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Chapter 5

Application of Mass Spectrometry to Analyse Protein Structure within a Live Cell

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1. Introduction

In depth understanding of molecular mechanisms of cellular processes requires protein structure-function correlation to investigate in its endogenous environment. Due to identical chromophoric groups present across all proteins within a cell, the different spectroscopic tools that are utilized to visualize protein structure cannot be used within a crowded molecular environment *in vivo*. Molecular mass is the molecular signature of a molecule. Thus, mass spectrometry that measures molecular mass, could be used to investigate a single molecular entity in an unpurified sample. In fact, mass spectrometry might be an appropriate biophysical technique that could be used to solve protein structure even in a heterogeneous molecular pool such as inside a live cell. *In vivo* application of mass spectrometry to monitor protein structure and stability is little older than a decade. In this chapter we highlighted following three different types of mass spectrometry based biophysical techniques that had been used to elucidate structure and conformational dynamics of proteins and to map protein-protein interactions within a live cell: Hydrogen deuterium exchange of back bone amide hydrogens of a protein, Free radical mediated fast photochemical oxidation and Cross linking of proteins.

2. Molecular Crowding *in vivo*

Proteins in biological cells are in a highly crowded environment. Molecular crowding

in a cell is essential for the biochemical processes such as protein folding, protein nucleic acid interaction, oligomerization etc [1]. Arthur Konerberg reported that the replication of oriC plasmid in a cell free environment became possible using high concentration of polyethylene glycol (PEG). In fact PEG occupies major portion of aqueous volume which results in restoration of the required crowded environment for the event [2]. Another important example of molecular crowding is polymerization of sickle hemoglobin (HbS) in sickle cell anemia. A very high concentration of hemoglobin in red blood cell results in a crowded environment that is essential for the polymerization of deoxy-HbS [3]. Theoretically, the rate of homogenous nucleation of sickle hemoglobin is expected to decrease by 10^{10} fold on reducing HbS concentration by 20%. In practice, the replacement of HbS with HbF by 20% results in reduced homogenous nucleation rate by a factor of 10^3 fold. Although HbF does not participate in polymerization but it preserves the molecular crowding inside RBCs resulting in a large difference (10^7 fold) in sickle hemoglobin polymerization [4]. A significant increase in the molecular crowding is observed with aging, which could be associated with a reduction in the cellular volume and retardation in the protein degradation rate [5]. One of the hypotheses for the brain cells being susceptible to Parkinson's disease with aging is increase in the rate of aggregation of α-synuclein [6]. Thus to understand a molecular mechanism in a biological system, it is important to explore the molecular interactions inside a living cell.

Function of a protein is completely guided by its structure. Various spectroscopic tools that are used to visualise protein structure namely circular dichroism, fluorescence, infrared, nuclear magnetic resonance utilize intrinsic chromophores of the protein molecule. A major limitation in using these spectroscopic methods in a living cell arises from the difficulty in differentiating the contribution of chromophoric groups that are similar among all proteins. Atomically-resolved structural information is obtained from X-Ray crystallography where the experimental molecule needs to be crystallized. High concentration of complex macromolecules results in a crowded environment in a biological cell [7]. Thus, it is impossible to compartmentalize a protein molecule from its surroundings within a live cell. Therefore, the classical approaches for most of the structural investigations of proteins using above mentioned techniques are restricted to purified molecules *in vitro*. Subsequently, *in vitro* observation obtained by spectroscopic methods is extrapolated *in vivo* for its functional correlation [8]. However, crowding of macromolecules represents a significant functional feature of cellular complexity and subsequently its importance on biochemical processes as mentioned earlier.

3. Mass Spectrometry Based Structural Analysis *in vivo*

To understand the molecular mechanism of a biological process it is crucial to analyze the macromolecular binding stoichiometry and conformational dynamics associated with the event *in vivo*. However, it is impossible to mimic the complex cellular environment *in vitro* [9]. Unlike the other molecular spectroscopic tools, mass spectrometry is specific to molecular

mass, which enables the technique to monitor individual molecular entity even in a crowded impure molecular milieu. The bottom line of mass spectrometry based protein structure analysis is to monitor the changes in molecular mass as the process progresses. To investigate the conformational dynamics of macromolecules, it is essential to label the experimental molecule with a molecular probe. Therefore, to monitor an event *in vivo* using mass spectrometry, the molecular probe must be permeable across the cell membrane and execute the required chemical modification of experimental molecule inside the live cell. A couple of mass spectrometry based methods have been used in last few years to investigate structure and conformational dynamics of proteins inside living cells.

In the conformation analysis using hydrogen deuterium exchange, the polar hydrogens of a protein molecule are replaced with deuterium from solvent D_2O [10]. Narayanan S. *et al.* showed that exploiting permeability of D_2O across cell membrane followed by the isotope exchange of polypeptide backbone amide hydrogens, the structure-function correlation of human hemoglobin can be studied within live red blood cells (RBCs) [11]. Ghaemmaghami S. *et al*. reported that allowing urea and D_2O to penetrate *E.Coli* cells, the stability of truncated N-terminal domain of λ repressor ($\lambda_{6\text{-}85}$) protein was measured through isotope exchange based mass spectrometry [12]. In free radical mediated oxidative modification, the structural changes of protein were monitored through radical induced oxidation of the constituent amino acid residues [13]. Using laser photolysis of hydrogen peroxide, the hydroxyl radical induced oxidation of cytoplasmic proteins in Vero cells were studied by Espino JA, *et. al* [14]. In cross linking method, a cross linker gets covalently bonded with the reactive functional groups of either two parts of a molecule or between parts of different molecules [15]. Using formaldehyde as a cross linker, protein-protein interactions network was mapped in yeast cells by Cortnie and co-workers [16]. In these methods the observed increase in mass of a protein is translated in terms of its structural changes. In this chapter we described the application of aforementioned techniques in the analysis of protein conformation within live cells (*in vivo*).

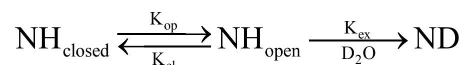
4. Hydrogen Deuterium Exchange in Protein

The vibrational degrees of freedom of a protein molecule results in breathing motion of the molecule that manifests as local unfolding thereby causing transient open conformations to exist [17,18]. During this transition, the polar hydrogens that are bonded covalently with oxygen, nitrogen and sulphur, become exposed to the solvents and subsequently undergo exchange with a rate that depends on differential solvent accessibility, inductive effect and field effect of neighbouring groups, pH and temperature [19]. The above exchange can be kinetically monitored using heavy water [D_2O] as solvent [20]. In practice, the isotope exchange of the peptide backbone amide hydrogens of a protein molecule that are involved in the formation of secondary structure of protein, α helices and β sheets, are studied [21]. Eventually, the isotope exchange kinetics of peptide backbone amide hydrogens of a protein can be translated

to its conformational dynamics in the solution phase [22].

5. Kinetics of Peptide Backbone Amide Hydrogen Deuterium Exchange

The local unfolding followed by the transient exposure of protein conformations and subsequent isotope exchange of backbone amide hydrogens from solvent can be explained by Linderstrom Lang's model [23,24]:



where, NH_{closed} and NH_{open} are backbone amide hydrogens in the closed and in the open conformation respectively. ND is the isotopically exchanged amide NH. k_{op}, k_{cl} and k_{ex} are rate constants of opening, closing and intrinsic isotope exchange steps respectively. The experimentally measured hydrogen deuterium exchange rate constant k_{HX}, can be expressed as

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

Thus, in the presence of large excess of D_2O, the isotope exchange reaction follows pseudo first order kinetics. In general the rate constant of isotope exchange reaction k_{ex} of peptide backbone amide hydrogens reaches minimum at pH 3. Additionally, every 10^0C rise in temperature increases k_{ex} by 2 to 3 folds [22].

Protein in its native state exists mainly in closed conformations and it might be approximated that under physiological condition the open conformation of a protein molecule is unstable and transient [25]. Thus, $k_{cl} >> k_{op}$ and k_{HX} is given by,

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

The rates of hydrogen deuterium exchange in a protein molecule are primarily determined by either the rate of opening of the closed conformation or by the intrinsic isotope exchange rate. These two conditions are referred as EX_1 mechanism and EX_2 mechanism respectively. Under EX_1 condition, $k_{ex} >> k_{cl}$, implying that the exchange occurs immediately upon opening event [26,27]

$$k_{HX}=k_{op}$$

In general EX_1 mechanism is observed on denaturation of protein molecules. Under EX_2 condition $k_{ex} << k_{cl}$, implying that several opening closing events occur before the exchange event.

$$k_{HX}=\frac{k_{op}\times k_{ex}}{k_{cl}}$$

or, $k_{HX} = K_{op} \times k_{ex}$

where $K_{op} = (k_{op}/k_{cl})$ is the equilibrium constant of opening event. In general, under physiologi-

cal condition protein exhibits the isotope exchange via EX_2 mechanism.

At the experimental temperature and pressure, the free energy change associated with the local fluctuation followed by the opening of native structure is given by [28]:

$$\Delta G_{HX} = -RT\ \log_e K_{op} = -RT\ \log_e\left(\frac{k_{HX}}{k_{ex}}\right) = -RT\ \log_e\left(\frac{1}{P}\right)$$

where P is protection factor providing information on the stability of the native conformer. The backbone amide hydrogens that are in the loop regions are unprotected and remain in the open conformation. Hydrogen deuterium exchange in these regions occurs rapidly with rate constant k_{ex}.

Permeability of D_2O across the cell membrane can be exploited to execute hydrogen/deuterium exchange inside a live cell [29]. The replacement of hydrogen with deuterium does not lead to change in the structure and chemical property of a molecule. The hydrogen deuterium exchange data of backbone amide hydrogens across the peptide backbone of a protein mirrors the conformational dynamics of a particular state [30]. Every isotope exchange reaction has its own kinetics. Thus to monitor the kinetics of a biological event, different intermediate states must be trapped and the hydrogen deuterium exchange must be executed for all those states separately. In practice, the isotope exchange reaction is quenched at different time points by reducing pH to 3 and temperature to 4^0C. Both the cell lysis and proteolytic digestion of isotope exchanged protein are performed under quenched condition to minimize the back exchange of deuterium. The number of deuterium incorporated 'D' in a peptide at a given time 't' can be calculated as follows [31]:

$$D_{(t)} = \left(\frac{M_t - M_0}{M_\infty - M_0}\right) \times N$$

where M_t is the observed isotope averaged centroid mass of deuterated peptide at time t, M_0 and M_∞ are the isotope averaged centroid mass of undeuterated and fully deuterated peptides respectively and N is the total number of backbone amide hydrogens in a peptide excluding amino terminal hydrogens. At a fixed pH and temperature and in presence of large excess of D_2O, the isotope exchange of each backbone amide hydrogens follows pseudo first order kinetics and D(t) can be expressed as

$$D_{(t)} = N - \sum_{i=1}^{N} \exp^{-k_i t}$$

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

In practice, backbone amide hydrogens of a peptide can be grouped into following three categories: fast, intermediate and slow exchanging [23]. Thus,

$$D_{(t)} = N - \left[P_A e^{-k_1 \times t} + P_B e^{-k_2 \times t} + P_C e^{-k_3 \times t}\right]$$

where, P_A, P_B, P_C are the population of fast, intermediate and slow exchanging amide hydrogens with average rate constants k_1, k_2, k_3 respectively. These kinetic parameters can be

obtained from D (t) vs. t plot.

6. *In vivo* Application of Hydrogen Deuterium Exchange

6.1. Conformational dynamics of protein

Structure-function correlation of protein *in vivo* might be explored by studying the change in conformational dynamics of a protein molecule accompanying ligand binding in-cell. Using continuous labeling method in hydrogen deuteriuim exchange Narayanan S. *et.al*., reported the structural transition of human hemoglobin associated with its oxygenation inside live Red Blood Cells [11]. Authors described the different step involved in hydrogen deuterium exchange in live cells followed by mass spectrometric data analysis in detail [11]. The cooperativity and allosteric regulation of oxygenation and the associated change in the conformational flexibility across various regions of globin chains in hemoglobin were monitored through hydrogen deuterium exchange of fully oxygenated and deoxygenated RBCs by incubating it in 300 mOsm D_2O buffer, isosmotic to human blood. To understand the change in conformational dynamics, a comparative analysis of six rate constants and six populations of oxy and deoxy states of hemoglobin are required. The analysis method was simplified by calculating the rate of exchange reaction for each category of amide hydrogens in both oxy and deoxy states of hemoglobin. The rate of pseudo first order hydrogen deuterium exchange reaction was calculated using the method of initial rates where the product of rate constant (k_i) and the respective population (P_i) of each group of backbone amide hydrogens were used as rate of exchange reaction. The algebraic summation of exchange rates across two states of hemoglobin was considered to assess whether the experimental region of the molecule became flexible or rigid in its conformational dynamics on deoxy to oxy transition.

The movement of heme Fe^{2+} towards the plane of porphyrin nucleus on O_2 binding to deoxy hemoglobin is transmitted to the proximal His residue resulting in the expulsion of penultimate Tyr residue from the pocket between F and H helices. Subsequently salt bridges of carboxyl group of the terminal residue and hydrogen bonds of penultimate residue are ruptured [32,33]. Disappearance of above interactions was reflected in the increased flexibility of a peptide β^{86}ATLSELHCDKLHVDPEN^{102} in the *in vivo* hydrogen deuterium exchange analysis [11]. The allosteric regulator 2,3-diphosphoglycerate is bound, one per deoxy hemoglobin tetramer, between two β-subunits through ionic interactions with βVal^{1}, βHis^{2}, βLys^{82} and βHis^{143} residues of both β globin chains [32]. Oxygenation causes contraction in the DPG binding pocket followed by the dissociation of interactions with all above residues and the associated structural transition was reflected in the increased flexibility in the *in vivo* hydrogen deuterium exchange of the peptide β1VHLTPEEKSAVTAL14 [11]. Another important factor in the cooperative oxygen binding to heme unit is Bohr effect, which is contributed by the imidazolium hydrogen of βHis^{146} and αHis^{122} and N-terminal NH_2 of αVal^{1}. Oxygenation results in the dis-

sociation of salt bridges involving those residues which in turn causes decrease in pK_a followed by the release of Bohr protons from above residues. Subsequently the released protons combine with bicarbonate to form carbonic acid [34]. The structural transitions associated with these crucial steps were reflected in the *in vivo* hydrogen deuterium exchange analysis through the change in the conformational dynamics of the peptides β^{130}YQKVVAGVANALHKYH^{146}, α^{110}AAHLPAEFTPAVHASLDKFLASVSTVLTSKYR^{141} and α^{1}VLSPADKTNVKAAWGKV GAHAGEYGAEALERMF^{33} respectively [11].

The structure function relationship of hemoglobin is well established from *in vitro* research over couple of decades. The hemoglobin content of RBCs is 95% of total protein. Thus RBCs might be considered as an *in vivo* system of pure hemoglobin. From the observed correlation between *in vitro* and *in vivo* results of structure-function correlation of human hemoglobin, authors claimed that *in vivo* hydrogen deuterium exchange method might be successful to monitor conformational dynamics of any protein in cell irrespective of its size, location and structural complexity. Mass spectrometry based visualization of proteins in a proteome is limited by the competition in the ionization between various proteins that depends on their ionization probability and abundance, dynamic range. Thus, the described *in vivo* method is limited by the coverage of cellular proteins which is primarily defined by the relative abundance within a live cell. For low abundant proteins in cell, liquid chromatography based prefractionation at low temperature and acidic pH might help in hydrogen deuterium exchange analysis.

6.2. Protein folding

The correlation between in cell stability measurement and degradation rate of full length protein provides important information on biological insights *in vivo*. The thermodynamic stability of a truncated N-terminal domain of λ repressor, $\lambda_{6\text{-}85}$, was measured within the E.Coli cells using SUPREX (Stability of Unpurified Proteins from Rates of hydrogen deuterium exchange) [12]. Both, heavy water (D_2O) and urea, a denaturant, were allowed to permeate across *E.Coli* cell membrane and subsequently the hydrogen deuterium exchange rate of unfolded protein was measured *in vivo*. The thermodynamic stability of the protein was calculated. In this experiment *in vivo* stability measurement of $\lambda_{6\text{-}85}$ was possible till 3M urea. With higher concentration of urea cell viability dropped drastically due to the denaturation of important proteins in cell.

In hydrogen deuterium exchange kinetics the observed rate constant of exchange for each hydrogen is given by,

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

Under the condition where both k_{close} and k_{open} are much greater than k_{ex},

$$k_{HX} = \frac{K_{op} \times k_{ex}}{K_{op} + 1}$$

where, $K_{op} = (k_{open} / k_{close})$. For hydrogen exchange following global unfolding mechanism where k_{ex} is similar for entire peptide backbone amide hydrogens, $K_{op} = (1/K_{fold})$,

$$k_{HX} = \frac{\langle k_{ex} \rangle}{[1 + K_{fold}]}$$

where $\langle k_{ex} \rangle$ is the average hydrogen deuterium exchange rate of unprotected amide hydrogens. The increase in mass as a function of time can be expressed as [35]

$$\Delta M = \Delta M + (\Delta M_0 - \Delta M_\infty) e^{-[\langle k_{ex} \rangle / (1 + K_{fold})]t}$$

where ΔM_0 and ΔM_∞ are the increase in mass before and after complete exchange respectively.

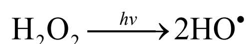
$$K_{fold} = e^{-(\Delta G_f + m[urea])/RT}$$

ΔG_f is the free energy change on folding and $m = (d[\Delta G_f] / d[urea])$. The above equation can be used to fit hydrogen deuterium exchange rates for both *in vivo* and *in vitro* experiments. From the obtained stability parameter of protein it was observed that *in vivo* stability of $\lambda_{6\text{-}85}$ in cell is very similar compared to that *in vitro*.

7. Hydroxyl Radical Labeling in Protein Footprinting

Hydroxyl radical mediated oxidative modification of proteins can be used as a probe for monitoring protein structure and dynamics [36]. Highly reactive hydroxyl radical forms covalent bonds with solvent exposed amino acid side chains. This strategy has been used in protein footprinting, an assay that traces protein conformational changes and ligand binding through the accessibility of backbone and side chain residues of amino acids [37]. In general there is incorporation of oxygen atom on modified amino acid residues. Differences in oxidative modification between multiple states of a protein, e.g., ligand bound versus unbound states provides residue level information on ligand binding sites [38,39]. Hydroxyl radical can be generated by various methods such as decomposition of H_2O_2 by Fenton chemistry, homolytic cleavage of H_2O_2 using UV radiation, radiolysis of water using synchrotron X-ray pulses or by γ-ray [40,41,42]. Optimized exposure of radicals is necessary to avoid any radical induced unfolding of the protein which might lead to the exposure of inaccessible regions of the protein molecule. Therefore, while employing the methods to generate hydroxyl radicals as covalent probes, considerable attention should be taken to ensure that oxidation reaction occurs prior to any changes in the protein conformation. Hambly and Gross demonstrated fast photochemical oxidation of protein (FPOP) where pulsed laser was used to generate hydroxyl radicals from H_2O_2 followed by oxidation of proteins to study the conformational changes and ligand binding sites *in vitro* [43]. Although, the hydroxyl radical induced unfolding of the protein occurs in a timescale of milli second or longer duration, the pulsed laser in FPOP takes an advantage of exposing the protein to the radical in less than a microsecond. Also, the presence of the

radical scavenger in the process helps in shortening the exposure lifetime of the radicals. This allows the labeling to occur in a microsecond time scale, ensuring that labeling occurs faster than the unfolding of most of the proteins [44,45]. Laser photolysis of H_2O_2 using UV-light induces a homolytic cleavage of H_2O_2 to generate highly reactive hydroxyl radical in an aqueous solution [46]. The primary quantum yield of H_2O_2 decomposition was observed to be close to 0.5 [47].



The hydroxyl radicals produced undergoes Haber-Weiss chain reaction which increases the total quantum yield of H_2O_2 decomposition to 1-2 [46],

$HO^\bullet$ undergoes diffusion controlled recombination to generate H_2O_2

Hydroxyl radical mediated footprinting of proteins involves the modification of amino acid residues either by an abstraction of hydrogen from saturated carbon atom or by the addition of hydroxyl group to an unsaturated carbon atom. Hydroxyl radical reaction via the hydrogen abstraction from C–H bond forms a carbon centered radical which then interacts with O_2 to form a peroxyl radical [48,49]. This peroxyl radical can get involved in a series of radical reactions resulting in major and minor products with respective mass shifts. The reaction on the amino acid residues with $HO^\bullet$, a week electrophile, is also significantly affected by the type of C-H ($3^0>2^0>1^0$), nature of neighbouring functional groups, stability of nascent radical and steric effect at the target site. Hydrogen abstraction is favoured when it is located adjacent to electron delocalising groups such as hydroxyl, carboxyl or amide groups that help in stabilization of the radical formed [50]. Due to steric hindrance, side chains of amino acids are preferentially attacked than α-carbon sites [51]. The peptide backbone can also be cleaved via the attack of hydroxyl radical at β-carbon atom [52]. In addition to the solvent accessibility, the reactivity of individual side chains is also determined by its chemical nature. Hydroxyl radical attack on the side chains takes place at a rate of 10 to 1000 folds faster than the hydrogen abstraction from the α-carbon atom at the peptide backbone [53].

The hydroxyl radical reaction on side chains is well characterized and developed using mass spectrometry based approach in both aerobic and anaerobic environments. Most of the amino acid residues on reaction with hydroxyl radical showed a mass shift of +16 Da on addition of hydroxyl group (A, R, N, D, E, Q, H, I, L, K, M, F, P, W, Y, V, S, T) [46]. Carbonylation (+14 Da) that leads to a mass increment of 14 Da, was observed for the following amino

acid residues: L, I, Q, R, V, P and K [46]. In addition to +16 Da, acidic amino acids, D and E might undergo loss of CO_2 (-44 Da); decarboxylation (-30 Da) and carbonylation (+14 Da). Similarly, a series of additional products with a mass shift of +5 Da, -22 Da, -23 Da, and -10 Da were observed on oxidation of His residue [46]. In addition to +16 Da and +14 Da, Arg showed a reduction in mass -43 Da by deguanidination [46]. Aromatic amino acids, Trp, Tyr, Phe, on oxidation results in multiple numbers of major and minor products with a mass shift of +16 Da, +32 Da, +48 Da etc [46]. Formation of sulfonic acid, sulfinic acid on oxidation of Cys residue results in a mass shift of +48 Da, +32 Da respectively [46]. Met on oxidation generates methionine sulfoxide, methionine sulfonate or a minor product with a mass shift of +16 Da, +32 Da or -32 Da respectively [46]. A summary of mass shift accompanied by a change in the composition for most of the amino acid residues on hydroxyl radical mediated modification is given in the **Table 1**.

The relative reactivity of amino acid side chains under aerobic condition through MS detection was observed to follow an order: Cys > Met > Trp > Tyr > Phe > His > Leu ~ Ile > Arg ~ Lys ~ Val > Ser ~ Thr ~ Pro > Gln ~ Glu > Asp ~ Asn > Ala > Gly [39]. Lower reactivity of the amino acid residues Asp, Asn, Ala and Gly in the side chains makes it less effective as probes in the experiment [39]. Modifications of Ser and Thr were found to be difficult to detect. Therefore among 20 natural amino acids 14 of them can be used as probes. These 14 amino acid residues comprises of ~65% of the total protein molecule which makes the footprinting technique to be reasonably good for probing the conformational studies of a protein molecule [54]. Following the irradiation of protein molecule with H_2O_2, the proteome is subjected to proteolytic digestion for the characterization of protein through peptide fragmentation.

8. *In vivo* Application of $HO^\bullet$ Labeling

The permeability of H_2O_2 across cell membranes via diffusion and/or through channel proteins made the *in vivo* application of FPOP feasible. In order to study the protein conformation in a living cell, FPOP footprinting method was used in cell and the oxidative modification of proteins in various subcellular compartments was studied in African green monkey kidney cells (Vero cell) [14]. After optimum incubation of Vero cells in H_2O_2 followed by pulsed laser exposure, the cells were lysed and proteome pool was subjected to proteolytic using trypsin digestion. LC/MS analysis was performed to identify and characterize the site specific modification and quantification of oxidized proteins in cell. To probe solvent accessibility of protein within the crowded environment in cell, oxidative modification of actin was investigated. The obtained profile of oxidation pattern of different residues of actin was compared with previously reported *in vitro* results. To study the actin conformation in cell, the solvent accessible surface area (SASA) of both open and closed conformations of the homologous (99%) bovine actin were compared with the obtained in cell FPOP data. Although actin is likely to present in multiple conformations inside a cell but the in cell FPOP data and its comparison with SASA

of actin suggested that most abundant conformation of actin in cell is open structure rather than the tightly packed one [14].

Hydroxyl radical labeling is fast and irreversible in nature. Thus, the labeling signature is retained in all post-labeling analytical steps. The conformational dynamics of membrane bound proteins which are difficult to isolate even, can be studied successfully using in cell FPOP method. However, the preferential oxidation of specific residues like Cys, Met limits the applicability of in cell FPOP technique to selective protein sequences. Prolonged exposure of H_2O_2 might turn out to be toxic to a biological cell. Therefore optimum time exposure and concentration of H_2O_2 to use in FPOP method in cell must be standardized very carefully.

9. Protein Crosslinking

Crosslinking is a process of chemical modification which involves the formation of covalent bonds between two molecules or two parts of a molecule through a cross-linker [55]. In protein crosslinking, mass spectrometric based identification of crosslinked peptides has become an amenable toolkit to obtain information on protein structure and topology of a protein complex [56,57]. Despite the growing application of protein crosslinking, mass spectrometry based identification of crosslinked sites was majorly restricted to *in vitro* studies [58]. However, a handful of experiments have been developed by employing cell permeable chemical crosslinkers for investigation of protein crosslinking *in vivo* [59]. Majority of the *in vivo* studies utilize formaldehyde as a crosslinker which is permeable to the cell membrane [60]. In addition, membrane permeable crosslinker, azide-tagged, acid-cleavable disuccinimidyl bis-sulfoxide was recently introduced by Robyn et al., to study protein-protein interaction in an endogenous environment [61].

The efficiency of linking two molecules using cross-linker depend on their chemical specificity (amino, sulfhydryl, carboxyl, guanidinyl etc.) and the distance between the two functional groups (spacer arm) [62]. The most widely used crosslinking reagents in protein crosslinking mass spectrometry as reviewed by Andrea Sinz are amine, sulfhydryl and photoreactive crosslinkers [63]. In general chemical crosslinkers can be broadly classified into homobifunctional, heterobifunctional and trifunctional crosslinkers. Homobifunctional crosslinkers contains identical functional groups on either sides of the spacer arm such as bis(sulfosuccinimidyl) suberate. Heterobifunctional crosslinkers comprises of two different reactive sites on either ends of the spacer arm, e.g., N-Sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate [64,65]. Trifunctional crosslinkers possess three different reactive sites where the third reactive group can link to a protein molecule or can be used for affinity purification of cross linked molecules [66]. Based on the location of the reaction, a crosslink can be formed within a single polypeptide chain, or between different polypeptide chains resulting in intramolecular or intermolecular crosslinking respectively [67,68]. Intra-

molecular cross-linking provides the information of the amino acid residues responsible in preserving the protein folds whereas intermolecular cross-linking between various proteins gains insights on specificity of the surface residues involved in protein-protein interaction [69].

10. Formaldehyde as Cross-linker for *in vivo* application

Formaldehyde is a water soluble, highly reactive polar compound which is used as a bifunctional cross-linker with a short spacer length of 2.3-2.7 Å [60]. Due to its membrane permeability, it finds more application in protein crosslinking *in vivo*. The chemical modification of protein molecules induced by formaldehyde as a cross-linker involves two steps. In a protein molecule, the nucleophilic amino group attacks the carbonyl carbon atom of formaldehyde forming an unstable carbinol intermediate which subsequently forms a methylene product on dehydration (**Scheme 1**). In the next step, nucleophilic attack on methylene carbon by another nucleophilic amino group of a protein results in cross-linking between two protein molecules (**Scheme 1**). The above reaction can be contributed by a series of side chain residues of the following of amino acids: lysine, histidine, asparagine, tryptophan, tyrosine, arginine; and α-amino group at the N-terminus of a protein molecule [60]. In the study of protein–protein and/or protein-DNA interaction using crosslinking strategy with formaldehyde as a cross linker, less than 1% formaldehyde v/v and limited time period of exposure of the experimental molecules to the cross linker are generally used. However in the histological analysis, formaldehyde with a concentration of more than 1% v/v and the duration of the crosslinking reaction from hours to days are followed to restore the localization of proteins in the tissue samples. It has been reported that use of lower concentration of formaldehyde and shorter time period, the crosslinking reaction is largely limited to the side chains of lysine and trptophan residues and the amino termini of protein molecule.

The methylene bridge that is formed between the two amino acid residues that are cross-linked, results in a mass increment by 12Da. The shorter spacer arm of formaldehyde allows cross linking between two amino acid residues of protein molecules that are located in a very close proximity [70]. In the subsequent analysis step the crosslinked proteins or peptides are purified using affinity chromatography and are subjected for proteolytic digestion for mass spectrometric analysis. Although lysine residues of proteins get crosslinked via ε-amino side chain group, it has been reported that crosslinked proteins are still left with sufficient number of unreacted lysine and arginine residues of proteins to provide global enzymatic digestion using trypsin as a proteolytic enzyme followed by fragmentation of proteolytic peptides for protein identification. In addition, most of the proteomic analysis search engine tools allow one missed cleavage in the proteolytic peptides to process the proteomics data for protein identification against the respective proteome database. Besides trypsin, other proteolytic enzymes such as GluC, chymotrypsin can also be used in protein identification [60].

Cortnie *et al*., used formaldehyde as an *in vivo* cross linker to map the proteins involved in both stable and transient interactions in 26 S proteasome network in yeast cells [16]. In this study formaldehyde was incubated with yeast cells and the cross linking reaction was quenched using glycine as quencher. The cross-linked proteins were isolated using affinity-based purification and interacting partners were characterised by mass spectrometry. Authors were successful to identify 64 proteasome-interacting proteins in yeast 26 S proteasome complex where 42 interactions were found to be novel. Azide-A-DSBSO (Azide-tagged disuccinimidyl bis-sulfoxide), a membrane permeable cross linker was synthesized by Robyn and coworkers for mapping protein-protein interactions in mammalian cells (HEK 293 cells). The synthesized chemical cross linker was incubated with HEK 293 cells and the cross linking reaction was quenched with glycine. The cross linked proteins were purified and identified through affinity purification and tandem mass spectrometry respectively. In this study, 54 crosslinked proteins were identified *in vivo* including both inter and intra-subunit novel crosslinks [61].

In vivo crosslinking coupled to mass spectrometry is a powerful technique to map protein-protein interactions in a crowded cellular environment. In formaldehyde based *in vivo* crosslinking the potential participating amino acid residue is lysine. Thus in mapping of protein-protein interaction depends on physical location of lysine also. Therefore mass spectrometry based *in vivo* cross linking is limited by the event that failure to observe successful cross linking between two proteins in the experimental results does not always mean that there is no interaction between them *in vivo*.

11. Conclusion

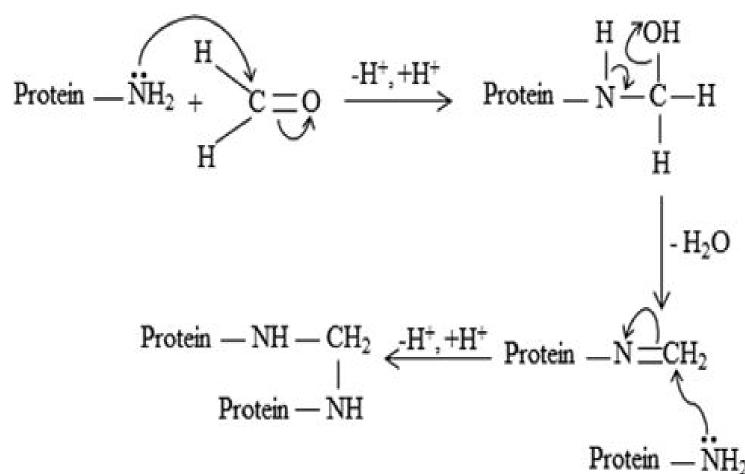
Mass spectrometry based hydrogen deuterium exchange, fast photochemical oxidation and crosslinking of different amino acid residues in a protein molecule enable to explore molecular mechanisms associated with complex biological events *in vivo*. Permeability of molecular probes, D_2O, H_2O_2 and H_2CO across the cell membrane is the crucial step behind above mentioned approaches to be successful. Molecular specificity of mass spectrometry made it feasible to investigate the structural stability, conformational dynamics of a protein and interaction network of a protein in a crowded cellular environment. However, reversible nature of hydrogen deuterium exchange, probability of oxidation induced protein unfolding and surface localization of reactive functional groups that gets crosslinked are the limitations of three aforementioned techniques respectively. Liquid chromatography based pre-fractionation at low temperature and acidic pH might help in visualizing low abundant proteins in cell. Reduced incubation period of a live cell with hydrogen peroxide might help to understand the structure of the protein in its native form. Optimal concentration of crosslinkers as well as surface exposed reactive functional groups of proteins is crucial while exploring the protein interaction network *in vivo*. Rapid advancement of mass spectrometry might provide answer in understanding the mechanisms of many complex cellular and subcellular biological processes

near future.

Table 1: A list of probable chemical modifications and the corresponding mass shifts of various amino acid residues in FPOP

Amino acid residues	Modification of side chains	Respective Mass shifts (Da)
Cysteine	sulfonic acid, sulfinic acid, hydroxy	+48, +32, -16
Methionine	sulfoxide, sulfone, aldehyde	+16, +32, -32
Phenylalanine	hydroxy	+16, +32, +48, etc.
Trptophan	hydroxy, pyrrol ring open	(+16, +32, +48, etc.), (+32, etc.)
Tyrosine	hydroxy	+16, +32, +48, etc.
Histidine	oxo, ring opening	(+16), (-22, -23, +5, -10)
Arginine	deguanidination, hydroxy, carbonyl	-43, +16, +14
Leucine	hydroxy, carbonyl	+16, +14
Isoleucine	hydroxy, carbonyl	+16, +14
Valine	hydroxy, carbonyl	+16, +14
Proline	hydroxy, carbonyl	+16, +14
Lysine	hydroxy, carbonyl	+16, +14
Glutamine	hydroxy, carbonyl	+16, +14
Serine	hydroxy, carbonyl	+16, -2
Threonine	hydroxy, carbonyl	+16, -2
Glutamic acid	decarboxylation, hydroxy, carbonyl	-30, +16, +14
Aspartic acid	decarboxylation, hydroxy	-30, +16
Asparagine	Hydroxy	+16
Alanine	Hydroxy	+16

Scheme 1: Formaldehyde mediated crosslinking through primary amino groups



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