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SPECIAL FEATURE:
PERSPECTIVE

Probing the Non-covalent Structure of Proteins by Amide Hydrogen Exchange and Mass Spectrometry

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The rates at which hydrogens located at peptide amide linkages in proteins undergo isotopic exchange when a protein is exposed to D_2O depend on whether these amide hydrogens are hydrogen bonded and whether they are accessible to the aqueous solvent. Hence, amide hydrogen exchange rates are a sensitive probe for detecting changes in protein conformation and dynamics. Hydrogen exchange rates in proteins are most often measured by NMR or Fourier transform IR spectroscopy. After a brief introduction to model kinetics used to relate amide hydrogen exchange rates to protein structure and dynamics, information required to understand and implement a new method that uses acid proteases and mass spectrometry to determine amide hydrogen exchange rates in proteins is presented. Structural and dynamic features affecting isotopic exchange rates can be detected and localized from the deuterium levels detected by mass spectrometry in proteolytic fragments of the protein. Procedures used to adjust for isotopic exchange occurring during the analysis, to extract isotope exchange rate constants from mass spectra and to link bimodal isotope patterns to protein unfolding and structural heterogeneity are also discussed. In addition, the relative merits of using mass spectrometry or NMR combined with amide hydrogen exchange to study protein structure and dynamics are discussed. The spatial resolution of hydrogen exchange results obtained by this method is typically in the range of 1–10 residues, which is substantially less than that obtained by high-resolution NMR, but sufficient to detect many functionally significant structural changes. Advantages in the areas of sensitivity, protein solubility, detection of correlated exchange and high molecular mass proteins make this approach particularly attractive for a wide range of studies. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

The rate at which tritium exchanges with hydrogen located on peptide linkages has been used to detect structural changes in proteins for over 30 years.[1–3] Increased or decreased rates of isotopic exchange were ascribed to loosening or tightening of the folded structure. For these experiments, as well as for experiments in which infrared or ultraviolet absorption spectroscopy is used to measure hydrogen–deuterium exchange, 'isotopic exchange rate' refers to a phenomenological rate averaged over all of the amide hydrogens in a polypeptide. Although one-dimensional NMR facilitated measurement of isotopic exchange rates of hydrogens located at specific peptide linkages, its impact was limited because the resonance signals for most amide hydrogens fall in a narrow frequency band and are not completely resolved, even for very small proteins.[4–6] Interest in amide hydrogen exchange as a probe of protein structure and dynamics accelerated dramatically with the use of high-resolution two-dimensional NMR because it significantly increased the number of amide hydrogens that could be resolved, thereby increasing the number of peptide linkages that could be used to sense structural changes in proteins.[7,8] The combination of multi-dimensional NMR and hydrogen exchange has now become an important tool for investigating structural and dynamic features of proteins. Examples of recent investigations include studies of protein–ligand binding,[9–13] protein folding and unfolding,[14–21] mutants[22–24] and functional variants.[25–27]

Although mass spectrometry (MS) now plays a defined and important role in studies of the primary (i.e. covalent) structures of proteins, its role in studies of non-covalent features of proteins is less developed.[28] The ability to detect molecular ions of binary and ternary complexes sprayed from neutral solutions[29–36] demonstrates that electrospray ionization mass spectrometry (ESIMS) will be useful for determining the stoichiometry of components joined by non-covalent forces to form complexes. A linkage between the structures of proteins in solution and their charge distributions in ESI mass spectra has also been demonstrated.[37–40]

The history of mass spectrometry in the field of stable isotope analysis is long and distinguished. This legacy suggests that mass spectrometry might be joined with

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amide hydrogen exchange to form the basis of a new and powerful analytical method for investigating non-covalent structural features of proteins in solution. Given the availability of mass spectrometric methods for the accurate determination of deuterium levels in peptides and proteins from their molecular masses, the principal challenge to joining mass spectrometry and solution-phase hydrogen exchange was the development of methods for preventing artifactual isotopic exchange of labile amide hydrogens during the analysis. Although gaseous-phase hydrogen exchange circumvents this problem, it precludes studies of proteins in solution.[41] The linkage between mass spectrometry and solution-phase hydrogen exchange has been developed to the point that the deuterium levels at peptide amide linkages in peptides and intact proteins can now be determined.[42–50] Furthermore, these methods have been extended to include proteolytic fragmentation of partially labeled proteins, thereby facilitating the determination of deuterium levels in specific regions of proteins.[51–59] With the methodology in place for accurately determining localized hydrogen exchange rates in proteins by mass spectrometry, it is now possible to pursue experiments which will complement results obtained by NMR and to extend the hydrogen exchange technique to proteins that are much too large to be analyzed by NMR. The goal of this paper is to provide: (i) an introduction to the use of amide hydrogen exchange as a probe of the non-covalent structural features of proteins; (ii) technical details of methods used to determine amide hydrogen exchange rates by mass spectrometry and (iii) a comparison of NMR and mass spectrometry for determining amide hydrogen exchange rates.

WHAT ARE CONFORMATIONAL CHANGES IN PROTEINS?

The three-dimensional structures of folded proteins result from a combination of non-covalent interactions including short-range repulsion, electrostatic forces, van der Waals interactions and hydrogen bonding. Localized as well as global structural changes often accompany protein functions such as catalysis and electron or oxygen transport. Characterization of these structural changes in terms of atomic locations, thermodynamics and dynamics is the basis for our understanding of how a protein performs its function. Functionally significant structural changes may be very small, as indicated by the structures of oxidized and reduced cytochrome c determined by x-ray diffraction.[60,61] These two forms of this protein, which usually has $\sim$104 residues and functions to transport electrons on the inner mitochondrial membrane, differ principally in the oxidation state of the iron atom located on the heme. The root mean square difference in the positions of the atoms of oxidized and reduced cytochrome c is 0.5 Å, suggesting that their backbones are nearly superimposable. The largest structural changes accompanying oxidation of iron include a 0.15 Å movement of the heme out of the pocket and a 1.0 Å movement of one water molecule toward the heme. Differences in the polypeptide backbone positions of these two forms of cytochrome c are illustrated in Fig. 1. The structures have been positioned to achieve the highest level of overlap, then oriented to illustrate regions in which the structural differences are greatest. Particularly large redox-dependent changes in $\phi\psi$ angles are noted for Gly56 and Ile57 (upper center of Fig. 1) and Lys27 and Val28 (lower right of Fig. 1). Despite the very small structural changes differentiating oxidized (red) and reduced (blue) cytochrome c, their amide hydrogen exchange rates are substantially different.[8,25,62]

Other proteins undergo large conformational changes. Interconversion between these conformers may require overcoming substantial energy barriers. There are several tyrosine kinases that are located within or attached to cell membranes and required for cellular response to extracellular stimuli. The five domains comprising the structure of the Src family of tyrosine kinases,[63] which usually has more than 500 residues, are illustrated schematically in Fig. 2. The activity of these kinases is regulated by shielding or exposing the active site located in the kinase domain. Inactivation begins with phosphorylation of a tyrosine (yellow) near the C-terminus (blue) which binds to the SH2 domain (green), shielding the kinase domain from its substrate. Although high resolution three-dimensional structures of the SH3 and SH2 domains have been determined by NMR and x-ray diffraction, structures for the intact protein have not been reported. Much of our current understanding of the structure–function relationship for these kinases is based on site-directed mutagenesis studies. To understand in more detail the functional roles of these domains, as well as domain-domain interactions, additional structural information is required. For example, how similar are the structures of the kinase domains in the active and inactive forms? Is the SH3 domain involved with binding of the phosphorylated tyrosine to the SH2 domain? How might one design ligands to alter the kinase activity? Preliminary studies demonstrate that amide hydrogen exchange rates in isolated SH3 and SH2 domains are useful probes for structural changes, suggesting that this approach may be used to study structural changes in the intact protein.[64]

AMIDE HYDROGEN EXCHANGE AS A PROBE OF THE NON-COVALENT STRUCTURES OF PROTEINS

Of the many exchangeable hydrogens present in polypeptides, only those located on peptide amide linkages are used for most hydrogen exchange studies because they have exchange rates in the range that can be measured readily. Exchangeable hydrogens located on amino acid side-chains, as well as those on the N- or C-terminus exchange too rapidly to be measured by most techniques. Part of the value of amide hydrogens as structural probes stems from the fact that they form a continuous string of sensors extending the entire length of a polypeptide chain. The significance of this near continuum of sensors can be appreciated when one considers that structural changes in proteins are often

studied using tryptophan fluorescence.[65,66] Since tryptophan is one of the less common amino acids, tryptophan fluorescence may not be affected by many localized structural changes.

Isotopic exchange of peptide amide hydrogens is catalyzed by acid and base. Hence, the rate constant for hydrogen exchange, k_{ex} can be expressed as the sum of two terms, as indicated in the equation

$$k_{ex} = k_H[H^+] + k_{OH}[OH^-] \quad (1)$$

where k_H and k_{OH} are the rate constants for acid-and base-catalyzed exchange, respectively. Detailed studies of amide hydrogen exchange in polyalanine model compounds indicate that k_H and k_{OH} have values of 41.7 and 1.12×10^{10} M^{-1} min^{-1}, respectively, at 20 °C and low concentrations of salt.[67,68] The isotopic exchange rate, k_{ex}, for polyalanine is given in Fig. 3 as a function of pH. The high pH sensitivity of isotopic exchange rates dictates careful control of pH in all hydrogen exchange experiments. Furthermore, this sensitivity is the basis for quenching isotopic exchange, thereby facilitating determination of exchange rates by mass spectrometry.

In addition to their pH sensitivity, exchange rates of amide hydrogens are affected by adjacent amino acid side-chains, but relatively insensitive to more distant side-chains. The sensitivity to neighboring side-chains has been quantitatively assessed using model dipeptides of the naturally occurring amino acids.[67,68] Both inductive and steric effects appear to influence amide hydrogen exchange rates. The effects of neighboring side-chains are generally additive, thereby forming the basis for estimating the amide hydrogen exchange rates in peptides of any amino acid sequence as a function of pH.[68] Furthermore, the activation energies for k_H and k_{OH} [14 and 17 kcal mol^{-1}, respectively (1 kcal = 4.184 kJ)] can be used to predict amide hydrogen exchange rates in unstructured polypeptides at different temperatures.[56,68] Protection factors, the exchange rate constant calculated for a particular peptide amide linkage when the protein is unfolded divided by the exchange rate constant measured for the same amide hydrogen in the folded protein, are a quantitative measure of the extent to which the secondary and tertiary structure of a folded protein decreases the rate of isotopic exchange. The ability to calculate the isotopic exchange rate of any amide hydrogen in an unstructured polypeptide has been used extensively to determine protection factors for amide hydrogens in folded polypeptides.[3,25,69,70,71]

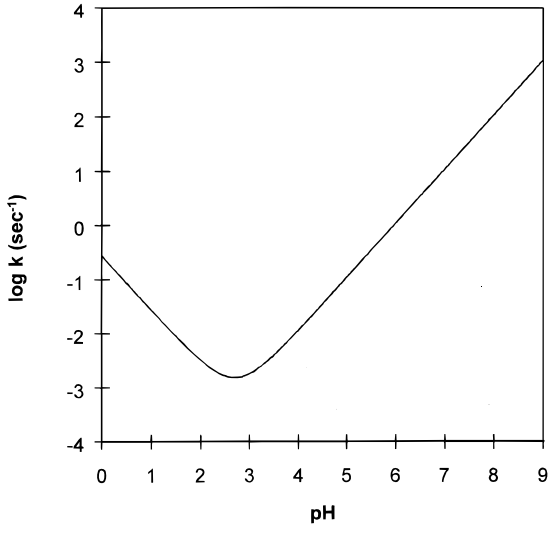

Figure 3. Rate constant for isotopic exchange of hydrogen located on peptide amide linkages in polyalanine presented as a function of pH. Results were calculated using Eqn (1) and values of k_H and k_{OH} given in the text.[68]

Although side-chain effects in peptides of different amino acid sequences alter the amide hydrogen exchange rates by as much as tenfold, secondary and tertiary structural features of folded proteins may decrease amide hydrogen exchange rates by as much as 10^8. It is this large reduction in isotopic exchange rates that facilitates use of amide hydrogen exchange as a sensitive probe for detecting and locating conformational changes in proteins.

Following incubation of a folded polypeptide in D_2O, one may find an increased level of deuterium at peptide amide linkages. The time dependence of the deuterium level can be translated into a phenomenological isotopic exchange rate constant, k_{ex}. For the simplest interpretation of hydrogen exchange results, finding different exchange rates for different samples or forms of a protein is evidence that the protein is present in different conformations. For a more profound understanding of factors responsible for different conformers, various kinetic models have been developed to describe amide hydrogen exchange in folded proteins.[3,5,70,72,73] Most of the results for amide hydrogen exchange in folded proteins can be explained through a two-process model, as illustrated in the equations

$$F(H) \xrightarrow{k_f} F(D) \quad (2)$$

$$F(H) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U(H) \xrightarrow[D_2O]{k_2} U(D) \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} F(D) \quad (3)$$

where F and U refer to folded and unfolded forms and H and D refer to hydrogen and deuterium. The process described by Eqn (2) allows for isotopic exchange directly from the folded protein. Although the compact nature of folded proteins would seem to preclude direct access of most amide hydrogens to the deuterated aqueous medium and OD^- catalyst,[3] many amide hydrogens do appear to undergo isotopic exchange without the aid of large structural changes in the protein. The rate constant for isotopic exchange by this process is designated here as k_f. Hydrogen exchange directly from the folded protein is expected to dominate for amide hydrogens located on peptide linkages near the surface or open channels within a folded protein. Furthermore, amide hydrogens that are involved in intramolecular hydrogen bonding (e.g. in α-helices or β-sheets) are unlikely to undergo isotopic exchange directly from the folded protein. A competing process, described by Eqn (3), delegates major responsibility for isotopic exchange to protein dynamics, which allows for a rapid unfolding and refolding of small regions (localized unfolding), as well as the entire protein (global unfolding). Rate constants for unfolding and refolding are designated here by k_1 and k_{-1}, respectively. According to this model, isotopic exchange can occur only

after unfolding, which breaks intramolecular hydrogen bonds, exposing amide hydrogens to the deuterated aqueous medium. Protection factors are calculated by assuming that the rate constant describing isotopic exchange from the momentarily unfolded protein is the same as the rate constant for isotopic exchange from an unstructured peptide.

Hydrogen exchange involving protein unfolding [Eqn (3)] is of special importance because it provides a direct measure of the rates of protein unfolding (k_1) and refolding (k_{-1}), as well as a link to the free energy change accompanying protein folding. For most proteins at neutral pH and in the absence of denaturants, $k_{-1} \gg k_2$ and the phenomenological rate constant for isotopic exchange is given by

$$k_{ex} = Kk_2 \quad (4)$$

where K is the equilibrium constant describing the unfolding process and k_2 is the exchange rate constant for the amide hydrogen if the polypeptide were completely unfolded.[1,73] Measuring k_{ex} and calculating k_2 leads to direct determination of the equilibrium constant and hence ΔG for the protein unfolding process, which may be localized to a particular element of secondary structure or regional domain, or it may include the entire molecule.[3,19,69,70,72,74] Under some destabilizing conditions (e.g. addition of denaturants, extreme pH, high temperature) $k_2 \gg k_{-1}$ and the measured isotope exchange rate constant, k_{ex}, may be used to determine the rate of protein unfolding, k_1:[75,76]

$$k_{ex} = k_1 \quad (5)$$

The two kinetic limits for hydrogen exchange, represented by Eqns (4) and (5), are often referred to as EX2 (uncorrelated exchange) and EX1 (correlated exchange) mechanisms.[1,73] In this context, 'correlated exchange' refers to conditions for which all of the amide hydrogens in a region undergo isotopic exchange during one unfolding event in this region. As will be discussed below, correlated and uncorrelated exchange give characteristic isotope distributions which may be evident in mass spectra of the proteins and their proteolytic fragments.

MEASURING AMIDE HYDROGEN EXCHANGE RATES BY MASS SPECTROMETRY

The technical details that must be considered when using mass spectrometry to measure amide hydrogen exchange rates in polypeptides depends on the goals of the study. Does one want to know the exchange rate averaged over all peptide amide hydrogens in the protein or in short segments of a protein that was labeled in the intact form? The former may be used to detect global changes, while the latter may be used to detect global as well as localized structural changes. The procedure used in our laboratory to measure amide hydrogen exchange rates in short segments of intact proteins is presented here because this approach, which has been called the protein fragmentation/mass spectrometry method, gives the most information. The simpler, but nevertheless useful, procedure required to measure hydrogen exchange rates averaged over all peptide linkages of intact proteins will be evident.

Although our group appears to be the first to combine proteolytic fragmentation with mass spectrometry,[51] the general approach can be traced to studies first reported by Rosa and Richards.[77,78] Fundamental aspects of the method were subsequently described by Englander *et al.*[79] The general procedure used in the protein fragmentation/MS approach for determining amide hydrogen exchange rates in intact proteins is illustrated in Fig. 4. Isotopic exchange occurs while the folded protein is incubated in D_2O buffered to the desired pD. Following the deuterium exchange-in step, isotopic exchange at peptide amide linkages is quenched by decreasing the pD to 2.4 and the temperature to 0 °C. Note that isotopic exchange in unfolded polypeptides is slowest for pH 2–3, as indicated in Fig. 3. If isotopic exchange were performed using typical conditions of neutral pH, decreasing the pH to 2–3 would decrease the amide hydrogen exchange rate in an unfolded polypeptide by $\sim 10^4$. Furthermore, decreasing the temperature from 20 to 0 °C lowers the exchange rate by an additional tenfold. For these quench conditions, the half-life for isotopic exchange at peptide amide linkages is 30–120 min.[68,79] To minimize artifactual isotopic exchange, it follows that isotopic analysis should be completed within several minutes.

To determine isotopic exchange rates within small, defined regions of a protein, the acid protease pepsin is used to fragment the protein into peptides. Since isotopic quench conditions must be maintained during the digestion, a large amount of pepsin (substrate:enzyme ratio of 1:1) is used to reduce the required digestion time to <10 min. The peptic digest is analyzed by directly-coupled high-performance liquid chromatography (HPLC)/MS to determine the molecular masses of the peptic fragments, from which the levels of deuterium present in the corresponding segments of the

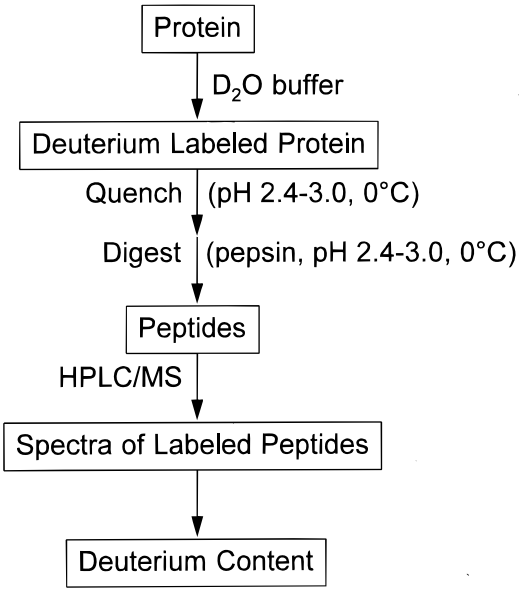

Figure 4. Procedure used to determine deuterium levels at peptide amide linkages in short segments of intact proteins incubated in D_2O.[51]

protein are determined. Although continuous-flow fast atom bombardment MS was used in our initial studies,[51,52,55,56] more recent studies,[53,57,59] have benefited from the advantages of ESIMS. The peptides are separated from the buffer salts and fractionated by their hydrophobicity in the chromatographic step. In addition, since a protiated mobile phase is used for HPLC, deuterium located at rapidly exchanging sites, such as on the side-chains or on the N-and C-termini of the peptides, are replaced with protium. Hence, the increment in molecular mass is due to deuterium located at peptide amide linkages. Although a recent study[68] using model peptides confirms that the back-exchange step works as predicted,[79] this study also suggests that the NδH deuterium in the side-chain of arginine may not be completely replaced with protium for some conditions. It is also important to note that the HPLC step, which is performed using perfusion chromatography,[80] is complete within 4–7 min. Similar analyses performed using columns packed with conventional silica supports required $\sim$20 min.[51,52]

Typical MS results obtained using a Micromass Autospec magnetic sector mass spectrometer equipped for ESI and array detection are given in Fig. 5. Approximately 350 pmol of rabbit muscle aldolase with the natural abundance of isotopes were placed in quench conditions and digested with pepsin (substrate : enzyme ratio 1 : 1) for 5 min. The elution profiles of three peptic fragments that include peptide amide linkages 18–30 (m/z 672–675), 120–138 (m/z 686–688) and 58–62 (m/z 692–696) are indicated by selected ion plots in Fig. 5. These, as well as several other peptides, elute in a 30 s time interval at an HPLC elution time of 3 min. The number of peptides eluting during this time interval is indicated by the complexity of the composite mass spectrum, created from the sum of four scans taken during the 30 s time interval and covering an m/z range of 400–1400 presented in the middle of Fig. 5. Although peptic digestion of aldolase was only about 10% complete under these conditions, signals with good signal-to-noise ratios were obtained for all but the least abundant fragments. The fidelity of weak signals in this mass spectrum is illustrated for the same three segments in the lower part of Fig. 5. The molecular masses averaged over all isotopes are the information required for studies of hydrogen exchange kinetics. This information

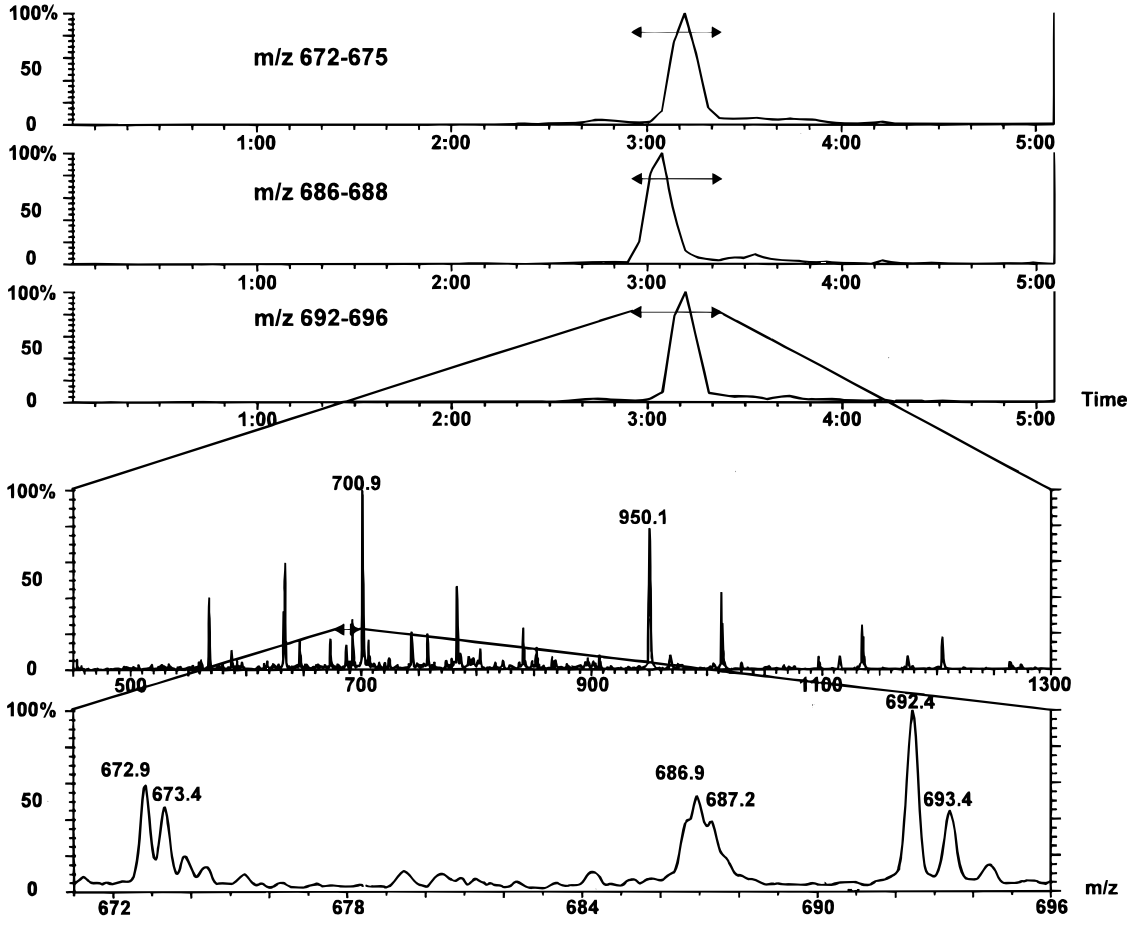

Figure 5. Representative ESI mass spectrum obtained for analysis of a digest of 300–400 pmol of rabbit muscle aldolase (M_r 38000). The top section illustrates the selected ion plots for three m/z ranges, which include the molecular ions for three peptic fragments representing linkages 58–62 (m/z 692.4), 120–138 (m/z 686.9) and 18–30 (m/z 672.9). Arrows indicate the four scans that were summed to give the complete mass spectrum presented in the middle section. The arrow in the middle section indicates the m/z range that was expanded to give the molecular ion peaks for three peptides, illustrated in the bottom section. Mass spectrometric analyses were performed with a Micromass Autospec high-resolution mass spectrometer equipped with a standard Micromass ESI source and a microchannel array detector. Mass spectra were acquired over the m/z range 400–1400. Each scan required a total of 3.2 s and consisted of 20 exposures, where 7.5% of the central beam m/z was recorded in each exposure. The resolution was 1900 (50% valley).

can be determined from molecular ion signals even though the isotope peaks are not fully resolved. Resolution of isotopic peaks for the three peptides presented in Fig. 5 is variable because molecular ions with +1 (m/z 692), +2 (m/z 672) and +3 (m/z 686) have been used for this illustration.

The accuracy with which the deuterium levels in peptides is determined is important because it sets the threshold for detecting structural changes in the protein. Application of the original version of the protein fragmentation method using tritium[77–79] was substantially limited because it required determining the quantity of tritium per peptide from the UV absorbance and radioactivity present in peptides isolated from the peptic digest by HPLC. Given the large number of peptides present in the peptic digests of large proteins and the requirement to purify the peptides rigorously within minutes before substantial back-exchange occurs, accurate isotope levels were usually determined for only a few fragments. Much of the power of the protein fragmentation/MS approach can be attributed to the ability to determine isotope levels in peptides that have not been rigorously isolated. The deuterium level is determined by mass spectrometry from the average molecular mass of a segment, which depends on both the mass and the intensity of the isotopic peaks. For a properly calibrated instrument tuned for maximum sensitivity, but without compromising general conditions required for hydrogen exchange studies, the uncertainty in the average molecular masses of peptides is 0.02–0.2 Da. As is often the case in mass spectrometry, the uncertainty in the results depends more on sample preparation than the mass spectrometric measurement.

Pepsin is used to fragment the protein because it has maximum activity at pH 2–3 where the amide hydrogen exchange rate is slowest. Given the power of mass spectrometry for identifying peptides in complex mixtures, the low specificity of pepsin is advantageous because it usually leads to formation of many peptides. For example, peptic digestion of aldolase, a relatively large protein with 363 residues, for 5 min under standard quench conditions (0 °C, pH 2.5) gave over 100 peptides.[51] A 64 peptide subset could be related back to 84% of the aldolase backbone. The average segment length in this subset was 13 peptide linkages, with the shortest and longest segments spanning three and 26 linkages, respectively. When the spatial resolution was increased by analyzing the difference in deuterium levels in overlapping peptides, the effective average segment length was five linkages. The sites at which pepsin cleaves a protein cannot be predicted with certainty, because it can cleave at several different residues and because it may cleave on either the N-terminal or C-terminal side of these residues. Careful analysis of peptides found in peptic digests of four proteins, performed under standard quench conditions, indicates a following priority for cleavage of Met– > Phe– > Leu– > –Tyr > –Phe > Glu– > Cys– > Tyr– > Ala–, where the '–' on the left or right indicates cleavage on the N-terminal or C-terminal side of the indicated residue, respectively. Cleavage at other sites has also been observed.[81] Although high levels of pepsin are used, we have found little evidence for proteolytic fragmentation of pepsin.

Deuterium loss during analysis

The most successful applications of the protein fragmentation/MS approach usually require optimizing all experimental parameters to minimize artifactual isotopic exchange during analysis. Several important experimental parameters have been investigated. For example, the deuterium levels in 13 fully deuterated peptides derived from aldolase have been determined after varying the trifluoroacetic acid (TFA) levels used in the HPLC mobile phase from 0.02 to 0.1%. Although different TFA levels were required for maximum recovery of deuterium in different peptides, a TFA level of 0.05% was the best compromise for the 13 peptides studied. This parameter, as well as the pH used to quench isotopic exchange, may be optimized for maximum recovery of deuterium in specific peptides. Replacing conventional silica support with high-speed perfusive support in the HPLC columns increased the recovery of deuterium from 64 to 88%.[81] Considerable isotopic exchange can also occur as peptides pass through the ESI interface. However, little or no exchange occurs in the Micromass electrospray source following judicious tuning, which includes using source temperatures below 70 °C.

Having chosen experimental parameters to minimize isotopic exchange at peptide amide linkages during analysis, recoveries averaged over many different peptides are expected to be in the range 80–90%. The fundamental basis of a relatively simple method that may be used to compensate for the 5–40% losses of deuterium in specific segments has been described.[51] This treatment recognizes that some isotopic exchange occurs during both the digestion and HPLC steps. Furthermore, the deuterium content of the peptides may increase or decrease during digestion, depending on the D_2O level in the digestion solution. Since the HPLC mobile phase contains no D_2O, the peptides can only lose deuterium during HPLC. Detailed analysis of the kinetics of isotopic exchange under quench conditions leads to the equation

$$D_0 = \frac{\langle m \rangle - \langle m_{0\%} \rangle}{\langle 100_{100\%} \rangle - \langle m_{0\%} \rangle} \times N \qquad (6)$$

which can be used to adjust measured deuterium levels for artifactual isotopic exchange occurring during digestion and HPLC fractionation. In this expression, D_0 is the number of deuteriums present in a particular segment of the protein after incubation in D_2O and $\langle m_{0\%} \rangle$, $\langle m \rangle$ and $\langle m_{100\%} \rangle$ are the average molecular masses of a peptide obtained by analysis of non-deuterated, partially deuterated and fully deuterated samples, respectively. Thus, accurate determination of the deuterium level at peptide amide linkages will include analysis of the non-deuterated and fully deuterated forms of a protein.

Application of Eqn (6) can be demonstrated using results presented in Fig. 6, which shows the isotope peaks for the molecular ion of the segment including peptide linkages 327–336 of rabbit muscle aldolase that contained no deuterium [Fig. 6(A)], aldolase that had been incubated in D_2O for 0.5 h [Fig. 6(B)] and aldolase that had all exchangeable hydrogens replaced with

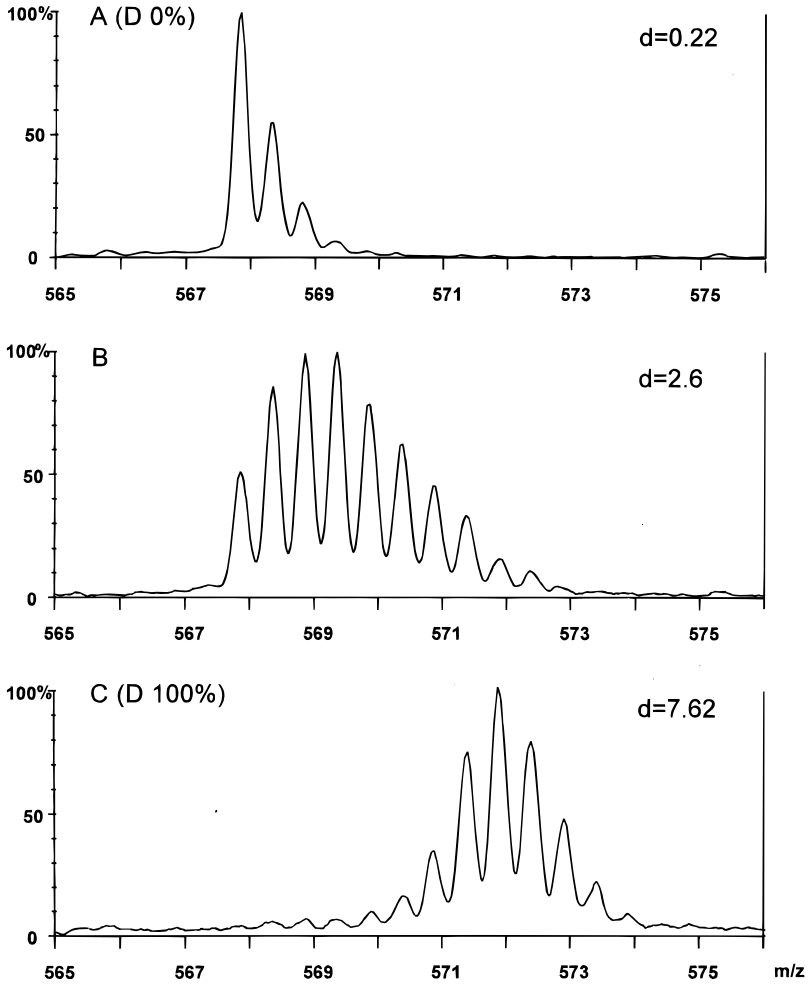

Figure 6. Molecular ion region of the ESI mass spectrum of the peptide fragment of aldolase including peptide linkages 327–336 derived from (A) non-deuterated, (B) partially deuterated and (C) completely deuterated rabbit muscle aldolase. The operating conditions were similar to those used for Fig. 5.

deuterium [Fig. 6(C)]. The deuterium levels in this segment of variously labeled aldolase was determined from the difference between the average molecular masses of these peptides and the average molecular mass calculated for this peptide if it had the natural abundance of isotopes. It is significant that 0.2 mol of deuterium was found in this segment when derived from aldolase that initially had no deuterium [Fig. 6(A)]. This level of deuterium was due to isotopic exchange (deuterium exchange-in) occurring during the 5 min digestion, which was performed in an aqueous solution that was 90% D_2O. When this segment was derived from completely deuterated aldolase [Fig. 6(C)], it had 7.6 mol of deuterium. Since this segment has nine peptide linkages, 2.4 mol of deuterium were apparently lost during analysis. Hence, analysis of completely labeled aldolase provides a quantitative measure of deuterium loss occurring during the digestion and HPLC steps. The molecular masses found for this segment in unlabeled and completely labeled aldolase are substituted for $m_{0\%}$ and $m_{100\%}$, respectively, in Eqn (6). Since the extent of isotopic exchange occurring during analysis depends on the amino acid sequence of the segment, deuterium recoveries are determined for each segment that will be used in a study. Frequent analysis of nondeuterated and completely deuterated forms of the protein to verify that isotopic exchange during analysis has been minimized is recommended.

It is important to note that the adjustment indicated by Eqn (6) is not perfect for all peptides because the derivation assumes that the isotopic exchange rates at all peptide linkages are equal. Statistical analysis of 3000 peptides with random sequences and 5–25 peptide linkages indicated that the average error is 5.5% of the adjustment. Furthermore, the error in the adjustment was $<10\%$ for 86% of the peptides, but $>25\%$ for 1% of the peptides. Since the adjustment is usually only 10–20% of the measured deuterium level, this treatment indicates that the adjustment is appropriate for nearly all (at least 99%) amino acid sequences.

Extracting hydrogen exchange rate constants from deuterium levels in peptides

Structural differences in short segments of protein mutants, as well as in proteins exposed to different environments, can be determined by the protein fragmentation/MS approach. Although structural changes may be detected following incubation of a protein in D_2O for a specified time, more complete information can be obtained using the widest possible

range of incubation times. Deuterium incorporation into the segment including peptide linkages 59–64 of aldolase incubated in D_2O at pD 6.53 for 2.5 min to 44 h is illustrated in Fig. 7. If the protein were not folded during the deuterium exchange-in step, all six peptide amide hydrogens would have been replaced with deuterium in less than 10 s. However, the high-order structure of aldolase decreases the rate of isotope exchange so that only about four deuteriums are found after the 44 h of incubation. These results have been adjusted to compensate for isotopic exchange that occurred during analysis using Eqn (6). Although these results do not give the isotopic exchange rate at specific amide linkages, they can be fitted to the expression presented in the equation

$$D = N - \sum_{i=1}^{N} \exp(-k_i t) \quad (7)$$

to determine quantitatively the range of exchange rate constants at peptide linkages in this segment. In this expression, D is the deuterium level found in a specific segment with N peptide linkages following incubation of the intact protein in D_2O for time t and k_i are the pseudo-first-order rate constants for isotopic exchange at each peptide linkage. The line through the data points in Fig. 7 was drawn using Eqn (7) after the rate constants had been varied to optimize the fit. This analysis shows that one peptide amide hydrogen in this segment exchanges with a rate constant of 720 h^{-1}. It is important to note that, although this rate constant is the maximum that could be measured for the incubation times used in this experiment, it is much less than it would be if there were no high-order structure in this segment. A flow-quench apparatus suitable for determining isotope exchange rate constants for the most rapidly exchanging peptide amide hydrogens in proteins has been described.[57] Further analysis of the data in Fig. 7 shows that the amide hydrogens located at other peptide linkages in the 59–64 segment have rate constants of 30, 1.8 and 0.6 h^{-1}. Two of these hydrogens have rate constants less than 0.12 h^{-1}, which is the slowest rate that could be determined for the incubation times used in this study. The exchange rate constants for peptide amide hydrogens slowest to exchange under these experimental conditions can be determined by using longer incubation times.

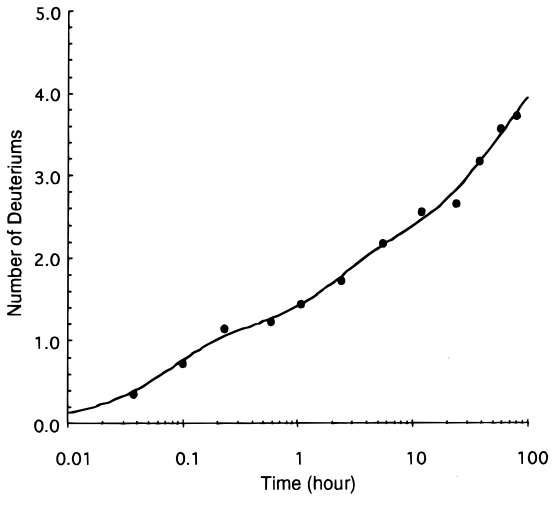

Figure 7. Deuterium level in the segment including peptide linkages 59–64 of aldolase following incubation of the intact protein in D_2O for 2.5 min–44 h. The line was drawn by optimizing isotope exchange rate constants in Eqn (7) to obtain the best fit.

Isotope patterns and amide hydrogen exchange

Isotope patterns in the mass spectra of proteins and their fragments are an important source of information in hydrogen exchange studies because they indicate the distribution of deuterium among all of the molecules in the sample. The distribution of heavy isotopes (^{13}C, ^{15}N, ^{18}O, etc.) among the molecules in a sample is normally random. Neglecting significant isotope effects occurring during synthesis, specific atomic positions in all copies of a peptide have the same probability of being occupied by a heavy isotope. For such a random distribution of isotopes, the intensities of isotope peaks in mass spectra are described by expansion of the appropriate binomials.[82,83] Similar considerations lead to new applications of amide hydrogen exchange when detected by mass spectrometry. If exchange at any particular amide linkage is the same for all molecules in a sample and independent of exchange at other linkages in the same molecule, deuterium will be distributed randomly among all copies of a protein in a sample and the relative intensities of the isotope peaks of the intact protein or its fragments will form one envelope of peaks. That is, a single envelope of isotope peaks is expected if the sample is structurally homogeneous and if exchange is uncorrelated (i.e. $k_{-1} \gg k_2$). Such a pattern is illustrated in Fig. 6. However, if the molecules in a sample belong to two structurally different groups that have different amide hydrogen exchange rates in some regions, mass spectra of peptides derived from these regions may have bimodal isotope patterns. Likewise, isotopic exchange occurring through a correlated exchange mechanism (i.e. $k_2 \gg k_{-1}$) may give bimodal isotope patterns.

The ability to detect variations in the secondary and tertiary structures of proteins from bimodal isotope patterns can be illustrated with rabbit muscle aldolase that was destabilized by acid. The protein was dissolved in D_2O at pD 6.52, 4.04 or 3.00 for 0.021, 6.29 or 56 h, respectively. Isotopic exchange was quenched by decreasing the pD and temperature, as described above. The incubation times were selected to compensate for the fact that k_2 also depends on pD (see Fig. 3). Details of the general approach used to relate changes in the equilibrium constant describing the distribution of folded and unfolded forms of a protein [Eqns (3) and (4)] to amide hydrogen exchange results is discussed elsewhere.[56] Mass spectra obtained for the segment including residues 257–269 derived from the intact protein are given in Fig. 8(A)–(C). Doubly charged ions would be found at m/z 700.9 if this segment had no deuterium. The multiple peaks in Fig. 8(A) are separated by 0.5 m/z units, as expected for doubly charged ions with various numbers of ^{13}C and deuterium. The molecular mass of this segment, averaged over all molecules in the sample, was determined from the centroid of the isotope

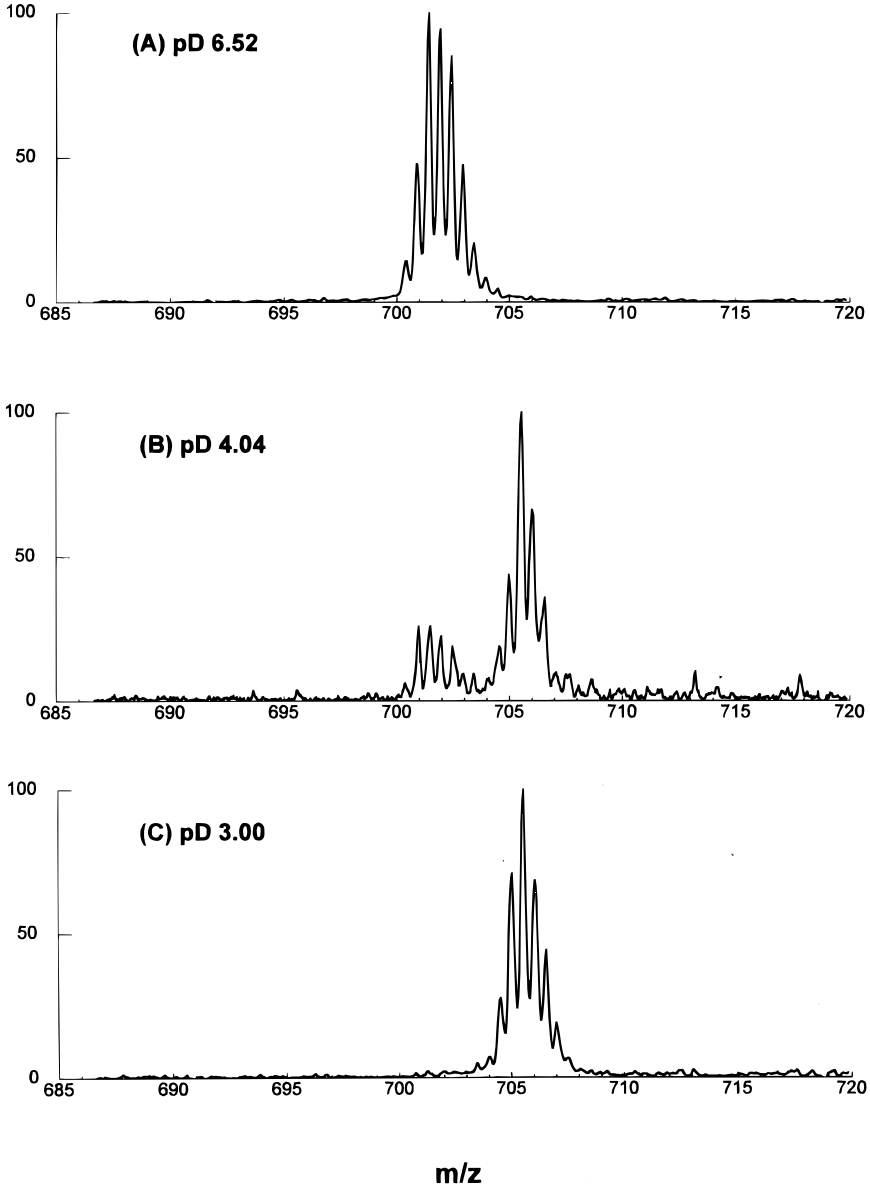

Figure 8. ESI mass spectra of the doubly charged molecular ion of the peptide derived from the 257–269 segment of aldolase following incubation of the intact protein in D_2O at (A) pD 6.52, (B) pD 4.04 and (C) pD 3.00.

peaks. After adjustment for the artifactual gain in deuterium during digestion, the deuterium level in this segment was found to be less than 0.2 mol. The low level of deuterium in this segment following incubation of aldolase in D_2O for 0.021 h is consistent with the results of Zhang *et al.*,[55] who reported that the amide hydrogens in this region have isotopic exchange rate constants of $<0.002\ h^{-1}$. The low rate of hydrogen exchange shows that the region including residues 257–269 of aldolase remained tightly folded during the 0.021 h that the intact protein was incubated in D_2O.

When adjusted for the gain and loss of deuterium during analysis, the centroid of the isotope pattern in Fig. 8(C) indicates that the amide hydrogens at all ten peptide linkages in the 257–269 segment are completely deuterated following incubation of intact aldolase in D_2O at pD 3.00. The dramatic increase (from 0 to 100%) in deuteration in this segment as the pD was decreased from 6.52 to 3.00 is attributable only to acid-induced unfolding of aldolase because the incubation time was increased to quantitatively compensate for the decrease in k_2 with pD. This high rate of hydrogen exchange is because aldolase has little or no secondary and tertiary structure at pD 3.00.

The bimodal isotope pattern obtained for the 257–269 segment of aldolase following incubation of the intact protein at the intermediate pD of 4.04 [Fig. 8(B)] is of particular interest to understanding processes associated with acid denaturing because it shows that hydrogen exchange has been very slow in this segment of some aldolase molecules and very fast in other molecules. The intensity of the low-mass envelope of isotope peaks indicates the fraction of aldolase molecules that were folded during the incubation time, while the intensity of the high-mass envelope of isotope peaks indicates the fraction of molecules that were

unfolded during the incubation period. Comparison of the relative intensities of the peaks in the two envelopes at pD 4.04 indicates that 78% of the aldolase molecules were unfolded in the region including residues 257–269. These results demonstrate that bimodal isotope patterns obtained with the protein fragmentation/MS method can be used to determine the pH at which proteins become unstable.

Results of exploratory studies in this area suggest that isotope patterns may be useful for a wide variety of protein structural and dynamic studies. Miranker *et al.*[46] have used bimodal isotope patterns to identify intermediates in the folding of lysozyme. Zhang *et al.*[55] found bimodal isotope patterns for peptides derived from three segments of aldolase. Although these segments are located in different regions of the aldolase backbone, the x-ray crystal structure of aldolase indicates that all are located in α-helices which are in close proximity. These results were interpreted to indicate structural heterogeneity in this region. When isotopic exchange is correlated (i.e. $k_2 \gg k_{-1}$), exchange is complete within the unfolding region following the first unfolding event. This situation is common for proteins that are inherently unstable or have been purposely destabilized with denaturants. The isotope patterns of peptides derived from segments in which hydrogen exchange is correlated may appear as two envelopes. For example, bimodal isotope patterns have been found when urea was used to destabilize aldolase.[76] As the incubation time increased, the relative intensity of the low-mass envelope decreased in concert with increasing intensity of the high-mass envelope. From the relative intensities of these envelopes, the relative abundances of the folded and unfolded forms of particular segments of a protein can be determined and used with Eqn (5) to determine the unfolding rates of specific regions within a protein. Although various spectroscopic methods (e.g. circular dichroism, tryptophan fluorescence, NMR) have traditionally been used to detect protein unfolding, the combination of hydrogen exchange and mass spectrometry is unique because it can be used to determine rate constants describing unfolding of specific regions of large proteins.

DETERMINE AMINE HYDROGEN EXCHANGE RATES BY MS OR NMR?

Whether to use MS or NMR to monitor amide hydrogen exchange in proteins is an important consideration for new practitioners. Although IR and UV absorbance spectroscopy are used to follow hydrogen exchange in proteins, only NMR and mass spectrometry are suitable for determining exchange rates in specific regions of proteins. Since NMR has been used extensively in hydrogen exchange studies, it is the standard with which mass spectrometry should be compared. The principal advantage of NMR rests in its ability to determine isotope exchange rates of amide hydrogens located at specific peptide linkages in folded proteins. In contrast, analysis by mass spectrometry gives the deuterium levels in short segments, from which the distribution of rate constants within these segments can be determined. The length of these segments and hence the spatial resolution of the hydrogen exchange results depends on the amino acid sequence and specificity of the acid protease used to fragment the protein. Although the average length of peptic fragments may be 10–15 linkages, the spatial resolution may be increased to approximately five peptide linkages if differences in deuterium levels of overlapping peptides are considered.[51,55] The validity of MS/MS as a means to quantify deuterium levels at specific peptide linkages along polypeptide backbones has yet to be determined.[84,85]

Mass spectrometry has undisputed advantages over NMR in the areas of sensitivity, protein solubility and molecular mass. Time course studies performed by high-resolution NMR require ~50 μmol of protein, while similar studies by mass spectrometry require only a few nanomoles. Furthermore, if the protein fragmentation/MS approach were optimized for sensitivity, the quantity of protein required could be decreased to the low picomole range. The quantity required for analysis by NMR can be reduced to ~5 μmol if ^{15}N-labeled protein is available. Additional material is normally required for preliminary studies using either NMR or mass spectrometry. For NMR, the peptide NH resonances must be assigned, whereas for mass spectrometry, the peptide fragments must be identified. The barriers presented by these necessary tasks in well equipped, experienced laboratories seem to be smaller for mass spectrometry. For studies of proteins that are only moderately soluble or proteins that aggregate at high concentrations, mass spectrometry is also advantageous because it requires only micromolar concentrations while NMR requires millimolar concentrations. With respect to molecular mass, NMR is limited to studies of proteins with M_r less than 30000, while mass spectrometry has been used to study amide hydrogen exchange in proteins with molecular masses approaching 1000000.[52] It appears that there is no limit to the size of proteins studied by the protein fragmentation/MS method because acid proteases are used to fragment the proteins into peptides. It should be noted, however, that peptic digests of large proteins have an extremely large number of peptides and that interfering signals from different peptides may limit the extent of backbone coverage in large proteins. In such cases, chromatographic speed may be compromised to improve the chromatographic separation, thereby minimizing the number of interfering signals in the mass spectrum.

The time and the conditions under which analyses are performed are important and generally unappreciated advantages inherent to mass spectrometry. As discussed above, the time scale for isotopic exchange of amide hydrogens at neutral pH spans a range from seconds to months. Deuterium levels are determined by NMR and mass spectrometry only after the isotopic exchange reaction has been quenched. For analysis by NMR, isotopic exchange is quenched by analyzing the protein in a folded state where chemical shifts allow detection of amide hydrogens located at specific peptide linkages. Because isotopic exchange is base catalyzed (see Fig. 3), the lowest pH consistent with maintaining the protein in a folded state is used. Hence most NMR measure-

ments are performed at pH 5–6. Since 2D NH-C$^{\alpha}$H measurements require 8–12 h, exchange rates of only the more slowly exchanging amide hydrogens ($t_{1/2} > 10$ h) can be determined. As a result, structural changes affecting primarily amide hydrogens that are not participating in intramolecular hydrogen bonding as well as hydrogens located on the surfaces of proteins may not be detected. It is noted that the NMR analysis time can be reduced to less than 1 h if a protein is labeled with ^{15}N. For analysis by mass spectrometry, isotopic exchange is quenched by lowering the pH and temperature, as discussed above. For typical conditions of pH 2.5 and 0 °C, the half-lives of all hydrogens located on peptide amide linkages are sufficiently long for detection within 15 min. Hence, exchange at all peptide linkages is sensed by mass spectrometry, although with less spatial resolution than NMR. Furthermore, a high-speed flow-quench apparatus may be used to effect deuterium exchange-in and quench, so exchange rates of the most rapidly exchanging amide hydrogens in polypeptides can be determined by mass spectrometry.[46,57,86] It should be noted that exchange rates of rapidly exchanging amide hydrogens have been estimated from relaxation times[27] and that recent advances in NMR may facilitate future studies of amide hydrogen exchange shorter time-scales.[87,88]

CONCLUSIONS

Until recently, amide hydrogen exchange rates in large proteins ($M_r > 30000$) were measured as the average of the exchange rates at all peptide amide linkages. By combining hydrogen exchange with acid proteolysis and directly coupled HPLC/MS (i.e. the protein fragmentation/MS method), it is now possible to determine isotope exchange rates of hydrogens located on peptide amide linkages in short segments of large proteins. The ability to determine exchange rates of hydrogens located at specific peptide linkages by NMR makes it the preferred method for studies of small proteins. However, advantages of mass spectrometry in the areas of sensitivity, protein molecular mass and solubility, as well as the ability to detect correlated exchange within short segments directly makes the protein fragmentation/MS approach uniquely suited for a wide range of protein structure studies. For example, the effects of site specific mutagenesis on local as well as global dynamics and structure may be studied. Similarly, structural changes induced by ligand binding may be determined. For experimental conditions that give bimodal isotope patterns, amide hydrogen exchange and mass spectrometry form the basis of a new method for determining rate constants for protein unfolding. While mass spectrometry may be used to augment NMR studies of small proteins, it is uniquely suited for hydrogen exchange studies of large proteins.

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