Mass spectrometry-based methods to study protein architecture and dynamics

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Abstract: Mass spectrometry is now an indispensable tool in the armamentarium of molecular biophysics, where it is used for tasks ranging from protein sequencing and mapping of post-translational modifications to studies of higher order structure, conformational dynamics, and interactions of proteins with small molecule ligands and other biopolymers. This mini-review highlights several popular mass spectrometry-based tools that are now commonly used for structural studies of proteins beyond their covalent structure with a particular emphasis on hydrogen exchange and direct electrospray ionization mass spectrometry.

Keywords: protein folding; protein conformation; conformational dynamics; non-covalent interactions; electrospray ionization; hydrogen/deuterium exchange

Introduction

Mass spectrometry (MS) has firmly established itself as an indispensable analytical tool in experimental biophysics and biochemistry, where its applications range from rather mundane tasks of measuring masses and providing information on covalent structure of biological molecules to large-scale studies of whole proteomes. One common feature shared by most sophisticated proteomic studies and relatively straightforward experiments aimed at measuring protein mass is the implicit assumption that a protein is defined by its covalent structure (amino acid sequence and post-translational modifications, PTMs). However, covalent structure alone does not provide an exhaustive characterization of the protein molecule, as its biological activity is usually deter-

mined by the higher order structure. The latter is a result of a delicate balance among multiple interactions, which include (in addition to covalent bonds) noncovalent interactions, both intramolecular and external (with solvent molecules, cofactors, other biopolymers, etc.). Among the impressive arsenal accumulated by experimental biophysics to study protein higher order structure, MS plays a very visible role, and its influence continues to grow. In this mini-review, we present a few MS-based techniques that are used to obtain valuable information on various aspects of protein higher order structure, dynamics, and interaction with other biomolecules, with a particular emphasis on those used in our laboratory.

Characterization of Noncovalent Interactions by Direct Electrospray Ionization MS

Although biological MS has been practiced for several decades, the incredible success enjoyed by this technique in the Life Sciences is unquestionably credited to the introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/

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ionization (MALDI). These two ionization techniques allow intact biopolymers to be transferred from a condensed phase to the vacuum without damaging their covalent structure, although it is possible to induce ion fragmentation in the gas phase (if so desired). ESI MS is particularly useful for characterizing intact proteins, as it produces multiply charged ions whose m/z values are significantly lower than their masses. As a result, detection of these ions can be accomplished using a variety of mass analyzers with relatively low m/z limits. For example, an ESI mass spectrum of a 22 kDa polypeptide (light chain of human ferritin) shown in Figure 1(A) consists of a number of peaks of multiply charged ions, whose m/z values are confined to a very narrow m/z range 800–2000 accessible by most types of mass analyzers, whereas MALDI MS detection of this polypeptide would require utilization of a time-of-flight mass analyzer (currently the only commercially available analyzer capable of measuring ionic signal in m/z region beyond 20,000). Another advantageous feature of multiply charged ions is the ease of inducing their fragmentation (if so desired). For example, collision-induced dissociation of ions is caused by their collisions with neutral atoms or molecules (typically He, N₂, Ar, or Xe), and its efficiency depends on the collision energy, giving multiply charged ions an obvious advantage (prior to collision, ions are typically accelerated in an electrostatic field, and the amount of kinetic energy acquired during acceleration is proportional to the ionic charge). An example of such processes is shown in Figure 1(B), where collisions of accelerated multiply charged ions of ferritin with molecules of residual gas in the ESI MS interface region lead to efficient collisional activation and, subsequently, to ion fragmentation. The ionic peaks labeled in Figure 1(B) represent species produced by cleavage of amide bonds, giving rise to a ladder of N-terminal ions (the so-called b -ions). As each two neighbors in the ladder are related to one another by a loss of a single amino acid residue, and 19 out of 20 natural amino acids have unique masses, measuring the spacing between adjacent peaks in the ladder provides an easy way to read the amino acid sequence of the protein, as indicated at the top of Figure 1(B). Apart from collisional activation, interactions with electrons or anions present another popular way to generate structurally diagnostic fragments of protein ions (the so-called electron-capture dissociation, ECD, or electron-transfer dissociation, ETD). By definition, MALDI-generated protein and peptide ions are poorly suited for interrogation by ECD or ETD (indeed, most of them are singly charged ions, and reaction with either an electron or an anion would lead to formation of neutral species, which are invisible to MS). Contrary to that, ESI-generated multiply charged ions are perfect targets for both ECD and ETD not only because

Figure 1. ESI mass spectra of human ferritin (light chain) acquired under the conditions described below. A: Solution (10 μ M) in water/methanol/acetic acid, 48:50:2 (v:v:v) and minimal collisional activation in the ESI MS interface. B: Same solution conditions as above and significant collisional activation of the protein ions in the ESI MS interface. C: Solution (10 μ M) in 20 mM aqueous ammonium acetate and minimal collisional activation in the ESI MS interface. Fragment ion labeling in panel B uses Biemann's nomenclature of polypeptide ion fragmentation.¹

reduction of a single positive charge and ensuing fragmentation of a radical polycation produce a plethora of charged fragments but also because the

Figure 2. ESI mass spectra of 0.9 mg/mL rhASA in 100 mM ammonium acetate at pH 7.5 and 5.0. The inset shows relative abundance of ionic signals corresponding to dimeric, tetrameric, and octameric species as a function of pH. Adapted with permission from Abzalimov et al., *Anal Chem*, 2013, 85, 1591–1596.⁹

cross-section of electron capture by a polycationic species increases in concert with its charge. Finally, a great advantage of ESI MS is its ability to generate ions directly from solutions kept under nondenaturing conditions, which (if combined with gentle handling of protein ions in the ESI MS interface) frequently allows weak noncovalent interactions to be preserved and recorded in the mass spectra. An example is shown in Figure 1(C), where an ESI mass spectrum of ferritin acquired under nondenaturing conditions in solution fails to reveal ionic species corresponding to 22 kDa individual polypeptides [as observed under denaturing conditions—see Fig. 1(A)], but instead an abundant ionic signal of multiply charged ions whose mass corresponds to a 24-unit assembly of identical polypeptides forming an intact ferritin molecule.

The two parameters that are most critical for the survival of noncovalent complexes upon transition from solution to vacuum are the ESI MS interface temperature and the strength of the electrostatic field in the ion desolvation region, which determines the average kinetic energy of ions undergoing frequent collisions with neutral molecules in this region. Keeping these parameters at relatively low levels allows the composition and stoichiometry of macromolecular assemblies to be determined reliably and with minimal sample consumption. The ability of ESI MS to preserve noncovalent interactions has been used in the past two decades in numerous studies aimed at establishing quaternary structure of protein complexes.² These range from relatively modest structures to large macromolecular

assemblies whose molecular weight exceeds 1 MDa, such as intact ribosomes³ and viral capsids.⁴ In addition to studying ordered association (such as protein/receptor binding⁵ or assembly of multiunit proteins to their functional form^{6,7}), ESI MS can also be used to monitor early stages of aberrant assembly processes, such as protein aggregation,⁸ which will be discussed in more detail in the following section.

Using ESI MS to determine the stoichiometry of multiprotein complexes is particularly useful when multiple species are present in solution under equilibrium, which makes application of many other biophysical techniques problematic. ESI MS typically generates distinct signals for various assemblies, as shown in Figure 2 for a lysosomal enzyme arylsulfatase A, which converts from a dimeric to an octameric form upon mild acidification of solution (octamer formation is very important for this enzyme's survival in the acidic environment of the lysosome, where lower oligomeric states are rapidly degraded by cathepsin). Interestingly, application of X-ray crystallography to determine quaternary structure of this enzyme under both neutral and lysosomal pH yields octameric structures.¹⁰ Although size exclusion chromatography (SEC) does provide very different elution profiles for the neutral and the acidic forms of the enzyme (consistent with the dimeric and octameric forms), SEC analysis of the protein quaternary structure at intermediate pH fails to provide definitive answer due to the rapid interconversion between the dimer and the octamer on the separation time scale.⁹ ESI MS is unique, as it not only allows a clear distinction to be made between the dimeric and the octameric forms of this enzyme at any point when the two coexist under the equilibrium but also is clearly capable of detecting other (minor) species of the protein, and tracking their abundance as a function of solution conditions (Fig. 2).

One concern that is frequently expressed when ESI MS is used to probe noncovalent assemblies of proteins and other biopolymers in solution is related to the possibility of artifacts caused by dissociation of macromolecular assemblies in the gas phase. Occurrence of fragmentation in the gas phase may indeed lead to incorrect conclusions *vis-à-vis* composition of protein complexes in solution. In most cases, however, occurrence of gas phase dissociation events can be easily detected, as its mechanism (asymmetric charge partitioning¹¹) gives rise to very distinct dissociation patterns that are very different from the disassembly pathways in solution. Asymmetric charge partitioning proceeds through ejection of a single polypeptide chain from a metastable complex, with the ejected unit carrying a disproportionately high number of charges.¹² An example of such a process is shown in Figure 3, where mild collisional activation of large protein complexes (the 14-mer molecular chaperone GroEL) in the ESI

Figure 3. ESI mass spectrum of GroEL acquired under near-native conditions in solution (100 mM ammonium acetate) and mild collisional activation in the ESI interface. The low m/z region of the spectrum is shown in the inset. Highly charged monomers and low charge density tridecamers are products of dissociation of tetradecameric structures in the gas phase. Oligomerization of GroEL tetradecamers (formation of $2M_{14}$ species) is likely caused by increased protein concentration in ESI droplets as a result of solvent evaporation. Adapted with permission from Kaltashov and Abzalimov, *J Am Soc Mass Spectrom*, 2008, 19, 1239–1246.¹³

interface results in ejection of a highly charged monomeric ion from the complex. The remainder of the complex (13-mer) retains a disproportionately low fraction of the initial number of charges (average charge +43), giving rise to ionic signal at the high end of the m/z scale. The anomalous charge density of the products of dissociation is a clear indication that these species are generated in the gas phase rather than in solution.¹⁴ In general, dissociation of protein assemblies in the gas phase almost always manifests itself in ESI mass spectra by giving rise to ionic species of either atypical composition or charge density.¹⁵ Therefore, in most cases, it is not too difficult to make a clear distinction between the dissociation processes occurring in solution and in the gas phase, and the experimental conditions can be adjusted to minimize or completely eliminate the latter.

Although most of the studies of noncovalent protein complexes with ESI MS aim at establishing the binding stoichiometry (by measuring the mass of the assembly and relating it to the masses of its constituents), this technique also provides an opportunity to obtain other useful information by analyzing the average number of charges carried by the ions. The relationship between the charge on a protein ion and its size (geometry) has been the focus of numerous investigations. One particularly important observation made by Fenselau *et al.*,¹⁶ and later confirmed by De la Mora,¹⁷ Heck,² and Nesatyy,¹⁸ is that there is a strong correlation between the aver-

age number of charges accommodated by protein ions generated under native conditions in solution and the surface area of the native conformation in solution.

The extent of multiple charging also depends on certain experimental parameters (geometry and ion optics settings in the ESI and the interface region, mass bias function of the mass analyzer, etc.), but if these parameters are kept constant, the average number of charges N accommodated by a protein molecule in ESI MS appears to follow the power function¹⁹:

$$\ln(N) = A + \alpha \cdot \ln(S), \quad (1)$$

where S is the solvent-accessible surface area of the protein, $\alpha = 0.69 \pm 0.02$, and A is an empirically determined constant. Very similar correlations were obtained in a recent comprehensive analysis of the published data.²⁰ This charge–surface correlation can be used to provide reasonable estimates of protein surface areas in solution. Although it cannot rival the established techniques in terms of measurement precision, it is very useful for characterizing protein assemblies that are not amenable to analysis using traditional biophysical tools due to their transient nature or heterogeneous character.

Analysis of Large-Scale Conformational Dynamics by Monitoring Protein Ion Charge-State Distributions in ESI MS

As the extent of multiple charging of protein ions in ESI MS is determined by their solvent-exposed area, the charge-state distributions should depend on conformation in solution. Native conformers should give rise to ions with relatively low charge density, while partially or fully unstructured protein molecules should be able to accommodate a significantly higher number of charges due to an increased surface area. Indeed, such observations had been made shortly after ESI MS was introduced.^{21,22} Protein ion peaks in ESI mass spectra acquired under near-native conditions (i.e., in aqueous solutions kept at neutral pH in the absence of denaturants) typically appear in the high m/z regions of their ESI mass spectra, and the charge-state distributions in such cases are almost always narrow. On the contrary, non-native (partially or fully unfolded) protein conformers give rise to ions with a significantly higher charge density, as many more charges can be accommodated on the surface of a protein once it loses its compactness. If both native and denatured states of the protein coexist at equilibrium in solution, the protein charge-state distributions are bimodal (Fig. 4). Therefore, dramatic changes of protein charge-state distributions serve as gauges of large-scale conformational changes.²³ This feature is widely used to study large-scale protein dynamics in processes

Figure 4. ESI mass spectra of porcine pepsin acquired under near-native (pH 1.6, black trace) and denaturing (pH adjusted to 9.5, 50% MeOH by volume, blue and red traces) conditions. The mass spectra are acquired in the positive (black and blue traces) and negative (red) ion modes. The inset shows the crystal structure of pepsin, where acidic and basic residues are colored in red and blue, respectively. Adapted with permission from Kaltashov and Abzalimov, *J Am Soc Mass Spectrom*, 2008, 19, 1239–1246.¹³

ranging from folding^{24–26} to ligand binding^{27–30} to protein assembly (both ordered association^{6,31} and disordered aggregation⁸).

The example of protein unfolding presented in Figure 4 is also interesting in another respect. Pepsin is an acidic protein, which has only four basic residues; however, its unfolding leads to generation of protein ions that accommodate over 31 protons, more than six times the number of basic sites on the polypeptide chain. Furthermore, the extent of multiple charging of pepsin polyanions in negative ion ESI MS is very similar to that of multiply protonated species in positive ion ESI MS (compare the blue and red traces in Fig. 4), despite more than a 10-fold excess of acidic residues in this protein. This observation provides strong evidence that it is the physical dimension of the unstructured protein species (and not the balance of acidic and basic sites) that ultimately dictates the extent of multiple charging in ESI MS and suggests that charge-state distributions of protein ions may be used to evaluate the geometry of non-native protein conformations in solution. A recent analysis of a compilation of ESI MS data for a range of unfolded and intrinsically unstructured proteins did reveal a strong correlation between the calculated surface area in solution and the extent of multiple charging,²⁰ similar to that established earlier for natively folded protein ions [see Eq. (1)].

As long as a fraction of protein molecules in solution retain their native fold, the ion signal generated for these molecules is characterized by a nearly constant average charge, unless certain gas-phase processes cause distortion of the charge-state distributions (*vide infra*). On the contrary, the portion of ion signal representing non-native (less compact) states is much more heterogeneous (e.g., compare the red and blue traces in Fig. 4) and evolves as the solution conditions change. The reason for such behavior is that most proteins have multiple non-native conformations, not just a single random coil state, but the differences in the solvent-exposed surface areas among such states are frequently not sufficient to generate distinct ion signals in ESI mass spectra. As a result, charge-state distributions corresponding to various protein conformational isomers may be unresolved or poorly resolved (i.e., two or more different conformers may generate ions that carry the same number of charges in the gas phase).

The problem of overlapping ion signals can be circumvented using chemometric tools.^{32–34} This is done by acquiring an array of ESI MS data over a range of near-native and denaturing conditions to ensure adequate sampling of various protein states and significant variation of their fractional concentrations in solution. A total number of protein conformers sampled in the course of the experiment is determined by subjecting the entire set of collected mass spectra to singular value decomposition, SVD.³⁵ SVD of the data array yields the number of independent components, which (apart from the noise) are responsible for the observed variations of protein ion charge-state distributions. This number is equal to a number of protein conformers whose geometries are sufficiently different to allow at least some distinction to be made as far as their individual contributions to the overall charge-state distributions. A set of basis functions is then constructed, each representing a “pure signal” of a certain conformer, and the entire data array is fitted using this set, yielding ionic profiles of individual protein states over the range of solution conditions. Contribution of each conformer to the total ionic signal is determined using a supervised minimization routine. This procedure allows any set of asymmetric charge-state distributions to be represented as sums of a limited number of basis functions, each corresponding to a particular protein conformer.

This procedure is illustrated in Figure 5, where SVD is used to analyze charge-state distributions in ESI MS of apo-myoglobin (aMb), which is a single-chain 153 amino acid residue-long protein free of a heme group. Four states are known to exist both kinetically (in refolding experiments) and at equilibrium, commonly termed N (native, or native-like), I (so-called pH 4 intermediate), E (extended), and U (unfolded).³⁶ SVD of the entire data array (a 22×12

Figure 5. Chemometric analysis of the protein ion charge state distributions in ESI mass spectra of aMb acquired over a wide range of near-native and nondenaturing conditions (acid unfolding). A: Plots of singular values w_i (the inset in this panel shows cumulative variance accounted for by the first i singular values as a function of i). B and C: Normalized projection coefficients p_{ik} , $\mathbf{P} = \mathbf{V}\mathbf{T}$ obtained from SVD of the ESI MS data array (projection coefficients corresponding to only two representative significant [$i = 1$ and 4] and two insignificant [$i = 6$ and 8] singular values are shown for clarity). D–F: Fitting for representative charge state distributions of aMb, the spectra were acquired in 10 mM $\text{CH}_3\text{CO}_2\text{NH}_4$ aqueous solutions whose pH levels were adjusted to 7.4 (D), 4.5 (E), and 2.5 (F). G: Quality of the aMb ion signal reconstruction using first M singular values as a function of the dataset size K . The curves represent cumulative variance accounted for by the first M singular values within K ESI spectra of aMb ($M = 2-8$).

matrix representing 12 ESI mass spectra acquired in the pH range 2.5–10.0) yields four significant singular values, together accounting for 96% of the total signal variance (Fig. 5). This suggests that evolution of the charge-state distributions of *aMb* ions during acid unfolding of the protein can be reconstructed using four independent components, that is, four conformers become populated during such process, whose ionic signals are shown in Figure 5 as shaded bell curves. The example of *aMb* shown in Figure 5 is interesting in that the protein does not appear to be locked in a single (native) conformation even under the near-native conditions [Fig. 5(D)], always displaying the presence of non-native states. In this particular case, such behavior is caused by the heme removal from the protein, which is known to destabilize its conformation.³⁷ There are some other cases where a protein fails to fold into a unique compact structure under physiological conditions. Such proteins, collectively referred to as intrinsically unstructured proteins, display remarkable conformational heterogeneity, which manifests itself in ESI MS via broad charge-state distributions.^{38–40} Remarkably, analysis of charge-state distributions in ESI MS of intrinsically disordered proteins frequently provides evidence that at least a fraction of these species adopt very compact conformations in solution, although they always coexist at equilibrium with other, more unstructured states.^{38,41}

The ability of ESI MS to visualize protein conformers coexisting in solution under equilibrium is unique, as all other biophysical techniques commonly used to study protein structure provide information averaged across the entire ensemble of states. It must be noted, however, that the relative abundance of ionic signals representing different conformers (e.g., areas of the shaded curves in Fig. 5) is not necessarily equal to the fractional concentrations of respective protein states in solution. A thorough investigation of the relationship between the fractional concentrations of different conformers and relative abundance their respective ionic signals provided evidence that non-native polypeptide chains generate higher signal response compared to the natively folded species, and in some unfavorable cases the ionic intensity ratios may deviate from the actual concentration ratios by as much as two orders of magnitude.⁴² The higher ionization efficiency of unfolded proteins is attributed to their increased hydrophobicity, which increases their surface activity (as a result, these species are much more likely to be located at or near the solvent/air interface in the charged droplets produced by electrospray and will have a significantly higher probability to be transferred to the ion-producing progeny droplets during Coulomb explosion events). Fortunately, the same study also found that suppression of the ionic

signal corresponding to the natively folded protein species can be minimized if the experiments are carried out in a charge surplus regime, that is, if the protein concentration is kept in low or sub- μ M range.⁴²

Adding another cautionary note, it must be mentioned that characterization of protein dynamics in solution using charge-state distributions is accurate and reliable only if the observed changes in the extent of multiple charging are due to the changes in the protein shape. However, the number of charges accommodated by a protein upon transition from solution to the gas phase can also be affected by a variety of other factors, including changes in protein ion desorption conditions and ion-molecule chemistry in the ESI interface. Ignoring these factors may lead to a significant systematic error in evaluating protein conformational heterogeneity and dynamics in solution. Proton transfer, asymmetric charge partitioning, and other gas-phase processes can obviously affect protein ion charge-state distributions. One particularly common situation is the reduction of the number of protons carried by protein ions in the gas phase. Such a reduction is frequently encountered when acid unfolding of proteins in solution is induced by adjusting the pH of weak buffer solutions (such as ammonium acetate, $\text{CH}_3\text{CO}_2\text{NH}_4$) with acids (such as acetic acid, $\text{CH}_3\text{CO}_2\text{H}$). Evolution of charge-state distributions following mild acidification (down to $\text{pH} = \text{pK}_a$ of acetic acid) does not reveal any noticeable contributions of gas-phase ion chemistry.⁴³ However, continuous acidification of protein solution (below the pK_a) often results in a detectable decrease of the average charges of ionic species representing native protein conformations.⁴³ This is due to protein–anion adduct formation in solution (e.g., $\text{MH}_n^{n+} \dots \text{CH}_3\text{CO}_2^-$). At a pH equal to that of the pK_a of acetic acid, the $\text{CH}_3\text{CO}_2^-/\text{CH}_3\text{CO}_2\text{H}$ pair acts as an ideal buffer, and large quantities of $\text{CH}_3\text{CO}_2\text{H}$ must be added to cause even a relatively small pH decrease. Such large additions would cause a dramatic increase of CH_3CO_2^- concentration, resulting in more efficient $\text{MH}_n^{n+} \dots \text{CH}_3\text{CO}_2^-$ complex formation. This complex dissociates in the gas phase to produce $\text{MH}_{(n-1)}^{(n-1)+}$ and neutral $\text{CH}_3\text{CO}_2\text{H}$, given that a charge separation in the gas phase (to produce MH_n^{n+} and CH_3CO_2^- ions) would carry a very significant enthalpic penalty. The result of these processes is a reduction of charge for protein ions, which would be incorrectly interpreted (if the gas-phase ion chemistry is ignored) as a tightening of protein structure in solution.⁴³

Another gas-phase process that may affect charge-state distributions of protein ions in ESI and potentially lead to misinterpretation of experimental data is dissociation of metastable protein complexes and aggregates.¹⁵ As already mentioned earlier, such dissociation often proceeds via asymmetric charge partitioning, when a highly charged

monomer is ejected from the complex.^{11,44} The presence of such ionic species in ESI mass spectra may be (incorrectly) interpreted as a manifestation of protein unfolding in solution.⁴⁵ An example of such a process can be seen in the previously discussed Figure 3, where mild collisional activation of large protein complexes (14-meric molecular chaperon GroEL) in the ESI interface results in ejection of a highly charged monomeric ion from the complex. The remaining part of the complex (13-mer) retains a disproportionately low fraction of the initial number of charges (average charge +43), giving rise to ionic signal at the high end of the m/z scale. The monomeric products of dissociation populate the low end of the m/z scale (average charge +29), and their presence in the mass spectrum alongside the low charge density monomeric ions (average charge +16) may falsely suggest the existence of unstructured monomers in solution at equilibrium with the natively folded species if the dissociation of the non-covalent complex is not taken into account.

Selection of the data acquisition parameters in ESI MS may (and almost always does!) also introduce bias in the ionic charge-state distribution by altering the transmission/detection efficiency as a function of m/z . Obviously, analysis of charge-state distributions of protein ions in ESI MS provides reliable information on protein conformational dynamics only if the observed changes in the extent of multiple charging are due to the changes of protein compactness in solution. Ignoring the gas phase processes that may alter the ionic charge distributions can introduce a significant systematic error in the evaluation of protein conformational heterogeneity and solution dynamics.

Hydrogen/Deuterium Exchange MS

One of the shortcomings of the direct ESI MS methods of probing protein higher order structure in solution described in the preceding sections is that such studies can only be carried out in solutions compatible with the ESI process. Even though physiologically relevant pH and ionic strength can be used in such experiments, the choice of buffers and salts is limited to volatile electrolytes (such as ammonium acetate or ammonium bicarbonate). In some cases, this limitation may not be acceptable (e.g., in a situation when behavior of a protein drug must be characterized in the context of its formulation buffer). Another limitation of the direct ESI MS methods of protein structure analysis is that they almost never provide sufficient structural detail. For example, when protein/protein interactions are studied with ESI MS, only the composition of the noncovalent complexes and the binding stoichiometry can be determined, while very little can be said about the location of the binding interfaces. Likewise, when ESI MS is used to monitor conformational

transitions in solution, it can detect the onset of protein unfolding, but does not provide any detail vis-à-vis localization of protein segments that lose their native structure. All these limitations are overcome by hydrogen/deuterium exchange (HDX), another technique that is frequently used in conjunction with MS to probe higher order structure and dynamics of protein molecules. Although MS is not the only means of detection that can be used for HDX measurements (high-resolution NMR is another popular choice), MS does offer several important advantages, namely: a faster time scale, tolerance to high-spin ligands and cofactors, ability to monitor the exchange in a conformer-specific fashion as well as much more forgiving molecular weight limitations. Another advantage offered by MS is its superior sensitivity, which allows many experiments to be performed using only minute quantities of proteins.

HDX targets all labile hydrogen atoms (i.e., those attached to nitrogen atoms at the backbone amides and heteroatoms at polar/charged side chains), although many labile hydrogen atoms do not readily undergo HDX due to their involvement in hydrogen bonding networks or sequestration from the solvent in the protein interior. Therefore, protein HDX involves two different types of reactions: (i) reversible protein unfolding that disrupts the H-bonding network and/or exposes buried segments to the solvent and (ii) isotope exchange at individual unprotected amides. As protein unfolding (either local or global) is a prerequisite for exchange at the sites that are protected in the native conformation, HDX reactions serve as a reliable and sensitive reporter of the unfolding events. However, conformation and dynamics are not the only determinants of the HDX kinetics. Even in the absence of any protection, the exchange kinetics of a labile hydrogen atom is strongly dependent on the nature of the functional group. Furthermore, the exchange rate of an unprotected hydrogen atom (often called intrinsic or chemical exchange rate, k_{int}) is strongly influenced by a variety of extrinsic factors, most notably solution pH and temperature, and the intrinsic rate constant can be expressed as⁴⁶:

$$k_{\text{int}} = k_{\text{acid}}[\text{H}^+] + k_{\text{base}}[\text{OH}^-] + k_{\text{W}}, \quad (2)$$

where k_{acid} , k_{base} , and k_{W} represent rate constants of exchange catalyzed by acid, base, and neutral water molecules.

Backbone amide hydrogen atoms constitute a particularly interesting class of labile hydrogen atoms due to their uniform distribution throughout the protein sequence, which makes them very convenient reporters of protein dynamics at the amino acid residue level (proline is the only naturally occurring amino acid lacking an amide hydrogen atom). Therefore, the majority of HDX MS

experiments are concerned with the exchange of the backbone amide hydrogen atoms. The mathematical description of HDX kinetics of backbone amides was introduced several decades ago and is based upon a simple two-state kinetic model⁴⁷:

where k_{op} and k_{cl} are the rate constants for the opening (unfolding) and closing (refolding) events that expose/protect a particular amide hydrogen to/from exchange with the solvent.

In most HDX studies, the exchange-incompetent state of the protein is its native state, whereas the exchange-competent state is either a fully unfolded (random coil) or a partially unfolded (intermediate) state. Alternatively, it can represent a structural fluctuation within the native conformation, which exposes an otherwise protected amide hydrogen to solvent transiently without large-scale structure loss.^{48,49} Transitions between different non-native states under equilibrium conditions are usually ignored, as the majority of HDX measurements are carried out under native or near-native conditions when the Boltzmann weight of such states is negligible when compared with the native state.

HDX MS measurements can provide information on global protection by measuring the deuterium content of the entire protein, although the data interpretation often uses the kinetic model⁴ by making an implicit assumption that **NH(protected)** and **NH(unprotected)** represent groups of amides, rather than individual amides that become unprotected upon transition from one state to another. Two extreme cases are usually considered: a situation when $k_{\text{cl}} \gg k_{\text{int}}$ and $k_{\text{cl}} \ll k_{\text{int}}$. The former case (referred to as the EX2 exchange regime) is commonly observed under native or near-native conditions, when each unfolding event is very brief, and its lifetime ($1/k_{\text{cl}}$) is much shorter than the characteristic time of exchange of an unprotected labile hydrogen atom ($1/k_{\text{int}}$). In this case, the probability of exchange for even a single amide during an unfolding event will be very low, and the overall rate of exchange will be defined by both the frequency of unfolding events (k_{op}) and the probability of exchange during a single opening event:

$$k^{\text{HDX}} = k_{\text{op}} \cdot (k_{\text{int}}/k_{\text{cl}}) = k_{\text{int}} \cdot K, \quad (4)$$

where K is an effective equilibrium constant for the unfolding reaction, which is determined by the free energy difference between the two states of the protein. The overall exchange rate constant k^{HDX} in this case is a cumulative rate of exchange, that is, an ensemble-averaged rate of deuterium incorpora-

Figure 6. Time evolution of the ion peak (charge state +14) of cellular retinoic acid binding protein I in H₂O solution at pH 3.5 (the protein was completely deuterated and kept in D₂O-based solution prior to the measurement, and the HDX reactions were induced by rapid dilution of the stock solution in H₂O). The dashed line on the left indicates the position of the fully exchanged protein ion peak. Reproduced with permission from Eyles et al., *J Mass Spectrom*, 1999, 34, 1289–1295.⁵⁰

tion into a molecule, and is measured as a mass shift of the isotopic cluster of the protein ion as a function of HDX time.

The opposite extreme ($k_{\text{cl}} \ll k_{\text{int}}$) is observed either when the protein is placed under denaturing conditions (which dramatically decreases the refolding rate k_{cl}) or by increasing the intrinsic exchange rate (e.g., by elevating the protein solution pH). As a result, the lifetime of the unprotected states become long enough to allow all exposed labile hydrogen atoms to be exchanged during a single unfolding event. In this case (commonly referred to as the EX1 exchange regime), the exchange rate will be determined simply by the rate of protein unfolding:

$$k^{\text{HDX}} = k_{\text{op}} \quad (5)$$

HDX MS measurements performed under the EX1 conditions typically give rise to bimodal or multimodal isotopic distributions, where the deuterium content of each part reflects the backbone protection levels of distinct protein conformers. This gives HDX MS the unique ability to visualize and track multiple protein states that may coexist in solution under equilibrium (Fig. 6).

Although the experiment depicted in Figure 6 was performed on-line (protein solution undergoing exchange was continuously infused in the ESI source), most measurements are performed following rapid acidification of the protein solution to pH 2.5–3

Figure 7. Schematic representation of HDX MS work flow to examine protein higher order structure and conformational dynamics. The exchange is initiated by placing the unlabeled protein into a D_2O -based solvent system (e.g., by a rapid dilution). Unstructured and highly dynamic protein segments undergo fast exchange (blue and red colors represent protons and deuterons, respectively). Following the quench step (rapid solution acidification and temperature drop), the protein loses its native conformation, but the spatial distribution of backbone amide protons and deuterons across the backbone is preserved (all labile hydrogen atoms at side chains undergo fast back-exchange at this step). Rapid clean-up followed by MS measurement of the protein mass reports the total number of backbone amide hydrogen atoms exchanged under native conditions (a global measure of the protein stability under native conditions), as long as the quench conditions are maintained during the sample work-up and measurement. Alternatively, the protein can be digested under the quench conditions using acid-stable protease(s), and LC/MS analysis of masses of individual proteolytic fragments will provide information on the backbone protection of corresponding protein segments under the native conditions. Reproduced with permission from Bobst and Kaltashov, *Curr Pharm Biotechnol*, 2011, 12, 1517–1529.⁵¹

and lowering the temperature to 0–4°C, which results in significant deceleration of the chemical (intrinsic) exchange rates of backbone amide hydrogen atoms. These conditions, known as quench or slow exchange conditions, also result in unfolding of most proteins. As the intrinsic exchange rates of labile side chain hydrogen atoms are not decelerated as significantly as those for backbone amides, all information on the side chain protection is generally lost during this step, leaving a single HDX reporter for each amino acid residue (again, with the exception of proline residues). In addition to characterizing protein conformation and stability globally, the protein can be digested with an acidic protease (e.g., pepsin) under the slow exchange conditions, and MS (usually following quick

desalting and fast LC separation) can be used to measure the deuterium content of each proteolytic fragment. A typical workflow diagram of an HDX MS experiment is shown in Figure 7.

An example of using HDX MS to probe protein conformation and dynamics as well as to identify protein segments involved in ligand binding is shown in Figure 8, where HDX kinetics are measured for apo- and holo-forms of the N-lobe of human serum transferrin. The peptic fragment whose deuterium uptake pattern is shown in this Figure is located in the iron-binding cleft of this protein, and this segment becomes less accessible to solvent upon metal binding, as is clearly demonstrated by reduced deuterium uptake by this peptide derived from the

Figure 8. Local HDX MS data for the N-lobe of human serum transferrin (a polypeptide segment represented by a peptic fragment 205–211). The top diagrams show localization of this segment within open (left) and closed (right) conformation of the protein (apo- and holo-forms, respectively). The mass spectra in the lower left panel show evolution of the isotopic distribution of this peptide throughout the course of exchange reactions at neutral pH. The red and blue traces correspond to peptides derived from holo- and apo-forms of the protein, respectively. The diagram in the lower right panel shows kinetics of deuterium incorporation within this peptide at neutral pH. Adapted with permission from Kaltashov et al., *Biochim Biophys Acta*, 2012, 1820, 417–426.⁵²

holo-form of the protein. In addition to probing small ligand binding to proteins,^{53,54} this approach can also be used for mapping interface regions in multi-protein complexes.^{55–64} HDX MS is also an excellent tool for probing conformational changes induced in proteins by enzymatic PTMs,⁶⁵ chemical modification,^{66,67} and mutations.⁶⁸

Spatial resolution offered by HDX MS is limited by the extent of proteolysis, which must be performed relatively quickly under the slow exchange conditions to avoid occurrence of significant back-exchange prior to MS measurements of the deuterium content of individual peptide fragments. In general, a large number of proteolytic fragments, particularly overlapping ones, would lead to greater

spatial resolution, and hence more precise localization of the structural regions which have undergone exchange. In some cases, this may allow the backbone amide protection patterns to be determined at a single-residue resolution,⁶⁹ although such instances remain very rare. Supplementation of enzymatic digestion with peptide ion fragmentation in the gas phase may also enhance the spatial resolution of HDX MS measurements,^{64,70–72} although extreme care must be taken to avoid possible gas phase artifacts leading to hydrogen scrambling within the peptide ion prior to its fragmentation.⁷³ In addition to limited spatial resolution, HDX MS measurements frequently suffer from incomplete sequence coverage, especially when applied to larger and extensively

glycosylated proteins. Proteins with multiple disulfide bonds constitute another class of targets for which adequate sequence coverage is difficult to achieve, although certain changes in experimental protocol can alleviate this problem, at least for smaller proteins.⁷⁴ Typically, an 80% level of sequence coverage is considered good, although significantly lower levels may also be adequate, depending on the context of the study.

An alternative method to probe HDX kinetics locally does not rely on proteolytic fragmentation prior to MS analysis, but instead takes advantage of the ability of modern mass spectrometers to induce fragmentation of intact protein ions in the gas phase followed by detection and identification of all fragment ions [the so-called top-down approach to MS/MS similar to that shown in Fig. 1(B)]. One unique advantage of the top-down HDX MS/MS measurements that cannot be matched by the classic bottom-up type experiments is the ability to obtain protection patterns in a conformer-specific fashion. This can be accomplished by fragmenting subpopulations of protein ions, which are mass-selected to include species with deuterium content representative of a certain protein conformer (this, of course, can be accomplished only under conditions favoring EX1 exchange regime in solution, so that different protein conformers can be visualized based on different levels in deuterium incorporation). Despite the great promise of top-down HDX MS,⁷³ applications of this technique have been limited so far due to concerns over the possibility of hydrogen scrambling accompanying dissociation of protein ions in the gas phase.⁷⁵ Several recent studies demonstrated that the extent of scrambling is indeed negligible when ECD⁷⁶ or ETD⁷⁷ is used as a means of generating fragment ions in top-down HDX MS experiments, while collisional activation of the protein ions is minimized. In addition to allowing hydrogen scrambling to be eliminated in the top-down HDX MS experiments, both ECD and ETD appear to be superior to collisional activation in terms of sequence coverage,⁷⁸ allowing both better sequence coverage and enhanced spatial resolution to be achieved. In fact, in some cases, it becomes possible to generate patterns of deuterium distribution across the protein backbone down to the single residue level.^{76,79,80}

Problem Areas in MS

The achievements of MS in the biophysical arena and in the studies of protein architecture and dynamics in particular are unquestionably impressive; however, several areas remain where progress has been less spectacular. As a detailed assessment of all challenges faced by MS is outside of the scope of this review, only two specific areas relevant to studies of protein conformation and dynamics will be considered here. One particularly limiting restriction is

the requirement that only “ESI-friendly” solvents be used in direct ESI MS measurements. Although these systems (volatile electrolytes, such as ammonium acetate or ammonium bicarbonate) can be readily used to perform measurements under physiological pH and ionic strength, the effective ban on the use of nonvolatile electrolytes means that a range of buffers and salts commonly used in biochemistry cannot be used in direct ESI MS measurements. Although relatively small amounts of nonvolatile electrolytes can be tolerated by modern high-performance ESI sources, physiologically relevant concentrations remain outside of the reach of direct ESI MS. Another area where the utility of MS remains limited is analysis of highly heterogeneous systems, such as extensively glycosylated proteins, protein-polymer conjugates, and so forth. Such highly heterogeneous systems give rise to broad mass distributions, which in the case of ESI MS frequently leads to poorly resolved or completely unresolved charge-state distributions, making even the mundane task of mass measurement an extremely challenging undertaking. As the interest in such systems continues to intensify (e.g., due to continuous adoption of MS-based tools in the biopharmaceutical sector), extensive efforts are now invested into solving this problem, and several recent developments (e.g., use of ion chemistry in the gas phase as a means of addressing the problem of structural heterogeneity⁸¹) give reason for optimism.

Conclusions

The focus of this review was on direct ESI MS methods and HDX MS, but there is also a host of other powerful MS-based techniques that can be used for probing protein higher order structure (such as chemical cross-linking and chemical labeling). MS has truly become a routine analytical tool for analysis of conformation and dynamics of proteins, frequently revealing details that cannot be provided by other experimental biophysical techniques. It is now and will continue to be a very dynamic area of research, which will certainly continue to advance the fields of biophysics and structural biology. Importantly, studies using MS-based methods of protein characterization beyond covalent structure analysis are no longer confined to the academic labs, but have become accepted tools in biotechnology and biopharmaceutical industry,^{51,82,83} where their role will also undoubtedly continue to expand.

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