presence of the probes (Rindermann et al. 2011). NMR spectroscopy has contributed to solving the structures of low molecular weight proteins (<30 kDa), but not the kinetics of conformational dynamics. Furthermore, it requires high protein concentrations, which could result in protein aggregation (Montelione et al. 2000). In contrast, X-ray crystallography is the gold standard for investigating the structures of higher order protein complexes at high atomic resolution. However, this technique has also limitations such as requirement for large amounts of samples, difficulties in monitoring protein dynamics, and in preparing protein crystals (Hassell et al. 2007).

Mass spectrometry (MS) has become highly useful not only for identifying protein sequences and PTMs (Na et al. 2008), but also for examining their structures, folding and dynamics (Kaltashov and Eyles 2002). With the discovery of electrospray ionization (ESI) in 1980s, ESI–MS made it possible to analyze non-volatile biological macromolecules in the gas-phase and to identify proteins and many PTMs with peptide sequencing with extraordinary speed and sensitivity (Jeong et al. 2012b). Hydrogen/deuterium-exchange combined with ESI-MS (HDX-MS) was first used in probing protein conformational changes (Katta and Chait 1991). Following these improvements, MS analysis was employed in studies of protein structures and dynamics, because it requires a small amounts of sample, has no mass limit and allows rapid processing and can be applied in high-throughput analysis (Huang and Chen 2014; Pacholarz et al. 2012; Wei et al. 2014; Marciano et al. 2014). In addition to providing precise mass and sequence specific information, ion-mobility MS analysis also allows studies of higher-order protein complexes (Borysik et al. 2013). Moreover, investigating exchange rates on a wide time scale with HDX-MS can provide information about protein conformational transitions. Deuterium exchange kinetics reflects the accessibility of a protein to solvent and the dynamics of protein secondary structure. In this review, we discuss fundamental concept and experimental approaches of HDX-MS, recent applications of this technique in identifying the sites of interactions and conformational changes of proteins and its potential role in antibody-based drug discovery.

Basic concept and methodology of HDX-MS

HDX-MS is a widely used to explore protein conformation and protein conformational changes in response to protein- and ligand-interactions in solution, PTMs. HDX-MS methodology is based on the exchange of the protein backbone amide hydrogens with deuterium in D_2O. The backbone amide hydrogens located at the surface of the protein or those involved in weak hydrogen bonds, can exchange readily with deuterium, while those buried inside of the protein or involved in strong hydrogen bonds exchange more slowly. One can obtain information on protein dynamics and conformational changes by measuring hydrogen–deuterium exchange rates of backbone amide hydrogens. There are two main approaches for performing deuterium labeling in HDX experiments:continuous labeling and pulse labeling. Pulse labeling in HDX-MS is mainly used for characterizing protein folding. Most HDX-MS approaches employ continuous labeling for characterization of protein folding and conformational changes by observing differential HDX kinetics. Continuous labeling enables the monitoring of deuterium incorporation into the protein structure as a function of time, providing information on the structural dynamics of protein under each condition. As illustrated in Fig. 1, the differential HDX-MS experiment performed by continuous labeling comprises four steps; (A) deuterium exchange, (B) quenching and protease (pepsin) digestion, (C) LC separation of digested peptide and mass detection, and (D) data analysis.

Protein samples in the absence or presence of chemical or interaction proteins (compound ± or interacting protein ±) are diluted and incubated in the D_2O containing buffer to initiate the labeling process for various times. The excess deuterium insures that the exchange of hydrogen by deuterium is favored (Marcsisin and Engen 2010). Deuterium exchange time, normally seconds to hours, can be optimized depending on the folding characteristics of the protein in question. After allowing time for deuterium exchange, the reaction is quickly quenched with a quenching buffer which reduces pH to 2.5, denatures the protein, and reduces temperature to 0 °C. The quenching step also minimizes back exchange in the subsequent analysis step. Temperature and pH are tightly controlled in order to minimize H/D back exchange. The presence of denaturants such as guanidine HCl and disulfide reducing agents such as dithiotheitol (DTT) or tris(2-carboxyethyl)phosphine)) (TCEP) in the quenching step, ensures denaturation of the protein, which increases their susceptibility to proteolysis. Proteolysis is performed with acidic proteases such as pepsin in HDX-MS analyses because they are active under acidic quenching conditions (Huang and Chen 2014). Digestion with pepsin produces many overlapping peptides which helps increase information of HDX peptides by raising the peptide resolution. In order to increase sequence coverage, SEMSA (selectively excluded MS analysis) technology can be employed (Seo et al. 2008), and other proteases such as fungal protease XIII or XVIII can be used depending on the protein size and its amino acid sequence. The enzyme immobilized

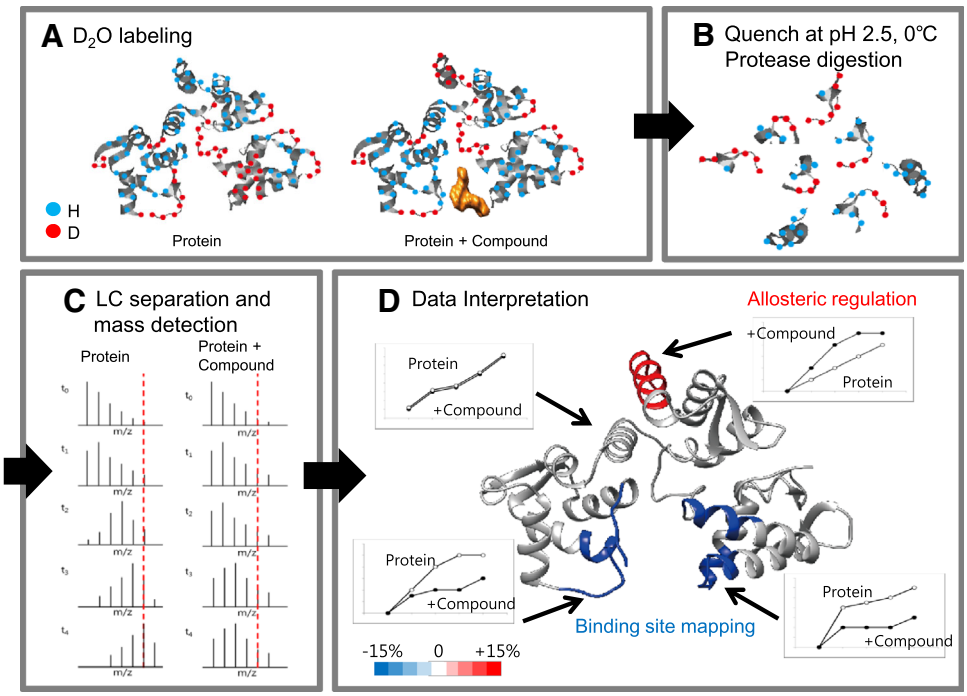

Fig. 1 Basic concept and methodology of HDX-MS

in beads, ensures that it is not injected into the LC/MS system, which can reduce the dynamic range. Following quenching and proteolysis, the peptides are separated by liquid chromatography (LC) coupled with MS. Chromatographic separation at low temperature requires control of back exchange. Short resolving time is critical to minimizing back exchange. It is necessary to identify each peptide released by digestion of the non-exchanged protein by tandem MS before performing the HDX-MS experiments. HDX-MS data analysis can be laborious, because of the large number of peptides generated and associated kinetic measurements. Measuring the percent deuterium exchange for each of the released peptides is achieved by calculating the average mass to charge ratio (m/z) of the peptide ion isotope cluster (centroid approach), or by fitting theoretical isotope distribution to experimental data (Wei et al. 2014). Recently, an user friendly HDX-MS software has become available from Deuterator, HD desktop, DXMS, and HD Express (Chalmers et al. 2011). HDX-MS results can be visualized the difference of deuterium uptake kinetics curve of each peptide (Fig. 1D in box), and the kinetic difference of each peptide are mapped onto crystal structure (Fig. 1D) which makes it possible to find the region of structural change in response to protein-, ligand-interaction or PTMs,

Application of HDX-MS

HDX helps detect conformational changes occurring in proteins during their interactions with other proteins, small molecules and when they are chemically modified biologically or experimentally.

Protein–Protein interactions

Protein–protein interactions occur in many biological processes. Especially understanding antigen–antibody interactions and identifying the epitopes are important in protein therapeutics, because therapeutics with antibody-based drugs are fast growing area. More than 40 antibodies and derivatives are approved for use and more than 300 are being employed in clinical trials. However, it is difficult to identify the interacting domain between antibody and antigen. X-ray crystallography provides the best resolution of the structure of a protein complex, but this technique is possible only if the protein crystal complexes which are difficult to obtain, are stable.

In efforts to discover effective antibody based therapeutics, it is important to have a clear understanding of the science underlying antigen–antibody interactions and identifying the epitope region. Mapping the epitope regions of therapeutic antibodies is required to ensure intellectual properties. High resolution HDX-MS is a cutting edge tool for analyzing protein–protein and protein–ligand interactions under physiological conditions using small amounts of proteins. HDX-MS has been useful in studying protein-proteninteractions (Zhang et al. 2011), and has been significantly extended to studies of antigen–antibody interactions of various kinds (Wei et al. 2014). HDX-MS has also become the most effective method for rapidly

characterizing epitope structure (Malito et al. 2013). This study has examined the binding between monoclonal antibody against factor H binding protein, the vaccine antigen of *Neisseira meningitides,* employing various technologies including peptide arrays, phage display, X-ray crystallography, and HDX-MS. HDX-MS was found to be the best methodology for very rapid epitope characterization with small amounts of sample. HDX-MS is now indispensable tool for examining protein–protein interactions.

G-protein-coupled receptors (GPCRs) are significantly regulated by β-arrestin, which desensitizes G-protein signaling and initiates a G-protein independent signaling. HDX-MS was employed to examine how activated β2 adrenergic receptor (β_2AR)-G-protein recruits β-arrestin. Two separate sets of interactions occur in this case. In the first, phosphorylated carboxy terminus of β_2AR interacts with N-terminal domain of β-arrestin; this is followed by a second interaction that involves the insertion of finger loop of β-arrestin into the receptor core (Shukla et al. 2014), The dynamic and structural information forms the basis for understanding how GPCR is regulated by β-arrestin.

Protein-small molecule interactions

Understanding how proteins interact with small molecules (<800 Da) is necessary for unveiling the effect of small molecules on protein function. In order to determine the modes of action of small molecule therapeutics on target proteins, analysis using X-ray crystallography of co-crystal is needed. Recently, HDX-MS has been extensively employed to verify the binding of small molecules such as therapeutic drugs, natural substrates, metabolites, and ligands to target proteins (Sowole and Konermann 2014). These studies demonstrated that binding to small molecules causes significant structural changes. Different conformational perturbations in proteins have been shown to occur in proteins in following binding to nucleotides and synthetic small molecule activators employing HDX-MS (Landgraf et al. 2013). For example, AMP-activated protein kinase (AMPK) is known to be activated by nucleotides and synthetic ligands. AMP binding induces conformational changes mainly in the γ subunit of AMPK, while binding to synthetic activators causes significant changes in the glycogen binding module of the β subunit. These findings demonstrate that HDX-MS is a useful tool for identifying the conformational changes that occur in proteins following protein-small molecule interactions. HDX-MS was also employed in studies of conformational changes in estrogen receptor α (Goswami et al. 2014), PPARγ and retinoid X receptor α (Carson et al. 2014). Modulation of the vitamin D receptor (VDR) with a ligand has the potential to be useful for the oral treatment of osteoporosis. In order to find lead compounds, screening was carried out to interact with synthetic ligands with VDR using HDX-MS. Significant protections of HDX with synthetic ligands were observed in helixes 3, 7, and 8 of the ligand binding domain, regions which are similar to those seen for the natural hormone VD3. This information provides viable starting points for synthetic expansion (Carson et al. 2014). These studies establish the potential of HDX-MS in small molecule therapeutics.

Protein structural changes by post-translational modifications

Proteins carryout their manifold biological functions by changing their tertiary and quaternary structures during their translational and/or posttranslational genesis. PTMs variously affect the protein's stability, cellular location, and ability to interact with other molecules thereby promoting several biological processes. Post translational structural changes can occur in proteins from various events including mutations, alternative splicing, proteolytic cleavage, chemical and enzymatic modifications in amino acid side chains, which can alter the charge, polarity, spatial features, and induce conformational changes (Seo and Lee 2004). Since PTM-induced conformational changes dynamically vary depending on the nature and degree of modification, highly sensitive and flexible analytical tools are necessary for identifying structural changes they produce. HDX-MS is now recognized as a suitable method for identifying the conformational changes of alternately spliced proteins (Kim et al. 2014), and oxidized proteins (Kim et al. 2013), as like X-ray crystallography for crystallized proteins, and NMR spectroscopy for solution structure of low molecular weight proteins.

Mass spectrometry with hydrogen/deuterium exchange (HDX-MS) was successfully employed for assessing structural changes in proteins such as ubiquitin C-terminal hydrolase-L1 (UCH-L1) in which residues exposed to surface more readily exchange deuterium allowing their mass increases to be detected. UCH-L1 is known as key molecule in neurodegenerative disease (Lowe et al. 1990) and cancer metastasis (Kim et al. 2009). HDX-MS helped determine the structural alterations produced in UCH-L1 by truncation of its N-terminal 11 amino acids (exon 1 deletion). A recent study identified a variant of UCH-L1 lacking N-terminal 11 amino acids (N-terminal truncated UCH-L1, NT-UCH-L1) and found that NT-UCH-L1, in contrast to UCH-L1, is aggregation prone and plays a protective role in an animal model of Parkinson Disease (PD) (Kim et al. 2014). Since NT-UCH-L1 has no enzyme activity, N-terminal 11-peptide seems necessary for catalytic activity. In order to understand the structural basis of these differences, HDX-MS of intact proteins without

proteolysis was employed, in which residues exposed to surface more readily exchange deuterium allowing their mass increases to be detected. HDX occurred slowly in UCH-L1, taking up to 16 h to complete, while in NT-UCH-L1, this exchange was completed in just about 2 min (Fig. 2A, B). This indicates that NT-UCH-L1 has a more open structure than UCH-L1. The flexible regions were identified by analyzing HDX in pepsin-digested peptides (Fig. 2C). More deuterium exchange occurred in the N- and C-termini of NT-UCH-L1 than of UCH-L1, and the peptide containing active site cysteine (^{82}MKQTIGNSCGTIGL^{95}) in NT-UCH-L1 exchanged less deuterium than in UCH-L1.

HDX-MS has also been found valuable in solving the complicated mechanisms involved in oxidative regulations. Oxidative modifications are closely involved in the regulatory functions of many proteins. The highly reactive cysteine residues in redox-regulated proteins play key roles by forming disulfide bonds and quickly change their structures into active or inactive conformations. As a consequence of disulfide formation, OxyR (Choi et al. 2001) and Hsp33 (Janda et al. 2004) are activated and RsrA (Zdanowski et al. 2006) and IpaH9.8 E3 ligase (Seyedarabi et al. 2010) are inactivated. Cysteine oxidation to sulfenic, sulfinic, sulfonic acid, dehydroalanin, serine, and thiosulfonic

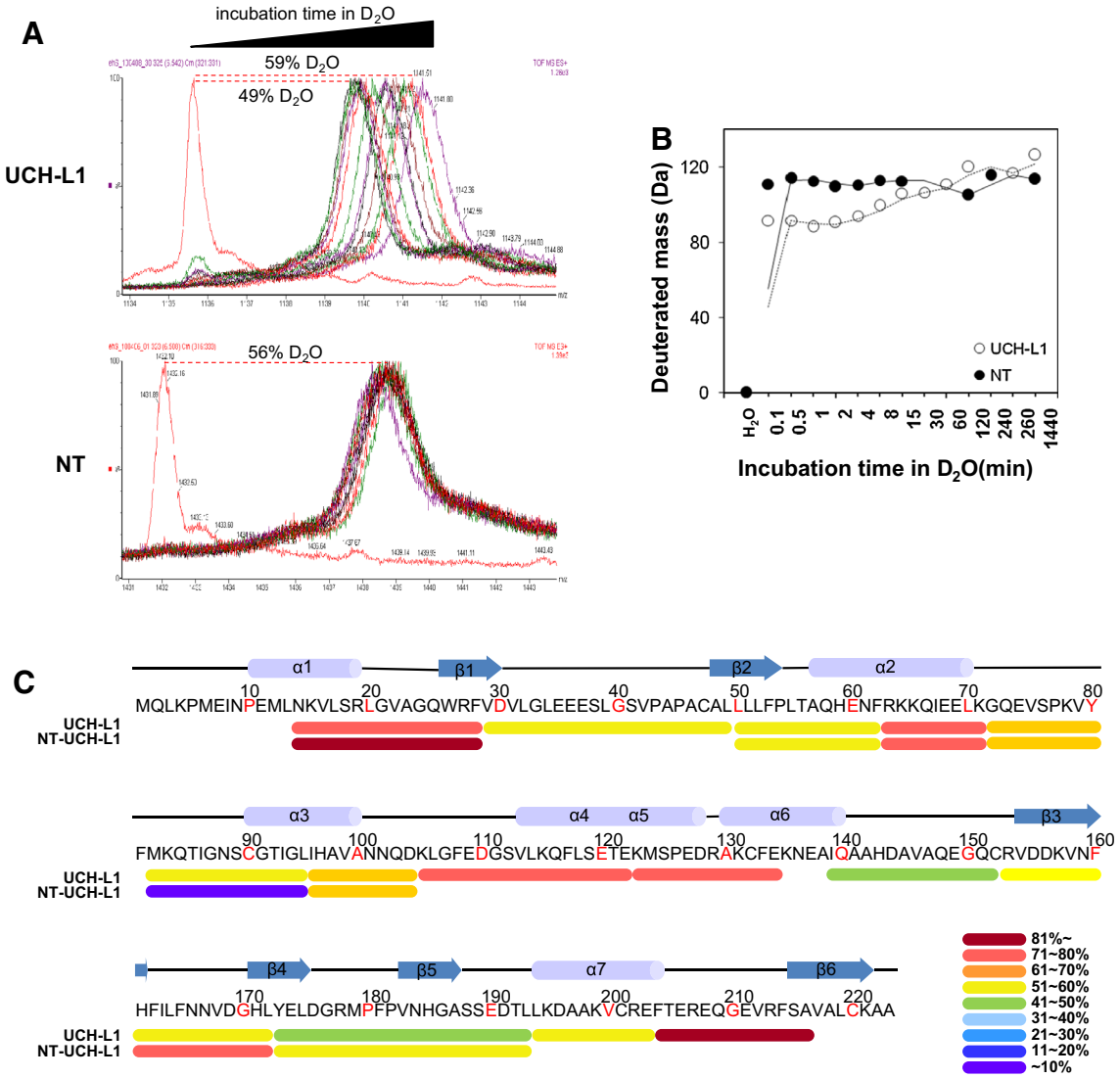

Fig. 2 Structural differences between UCH-L1 and NT-UCH-L1. **A**, **B** Purified UCH-L1 and NT-UCH-L1 were subjected to HDX studies. UCH-L1 and NT-UCH-L1 were incubated with D_2O exchange buffer at 25 °C for the indicated times and analyzed using nanoAcquity^{TM}/ESI/MS. HDX spectra of UCH-L1 and NT-UCH-L1 and deuterium exchange rates were represented by % exchange (**A**) and mass increase (**B**). **C** Recombinant UCH-L1 and NT-UCH-L1were incubated with D_2O exchange buffer at 25 °C for 30 min, digested with pepsin and analyzed using nanoAcquity^{TM}/ESI/MS. Deuterium exchange rates were represented by % exchange and colored accordingly (Cited from Kim et al. 2014)

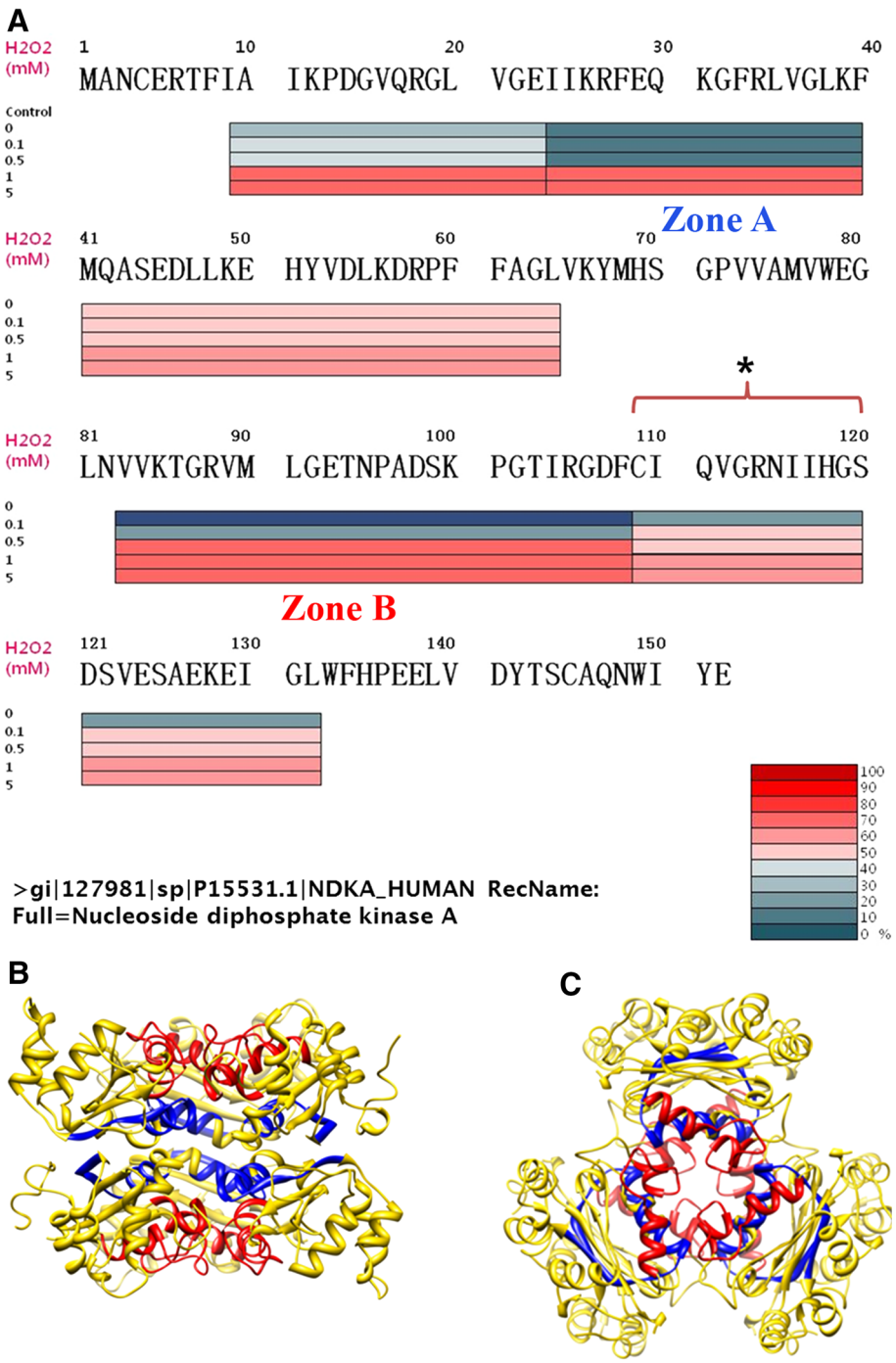

Fig. 3 HDX-MS of Nm23-H1. **A** HDX ratio of peptic peptides of native and oxidized Nm23-H1 in response to H_2O_2 (0–5 mM). Zone B is readily exposed to surface in lower H_2O_2 concentration and Zone A is exposed in higher H_2O_2 concentration. **B**, **C** Hexameric structure of Nm23-H1 in side view (**B**) and top view (**C**). *Red* color presents Zone B region and *blue* color, Zone A. (Cited from Kim et al. 2013)

acid (Jeong et al. 2011; Kim et al. 2015) is known to inactivate peroxiredoxins (Hall et al. 2009; Jeong et al. 2012a), GAPDH (Hwang et al. 2009), and phosphatases (van Montfort et al. 2003).

Nm23-H1/NDPK-A is another redox sensitive protein (Song et al. 2000). Nm23-H1/NDPK-A is a multifunctional housekeeping enzyme having nucleoside diphosphate kinase activity as a hexamer (Min et al. 2002) and plays a key role as a tumor metastasis suppressor (Rosengard et al. 1989). Oxidative modification of Cys109 promotes dissociation of Nm23-H1 from a hexamer into dimer (Song et al. 2000), loses its tumor metastasis suppressor activity as well as its enzymatic activity. Cys4 and Cys145 form a disulfide bond under oxidative condition, and the oxidized Nm23-H1 is a substrate of the NADPH-thioredoxin reductase 1-thioredoxin (NADPH-TrxR1-Trx) system and readily

reduced and recovered by this system (Lee et al. 2009; Choi et al. 2010). This suggests that Nm23-H1 is tightly regulated in oxido-reduction. However, the mechanism of the redox regulation is not well understood at the molecular level. Combining HDX-MS with X-ray crystallography demonstrate that formation of disulfide bond between Cys4 and Cys145 initiates a conformational change and expose Cys109 residue which is then readily oxidized to sulfonic acid as a stepwise oxidation and destabilizes hexameric state (Kim et al. 2013). The dissociation of hexameric state in response to H_2O_2 is determined employing HDX-MS. HDX ratio depending on H_2O_2 concentrations was monitored by MS. The HDX ratio increased and finally became saturated in 1 mM H_2O_2 and HDX ratio of each peptic peptides were shown in Fig. 3A. Most dramatic changes between control and oxidized Nm23-H1 are presented in Zone A and B. Zone B is readily expose to surface below 0.5 mM H_2O_2 and Zone A is exposed over 0.5 mM. These regions are presented in Fig. 3B, C. At low H_2O_2, dimer surface (red) is exposed and then monomer surface (blue), which indicates that hexamer is dissociated into dimer by forming disulfide between Cys4 and Cys145, and then into monomer. HDX-MS makes it possible to examine the dynamic changes of oligomeric states in response to oxidative stress. This is the first example of regulation of molecular and cellular functions of enzymes through stepwise oxidations. Since recent studies showed numerous oxidative modifications, this finding employing HDX-MS is valuable to solve the complicated mechanism of oxidative regulations.

Conclusion

This review demonstrates the basic concept, methodology and applications of HDX-MS, which is a cutting edge technology for determining protein conformational changes in response to protein–protein and protein–chemical interactions and PTMs of proteins. Although the resolution of HDX-MS is not as good as X-ray crystallography having single amino acid resolution, HDX-MS has many advantages for measuring conformational changes using small amounts of sample with high sensitivity and speed, and will greatly expand the applications to understand the mode of action of small chemical and biopharmaceutical therapeutics. We expect future improvements in the methodology, which will make it possible to speed up the analysis using better software for analyzing the HDX results and to increase the resolution up to single amino acid. Combining HDX-MS with computational modeling and docking will help drug discovery process, by leading to analytical technology for understanding interactions between target proteins and chemicals and biopharmaceuticals interactions in general.

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