Mass spectrometry studies of protein folding

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Mass spectrometry has become a powerful method to study the structure and dynamics of proteins. Hydrogen/deuterium exchange in conjunction with mass spectrometry is now becoming more widely used to monitor conformational changes in proteins, and when combined with proteolytic digestion or gas-phase dissociation, it can provide spatial resolution of structural regions which participate in conformational change. The biggest advantage offered by mass spectrometry is that it can distinguish different conformations of a protein even when they are present together, and this has made it an indispensable tool for studying the heterogeneity inherent in protein folding and unfolding reactions. This review surveys the application of mass spectrometry to study protein folding and unfolding reactions, and describes the important insights obtained from these studies. It also briefly examines the use of mass spectrometry to study the assembly and disassembly of large multimeric protein complexes, and to obtain structural information on amyloid protofibrils and fibrils.

Keywords: Hydrogen/deuterium exchange, mass spectrometry, protein folding and aggregation, macromolecular assembly.

Introduction

PROTEIN molecules are polymers typically constituted of a few hundred amino acid residues connected in a one-dimensional chain. Unlike other types of polymers which can arrange themselves in space in an almost infinite number of ways, a protein adopts a unique three-dimensional fold under native conditions, which confers to it a particular function. Even natively unfolded proteins fold to specific functional structures upon binding to specific partners[1–3]. The main question to be answered in protein folding studies is: How does a protein attain a unique structure out of nearly an infinite number of possible conformations, within the timescale of other cellular processes? Answering this question will lead to an understanding of the relationship between the amino acid sequence and the mechanism of protein folding, and will facilitate the prediction of the three-dimensional structure of a protein from its amino acid sequence.

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Based on extensive experimental studies, several phenomenological models have been proposed for describing the process of protein folding. The *nucleation model* envisages that a few adjacent residues of the unfolded polypeptide chain arrange themselves in a small secondary structural unit, which acts as a nucleus, and the growth of the rest of the structure propagates from the nucleus[4]. The *hydrophobic collapse* model suggests that the unfolded polypeptide chain undergoes an entropically driven transition in polar solvents, giving rise to a collapsed intermediate in which further structure evolves[5–7]. The *framework model* describes hierarchical formation of protein structure; secondary structural elements are formed independently, and they then arrange themselves in the final tertiary fold[8–10]. The *nucleation–condensation model* has both consolidation of the nucleus and formation of structure taking place concurrently; partially folded conformations are not populated[11–13]. These models suggest different ways by which the conformational search for the acquisition of the structure is restricted, so that folding occurs on a biologically relevant timescale.

Theoretical and computational studies describing the free energy surface over which folding reactions occur, have contributed significantly towards bettering our understanding of the mechanism of protein folding. These models suggest that protein folding pathways more closely resemble 'funnels than tunnels in configuration space'[14]. The funnel picture of folding depicts how conformational entropy (the number of accessible conformations) may decrease with decrease in an effective potential energy averaged over solvent interactions[15,16]. The potential energy gradient ensures that the search for the native state, which has the lowest effective energy, is not a random, unbiased search. The roughness of the free energy landscape is associated with partially folded conformations which are considered to be kinetic traps, and the different microscopic routes taken by different molecules to fold represent one or more macroscopic pathways for folding[15,16].

Most models proposed to describe the mechanism of protein folding envisage that partially folded intermediates are populated during protein folding reactions. Folding intermediates act as milestones defining the progress of the folding reaction, and can be used to delineate one folding pathway from another. Importantly, their presence facilitates folding because then entropy and energy changes occur stepwise, thereby making it possible for the free energy barriers to be smaller than they would be

if folding occurred in one step. But a number of proteins have been reported to fold by a simplistic 'two-state' mechanism[17], according to which a single, dominant, free energy barrier arises because of a mismatch on the free energy surface between potential energy favouring folding and the conformational entropy disfavouring folding. Apparent two-state folding behaviour is observed because partially folded intermediates are populated too sparsely to be detected by commonly used experimental probes. Nevertheless, for many 'two-state' folding proteins, partially folded conformations have now been detected, either indirectly[18] or directly[19,20], and intermediates can be made to manifest themselves by effecting a change in the folding or unfolding conditions[21–23]. The detection of folding intermediates requires the appropriate choice of folding conditions in which they get stabilized, and the appropriate choice of a probe sensitive to their presence.

A major reason why many proteins have been reported to fold in a 'two-state' manner is because of the types of probes that have been used to monitor folding and unfolding transitions. Optical probes such as steady-state fluorescence and circular dichroism (CD), which have been commonly used, provide ensemble-averaged signals and cannot distinguish between the different coexisting conformations of a protein. Hence, protein folding and unfolding reactions appear simple, similar to chemical reactions. But in contrast to chemical reactions, where there is formation or breakage of one or a few covalent bonds, hundreds of non-covalent interactions are broken and formed in protein folding reactions. Although commonly done, equating protein folding reactions with small-molecule chemical reactions may result in an improper understanding of the principles governing the mechanism of protein folding. The complexity of protein folding reactions becomes apparent when high-resolution probes such as nuclear magnetic resonance (NMR), single-molecule methods and time-resolved fluorescence resonance energy transfer (TR-FRET) methods, or hydrogen/deuterium (HX) exchange in conjunction with NMR or mass spectrometry (MS) are used[24]. NMR can provide residue-specific information, whereas TR-FRET and HX-MS can distinguish between populations of molecules present together in different conformations[25,26]. In this review, the application of MS to the studies of protein folding and unfolding, and the insights obtained into the mechanism of protein folding from these studies are presented.

Mass spectrometry in the study of protein folding and unfolding

Mass spectrometry has been used to study protein folding for several years now. Before describing the application of MS to the study of protein folding, it is important to describe briefly pertinent aspects of the technique. Among the several ionization methods available, electrospray ionization (ESI) has been commonly used to study the dynamics of proteins and protein–protein complexes. A typical ESI mass spectrum consists of a distribution of charge states with different mass-to-charge ratios. In electrospray ionization mass spectrometry (ESI-MS), ions are generated directly from solution. Detection of protein molecules on the basis of the mass-to-charge ratio, makes it possible to detect high-mass proteins and protein–protein complexes. Each charge state seen in an ESI mass spectrum is a distribution of masses, arising due to the natural abundances of atomic isotopes like ^{13}C, ^{15}N, etc. The natural isotope abundances determine the width of the mass distribution. High-resolution mass spectrometers (< 10 ppm resolution) can resolve different isotopic peaks of a charge state, and this can provide valuable information about the molecule of interest. The multiple charge states in an ESI mass spectrum, when deconvoluted to mass, provide mass measurements of a higher accuracy than does a matrix-assisted laser desorption ionization (MALDI) mass spectrum, which typically contains only one or two charge states.

The application of MS to the study of biomolecules has received a tremendous boost because of advances in MS technology[27–31]. The current list of applications of MS includes the identification of covalent modifications of proteins, study of protein–protein complexes, determination of amino acid sequences of proteins, identification of segments of a protein involved in protein–protein interactions, and the study of the conformational dynamics of proteins and protein–protein complexes[32–40].

Direct mass spectrometry studies of protein conformations

When acquired in denaturing solvent conditions, ESI mass spectra of proteins generally show higher charge state distributions (CSDs) than when acquired under solvent conditions favouring the folded conformation[41]. Several studies have attributed this phenomenon to changes in the solvent accessibility of the possible protonation sites of charged amino acid residues, and to changes in their specific pK_a values[42,43]. Another model postulates that the shift of the CSD towards higher values upon unfolding, arises mainly due to the increased surface area of the unfolded polypeptide[44].

To elucidate the structural changes in proteins which give rise to different CSDs, the acid-induced unfolding of cytochrome *c* in different concentrations of methanol was monitored by fluorescence and CD in addition to ESI-MS. In 50% methanol, the tertiary structure (monitored by fluorescence) is lost at ~pH 4, but no significant change in the helical content (monitored by CD at 222 nm) was observed. Both tertiary as well as secondary structures were lost in a similar fashion upon pH-induced unfolding in 3% methanol. Despite the difference in the secondary structure contents of the acid denatured states in the two methanol concentrations, the CSDs in the ESI mass

spectra were found to be similar. This led to the conclusion that in the case of cytochrome *c*, the CSD in the ESI mass spectrum changes with a change in the tertiary structure and is not sensitive to change in the secondary structure[45]. A comparison of the CSDs of proteins in solvents with different surface tension revealed that the CSD is not sensitive to the surface tension of the solvent[46].

Alcohol and acid-induced unfolding of the several proteins has been monitored by the change in their CSDs, and partially unfolded conformations populated during unfolding have been detected[47–49]. The acid-induced unfolding of myoglobin was studied by time-resolved ESI-MS using a continuous-flow mixing technique. An intermediate, which is largely unfolded but which has intact heme–protein interactions was detected[47]. This approach was also used to study the effect of metal ion binding[50], and the influence of mutations on the conformational stability of a protein[51].

Optical spectroscopic techniques cannot dissect out the conformational changes in each component of a multicomponent system. On the other hand, changes in the CSD reflect changes in individual components. For example, the assembly of tetrameric hemoglobin was studied by examining the change in its CSD; seven different species populated during assembly could be detected[52]. Acid and organic co-solvent-induced unfolding of hemoglobin monitored by ESI-MS revealed that the loss of heme from both the α- and β-chains occured in parallel, suggesting a symmetric mechanism of denaturation[53].

Measurement of the change in the CSD has been used to monitor the conformational dynamics of many proteins, but it provides largely qualitative or semi-quantitative information. Factors which affect the desorption of proteins, or gas phase ion chemistry, can alter the CSD. Hence, appropriate care must be taken while interpreting the changes in a CSD. It should also be noted that comparative studies of positive- and negative-ion CSDs on several small polypeptides led to the conclusion that only positively charged ions can provide information on the structures of proteins[54]. Hence, it is important to develop a good understanding of a protein CSD and to have a control on it. Continuous flow-extractive desorption ESI developed recently is an ESI arrangement by which the CSD of a protein can be controlled[55]. In a typical ESI-MS, a change in the CSD of the protein requires a change in the pH of bulk solution; however, exposure of electrospray droplets to acid vapours provides a fast means of changing the CSD independent of the pH of bulk unbuffered solution[56].

Mass spectrometry in conjunction with hydrogen/deuterium exchange

In an unfolded polypeptide, the amide hydrogens continuously exchange with solvent protons with an intrinsic exchange rate, k_{int}. The value of k_{int} of an amide hydrogen depends on the pH, temperature and nature of the neighbouring amino acid residues[57]. The intrinsic exchange rate has a minimum value around pH 2.5. The mechanism of exchange at pH values above this minimum involves the abstraction of a proton, which is catalysed by OH^-, and k_{int} increases by one order of magnitude for every unit increase in pH. Below pH 2.5, the intrinsic exchange rate also increases and exchange is catalysed by H_3O^+. In general, the exchange rate doubles for every 10°C rise in temperature. The inductive effect exerted by neighbouring amino acids can change the pK_a value of the amide hydrogen, and thus affect the intrinsic exchange rate[57]. In the case of folded proteins, the observed exchange rate (k_{HX}) is slower than the k_{int} measured in an unfolded polypeptide because: amide hydrogens (i) buried and shielded from the solvent, and (ii) involved in hydrogen bonding in the secondary structure.

The folded conformation of a protein is not a rigid static state, but undergoes conformational transitions of varying magnitudes, from small local fluctuations to complete unfolding. These conformational transitions result in the transient exposure of buried and hydrogen-bonded amide hydrogens to the solvent. They may be small, leading to the exchange of only a single amide hydrogen, or at the other extreme, they may be large enough to expose all amide hydrogens to the solvent. Thus, the exchange at amide hydrogen sites in a folded protein depends upon unfolding and folding events having rate constants of k_u and k_f, respectively. This can be represented for an amide hydrogen as:

$$\text{NH (folded)} \underset{k_f}{\overset{k_u}{\rightleftarrows}} \text{NH (unfolded)} \overset{k_{int}}{\rightarrow} \text{ND (exchanged).}$$

k_{HX} is given by

$$k_{HX} = \frac{k_u \cdot k_{int}}{k_u + k_f + k_{int}}.$$

In the EX1 limit, where $k_u < k_f << k_{int}$, every unfolding event leads to the exchange of one or more hydrogens, and the observed exchange rate reduces to $k_{HX} = k_u$. In the EX2 regime, where $k_f >> k_{int} >> k_u$, every unfolding event does not result in exchange, and an equilibrium gets established between the folded and exchange-competent unfolded states. In this case $k_{HX} = (k_u/k_f)\cdot k_{int}$. Also, k_{HX} increases with pH in the EX2 limit, whereas it is independent of pH in the EX1 limit. When k_f is comparable in value to k_{int}, the exchange mechanism falls neither in the EX1 limit nor in the EX2 limit, but in the mixed EXX limit[58].

Monitoring HX reactions in proteins

The HX reactions of proteins are most commonly monitored by NMR, which gives the average proton occupancy at each amide site at each time point of the folding

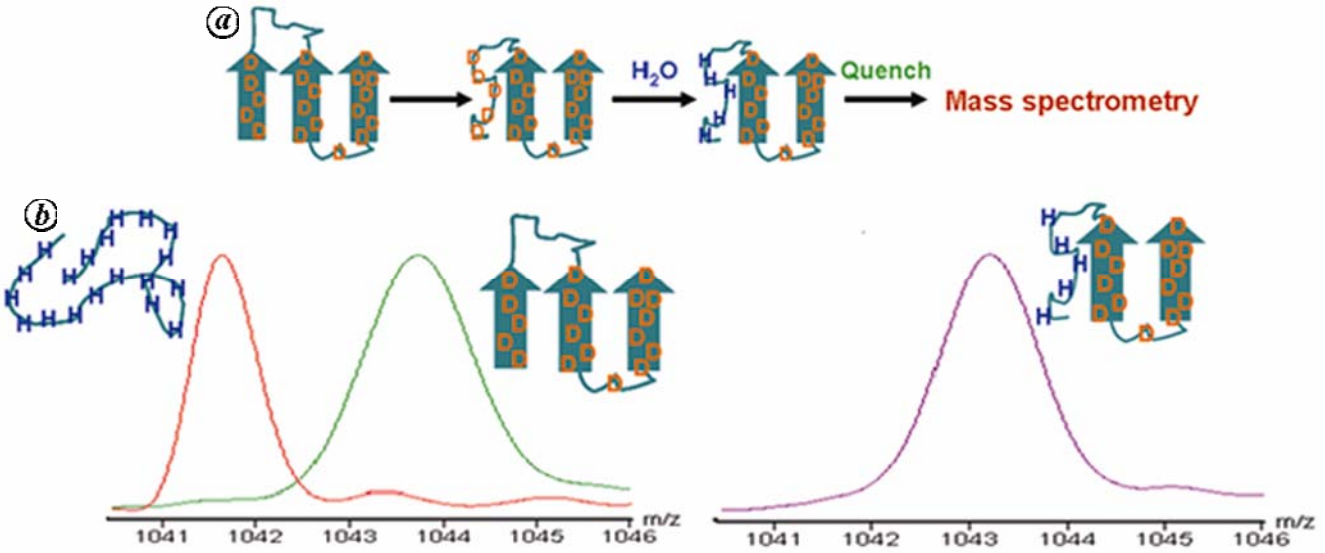

Figure 1. Schematic description of the principle of HX-MS to monitor conformational changes during protein unfolding. ***a***, The deuterated protein is briefly exposed to an excess of water after increasing time intervals of unfolding. The segments which have unfolded exchange their amide deuterium with solvent protons. The exchange is quenched by lowering the pH. The decrease in the mass of the protein, determined by the extent of exchange/unfolding, is measured by mass spectrometry. ***b***, The isotopic distributions (from the same charge state) of different conformations of a protein are centred at different *m*/*z* values in a mass spectrum. Hence, they can be distinguished from each other. The folded protein with the maximum number of protected deuterium is represented by the highest *m*/*z* distribution, whereas the completely unfolded state having all amide sites protonated is represented by the lowest *m*/*z* distribution. The *m*/*z* distribution corresponding to the partially unfolded conformation lies in between the folded and unfolded protein mass distributions, and is centred at a *m*/*z* value which depends on the extent of exchange (unfolding).

reaction. By measuring proton occupancy at each amide site as a function of the time of folding, a kinetic curve for the gain of protection against HX can be constructed, from which the structural status of the amino acid residue at any time during folding can be inferred. A major limitation of HX-NMR arises from the fact that it gives an ensemble-averaged signal, and hence cannot distinguish molecules having an amide site protonated from molecules having the same amide site deuterated, when both are present in the same sample. MS can differentiate between different molecules on the basis of their masses. Hence, it can distinguish between populations of molecules having different numbers of deuterium incorporated, even when all these molecules are present together. Another advantage of MS is that the protein concentration needed is typically a few hundred-fold lower than that required for NMR measurements. In addition, large proteins as well as protein–protein complexes can be studied by MS, but not by NMR. Unlike NMR, however, MS cannot readily provide residue-level information, but this apparent drawback can be circumvented by proteolytic digestion and MS/MS analysis of exchanged samples.

Figure 1 explains how HX-MS can be used to monitor conformational changes in proteins. The deuterated protein is exposed to an excess of H_2O. The molecules which have undergone a conformational change resulting in the exposure of some amide deuteriums to the solvent will exchange these deuteriums with the solvent protons. Consequently their mass will be lower than the molecules that do not undergo the conformational change. For example, in the case of protein (un)folding, the folded protein will typically have a certain fraction of its amide deuteriums protected against exchange, the completely unfolded conformation will exchange almost all amide deuteriums, and a partially unfolded conformation will afford protection to only a subset of the amide deuteriums protected in the folded form. Different extents of unfolding in different conformations of a protein result in the exchange of different numbers of amide deuterium. Hence, the different conformations have different masses, and are therefore distinguishable by MS, even when they coexist (Figure 1 *b*).

ESI HX-MS spectra are not often as simple as shown in Figure 1 *b*, and if multiple conformations of a protein coexist, the analysis of the spectrum becomes complicated. A HX mass spectrum can provide answers to several questions: (1) How many conformations of a protein are present in a given sample? What is the extent of exchange in each conformation? Which segments have undergone exchange in each conformation? What is the rate of inter-conversion among different conformations? In most cases, HX-MS spectra are analysed manually; however, some efforts have been made to develop software that can be used to analyse HX-MS data[59–65].

Deducing the structure of partially (un)folded conformations

Although, HX-MS offers several advantages over HX-NMR, it falls behind the latter in not being able to pro-

vide residue-level information on the different conformations sampled by a protein. Zhang and Smith[66] developed a proteolytic digestion/MS methodology which can be used to determine the pattern of isotope exchange along the primary sequence of a protein. The regions of a protein which transiently sample the unfolded conformation, or remain in the unfolded state, undergo exchange, whereas folded regions protect their amide sites from exchange. Hence, from the pattern of exchange, derived from an analysis of proteolytic fragments created after HX is complete, the structural status of a sequence segment can be inferred. This methodology can be applied to equilibrium and kinetic studies of protein folding and unfolding.

The details of the technique have been reviewed earlier[67–69]. Briefly, exchanged and quenched samples are digested with pepsin, either by adding pepsin directly to the solution or by passing the samples through a column containing immobilized pepsin on agarose beads. Since pepsin remains active even at pH 3, it is suitable for the digestion of proteins at low pH, where the intrinsic exchange rate is at its minimum value. The separation of peptides is carried out by reverse phase chromatography using a high performance liquid chromatography (HPLC) system connected to the source of the mass spectrometer. The separation of peptides by HPLC can be bypassed by directly injecting the digested sample into the mass spectrometer if: (1) the amount of sample is not limiting; (2) digestion is carried out after desalting, and (3) the resolution of the mass spectrometer is sufficient to confidently resolve the different peptides. The peptides are identified by exact mass measurement and collision-induced dissociation (CID) MS/MS.

A comparison of the masses (which give the number of amide sites exchanged) of the peptides obtained from a sample allowed to undergo exchange for a defined period of time (or from a particular denaturant concentration in the case of equilibrium unfolding experiments) to those of the corresponding native and unfolded reference peptides, yields the extent of exchange that occurred in the protein segments corresponding to the peptides, which in turn can be used to infer the extent of folding. The extent of proteolysis and the number of overlapping peptides generated by proteolysis determines the spatial resolution offered by this method, which usually narrows down to a few amino acid residues[70]. Exchange information even at the single residue level can potentially be obtained by the CID MS/MS of proteolytic fragments[71].

The proteolytic digestion/MS methodology has been used to study the folding and unfolding pathways of several proteins[72–74]. In the case of tryptophan synthase, an equilibrium intermediate is populated in 3 M urea. Analysis of peptic fragments (Figure 2 *a*) from samples corresponding to this intermediate revealed that most of the N-terminal region (residues 20–130) of the protein is structured, whereas the C-terminal region is unfolded, and therefore unable to afford protection against exchange[73]. Figure 2 *b* shows the three-dimensional structure of the protein, onto which are mapped segments of the protein which are unfolded in the partially unfolded intermediate. During kinetic refolding studies of tryptophan synthase, an intermediate sampled by the entire population of unfolded molecules during refolding was detected by HX-MS. The HX protection pattern of this kinetic intermediate was similar to that of the equilibrium intermediate, indicating that both the intermediates have the same secondary structure and hence are likely to be the same[75].

Partially unfolded conformations sampled by SH3 domains from various proteins under native conditions have been detected by HX-MS. The segments which are involved in partial unfolding were identified by proteolytic digestion/MS[76]. In the case of the SH3 domain of PI3 kinase, the sequence segments which first exchange out under native conditions are the same as those which unfold (and exchange) first during unfolding in the presence of high GdnHCl concentration. This implies that the unfolding of a protein in the presence of denaturant is initiated by native-state fluctuations, and that the unfolding pathways in the absence and presence of denaturant are the same (Figure 2 *c*)[77].

The applicability of this technique to study the conformational dynamics of proteins too large to be studied by NMR has been demonstrated by Smith and co-workers[78]. HX rates of different segments of rabbit muscle aldolase, a homotetramer with M_r = 157 kDa, were measured by this method, and a good correlation was observed between the crystallographic B-factor and the observed HX rates. This suggested that the HX involved localized, low-amplitude unfolding events. The segments which showed lower exchange rates than expected were identified as those involved in inter-subunit interactions[79].

This approach, however, has been challenged by observations that back-exchange can take place during enzymatic digestion and scrambling of deuterium can take place during gas phase fragmentation of peptides[80]. Top-down fragmentation of electrosprayed ions provides an alternative method to prevent the undesired back-exchange during enzymatic digestion. Top-down fragmentation by electron capture dissociation (ECD) has also been shown to minimize the scrambling of amide deuterium[81]. When structural transitions of horse myoglobin were studied by HX-MS using top-down ECD, the results obtained were consistent with that obtained by NMR studies. This study also demonstrated that ECD can pin-point the location of a protected amide deuterium with an average spatial resolution of less than two residues[82,83].

Native state exchange

Native state exchange (NHX) is usually carried out using a continuous labelling method in which fully deuterated

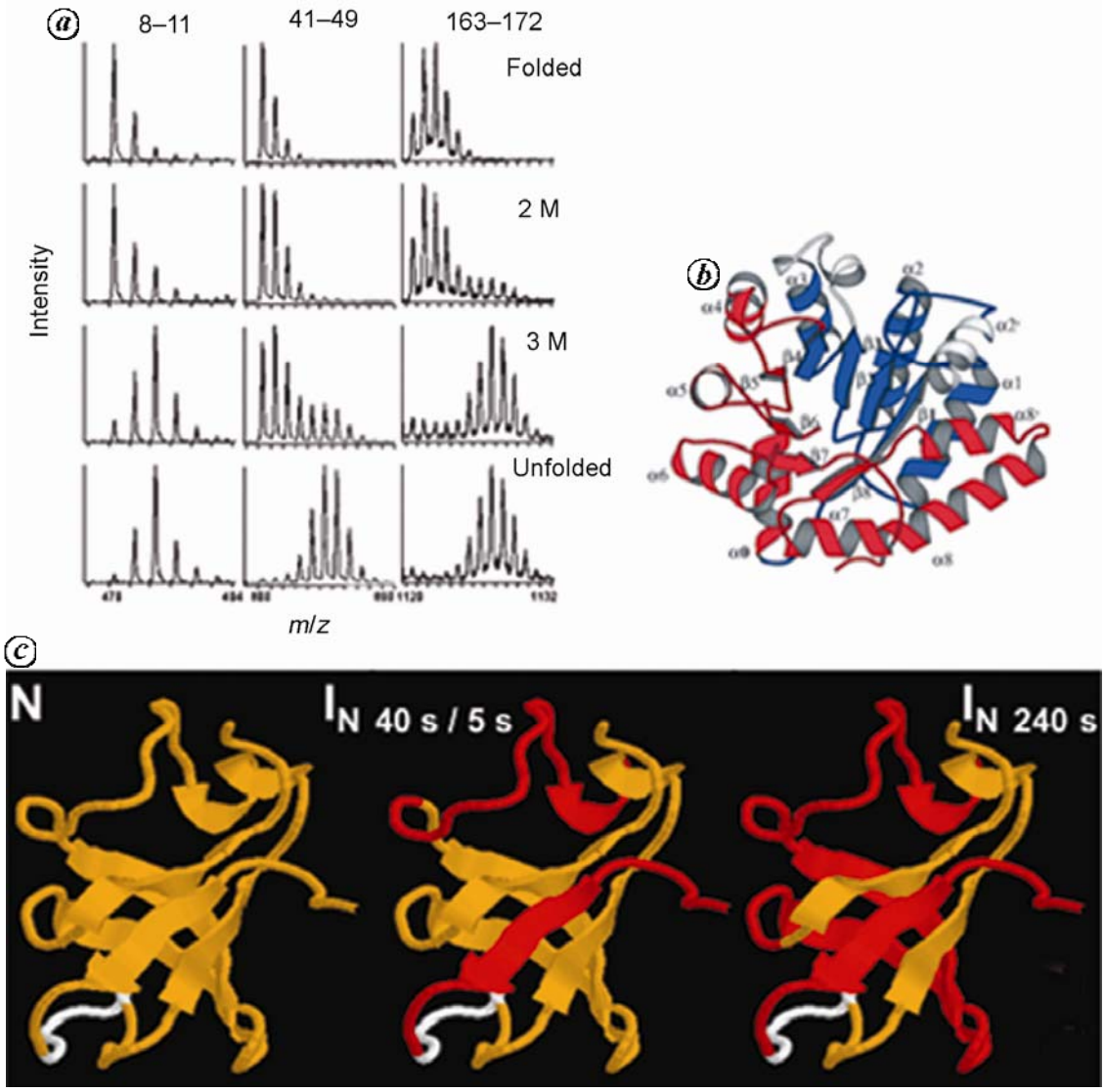

Figure 2. Structure of partially unfolded conformations. ***a***, ESI-mass spectra of peptic fragments derived from the intact protein equilibrated in different concentrations of urea. Reference spectra indicating the extent of deuteration in the folded and unfolded conformations are shown at the top and bottom respectively. Samples were labelled for 5 s in urea/D_2O before quenching and digestion by pepsin. In 2 M GdnHCl all the three fragments in most of the molecules have mass spectra similar to those of the corresponding fragments from the folded sample, implying that these segments are folded. In 3 M GdnHCl, the mass spectra of fragments 8–11 and 163–172 are similar to those of the corresponding fragments from the unfolded protein, indicating that these fragments are unfolded in most of the molecules. But in the case of fragments 41–49, only 33% of molecules have become unfolded in 3 M GdnHCl, which is apparent from the bimodal distribution. ***b***, Three-dimentional structure of the protein depicting unfolded (red) and folded (blue) regions in the equilibrium unfolding intermediate. (Reprinted with permission from Rojsajjakul *et al.*[73]). ***c***, Results of HX-MS studies of the unfolding of the SH3 domain of PI3 kinase. Shown are the locations of protected deuteriums in the native state, N; partially unfolded conformation, I_N at 40 s in zero denaturant (I_N (40 s)) or at 5 s in 1.8 M GdnHCl (I_N (5 s)), and I_N at 240 s of exchange (I_N (240 s)) in zero denaturant. Protected deuteriums are distributed throughout the folded protein. The segments coloured yellow have their deuterium protected, while segments which have exchanged all or some of their protected deuterium are shown in red. No peptide could be identified for the region shown in white.

protein is diluted in H_2O or fully protonated protein is diluted in D_2O, and the extent of exchange is followed as a function of the time of incubation in water. Continuous labelling gives a cumulative picture of the population of different conformations that are sampled by the protein molecules. This makes the method ideal to determine unfolding rate constants under conditions where partially unfolded and completely unfolded states are sampled only transiently[84], provided that the exchange mechanism is EX1.

Native state exchange studies can be of two types: kinetic NHX or equilibrium NHX. Kinetic NHX experiments differentiate partially unfolded intermediates on the basis of the rates of the unfolding processes creating them, regardless of whether the intermediate is stable or not. On the other hand, equilibrium NHX distinguishes

partially unfolded conformations on the basis of their free energies of unfolding (ΔG), and the dependences of the free energies of unfolding on denaturant concentration. While equilibrium NHX studies provide information on the stability of an intermediate, kinetic NHX studies yield the temporal order in which different intermediates get populated. For kinetic NHX studies, exchange must occur in the EX1 limit, and for equilibrium NHX studies, exchange must occur in the EX2 limit. The exchange mechanism can be switched from EX2 to EX1 by increasing the pH or temperature, or by adding denaturants[85,86].

For most proteins under native conditions, exchange occurs by the EX2 mechanism. When the exchange of fully deuterated protein in water is monitored by ESI-MS, a single peak, shifting gradually towards decreasing mass with the time of incubation in water is observed. The unimodal profile of the mass spectrum arises because only one or two deuteriums exchange at a time, resulting in the shifting of the centre of mass to lower values. For the apparent two-state folder, chymotrypsin inhibitor 2, CI2, analysis of the deuterium content as a function of the time of incubation in water reveals that its exchange reaction cannot be described by single exponential kinetics; a bi-exponential exchange kinetics is observed[87]. In the case of *Escherichia coli* thioredoxin, more complicated exchange kinetics has been observed. The protected deuteriums exchange in five different kinetic phases, with rate constants that differ by several orders of magnitude[23].

The above observations give rise to several questions: (1) Why is biphasic exchange kinetics observed for the 'two-state' folding protein CI2? (2) Why do different amide deuterium exchanges take place independently of each other at different times of exchange? (3) Why does a subset of deuteriums exchange in one particular phase? An amide deuterium can exchange when it becomes accessible to solvent and is no longer involved in hydrogen bond formation. These conditions are met when an unfolding event caused by the breaking of a hydrogen bond leads to the exposure of the amino acid residue to the solvent. This implies that the exchange of one or two amide deuteriums at a time can arise due to small, local unfolding events. These local unfolding events are more probable than large global unfolding processes under native conditions where exchange takes place usually by the EX2 mechanism. Different protection factors (arising due to differences in hydrogen bonding and burial from the surface) of amide deuteriums result in differences in the exchange kinetics of different amide deuteriums. The exchange of different subsets of amide deuteriums in different kinetic phases can be explained as a process in which molecules sample, sequentially, conformations that afford protection to increasingly fewer subsets of deuteriums. The observed rate constants of exchange monitored by HX-MS under EX2 conditions may represent the average frequency of local fluctuations. Partially unfolded conformations sampled by proteins under native or near-native conditions have also been revealed by HX-NMR. These measurements have yielded the free energies of exchange of amide hydrogens. On the basis of these free energies of unfolding/exchange, different amino acid residues can be classified into subsets that comprise folding units called 'foldons'[88].

In order to carry out kinetic HX studies of a protein, exchange should occur in the EX1 limit. This is achieved by carrying out the exchange at high pH, so that unfolding, and not the intrinsic rate of exchange is rate limiting. In the case of muscle acylphosphatase[58] and the SH3 domain of PI3 kinase, this condition is met at physiological pH, but the same is not the case with most other proteins. In the case of the SH3 domain of PI3 kinase, HX in the EXI limit at pH 7.2 was monitored by ESI-MS. The 19 deuteriums protected in the native state exchanged out in two kinetic phases. In the first phase, five of these exchanged out leading to the formation of a partially unfolded form, I. In the second phase, the 14 deuteriums protected in I exchanged out as all the molecules transiently sampled the completely unfolded state. In both kinetic phases, the mean of each mass distribution shifted towards lower m/z values with increase in the time of exchange, indicating the sampling of conformations having fewer protected deuteriums. These results led to the conclusion that the SH3 domain of PI3 kinase samples several partially unfolded conformations, in the course of sampling the completely unfolded state (Figure 3)[77]. The NHX of several other SH3 domains has also been studied by MS. Partially unfolded conformations were observed, but an unfolding mechanism could not be established because HX occurred in the EX2 regime[76].

Excursions to partially unfolded conformations under native conditions, as captured by HX-MS have been implicated in the functions of several proteins[89–92]. The segment of the hematopoetic kinase SH3 domain required for the binding of a prolyl-rich peptide has been mapped by proteolytic digestion of the exchanged samples, followed by their analysis using ESI-MS[93]. Such transient unfolding events can also produce aggregation-competent species, capable of aggregating into amyloid protofibrils and fibrils[94].

Equilibrium unfolding

The characterization of partially unfolded conformations populated during the folding and unfolding of proteins is important for understanding the mechanism of protein folding. Partially folded conformations are usually not stable under native conditions, and hence do not get populated to the extent necessary to be detected. In order to induce partial unfolding, proteins are incubated with denaturing agents such as chemical denaturants or organic solvents which differentially destabilize the folded protein

and partially unfolded conformations. Several techniques based on optical spectroscopy and NMR have been used to study partially unfolded conformations. The observation of non-coincident equilibrium unfolding transitions monitored by different probes, which report on different structural aspects of the protein, is indicative of the presence of equilibrium unfolding intermediates[9,23,95,96]. But when partially unfolded conformations are populated to a very low extent, they cannot be detected by optical measurements. Furthermore, estimation of the fractional population of an intermediate populated under a given set of conditions by optical methods is not straightforward.

Two equilibrium unfolding intermediates populated during the acid-induced unfolding of cytochrome *c* were detected and distinguished by the CSDs they produced in ESI mass spectra. The methanol-induced molten-globule state in the pH range 2.6–3.0 was observed to consist of two partially unfolded species which were also observed at the mid-point of the pH-induced unfolding transition[97]. During acid-induced unfolding of apo-myoglobin, a gradual shift in the maximum of the CSD towards lower *m*/*z* values was observed, which indicated a non-cooperative unfolding reaction involving several partially unfolded conformations[98].

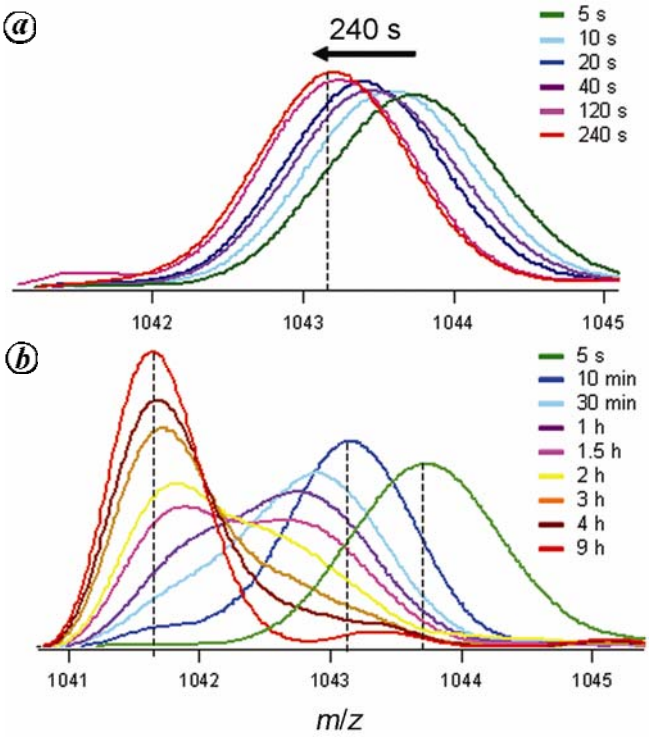

Figure 3. Kinetics of native-state exchange of the SH3 domain at pH 7.2. Exchange was allowed to proceed for different times in 0 M GdnHCl. The native state spectra shown in green in (***a*** and ***b***) were obtained by quenching the exchange after 5 s of labelling. The mass spectrum shown by the red line in (***b***) corresponds to the completely unfolded state, which retains two deuteriums due to the presence of residual (7%) D_2O during labelling. ***a***, Unimodal mass spectra for exchange of the first five deuteriums, at different early times of labelling. The mass spectra shift gradually to the *m*/*z* value of partially unfolded conformation, I_N during the time indicated above the arrow. ***b***, Representative mass profiles for the exchange of the remaining 14 protected deuteriums, at different times of labelling. The mass spectra in both the panels correspond to the + 9 charge state.

Equilibrium unfolding intermediates populated during the unfolding of proteins by chemical denaturants such as GdnHCl and urea have been detected by HX-MS. An equilibrium unfolding intermediate, stable at low pH but not at neutral pH was observed during the GdnHCl-induced equilibrium unfolding of *E. coli* thioredoxin. The structure in the folded form protected 33 ± 3 deuteriums from exchange with solvent protons, whereas unfolded molecules exchanged deuteriums from all exchangeable sites. The intermediate manifested itself as a mass distribution spanning between the isotopic envelopes corresponding to the folded and unfolded molecules, indicating that the molecules in the intermediate state are protected against HX at only a subset of the 33 ± 3 amide sites protected in the folded protein. The broad isotopic distribution observed for the intermediate state indicates that this state is an ensemble of conformations rather than a discrete state[23].

Urea-induced equilibrium unfolding of *Staphylococcus aureus* aldolase was monitored by HX-MS, and two equilibrium unfolding intermediates were detected. The fractions of folded, partially unfolded and unfolded protein were calculated and their free energies of unfolding were estimated. The mapping of the exchanged segments of sequence in the intermediate onto the three-dimensional native structure of the protein led to the conclusion that the intermediates arise due to sequential unfolding of the three domains of the protein[72]. Figure 4 shows representative mass spectra obtained in different concentrations of urea. In the case of tryptophan synthase, similar studies revealed the secondary structure content of two equilibrium unfolding intermediates populated upon urea-induced unfolding[73].

Hydrogen exchange in conjunction with MALDI-MS has been used to determine the stabilities of unpurified proteins[99]. The method, called 'Stability of Unpurified Proteins from Rates of H/D Exchange' (SUPREX) estimates the stability of an unpurified or purified protein by measuring the extent of H/D at a specified time of incubation in an exchange buffer containing increasing concentrations of denaturant. The amount of deuterium incorporated is determined by analysis of the samples using MALDI-MS. The SUPREX curves are generated by plotting the change in mass (with respect to the fully protonated protein) as a function of denaturant concentration. The observation of different surface area changes (*m*-values) associated with unfolding in low denaturant concentrations (determined by SUPREX) compared to the values in higher denaturant concentrations (determined by optical methods) indicated the presence of high-energy intermediates populated at low denaturant concentrations[100].

Kinetic studies

Equilibrium unfolding studies have provided a wealth of information about partially unfolded conformations sampled by proteins. Equilibrium studies cannot, however, define the sequence of events that occur on the folding or unfolding pathway of a protein. Kinetic studies can yield the temporal order of structural events during folding or unfolding. Partially (un)folded intermediates, however, get populated only transiently during this process, which makes their characterization difficult. Only under equilibrium conditions, if they are stable and get populated significantly, can intermediates be studied easily. Although kinetic intermediates may not always be identical to equilibrium intermediates, similarities in the structures and stabilities of the equilibrium and kinetic intermediates have been shown for several proteins[20,95,101].

HX methods have proven to be extremely powerful for studying protein folding kinetics[10,102]. The detection and structural characterization of kinetic folding intermediates still remains a difficult task. In contrast to other probes commonly used to monitor protein folding, HX-MS provides direct evidence for the presence of partially folded intermediates. HX-MS monitored kinetic refolding studies of various proteins have revealed the presence of parallel folding pathways and obligatory intermediates populated during folding[75,102–106]. In the case of triosephosphate isomerase, a pulse labelling methodology was used to study the kinetics of refolding, and a partially folded conformation was detected. The intermediate manifested itself in the mass spectrum as a mass distribution in between the isotopic distributions corresponding to the folded and completely unfolded conformations. Proteolytic digestion of exchanged samples revealed that the C-terminal region of the protein folds first, giving rise to a partially folded conformation which subsequently attains the native conformation upon folding of the N-terminal region[107].

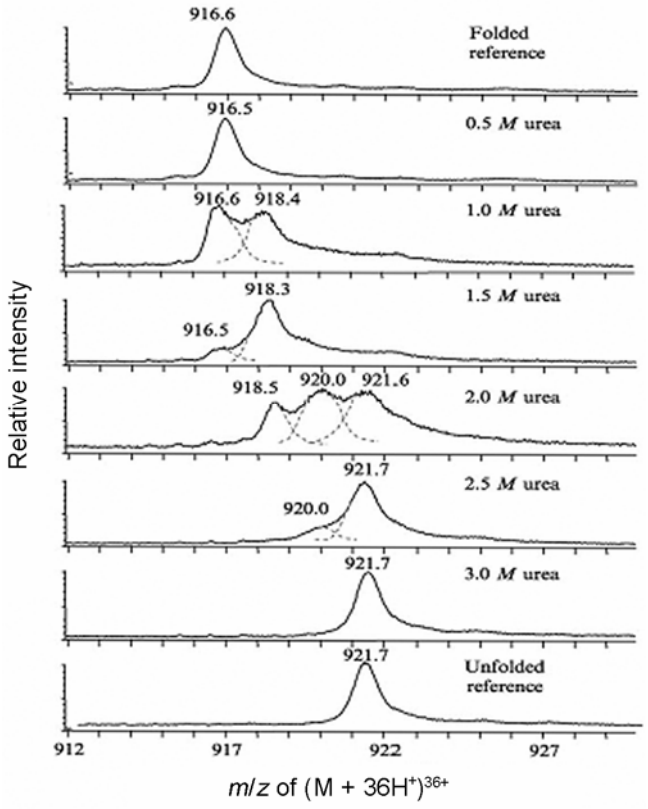

Figure 4. Equilibrium unfolding of aldolase. ESI – mass spectra of intact aldolase (+36 charge state) equilibrated in various concentrations of urea. The dashed lines indicate the envelopes of isotope peaks representing specific structural forms of the protein. Each sample was labelled for 5 s in urea/D_2O prior to quenching the HX. The spectra of the folded and unfolded protein references (top and bottom respectively) indicate the deuterium levels expected for folded and unfolded aldolase. (Reprinted with permission from Pan and Smith[72].)

A competition labelling method or a pulse-labelling method can be used to study the folding kinetics of a protein by HX-MS. In conventional pulse-labelling experiments, samples are exchanged and quenched off-line, either by manual mixing or by using a quenched-flow device. The quenched samples need to be desalted and sometimes concentrated before injecting into the mass spectrometer. During this processing, some of the information might be lost due to exchange at some of the protected amide sites, and back exchange can further complicate the analysis. An on-line pulse-labelling method, developed by Konermann and co-workers[108] employs a mixing-cum-labelling apparatus coupled to the source of the ESI mass spectrometer. The folding (or unfolding) reaction is initiated in the first mixer of the apparatus, followed by a brief labelling pulse after a specified time interval in the second mixer, after which the mixture enters into the mass spectrometer, where exchange is quenched by desorption. In addition to enabling a rapid analysis of the labelled samples, this method also gives the CSD of the species present at the time of labelling, from which additional information about the folding process can be inferred.

The refolding of acid/methanol denatured ubiquitin was initiated by a pH jump and monitored by on-line pulse-labelling[108]. An intermediate with a CSD similar to that of the folded protein, but having non-native protection against hydrogen exchange was observed. This method has also been applied to study the folding and assembly of holomyoglobin[109] and the S100A1 protein[110]. In both cases, intermediate states populated during folding were detected by measurement of the CSD as well as the protection afforded against hydrogen exchange. This method may not, however, be readily applicable to study refolding reactions which require the desalting of samples prior to MS analysis, for example, refolding reactions initiated from denatured states in chemical denaturants like urea or GdnHCl.

Transiently populated intermediates have not only been detected during refolding, but also during the unfolding

of proteins[111,112]. Unfolding studies can sometimes yield information about the mechanism of folding, which cannot be obtained from folding studies[20]. Pulse-labelling HX exchange has also been applied to monitor the unfolding of several proteins[23,107,113]. A two-state unfolding process was observed for triosephosphate isomerase[107]. In the case of *E. coli* thioredoxin, bi-exponential kinetics was observed when the kinetics of unfolding was monitored by intrinsic fluorescence and CD. Usually the observation of double exponential kinetics is attributed to the population of a transient intermediate species. HX-MS studies, however, revealed that a fraction of the molecules unfolds rapidly to the completely unfolded state, giving rise to a fast unfolding phase, and the rest of the molecules having peptidyl-prolyl bonds in the *cis* configuration are held back and unfold slowly, giving rise to the slower unfolding phase. Hence, the application of HX-MS in these kinetic experiments allowed delineation of the unfolding reaction from the proline isomerization reaction to which it is coupled[23]. In the case of aldolase, a tetrameric protein, a sequential unfolding pathway involving two partially unfolded intermediates was deduced from HX-MS monitored kinetic unfolding studies. A detailed MS analysis showed that each unfolding domain consists of 107 residues, and three unfolding domains were mapped onto the folded three-dimensional structure of the protein[78].

In the case of a protein which is active only when it exists in a multimeric form, it becomes important to study assembly and disassembly in order to understand the factors which stabilize the multimeric state, and hence regulate the activity of the protein[114]. Hemoglobin is believed to exist as only a heterotetramer under equilibrium conditions. However, ESI mass spectra showed, signals corresponding to dimeric, hexameric as well as octameric states, implying the existence of heterogeneity under equilibrium conditions. Acid-induced unfolding studies of hemoglobin showed that the tetrameric form consists of two subpopulations which differ in their rate of disassembly and in their CSDs. The tetramer with the higher CSD dissociated via a dimeric intermediate, whereas the tetrameric form with the lower CSD dissociated directly into monomers. A complex unfolding mechanism involving several intermediate states was deduced by global analysis of the kinetic profiles of various species[115]. Temperature-induced unfolding of a dimeric protein, NAD(+) sythetase monitored by hydrogen exchange and native ESI-MS revealed that NAD(+) synthetase undergoes reversible dissociation before monomer unfolding, both at the temperature where monomer unfolding is not observed as well as at the temperature where monomer unfolding is observed[116].

Mass spectrometry and other labelling methods

In addition to H/D exchange, a variety of other labelling methods have been used to probe the dynamics of proteins, protein–protein complexes and protein–ligand complexes[117–119]. The reactivity of a thiol group of a free cysteinyl residue has been exploited in chemical cross-linking methods used to distinguish different conformations of proteins. Solvent-accessible cysteine thiols of a protein can be labelled with thiol-specific labelling reagents such as methyl methanethiosulfonate (MMTS) or 5-5 dithiobis(2-nitrobenzoic acid) (DTNB) in milliseconds at alkaline pH[120]. This method can provide site-specific information if the proteins contain only one cysteine residue, which can be achieved by generating suitable mutant variants of the protein[121].

The refolding kinetics of various single cysteine-containing mutant forms of a small protein, barstar was monitored by pulsed MMTS labelling in conjunction with ESI-MS. Urea-denatured protein was refolded by diluting the denaturant, and a 4 ms pulse of MMTS was applied at different time points of refolding using a quenched-flow device. The labelling reaction was stopped by adding excess of cysteine at low pH. The samples were desalted and analysed by ESI-MS. The fraction of labelled protein present at each time point of refolding, which depends upon the extent of refolding, was determined from the relative ion abundances of the mass distributions corresponding to the labelled and unlabelled proteins. Figure 5 shows representative mass spectra obtained at different time points of refolding, and the change in the fraction of labelled protein with the time of refolding. The apparent rate constants for the burial of cysteine residues were site-specific, and different from the refolding rate constants obtained from fluorescence-monitored refolding studies. This indicated that side-chain burial is not synchronized across different regions of the protein molecule[122]. Pulsed MMTS labelling in conjunction with ESI-MS (SX-MS) has also been employed to study the origin of multi-exponential folding kinetics of single-chain monellin, observed by changes in intrinsic optical signals. Two-exponential kinetics of protection of the Cys 42 thiol was observed. Varying the strength of the labelling pulse ruled out the possibility of a sequential folding mechanism, and led to the conclusion that two parallel folding pathways are operational during the folding of monellin[123].

Mass spectrometry-detected cyanylation of free sulphydryl groups has been used to study the oxidative refolding of disulphide-containing proteins. Disulphide bond intermediates populated during the folding of ribonuclease A and ribonuclease T1 were detected when folding was monitored by ESI-MS in conjunction with glutathione labelling. Two disulphide-bond-containing intermediates were predominant in the case of ribonuclease A, while one disulphide bond intermediate was populated in the case of ribonuclease T1[124,125]. Intermediates with native and non-native disulphide bonds were also detected during the folding of human epidermal growth factor[126]. An integrated approach utilizing HX and

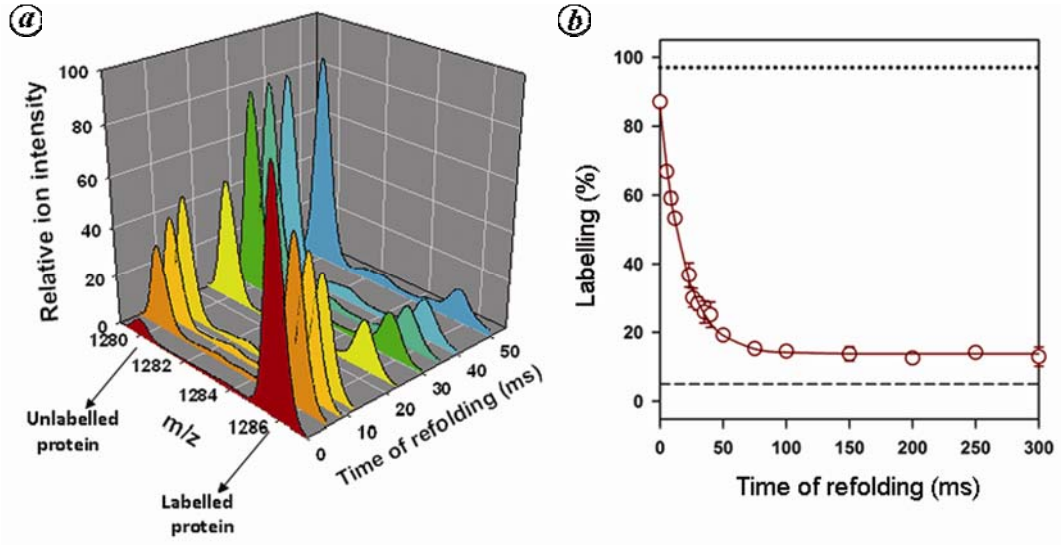

Figure 5. Kinetics of the change in cysteine accessibility during the refolding of barstar (having a cysteine at residue 3) in 0.6 M urea at pH 9.2. The protein was unfolded in 6 M urea and refolding was initiated by diluting the urea concentration to 0.6 M. A 4 ms MMTS pulse was applied at different times of refolding, and the labelling reaction was quenched by adding an excess of cysteine in 1% formic acid. ***a***, Representative mass spectra obtained at different times of refolding. ***b***, Change in percentage labelling (red circles), determined from relative ion intensity, with the time of refolding. The dashed and dotted lines represent the percentage labelling in the native and unfolded proteins respectively. The kinetics of the change in labelling represents the kinetics of the burial of cysteine 3 during refolding (Reprinted from Jha and Udgaonkar[122]).

cyanylation was employed to characterize the disulphide intermediates of long Arg3 insulin-like growth factor-1. HX results showed an increase in structure as a function of disulphide bond formation[127].

Cysteine labelling has several advantages over H/D exchange: (1) The label is covalently attached, and samples are therefore stable enough to be stored for longer durations before being analysed by MS. (2) In the case of HX, side-chain deuteriums exchange fast and information is mainly obtained from backbone amide hydrogens. On the other hand, cysteine labelling reports on side-chain packing. (3) Amide hydrogens belonging to the surface residues of the protein exchange rapidly with solvent protons, and cannot be used to probe folding or unfolding reactions, but cysteine labels even at the surface of a protein are not lost once attached to a thiol group. The cysteine labelling experiment, however, involves extensive protein engineering to generate a library of single cysteine-containing mutant proteins, which is not required for HX.

Hydroxyl radicals generated by the radiolysis of water upon exposure to synchrotron X-rays can oxidize amino acid side chains accessible to the solvent. These radicals are formed in microseconds, and can react with target groups on the microsecond timescale. The reactivities of the hydroxl radical with different side chains vary by two orders of magnitude. The cysteine side chain is the most reactive, whereas glycine is the least reactive[128]. The sites of oxidation are identified by MS, and the extent of oxidation at each site can also be quantitatively estimated by MS. A close correlation has been observed between the surface accessibilities of different target residues and their oxidation rates[129]. This technique has been used to monitor the urea-induced unfolding of apomyoglobin at pH 7.8. Cooperative unfolding was observed for helices A to C with the values of thermodynamic parameters similar to those obtained from fluorescence data. Local unfolding was, however, observed for helix G[130]. In another study, this methodology was used to monitor the folding and dimerization of a heterodimeric protein, S100A11. A monomeric intermediate was observed at 10 ms, which led to a completely associated but partially disordered state at 800 ms, before the final consolidated state[131]. MS-monitored hydroxyl labelling coupled to temperature jump-induced folding has been used to study the submillisecond refolding reaction of barstar[132]. This study demonstrated for the first time that photochemical oxidation-induced hydroxyl labelling monitored by MS can be used to study protein folding/unfolding reactions on the sub-millisecond timescale[132,133].

Hydroxyl radical labelling in conjunction with MS yields information about the packing of the amino acid residue side chains, while HX-MS reports on backbone structure; hence, the two methods are complementary to each other. In the case of cytochrome *c*, the results obtained by hydroxyl radical labelling folding were consistent with the foldon model of cytochrome *c* folding obtained by HX[134].

Application of MS in conjunction with different labelling methods to the study of membrane proteins has been

recently reviewed by Barrera and Robinson[135]. Oxidative labelling of methionine residues engineered at different locations of the membrane protein bacteriorhodopsin has been employed to characterize the partially collapsed state attained by this protein in the presence of sodium dodecyl sulphate (SDS). The solvent-accessible core composed of different partially intact helices was retained in SDS-induced state[136,137]. The application of this methodology to the study of the refolding of bacteriorhodopsin revealed a sequential folding mechanism[138].

Lessons for protein folding

Multiple pathways

It has been difficult to determine whether proteins avail of multiple pathways for folding and unfolding. Multiple folding pathways can arise if there is heterogeneity in the unfolded state, and when different members of the unfolded state ensemble interconvert on a timescale much slower than their folding time[24]. The observation of single exponential kinetics does not mean that all the unfolded molecules fold via the same route. Single exponential kinetics can also be observed when multiple folding routes operate in parallel[139]. Interrupted refolding experiments can distinguish between different species populated during refolding on the basis of their different unfolding rate constants and hence can dissect out mechanisms of folding involving one or more pathways[140]. But such experiments involve complex kinetic analysis. HX-MS was first used to demonstrate the existence of multiple pathways by Miranker *et al.*[102], when they monitored the refolding of lysozyme by pulse-labelling HX-MS. The results showed that a fraction of the unfolded lysozyme molecules refolds very fast on the timescale of 10 ms, whereas the rest of the molecules refold via a slower pathway sampling a partially folded intermediate. This conclusion was based on the unique ability of HX-MS to distinguish co-existing protein populations that differ in their level of exchange. In the case of single-chain Fv (scFv) too, two folding pathways operating in parallel were confirmed by HX-MS refolding studies[141]. SX-MS studies have also been used recently to show that monellin also refolds via multiple pathways[123].

Obligatory intermediates

Is the formation of an intermediate during folding of a protein an obligatory step or can some molecules bypass this step and fold directly to the native state? Does the structure of an intermediate determine how it will fold to the native state? The kinetic criterion for an obligatory intermediate is the observation of a lag phase in the formation of the native protein. Kinetic refolding studies of interleukin-1β, apomyoglobin and the α-subunit of tryptophan synthase, in which refolding was monitored by HX-MS, provided direct evidence that the entire population of the unfolded molecules proceeds through an intermediate state while refolding to the native state[75,103–105]. In the case of interleukin-1β, HX-MS results revealed that no native protein is formed for the first 400 ms of refolding, and during this time, the intermediate becomes maximally populated (Figure 6). Hence, a lag phase was observed in the formation of the native protein, which was direct evidence showing that the formation of this intermediate is an obligatory step during refolding[103].

During the refolding of apomyoglobin, a single isotopic distribution at a mass value falling in between the mass values corresponding to the native and unfolded proteins was observed after a few milliseconds of refolding. At this earliest time of refolding, no signal corresponding to the native and unfolded protein could be detected, indicating that all the unfolded apomyoglobin

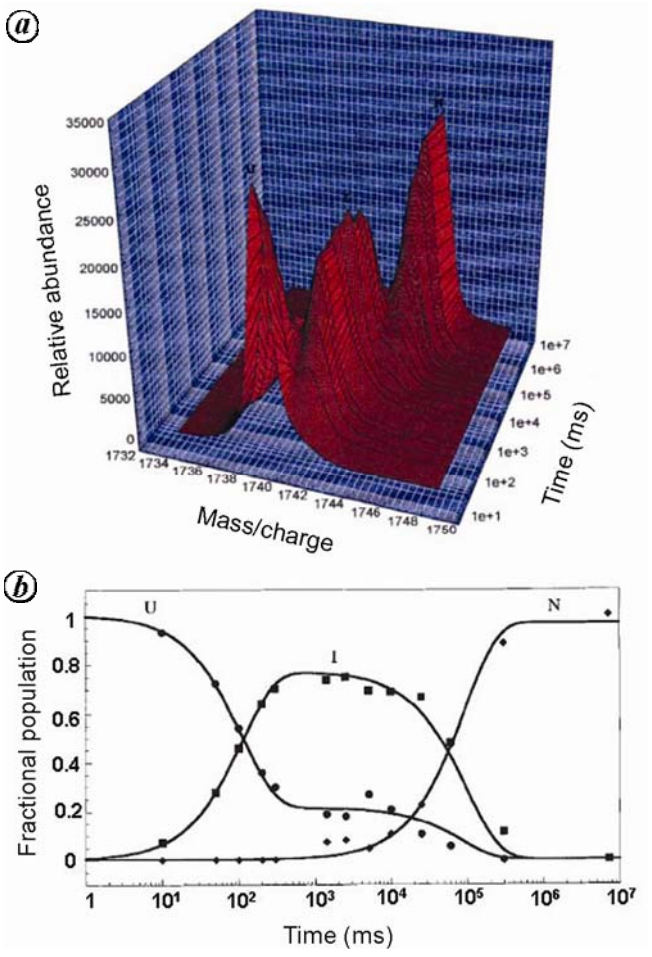

Figure 6. Refolding kinetics of interleukin-1β (IL-1β) monitored by HX-MS. ***a***, Three-dimensional plot showing the relative abundances of different species populated during the folding of IL-1β as a function of mass/charge ratio and folding time. ***b***, Relative populations of molecules in the native state (N), the unfolded state (U) and the intermediate conformation as a function of refolding time. In (***a***) no signal corresponding to N is observed for the initial period of folding. Hence, a lag phase in the formation of N is observed (Reprinted from Heidary *et al.*[103]).

molecules had adopted the partially folded conformation while refolding to the native state. With an increase in the time of refolding, the mass distribution corresponding to the native protein gained in intensity at the cost of the intensity of the mass distribution corresponding to the intermediate state[104,105].

A comparison of the refolding kinetics of isolated V_H and V_L domains with scFv, revealed that the isolated domains attain native-like protection against exchange within 10 s of refolding, whereas scFv attains full protection on the timescale of minutes. At the earliest time of refolding (10 s), fewer deuteriums are protected in scFv than in the two isolated domains together. The slower refolding and lower protection in scFv was attributed to premature association of domains in a non-native form, which acted as a kinetic trap[142]. The HX-MS kinetic refolding studies demonstrated the power of this technique to determine whether an intermediate is an obligatory on-pathway species or not.

Heterogeneity and quantification

The number and amount of different intermediates populated during the folding of a protein are two important parameters required to establish a mechanism of folding. It is not easy to determine these two parameters precisely when folding is monitored by optical spectroscopy or NMR, both of which report ensemble-averaged signals. Mass spectrometry can, however, distinguish between several conformations of a protein, even when they are present together. In the case of the unfolding of aldolase, four different conformations, i.e. the native state, two partially unfolded intermediates, and the unfolded state populated at the same time could be distinguished on the basis of their extent of exchange in a single mass spectrum[78]. The relative amounts of different species populated during the folding or unfolding of a protein can be determined easily from their relative ion intensities, or from the relative areas occupied by the different species in the mass spectrum. When the different mass distributions are not well resolved, the mass spectra need to be deconvoluted using a Gaussian fitting procedure, and the relative populations of different forms can then be estimated from the relative area under each distribution. For several proteins whose folding or unfolding has been studied by MS, the estimation of the relative amounts of different conformations populated at different times makes it possible to determine the rate constants with which the populations of the different conformations change as the reaction proceeds[23,77,78,102].

Chaperone-mediated folding

Molecular chaperones are proteins which help in preventing newly synthesized proteins in non-native conformations from aggregating. Structural and mechanistic studies of chaperones over the past several years have provided detailed insights into the mechanisms of action of these proteins, and have led to different models which explain how they work[143–145]. A proper understanding of how chaperones perform their function requires an understanding of the conformational changes that occur both in the chaperone and in the substrate polypeptide. It has been a challenge to study chaperones due to their large size, and to characterize the relatively small substrate polypeptides in the presence of large chaperones.

The GroEL–GroES chaperonin system has been studied extensively by a wide variety of techniques[146–148]. Robinson and co-workers[149] were the first to demonstrate the utility of HX-MS to study the functioning of GroEL. In studies of the folding of α-lactalbumin, they showed that it was possible to obtain information on the chaperone and the substrate at the same time from the same mass spectrum. The HX protection pattern of α-lactalbumin bound to GroEL resembles that of the free molten globule state of the three-disulphide protein. The molten globule state of the four-disulphide protein, which presumably is more ordered than the three-disulphide protein molten globule, does not, however, bind to GroEL. These studies supported the idea that GroEL binds to relatively disordered states formed during the early stages of refolding, and they also showed that there is a major conformational change in GroEL upon dissociation of the substrate. This conformational change resulted in the exchange out of more than 195 deuterons from each GroEL monomer[149].

Other studies of GroEL-mediated folding monitored by HX-MS suggested that GroEL helps partly folded, trapped conformations of proteins to fold by causing them first to partially unfold in response to a mechanical stretching force[150]. On the other hand, proteolytic HX-MS-monitored folding studies of malate dehydrogenase (MDH) in the presence of GroEL showed that there is only a modest deprotection in MDH upon the addition of ATP and GroES binding, and that the deprotection is distributed throughout the protein. This study concluded that deprotection/unfolding arises due to the breaking of contacts between MDH and the cavity wall, rather than because of mechanical unfolding[151]. Tandem mass spectrometry of GroEL–substrate complexes has demonstrated that upon binding to the *cis* ring, different substrate proteins can induce different conformational changes in the *trans* ring[152]. In the case of Hsp90, the results from fluorescence spectroscopy and HX-MS-monitored conformational dynamics could resolve ATP-induced conformational changes, both spatially and temporally. The conformational change initiates from near the nucleotide-binding site, through the N-terminal region and finally to the middle domain[153].

Small heat shock proteins (sHSPs) constitute an ubiquitous class of molecular chaperones involved in various

cellular processes. They are present in almost all organisms from *E. coli* to humans. Several studies have employed HX-MS to study the conformational dynamics of sHSPs upon binding to their substrate proteins, as well as the conformational dynamics of the substrate proteins when they bind to sHSPs[154–156]. Complex formation between pea PsHsp18.1 and TaHsp16.9 with the substrates MDH and luciferase, monitored by HX-MS, revealed that there were no significant changes in the dynamics of the sHsps upon binding to these substrate proteins. Both the substrate proteins attained a partially unfolded form only in the presence of the sHsps. In the case of MDH, the same segments were found structured when present in the complex with either of the sHsps, despite the difference in the sizes of the two sHsps. In that study, it was concluded that the sHsps do not adopt new structures upon binding to the substrates, and that the substrates are tethered to the sHsps at multiple positions[155].

Structural characterization of protein aggregates

Misfolded proteins can self-assemble to form higher order oligomeric intermediates[157–159], which can then further associate to form mature amyloid fibrils, which are unbranched fibrillar structures having a cross-β spine[160]. The formation of amyloid fibrils by specific proteins is associated with specific human diseases[161]. There is mounting evidence that the transient oligomeric forms, and not the mature amyloid fibrils, are toxic[162–164]. Hence, it is important to study the mechanism of formation of amyloid fibrils to understand the structural basis of protein aggregate toxicity.

Structural studies of amyloid fibrils, transient oligomeric intermediates and protofibrils are challenging due to several reasons. (1) Amyloid fibrils are high molecular weight structures, which makes their structural characterization difficult by the techniques used to study globular proteins. (2) They are insoluble and noncrystalline. (3) The transient nature of oligomeric intermediates and protofibrils is a big obstacle to their characterization. Several structural biology techniques such as electron microscopy, atomic force microscopy, X-ray fibre diffraction, solid-state NMR, Fourier transform infrared spectroscopy and CD have provided important insights into the structural details of amyloid fibrils and protofibrils[165–171].

HX-MS offers the important advantage that it can be applied to study the secondary structure contents of all the species (from misfolded monomers to mature amyloid fibrils) populated during amyloid fibril formation. In addition, HX-MS can distinguish between different species formed during amyloid fibril formation, even when they are present together, on the basis of their different patterns of exchange protection. Protofibrils and fibrils formed by the Aβ(1–40) peptide have been studied by HX-MS. The methodology employed and the major results of these studies have been reviewed recently[172,173]. Protofibrillar structures formed transiently during the aggregation of Aβ(1–40) were stable for days in the presence of calmidazolium chloride, which made them amenable to be studied by HX-MS[174]. Proteolytic HX-MS studies of Aβ(1–40) protofibrils and fibrils showed that both the N- and C-terminal segments exchange readily, and are therefore not involved in the core structure. The segment 20–34 is highly protected from exchange in fibrils, but to a smaller extent in protofibrils. These studies showed that β-sheet structures present in the mature fibrils have already formed in the protofibrils, but expand to adjacent residues upon conversion of the protofibrils to fibrils[175]. In another study of Aβ(1–40) aggregation, a gradual decrease in solvent accessibility from the C-terminal region to the N-terminal region was observed as the aggregation proceeded towards more aggregated states. The greater extent of solvent-exposed structure in the early aggregates compared to that in the mature fibrils might explain the structural basis of the toxicity of oligomeric intermediates[176]. Application of HX-MS to the study of Aβ(1–40) and Aβ(1–42) fibrils has shown that these fibrils are not static structures, and that monomers comprising these fibrils continuously dissociate and reassociate, resulting in molecular recycling within the fibril population. The monomers in Aβ(1–40) fibrils were found to recycle much faster than those in Aβ(1–42)[177]. The HX-MS-detected molecular recycling with amyloid fibrils was first reported in the case of fibrils formed by the SH3 domain of PI3 kinase[178]. This insight into the dynamic nature of amyloid fibrils might have important implications for drug design.

The application of HX-MS to the study of the aggregation of several other proteins such as the prion protein, apolipoprotein C-II and insulin have led to the identification of sequence segments which comprise the cores of amyloid fibrils[179–182]. Direct MS studies have also provided important insights into the process of formation of the amyloid fibrils[183,184]. Mass spectrometry has also been used to detect and identify the types of amyloid deposits in patient tissues[27,185].

Ion mobility (IM) is a gas phase electrophoretic technique that separates the molecules in an infused sample according to their size, shape and charge. Although developed in the early 19th century, IM has been only recently coupled to MS. The development of ion mobility mass spectrometry (IMMS) has been an important addition to the tool kit used for studying proteins[186]. IMMS-monitored aggregation of β_2-microglobulin has revealed that monomers to tetramers are populated during the lag phase of aggregation with no significant population of larger oligomers. The study also revealed that β_2-microglobulin oligomers are arranged in elongated assemblies in contrast to ring-like arrangements formed

during Aβ aggregation[184]. IMMS has also been used to study the conformational stability of the prion protein[187], the oligomerization of amyloid-β[188], and the mechanism of polymerization in serpinopathies[189].

Macromolecular assembly

The realization that non-covalent complexes can survive the ionization process and remain intact in the mass spectrometer gave rise to new facets of mass spectrometry-based research[190]. Macromolecular non-covalent complexes are usually studied by ESI hybrid quadrupole time-of-flight instruments (Q-Tof), originally designed for peptide sequencing in proteomics. Many of the older quadrupole instruments could transmit ions of up to about 4000 *m*/*z*, which allowed the detection of protein complexes having molecular masses of about 50–60 kDa. New developments in quadrupole design have led to an increase in the *m*/*z* range up to 32,000 (ref. 191), making it possible to study complexes of mega-Dalton molecular mass[192].

Some modifications are required to increase the transmission of intact macromolecular ions from the ion source to the detector. The difference in pressure between different sections of the mass spectrometer is the main threat to the survival of non-covalent complexes. The pressure gradient can be manipulated either by introducing collision gas, or by reducing the pumping at various stages along the path of the ions[191]. A flow-restricting sleeve fitted at the interface of the source and the first ion guide can increase the pressure locally in the first ion guide[193]. Decreasing source parameters such as the desolvation temperature, source temperature and capillary voltage can sometimes also be useful.

The application of MS to study the stoichiometry, heterogeneity, dynamics and architecture of macromolecular complexes has been reviewed recently by Sharon and Robinson[190]. Typical examples of macromolecular complexes whose assembly has been studied by MS include the molecular chaperone GimC/prefoldin, the bacterial 20S proteosome and the 60S ribosomal subunit complex[194–196]. In the case of GimC/prefoldin, mass spectra were collected throughout the time course of assembly. No significantly populated intermediates could be observed, implying that the assembly process is highly cooperative[194]. Mass spectrometry-monitored assembly of the 20S proteosome revealed the population of an early heterodimeric intermediate as well as an unprocessed half proteosome particle. Two half proteosome particles were then observed to combine to form the mature 20S proteosome. This study also determined the sites that are cleaved in the β-subunit during different stages of the assembly process[195].

A pulse-chase based methodology in conjunction with quantitative mass spectrometry (PC/QMS) developed by Willianson and co-workers[197–201] was used to monitor assembly of the 30S ribosomal subunit of *E. coli*. The binding rates of different 30S proteins determined by PC/QMS were found to be distributed within two orders of magnitude. The study of the temperature dependence of the binding kinetics revealed that different events throughout the assembly process have similar activation energies, leading to the conclusion that there are multiple parallel pathways available for the assembly of the 30S ribosomal subunit[197]. This conclusion contradicted the prevailing view that the 30S assembly takes place along a pathway proceeding through global rate-limiting conformational change[202].

MS/MS studies of various protein complexes suggest that the subunits which dissociate first are peripheral and more exposed, whereas those occupying the core of the complex are retained in the stripped complex[192–194]. These results provide some insights into the topologies of macromolecular complexes. CID MS/MS of the 20S proteosome from *Rhodococcus* resulted in the dissociation of the α-subunits, consistent with their peripheral location[203]. However, a half proteosome complex, in which both the α- and β-subunits are equally exposed, yielded β-subunits upon CID dissociation; implying that the contact area (more between the α-subunits than between the β-subunits) might be the dominant factor in determining the pattern of dissociation[204]. It should be noted that other factors like the nature of inter-subunit interactions can also determine the dissociation pattern of a heterosubunit macromolecular complex.

IMMS has also been used to study protein–protein complexes and provides information on their stoichiometry, topology and cross-section[205]. It can also provide information about the influence of subunit packing and charge on the dissociation of protein–protein complexes[206]. Complexes as large as viruses such as the Hepatitis virus B capsid can be analysed[207], but experimental parameters are not easily optimized[208].

Concluding remarks

Unravelling the conformational heterogeneity in protein folding and unfolding reactions is crucial for understanding the mechanism of protein folding. To distinguish between, and to characterize the different conformations sampled by an unfolded polypeptide chain while attaining the native three-dimensional structure has been one of the biggest challenges in protein folding studies. HX-MS provides the opportunity to distinguish between different coexisting conformations of a protein on the basis how they differ in the extent of protection they confer against exchange. It has not only provided direct evidence for the population of partially folded conformations populated during protein folding and unfolding reactions, but it has also made it possible to quantify their relative amounts at any given time of reaction. The application of this

methodology to protein folding has also shown that there may be parallel pathways of folding, and that transitions via partially folded conformations may be obligatory during folding.

Another important advantage offered by HX-MS when combined with proteolytic digestion and CID is that it can identify the segments of a protein which are structured in partially unfolded intermediates, and hence yield structural details about intermediate states. This method has also been applied to identify the regions of a protein involved in protein–protein interactions like in the case of multi-subunit complexes or in the case of protein aggregates. Hence, HX-MS can provide a wealth of information about the mechanistic aspects of protein folding/unfolding reactions, the assembly of multisubunit complexes, and the aggregation of proteins into amyloid fibrils.

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