Hydrogen/Deuterium Exchange Electrospray Ionization Mass Spectrometry: A Method for Probing Protein Conformational Changes in Solution

Viswanatham Katta† and Brian T. Chait*

Contribution from The Rockefeller University, 1230 York Avenue, New York, New York 10021

Received January 27, 1993

Abstract: Hydrogen/deuterium exchange electrospray ionization mass spectrometry is demonstrated to be effective in probing changes in the conformation of proteins in solution. The method is based on the mass spectrometric measurement of the extent of hydrogen/deuterium exchange that occurs in different protein conformers over defined periods of time. Results are presented in which the method is used to probe conformational changes in (a) bovine ubiquitin induced by the addition of methanol to aqueous acidic solutions and (b) chicken egg lysozyme caused by the reduction of the intramolecular disulfide cross-linkages.

Introduction

In their native states, globular proteins are tightly folded compact structures that can be denatured and caused to unfold by subjecting them to high temperatures, extremes of pH, detergents, and solutions containing high concentrations of compounds such as urea, guanidinium chloride, and organic solvents.[1] The native proteins exhibit different physical and chemical properties compared with the corresponding denatured proteins. An array of techniques, which include optical rotation, spectrophotometry, viscometry, fluorescence, circular dichroism, hydrogen exchange, and nuclear magnetic resonance (NMR), have utilized these differences to probe conformational changes in proteins.[2] Recently, it has been demonstrated that the conformational states of certain proteins in solution have a profound influence on the distribution of charge states observed in the electrospray mass spectra of these proteins,[3-9] providing a new means for probing such conformational changes.

For protein molecules in solution, the net positive charge on a tightly folded native conformer is generally smaller than that on the unfolded form because some of the basic groups (especially histidines) are buried or involved in interactions and are not available for protonation. In addition, the effective pK values of the acidic side chains (aspartic and glutamic acids) are lowered due to electrostatic interactions, like salt bridges, that are specific to the native state.[10,11] As a result, the net positive charge on the native protein is lower than would be predicted from the intrinsic pK values of the side chains. Denaturation results in an unfolding of the protein, in which state the charged side groups are more exposed to the solvents and their effective pK values are closer to their intrinsic values. Thus, the protein conformation may be probed by the extent to which the protein is observed to be protonated in solution.[10]

Electrospray is a gentle method of ionization that produces intact, multiply protonated gas phase ions from protein molecules in solution.[12] The net charge on the multiply protonated protein ions observed in the positive electrospray mass spectra has been shown to reflect, to some extent, the degree of protonation of proteins in solution.[3-9,11] Electrospray ionization mass spectrometry can therefore be used to probe changes in protein conformations. For example, the electrospray mass spectrum obtained from an aqueous bovine cytochrome c solution at pH 2.6 (where the protein is known to be denatured) exhibited a charge distribution centered at 16+, whereas the spectrum obtained from a solution at pH 3.0 showed an additional distribution centered at 8+, indicating the coexistence of a tightly folded conformer in solution at the latter pH value.[3] During the course of a series of experiments designed to test rigorously our hypothesis that the observed changes in charge distributions indeed reflect changes in the solution-phase tertiary structure of the proteins, we developed a new technique for probing conformational changes, i.e. hydrogen/deuterium exchange electrospray ionization mass spectrometry, which we have described previously in a short communication.[4]

In small peptides, all the labile hydrogen atoms are readily accessible to the solvents in which the peptides are dissolved. The rates of exchange differ widely between different labile hydrogen atoms in a peptide and are a strong function of pH.[13] Generally, the backbone amide hydrogens are the slowest to exchange compared to the labile hydrogens on the side chains. For simple peptides dissolved in D_2O (pH > 5) at room temperature, exchange can be complete within minutes even for the slowly exchanging amide hydrogens.[13] In the case of proteins, the rates of exchange also depend on the conformational state. For many proteins in unfolded conformations (denatured states), the exchange rates are similar to those in simple peptides, and a high degree of

* Author to whom correspondence should be addressed.
† Present address: Protein Structure Group, Amgen Inc., Thousand Oaks, CA 91320.

(1) Creighton, T. E. *Proteins, Structures and Molecular Principles*; W. H. Freeman: New York, 1984. Creighton, T. E. *Protein Structure a Practical Approach*; Creighton, T. E., Ed.; IRL Press: Oxford, England, 1990.
(2) Ghelis, C.; Yon, J. *Protein Folding*; Academic Press: New York, 1982. Lapanje, S. *Physicochemical Aspects of Protein Denaturation*; Wiley-Interscience: New York, 1978.
(3) Chowdhury, S. K.; Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1990**, *112*, 9012–3.
(4) Katta, V.; Chait, B. T. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 214–7.
(5) Loo, J. A.; Loo, R. R. O.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 101–5.
(6) Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1991**, *113*, 8534–5.
(7) (a) Feng, R.; Konishi, Y. *Proc. 40th ASMS Conference on Mass Spectrometry and Allied Topics*; Washington, DC, 1992; pp 1635 and 625. (b) Feng, R.; Konishi, Y. *Proc. 39th ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, 1991; p 1432.
(8) LeBlanc, J. C. Y.; Beuchemin, D.; Siu, K. W. M.; Guevremont, R. *Org. Mass Spectrom.* **1991**, *26*, 831.
(9) Mirza, U. A.; Cohen, S. L.; Chait, B. T. *Anal. Chem.* **1993**, *65*, 1–6.
(10) Matthew, J. B.; Gurd, F. R. N. *Methods Enzymol.* **1986**, *130*, 413–35.
(11) Guevremont, R.; Siu, K. W. M.; LeBlanc, J. C. Y.; Berman, S. S. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 216.
(12) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64–71. Smith, R. D.; Loo, J. A.; Loo, R. R. O.; Busman, M.; Udseth, H. R. *Mass Spectrom. Rev.* **1991**, *10*, 359–451. Covey, T.; Bonner, R. F.; Shushan, B. I.; Henion, J. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249.
(13) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley-Interscience: New York, 1986.

0002-7863/93/1515-6317$04.00/0

exchange can be expected in a short period of time. On the other hand, proteins in tightly folded conformations (native states) have a considerably lower number of labile hydrogens that are readily accessible to the solvents and available for exchange.[14] The inaccessibility of the remaining exchangeable hydrogens is attributed to their presence in the hydrophobic core of the protein or involvement in hydrogen bonds and in salt bridges. Thus, the rates of exchange of certain labile hydrogens can differ by many orders of magnitude depending on the protein conformation, and complete exchange of all the labile hydrogens in a tightly folded protein can be extremely slow.[14] Such differences in exchange behavior of folded and unfolded proteins have previously been used, for example, to identify intermediates in protein folding by NMR.[15]

Electrospray mass spectra can be obtained with sufficient speed and mass accuracy that differences in exchange behavior can be readily measured. A combination of hydrogen/dueterium exchange with electrospray ionization mass spectrometry appears to be a natural choice for protein conformational studies because the mass spectra obtained from proteins in folded and unfolded states should exhibit differences in both the molecular masses and the charge distributions.[4] The high speed of this approach has recently enabled its application to an investigation of the secondary structures of certain peptides.[16] Most recently, hydrogen/deuterium exchange has been applied to a study of gas-phase conformations of multiply protonated protein ions produced by electrospray ionization.[17,18] In the present paper, we give details of the hydrogen-exchange electrospray ionization mass spectrometry approach[4] to the study of conformational changes in bovine ubiquitin and chicken egg lysozyme.

Experimental Section

Bradykinin, bovine ubiquitin, chicken egg lysozyme, bovine carbonic anhydrase II, equine myoglobin, bovine hemoglobin, dithiothreitol, D_2O (99.9 atom % D), CH_3COOD (98 atom % D), and CH_3OD (99.5 atom % D) were obtained from the Sigma Chemical Corp. (St. Louis, MO). High-purity water and methanol were obtained from Burdick & Jackson, and ultrapure acetic acid was obtained from J. T. Baker (Phillipsburg, NJ).

The electrospray ionization mass spectrometer used in these experiments was constructed in our laboratory and has been described earlier.[19] Briefly, the charged droplets produced by electrospray at atmospheric pressure are focussed and transported through a 20 cm long stainless steel capillary tube (0.5-mm i.d.) into a region maintained at 1–10 Torr. Desolvation of the analyte ions is achieved in part by controlled heating of the capillary and in part by collisional activation brought about by an electrostatic field in the intermediate pressure region (1–10 Torr) between the capillary exit and a coaxial skimmer. All the solutions used in the present study were electrosprayed at rates of 0.5–1.0 μL/min using a stainless steel syringe needle (two types were used: one with a 90° cut, 100-μm i.d., 228-μm o.d.; the other with a 500-μm o.d., 150-μm i.d. tip sharpened to about 150 μm by electrochemical etching).[4,20]

The hydrogen exchange experiments conducted on bradykinin were made using H_2O or D_2O, without the addition of any acids or buffers. For experiments in which it was desired that the ubiquitin be maintained in a tightly folded state, the spray solution was prepared by dissolving the protein, at concentrations of 10–30 μM, in 1% CH_3COOD in D_2O. Mass spectra were then obtained as a function of time after dissolution of the protein. For investigations of the unfolded states of ubiquitin, an equal volume of CH_3OD was added dropwise to the aqueous protein solution (1% CH_3COOD in D_2O) immediately after dissolution of the protein. Mass spectra were then obtained as a function of time after dissolution.

The native state of chicken egg lysozyme was investigated by dissolving the unreduced protein in 1% CH_3COOD in D_2O. Reduction of the disulfide bonds in lysozyme was carried out by adding a 50-fold molar excess of dithiothreitol to the protein in 0.05 M NH_4HCO_3 solution (pH 8.0) and maintaining the solution at 37 °C for 2 h. The mixture was subsequently lyophilized and redissolved in deuterated solvents.

In the case of bovine ubiquitin, the protein conformations were also investigated by circular dichroism on a AVIV Model 62DS (Aviv Associates, Lakewood, NJ) CD spectrometer using a 1 mm path length cuvette. Bovine ubiquitin solutions (~0.5 μg/μL) for the CD experiments were prepared in the following solvents: (a) 1:1 (v/v) aqueous HCl (pH 4.8):H_2O, (b) 1:1 (v/v) aqueous HCl (pH 4.8):MeOH, (c) 1:1 (v/v) aqueous HCl (pH 2.3):H_2O, and (d) 1:1 (v/v) aqueous HCl (pH 2.3): MeOH.

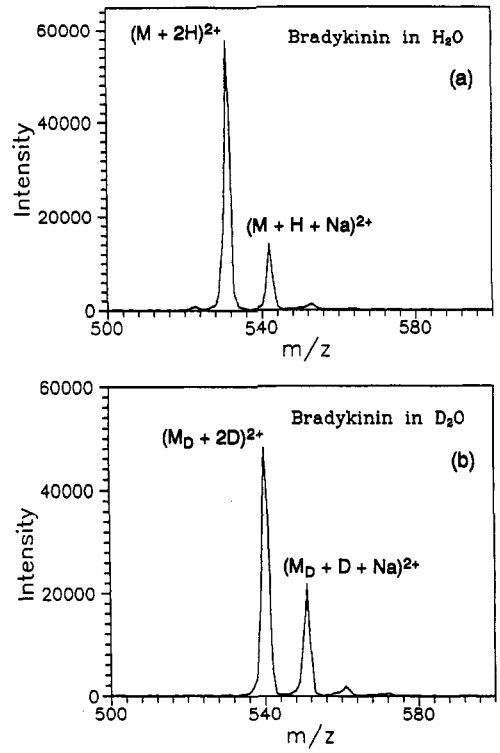

Figure 1. Electrospray ionization mass spectra of bradykinin obtained from (a) H_2O, showing the dominant peak from the $(M + 2H)^{2+}$ ion at m/z 531.1, and (b) D_2O, showing the dominant peak from the $(MD + 2D)^{2+}$ ion at m/z 540.4, indicating an exchange of 97% of the total of 17 labile hydrogens present on the neutral molecule (see text). The spectra were obtained by spraying the peptide solution into a dry air atmosphere.

Results and Discussion

Hydrogen Exchange in Peptides. To establish a reliable method for measuring hydrogen/deuterium exchange with electrospray ionization, we selected a test compound in which all the labile hydrogens are readily accessible to the solvent. This compound was the nine-residue peptide bradykinin (sequence ArgProPro-GlyPheSerProPheArg, molecular mass 1060.2 u), containing a total of 17 labile hydrogens in the neutral molecule (12 on the side chains and termini and 5 on the amide backbone). For bradykinin in H_2O, electrospray ionization produced a dominant $(M + 2H)^{2+}$ ion at m/z 531.1 (see Figure 1a). Contrary to expectations, our first electrospray ionization measurements of bradykinin in D_2O yielded masses that indicated incomplete

(14) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophysics* **1984**, *16*, 521–655. Roder, H. *Methods Enzymol.* **1989**, *176*, 446–73. Englander, S. W. In *Biomolecular Stereodynamics*; Sarma, H. R., Ed.; Adenine Press: New York, 1982.

(15) Roder, H.; Elove, G. A.; Englander, S. W. *Nature* **1988**, *335*, 700–4. Hughson, F. M.; Wright, P. E.; Baldwin, R. L. *Science* **1990**, *249*, 1544–8; Englander, S. W., Mayne, L. *Ann. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 243–65.

(16) Anderegg, R. J.; Stevenson, C. L.; Borchardt, R. T. *Proc. 40th ASMS Conference on Mass Spectrometry and Allied Topics*; Washington, DC, 1992; p 475.

(17) Winger, B. E.; Light-Wahl, K. J.; Rockwood, A. L.; Smith, R. D. *J. Am. Chem. Soc.* **1992**, *114*, 5897–8.

(18) Suckau, D.; Shi, Y.; Quinn, J. P.; Senko, M. W.; Zhang, M.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 790–793.

(19) Chowdhury, S. K.; Katta, V.; Chait, B. T. *Rapid Commun. Mass Spectrom* **1990**, *4*, 81–7.

(20) Chowdhury, S. K.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1660–4.

exchange (typically <75% even after 1 h). We found that this observed incomplete exchange was the result of rapid back-exchange of labile deuterium atoms in the peptide with hydrogen in the water vapor of the ambient laboratory atmosphere.[21] In our instrumental configuration, we estimate that after the solutions are electrosprayed at atmospheric pressure, time intervals of the order 2–5 ms are required before the ions enter the high vacuum of the mass analyzer. This short duration is sufficient for back exchange of certain of the labile hydrogens, especially those attached to heteroatoms on the side chains (intrinsic exchange rates of the order of 10^3–10^7 s^{-1} at pH ~7[13]). In order to prevent this back exchange from occurring, the spray area was enclosed (with a glass enclosure so that the spray could still be visually observed) and flushed with dry air (Matheson, E. Rutherford, NJ) continuously at rates of 2–3 L/min (typical gas load through the heated metal capillary tube is about 1 L/min). This arrangement enabled us to measure much higher exchange levels with D_2O. For example, after a 5 min incubation of bradykinin in D_2O, the original $(M + 2H)^{2+}$ ion at m/z 531.1 is completely replaced by a new $(M_D + 2D)^{2+}$ ion peak at m/z 540.4 (Figure 1b). The measured molecular mass of this exchanged species, M_D, is (540.4 × 2) − (2 × 2) = 1076.8 u, an increase of 16.6 u over the molecular mass of unexchanged bradykinin (1060.2 u). The predicted mass increase for complete exchange of all 17 labile hydrogens is 17.1 u, implying a measured exchange level of 16.6/17.1 = 97%.

Circular Dichroism of Ubiquitin. Before applying the hydrogen exchange method to probe conformational changes in ubiquitin, it was necessary to establish conditions under which the protein is completely denatured. Bovine ubiquitin in its native form is a small, tightly folded protein (76 residues, no disulfide bonds, 13 basic sites, 12 acidic sites, molecular mass 8565 u) that is very resistant to denaturation.[22,23] It is known to withstand extremes of pH, temperature, and high concentrations of guanidine hydrochloride. It is resistant to digestion by trypsin except at arginine-74, close to the carboxyl terminus. Only the enzyme pepsin is known to digest the protein at low pH.[22]

Circular dichroism (CD) is an established method for monitoring conformational changes in proteins.[24] CD spectra of ubiquitin were obtained at high and low pH values with and without the addition of methanol (Figure 2). In agreement with earlier NMR and CD measurements, we found that ubiquitin exists in a tightly folded native state (Figure 2) even at low pH (pH 2.3) in aqueous solutions.[25,26] Although the electrospray solutions were generally acidified with acetic acid, this acid was not used in the CD experiments because of its strong absorption at ultraviolet wavelengths. Instead hydrochloric acid was used for the CD experiments. In these experiments, the protein concentration in the various solutions was kept constant. Addition of methanol (final concentration 50%) to an aqueous solution at pH 4.8 did not cause any significant change in the CD spectrum, indicating that at pH 4.8 the addition of methanol was ineffective in causing denaturation. The CD spectrum of ubiquitin in an aqueous solution at pH 2.3 also indicated that the protein did not undergo denaturation. However, addition of methanol to this pH 2.3 solution caused a dramatic change in the CD spectrum indicating profound changes in the conformation of the protein

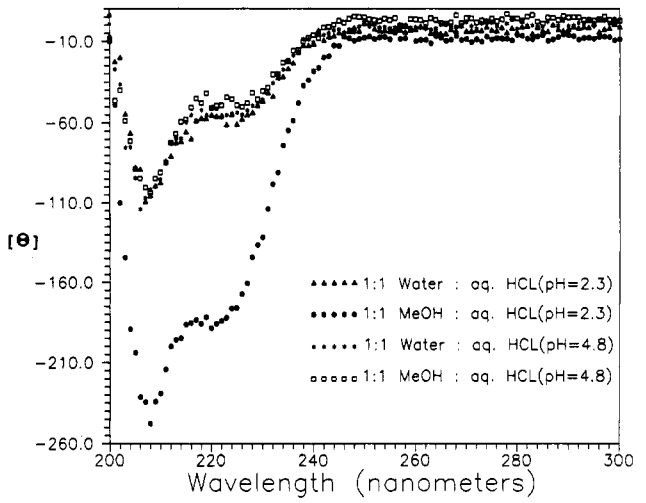

Figure 2. Circular dichroism spectra of bovine ubiquitin obtained from aqueous solutions at pH 4.8 and 2.3, with and without the addition of methanol. The final protein concentration is the same in all cases (~60 μM). The cumulative effect of low pH and the presence of methanol is needed to unfold the protein.

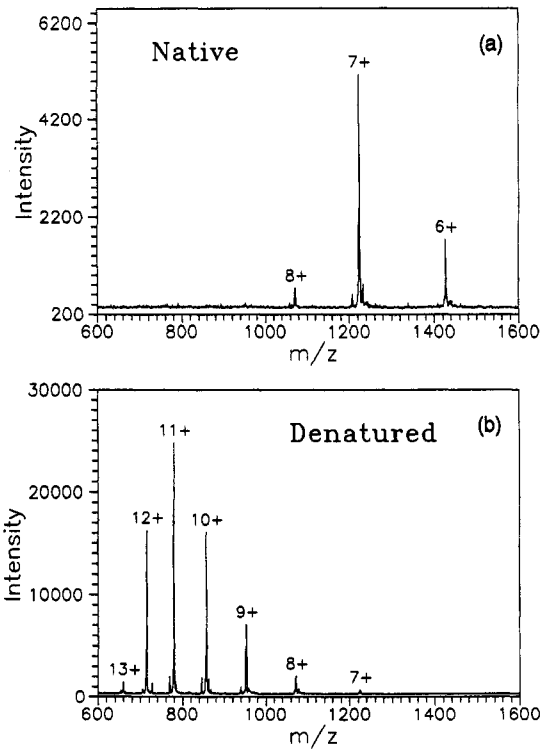

Figure 3. Electrospray ionization mass spectra of bovine ubiquitin obtained (a) from 0.1% aqueous acidic acid (pH 3.2) and (b) 1:1 (v/v) methanol: aqueous HCl (pH 2.3). The labels indicate the protonation states. The two spectra show significant differences in their charge distributions.

(see Figure 2). The cumulative effects of both low pH and the presence of methanol were required to effect the denaturation of ubiquitin.

Charge Distributions. The electrospray mass spectra obtained from ubiquitin solutions in which the protein is respectively in the native and denatured forms showed marked differences (Figure 3). The spectrum obtained from tightly folded ubiquitin (0.1% aqueous acetic acid, pH 3.2) is dominated by the $(M + 8H)^{8+}$, $(M + 7H)^{7+}$, and $(M + 6H)^{6+}$ ions (abbreviated as 8+, 7+, and 6+). This result is in concert with the expectation that native proteins will acquire a net positive charge lower than would be expected from the intrinsic p*K* values of the side chains. Figure 3b shows the electrospray mass spectrum obtained from a solution

(21) McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 329. Verma, S.; Pomerantz, S. C.; Sethi, S. K.; McCloskey, J. A. *Anal. Chem.* **1986**, *58*, 2898–2902.
(22) Wilkinson, K. D. In *Ubiquitin*; Rechsteiner, M. Ed.; Plenum Press: New York, 1988.
(23) Vijaya-Kumar, S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531–44.
(24) Neumann, N.; Snatzke, G. In *Proteins: Form & Function*; Bradshaw, R. A., Purton, M., Eds.; Elsevier Trend Journal: Cambridge, 1990.
(25) Wilkinson, K. D.; Mayer, A. N. *Arch. Biochem. Biophys.* **1986**, *250*, 390–99.
(26) Cary, P. D.; King, D. S.; Crane-Robinson, C.; Bradbury, M.; Rabbani, A.; Goodwin, G. H.; Johns, E. *Eur. J. Biochem.* **1980**, *112*, 577–88.

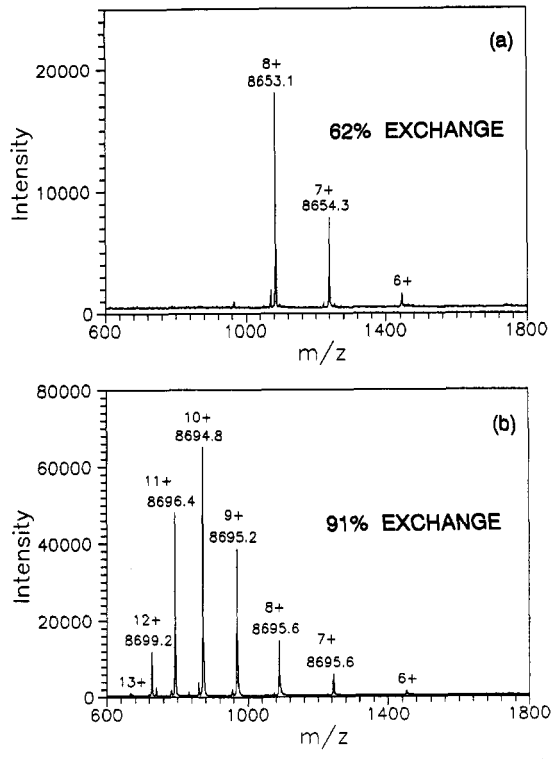

Figure 4. Electrospray ionization mass spectra of bovine ubiquitin (in deuterated solvents) obtained by spraying the protein solution into a dry air atmosphere. The labels on the peaks give the measured molecular mass derived from the protonation state indicated. The increase in molecular mass measured over the molecular mass of ubiquitin provides a measure of the average number of hydrogens exchanged. (a) The spectrum obtained 20 min after dissolving the protein (30 μM) in 1% CH_3COOD in D_2O. (b) The spectrum obtained 23 min after dissolving the protein (30 μM) in 1:1 (v/v) CH_3OD:1% CH_3COOD in D_2O.

(1:1 (v/v) methanol:aqueous HCl (pH 2.3)) where ubiquitin is known to be fully denatured. The spectrum shows a wider distribution and a much higher average charge state than that obtained from the native protein. The distribution exhibited charge states from 13+ to 7+, with 11+ being the most intense.

Unfolding of a protein results in changes in the effective pK values of the side chains toward the intrinsic pK values.[10,11] As a result, the net positive charge on the denatured protein is predicted to be much higher than that on the native protein, and the mass spectrometric results shown in Figure 3 are in agreement with this prediction.[4,5] Indeed, the spectrum of denatured ubiquitin shows protonation states ranging all the way to 13+, consistent with protonation of all 13 basic sites and neutralization of all 12 acidic sites in a fraction of the ubiquitin molecules.

Hydrogen/Deuterium Exchange in Ubiquitin. Ubiquitin contains a total of 144 labile hydrogens with 72 on the side chains and 72 on the backbone. Figure 4a shows the electrospray mass spectrum obtained 20 min after dissolving the protein in 1% CH_3COOD in D_2O. The measured molecular masses deduced from the m/z values of the 8+ and 7+ ions agree closely (average molecular mass, 8653.7 u) and are higher than the molecular mass of ubiquitin (8565.0 u) by 88.7 u. These results indicate that an exchange of 88.7 hydrogens occurred in 20 min. As predicted for a tightly folded protein, the exchange is much less than complete, i.e. an exchange of only 62% of all labile hydrogens present in the protein. NMR experiments have previously demonstrated that some hydrogens in the native state exchange extremely slowly, and even after a several day incubation in D_2O at room temperature, the exchange was found to be incomplete.[26]

The mass spectrum shown in Figure 4b was obtained 23 min after dissolving the ubiquitin in deuterated denaturing methanolic solvent (1:99:100 (v/v/v) CH_3COOD:D_2O:CH_3OD). Although

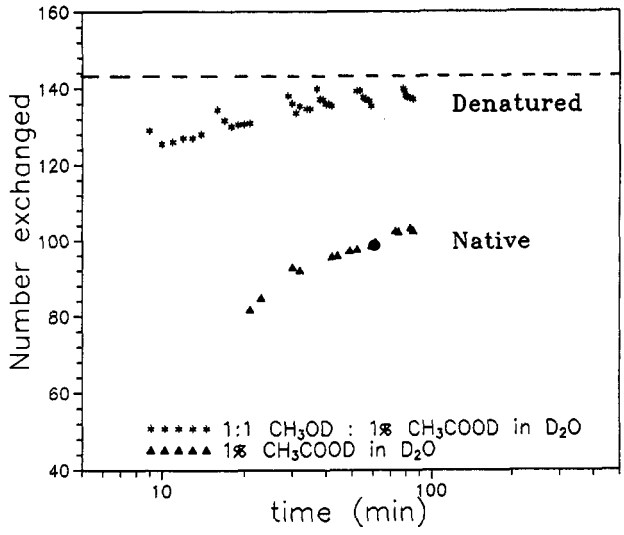

Figure 5. Plot of the number of hydrogen exchanges as a function of time occurring in bovine ubiquitin in (bottom curve) 1% CH_3COOD in D_2O and (top curve) 1:1 (v/v) CH_3OD:1% CH_3COOD in D_2O. The time zero refers to the time the protein was dissolved. The dashed line indicates the total number of exchangeable hydrogens in bovine ubiquitin.

the exchange time interval is close to that used in obtaining the spectrum shown in Figure 4a, a much higher level of exchange is evident (131 hydrogens, i.e. an exchange of 91% of all the labile hydrogens present in the protein). These results indicate that denaturation renders the majority of the labile hydrogens accessible to the solvents.

We were able to follow the total number of hydrogens exchanged as a function of time by measuring the m/z values at various intervals after dissolution of the protein (see Figure 5). For the native protein (bottom curve), a subpopulation of ubiquitin hydrogens exchange rapidly (89 hydrogens or 62% of the exchangeable hydrogens in the first 20 minutes). This subpopulation is expected to consist mainly of side chain hydrogens and solvent-exposed amide hydrogens. After this interval, the exchange slowed dramatically, levelling off to 105 exchanges (73%) in 90 minutes. Even after leaving the solution in an airtight vial for a week at room temperature, only 130 exchanges (90%) were measured. This slow exchange behavior confirms that the ubiquitin molecules in the solution used to obtain Figure 4a are present in a tightly folded form. The top curve of Figure 5 shows results obtained from the denatured protein. The measured rate of exchange is much higher (131 hydrogens or 91% of all the labile hydrogens in the first 23 minutes) than that obtained with the tightly folded form of the protein. The exchange level reached 96% in one hour and thereafter did not change significantly.

Hydrogen/Deuterium Exchange in Lysozyme. Chicken egg lysozyme is a protein (molecular mass 14 305 u) with four disulfide bonds that infer upon it resistance to denaturation. The protein contains 129 amino acid residues (19 basic groups, 10 acidic residues) and a total of 255 labile hydrogens with 126 on the amide backbone. Results from NMR experiments indicate that, in the unreduced native protein, some of the labile hydrogens are not exchanged even after incubation in solution (pH 4.2) at room temperature for 25 days.[27] The electrospray spectrum obtained from chicken egg lysozyme obtained 30 min after dissolving the protein in 1% CH_3COOD in D_2O (temperature 23 C) is shown in Figure 6a. The spectrum is dominated by the protonation state 10+ (range 8+ to 11+) even though the protein has 19 basic sites, and is similar to spectra reported previously.[5,28] The molecular mass measured from the m/z values of these peaks is

(27) Delepierre, M.; Dobson, C. M.; Howarth, M. A.; Poulson, F. M. *Eur. J. Biochem.* **1984**, *145*, 389–95.

(28) Loo, J. A.; Edmunds, C. G.; Udseth, H. R.; Smith, R. D. *Anal. Chem.* **1990**, *62*, 693–8.

Table I. A Comparison of the Percentage of Labile Hydrogens Exchanged in 30 min for Five Proteins[a]

protein	solvent	no. of possible exchanges	% exchanged in 30 min	state
bovine ubiquitin	1% CH_3COOD in D_2O	144	65	native
bovine ubiquitin	CH_3OD:1% CH_3COOD in D_2O (1:1)	144	94	denatured
C.E. lysozyme (native)[b]	1% CH_3COOD in D_2O	255	62	native
C.E. lysozyme (reduced)[b]	1% CH_3COOD in D_2O	263	96	denatured
bovine carbonic anhydrase	2% CH_3COOD in D_2O	450	99	denatured
equine myoglobin	1% CH_3COOD in D_2O	262	96	denatured
bovine hemoglobin	CH_3OD:2% CH_3COOD in D_2O (1:1)	228 (α)	97	denatured
		230 (β)	94	denatured

[a] The solvents used and the protein conformational state are also given. [b] C.E. lysozyme designates chicken egg lysozyme.

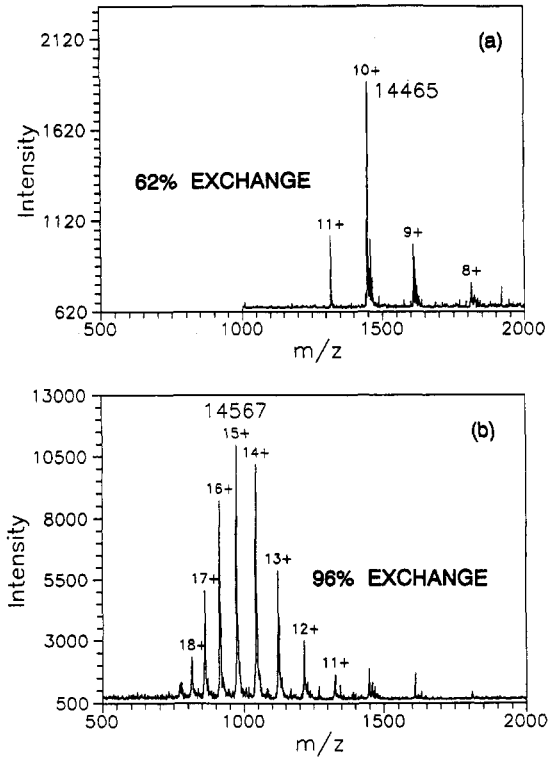

Figure 6. Electrospray ionization mass spectra of chicken egg lysozyme (a) 30 min after dissolving the native protein in 1% CH_3COOD in D_2O and (b) 30 min after dissolving the disulfide bond reduced protein in 1% CH_3COOD in D_2O. The labels on the peaks give the measured molecular mass derived from the protonation state indicated. The spectra exhibit marked differences in charge state distribution and extent of H/D exchange.

14 465 u, indicating an exchange of 159 hydrogens, i.e. 62% of the total of 255 labile hydrogens. The charge distribution and hydrogen exchange data suggest that lysozyme in this solution exists in a tightly folded conformation. Addition of an equal volume of deuterated methanol to the solution neither changed the charge distribution nor increased the hydrogen exchange rates. The disulfide bonds present in lysozyme apparently provide sufficient additional stabilization to prevent the protein from unfolding under these conditions.

Figure 6b shows the electrospray mass spectrum obtained 30 min after dissolving the reduced form of lysozyme in 1% CH_3COOD in D_2O. The spectrum is markedly different from the one shown in Figure 6a, both in the observed distribution of charge states and in the hydrogen exchange levels. The charge distribution is centered at the protonation state 15+ (range 9+ to 19+), which is significantly higher than that observed for unreduced lysozyme.[28] The measured mass from these peaks is 14 567 u, which is higher by 253 u than the molecular mass of reduced lysozyme (14 313 u; reduction of the disulfide bonds increases the molecular mass by 8 u as well as creates an additional eight labile hydrogens on the protein). The increase in mass indicates an exchange of 96% of the total 263 labile hydrogens. Both the charge distribution and hydrogen exchange results suggest that the reduced lysozyme is in an unfolded state in this solution (1% CH_3COOD in D_2O).

Results of the application of the method to five different proteins are shown in Table I as percent exchanged in 30 min at room temperature (23 °C) after dissolving the proteins in deuterated solvents. Some of these proteins, like bovine carbonic anhydrase II, equine myoglobin, and bovine hemoglobin, were only analyzed in the denatured state because of limitations in the m/z range of our quadrupole instrument. The data show that for solution conditions under which the proteins were denatured, more than 94% of the labile hydrogens are exchanged within 30 min at room temperature.

Conclusion

The present results demonstrate that it is feasible to probe conformational changes in proteins by hydrogen exchange electrospray ionization mass spectrometry. The method is straightforward to implement and sensitive. At present, we cannot identify the precise locations of hydrogens in the folded protein that are or are not exchanged. One possible way of identifying such sites of exchange is to utilize methods of collision activated dissociation of the ions produced from an exchange-labeled protein in the folded state. Another possible method would be to digest the labeled protein in its folded state very rapidly by chemical or enzymatic means and then to determine the individual pieces in the digest by mass spectrometric means to identify the number of hydrogens exchanged in each peptide.[29]

Acknowledgment. We thank Dr. Francis Picart (Rockefeller University) for assistance with the operation of the circular dichroism instrument. This work was supported in part by Grants RR00862, RR07065, and GM38274 (NIH).

(29) Thevenon-Emeric, G.; Kozlowski, J.; Zhang, Z.; Smith, D. L. *Anal. Chem.* **1992**, *64*, 2456–8. Zhang, Z.; Smith, D. L. *Protein Science* **1993**, *2*, 522–531.