AC RESEARCH

# Heat-Induced Conformational Changes in Proteins Studied by Electrospray Ionization Mass Spectrometry

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**A simple and effective device for investigating heat-induced denaturation of proteins by electrospray ionization mass spectrometry is described. Results are presented for the denaturation as a function of temperature and solution pH of bovine ubiquitin and bovine cytochrome *c*. These results are in concert with and extend the earlier results of LeBlanc et al. (*Org. Mass Spectrom.* 1991, *26*, 831). The cooperative effects of pH and temperature on the denaturation of ubiquitin and cytochrome *c* were investigated. Electrospray ionization mass spectrometry is also shown to be a useful probe of the reversibility of heat-induced denaturation of proteins. Finally, it is demonstrated that heat-induced denaturation can be used to improve the mass spectrometric response of proteins that do not normally yield useful spectra when the solubilized protein is electrosprayed at ambient temperatures.**

## INTRODUCTION

In their native state, globular proteins are tightly folded, compact structures that can be denatured and caused to unfold by subjecting them to extremes of pH, denaturants such as guanidinium hydrochloride, organic solvents, detergents, and high temperatures.[1] A variety of techniques have been applied to the measurement of conformational changes of proteins in solution, including acid–base titrations, calorimetry, spectrophotometry, viscometry, circular dichroism, fluorescence, and nuclear magnetic resonance.[2,3] Recently, we and others have demonstrated that conformational changes of proteins can also be detected by electrospray ionization mass spectrometry.[4–8]

Electrospray is a gentle method of ionization that produces intact, multiply protonated gas-phase ions directly from protein molecules in solution.[9–12] The multiply charged ions observed in the positive-ion spectra are produced by proton attachment to basic and deprotonated acidic sites in the protein and reflect, to some extent, the degree of protonation in solution.[13] Because the accessibility and the effective p$K$ values of the acidic and basic side chains are determined by the precise conformation that the protein assumes under the conditions of study, the conformation can be probed by the extent to which the protein is observed to be protonated in electrospray ionization.[4–8]

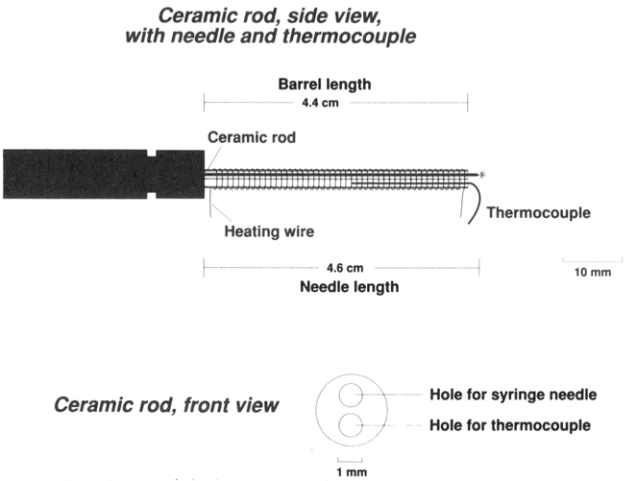


**Figure 1.** Apparatus for heating and controlling the temperatures of protein solutions just prior to electrospray.

Chowdhury, Katta, and Chait[4] demonstrated this new probe in an investigation of conformational changes in bovine cytochrome *c*, bovine ubiquitin, and yeast ubiquitin induced by changes in pH and by the addition of organic solvent denaturants. Dramatic differences were observed in the charge state distributions obtained from the native versus denatured proteins. The conformations of these proteins were

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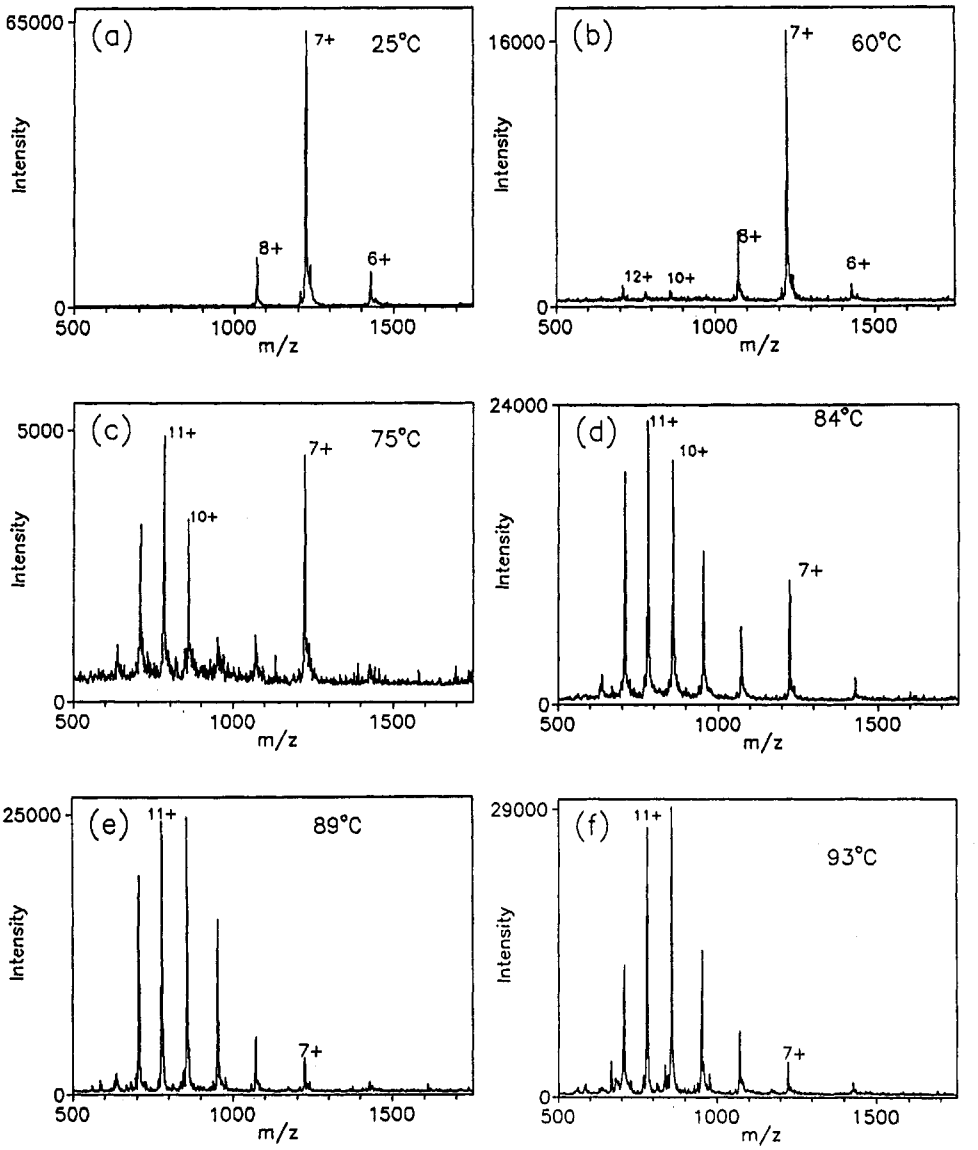


**Figure 2.** Electrospray ionization mass spectra of bovine ubiquitin in water and acetic acid (pH 2.8) obtained at a series of different temperatures. Protein concentration: $2 \times 10^{-5}$ M.

also probed by electrospray ionization mass spectrometric determination of the rate of hydrogen/deuterium exchange in solution.[5] The rate of hydrogen/deuterium exchange was observed to be much enhanced in the unfolded forms of the proteins versus the folded forms, and the rates of exchange correlated closely with the observed differences in charge-state distributions of the different forms as well as changes in conformation determined by circular dichroism measurements. Loo et al.[7] have used charge-state distribution determinations by electrospray ionization mass spectrometry to monitor changes in the higher order structures of hen egg lysozyme, bovine ubiquitin, and yeast ubiquitin induced by changes in pH and organic solvent composition and have noted significant changes in the charge-state distributions of the oxidized and reduced forms of several proteins. Le Blanc et al.[8] studied the effects of heat on the electrospray spectra of a number of globular proteins, observing a dramatic increase with increasing temperature of the charge states of ions produced from equine cytochrome *c* and chicken egg lysozyme as well as a strong increase of the ion intensity from cytochrome *c*. These workers attributed the changes in the observed charge states to heat-induced denaturation of the proteins, leading to an effective increase in the p$K_a$ values of the carboxylic acid groups. Allen and Vestal[14] have described an electrospray interface in which the spray chamber is heated. These authors have proposed that the special facility of their source for producing intense spectra from certain proteins that do not normally give intense electrospray ion peaks (e.g. subtilisin Carlsberg) may be related to the heating of the sample.

In the present study, we describe a simple and effective device for investigating heat-induced denaturation of proteins by electrospray ionization mass spectrometry. Results are presented for the denaturation as a function of temperature and solution pH of bovine ubiquitin and bovine cytochrome *c*. These results are in concert with and extend the earlier results of LeBlanc et al.[8] Electrospray ionization mass spectrometry is also shown to be a useful probe of the reversibility of heat-induced denaturation of proteins. Finally, it is demonstrated that heat-induced denaturation can be used to improve the mass spectrometric response of proteins (such as wheat germ agglutinin) that do not normally yield useful spectra when the solubilized protein is electrosprayed at ambient temperatures.

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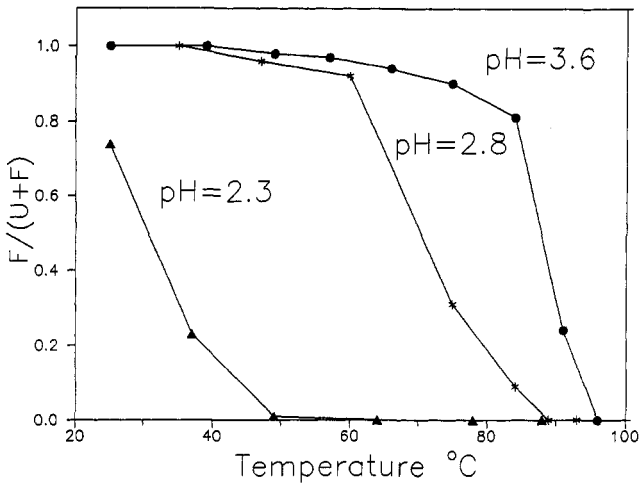


**Figure 3.** Denaturation transition curves for bovine ubiquitin plotted as a function of temperature for three different values of solution pH. *F* designates a tightly folded form of ubiquitin and *U* designates less tightly folded forms of the protein. See ref 20 for a discussion of the method by which the ratio $F/(U + F)$ was determined.

## EXPERIMENTAL SECTION

Figure 1 shows the apparatus that we constructed for the purpose of controlling and measuring the temperature of the solution just prior to electrospraying. It consists of a cylindrical ceramic rod (length 4.4. cm, diameter 3 mm) containing two holes (diameter 1 mm) running parallel to the long axis of the rod. A stainless steel electrospray syringe needle (length 4.6 cm, o.d. 0.7 mm, i.d. 0.15 mm, electropolished to produce a sharpened tip[15]) is inserted through one of the holes in the ceramic rod such that the syringe needle is covered along its length by the rod and protrudes from the ceramic rod for a length of 2 mm. An iron/constantan thermocouple (connected to an Omega Engineering Model DP-701 thermocouple gauge) is inserted into the second hole in the ceramic rod to provide a measure of the temperature of solutions that are pumped through the electrospray syringe needle. A chromel wire was wound tightly around the outside of the ceramic rod and cemented in place with a layer of high-temperature castable ceramic (Aremco Products Inc., NY). The temperature of the ceramic rod and the protein solution flowing through the electrospray syringe needle is controlled by passing an appropriate current through the chromel heating wire using a low-voltage power supply (Kepco Model JQE, 0–6 V, 0–10 A). Heating wire currents in the range 0–3.5 A produced electrospray syringe needle temperatures in the range 25–96 °C. Because the dead volume in the heated syringe needle is 1 μL and the flow rate of the protein solution is typically 0.5 μL/min, the solution is normally heated for at least 2 min at the controlled temperature prior to electrospray ionization. Temperature measurements were made prior to and after each electrospray ionization mass spectrometric measurement by inserting the thermocouple wire into the second hole in the ceramic rod. The temperatures prior to and after electrospray generally coincided to within ±1 °C. Temperature measurements were not normally made during the electrospray measurement to avoid the perturbing effect of the thermocouple wires on the field applied to the syringe needle. The walls of the ceramic rod provided adequate electrical insulation between the potentials applied to the spray capillary (<5 kV) and the heating wire (<5 V). The arrangement described above allowed protein solutions to be electrosprayed with temperatures ranging between 25 and 98 °C.

The electrospray ionization mass spectrometer and the sample preparation procedures have been described previously.[16] Briefly, the sample solution was pumped through the stainless steel syringe needle using a syringe pump (Harvard Model 2400-001) and electrosprayed in ambient laboratory air. The resulting highly charged droplets and solvated ions were transported into the vacuum of a quadrupole mass spectrometer (Vestec Model 201) through a 20-cm-long, 0.5-mm-i.d. heated capillary tube. The flow rate of the analyte through the spray needle (0.2–0.7 μL/min) was adjusted to produce stable electrospray and depended on the solvent composition and temperature of the spray solution. Electrospray was performed by applying a dc voltage of 3–5 kV to the syringe needle. The distance between the tip of the syringe needle and the capillary leading into the mass spectrometer vacuum ranged between 4 and 5 mm. The capillary leading into the mass spectrometer vacuum was sharpened by electropolishing to focus the field lines from the spray needle and improve the transport of charged droplets and ions into the capillary. The spectra were acquired using a commercially available data system (Tecknivent Vector II) on an IBM compatable computer. Data collection times ranged between 2 and 4 min.

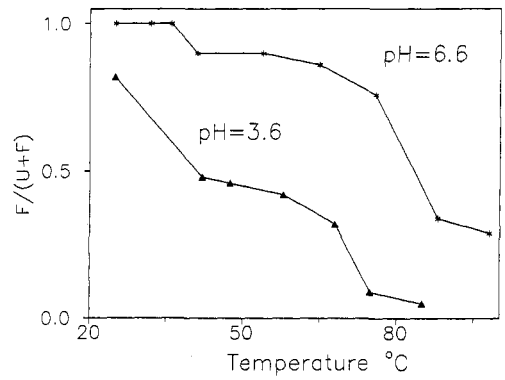


**Figure 4.** Denaturation transition curves for bovine cytochrome *c* plotted as a function of temperature for two different values of solution pH. *F* designates a tightly folded form of cytochrome *c* and *U* designates less tightly folded forms of the protein. See ref 20 for a discussion of the method by which the ratio $F/(U + F)$ was determined.

The protein samples were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. The samples, catalog numbers, and molecular masses (MM) are bovine ubiquitin (U-6253, MM 8565 u), bovine cytochrome *c* (C-3256, MM 12 229 u), chicken egg lysozyme (L-6876, MM 14 306 u), thermolysin from *Bacillus proteolysis* (P-1512, MM 34 420 u), subtilisin Carlsberg from *Bacillus subtilis* (P-5380, MM 27 300 u), and wheat germ agglutinin (L-9640, MM 17 000 u). The protein concentrations of the electrospray solutions were in the range 10–20 μM.

## RESULTS AND DISCUSSION

**Denaturation of Bovine Ubiquitin.** Our first studies of heat-induced conformational changes by electrospray ionization mass spectrometry were carried out on bovine ubiquitin. This protein was chosen because we have previously carried out extensive mass spectrometric investigations of conformational changes in ubiquitin brought about by changes in pH and organic solvent content of the spray solution[5] and because heat-induced conformational changes in ubiquitin have previously been investigated by nuclear magnetic resonance measurements.[17,18] Bovine ubiquitin is a tightly folded protein (molecular mass 8565 u, no disulfide bonds, 13 basic sites, 4 acidic sites) that is very resistant to denaturation.[18] The extreme stability of the protein has been attributed to the pronounced hydrophobic core and the fact that some 90% of the residues in the polypeptide chain appear to be involved in intramolecular hydrogen bonding.[19]

Figure 2(a–f) compares the electrospray ionization mass spectra of bovine ubiquitin obtained at a number of different temperatures of the spray solution. In each case, the protein

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(19) Kumar, S. V.; Bugg, C. E.; Cook, W. J. *Mol. Biol.* **1987**, *194*, 531.

was sprayed from a 1% acetic acid solution in water (pH 2.8) at a flow rate of 0.5 μL/min. Figure 2a shows the mass spectrum of ubiquitin obtained at the ambient laboratory temperature (25 °C). The spectrum is dominated by an ion corresponding to the intact protein containing seven additional protons and conferring on it a charge state of +7. Small contributions from the adjacent +6 and +8 charge states are also observed. From previous NMR measurements[18] and from our earlier circular dichroism electrospray ionization studies,[5] we conclude that Figure 2a represents the spectrum obtained from the native state of the protein. Raising the temperature of the spray solution to 60 °C produces little change in the distribution of charge states (Figure 2b). As the temperature is further raised to 75 °C (Figure 2c), the mass spectrum undergoes a substantial change and a second distribution of charge states (centered about +11) becomes apparent. We[4] and others[7] have attributed such bimodal charge-state distributions to the presence in solution of two populations of conformers, one population that is relatively tightly folded and one that is less tightly folded. Further heating of the protein solution leads to a shift of the bimodal distribution (Figures 2d–f) toward the higher charge-state component. Above 89 °C there remains little evidence for the presence of the lower charge-state component.

The data given in Figure 2 are presented in a different form in the central curve shown in Figure 3, where the proportion of ionized molecules in the tightly folded conformation ($F$) relative to the total in both tightly ($F$) and loosely ($U$) folded conformations is plotted against temperature (see ref 20 for a discussion of the calculation of the values of $F$ and $U$). We interpret this data as evidence for a two-state transition, where the transition temperature for half-denaturation of bovine ubiquitin in aqueous acetic acid at pH 2.8 is 71 ± 5 °C (see ref 21 for a discussion of the uncertainty in this measure). Transition curves obtained from analogous experiments performed at pH 2.3 and pH 3.6 are also given in Figure 3, yielding half-denaturation temperatures of respectively 31 ± 5 and 88 ± 5 °C. The half-denaturation temperatures are observed to fall off rapidly with decreasing pH in accord with the expectation that heat and low pH act as additive denaturants.[2] Our findings are in agreement with the results of Cary et al.[18] who investigated the effects of heat on the NMR spectra of ubiquitin. At pH 3.9, with no added salt, these workers observed denaturation to occur as a two-state process over the range 70–95 °C with a half-denaturation temperature of 85 °C.

**Denaturation of Bovine Cytochrome *c*.** We have previously investigated the effect of pH, at a constant solution temperature (25 °C), on the electrospray ionization spectra of bovine cytochrome *c*,[4] finding dramatic variations in the charge-state distributions as a function of pH. For example, at pH 2.6, a single distribution was observed centered about the +16 charge state, which we identified with a denatured form of the protein. At pH 3.0, on the other hand, a bimodal distribution was observed where, in addition to the distribution centered around +16, a second distribution was observed centered about +8. The latter distribution was identified with a more tightly folded form of the protein. In the present set of experiments, electrospray ionization mass spectra of bovine cytochrome *c* were obtained at pH 3.6 for temperatures ranging between 25 and 85 °C and at pH 6.6 between 25 and 98 °C. Again, bimodal distributions of charge states were observed, leading to the temperature transition curves given in Figure 4. The shapes of these temperature transition curves appear more complex than those obtained for ubiquitin (Figure 3), with an indication of two temperature transitions. At pH 3.6 (bottom curve of Figure 4), the first transition occurs between 25 and 40 °C and the second between 67 and 75 °C. At pH 6.6, corresponding transitions take place at temperatures some 10 deg above those observed in the pH 3.6 solution, in accord with the greater predicted stability of cytochrome *c* at pH values closer to neutrality. We note that transitions between more than two conformational states of cytochrome *c*, at acidic pHs, have previouslybeen inferred from spectroscopic,[22] circular dichroism,[23] and mass spectrometric measurements.[4]

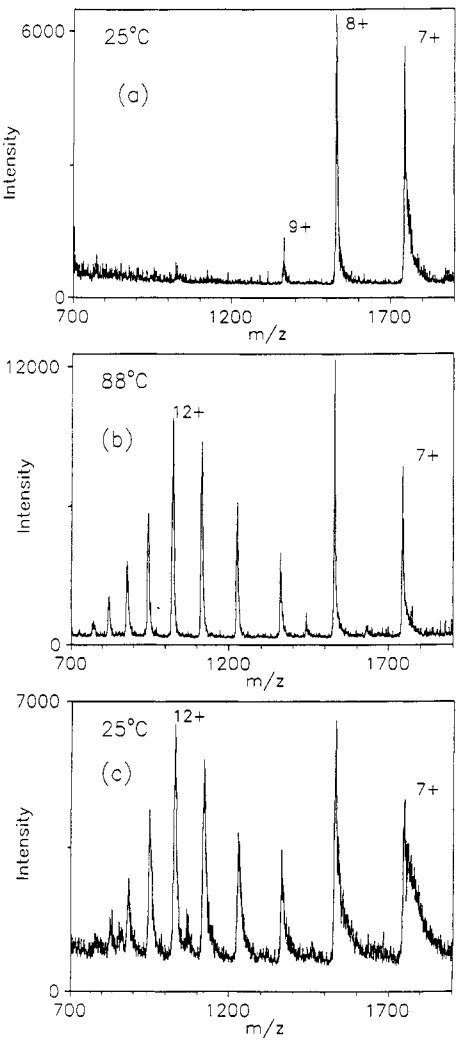


**Figure 5.** Illustration of a three step experiment designed to probe the reversibility of conformational changes in bovine cytochrome *c* by electrospray ionization mass spectrometry. The spectra were obtained by electrospraying bovine cytochrome *c* in water (pH 6.6, 5 mM ammonium acetate buffer) at a spray needle solution temperature of (a) 25 °C and (b) 88 °C. (c) Spectrum obtained after heating the protein solution at 90 °C for 4 min in a test tube and subsequently cooling the solution to 25 °C prior to electrospray.

**Probing the Reversibility of Conformational Changes by Mass Spectrometry.** Certain proteins, after being

(20) The values of $F$ (or $U$) were calculated by summing the heights of the spectral peaks corresponding to ions originating from proteins in tightly folded (or loosely folded) conformations. For example, the values of $F/(U+F)$ yielding the central curve in Figure 3 (pH = 2.8) were extracted from Figure 2 using the following assumptions: (i) The distribution of charge states obtained at room temperature (Figure 2a) originated completely from proteins in a tightly folded conformation. (ii) The distribution of charge states at 93 °C (Figure 2f) originated completely from proteins in a loosely folded conformation. At intermediate temperatures, the values of $U$ and $F$ were obtained by unfolding the distribution of charge states corresponding to proteins in tightly folded conformations from that corresponding to proteins in loosely folded conformations.

(21) The largest source of uncertainty in determining the half-denaturation temperatures arises from uncertainties in the efficiency of the mass analyzer as a function of $m/z$. For the present measurements we assume that the efficiency for the analysis and detection of ions between $m/z$ 800 and $m/z$ 1200 varies by less than a factor of 2.

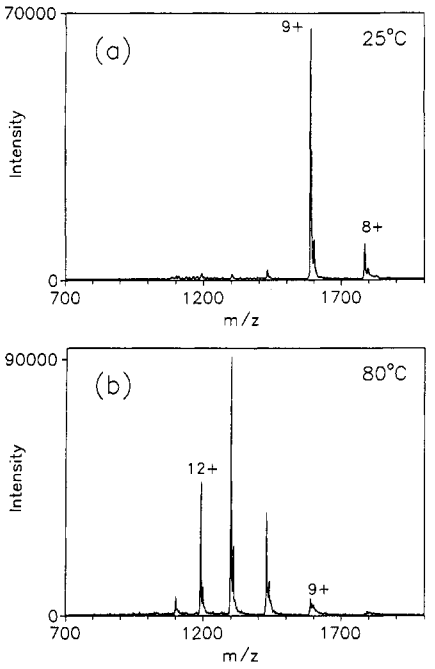


**Figure 6.** Electrospray ionization mass spectra of chicken egg lysozyme in water and acetic acid (pH 3.1), obtained at two different temperatures: (a) 25 °C; (b) 80 °C. Protein concentration: $1 \times 10^{-5}$ M.

subjected to heat-induced denaturation, are able to refold into their native forms when the solution temperature is lowered; others are irreversibly denatured.[2] To investigate the potential of electrospray ionization as a probe of the reversibility of conformational changes in proteins, the three-step experiment described below and illustrated in Figure 5 was carried out on a solution of bovine cytochrome *c* maintained at pH 6.6.

*Step 1.* A mass spectrum was obtained from a solution sprayed at 25 °C (Figure 5a). At this temperature, the protein is largely in a tightly folded conformation (see the top curve of Figure 4).

*Step 2.* A mass spectrum was obtained from a solution sprayed at 88 °C (Figure 5b). At this temperature, both folded and denatured forms of the protein are present in solution (see top curve of Figure 4).

*Step 3.* A solution of the protein was heated to 90 °C, in a test tube, maintained at this elevated temperature for 4 min, recooled to 25 °C, and then electrosprayed to give the spectrum shown in Figure 5c.

The spectrum obtained after recooling the solution to 25 °C (Figure 5c) has the same distribution of charge states as that obtained by electrospraying the solution at 88 °C, indicating that the protein has not renatured in the time required for cooling (25 min). A similar indication of irreversible denaturation was observed for a solution of cytochrome *c* maintained at pH 3.6 where the temperature was raised to 75 °C (data not shown). By contrast, an analogous experiment performed on bovine ubiquitin (pH 3.0) demonstrated complete reversibility of the denaturation observed upon heating the solution to 92 °C (data not shown). These results demonstrate that electrospray ionization provides a useful probe of the reversibility of heat-induced conformational changes in proteins.

**Improving the Mass Spectra of Proteins by Heating the Electrospray Solutions.** We have previously experienced considerable difficulty in obtaining useful electrospray ionization mass spectra of some members from certain classes of protein,[12] including those that contain a large number of disulfide bonds or are otherwise highly stabilized against unfolding. Because our analyzer has an upper $m/z$ limit of 2000, we hypothesized that one possible reason for this lack of success was the low number of charges that attach to these proteins.

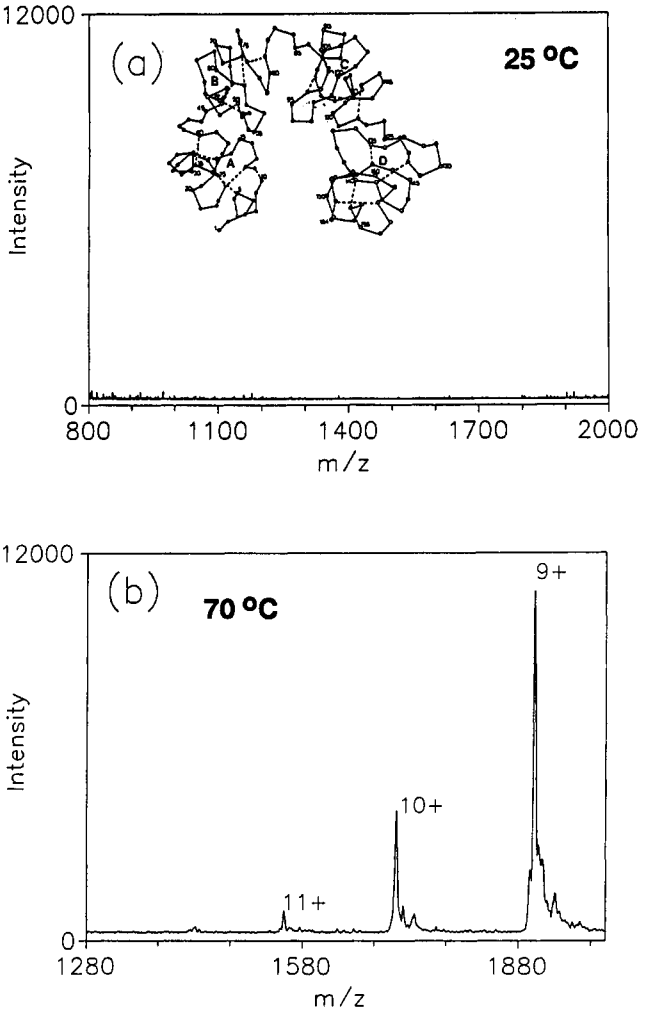


**Figure 7.** Electrospray ionization mass spectrum of wheat germ agglutinin in 45% water, 50% methanol, and 5% acetic acid obtained at two different temperatures: (a) 25 °C; (b) 70 °C. Protein concentration: $1 \times 10^{-5}$ M. The structure of wheat germ agglutinin is indicated in the top panel.

Chicken egg lysozyme is a highly basic protein($pI$ = 11.1) containing 4 disulfide bonds. Even though this protein contains a large number (19) of basic groups, the dominant ion in the mass spectrum obtained at 25 °C from an acidic solution (pH 3.1) of lysozyme contains only 9 positive charges and has a $m/z$ of approximately 1600 (Figure 6a). It is readily seen that a mass analyzer having a $m/z$ upper limit of less than 1600 would fail to yield a useful mass spectrum under these circumstances. Such a limitation could be eliminated by shifting the charge-state distribution to higher values, and as we have seen heat provides an effective means for bringing about such shifts. Figure 6b shows the spectrum of chicken egg lysozyme obtained by heating the spray solution to 80 °C, where the dominant ion species are now shifted into the $m/z$ range 1200–1400 (+12 to +10). The present result for lysozyme is in concert with that previously obtained by Le Blanc et al. using heat-induced denaturation[8] and Loo et al. using acid- and methanol-induced denaturation.[7]

A somewhat similar but more challenging example is provided by wheat germ agglutinin, a 171 amino acid residue

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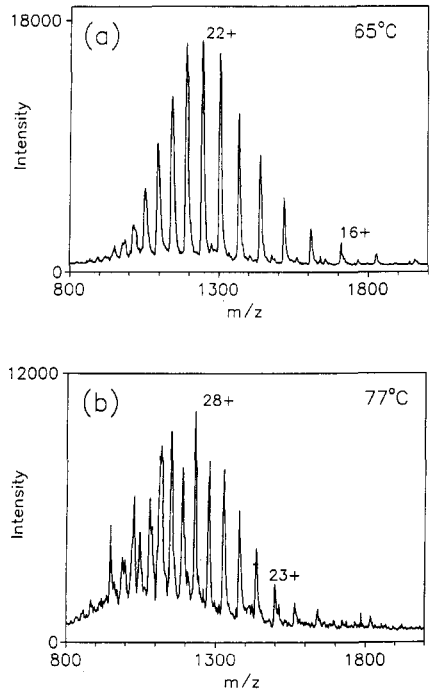


**Figure 8.** Electrospray ionization mass spectra of (a) subtilisin Carlsberg in 45% water, 50% methanol, and 5% acetic acid (pH 2.3) obtained at 65 °C and (b) thermolysin in 45% water, 50% methanol, and 5% acetic acid (pH 2.3) obtained at 77 °C. Protein concentration: $1 \times 10^{-5}$ M.

lectin containing only 12 basic groups. The protein contains an unusually high density of disulfide bridges (16), which confers upon it a high degree of stabilization against denaturation (Figure 7a). Thus, at 25 °C even when 50% methanol is added to a highly acidified (pH 2.4) spray solution, no discernable protonated molecule ions are observed with $m/z$ value less than 2000 (Figure 7a). However, when this spray solution is heated to 70 °C, intense ions are observed that correspond to the +9, +10, and +11 charge states of the intact lectin (Figure 7b). The elevation in temperature apparently induces denaturation, leading to an elevation in the number of charges that attach to the protein and allowing for the collection of an analytically useful mass spectrum.

Subtilisin Carlsberg and thermolysin are additional examples of proteins having high conformational stability that yield very weak electrospray ionization spectra at room temperature even from acidified spray solutions containing 50% methanol. Figure 8 shows that intense mass spectra can be obtained from these two intractable enzymes when the temperature of the spray solution is raised substantially, confirming the earlier result of Allen and Vestal[14] with these compounds.

## CONCLUSIONS

The effects of heat on the electrospray ionization mass spectra of several proteins were investigated using a simple and effective new apparatus. The described measurements demonstrate that heat provides an effective means for inducing denaturation of proteins prior to electrospray ionization in concert with previous results obtained by LeBlanc et al.[8] and that denaturation transition curves can be deduced from the resulting mass spectrometric data. Different transition temperatures obtained for the heat-induced denaturation of bovine ubiquitin and cytochrome c at different values of the solution pH indicate the cooperative effect of pH and temperatures on the unfolding of these proteins. The reversibility of heat-induced denaturation and refolding of proteins can also be probed by the present technique. Finally, the heat-induced denaturation technique is found to be useful for obtaining mass spectra of proteins (e.g., wheat germ agglutinin, subtilisin Carlsberg, thermolysin) that do not yield analytically useful electrospray ionization mass spectra under more conventional electrospray conditions.

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