Determination of amide hydrogen exchange by mass spectrometry: A new tool for protein structure elucidation

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Abstract

A new method based on protein fragmentation and directly coupled microbore high-performance liquid chromatography–fast atom bombardment mass spectrometry (HPLC-FABMS) is described for determining the rates at which peptide amide hydrogens in proteins undergo isotopic exchange. Horse heart cytochrome c was incubated in D_2O as a function of time and temperature to effect isotopic exchange, transferred into slow exchange conditions (pH 2–3, 0 °C), and fragmented with pepsin. The number of peptide amide deuterons present in the proteolytic peptides was deduced from their molecular weights, which were determined following analysis of the digest by HPLC-FABMS. The present results demonstrate that the exchange rates of amide hydrogens in cytochrome c range from very rapid ($k > 140$ h^{-1}) to very slow ($k < 0.002$ h^{-1}). The deuterium content of specific segments of the protein was determined as a function of incubation temperature and used to indicate participation of these segments in conformational changes associated with heating of cytochrome c. For the present HPLC-FABMS system, approximately 5 nmol of protein were used for each determination. Results of this investigation indicate that the combination of protein fragmentation and HPLC-FABMS is relatively free of constraints associated with other analytical methods used for this purpose and may be a general method for determining hydrogen exchange rates in specific segments of proteins.

Keywords: cytochrome c; hydrogen exchange; mass spectrometry; proteins

The rates at which peptide amide protons in proteins undergo isotopic exchange depend on whether they are participating in intramolecular hydrogen bonding and on the extent to which they are shielded from the solvent. Because intramolecular hydrogen bonding and solvent shielding are directly related to protein structure, it follows that amide hydrogen exchange rates may be used as a sensitive probe of protein structure. For example, hydrogen exchange has been used to investigate structural features accompanying protein folding and unfolding (Roder et al., 1985b, 1988; Loftus et al., 1986; Jeng et al., 1990), functionally different forms of proteins (Wand et al., 1986; Muga et al., 1991; Englander et al., 1992), and protein–protein complexation (Brandt & Woodward, 1987; Paterson et al., 1990). Determining specific conditions (pH, temperature, etc.) under which proteins un-

dergo structural changes has been the principal goal of many investigations using hydrogen exchange as a probe. In such investigations, experimental methods used to determine the extent of hydrogen exchange, such as tritium exchange-in or infrared (Hvidt & Nielsen, 1966; Osborne & Nabedryk-Viala, 1982) and ultraviolet (Englander et al., 1979) spectroscopy, proved satisfactory. However, to understand the relation between protein structure and function, it is important to know which regions within a protein are participating in conformational changes. Some highly detailed information of this type has been obtained for a few small proteins by using high field one- and two-dimensional NMR to measure isotopic exchange rates of individual amide hydrogens (Wagner & Wüthrich, 1982; Delepierre et al., 1987; Tüchsen & Woodward, 1987) and by neutron diffraction (Kossiakoff, 1982; Bentley et al., 1983).

Despite the success of methods currently used for determining amide hydrogen exchange rates, none satisfy the minimum requirements of a general method for ob-

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taining spatially resolved hydrogen exchange information in proteins of modest size. Sensitivity, protein solubility, lengthy analysis time, molecular weight, and spectroscopic assignment of individual amide hydrogens are common hurdles for NMR spectroscopy. Rosa and Richards (1979) as well as Englander et al. (1985) described a method that has intermediate spatial resolution and potential applicability to large proteins. In this method, the amount of tritium present in a protein is determined by fragmenting the protein with an acid protease, isolating the peptides, and determining their specific activity. We wish to report on an extension of their method in which deuterium is substituted for tritium, and the deuterium content of peptides is determined by directly coupled microbore high-performance liquid chromatography–fast atom bombardment mass spectrometry (HPLC-FABMS). Horse heart cytochrome c has been used to demonstrate this new methodology.

Results and discussion

Experimental design

The general procedure used in this study to determine amide hydrogen exchange rates, illustrated in Figure 1, is based on a protein fragmentation method described previously (Rosa & Richards, 1979; Englander et al., 1985). However, in the present procedure, deuterium is used in place of tritium and is detected by directly coupled HPLC-FABMS. The microbore HPLC system, including its interface to the mass spectrometer via a continuous-flow probe, is illustrated in Figure 2. Results presented here were obtained by incubating cytochrome c in D_2O (pD 6.8) for different times and at different temperatures. Ten-nanomole aliquots of the incubate were removed, adjusted to pH 2.4 and 0 °C to quench hydrogen exchange, and briefly digested with pepsin. The proteolytic digests were analyzed by HPLC-FABMS to determine the deuterium content of the peptides.

In the present experiments, the digestion was performed in a mixture that was 44% D_2O because protiated acid was used to quench the exchange-in reaction. Amide hydrogens in peptides derived from the test protein underwent isotopic exchange with the solvent during digestion and fractionation by HPLC. The extent of back-exchange was minimized by performing the digestion and HPLC fractionation at 0 °C and pH 2.5 (Englander & Poulsen, 1969). Similar procedures have been used to determine that the half-life for back-exchange of peptide amide hydrogens from peptides in these slow-exchange conditions is 40–50 min (Thévenon-Emeric et al., 1992). Deuterons located on side chains and on the N- and C-termini of the peptides are completely back-exchanged to protons during HPLC fractionation because the exchange rates of these hydrogens are several orders of magnitude faster than the exchange rates of peptide amide hydrogens. As
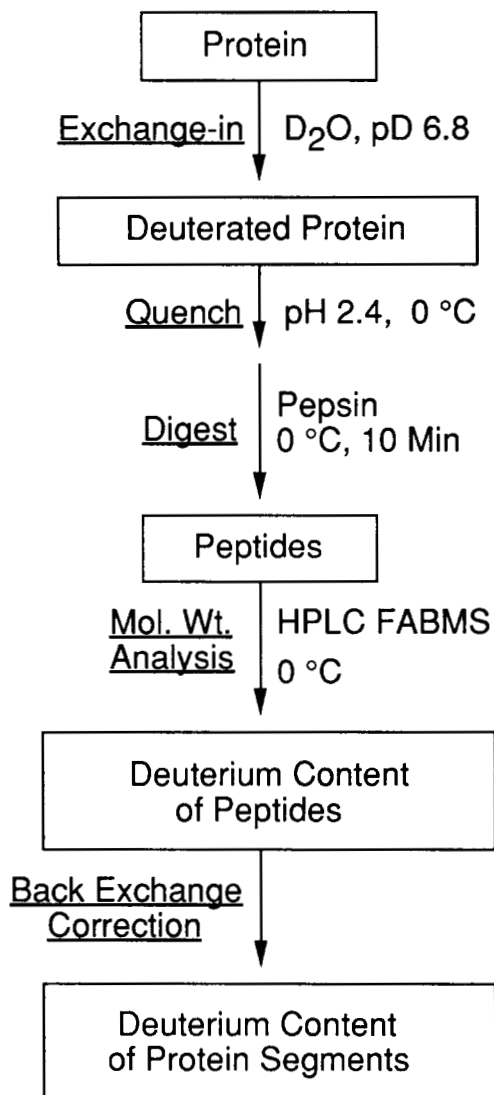

Fig. 1. Diagram of the general procedure used to exchange deuterium into proteins, to fragment the proteins into peptides, and to determine the deuterium content of the peptides.

a result, the increase in the molecular weights of peptides analyzed by this procedure is a direct measure of the number of peptide amide deuterons present in the peptide. Following a small correction for loss of deuterium during digestion and analysis, the deuterium content of the peptide is a direct measure of the deuterium content of the corresponding segment of the protein.

Pepsin was used to fragment the protein into peptides because it has maximum activity in the pH range 2–3, where amide hydrogen exchange is slowest. In addition, pepsin usually cleaves at many points to give small peptides (3–30 residues), which are a convenient size for analysis by FABMS. Small peptides consisting of only a few residues are desirable because they more accurately define the regions of the protein that have undergone isotopic
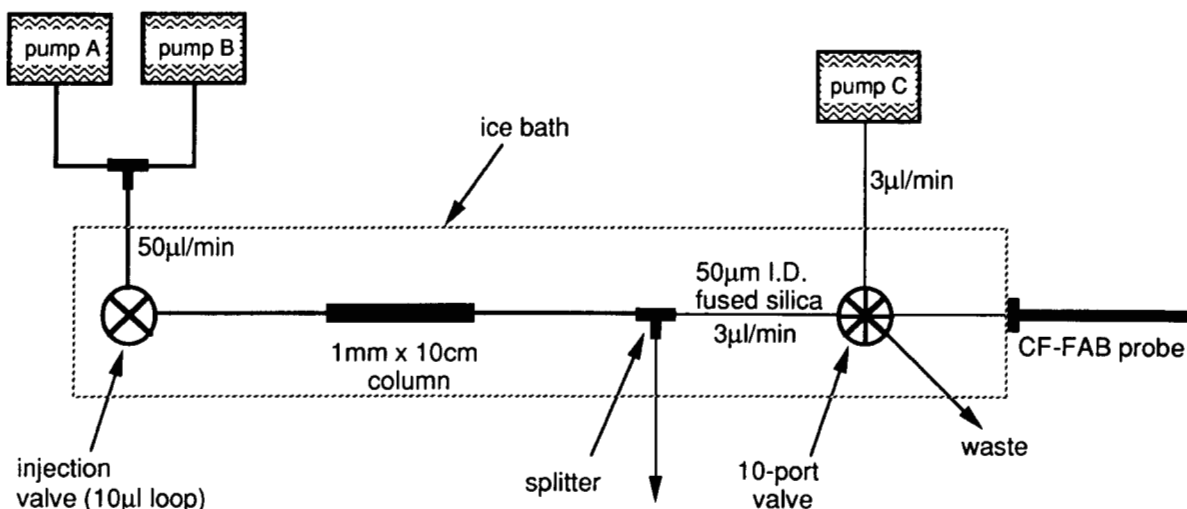
Fig. 2. Microbore high-performance liquid chromatography system and associated apparatus used to admit peptides into the mass spectrometer for molecular weight analysis.

exchange. For the present conditions, two peptides derived from the N-terminus (including residues 1–36) and seven peptides from the C-terminus (including residues 67–104) will be used to illustrate the method. These peptides are identified in Table 1.

Quantification of peptide amide deuterium

As peptides elute from the reversed-phase HPLC column, they are transmitted to the FABMS probe tip where they are desorbed with high energy xenon atoms and analyzed by mass spectrometry to determine their molecular weights (MH^+). Typical FAB mass spectra of the 95–104 segment of cytochrome c after (1) no incubation in D_2O, (2) incubation of the intact protein in D_2O for 1 h, and

Table 1. *Number of amide protons (N_{1-3}) and their exchange rate constants (k_{1-3}, h^{-1}) determined by applying a three-component model (Equation 2) to exchange-in data, as illustrated in Figure 4 for the 95–104 segment of cytochrome c*

Peptide	N_1	k_1	N_2	k_2	N_3	k_3
1–10[a]	4.9	72	2.4	0.8	2.7	0.007
22–36	3.9	70	1.1	0.5	8.0	0.006
67–80	5.0	>140	2.5	1.0	3.5	<0.002
67–82	7.8	140	1.5	1.5	3.7	0.007
81–94	4.3	78	1.3	4.3	7.4	0.007
81–96	3.3	>140	1.7	5.2	10.0	0.005
83–94	3.6	79	1.3	1.1	6.1	0.005
83–96	3.0	95	1.1	4.1	8.9	0.005
95–104	1.7	38	2.5	1.1	4.8	<0.002

[a] The 1–10 segment has 10 peptide amide hydrogens because the N-terminus of cytochrome c is acetylated.

(3) complete deuteration are presented in Figure 3. Each of the molecular ion patterns in Figure 3 have contributions from the natural abundance of isotopes (^{13}C, etc.) and an excess of deuterium. Although the mass spectrum presented in Figure 3A is for cytochrome c that was not incubated in D_2O, the peptides have an excess of deuterium because the digestion was performed in a mixture of H_2O and D_2O. Hence, a small number of deuterons were both gained and lost by the peptides during the 10-min digestion. Although HPLC conditions have been chosen to minimize hydrogen exchange, deuterated peptides lose some peptide amide deuterons during HPLC separation.

The deuterium content of the peptide comprising residues 95–104 was determined from the centroid of the molecular ion isotope peaks illustrated in Figure 3B. The deuterium content of the 95–104 segment in the protein (D_0) was deduced from the deuterium content of this peptide, after adjustment for deuteron gain/loss during digestion and HPLC fractionation. To make this adjustment, nondeuterated as well as fully deuterated cytochrome c was analyzed by the same procedure. The deuterium levels of the same peptide derived from these references were used to adjust for deuterium gain/loss, as illustrated in Equation 1:

$$D_0 = \frac{\langle m \rangle - \langle m_{0\%} \rangle}{\langle m_{100\%} \rangle - \langle m_{0\%} \rangle} \cdot N, \tag{1}$$

where D_0 is the average number of peptide amide deuterons in the 95–104 segment of the protein after incubation for time t, and $\langle m_{0\%} \rangle$, $\langle m \rangle$, and $\langle m_{100\%} \rangle$ are the isotope-averaged centroids of the molecular ion peaks in Figure 3A–C, respectively, and N is the total number of peptide amide hydrogens in the peptide. A derivation and error analysis of this expression is given on the Diskette
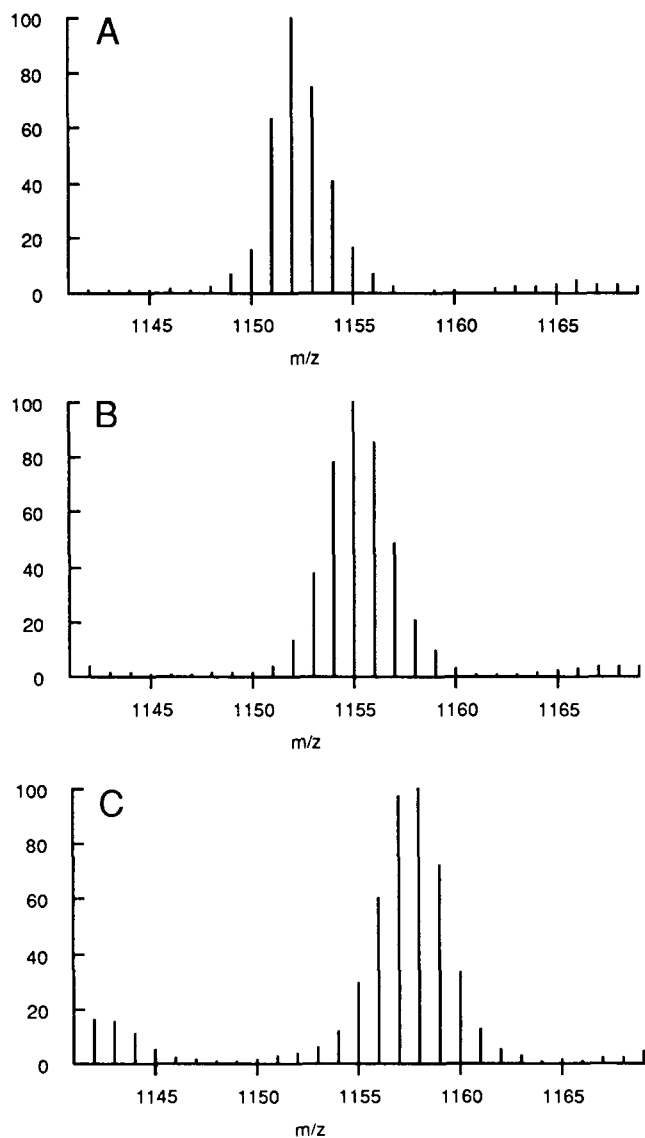
Fig. 3. Fast atom bombardment mass spectra of the molecular ion region of the 95–104 segment (m/z 1,151) of cytochrome c that was (A) not incubated in D_2O, (B) partially deuterated by incubating in D_2O for 1 h at 25 °C, and (C) completely deuterated by incubating in D_2O for 3 h at 75 °C. All incubations were performed at pD 6.8.

Fig. 4. Deuterium exchange into the 95–104 segment of cytochrome c (expressed as % of peptide amide positions deuterated) as a function of incubation time (pD 6.8; 25 °C, time = 1, 3, 8, 24, min/1, 3.6, 26.5, 47.1 h) before (O) and after (□) adjustment for deuterium gain/loss during digestion and analysis.

practice, one often measures only changes in deuterium incorporation as a function of some variable. In such cases, the deuterium gain/loss during analysis is constant, making correction for deuterium gain/loss less important.

Hydrogen exchange rates

The present protein fragmentation method for determining hydrogen exchange gives only the number of deuterons in a specific segment of the protein but no direct information about the deuteron content of specific amide linkages. Qualitative analysis of the results for the 95–104 segment presented in Figure 4 indicates that approximately half of the amide hydrogens in this segment are fully exchanged within 3 h, whereas the others resist exchange for 47 h. A more quantitative assessment of hydrogen exchange rates was made by fitting the data in Figure 4 to the following expression, which represents a three-component model.

$$D = N_1[1 - \exp(-k_1 t)]$$
$$+ N_2[1 - \exp(-k_2 t)] + N_3 k_3 t. \qquad (2)$$

According to this model, the total number of amide hydrogens, 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. Values for N_{1-3} and k_{1-3} for each of the nine segments of cytochrome c analyzed in this study are given in Table 1. For the N-terminal peptide of cytochrome c, consisting of residues 1–10, there is a total of 10 amide hydrogens in the backbone; 4.9 exchange with rate constants equal to 72 h^{-1}, 2.4 exchange with rate constants equal

Appendix. Hydrogen exchange results presented below for other segments in cytochrome c were obtained using the procedures described here for the 95–104 segment.

Because the digestion and analysis were performed under slow-exchange conditions, adjustments for gain/loss of deuterons is relatively small, as illustrated in Figure 4 for the 95–104 segment of cytochrome c incubated in D_2O for 1 min to 47 h. One set of data corresponds to the deuterium content of this peptide with no correction for deuterium gain/loss, while the other corresponds to the deuterium content after adjustment for deuterium gain/loss during analysis. From these results it is evident that this adjustment is significant, but relatively small. In
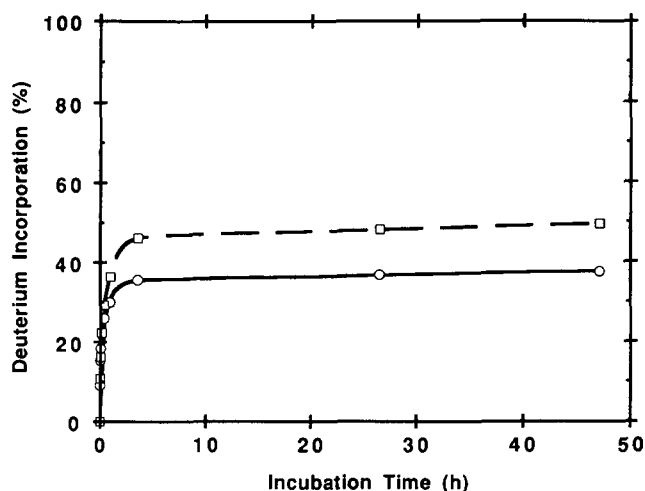
to 0.8 h^{-1}, and 2.7 exchange very slowly with rate constants equal to 0.007 h^{-1}. Because only the total number of peptide amide deuterons in this segment can be determined from the molecular weight of the peptide, it is not possible to determine exchange rates for specific amide hydrogens. However, this type of analysis may be a sensitive probe for detecting and locating conformational changes in proteins.

The rate constants for amide hydrogen exchange in proteins may be reduced by a factor of 10^8 or more from their exchange rates in unstructured peptides. Although the basis for reduced hydrogen exchange rates in proteins remains an active area of research, it has generally been attributed to a combination of solvent shielding and intramolecular hydrogen bonding (Woodward et al., 1982; Englander & Kallenbach, 1984; Wand et al., 1986). 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. From the hydrogen exchange rate constants in Table 1, the number of amide hydrogens that exchange slowly (defined by $k < 5$ h^{-1}) in each of the nine segments sampled in this study was compared with the number of amide hydrogens located in α-helices (Bushnell et al., 1990). These results are given in Table 2. For eight of the nine 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 α-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, which is also given in Table 2. 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 α-helices (Bushnell et al., 1990).

Table 2. *Correlation between the number of slow-exchanging amide hydrogens (k < 5 h^{-1}) and the number of α-helix amide hydrogens in each of the peptides[a]*

Peptide	$k > 5$ h^{-1}	$k < 5$ h^{-1}	α-Helix	H-bond
1–10	4.9	5.1	5	3
22–36	3.9	9.1	0	5
67–80	5.0	6.0	6	9
67–82	7.8	5.2	6	9
81–94	4.3	8.7	8	5
81–96	5.0	10.0	10	7
83–94	3.6	7.4	8	5
83–96	3.0	10.0	10	7
95–104	1.7	7.3	7	7

[a] The numbers of peptide amide hydrogens that participate in intramolecular hydrogen bonding are also given (Bushnell et al., 1990).

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, which will be discussed below.

A general method for quantifying hydrogen exchange will be most useful if rates of both very slowly and very rapidly exchanging hydrogens can be determined. Rate constants for very slowly exchanging hydrogens can be determined by extending the incubation time indefinitely. For the present investigation, the longest incubation time was 47 h, limiting meaningful measurements to exchange reactions with half-lives less than approximately 350 h ($k > 0.002$ h^{-1}).

Factors limiting measurement of the most rapidly exchanging amide hydrogens are less obvious. Thévenon-Emeric et al. (1992) demonstrated that apparatus and procedures similar to those used in the present investigation could be used to accurately determine amide hydrogen exchange rates for random coil peptides. It follows that the ability to measure rate constants for rapidly exchanging hydrogens in proteins is not limited by HPLC-FABMS, but rather by the minimum deuterium exchange-in time and the time required to quench the protein into slow-exchange conditions. For exchange-in at pH 7 and 25 °C, amide hydrogens in random coil peptides exchange with a half-life on the order of 0.1 s (Molday et al., 1972). Roder et al. (1988) have described a pulse labeling method that was used to limit exchange-in time to 0.05 s. Hence, it should be possible to measure exchange rate constants for the most rapidly exchanging hydrogens, even when exchange-in is carried out at high pH and temperature. In the present investigation, the minimum exchange-in time was 1 min (pD 6.8, 25 °C), limiting measurements to hydrogens with half-lives greater than approximately 0.3 min ($k < 140$ h^{-1}). Following exchange-in reactions with half-lives of 0.1 s with an analysis time of approximately 20 min is possible because the exchange rates decrease by approximately five orders of magnitude when the protein is quenched into the slow-exchange conditions used for analysis (Englander & Poulsen, 1969).

Hydrogen exchange mechanisms

Solvent penetration and local unfolding models have been developed to describe hydrogen exchange in proteins at the molecular level (Woodward et al., 1982; Englander & Kallenbach, 1984). According to the solvent penetration model, hydrogen exchange catalysts (OH$^-$ or H$^+$) must penetrate the tertiary structure to reach amide hydrogens, whereupon exchange may occur. As a result, amide hydrogens located in the interior of a protein are expected to exchange more slowly than amide hydrogens located on the surface. According to the local unfolding model, small segments of 5–10 residues momentarily unfold, breaking the hydrogen bonds and exposing the amide hydrogens to the solvent and hydrogen exchange catalysts.

The hydrogen exchange process, as viewed in either model, is described by the following expression:

$$NH + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NH \cdots D \overset{k_2}{\longrightarrow} ND + H,$$
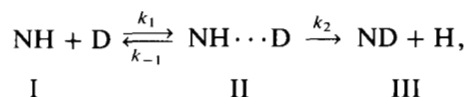
$$\text{I} \qquad\qquad \text{II} \qquad\qquad \text{III}$$

where I is the state in which the amide hydrogen and catalyst are separated, II is the state where the catalyst and the amide hydrogen are together, and III is the state following hydrogen exchange. According to the solvent penetration model, the first-order rate constant for hydrogen exchange, k_{ex} is given by the expression:

$$k_{ex} = (k_1/k_{-1})k_2 = Kk_2, \tag{3}$$

where K is the equilibrium constant describing the penetration of hydrogen exchange catalyst to the amide hydrogen (Woodward et al., 1982; Englander & Kallenbach, 1984).

In the local unfolding model, the folded state is strongly favored (i.e., $k_{-1} \gg k_1$) and the exchange rate constant is given by Equation 4 (Englander & Kallenbach, 1984):

$$k_{ex} = (k_1 k_2)/(k_{-1} + k_2). \tag{4}$$

If the rate of intrinsic hydrogen exchange is much larger than the refolding rate ($k_2 \gg k_{-1}$), the exchange rate is equal to the unfolding rate:

$$k_{ex} = k_1. \tag{5}$$

In this case, all of the amide hydrogens within a small segment of protein undergo exchange while the segment is open. Roder et al. (1985a) referred to this type of exchange as correlated. When this model is appropriate, the rate constant for hydrogen exchange is a direct measure of the rate of protein unfolding.

Alternatively, the refolding rate may be much faster than the rate of intrinsic hydrogen exchange ($k_{-1} \gg k_2$). In this case, a particular segment will unfold and refold many times before the amide hydrogens in this segment have been completely replaced by deuterium. Because hydrogen exchange occurs residue by residue, rather than segment by segment, this type of exchange is referred to as uncorrelated. The rate constant for hydrogen exchange is given by the product of the equilibrium constant for unfolding, K, and the rate constant for intrinsic hydrogen exchange, k_2:

$$k_{ex} = Kk_2. \tag{6}$$

When this model is appropriate, the hydrogen exchange rate constant can be related to the equilibrium constant for unfolding, K, since the rate constant for intrinsic hy-

drogen exchange can be calculated (Englander & Poulsen, 1969; Molday et al., 1972).

Rate constants for hydrogen exchange can be directly related to protein structure and dynamics only when the mechanism of exchange is known. Roder et al. (1985b) used two-dimensional nuclear Overhauser enhancement spectroscopy to investigate hydrogen exchange mechanisms in bovine pancreatic trypsin inhibitor. They found that hydrogen exchange in this protein is usually uncorrelated, and proposed that hydrogen exchange rate constants should be linked to protein dynamics via Equation 6, above. The molecular ion isotope patterns of peptides (Fig. 3) for which approximately 50% of the amide hydrogens have been exchanged are a direct indication of whether hydrogen exchange occurred in a correlated manner. In the simplest case of correlated exchange (Equation 5), where a peptide is derived entirely from one contiguous unfolded segment, half of the peptides will be fully deuterated and half will be fully protiated, giving rise to a bimodal isotope pattern. If hydrogen exchange were uncorrelated (Equation 6), the isotope pattern would be binomial. Even for the more realistic case where peptides are not derived entirely from a single unfolded segment, correlated exchange should normally give rise to a bimodal distribution of isotopes. All of the molecular ion isotope patterns of peptides used in this investigation were approximately binomial (Fig. 3), indicating that hydrogen exchange was uncorrelated and that Equation 6 is appropriate for relating the hydrogen exchange rate constants to the structure and dynamics of cytochrome *c*.

Thermal denaturation of cytochrome c

One of the potentially most important applications of hydrogen exchange as a probe of protein structure is the detection and location of conformational changes in proteins. To demonstrate that the present method can be used for this purpose, hydrogen exchange into cytochrome *c* was determined for a range (30–80 °C) of incubation temperatures. The isotope patterns of the molecular ions of the peptides suggested that exchange was uncorrelated. 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 (Equation 6). Because the temperature dependence of the intrinsic rate of hydrogen exchange can be calculated from its activation energy (Englander & Poulsen, 1969; Englander et al., 1979), the temperature dependence of the equilibrium constant, including associated thermodynamic information, can be derived from the temperature dependence of the hydrogen exchange rate constant (Englander & Kallenbach, 1984; Roder et al., 1985a).

The extent of deuterium incorporation into eight of the nine peptides listed in Tables 1 and 2 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 Equation 7:

$$d = 1 - \exp(-k_{ex}t) = 1 - \exp(-Kk_2t), \qquad (7)$$

where d is the deuterium level, and k_{ex}, K, and k_2 are defined in Equation 6. Assuming an activation energy of 17.4 kcal/mol for intrinsic hydrogen exchange (Englander et al., 1979), the temperature dependence of k_2 can be calculated. The incubation time, t, was adjusted to compensate for changes in k_2.

The 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. 5) are a direct indication of the temperature dependence of unfolding in specific regions of the protein. Deuterium incorporation into the 95–104 segment was approximately 53–55% as the temperature was increased from 30 to 55 °C. However, the deuterium content of this peptide increased to 100% as the temperature was increased from 58 to 64 °C. These results indicate that in the temperature range 58–64 °C, amide hydrogen bonds in this region are substantially weakened, causing an increase in the equilibrium constant for unfolding of the 95–104 segment. Deuterium incorporation into several other segments of cytochrome c (Fig. 5) also increases sharply in the same temperature range, suggesting a conformational change in cytochrome c when heated to approximately 60 °C. Previous investigations of thermal denaturation of cytochrome c also indicate a conformational change in this temperature range
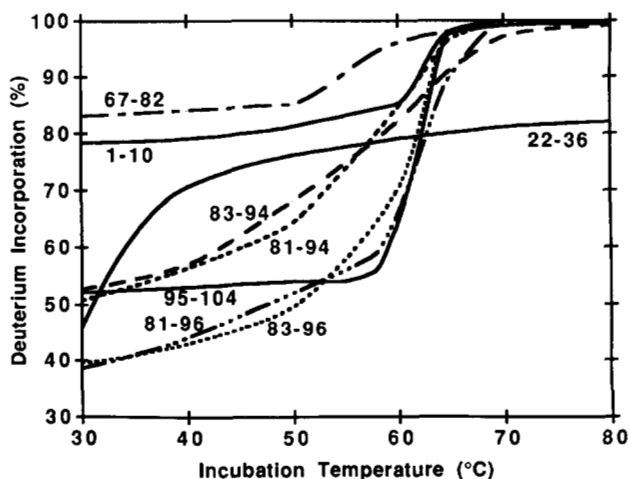

Fig. 5. Plot of deuterium incorporation into specific segments of cytochrome c as a function of the incubation temperature. The incubation time was adjusted to minimize the effect of temperature on the intrinsic rate of hydrogen exchange.

(Myer, 1968; Myer et al., 1980; Muga et al., 1991). It is interesting to note that Myer (1968) attributed the conformational change around 60 °C to loosening of the heme crevice, whereas the present results indicate that hydrogen bonding in many regions of cytochrome c is abruptly weakened at this temperature. The present results are also consistent with the results of Muga et al. (1991) 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.

Although the rate of deuterium incorporation into most (seven of eight) of the segments of cytochrome c measured in this study indicate a major conformational change at 60 °C, the increasing levels of deuterium between 30 and 60 °C of some of the peptides indicate a general loosening of the structure in some regions at lower temperatures. For example, deuterium incorporation into the four segments 83–94, 81–94, 83–96, and 81–96 increased substantially as the incubation temperature was increased from 30 to 55 °C. Whether this loosening of structure below 60 °C occurs in all parts of cytochrome c, or whether it is predominantly in the regions outside the α-helices may be determined from time-course measurements of hydrogen exchange at each temperature. However, the relatively sharp increase in the exchange rate for the 95–104 segment, which is also the segment with the highest fraction of α-helix, suggests that the exchange rates of amide hydrogens in α-helices do not increase with incubation temperature until the obvious onset at 60 °C.

The temperature dependence of isotopic exchange into the 22–36 segment is very different from the other segments illustrated in Figure 5. Not only is there a sharp increase in the isotopic exchange rate around 30–40 °C, but the maximum incorporation of deuterium is only 80%, even for an exchange-in temperature of 80 °C. As discussed above, isotopic exchange in this segment is very slow, even though it is not in an α-helix. The structure of albacore tuna heart cytochrome c (Takano & Dickerson, 1980) is given in Kinemage 1. It is evident from this structure that a portion of the 22–36 segment, specifically residues 29–33, are adjacent to the heme and are shielded from the solvent by a segment composed of residues 20–28. Thus, the anomalous hydrogen exchange behavior of the 22–36 segment may be due to limited access to the solvent. The temperature dependence of hydrogen exchange in this region suggests that the extent of shielding decreases with increasing temperature but is not totally eliminated, even at temperatures as high as 80 °C.

Localized hydrogen exchange

To investigate dynamic and structural features of proteins in detail, one would like know the isotopic exchange rates of specific amide hydrogens. This type of detailed information will likely prove to be an advantage unique to

NMR. Although the present protein fragmentation method is generally limited to determining isotopic exchange rates in relatively small segments of proteins, it can be used to obtain more detailed information in some instances. For example, the time-course data may fit a single exponential function, indicating that all of the amide hydrogens in this segment exchange at similar rates. Alternatively, there may be several overlapping peptides from the same segment of the protein. Differences in the deuterium contents of such peptides can be used to increase the spatial resolution of the method. For example, segments 83–94 and 83–96 differ by only two residues, but show very different isotopic exchange rates (Fig. 5). The 81–94 and 81–96 segments form a similar pair. The difference in deuterium incorporation into these two pairs of segments indicates deuterium exchange into amide hydrogens located on residues 95 and 96. Results for the 81–96/81–94 pair are given in Figure 6, illustrating this method for achieving enhanced spatial resolution. The temperature profile for hydrogen exchange into this two-residue segment has an abrupt increase at 60 °C, as was found for the 95–104 segment, which is also derived entirely from the same α-helix. Similar analysis of the related pair of peptides (83–96/83–94) gave the same result, demonstrating that the spatial resolution available with the protein fragmentation method may be substantially increased by using overlapping peptides. This approach to enhanced spatial resolution may be extended even further by using other acid proteases to fragment the protein.

Summary

Results described here demonstrate that deuterium incorporation into proteins can be determined by combining protein fragmentation and directly coupled HPLC-FABMS.
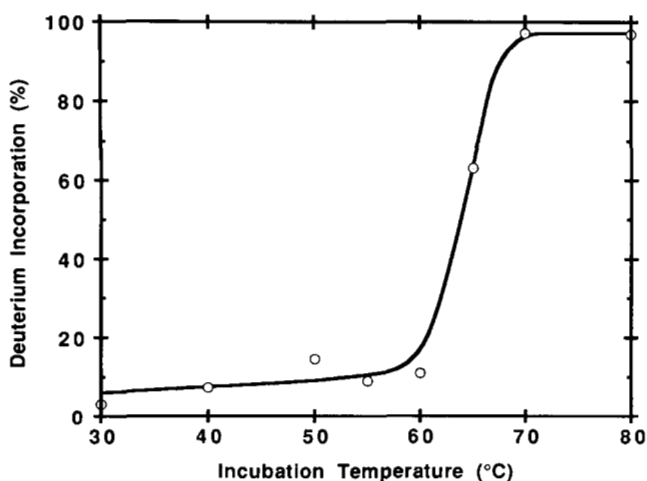

Fig. 6. Difference in amide deuterium content of the 81–96 and 81–94 segments, illustrating isotopic exchange into the 95–96 segment of cytochrome *c* as a function of incubation temperature.

For the present experimental conditions, hydrogen exchange reactions with half-lives between 0.3 min and 350 h were determined. Reproducibility of the measurements indicated that the deuterium content of peptides could be determined with an uncertainty of approximately 5%. Although the spatial resolution achieved by this method is not at the single residue level, exchange rates within small segments, sometimes as short as two residues, can be determined. The major disadvantage will likely be resistance of some proteins to fragmentation by acid proteases. These results indicate that protein fragmentation and HPLC-FABMS may lead to the realization of amide hydrogen exchange as a general tool for investigating protein structure and dynamics.

Materials and methods

Materials

Horse heart cytochrome *c* (type III), pepsin, carboxypeptidase Y, carboxypeptidase A, anhydrous monobasic sodium phosphate, and monothioglycerol were purchased from Sigma; anhydrous dibasic sodium phosphate and glycerol were purchased from Mallinckrodt; and deuterium oxide (99.9 atom % D) and trifluoroacetic acid were purchased from Aldrich. All materials were used without further purification.

Apparatus

The microbore HPLC system, as well as the continuous-flow probe used to interface it to the mass spectrometer are illustrated in Figure 2. The HPLC system consisted of two Rainin pumps (A and B), a Rheodyne 8125 injector fitted with a 10-μL sample loop, and a 0.1 × 10-cm reversed-phase column (Aquapore RP300, C-8, 7 μm). Peptides were eluted with a gradient (0–50% acetonitrile in 20 min) where both mobile phases contained trifluoroacetic acid, glycerol, and monothioglycerol (0.1%, 3%, and 3%, respectively). The flow rate through the column was maintained at 50 μL/min. The effluent from the column was split (17:1), reducing the flow to the mass spectrometer to 3 μL/min. A 10-port valve (Valco C10U) was used to substitute the flow (3 μL/min of phase A) from pump C (Applied Biosystems) for the column effluent. This feature was used to divert involatile buffer salts from the mass spectrometer and for postcolumn direct injection of standard peptides used to tune the mass spectrometer. All components of the HPLC system, from the injector to the continuous-flow FAB probe, were submerged in an ice bath to minimize hydrogen exchange.

Mass spectrometric analyses were performed with a Kratos MS-50 FAB mass spectrometer, which was operated with xenon and an accelerating potential of 8 kV. The instrument was tuned for a static resolution of 2,500 and scanned over the mass range from 2,200 to 1,100 at a rate

of 30 s/decade of mass. Mass spectra were recorded with a Kratos DS-90 data acquisition system, transferred to a Macintosh SE computer, and analyzed by software written in this laboratory. The continuous-flow FABMS probe has been described previously (Smith, 1990).

Procedures

Prior to the hydrogen exchange experiments, peptic digestion of cytochrome c was investigated by off-line HPLC and FABMS. Cytochrome c was dissolved in 0.1 M phosphate buffer to a concentration of 0.5 nmol/μL and digested with pepsin (5:1 substrate:enzyme, pH 2.6, 0 °C, 10 min). The digest was fractionated by reversed-phase HPLC. Fractions were collected, dried, and analyzed by FABMS to determine the molecular weights of the peptides. The identities of the peptides were determined by computer-assisted analysis of the molecular weight information, and confirmed by C-terminal sequencing with carboxypeptidases (Caprioli & Fan, 1986; Smith et al., 1992).

Isotopic exchange experiments were initiated by dissolving horse heart cytochrome c in D_2O buffer (0.1 M phosphate, pD 6.8) to a concentration of 1 nmol/μL. Values given in the text for pH and pD were taken directly from the pH meter and were not corrected for isotopic effects (Englander et al., 1985). Time and temperature were treated as incubation variables. Ten-microliter aliquots of the incubate were taken at different times and combined with 7.5 μL of 0.1 M HCl in H_2O to quench the exchange reaction. These samples were stored at −20 °C until analyzed by HPLC-FABMS. To determine the extent of deuterium exchange-in, the pH of the sample was brought to 2.4 by addition of 2.5 μL of 0.1 M HCl, followed by addition of 2.5 μL of 10 μg/μL pepsin solution (5:1 substrate:enzyme, pH 2.6). Digestion was carried out for 10 min at 0 °C. Ten microliters (4.4 nmol of digested cytochrome c) were analyzed by microbore HPLC-FABMS. Because the column effluent was split 17:1, only 0.26 nmol of digest was actually analyzed by the mass spectrometer.

Two different approaches were used to analyze the data. To determine whether the isotopic patterns were binomial or bimodal, FAB mass spectra of peptides that contained no deuterium were used to remove effects of the natural abundance of isotopes from the isotope patterns of the deuterated peptides. To determine the deuterium content of peptides, two standards (0% and 100% deuterated) were used with Equation 1 to correct for deuterium loss/gain occurring during digestion and analysis. The 100% deuterated standard was prepared by incubating cytochrome c in phosphate buffer at 75 °C for 3 h and analyzing by procedures described above. A more complete description of the data analysis is given on the Diskette Appendix.

Acknowledgments

References

Bentley, G.A., Delepierre, M., Dobson, C.M., Wedin, R.E., Mason, S.A., & Poulsen, F.M. (1983). Exchange of individual hydrogens for a protein in a crystal and in solution. *J. Mol. Biol. 170*, 243–247.

Brandt, P. & Woodward, C. (1987). Hydrogen exchange kinetics of bovine pancreatic trypsin inhibitor β-sheet protons in trypsin–bovine pancreatic trypsin inhibitor, trypsinogen–bovine pancreatic trypsin inhibitor, and trypsinogen–isoleucylvaline–bovine pancreatic trypsin inhibitor. *Biochemistry 26*, 3156–3162.

Bushnell, G.W., Louie, G.V., & Brayer, G.D. (1990). High-resolution three-dimensional structure of horse heart cytochrome c. *J. Mol. Biol. 214*, 585–595.

Caprioli, R.M. & Fan, T. (1986). Peptide sequence analysis using exopeptidases with molecular analysis of the truncated polypeptides by mass spectrometry. *Anal. Biochem. 154*, 596–603.

Delepierre, M., Dobson, C.M., Karplus, M., Poulsen, F.M., States, D.J., & Wedin, R.E. (1987). Electrostatic effects and hydrogen exchange behavior in proteins: The pH dependence of exchange rates in lysozyme. *J. Mol. Biol. 197*, 111–130.

Englander, J.J., Calhoun, D.B., & Englander, S.W. (1979). Measurement and calibration of peptide group hydrogen–deuterium exchange by ultraviolet spectrophotometry. *Anal. Biochem. 92*, 517–524.

Englander, J.J., Rogero, J.R., & Englander S.W. (1985). Protein hydrogen exchange studied by the fragmentation separation method. *Anal. Biochem. 147*, 234–244.

Englander, S.W., Englander, J.J., McKinnie, R.E., Jurner, G.J., Westrick, J.A., & Gill, S.J. (1992). Hydrogen exchange measurement of the free energy of structural and allosteric change in hemoglobin. *Science 256*, 1684–1687.

Englander, S.W. & Kallenbach, N.R. (1984). Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys. 16*, 521–655.

Englander, S.W. & Poulsen, A. (1969). Hydrogen–tritium exchange of the random chain polypeptide. *Biopolymers 7*, 379–393.

Hvidt, A. & Nielsen, S.O. (1966). Hydrogen exchange in proteins. *Adv. Protein Chem. 21*, 287–385.

Jeng, M.F., Englander, S.W., Elöve, G.A., Wand, J., & Roder, R. (1990). Structural description of acid-denatured cytochrome c by hydrogen exchange 2D NMR. *Biochemistry 29*, 10433–10437.

Kossiakoff, A.A. (1982). Protein dynamics investigated by the neutron diffraction–hydrogen exchange technique. *Nature 296*, 713–721.

Loftus, D., Gbenle, G., Kim, P.S., & Baldwin, R.L. (1986). Effects of denaturants on amide proton exchange rates: A test for structure in protein fragments and folding intermediates. *Biochemistry 25*, 1428–1436.

Molday, R.S., Englander, S.W., & Kallen, R.G. (1972). Primary structure effects on peptide group hydrogen exchange. *Biochemistry 11*, 150–158.

Muga, A., Mantsch, H.H., & Surewicz, W.K. (1991). Membrane binding induces destabilization of cytochrome c structure. *Biochemistry 30*, 7219–7224.

Myer, Y.P. (1968). Conformation of cytochromes. III. Effect of urea, temperature, extrinsic ligands, and pH variation on the conformation of horse heart ferricytochrome c. *Biochemistry 7*, 765–776.

Myer, Y.P., MacDonald, L.H., Verma, B.C., & Pande, A. (1980). Urea denaturation of horse heart ferricytochrome c, equilibrium studies and characterization of intermediate forms. *Biochemistry 19*, 199–207.

Osborne, H.B. & Nabedryk-Viala, E. (1982). Infrared measurement of peptide hydrogen exchange in rhodopsin. *Methods Enzymol. 88*, 676–680.

Paterson, Y., Englander, S.W., & Roder, R. (1990). An antibody binding site on cytochrome c defined by hydrogen exchange and two-dimensional NMR. *Science 249*, 755–759.

Roder, H., Elöve, G.A., & Englander, S.W. (1988). Structural characterization of folding intermediates in cytochrome *c* by H-exchange labelling and proton NMR. *Nature 335*, 700–704.

Roder, H., Wagner, G., & Wüthrich, K. (1985a). Amide proton exchange in protein by EX_1 kinetics: Studies of the basic pancreatic trypsin inhibitor at variable p^2H and temperature. *Biochemistry 24*, 7396–7407.

Roder, R., Wagner, G., & Wüthrich, K. (1985b). Individual amide proton exchange rates in thermally unfolded basic pancreatic trypsin inhibitor. *Biochemistry 24*, 7407–7411.

Rosa, J.J. & Richards, F.M. (1979). An experimental procedure for increasing the structural resolution of chemical hydrogen-exchange measurements on proteins: Application to ribonuclease S peptide. *J. Mol. Biol. 133*, 399–416.

Smith, D.L. (1990). Analysis of low-polarity substances. In *Continuous-Flow Fast Atom Bombardment Mass Spectrometry* (Caprioli, R.M., Ed.), pp. 137–149. Wiley & Sons, New York.

Smith, J.B., Sun, Y., Smith, D.L., & Green, B. (1992). Identification of the posttranslational modifications of bovine lens αB-crystallins by mass spectrometry. *Protein Sci. 1*, 601–608.

Takano, T., & Dickerson, R.E. (1980). Redox conformation changes in refined tuna cytochrome *c*. *Proc. Natl. Acad. Sci. USA 77*, 6371–6375.

Thévenon-Emeric, G., Kozlowski, J., Zhang, Z., & Smith, D.L. (1992). Determination of amide hydrogen exchange rates in peptides by mass spectrometry. *Anal. Chem. 64*, 2456–2458

Tüchsen, E. & Woodward, C. (1987). Hydrogen exchange of primary amide protons in basic pancreatic trypsin inhibitor: Evidence for NH_2 group rotation in buried asparagine side chains. *Biochemistry 26*, 8073–8078.

Wagner, G. & Wüthrich, K. (1982). Amide proton exchange and surface conformation of the basic pancreatic trypsin inhibitor in solution. *J. Mol. Biol. 160*, 343–361.

Wand, A.J., Roder, H., & Englander, S.W. (1986). Two-dimensional ^1H NMR studies of cytochrome *c*: Hydrogen exchange in the N-terminal helix. *Biochemistry 25*, 1107–1114.

Woodward, C., Simon, I., & Tüchsen, E. (1982). Hydrogen exchange and the dynamic structure of proteins. *Mol. Cell. Biochem. 48*, 135–160.