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# Amide hydrogen exchange and mass spectrometry: A probe of high-order structure in proteins

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***Abstract***

A new analytical method which facilitates peptide amide hydrogen as a tool for detecting conformational changes and probing high-order structure in large proteins is described. Following a period of deuterium exchange-in, the protein is placed in slow exchange conditions and fragmented into peptides with pepsin. The peptides are analyzed by directly-coupled HPLC fast atom bombardment mass spectrometry to determine their deuterium content. Results presented here demonstrate that this method can be used to determine rate constants for peptide amide hydrogen exchange, and to detect the thermal denaturing of cytochrome c.

## INTRODUCTION

Although many approaches have been proposed for predicting the secondary structures of proteins from their amino acid sequences, none have achieved the level of success required for linking protein structure and function. Hence, X-ray and NMR spectroscopies are used extensively to determine the high-order structure of proteins directly. Despite the tremendous success of these methods, our understanding of the structure/function relationships of many proteins remains incomplete, either because no single method gives a complete picture or because of physical limitations, such as the failure of a protein to crystalize. The rates at which amide hydrogens undergo isotopic exchange is another method that has been used to probe the high-order structure of proteins (refs. 1-5). The protein backbone is a series of amide linkages, each with a single amide hydrogen. Linkages in which proline is the C-terminal residue have no amide hydrogens and are the only exception. These peptide amide hydrogens form a complex network of hydrogen bonds that extends throughout a protein, thereby playing an important role in stabilizing the high-order structure. As proteins change conformation, the network of hydrogen bonds is altered, causing changes in the rates at which amide hydrogens undergo isotopic exchange. It is these changes in isotopic exchange rates that facilitate hydrogen exchange as a probe of conformational change.

Peptide hydrogen exchange in proteins has been detected and quantified by several different techniques. One approach is to simply incubate the protein in tritiated water. Rapidly exchanging hydrogens are replaced with protium/tritium from the solution within seconds, while more slowly exchanging peptide amide hydrogens may require minutes to years to undergo exchange. Following the isotopic exchange-in period, the sample is briefly subjected to exchange-out in water, leaving only the peptide amide positions tritiated. The activity in the protein is a direct measure of the extent of hydrogen exchange that took place during exchange-in. Kaminsky and Richards recently used this approach to distinguish between the oxidized and reduced forms of thioredoxin (ref. 6). UV and IR spectroscopies have also been used to quantify hydrogen/deuterium exchange in proteins. Loftus et al. used UV spectroscopy to study marginally stable forms of the S-protein fragment of ribonuclease A (ref. 7), and Muga et al. used FTIR to detect structural changes in cytochrome c that accompany its binding to a membrane (ref. 8). Although results obtained with these relatively simple approaches clearly demonstrate that peptide amide hydrogen exchange rates are a sensitive probe of the high-order structure of proteins, they cannot be used to determine which regions within a protein have undergone a conformational change.

The exchange rates of individual peptide amide hydrogens in small proteins have been determined by one- and two-dimensional NMR spectroscopy. For example, Brandt and Woodward showed that the exchange rates of certain amide hydrogens in bovine pancreatic trypsin inhibitor are reduced

when the protein is complexed with trypsin (refs. 9,10). Paterson et al. observed similar behavior for amide hydrogens in three segments that comprise an antibody binding site on cytochrome c (ref. 11). Robertson and Baldwin used hydrogen exchange to search for evidence for nonrandom structure in thermally denatured ribonuclease A (ref. 5), and Mau et al. used hydrogen exchange to investigate effects of DNA binding and metal substitution on the dynamics of the DNA-binding domain of GAL4 (ref. 12). Since high resolution NMR analysis of large proteins is generally impossible at this time, this approach will likely be restricted to highly detailed studies of small proteins and functional domains of larger proteins.

We wish to report on a new method, based on fast atom bombardment mass spectrometry (FABMS) and protein fragmentation, to facilitate hydrogen exchange as a probe of the high-order structure of proteins. This approach is an extension of a method described by Rosa and Richards (ref. 13) and Englander et al. (ref. 14), who determined hydrogen exchange rates by exchanging tritium into proteins, fragmenting the proteins into peptides, separating the peptides by HPLC, and quantifying the tritium in the peptides. We have used deuterium in place of tritium, and quantified its incorporation by directly-coupled continuous-flow HPLC fast atom bombardment mass spectrometry. Results presented here for hydrogen exchange in cytochrome c demonstrate that these modifications to the protein fragmentation method are a significant advancement in methodology, facilitating widespread use of hydrogen exchange as a probe of high-order structure of proteins.

## EXPERIMENTAL

The basis of the protein fragmentation method used in the present investigation is illustrated in Fig.1. Deuterium is exchanged into the protein at high pH where the intrinsic rate of hydrogen exchange is fast. The actual rate of exchange depends on the high-order structure of the protein, and may be fast or slow. After the desired exchange-in time, the exchange reaction is quenched by lowering the pH to 2.5 and decreasing the temperature to $0^{\circ}C$. The half-life for isotopic exchange of peptide amide hydrogens under these conditions is approximately 50 min (ref. 15). Brief digestion with an acid protease, such as pepsin, fragments the protein into peptides which are analyzed by directly-coupled HPLC FABMS to determine their deuterium contents.

During the exchange-in period, peptide amide hydrogens as well as hydrogens on many other functionalities (e.g., -OH, $-NH_2$) undergo isotopic exchange. Since the hydrogens on these other functionalities are much more acidic than peptide amide hydrogens, they undergo exchange much more rapidly. This disparity in exchange rates makes it possible to remove all but the peptide amide deuterons during HPLC analysis. As a result, the increase in molecular weight of deuterated peptides is a direct measure of the quantity of deuterium located at peptide amide linkages. The molecular weights of the peptides were determined by directly-coupled microbore HPLC continuous-flow FABMS. The time required for the analysis is only a small fraction of the half-life for back-exchange. Details of the analytical procedures are described elsewhere (ref. 16).

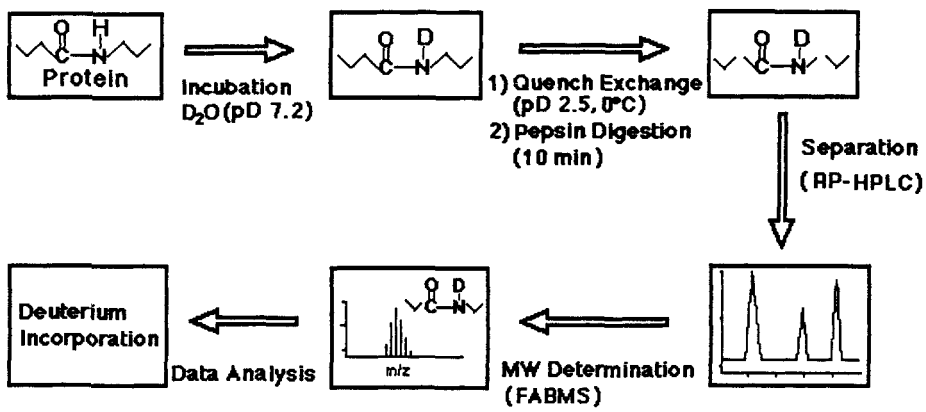


Fig. 1 Experimental procedure used to exchange deuterium into a protein, to fragment the protein into peptides, and to determine the deuterium content of the peptides by directly-coupled microbore HPLC fast atom bombardment mass spectrometry (FABMS).

## RESULTS AND DISCUSSION

The molecular weights of peptides are determined from the molecular ions ($MH^+$) in their FAB mass spectra. As peptides elute from the HPLC column, they are analyzed by the mass spectrometer. Mass spectra of the molecular ion region of the 83-96 segment of cytochrome c are given in Fig. 2 a and b for cytochrome c with no deuterium, and cytochrome c that was partially deuterated after incubation in $D_2O$ for 1 hr. Both spectra have the natural distribution of heavy isotopes ($^{13}C$, $^{18}O$ etc.), while the spectrum in Fig. 2b also has contributions from the exchange-in of deuterium during incubation in $D_2O$. The difference in the centroids of the two envelopes of molecular ions in Fig. 2a and b is a direct measure of the average number of deuteriums in the peptide. Since some deuterium has been lost from the peptide amide linkages during digestion and analysis, a minor adjustment is made using results for the same peptide derived from fully deuterated cytochrome c (ref. 16).

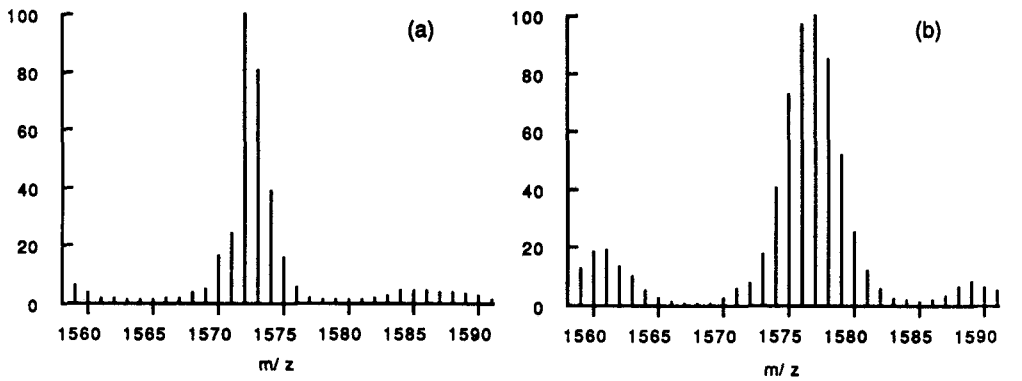


Fig. 2 The molecular ion regions of FAB mass spectra obtained for the 83-96 segment of cytochrome c that (a) contained no deuterium and (b) was partially deuterated by incubating in $D_2O$ for 1 hr.

Amide hydrogens in peptides undergo complete exchange in less than 1 sec at pD 7.2. Similarly, amide hydrogens in proteins that are neither hydrogen bonded nor distant from the solvent exchange rapidly. However, amide hydrogens that are participating in intramolecular hydrogen bonding, as well as hydrogens that are buried in the hydrophobic interior of a protein may require weeks or months to exchange. This wide variation in hydrogen exchange rates is reflected in the deuterium exchange-in time course results presented in Fig. 3, which is a plot of the percentage of peptide amide linkages that have been deuterated in two different segments 67-82 and 83-96 as a function of the time the protein was incubated in $D_2O$. These results indicate that 9 of the 12 peptide amide hydrogens in the 67-82 segment are replaced with deuterium after approximately 1 hr. Incubation in $D_2O$ for approximately 48 hr is required to replace 2 more hydrogens. Although the 83-96 segment also has both slow and fast exchanging amide hydrogens, only 4 hydrogens are replaced with deuterium after incubation in $D_2O$ for 1 hr.

Although the exchange rates of specific amide hydrogens cannot be deduced from these data, a more quantitative analysis is possible. The deuterium content of a peptide can be expressed as the sum of N terms, where each term represents the deuterium level at each of the N amide linkages. For small peptides ($N<3$) the exchange-in data can be fitted to an expression from which rate constants for exchange at each of the N linkages occurs. When applied to large peptides, this approach requires more variables and may lead to erroneous results. As a compromise, we have divided the amide hydrogens into three groups according to their rates of isotopic exchange (ref. 16). Although the amide hydrogens in a particular group must have similar exchange rate constants, they may be located anywhere along the backbone of a peptide. The lines drawn through the experimental data points in Fig.3 represent the deuterium levels calculated for this three-component model using Eq. 1.

$$D = N_1[1-\exp(-k_1t)] + N_2[1-\exp(-k_2t)] + N_3k_3t \qquad \text{Eq. 1}$$

According to this model, the total number of amide linkages in a peptide (N) is divided into three

groups, $N_1$, $N_2$, and $N_3$, with exchange rate constants $k_1$, $k_2$, and $k_3$, respectively. Since the theoretical curves obtained using the three-component model fit the experimental data well, data analysis using more complex models was not attempted. Application of Eq. 1 to exchange-in data for segment 67-82 shows that 7.8 of the amide hydrogens in this segment undergo isotopic exchange with an average rate constant of 141 $h^{-1}$; 1.5 amide hydrogens exchange with an average rate constant of 1.5 $h^{-1}$; and 5.7 hydrogens exchange with an average rate constant of 0.006 $h^{-1}$.

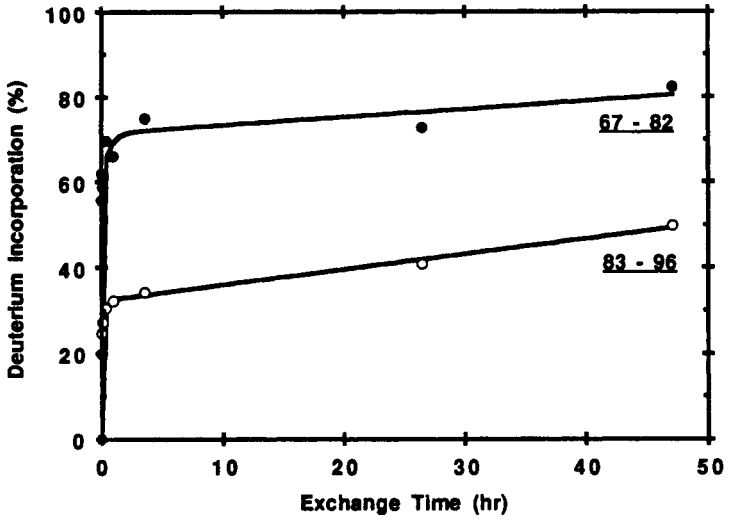


Fig. 3. Deuterium incorporation into peptide amide linkages in the 67-82 (•) and 83-96 (o) segments of cytochrome c incubated in $D_2O$.

The reduction of peptide amide hydrogen exchange rates by protein high-order structure is demonstrated by these results. If there were no high-order structure, the half-life for amide hydrogen exchange in these segments of cytochrome c would be 0.3 s at pH 7 (ref. 17). Although the half-lives of individual amide hydrogens in the most rapidly exchanging groups of these segments may differ substantially from their average, their average half-life in the protein is $10^2$ greater than in the random structure peptide. Furthermore, the average half-life of the slowest exchanging amide hydrogens in this segment of the protein is $10^6$ longer than in the free peptide. It is this reduction in hydrogen exchange rates in proteins that facilitates hydrogen exchange as a probe of high-order structure in proteins.

The present results demonstrate that rate constants for amide hydrogen exchange in proteins may be reduced by a factor of $10^6$ or more from their exchange rates in unstructured peptides. Reduced hydrogen exchange rates in proteins has generally been attributed to a combination of solvent shielding and intramolecular hydrogen bonding (refs. 18-20). It follows that the present protein fragmentation method may be useful for identifying those regions of a protein that have little contact with the solvent or where there is strong secondary structure. The number of amide hydrogens that exchange slowly (defined by $k < 5\ h^{-1}$) in each of 9 segments sampled in this study was compared with the number of amide hydrogens located in $\alpha$-helices (refs. 16,21). For 8 of the 9 segments studied, there is an excellent correlation between the number of peptide amide hydrogens with exchange rate constants less than 5 $h^{-1}$ and the number of peptide amide hydrogens located in $\alpha$-helices in cytochrome c. It is interesting to note that the number of slowly exchanging peptide amide hydrogens does not correlate well with the number of peptide amide hydrogens involved with intramolecular hydrogen bonding. The segment comprising residues 22-36 is anomalous in that 9.1 (75%) of the amide hydrogens exchange slowly, despite the fact that none are located in $\alpha$-helices (ref. 21). These results suggest that the 22-36 segment of cytochrome c is highly shielded from the solvent, consistent with the crystallographic structure of cytochrome c.

Detection and location of conformational changes in proteins may be an important application of hydrogen exchange as a probe of protein structure. To demonstrate that the present method can be

used for this purpose, hydrogen exchange into cytochrome c was determined for a range (30°C to 80°C) of incubation temperatures. The isotope patterns of the molecular ions of the peptides suggested that exchange was uncorrelated (ref. 16). The rate constant for hydrogen exchange could, therefore be equated with the product of the unfolding equilibrium constant and the intrinsic rate of hydrogen exchange (Eq. 2),

$$k_{ex} = Kk_{int} \qquad \text{Eq. 2}$$

where $k_{ex}$ is the experimentally determined rate constant for hydrogen exchange, K is the equilibrium constant describing the unfolding of a short segment of the protein prior to isotopic exchange (ref. 19), and kint is the rate constant for hydrogen exchange in a peptide with random structure (ref. 17). Since the temperature dependence of the intrinsic rate of hydrogen exchange can be calculated from its activation energy (ref. 22), the temperature dependence of the equilibrium constant, including associated thermodynamic information, can be derived from the temperature dependence of the hydrogen exchange rate constant (refs. 1,19).

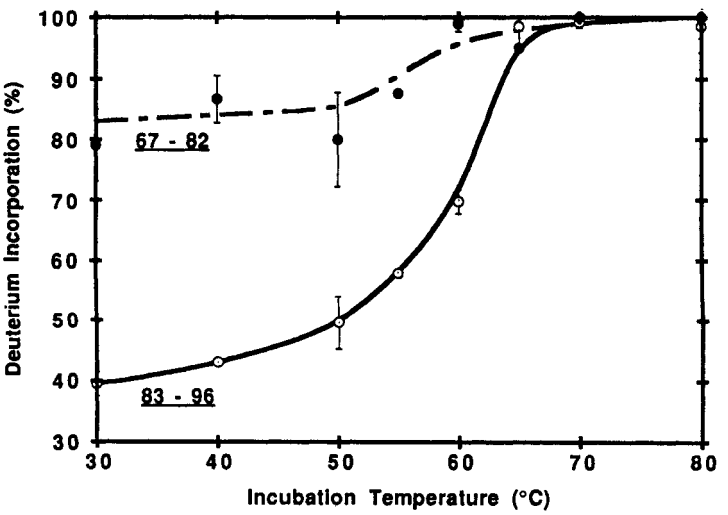


Fig. 4 Deuterium incorporation into the 67-82 and 83-96 segments of cytochrome c as a function of the incubation temperature. Adjustments for the effect of increased temperature on the intrinsic rate of hydrogen exchange were made by varying the incubation time.

The extent of deuterium incorporation into 8 peptides was determined for exchange-in temperatures from 30 to 80°C. To separate the effects of increased temperature on the equilibrium constant for unfolding and the rate constant for intrinsic hydrogen exchange, the incubation time was changed from 118 min to 2.0 min over the same temperature range. Justification for this adjustment in incubation time with temperature can be visualized with Eq. 3

$$d = 1 - \exp(-k_{ext}) = 1 - \exp(-Kk_{int}t) \qquad \text{Eq. 3}$$

where d is the number of deuteriums in a peptide, and $k_{ex}$, K, and $k_{int}$ are defined in Eq 2. Assuming an activation energy of 17.4 Kcal/mol for intrinsic hydrogen exchange (ref. 22,23), the temperature dependence of $k_{int}$ can be calculated. The incubation time, t, was adjusted to compensate for changes in $k_{int}$.

Plots of deuterium incorporation, after adjustment for the temperature dependence of the intrinsic rate of exchange, into specific segments of cytochrome c versus the incubation temperature (Fig. 4) are a direct indication of the temperature dependence of unfolding in specific regions of the protein. Deuterium incorporation into the 67-82 segment was approximately 83-86% as the temperature was increased from 30 to 50°C. However, the deuterium content of this peptide increased to 100% as the temperature was increased from 50 to 60°C. These results indicate that in the temperature range 50 to 60°C, amide hydrogen bonds in this region are substantially weakened, causing an increase in the equilibrium constant for unfolding of the 67-82 segment. A similar pattern was found for

deuterium incorporation into the 83-96 segment of cytochrome c, suggesting a conformational change in cytochrome c when heated to approximately 60°C. The present results are also consistent with the results of Muga et al. ( ref. 8) who reported a small (2-3 $cm^{-1}$) downward shift of the amide I band maximum with an apparent midpoint temperature of 60-62°C, suggesting increased accessibility of the polypeptide backbone to hydrogen exchange.

## CONCLUSIONS

Results described here demonstrate that deuterium incorporation into proteins can be determined by combining protein fragmentation and directly-coupled HPLC FABMS. When compared with other methods used for quantifying peptide amide hydrogen exchange in the entire protein, this approach is attractive because it can be used to determine which regions within the protein are undergoing a conformational change. This kind of information is essential, as we attempt to link protein structure and function. Although rate constants for hydrogen exchange of small groups of linkages located within a peptic peptide can be determined with high accuracy, it generally is not possible to determine exchange rate constants for individual amide hydrogens by this technique. Such high spatial resolution measurements can only be made by high resolution NMR spectroscopy. The method reported here will likely be most useful for investigations of large proteins which generally are not amenable to analysis by high resolution NMR spectroscopy. The success of continuous-flow HPLC FABMS is due to the fact that the deuterium content of peptides is determined on a time scale that is short relative to the time in which deuterium undergoes back-exchange.

The experimental methods described here may be useful for studying many different types of problems that are frequently encountered in protein structure/function research. For example, the rates of hydrogen exchange in regions participating in ligand binding may be reduced if the ligand decreases the access of the hydrogens to the solvent. Hydrogen exchange may also be useful for determining which segments of the primary structure of a protein form $\alpha$-helices or $\beta$-sheets, since the rate of hydrogen exchange within these units of secondary structure is much slower than in segments of random structure. These methods may also be useful for investigations of the quaternary structures of proteins where different subunits of a protein may have different accessibility to the solvent.

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