Advances in Biochemistry & Applications in Medicine

Chapter 6

Application of Hydrogen/Deuterium Exchange Mass Spectrometry in Structural Biology and Molecular Medicine

***Bindu Y Srinivasu*[1]*; Amit Kumar Mandal*[*]**

[1]*Clinical Proteomics Unit, Division of Molecular Medicine, St. John's Research Institute, St. John's National Academy of Health Sciences, 100ft road, Koramangala, Bangalore 560034, India.*

[*]***Correspondence to: Amit Kumar Mandal,*** *Clinical Proteomics Unit, Division of Molecular Medicine, St. John's Research Institute, St. John's National Academy of Health Sciences, 100ft road, Koramangala, Bangalore 560034, India.*

Email: amit@sjri.res.in; drakm123@gmail.com

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1. Introduction

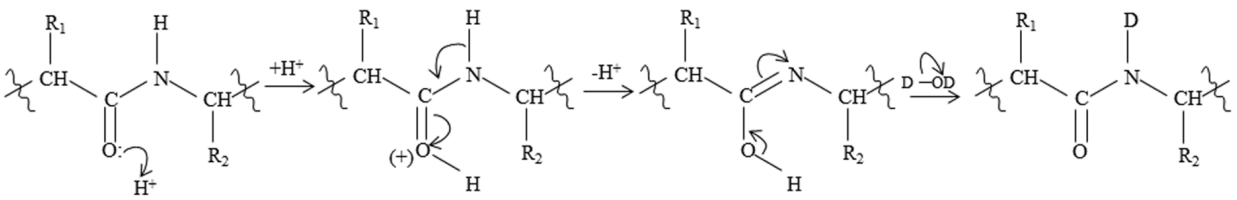
In last two decades soft ionization method in mass spectrometry has emerged as a powerful biophysical tool to study the structure of biological macromolecules such as proteins. Two ionization methods, electrospray ionization mass spectrometry (ESI-MS) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) were discovered independently by two research groups in 1980s. Although both of these methods have been extensively used in the analysis of primary structure of proteins in the domain of proteomics research, the isotope exchange chemistry of macromolecules monitored through soft ionization based MS made it feasible to study the functionally active three dimensional structure of proteins without any limit to its size, structural complexity and state of purity. The isotope exchange kinetics of polar hydrogen atoms of proteins with deuterium from solvent D_2O can be visualized using mass spectrometric platform. This isotope exchange kinetics monitored through MS is defined as hydrogen/deuterium exchange mass spectrometry (H/DX-MS). Subsequently the isotope exchange information can be used to investigate the change in conformational dynamics of a protein associated with a biological event such as protein-ligand interaction, protein folding etc. In addition, exploiting the permeability of D_2O across cell membrane, H/DX kinetics can be executed within a crowded molecular environment and the conformational dynamics of a

protein can be studied to understand the structure function correlation of a protein within a live cell.

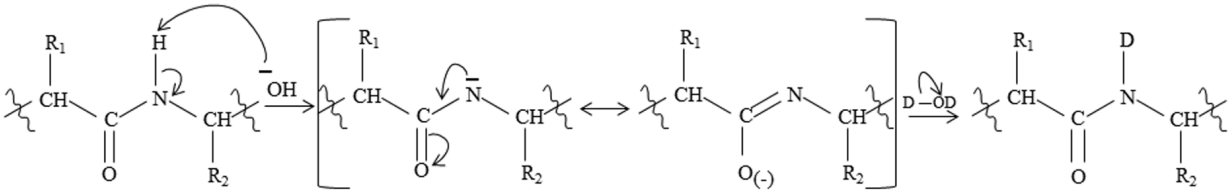
2. Fundamentals of H/DX Kinetics in Proteins

In solution phase, multiple vibrational degrees of freedom of a protein molecule provide flexibility in its conformation resulting in breathing motion of the molecule [1]. This conformational freedom results in local unfolding which causes transient open conformations to exist [2,3]. In general protein exists in a range of closely related conformations that are interconvertable at physiological temperature [4]. In a protein molecule hydrogen atoms bonded covalently to electronegative heteroatoms, e.g., oxygen, nitrogen and sulphur, are in continuous exchange with polar hydrogen atoms of the protonated solvent such as H_2O [5,6]. The rate of exchange depends on the differential solvent accessibility, inductive effect and field effect of neighbouring groups, pH and temperature [7,8]. Using mass spectrometry platform, the above exchange can be visualised by replacing the surrounding solvent water (H_2O) with heavy water (D_2O) [9-12]. In a protein molecule the polar hydrogen atoms present in the terminal groups and the side chain residues of amino acids exchange in the micro seconds scale and the kinetics of these fast isotope exchange steps cannot be monitored [13-15]. The time scale of the exchange reaction of the peptide backbone amide hydrogens is in the range of milli seconds, seconds, minutes and hours and thus it is possible to follow exchange kinetics in real time [16]. In general, the backbone amide hydrogens are involved in the formation of secondary structure of a protein, α helices and β sheets, and become less readily accessible in the exchange reaction to participate [17]. Eventually, the isotope exchange kinetics of backbone amide hydrogen can be translated to the conformational dynamics of a protein molecule [18,19]. The H/DX of the amide backbone can be catalyzed by both acids and bases [20,21]. Acid catalyzed mechanism initiates through the transient protonation of carbonyl oxygen atom followed by the removal of adjacent backbone amide hydrogen which gets replaced by the proton from solvent in the next step (**Scheme 1A**). In base catalysis, the hydroxide ion transiently abstracts the backbone amide hydrogen resulting in an imidate anion which gets subsequently protonated from the solvent (**Scheme 1B**).

Scheme 1A: Acid catalysis of hydrogen deuterium exchange



Scheme 1B: Base catalysis of hydrogen deuterium exchange



The rate constant of isotope exchange reaction kinetics k_{ex} can be expressed as follows:

$$k_{ex} = k_{H^+} x[H^+] + k_{HO^-} x[HO^-] + K_W \quad --------(1)$$

where, k_{H^+} and k_{H^-} are rate constants of acid and base catalyzed exchange reaction respectively and K_W is ion product of water. Differentiating both sides of equation (1) with respect to $[H^+]$ and applying the condition $dk_{ex}/d[H^+] = 0$, the H^+ ion concentration at which the exchange rate becomes minimum is as follows:

$$[H^+] = \left(\frac{k_{HO^-} x K_w}{k_{H^+}} \right)^{1/2} \quad -----(2)$$

For a typical backbone amide proton of proteins, k_{H^+} is 10^{-1} $M^{-1}s^{-1}$ and k_{HO^-} is 10^7 $M^{-1}s^{-1}$ at 25 °C [22]. Therefore k_{ex} reaches its minimum value at pH 3. For every 10 °C rise in temperature the amide exchange rate is increased by about 2 to 3 folds [23].

3. Kinetics of Peptide Backbone Amide Hydrogen Deuterium Exchange

According to Linderstrom Lang's model, the local unfolding or breathing motion of protein molecules results in a number of transient open conformations, with exchangeable amide hydrogens being transiently exposed to the solvent. In the next step the amide hydrogen gets exchanged with its isotope from the solvent [24,25]. The isotope exchange reaction of amide hydrogens of protein can be represented as follows [26-28]:

$$NH_{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} NH_{open} \xrightarrow[D_2O]{k_{ex}} ND \quad -----(3)$$

where, NH_{closed} and NH_{open} are the peptide backbone amide hydrogens in closed and open conformation respectively. ND is the isotopically exchanged amide NH. k_{op} and k_{cl} are rate constants of opening and closing events respectively and k_{ex} is the rate constant of intrinsic isotope exchange step.

The two states of the amide hydrogens NH_{closed} and NH_{open} can be collectively consid-

ered as $NH_{backbone}$. In presence of large excess of solvent D_2O the rate law of $NH_{backbone}$ exchange can be expressed as:

$$\frac{d[NH_{backbone}]}{dt} = -k_{ex}[NH_{open}] \text{-----(4)}$$

Rate of change in concentration of intermediate NH_{open}

$$\frac{d[NH_{open}]}{dt} = -(k_{cl} + k_{ex})[NH_{open}] + k_{op}[NH_{closed}] \text{-----(5)}$$

Applying steady state approximation,

$$\frac{d[NH_{open}]}{dt} = 0 \text{-----(6)}$$

Thus, $(k_{cl} + k_{ex})\,[NH_{open}] = k_{op}\,[NH_{closed}]$

or, $(k_{cl} + k_{ex})\,[NH_{open}] = k_{op}\,([NH_{backbone}] - [NH_{open}])$

$$[NH_{open}] = \frac{k_{op}}{k_{cl} + k_{ex} + k_{op}}[NH_{backbone}] \text{ -----(7)}$$

Therefore, using equation 4 and 7

$$\frac{d[NH_{backbone}]}{dt} = -\frac{k_{ex}.k_{op}}{k_{cl} + k_{ex} + k_{op}}[NH_{backbone}] = -k_{HX}[NH_{backbone}] \text{ -----(8)}$$

where, k_{HX} is the experimentally measured H/DX rate constant,

$$k_{HX} = \frac{k_{op}.k_{ex}}{k_{cl} + k_{ex} + k_{op}} \text{ -----(9)}$$

Thus, in presence of large excess of D_2O the isotope exchange reaction follows pseudo first order kinetics,

$$\left[NH_{backbone}\right] = \left[NH_{backbone}\right]_0 .\exp\left(-k_{HX}.t\right) \text{-----(10)}$$

where $[NH_{backbone}]_0$ is the initial concentration of exchangeable backbone amide hydrogens.

Protein in its native state exists mainly in its closed conformation [29]. Thus it might be assumed that under physiological condition open conformation is unstable and transient [30]. Thus exchangeable peptide backbone amide hydrogens predominantly exist in the closed conformation. Therefore, $k_{cl} >> k_{op}$ and using equation 9, k_{HX} is given by,

$$k_{HX} = \frac{k_{op}.k_{ex}}{k_{cl} + k_{ex}} \text{ -----(11)}$$

The rates of H/DX are primarily determined by the rate of opening of the closed conformation or by the intrinsic isotope exchange rate. These two regimes are referred as EX_1 mechanism and EX_2 mechanism respectively. Under EX_1 condition $k_{ex} >> k_{cl}$, implying that H/DX occurs immediately upon first opening event [31-35]. Therefore, using equation 11, k_{HX} can be written as,

$$k_{HX} = k_{op} \text{ --------(12)}$$

In general, EX_1 mechanism is observed at high pH or in the presence of denaturants such as urea, guanidinium hydrochloride where global unfolding or denaturation of a protein molecule occurs [36-38]. Also at alkaline pH the intrinsic exchange rate (k_{ex}) is very fast [36]. Under EX_2 condition $k_{ex} << k_{cl}$, implying that several opening closing cycles occur before the amide labeling [39]. Hence using equation 11, k_{HX} can be expressed as,

$$k_{HX} = \frac{k_{op}.k_{ex}}{k_{cl}} \text{ -----(9)}$$

$$\text{or, } k_{HX} = K_{op}\, k_{ex} \text{ ----------(13)}$$

where $K_{op} = (k_{op}/k_{cl})$ is the equilibrium constant of opening event. Under physiological condition protein demonstrates the isotope exchange through EX_2 mechanism. At the experimental temperature and pressure, the free energy change associated with local fluctuation and opening of native structure is represented as:

$$\Delta G_{HX} = -RT \log_e K_{op} = -RT \log_e (k_{HX}/k_{ex}) = -RT \log_e (1/P) \text{ -----(14)}$$

where $P = k_{ex}/k_{HX}$ is protection factor providing information on the stability of native structure [40]. The backbone amide hydrogens that are located in the loop regions are unprotected and remain in the open conformation. H/DX in those regions occurs rapidly with rate constant equals to k_{ex}.

H/DX reaction can be performed in two modes: continuous labeling and pulsed labeling methods. In the present chapter, applications of continuous labeling method in the advanced biochemistry research and in the molecular medicine have been described. In continuous labeling method, the native protein molecule is incubated in a buffer solution prepared using D_2O as solvent and the isotope exchange kinetics is monitored as a function of time. As the isotope exchange step has its own reaction kinetics, the dynamics of a biological event of a protein at different states along with their intermediates can be monitored using H/DX kinetics for all those states separately. The H/DX kinetics of the backbone amide hydrogens across a specific region of the polypeptide chain of a protein reflects the conformational dynamics of that region of the protein in its respective state [41,42]. In the continuous exchange method the

isotope exchange reaction is quenched at different time points by reducing pH of the solution to 3 and temperature to 4 °C. Subsequently, the proteolytic digestion of exchanged protein is performed under quenched condition so that the back exchange of the incorporated deuterium to solvent H_2O is minimum. In general, pepsin is used as the proteolytic enzyme in the H/DX experiments of proteins.

4. Data Analysis

The number of deuterium incorporated 'D' in a proteolytic peptide at a given time 't' is calculated as follows [43] :

$$D(t)=\frac{(M_t-M_0)}{(M_\infty-M_0)} \text{ x } N \quad \text{----(15)}$$

where M_t is the observed isotope average centroid mass of the deuterated peptide at time t, M_0 and M_∞ are the isotope average centroid mass of the undeuterated and fully deuterated peptides respectively and N is the total number of backbone amide hydrogens in a peptide excluding amino terminal hydrogens. As mentioned previously, at a fixed pH and temperature and in presence of large excess of D_2O, the H/DX of each amide hydrogen follows pseudo first order kinetics. Therefore, the number of amide hydrogen exchanged with deuterium at time t, ND of a peptide can be obtained as follows:

$$NH_{closed} + NH_{open} + ND = [NH_{closed}]_0 \quad \text{-----(16)}$$

or, $[NH_{backbone}] + ND = [NH_{closed}]_0$

As, $[NH_{backbone}]_0 = [NH_{closed}]_0$, equation 10 can be written as

$$[NH_{backbone}] = [NH_{closed}]_0 \,.\, \exp(-k_{HX} \,.\, t) \quad \text{-------(17)}$$

or, $$ND = [NH_{closed}]_0 \,.\, (1- e^{-k_{HX}.t}) \quad \text{-------(18)}$$

Considering $[NH_{closed}]_0$ is equal to the total number of peptide backbone amide hydrogens 'N' (excluding amino terminal amide hydrogen) and replacing ND by D

$$D(t) = N - Ne^{-k_{HX}.t} \quad \text{-----(19)}$$

In practice, every single backbone amide hydrogen has its characteristic isotope exchange rate constant.

Therefore, D (t) can be expressed as

$$\underset{i=1}{\overset{N}{D}}(t)=N-\sum \exp^{-k_i t} \quad \text{-----(20)}$$

where, k_i is the isotope exchange rate constant of i^{th} backbone amide hydrogen.

On the basis of magnitude of H/DX rate constants, backbone amide hydrogens of a peptide can be grouped into three categories as fast, intermediate and slow exchanging [44]. Thus, the expression of D(t) can be simplified as:

$$D(t) = N - \left(P_A e^{-k_1 .t} + P_B e^{-k_2 .t} + P_C e^{-k_3 .t}\right) \text{-----(21)}$$

where, P_A, P_B, P_C are the number of fast, intermediate and slow exchanging amide hydrogens and k_1, k_2, k_3 are average rate constants of the respective groups of amide hydrogens. The H/DX exchange rates and populations of different group of backbone amide hydrogens can be obtained by minimizing the sum of squared residuals (SSR) through performing multiple iterations steps using Microsoft Solver. The SSR for each data set can be calculated as given below:

$$SSR = \sum_i [(y_{i,obs} - y_{i,calc}) / y_{i,obs}]^2$$

$y_{i,obs}$ (observed data) and $y_{i,calc}$ (calculated data) can be calculated from equation 15 and 21 respectively. The combination of the kinetic parameters which gives the minimum SSR values is selected as the best-fit values of a given kinetic data set [45].

To understand the change in conformational dynamics of a protein associated with a biological event, a comparative analysis is required between six rate constants and six populations between initial and final states of the protein. In practice, it is very difficult to deal with twelve such kinetic parameters simultaneously. This complicated analysis step can be simplified by calculating the rate of isotope exchange reaction of different groups of amide hydrogens in both states of protein molecule by using the method of initial rate of a reaction [45]. The initial rate of pseudo first order H/DX reaction of a particular group of backbone amide hydrogens can be calculated from the product of rate constant (k_i) and the respective population of backbone amide hydrogens (P_i) of the group, which is the initial concentration of reactants in the isotope exchange reaction. It was hypothesized that the algebraic summation of the differential exchange rates across three groups of amide hydrogens between two states of a protein molecule mirrors the change in the conformational dynamics of the molecule associated with the biological event. A positive sign indicates increased flexibility and a negative sign indicates increased rigidity in the conformational dynamics associated with the state change of protein molecule.

5. Application of H/DX-MS in Structural Biology

5.1. Protein ligand interaction

In general most of the molecular interactions in a biological system are non-covalent in

nature such as protein-ligand interaction, where ligands can be small or large biomolecules including metal ions. These interactions are always driven by the binding affinity and the specificity of the ligand to the protein molecule. H/DX-MS is widely used for studying such interactions. This approach relies on the fact that binding of ligand to the protein restricts the solvent accessibility of the backbone amide hydrogens at the binding interface, thereby decreasing the H/DX rates of those backbone amide hydrogens. In addition, ligand binding to a protein might lead to the exposure or burial of backbone amide hydrogens in another part of protein molecule which modulates the conformational dynamics of the molecule resulting in an increase or decrease in the H/DX rates respectively. Thus, the comparison of these exchange rates of amide hydrogens between free and ligand bound states of the protein provides information on both the binding sites and the ligand induced conformational change of the protein molecule. Here we highlight few examples of the application of H/DX-MS in the structural characterization of protein-ligand interactions.

A recent study by Bennett and co-workers reported the unique binding site of laulimalide on microtubules using H/DX-MS. This study revealed that the binding site of laulimalide is located to the exterior of the microtubule on β-tubulin and also showed the structural perturbations of certain regions of proteins associated with the binding of laulimalide [46]. The ligand induced conformational change of human hemoglobin has been described from the difference in the H/DX kinetics of both oxygenated and deoxygenated states of hemoglobin [47,48]. Using H/DX-MS, Li *et al*. showed the binding region of the thermococcales inhibitor of the proliferating cell nuclear antigen (PCNA) designated as TIP, in PCNA-TIP complex [49].

Identification and characterization of an epitope in a specific antibody-antigen complex is a crucial step in developing therapeutic antibodies and vaccines. H/DX-MS is extensively used in the epitope mapping. Using H/DX-MS, Malito *et al.* mapped the binding of monoclonal antibody with the factor H binding protein (fHBp), the vaccine antigen of Neisseira meningitidis [50]. Similarly, Lu *et al*. reported that the open end of the β-barrel structure is the binding region of IL-1β to the Hu007, a humanized IgG1 monoclonal antibody [51]. Other examples include the epitope mapping of cytochrome C –E8, IL-13–CNTO607, and IL-17A–CAT-2200 [52].

5.2. Conformational stability of proteins

According to Anfinsen's thermodynamic hypothesis, the native structure of a protein is the one with lowest possible free energy in the physiological environment [53]. However, the native structure can undergo certain conformational changes attaining metastable states under some conditions in a living system [54]. Investigation of the stability of a protein provides important biological information. A decrease in the stability might indicate a tendency of a protein molecule to misfold and an increase in the stability might represent the bound state

with the ligand [55,56]. H/DX-MS has been immensely used to study the stability of proteins in solution phase. To illustrate this application, some of the examples are presented below.

Ghaemmaghami *et al.* developed a method, named as SUPREX (Stability of Unpurified Proteins from Rates of hydrogen deuterium Exchange) that involved the use of H/DX kinetics of an unpurified protein monitored through MS to study its stability. In this method, the protein was allowed to undergo H/DX at different concentration of the denaturant and the relative stability was measured. This study showed that the presence of maltose increased the stability of maltose binding protein in its native state [57]. Using H/DX-MS, Hodkinson and co-workers studied the conformational dynamics of β2-Microglobulin (β2m). This study showed that the amyloidogenic protein β2m on its dissociation from the major histocompatibility complex class 1 (MHC-1) becomes very unstable and exhibits a high conformational dynamics resulting in the formation of unfolding transitions with an increased tendency for aggregation [58]. The conformational dynamics of β2m measured by H/DX-MS showed that there was no significant correlation between the rate of EX_1 unfolding and fibril formation. Thus, authors concluded that existence of other fibril nucleation mechanisms besides global unfolding might have resulted in the formation of amyloid fibrils from β2m [59]. Two conformations of human growth hormone (hGH) at two different pH were investigated by Hamuro *et al*. The authors reported that in acidic pH hGH was more flexible and less stable when compared to its conformation in neutral pH [60]. In an another study McCammon *et al.* reported that the binding of the small molecule inhibitors to transthyretin would result in a substantial increase in the stability of the tetrameric protein molecule and thereby reducing its amyloidogenicity [61].

6. Application of H/DX-MS in Molecular Medicine

6.1. Biosimilar study

Monoclonal antibodies are emerging as potential drugs, therapeutic agents for various diseases [62]. A crucial step in the development of these biopharmaceuticals is the structural characterization of a particular protein. The functions of a protein is driven by its three dimensional structure. Thus, monitoring higher order three dimensional conformation of monoclonal antibodies become crucial in the development of biosimilars, the generic version of originator proteins as drugs. Alterations in the structure of biosimilars might cause significant changes in its function and eventually their use as therapeutics leading to side effects in the living system [63]. H/DX-MS platform has been used as a potential biophysical tool in the biosimilar studies. Using H/DX-MS, Nakazawa *et al*. analysed the stability of oligomers of the formulated insulin analogs with different pharmacokinetics. The study showed that the difference in the H/DX rates exist among insulin analogs and a correlation was observed between the pharmacokinetics parameters in humans and the H/DX kinetics for the formulated insulin analogs [64]. In an another study by Vissar *et al*. characterized the proposed biosimilar rituximab (GP2013)

and compared with the originator rituximab using H/DX-MS. The study showed no significant structural change on addition of drug conjugate to the antibody (IgG). Authors also showed that GP2013 was physiochemically and functionally similar to the originator rituximab [65]. Similar studies were performed in characterization of biosimilars of Trastuzumab which is a humanized immunoglobulin used in treating HER2-overexpressing breast cancer and Cetuximab, a chimeric mouse-human immunoglobulin monoclonal antibody that is used in treating colorectal cancer and squamous cell carcinoma of head and neck [66].

6.2. Discovery of drug target sites

In order to develop drugs that can arrest the process involved in the undesirable pathological condition, it is important to structurally characterize protein molecules to obtain the information about the molecular mechanism associated with the disease states. H/DX-MS is one of the molecular approaches that provide information on the probable drug target sites in the protein molecules [67]. For example, the main pathophysiology of sickle cell anemia involves energetically favoured polymerization of sickle hemoglobin in the deoxy state of hemoglobin. Using H/DX-MS, Das *et al*. showed that the change in the conformational dynamics of amino acid residues around hydrophobic patch, alteration in the conformation of the groove regions and the modulation of the important axial contacts within a single stranded polymer and the lateral interactions between the amino acid residues across the double strands in the oxy state of sickle hemoglobin molecule might be the molecular basis of inhibition of sickle hemoglobin polymerisation in its oxygenated state [68]. Authors proposed that these sites with altered conformation might be utilized to develop drugs that can inhibit hemoglobin polymerization in sickle cell anemia.

7. *In vivo* Application

Proteins are workhorse within a live biological cell. Function of a protein is defined by its three dimensional structure. Functionally active conformation of a protein is not static but dynamic. Structure-function correlation of a protein *in vivo* can be explored by studying the change in conformational dynamics of a protein molecule associated with the biological event in an extremely crowded molecular environment of a live cell. Exploiting the permeability of D_2O across the cell membrane, H/DX can be executed inside a live cell. Subsequently the structural information related to conformational dynamics and conformational stability of proteins can be extracted.

7.1. Conformational dynamics of a protein *in vivo*

Narayanan et. al. studied that the structural transition of human hemoglobin associated with its oxygenation inside live Red Blood Cells (RBCs) [45]. The cooperativity and allosteric regulation of oxygenation and the associated change in the conformational flexibility across

various regions of both α and β globin chains of human hemoglobin were monitored through H/DX-MS of fully oxygenated and fully deoxygenated RBCs in separate sets by incubating RBCs in 300 mOsM buffer prepared in D_2O, which is isosmotic to human blood. The different steps involved in the H/DX of live cells followed by mass spectrometric data analysis are shown in **Figure 1**.

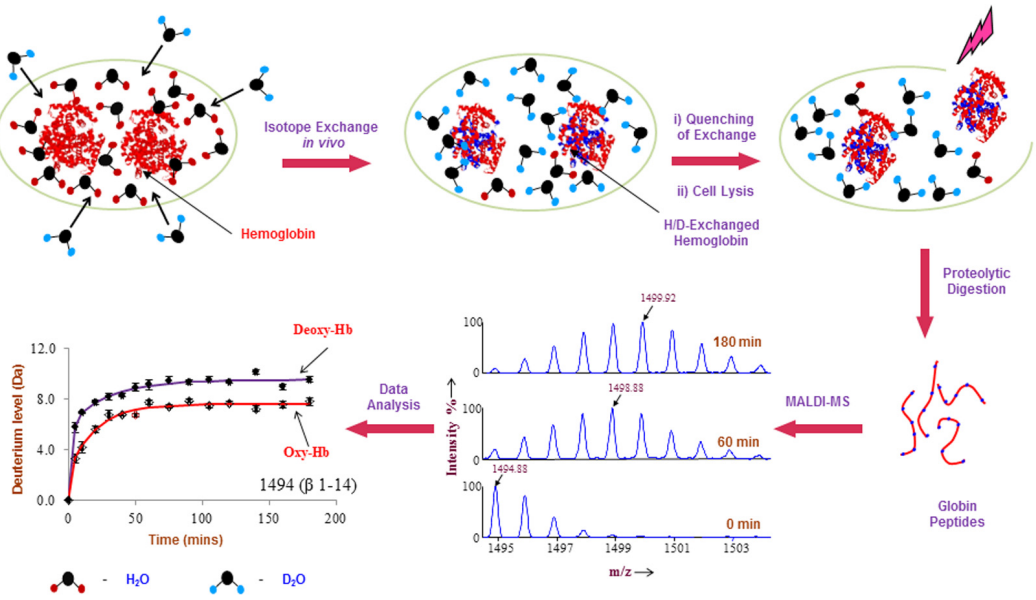


Figure 1: Schematic representation of hydrogen deuterium exchange in a living cell and subsequent steps involved in the analysis of exchange kinetics. ("Adapted with permission from (Anal Chem, 2015; 87: 11812-11818). Copyright (2015) American Chemical Society")

The rate of H/DX reaction of seven β globin peptides of human hemoglobin were calculated using the method of initial rates. The algebraic summation of the exchange rates of different parts of hemoglobin were used to understand the associated changes in the conformational dynamics of human hemoglobin inside live RBCs on deoxy to oxy transition. **Figure 2A** shows the changes in the conformational dynamics of β globin chain of hemoglobin associated with deoxy to oxy transition. The spatial orientation of those seven peptic peptides of β globin chain in human hemoglobin crystal structure is shown in **Figure 2B**. Hemoglobin concentration in RBCs is more than 95% of its total protein content. Thus the observed conformational change monitored through H/DX-MS was correlated with the literature information on the structural transition of human hemoglobin associated with its oxygenation *in vitro*. Authors proposed that this *in vivo* H/DX method might be successfully used to monitor conformational dynamics of any protein in cell irrespective of its size, location and structural complexity. For low abundant proteins in cell, liquid chromatography based pre-fractionation of isotope exchanged peptides at low temperature and acidic pH and subsequent online analysis of their mass might be helpful in the analysis.

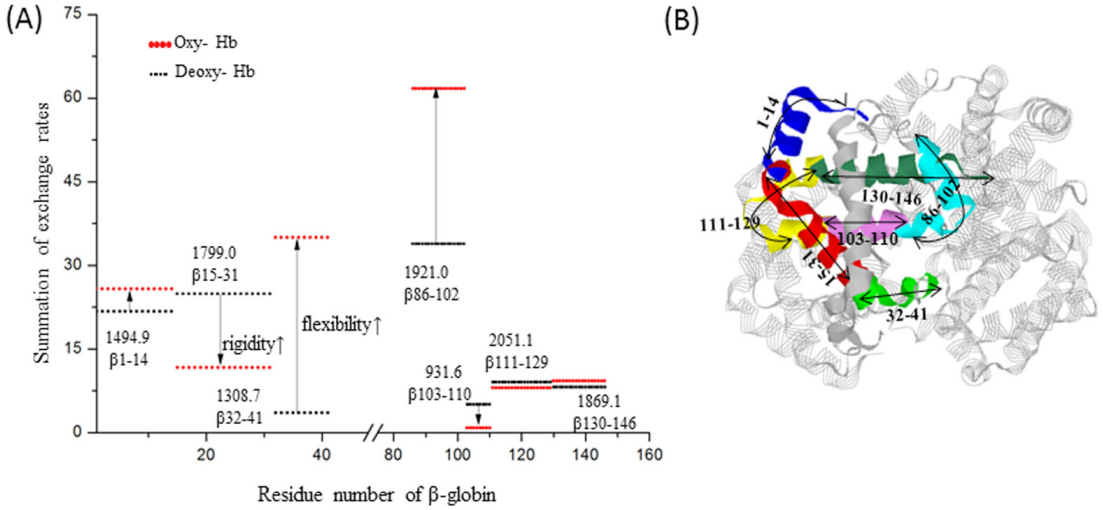


Figure 2: Conformational dynamics of β-globin chain of human hemoglobin on oxygen binding. (A) Hydrogen deuterium exchange rates of 7 different peptic peptides in both oxy and deoxy states of β-globin are shown. Direction of arrow indicates the increase in flexibility. ("Adapted with permission from (Anal Chem, 2015; 87: 11812-11818). Copyright (2015) American Chemical Society") (B) Orientation of 7 peptic peptides of β-globin chain is shown in the deoxy human hemoglobin crystal structure [PDB: 4HHB obtained from the protein data bank (www.PDB.org)].

7.2 Conformational stability of a protein *in vivo*

Ghaemmaghami *et al*., measured the thermodynamic stability of monomeric form of the N-terminal domain of repressor, $\lambda_{6\text{-}85}$, within the *E.Coli* cells using SUPREX [69]. Exploiting permeability of urea, a denaturant, and D_2O across the cell membrane, globally exchanging hydrogen exchange rate of the unfolded protein was measured and subsequently the thermodynamic stability of the protein in *E.Coli* cytoplasm was calculated. Record et al. reported that the presence of natural osmolyte at high concentration in the cytoplasm of *E.Coli* cell results in reduced water content, thereby increasing the stability of less hydrated native state [70,71]. In this study, *in vivo* measurement of H/DX was possible to monitor the stability of $\lambda_{6\text{-}85}$ till 3 M urea. Beyond 3 M urea cell viability dropped drastically, presumably due to irreversible denaturation of crucial proteins in cell.

According to the classical H/DX kinetics the observed rate constant of exchange for each hydrogen in terms of rate constants of various elementary steps of exchange reaction is given by equation 9,

$$k_{HX} = \frac{k_{op} . k_{ex}}{k_{cl} + k_{ex} + k_{op}}$$

Under EX_2 regime both k_{cl} and $k_{op} >> k_{ex}$. Thus,

$$k_{HX} = \frac{K_{op} . k_{ex}}{K_{op} + 1} \quad \text{-----(23)}$$

where, $K_{op} = (k_{op}/ k_{cl})$ is the equilibrium constant between closed and open conformations of protein. For hydrogen that is exchanging through global unfolding mechanism $K_{op} = (1/K_{fold})$,

$$k_{HX} = \frac{k_{ex}}{(1+K_{fold})} \quad \text{-----(24)}$$

For global unfolding the intrinsic isotope exchange rates (k_{ex}) is similar for entire peptide backbone amide hydrogens and the average H/DX rate of unprotected amide hydrogens is represented as $<k_{ex}>$. Thus the increase in mass as a function of time can be expressed as follows (7):

$$\Delta M = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})\, e^{-k_{HX}.t} \quad \text{-----(25)}$$

where ΔM_0 and ΔM_{∞} are the increase in mass before global exchange and after complete exchange respectively [57]. Thus,

$$\Delta M = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})\, e^{-[<k_{ex}>/(1+K_{fold})]t} \quad \text{-----(26)}$$

where $K_{fold} = e^{-(\Delta G_f + m[urea])/RT}$ -----(27)

ΔG_f is the free energy change on folding and m = (d[ΔGf] /d[urea]). Equation 26 was used to fit H/DX rates for both *in vivo* and *in vitro* experiments. From the measured stability parameter of protein it was concluded that in vivo stability of $\lambda_{6\text{-}85}$ in cell is very similar compared to its *in vitro* stability.

8. Conclusion

H/DX-MS can be used to monitor the change in the conformational dynamics of a protein associated with a biological event and to measure the conformational stability of a protein in solution phase. As the molecular mass is probed in an isotope exchange experiment, the method is applicable even in an extremely crowded molecular environment such as inside a live cell. Using this approach, structurally very complex protein molecules can be studied irrespective of its molecular size. The method has been increasingly used both in academic research as well as in molecular medicine such as in the biosimilar study. Advancement of liquid chromatographic separation of a complex mixture of proteolytic peptides with isotope exchange information retained, followed by online mass spectrometry based analysis might be an indispensable technique to understand innumerable cellular event at the molecular level in near future.

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