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Conformational Changes in Proteins Probed by Hydrogen-exchange Electrospray-ionization Mass Spectrometry

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Hydrogen-exchange electrospray-ionization mass spectrometry is demonstrated to be an effective new method for probing conformational changes of proteins in solutions. 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 hydrogen-exchange electrospray-ionization mass spectrometry is used to probe conformational changes in bovine ubiquitin induced by the addition of methanol to aqueous acidic solutions of the protein.

In their native state, globular proteins are tightly folded, compact structures. They 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] A variety of physical techniques has been applied to monitor these conformational changes including optical rotation, spectrophotometry, viscometry, fluorescence, circular dichroism, and nuclear magnetic resonance (NMR).[1–3] Some of these methods measure average changes in the overall conformation of proteins (e.g., circular dichroism). Others (e.g., NMR), yield information concerning the precise spatial relations of particular component atoms in the different protein forms.

In a recent Communication, we demonstrated that conformational changes in proteins can also be detected by mass spectrometry.[4] Conformation is probed by the extent to which proteins are observed to be protonated in electrospray ionization, which is a gentle method of ionization that produces intact multiply protonated gas-phase ions directly from protein molecules in solution.[5] The multiple protonation occurs as a result of proton attachment to the *available* basic sites in the molecule. The availability of ionizable basic sites is determined by the precise conformation the protein assumes under the conditions of study. In general, a protein in a tightly folded conformation is expected to have fewer basic sites available for protonation compared to the same protein in an unfolded conformation. During the course of a series of experiments designed to test rigorously our hypothesis that the observed changes in the charge distributions reflect changes in the solution-phase tertiary structure of the proteins, we developed a new technique for probing conformational changes, i.e., hydrogen-exchange electrospray-ionization mass spectrometry.

In small peptides, all the labile hydrogen atoms are readily accessible to the solvents in which the peptides are dissolved. Thus rapid hydrogen/deuterium exchange is expected to occur when such peptides are dissolved in D_2O.[3,6] Although the rates of exchange differ widely between different labile hydrogen atoms in a peptide and are a strong function of pH, the exchange should be complete within minutes even for the slowly exchanging amide backbone hydrogens.[3] The same high degree of exchange is expected for many proteins in unfolded conformations (denatured states) where the exchange of all the labile hydrogens with deuterium can be a rapid process.[7] On the other hand, proteins in tightly folded conformations (native states) have a considerably lower number of hydrogens that are readily accessible to the solvent and available for exchange. The inaccessibility of the remaining exchangeable hydrogens arises because some are buried in the hydrophobic core of the protein or are involved 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. These differences in exchange behavior of folded and unfolded proteins have been used to identify intermediates in protein folding by NMR.[8]

A combination of hydrogen exchange with electrospray-ionization mass spectrometry would thus appear 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 masses as well as in charge distributions. We have successfully achieved this combination of techniques and in this Communication, report a study of conformational changes in bovine ubiquitin induced by the addition of methyl alcohol to aqueous acidic solutions of the protein.

EXPERIMENTAL

Bradykinin, bovine ubiquitin (catalog no. U6253), D_2O (99.9 atom% D), CH_3COOD (98 atom% D) and CH_3OD (99.5 atom% D) were obtained from the Sigma Chemical Co. (St Louis, MO, USA). High-purity water and methanol were from Burdick and Jackson (Muskegon, MI, USA), and ultrapure acetic acid was from J. T. Baker (Phillisburgh, NJ, USA).

The electrospray-ionization mass spectrometer used

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in these experiments was constructed in our laboratory and has been described previously.[9] Briefly, the charged droplets produced by electrospray at atmospheric pressure, are focused and transported through a 0.5 mm ID, 20 cm long stainless-steel capillary tube into a region maintained at 1–10 Torr. Desolvation of the analyte ions is achieved by controlled heating of the capillary tube as well as by collisional activation induced by an electrostatic field in the intermediate pressure region (1–10 Torr) between the capillary exit and a coaxial skimmer. All the solutions (aqueous acidic with or without the addition of methanol) were electrosprayed from a stainless-steel syringe needle (90° cut, 100 μm ID, 228 μm OD) at rates of 0.5–1.0 μL/min.[10]

The hydrogen exchange experiments conducted on bradykinin were made in H_2O or D_2O, without the addition of any acids or buffers. For experiments in which we desired to maintain the protein in a tightly folded state, the spray solution was prepared by dissolving bovine ubiquitin in D_2O containing 1% CH_3COOD at concentrations of 10–30 μM. Mass spectra were then obtained as a function of time after dissolution of the protein. For investigations of the unfolded states of bovine 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.

Circular dichroism (CD) experiments were carried out on an AVIV model 62DS (Aviv Associates, Lakewood, NJ, USA) CD spectrometer using a 1 mm pathlength cuvette.

RESULTS AND DISCUSSION

To establish a reliable method for measuring hydrogen/deuterium exchange with electrospray-ionization, we selected a test compound in which all the exchangeable hydrogens are readily accessible to the solvent. The test compound was the nine-residue peptide bradykinin (sequence RPPGFSPFR, molecular mass 1060.2 u), containing a total of 17 labile hydrogens in the neutral molecule. Electrospray-ionization of bradykinin from H_2O solutions produced a dominant $(M+2H)^{2+}$ ion at m/z 531.1. Contrary to our expectations, our first electrospray-ionization measurements of bradykinin in D_2O yielded masses that indicated incomplete exchange (typically <75% even after 1 h). We discovered that this incomplete exchange was the result of rapid back-exchange of labile deuterium atoms with hydrogen in the water vapour of the ambient laboratory air during spraying.[11] 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 observed) and flushed with dry air (Matheson, E. Rutherford, NJ, USA) continuously at rates of 2–3 L/min. This improvement enabled us to measure much higher exchange levels in D_2O. Thus, for example, after a 5 min incubation in D_2O, the original $(M+2H)^{2+}$ ion peak at m/z 531.1 was completely replaced by a new $(M_D+2D)^{2+}$ ion peak with m/z 540.4. The measured molecular mass of this exchanged species, M_D, is $(540.4\times2)-(2\times2)=1076.8$ u, an increase of 16.6 u over the molecular mass of unexchanged bradykinin (molecular mass 1060.2 u). The

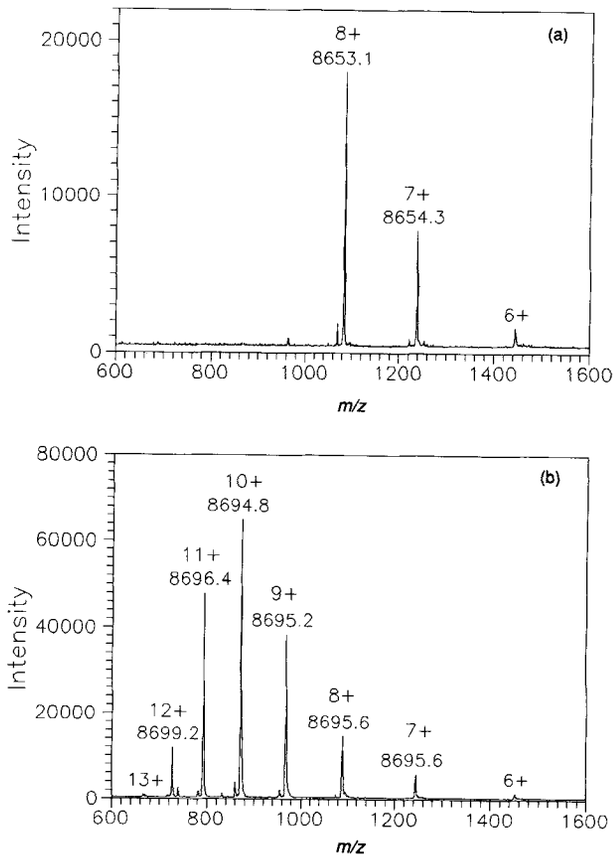

Figure 1. (a) Electrospray-ionization mass spectrum of bovine ubiquitin obtained 20 min after dissolving the protein in D_2O containing 1% CH_3COOD. (b) Electrospray-ionization mass spectrum of bovine ubiquitin obtained 23 min after dissolving the protein in a solution containing $CH_3COOD + D_2O + CH_3OD$ in a volume ratio 1:99:100. The labels on the peaks indicate the neutral molecular masses deduced from the measured m/z values of the individual peaks. The protonation state, $n+$, of each ion species is also given.

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%.

Next, we applied the method to a study of conformational changes in ubiquitin. Bovine ubiquitin, in its native form, is a small, tightly folded protein (76 residues, molecular mass 8565.0 u, no disulfide bonds, 13 basic sites) that is very resistant to denaturation.[12] Figure 1 compares the electrospray ionization mass spectra of bovine ubiquitin obtained (a) from a solution in which the protein is in a tightly folded conformation and (b) from a solution in which the protein is unfolded. The spectrum shown in Fig. 1(a) was obtained by electrospraying a solution of ubiquitin dissolved in $CH_3COOD + D_2O$ (1:99); exchange was allowed to occur at room temperature (23 °C) for 20 min prior to acquisition of the mass spectrum. NMR studies[13] and circular-dichroism measurements made by other workers[14] and by us (data not shown) confirm that, under these solution conditions, the protein is in a tightly folded conformation. The spectrum shown in Fig. 1(b) was obtained by electrospraying a solution of ubiquitin dissolved in $CH_3COOD + D_2O + CH_3OD$ (1:99:100). Exchange was allowed to occur at room temperature for 23 min prior to acquisition of the mass spectrum. Circular-dichroism measurements (data not shown) indicated that, under these solution conditions,

the protein undergoes a large conformational change and unfolds (see also Ref 14).

The spectra given in Fig. 1(a) and (b) are strikingly different with respect both to their charge distributions (i.e., the extent of protonation of the protein) and to their masses (i.e., the amount of hydrogen/deuterium exchange that has occurred).

Charge distributions

The observed charge distribution for tightly folded ubiquitin in Fig. 1(a) is dominated by the $(M+8H)^{8+}$ and $(M+7H)^{7+}$ ions (abbreviated 8+ and 7+), indicating that, on average, only 7 or 8 of the 13 basic sites are protonated. This finding is in concert with the known crystal structure of ubiquitin[12,15] where it has been found that the N-terminal amino group, Lys-48, Lys-29, Lys-27, and Lys-11 are involved in hydrogen bonding and/or salt bridges and may not be readily accessible to the solvents. Arg-74, Arg-72, Arg-42, Lys-63, Lys-33, and Lys-6 are clearly exposed to the solvent. His-68 and Arg-54 may only be partially exposed to the solvent. Thus, in the folded state of ubiquitin only 7 or 8 basic sites may be readily available for protonation.

On the other hand, the unfolded form of ubiquitin obtained from the methanolic solution (Fig. 1(b)) exhibits a charge distribution that ranges from 6+ to 13+, with 10+ the most intense charge state. The higher average charge confirms our hypothesis that the protein unfolds when methanol is added to the solution and renders more basic sites accessible for protonation. These findings concerning the dependence of the observed charge states on the conformation of ubiquitin are similar to our earlier findings with cytochrome c[4] in which the conformational changes were induced by altering the pH of the spray solutions.

Hydrogen/deuterium exchange

The spectrum shown in Fig. 1(a) was obtained from the tightly folded form of ubiquitin, 20 min after dissolution in the deuterated solvents. The measured molecular masses deduced from the m/z values of the 8+ and 7+ ions agree to within 1.2 u (average molecular mass 8653.7 u) and are higher than the molecular mass of unexchanged 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 (ubiquitin has a total of 144 labile hydrogen atoms with 72 on the side chains and 72 on the amide backbone). NMR experiments have previously demonstrated[13] that some hydrogens in native ubiquitin exchange very slowly, and even after a several-day incubation in D_2O at room temperature, exchange of all the hydrogens did not occur. We were able to follow the total number of hydrogens exchanged as a function of time by obtaining mass spectra at different intervals after dissolution of the protein. A subpopulation of ubiquitin hydrogens exchanged rapidly (89 exchanges or 62% of exchangeable hydrogens during the first 20 min) whereupon the exchange rate slowed dramatically, levelling off to 105 exchanges (73%) in 90 min. Even after a week at room temperature, the total number of exchanges was only 130 (90%). The large subpopulation of slowly exchanging hydrogens confirms that the ubiquitin molecules in the solution used to obtain Fig. 1(a) are present in a tightly folded form.

The mass spectrum shown in Fig. 1(b) was obtained 23 min after dissolving the protein in the deuterated denaturing methanolic solvent. Although the exchange time interval is close to that used to obtain the spectrum shown in Fig. 1(a), a much higher exchange of hydrogens is evident (131 hydrogens, i.e., an exchange of 91% of all labile hydrogens present in the molecule). The exchange level reached 96% in 1 h and thereafter changed very slowly. These results demonstrate that denaturation effected by the addition of CH_3OD exposes a large number of labile hydrogens to exchange as well as several additional basic groups to protonation.

CONCLUSION

The results presented here demonstrate that hydrogen-exchange electrospray-ionization mass spectrometry provides an effective new means for probing conformational changes in proteins. The average hydrogen/deuterium exchange rate can be followed as a function of time even for small quantities of protein (<100 pmol). At present we cannot identify the precise locations of hydrogens that have exchanged in the folded protein. One possible way of identifying these locations is to utilize methods of collisionally activated dissociation. Another possible method involves rapid digestion of the labelled protein in its folded state by chemical or enzymatic means and mass spectrometric identification of the peptide products to identify the number of hydrogens exchanged in each peptide. The protein/solvent systems that can be investigated effectively with the present technique are limited to those that produce intense electrospray-ionization mass spectra. This requirement imposes constraints on the solution compositions; for example, solutions containing high concentrations of salts or buffers cannot be used. The method also provides a measure of the fraction of basic sites on a protein in a given conformation that are available for protonation.

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