Comparison of Continuous and Pulsed Labeling Amide Hydrogen Exchange/Mass Spectrometry for Studies of Protein Dynamics

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In contrast to the rigid structures portrayed by X-ray diffraction, proteins in solution display constant motion which leads to populations that are momentarily unfolded. To begin to understand protein dynamics, we must have experimental methods for determining rates of folding and unfolding, as well as for identifying structures of folding and unfolding intermediates. Amide hydrogen exchange has become an important tool for such measurements. When urea is used to stabilize unfolded forms of proteins, the refolding rates may become slower than the rates of isotope exchange. In such cases, the intermolecular distribution of deuterium among the entire population of molecules may become bimodal, giving rise to a bimodal distribution of isotope peaks in mass spectra of the protein or its peptic fragments. When the protein is exposed continuously to D_2O, the relative intensities of the two envelopes of isotope peaks give an integrated account of populations participating in the folding/unfolding process. However, when the protein is exposed only briefly to D_2O, the relative intensities of the two envelopes of isotope peaks give an instantaneous measure of the folded/unfolded populations. Application of these two labeling methods to a large protein, aldolase, is described along with a discussion of specific parameters required to optimize these experiments. (J Am Soc Mass Spectrom 1999, 10, 675–684) © 1999 American Society for Mass Spectrometry

Proteins normally exist as mixtures of folded and unfolded forms. Individual molecules of large proteins may be completely unfolded in some regions and completely folded in others [1]. A variety of experiments have been used to determine the structures of variously unfolded forms of proteins, their stabilities, and their rates of folding or unfolding. In such experiments, the folding rate describes the process though which a population of unfolded molecules is transformed into folded molecules. Similarly, the unfolding rate describes unfolding of a population of folded molecules. The time required to fold a single molecule after it has begun to fold is usually much shorter due to the cooperativity of forces driving this microscopic process than the time required for half of the population to fold [2, 3]. Radioactive decay involves similar concepts. The time required for radiation to escape from a nuclide is short relative to the time required for half of the nuclides to decay. Although both forms of folding kinetics are of considerable interest, the present study involves the unfolding kinetics of a population of folded molecules. Both equilibrium and nonequilibrium experiments have been used to investigate this aspect of protein folding and unfolding. In nonequilibrium folding experiments, the population of the folded form increases with time until equilibrium is established. For example, protein folding has been studied by unfolding proteins in urea or guanidine, followed by diluting the denaturant to initiate folding [4, 5]. Under equilibrium conditions, the size of the populations of the variously unfolded forms is constant but all molecules comprising the system cycle though the various unfolded states [6–8].

Identification of these unfolded forms of proteins, as well as determination of their kinetic and thermodynamic properties, is essential for our understanding of protein stability and its numerous practical applications in biotechnology. Tryptophan fluorescence and circular dichroism are two of the most important methods used to investigate protein structure [9–11]. Though attractive for their simplicity and speed, these methods give only an overview of structure. For example, reduced fluorescence is normally accepted as evidence that the region around one or more tryptophan residues has become more unfolded. However, this approach is relatively insensitive to folding distant from tryptophan residues. Amide hydrogen exchange (HX) has become

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an important new tool for investigations of protein folding because unfolded regions of proteins undergo isotope exchange much faster than folded regions. This approach is particularly attractive for structural studies because it employs an essentially continuous series of sensors along the entire length of the protein backbone. Both nuclear magnetic resonance spectroscopy (NMR) [4, 5, 12] and mass spectrometry (MS) [1, 13–16] have been used to quantify HX in studies of protein folding and unfolding.

Hydrogen exchange studies normally use one of two general approaches to label proteins in their variously folded states [17]. In continuous-labeling experiments, the protein is exposed to D_2O while the populations of folded and unfolded states are changing. Molecules that are or become unfolded during the labeling time are completely deuterated and molecules that did not unfold during this time have less deuterium. Deuterium levels in proteins labeled continuously effectively integrate the number of molecules that unfold during the labeling time, which may be as short as milliseconds or as long as days. In pulsed-labeling experiments, the exposure of the protein to D_2O is short relative to the time scale of the folding/unfolding dynamics. Since little unfolding or folding occurs during the labeling step, the deuterium levels resulting from pulsed labeling indicate the instantaneous populations of folded and unfolded molecules. Although pulsed labeling has been used in several NMR studies [4, 5, 17], it has been used in only a few MS studies [1, 13–16].

Results presented here stem from our HX MS studies of the structure and dynamics of rabbit muscle aldolase [1, 18, 19]. This protein is a homotetramer in which each subunit has 363 amino acids. The three-dimensional structure of the subunits, as well as their positions in the tetramer, have been determined by X-ray diffraction [20, 21]. Aldolase has the classical α/β barrel structure in which the β-strands are joined by α-helices. When incubated in high concentrations of urea, aldolase begins to unfold. Results of recent pulse-labeling HX MS studies showed that aldolase consists of three unfolding domains which unfold at different rates [1]. We report here a comparison of continuous and pulsed labeling when used to investigate the unfolding of aldolase by amide hydrogen exchange and mass spectrometry. Important considerations for designing pulsed-labeling experiments that use mass spectrometry are also discussed.

HX/MS Background

Since details of the general models used to describe how amide hydrogens undergo isotopic exchange in proteins have been described elsewhere [6, 22–24], only a brief summary will be given here. Isotope exchange under conditions that unfold most molecules of a protein is similar to exchange in short peptides, models of which have been studied extensively by NMR [25]. Isotope exchange under conditions that favor folded forms of proteins is generally more complex, requiring at least two different models. These models, which differ fundamentally in whether the protein is folded or unfolded at the moment that isotope exchange occurs, are described by eqs 1 and 2.

$$F_H \underset{}{\overset{OD^-,\, D_2O}{\rightleftharpoons}} F_D \tag{1}$$

$$F_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_H \xrightarrow[OD^-,\, D_2O]{k_2} U_D \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} F_D \tag{2}$$

Exchange in the folded protein occurs when the D_2O and OD^- arrive at an amide linkage by some means that does not require substantial movement of the protein backbone. This process is most common for amide hydrogens located near the surfaces of folded proteins. The rate constant for exchange at any peptide amide linkage via this process is given by eq 3, where β is the probability that D_2O and OD^- are present at the peptide linkage and k_2 is the rate constant for isotope exchange at this peptide linkage in an unfolded peptide [25]. Isotope exchange in peptides and unfolded proteins, where the peptide amide hydrogens are exposed to the solvent, is described by eq 3 if β is equal to 1.

$$k_{exch} = \beta k_2 \tag{3}$$

Proteins are continuously unfolding and refolding. Even under physiological conditions that favor the folded protein, individual molecules spend a small fraction of time in various unfolded states. Significant isotope exchange may occur while these molecules are momentarily unfolded because exchange rates in unfolded proteins are typically several orders of magnitude greater than in folded proteins. Isotope exchange in an unfolded region of a protein that spends most of its time folded is described by eq 2 and illustrated in Figure 1. Rate constants k_1 and k_{-1} describe the unfolding and refolding processes, while k_2 is as described above. Explicit expressions for the rate constant describing isotope exchange via momentary unfolding have been presented previously [26]. When $k_2 \ll k_{-1}$, a protein must unfold many times before it becomes completely deuterated. The isotope exchange rate constant for this behavior, which is typical of most proteins under physiological conditions, is given by eq 4 where K_{unf} is the equilibrium constant describing the unfolding process.

$$k_{exch} = K_{unf}\, k_2 \tag{4}$$

For some conditions, k_2 may be much greater than k_{-1}. In this case, a molecule must unfold only one time to become deuterated. Whether the entire molecule or only a region of the backbone becomes deuterated

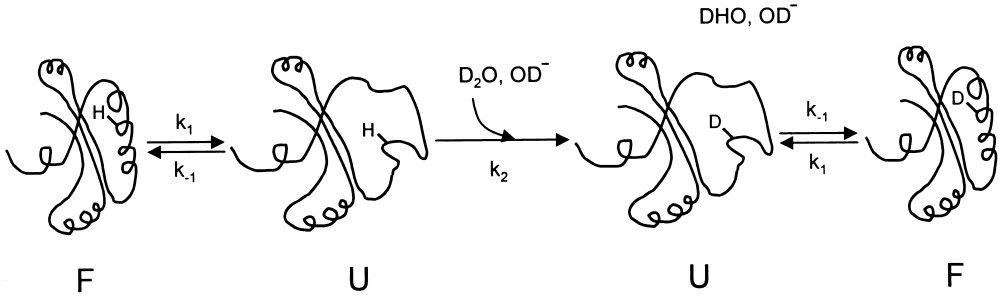

Figure 1. Pictorial representation of a folded (F) protein unfolding in a small region to facilitate partial or complete isotope exchange. The kinetics of the unfolding and refolding processes are described by rate constants k_1 and k_{-1}, respectively, and exchange from the unfolded polypeptide is described by k_2.

depends on whether all or part of the backbone participates in the unfolding event. The isotope exchange rate constant for this process is given in eq 5, where k_1 is the unfolding rate constant described in eq 2.

$$k_{\text{exch}} = k_1 \tag{5}$$

Mass spectrometry has been particularly effective in detecting hydrogen exchange by this mechanism because it leads to bimodal distributions of deuterium among the molecules, which appear as bimodal distributions of isotope peaks in mass spectra [1, 13, 15, 16].

Results and Discussion

Continuous Labeling

Use of continuous-labeling HX/MS to investigate protein dynamics will be demonstrated by examining the unfolding of rabbit muscle aldolase in 3 M urea. The procedure used to investigate the unfolding kinetics of aldolase by continuous-labeling HX/MS is illustrated in Figure 2. Aldolase was equilibrated in H_2O (phosphate buffer pH 7) and then transferred into D_2O (phosphate buffer, pD 7.0, 3 M urea). This transfer was accomplished using a column (1 × 3 cm) packed with Sephadex G-10. The column was saturated with the desired final solution (D_2O, phosphate buffer, pD 7.0, 3 M urea); the solution containing the protein in H_2O was placed on top of the Sephadex; the column was spun to elute the protein in the D_2O solution. The concentration of aldolase in the D_2O/urea solution was 2.5 mM. Following incubation times of 3 min to 48 h, isotope exchange was quenched by decreasing the pH to 2.5 and the temperature to 0 °C. Pepsin was added to fragment the protein into peptides, which were analyzed by HPLC ESIMS under conditions that minimized isotope exchange at the peptide amide linkages. Details of similar experiments have been described elsewhere [1, 24].

Electrospray ionization mass spectra of two peptic fragments, which include residues 180–187 and 299–307, will be used to illustrate two very different types of HX behavior found when intact aldolase was labeled continuously for 5, 30, and 480 min. These spectra are presented in Figure 3. Reference spectra of the same peptides taken from aldolase that contained no deuterium (0% ref) and aldolase that was completely exchanged in D_2O (100% ref) are presented in the top and bottom panels of Figure 3. These reference spectra were used to determine the extent of deuterium loss during digestion and HPLC. The mean deuterium loss for all detectable peptic fragments of aldolase was approximately 15%. In addition, the 0% ref and 100% ref spectra served as references for totally folded and unfolded states of the protein. To understand these experiments, it is important to recognize that these mass spectra give the distribution of peptide molecular weights, and hence the intermolecular distribution of deuterium in segments of aldolase represented by these peptides. However, these spectra do not give the intramolecular distribution of deuterium within the peptides.

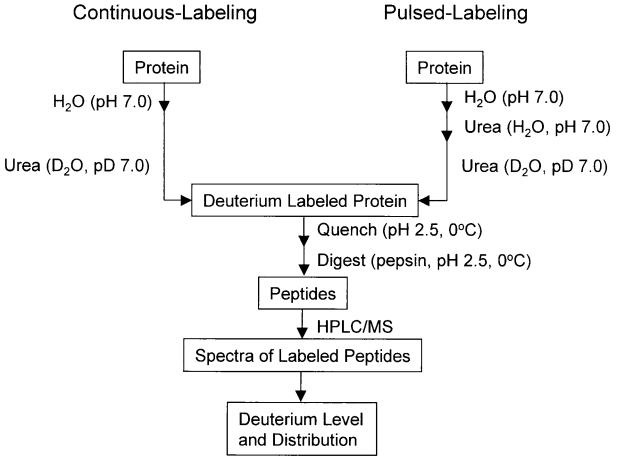

Figure 2. Experimental procedure for continuous and pulsed labeling of a protein destabilized in 3 M urea. Isotope exchange was quenched by decreasing the pH to 2.5 and the temperature to 0 °C. The pH or pD was controlled at each step using phosphate buffers. Pepsin (enzyme:substrate 1:1, digestion time 5 min) was used to fragment rabbit muscle aldolase into peptides which were analyzed by HPLC ESIMS (Micromass Autospec mass spectrometer), as described previously [1].

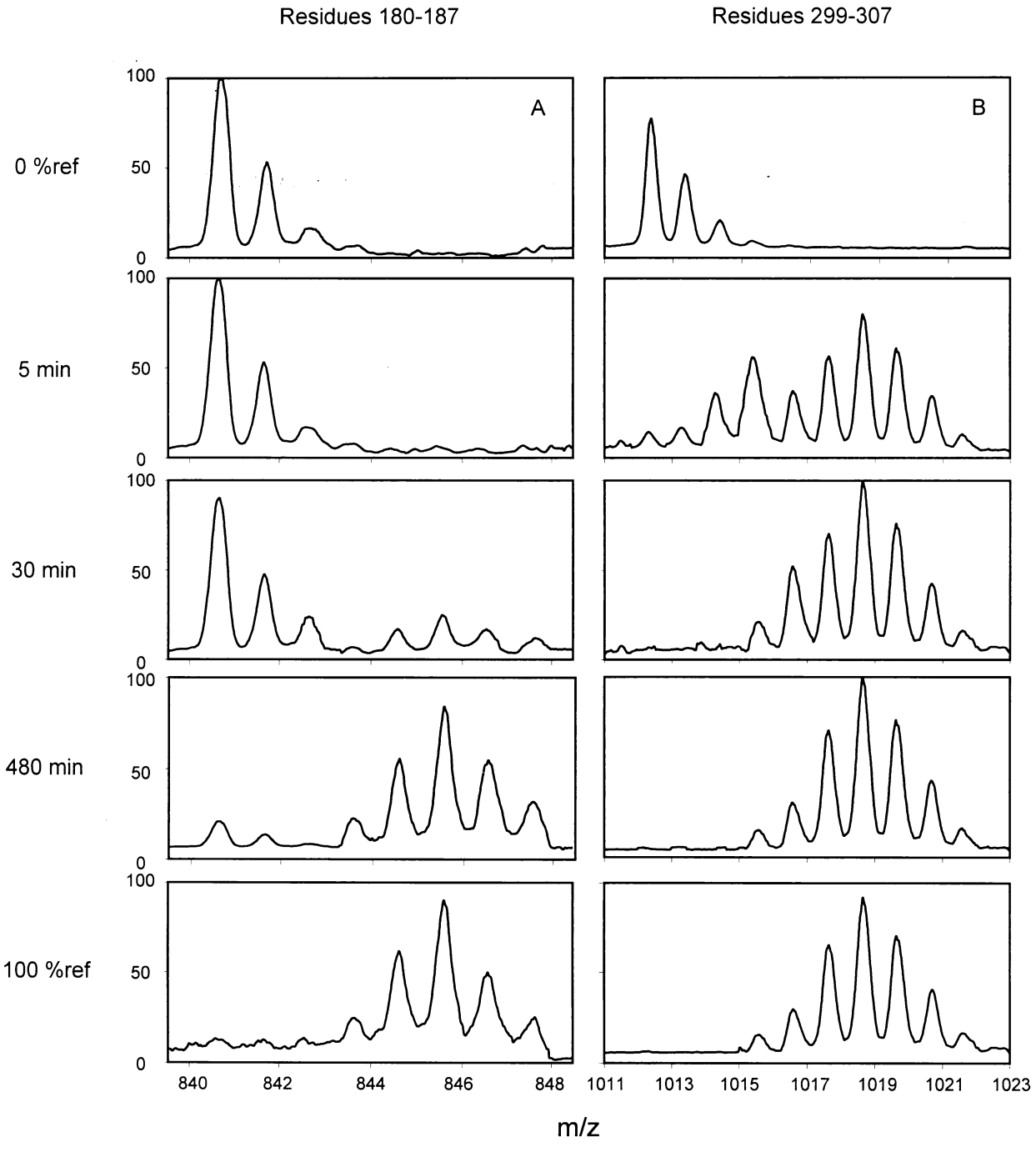

Figure 3. Electrospray ionization mass spectra of peptic fragments including residues 180–187 (**A**) and 299–307 (**B**) of rabbit muscle aldolase labeled continuously in 3 M urea/D_2O for 5, 30, and 480 min. Top and bottom spectra are for the same fragments of aldolase that contained no deuterium (0% ref) or aldolase that was completely exchanged in D_2O (100% ref).

Mass spectra for the peptic fragment including residues 180–187 exhibit one envelope of isotope peaks following a 5 min incubation in D_2O/urea and two envelopes of isotope peaks for incubation times greater than 30 min. The average *m/z* of the low *m/z* envelope was equal to that of the 0% ref and the average *m/z* of the high *m/z* envelope was equal to that of the 100% ref. This correlation shows that the amide linkages in these peptides, when extracted from intact aldolase subjected to continuous labeling, were completely protiated or completely deuterated. It is important to note that urea increases the equilibrium populations of unfolded forms of aldolase by increasing k_1 and decreasing k_{-1}. The bimodal isotope patterns found for this segment are consistent with a decreased refolding rate constant, k_{-1} (see eq 2), such that k_2 was much greater than k_{-1} and the rate of isotopic exchange in the unfolding population was determined by the unfolding rate constant, k_1

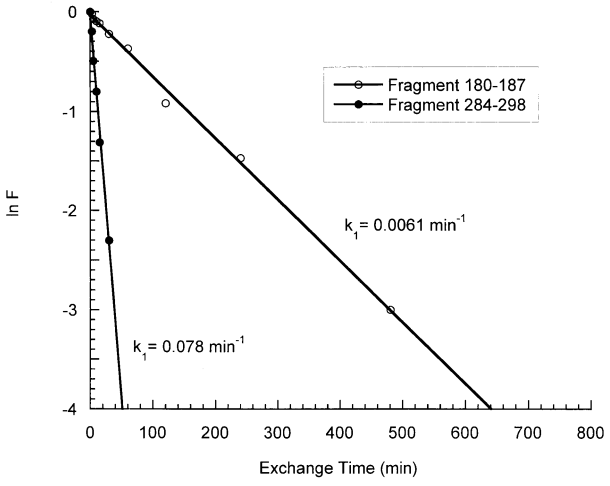

Figure 4. First order kinetic plots of the natural log of the fraction of aldolase molecules that remained folded in two segments including residues 180–187 and 284–298 when aldolase was continuously labeled in D_2O urea. The fraction of aldolase molecules that did not unfold was determined from the intensities of the low mass envelopes of isotope peaks, as illustrated for segment 180–187 in Figure 3A.

(see eq 5). It follows that, in 3 M urea, aldolase exhibits an unfolding process in which molecules unfolding in the region including residues 180–187 become completely deuterated (i.e., their average *m/z* is the same as that of the 100% ref).

The intensity of the low *m/z* envelope of isotope peaks relative to the high *m/z* envelope is a direct measure of the fraction of aldolase molecules that remained folded (F) in this segment (residues 180–187) during incubation in D_2O/urea. From eq 5 and first order kinetics, the rate at which this segment of aldolase unfolds in 3 M urea can be determined. This type of analysis is illustrated in Figure 4 where the natural logarithm of F for the peptic fragment including residues 180–187 is plotted versus the exchange time. The slope of this plot indicates that this segment of aldolase unfolds in 3 M urea with a rate constant of 0.0061 min^{-1}. Results for a peptic fragment taken from another region of the aldolase backbone, residues 284–298, show that this region of aldolase unfolds much faster. Thus, continuous labeling can be used to identify regions of proteins that unfold at different rates.

Continuous-exchange results for another segment of aldolase, residues 299–307, are presented in Figure 3B. Spectra for this segment derived from unlabeled (0% ref) and completely exchanged (100% ref) aldolase are also presented. Mass spectra for this segment of the aldolase backbone suggest a bimodal distribution of isotopes after incubation of the intact protein in D_2O for only 5 min, although the two envelopes are not completely resolved. This isotope distribution is very different from that of the nearby segment including residues 180–187 (Figure 3A) in that the average *m/z* of the low-mass envelope of isotopes is much greater than that of the 0% ref. In addition, the intensity of the high *m/z* envelope increases rapidly with incubation time. It follows that the unfolding dynamics of these two regions of the backbone of aldolase are very different. The bimodal isotope pattern indicates that this region unfolds in 3 M urea, and that the refolding rate, k_{-1}, is slow relative to the isotope exchange rate constant, k_2 (see eq 5). The rapid increase in the intensity of the high *m/z* envelope shows that unfolding in this segment is much faster than unfolding of the nearby segment including residues 180–187 (Figure 3A). The unfolding dynamics of the 299–307 region also differ in processes that lead to isotope exchange in the normally folded form (eqs 3 and 4), which are represented by the low *m/z* envelope of isotope peaks. The high levels of deuterium in this population of molecules after incubation in D_2O for only 5 min are attributed to large values of β or K_{unf} where the latter refers to unfolding processes for which the refolding rate, k_{-1} is much greater than k_2 (see eq 4). An increased deuterium level in the 299–307 segment via isotope exchange from folded aldolase (eqs 3 and 4) is consistent with the X-ray diffraction structure of aldolase, which indicates that half of the amide hydrogens in the 299–307 segment do not participate in intramolecular hydrogen bonding. This fast exchange contrasts with isotope exchange in the 180–187 segment, which is slow because all six of the peptide amide hydrogens in this segment participate in intramolecular hydrogen bonds. This form of hydrogen bonding is the principal factor responsible for slow hydrogen exchange in folded proteins [27]. Since the low and high *m/z* envelopes of isotope peaks in the mass spectra of the 299–307 segment are only poorly resolved, the unfolding rate constant cannot be determined accurately.

The results presented in Figure 3 demonstrate that continuous labeling can be used to determine rate constants for local unfolding of regions of large proteins, such as aldolase. However, this approach is successful only when isotope exchange in the normally folded form of the protein is slow, as was found for segment 180–187. Our analysis of over 50 peptic fragments of aldolase continuously labeled in D_2O/urea shows that only 3–4 fragments have well-resolved bimodal isotope distributions. Thus this approach generally cannot be used to determine unfolding rates along the entire backbones of proteins. It may be important to note that similar measurements could presumably be made under equilibrium conditions if the protein were equilibrated in H_2O/urea prior to transfer to D_2O/urea. This general approach, but without denaturant, has been used to determine the rate constant for the seemingly natural unfolding of a region of the SH3 functional domain of Hck tyrosine kinase [28, 29].

Pulsed Labeling

Important features of pulsed-labeling hydrogen exchange will also be illustrated using urea induced

unfolding of aldolase. The procedure used for this study, also illustrated in Figure 2, differs from that used for continuous labeling in that the protein was incubated in H_2O/urea in place of D_2O/urea for various times. Because H_2O was used in both solutions, a column was not used to transfer the protein to the urea solution. The aldolase concentration during incubation in H_2O/urea was 2.5 mM. During the incubation time, the population of folded forms decreased until the new equilibrium population satisfying the thermodynamic requirements of 3 M urea was established. This equilibration of folded and unfolded populations of the protein occurred in the presence of D_2O in the continuous-labeling experiment, but in the presence of H_2O in the pulsed-labeling experiment. Following this incubation period, the protein was exposed to D_2O/urea ($\times$ 20 dilution) for 10 s prior to quenching of isotope exchange and analysis, as described above for aldolase labeled continuously.

Mass spectra of peptides representing the same two segments of the aldolase backbone used to illustrate continuous labeling are presented in Figure 5. Pulsed-labeling results for the segment including residues 180–187 differ little from the continuous-labeling results presented for the same segment in Figure 3A. The bimodal isotope pattern appears after 30 min incubation in H_2O/urea and remains evident after incubation of the protein in 3 M urea for 480 min. The average *m/z*'s of the low and high *m/z* envelopes are similar to the average *m/z*'s of the 0% ref and 100% ref, respectively. As discussed above, finding this peptide with no deuterium, or completely deuterated, means that this segment of all of the molecules in solution was either folded or unfolded. This bimodal distribution of structure is a direct result of the cooperativity of the folding process generally thought to be typical of most proteins, but difficult to demonstrate experimentally. Quantitative interpretation of the relative intensities of the two envelopes of isotope peaks is different for peptides derived from continuous- and pulsed-label proteins. The intensity of the low *m/z* envelope for peptides derived from proteins labeled continuously indicates the sum of the number of molecules that remained folded during the incubation time. For pulsed labeling, the intensity of this envelope indicates the size of the population of molecules that were folded in this region of the backbone after incubation in urea for a specified time. The deuterium detected after pulsed labeling reflects only molecules that were unfolded during the brief labeling time and does not include molecules that unfolded then refolded during the incubation time prior to exposure to D_2O.

Mass spectra of segment 299–307 derived from pulsed-label aldolase are very different from those obtained by continuous labeling (Figure 3B). Pulsed labeling gives bimodal isotope distributions that are completely resolved. The low and high *m/z* envelopes are completely separated because little isotope exchange occurs in this segment of folded aldolase during its brief exposure of aldolase to D_2O. Mass spectra of over 30 peptic fragments of aldolase pulsed label with deuterium exhibited bimodal isotope patterns where the low and high *m/z* envelopes were well resolved.

This excellent separation of isotope envelopes facilitates determination of the folding status of short segments of the entire backbones of large proteins, such as aldolase. The folding status of the two segments of aldolase including residues 180–187 and 299–307 is presented in Figure 6 as the population of molecules remaining folded following incubation in 3 M urea for various times. For the shortest incubation times, the population of unfolded states was too small to be detected. After several minutes, the population of aldolase molecules remaining folded in these regions decreased (i.e., the intensity of the low *m/z* envelope decreased) until equilibrium populations were achieved. Results presented in Figure 6 demonstrate that different regions of aldolase unfold at different rates, as discussed by Deng and Smith [1].

Optimization of Pulsed-labeling Time

Although the aldolase was exposed to D_2O in the present experiments for 10 s, exposure times as short as 10 ms have been used to follow rapid folding of proteins [13–15]. Experiments requiring exposure times much greater than 10 s can also be envisioned. The optimum duration for exposure of the protein to D_2O in pulsed-labeling experiments depends primarily on the kinetics of the process of interest, pH, and temperature. Though generally less important, the rate of isotope exchange in the folded protein and its amino acid sequence should be considered when selecting an exposure time.

The minimum exposure time is dictated by the minimum time required to complete isotope exchange at the peptide amide linkages in the unfolded protein. This time, which depends on pH, temperature, and the amino acid sequence, can be estimated using parameters derived from studies of model peptides [25]. This approach was used to calculate the deuterium levels expected in the two peptic fragments comprised of residues 180–187 and 299–307 when exposed to D_2O at pD 7 and 25 °C. Results of this calculation, which are presented as dashed lines in Figure 7, indicate that significant deuteration occurs after only a few milliseconds, while complete deuteration requires 0.5–5 s. The different times required to completely deuterate these peptic fragments under identical conditions are due to steric and inductive effects of the amino acid side chains comprising the peptides [25]. These results suggest that unfolded regions of a protein can be completely deuterated at pD 7 and 25 °C in several seconds. Although pulsed labeling under these conditions can be complete within a few seconds, 10 s was used for the present experiments because it was compatible with manual mixing methods. For exposures of less than approximately 10 s, automated mixing is required [13, 14].

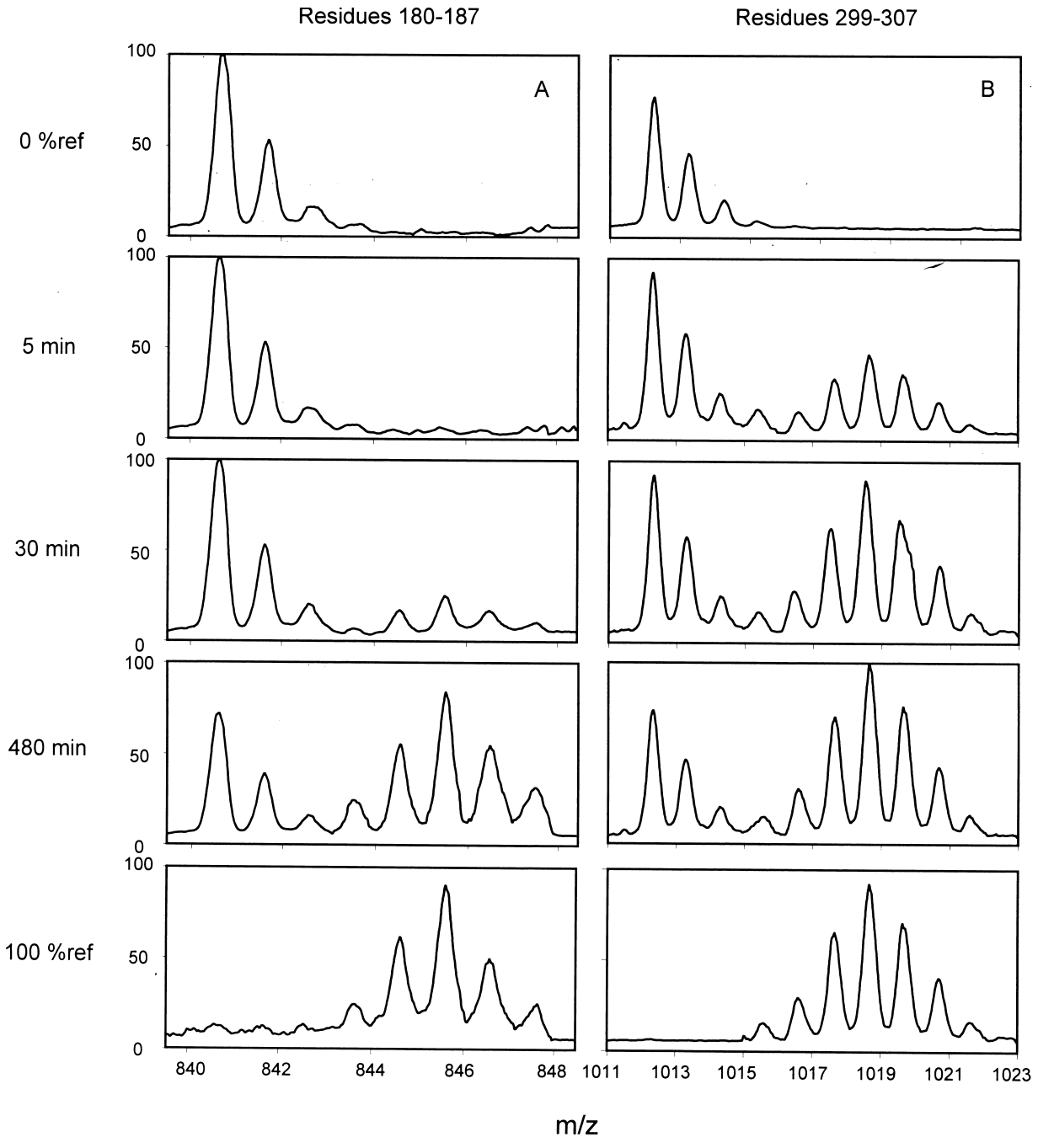

Figure 5. Electrospray ionization mass spectra of peptic fragments including residues 180–187 (**A**) and 299–307 (**B**) of rabbit muscle aldolase pulsed labeled in 3 M urea/D_2O for 10 s following incubation in 3 M urea/H_2O for 5, 30, and 480 min. Top and bottom spectra are for the same fragments of aldolase that contained no deuterium (0% ref) or aldolase that was completely exchanged in D_2O (100% ref).

To study unfolding dynamics, the isotope exchange rate in pulsed-labeling experiments must be faster than the folding/unfolding process of interest, as was the case for the unfolding processes described above. To study even faster processes, conditions that lead to faster isotope exchange must be used. Faster isotope exchange may be achieved by using higher pH. When the pH is greater than approximately 4, isotope exchange in unfolded polypeptides is proportional to $[OH^-]$, as illustrated in eq 6.

$$k_{exch} = k_2 = k_{OH}[OH^-] \quad (6)$$

The rate constant for base catalyzed exchange at a particular peptide amide linkage, k_{OH}, depends on the

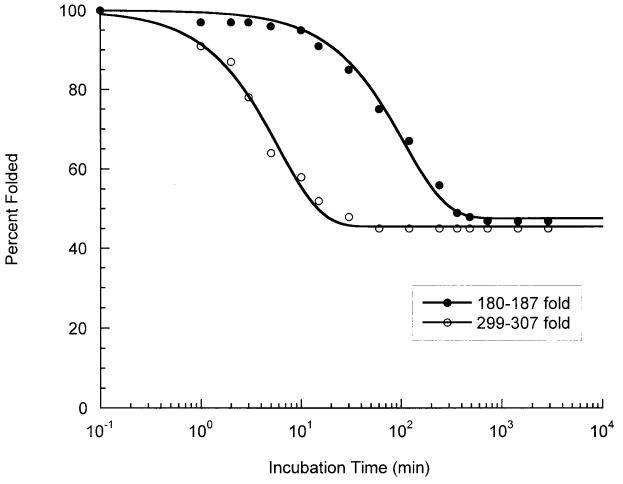

Figure 6. Fraction of aldolase molecules that remained folded in the segment including residues 180–187 and 299–307 at various times following incubation of aldolase in 3 M urea (H_2O). The experimental conditions were as described for Figure 5. The fraction of molecules that were folded was determined from the intensities of the low mass envelope of isotope peaks (Figure 5).

adjacent amino acid side chains and can be estimated empirically [25]. It follows from eq 6 that the rate of isotope exchange increases 10-fold per pH unit. To study protein folding dynamics on the millisecond time scale requires very short exposure times, which are effected only at high pH and with use of automated mixing [13, 14]. It is important to note that isotope exchange in normally folded proteins is also proportional to k_2 (see eqs 3 and 4) and hence $[OH^-]$. As a result, increasing pH increases isotope exchange rates for both folded and unfolded forms of the protein. Interpretation of results obtained by labeling at high pH may be complicated by structural changes induced by the high pH. However, structural changes sensitive to pH, which are described by changes in β and K_{unf}, can be studied by hydrogen exchange experiments designed for this purpose [24]. Temperature is another variable that can be used to alter both protein dynamics and isotope exchange rates. Based on the effective activation energy of 17 kcal/mol for isotope exchange in unfolded polypeptides, it can be shown that k_2 increases threefold for each 10 °C increase in temperature [25].

The D_2O exposure time must be short relative to the time scale of the dynamics of interest and the time required for isotope exchange in the folded form of the protein. Failure to meet the latter requirement may lead to poorly resolved envelopes of isotope peaks. Isotope exchange into our example segments (residues 180–187 and 299–307) of folded aldolase are presented as solid lines in Figure 7. As indicated in eqs 3 and 4, the rate of isotope exchange in the folded forms of proteins depends on β and K_{unf}. Because these parameters normally change dramatically and abruptly along the polypeptide backbone, they cannot be predicted accurately. The results presented in Figure 7 were determined from continuous-labeling experiments performed without urea [18]. Although urea has relatively little effect on the rate of hydrogen exchange in unfolded polypeptides (i.e., k_2) [30], exchange in 3 M urea may be somewhat faster because urea increases K_{unf}. Although deuterium levels for exposure times less than 150 s are only estimates, they illustrate the importance of optimizing the labeling time. The exposure time that leads to the greatest separation of the low and high m/z isotope envelopes is the time for which the differences between the dashed and solid lines in Figure 7 are greatest. While 3 s is required to completely deuterate the unfolded form of segment 180–187, only 0.5 s is required to deuterate the unfolded form of segment 299–307. Exposure times greater than 10 s will lead to significant deuteration of the folded form of segment 299–307, whereas exposure as long as 100 s does not give detectable deuterium in the 180–187 segment. As discussed above, isotope exchange into the folded form of segment 180–187 is slow because all of the amide hydrogens in this segment participate in intramolecular

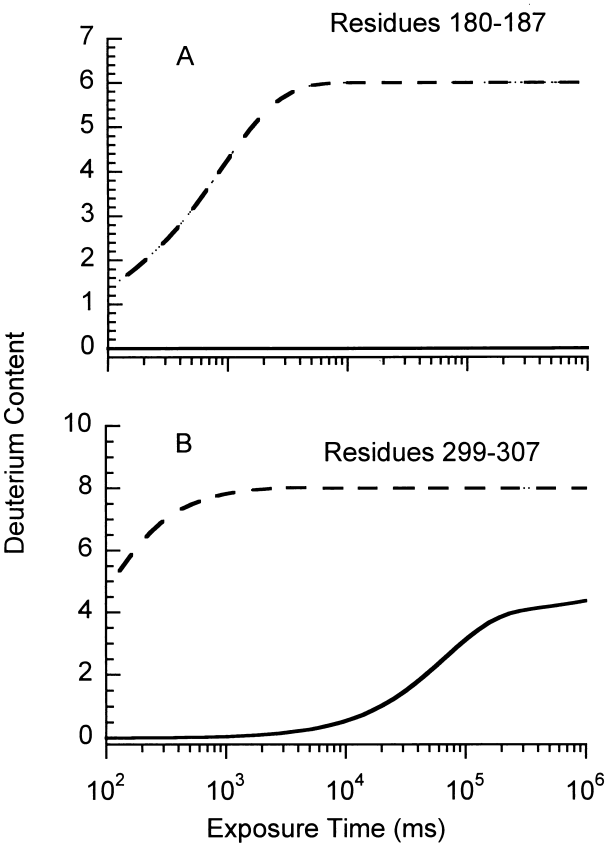

Figure 7. Plot of deuterium levels expected in backbone segments 180–187 (**A**) and 299–307 (**B**) of aldolase if these segments were completely unfolded (dashed line) or in a native state (solid line) at pH 7 and 25 °C. The deuterium levels expected for unfolded segments were calculated from parameters derived from studies of model peptides [25]. The deuterium levels expected for native aldolase are based on experimental results published by Zhang et al. [18].

hydrogen bonding. Nonoptimal choices of exposure time can usually be detected by comparing the average *m/z* of the low and high *m/z* envelopes of isotope peaks of peptides derived from pulsed-labeled protein with the average *m/z*'s of the appropriate 0% ref and 100% ref. If the average *m/z* of the low *m/z* envelope is greater than that of the 0% ref, the exchange time is probably too long; if the average *m/z* of the high *m/z* envelope is less than that of the 100% ref, the exposure time is too short. Of course, features of the protein dynamics may also complicate this interpretation of the average *m/z*'s of the isotope envelopes found for peptic fragments of proteins pulsed labeled with D_2O. For example, one peptic fragment may span two regions of the backbone that are folding/unfolding at different rates or out of phase. In addition, the isotope exchange in unstructured segments on the surface of proteins may closely resemble exchange in the unfolded form of this segment. The actual exposure time used in an experiment will be the same for all peptic fragments of the polypeptide backbone and will, therefore, be optimum only for some fragments.

Conclusions

Pulsed-labeling amide HX/MS has provided highly detailed new information about the kinetics of protein folding [13–15] and unfolding [1, 16]. This approach has led to determination of rate constants for unfolding and folding (k_1 and k_{-1} in Figure 1), to determination of the equilibrium constant for protein unfolding (K_{unf}) and to insight on the sequence of unfolding events in aldolase [1]. Results of the present study demonstrate that continuous labeling leads to direct determination of unfolding rates in segments where isotope exchange in the folded form of a protein is slow. This approach is attractive because it integrates the unfolded population over time, thereby facilitating detection of unfolding processes under conditions that strongly favor the folded protein. That is, continuous labeling can be used to detect unfolding processes where the population of unfolded species is less than 1%. This approach was used to detect slow unfolding in the SH3 domain of hematopoietic cell kinase [28, 29]. In the present study of aldolase, urea was used to increase artificially the population of unfolded states, thereby facilitating their detection by pulsed labeling. Continuous labeling is also attractive because the experimental procedure and data analysis are relatively easy.

Acknowledgments

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