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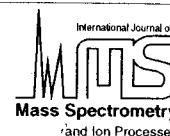
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Abstract

The experiments described in this paper were designed to address the question of whether proteins undergo denaturation on the millisecond timescale of the phase transition in electrospray ionization. The experiments show that it is relatively difficult to induce a change in charge in selected proteins by the application of heat to electrosprayed droplets during their transfer from atmospheric pressure to the vacuum of the mass spectrometer. These findings indicate that the net heat transfer to the droplets is insufficient for denaturation given the short lifetime of the droplets in the heated tube (0.6 ms or less) and provide a partial explanation as to why the charge distribution characteristic of a protein in equilibrium in solution is to some extent preserved into the gas phase. Our findings lead us to suggest general guidelines for improving the information about solution protein conformation and non-covalent associations obtained by electrospray ionization mass spectrometric measurements. © 1997 Elsevier Science B.V.

Keywords: Denaturation; Droplet evolution; Electrospray ionization; Protein

1. Introduction

It has been well documented that the distribution of charge states observed for protein ions by electrospray ionization mass spectrometry can provide information about the conformational state of proteins in solution just prior to electrospray ionization [1–10]. For example, we and others have demonstrated that heat-induced denaturation of proteins can be followed mass spectrometrically by assaying the distribution of charge states of the proteins in the gas phase as a function of the electrospray solution temperature [6,7]. Native proteins typically exhibit relatively tight distributions with low average charge, while the distributions characteristic of denatured

proteins are generally broader with a higher average charge. The conformational transition observed for heat-induced denaturation of bovine ubiquitin is illustrated in Fig. 1, where it is seen that the distribution of charge states flips from that characteristic of a tightly folded protein to that of the unfolded protein. In the transition region, where both the folded and unfolded forms of ubiquitin are present in solution, the charge distribution is bimodal. It appears that, during electrospray ionization, the charge distribution characteristic of a protein in equilibrium in solution is to some extent preserved into the gas phase [1–10]. Much less obvious is the degree to which this charge information is preserved during the phase transition and why it is preserved.

The transition of a protein originally in equilibrium in solution to an isolated highly charged

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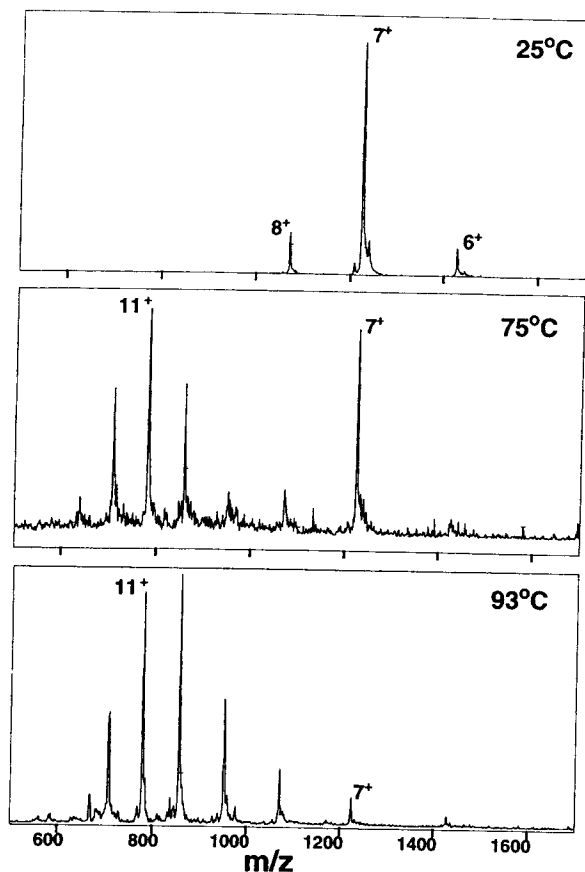


Fig. 1. Mass spectra of bovine ubiquitin electrospayed from aqueous acetic acid (pH 2.8) at three different spray solution temperatures. Experimental details are given in Ref. [7].

naked molecule in the gas phase involves a series of non-equilibrium steps, including the formation of highly charged liquid droplets, evaporation of volatile solvent from the droplets, evolution of smaller charged droplets from the larger droplets, emergence of isolated protein ions (perhaps still solvated) and, finally, desolvation of the protein ions [11–15]. During this complex non-equilibrium process, there is much opportunity for the loss of fidelity of the original protein charge state information characteristic of the equilibrated solution. For example, it appears likely that the pH of the solution in the rapidly shrinking droplets does not remain constant [16]. In addition, heat is frequently applied to the stream

of droplets and solvated proteins to assist the ion evolution and desolvation process [13,17]. If the temperature of the droplets is raised sufficiently during desolvation, heat-induced denaturation may occur, leading to a further possible alteration of the charge state distribution during droplet evolution.

The experiments described in this paper were designed to address the question of whether proteins undergo denaturation on the millisecond timescale of the phase transition in electrospray ionization.

2. Experimental details

Proteins were obtained from Sigma Chemical Company (St. Louis, MO): bovine cytochrome c (catalog number C-2037; molecular mass (MM) = 12 231 Da), bovine ubiquitin (U-6253; MM = 8565 Da), chicken egg lysozyme (L-6876; MM = 14 306 Da), equine myoglobin (M-0630; MM = 17 568 Da) and equine apomyoglobin (A-8673; MM = 16 952 Da). Protein concentrations in the electrospray solutions were in the range 10–20 μM . Solution pH measurements were made with a PHM 95 pH meter (Radiometer, Copenhagen), without correction for the presence of organic solvents.

Mass spectra were obtained with a TSQ-700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) fitted with a standard Finnigan heated capillary electrospray ionization source. The sample solution was delivered to the source by infusion at a rate of 3–4 $\mu\text{l min}^{-1}$ through a 100 μm i.d. fused silica capillary. The nebulizing sheath gas (nitrogen) flow pressure was maintained at 40 lbf in^{-2} . Electrospray was carried out in an environment of dry nitrogen, unless otherwise indicated. Electrospray droplets formed at the tip of the fused silica capillary were directed towards a stainless steel tube (length, 10 cm; i.d., 0.4 mm) through which a fraction of the droplets and solvated proteins was transported by the flow into the vacuum of the mass

spectrometer. This droplet/ion transfer tube was surrounded by a heated housing whose temperature could be varied between 25 and 350 °C to assist in the evaporation of the droplets and desolvation of the proteins. It is important to note that the temperature of the flowing gas and entrained droplets does not reach equilibrium with the temperature of the housing, and that the housing temperature provides only a measure of the relative energy input. The mass spectra were recorded with a Finnigan ICIS data system operating on a DEC 5000/120 computer. Each spectrum was obtained by averaging 16 individual scans, collected at 3 s per scan.

3. Results and discussion

Fig. 1 shows the electrospray mass spectra of bovine ubiquitin obtained from aqueous acetic acid solutions (pH 2.8) sprayed at three different spray solution temperatures while maintaining constant the temperature of the heated stainless steel tube used to transfer the droplets and ions into the mass spectrometer [7]. At the ambient temperature of 25 °C (top panel), the dominant charge state observed is 7+ and the charge distribution is composed of just three components. As the temperature of the spray solution is increased to 75 °C (middle panel), a bimodal charge distribution emerges, characteristic of a mixture of both folded and unfolded forms of the protein. The broader, more highly charged, component of the distribution centered around the 11+ charge state is characteristic of unfolded/denatured ubiquitin. Further elevation of the temperature (bottom panel) leads to complete denaturation of the protein and exclusive population of the more highly charged component of the distribution. These and similar measurements allowed us to determine a half-denaturation temperature of 71 ± 5 °C at pH 2.8 [7]. As we lowered the pH to 2.3, the half-denaturation temperature dropped to 31 ± 5 °C, in accord with the

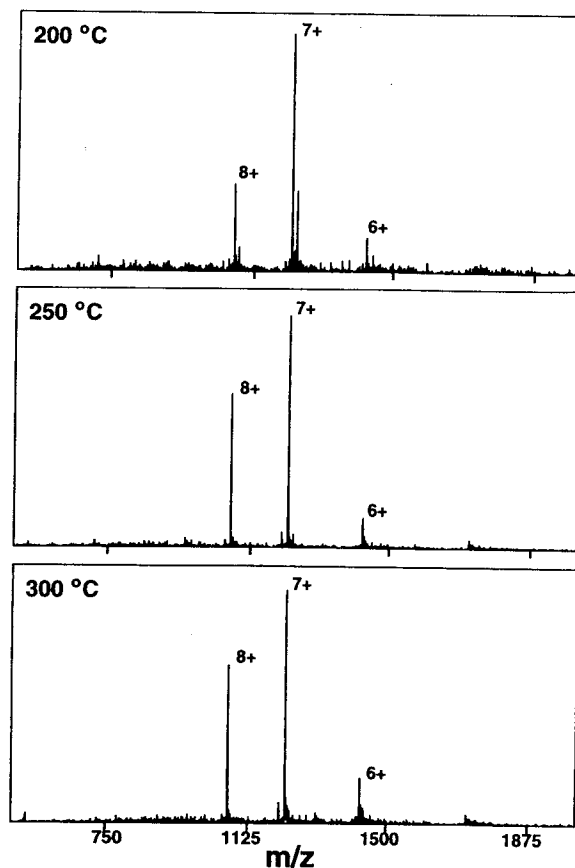


Fig. 2. Mass spectra of bovine ubiquitin electrosprayed from aqueous acetic acid (pH 2.4) at three different transfer tube temperatures. The spray solution temperature was maintained constant at 25 °C.

expectation that heat and low pH act as additive denaturants [7].

To investigate the possibility that proteins, such as ubiquitin, can unfold during their evolution to naked ions from the electrosprayed droplets, we carried out a series of experiments in which we maintained the temperature of the spray solution constant (at 25 °C) and varied the temperature of the droplet/ion transfer tube (Fig. 2). As the temperature of the droplet/ion transfer tube is increased, we expect that the temperature of the evolving droplets will rise, and the propensity for heat-induced denaturation will increase. The experiment was carried out with an aqueous acetic acid spray solution maintained

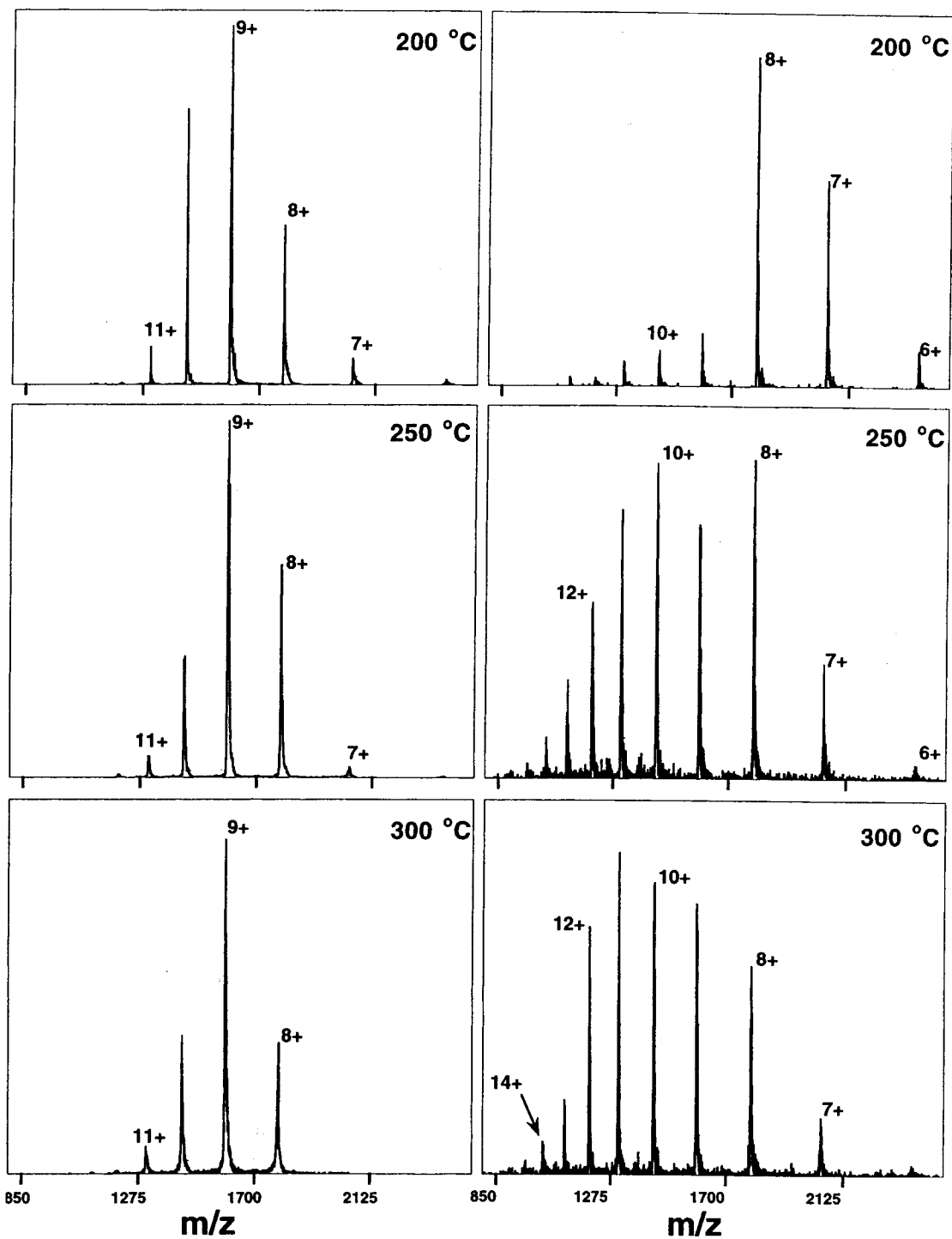


Fig. 3. Electrospray ionization mass spectra of chicken egg lysozyme obtained at three different transfer tube temperatures. Left-hand panel: spray solution water/methanol (1:1, v/v), pH 6. Right-hand panel: spray solution water/methanol (1:1, v/v) with the addition of 5 mM ammonium acetate (pH 6.9).

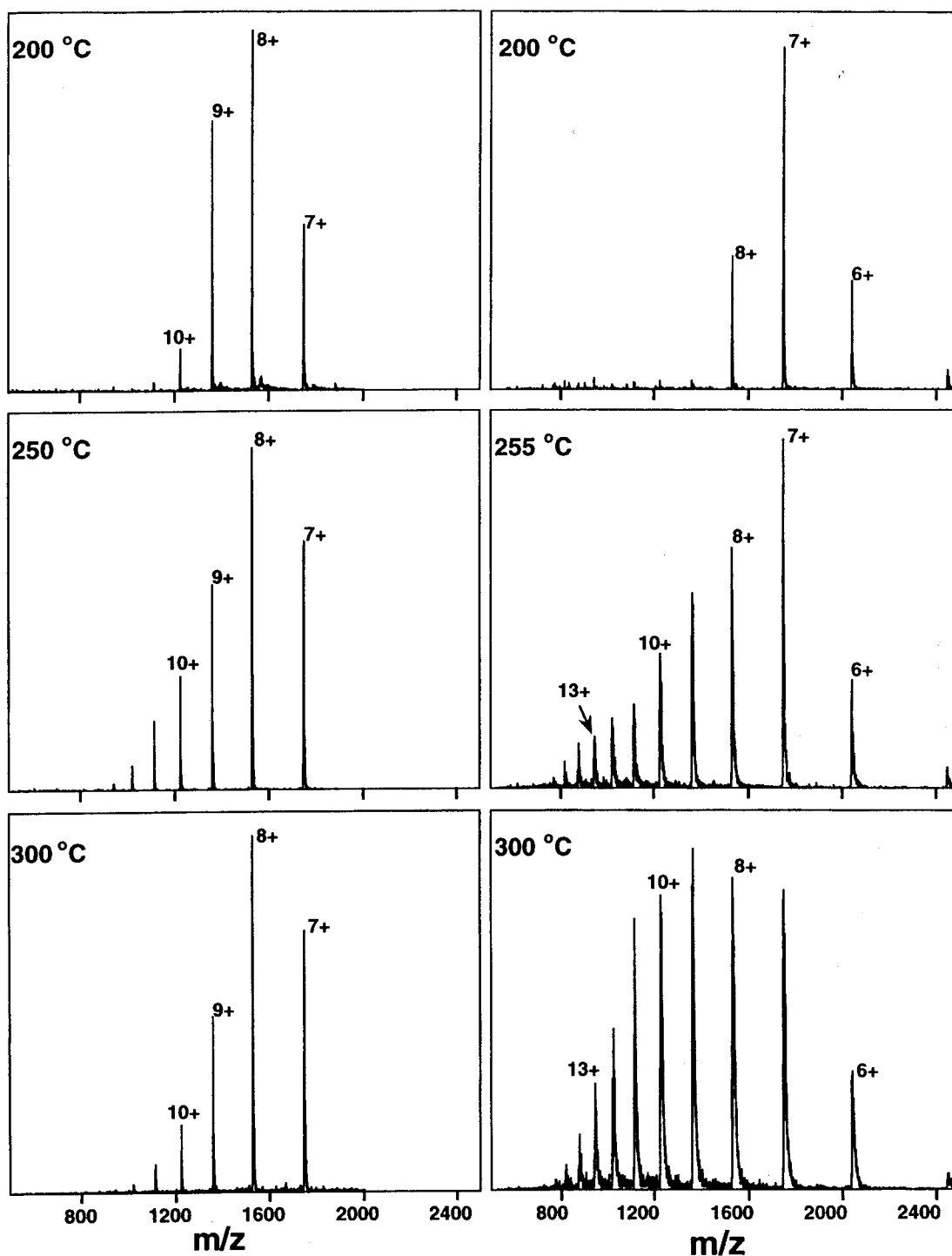


Fig. 4. Electrospray ionization mass spectra of bovine cytochrome c obtained at three different transfer tube temperatures. Left-hand panel: spray solution water/methanol (1 : 1, v/v). Right-hand panel: spray solution water/methanol (1 : 1, v/v) with the addition of 10 mM ammonium acetate.

at pH 2.4 to maximize the potential for heat-induced denaturation (see above). At the lowest transfer tube temperature investigated (200 °C), the charge distribution was characteristic of that previously observed for folded ubiquitin (see Fig. 1, top panel). Further increases in the transfer tube temperature (up to 300 °C) produced relatively little change in the observed charge distributions. From this experiment, we conclude that the elevation of the temperature in the transfer tube either does not induce denaturation of ubiquitin in the droplets or naked (or relatively lightly solvated) ubiquitin ions are formed at atmospheric pressure prior to entering the transfer tube. The latter possibility was deemed unlikely because reduction of the transfer tube temperature to below 100 °C was sufficient to cause almost total loss of the ion signal. Results comparable with those shown in Fig. 2 were obtained with electrosprayed ubiquitin in 7:3 (v/v) water/methanol (data not shown), lysozyme, either in aqueous acetic acid (pH 2.9) (data not shown) or 1:1 (v/v) methanol/water (Fig. 3, left-hand panels), and cytochrome c in 1:1 (v/v) methanol/water (Fig. 4, left-hand panels), i.e. no significant shifts in the charge state distributions were observed from those characteristic of the native structures. Again, it appears that elevation of the temperature in the transfer tube does not induce denaturation of the protein in the rapidly drying droplets.

Interestingly, different results were observed for bovine myoglobin electrosprayed from an aqueous methanolic (30%) solution (Fig. 5). At a transfer tube temperature of 150 °C, the spectrum is dominated by ions having relatively low charge (7+, 8+ and 9+) corresponding to the intact globin-heme complex (Fig. 5, top panel). Increasing the transfer tube temperature to 300 °C effects a dramatic change in the mass spectrum (Fig. 5, center panel); we observe a loss of the signal corresponding to intact myoglobin and an increase in the signal arising from myoglobin minus the non-covalently attached heme group (i.e. apomyoglobin). The distribution of

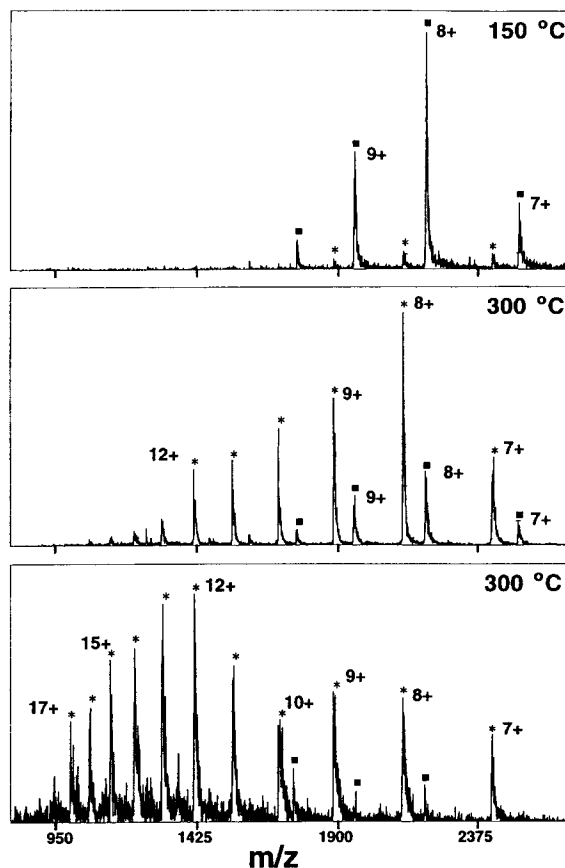


Fig. 5. Electrospray ionization mass spectra of equine myoglobin obtained from water/methanol (7:3, v/v) at three different transfer tube temperatures. Top and middle panels: no salt added to the spray solution. Bottom panel: 10 mM ammonium acetate salt added to the spray solution. The symbol ■ denotes myoglobin and * denotes apomyoglobin.

charge states for these apomyoglobin ions is considerably wider than that observed for myoglobin at the lower transfer tube temperature, and the mean charge state is shifted to a higher value. It appears that the additional heat applied at the higher transfer tube temperature causes sufficient denaturation of myoglobin in the droplets to result in the release of the heme group and an upward shift in the charge state distribution of the apomyoglobin product. However, it should be noted that the observed charge distribution of these apomyoglobin ions is centered at a much lower value ($Z_{\text{mean}} = 9.4$) than that observed

for fully denatured apomyoglobin ($Z_{\text{mean}} = 18$) (data not shown). Thus, while it appears possible to unfold selected proteins partially by the addition of heat during the ion desolvation process, denaturation in the droplets does not occur readily; indeed, as shown above, denaturation only occurs at transfer tube temperatures higher than those normally used in the mass analysis of proteins. This finding provides a partial explanation of why the charge distribution characteristic of a protein in equilibrium in solution is to some extent preserved into the gas phase.

A detailed understanding of the denaturation of proteins in electrosprayed droplets during transit through the heated transfer tube requires a knowledge of the rate of heat transfer to the droplets (after entering the tube) as well as the lifetime of the droplets. We have not determined either of these quantities, although we can set an upper limit on the lifetime of the droplets (after entering the transfer tube) as their transit time through the tube. Assuming viscous flow through the long tube and the applicability of Poiseuille's equation [18], we calculate the transit time to be approximately 0.6 ms. Thus the lifetime of the droplets in the tube is 0.6 ms or less. Our observation that it is relatively difficult to induce a change in charge (for ubiquitin, lysozyme and cytochrome c) by the application of heat to the droplets indicates that the net heat transfer to the droplets is insufficient for denaturation given the short lifetime of the droplets in the heated tube. Previous measurements of the unfolding rate constant of lysozyme (at pH 3.0) as a function of temperature showed that rate constants of 10^3 s^{-1} or greater could only be achieved at temperatures above approximately 90°C [19].

The addition of ammonium acetate (5 mM) to the spray solution of lysozyme used to obtain the spectra shown in the left-hand panels of Fig. 3 produced dramatic changes in the charge distribution as a function of temperature (Fig. 3, right-hand panels). Comparison of the left- and right-hand panels shows two significant

differences in the charge distributions. The first difference is apparent at the lowest transfer tube temperature investigated, 200°C , where the dominant components of the charge distribution are seen to decrease by approximately one charge state. This finding is in accord with our previously described anion neutralization effect [20]. The second difference is the occurrence of a second charge state distribution, which probably arises from denatured lysozyme [7]. This second, higher, charge state distribution becomes increasingly dominant as the temperature of the transfer tube is raised, consistent with an increasing level of denaturation. Similar phenomena were observed when ammonium acetate was added to the solutions used to spray cytochrome c (Fig. 4, right-hand panels) and myoglobin (Fig. 5, bottom panel). In both cases, the addition of ammonium acetate resulted in denaturation of the protein during droplet evolution.

The apparent increase in the propensity for heat-induced protein denaturation with the addition of salt may at first appear counterintuitive, since higher order structures are often stabilized in solution by the addition of moderate concentrations of salt. We speculate that the addition of ammonium acetate has the effect of increasing the lifetime of the electrosprayed droplets, thus increasing the time available for heat transfer and denaturation. Possible reasons for such an increase in droplet lifetime include an increase in the average droplet size, a decrease in the rate of solvent evaporation from the droplets and a decrease in the rate of Coulomb repulsion-induced droplet fission events [13–15]. The relative importance of these various possibilities remains to be investigated. However, to test our droplet lifetime/heat transfer hypothesis, we carried out an experiment in which we sought to increase the lifetime of the droplets and the heat transfer by a different procedure. Here, we changed the environment in which electrospray was carried out from the dry nitrogen normally used to nitrogen saturated with water vapor at 25°C . We reasoned that the water vapor-saturated spray

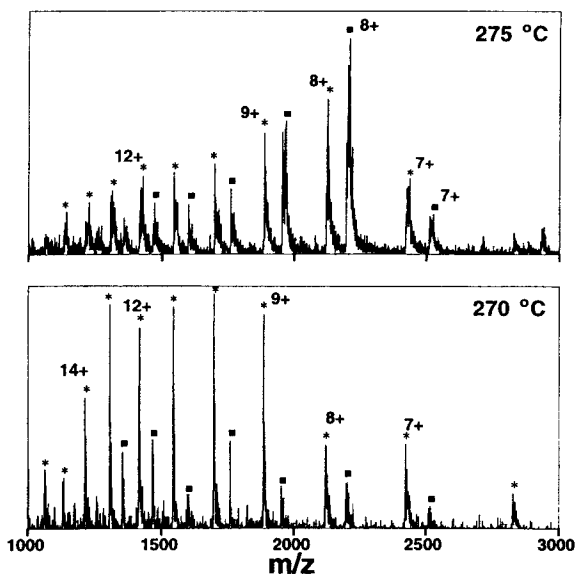


Fig. 6. Electro spray ionization mass spectra of equine myoglobin obtained from 7:3 (v/v) water/methanol. Electro spray atmosphere was dry nitrogen (top panel) and nitrogen saturated with water vapor (bottom panel). The symbol ■ denotes myoglobin and * denotes apomyoglobin.

environment would decrease the rate of solvent evaporation from the droplets and thus increase the droplet lifetime. The results of such an experiment are shown in Fig. 6 for myoglobin electro sprayed into the transfer tube maintained at constant temperature. The top panel shows the spectrum obtained from myoglobin electro sprayed from a dry nitrogen atmosphere (transfer tube temperature, 275 °C). Although numerous peaks arising from apomyoglobin are seen, the most intense peak in the spectrum arises from intact myoglobin. The bottom panel shows the spectrum resulting from the same solution electro sprayed from a water vapor-saturated nitrogen atmosphere (transfer tube temperature, 270 °C). The peaks corresponding to intact myoglobin have decreased in intensity with a concomitant increase in those arising from apomyoglobin, as well as an increase in the mean charge states of both species. Thus electro spray in a water vapor-saturated atmosphere leads to an increased level of protein denaturation, supporting our hypothesis.

4. Conclusions

We have determined experimentally that it is relatively difficult to induce a change in charge in selected proteins by the application of heat to electro sprayed droplets during their transfer from atmospheric pressure to the vacuum of the mass spectrometer. These findings indicate that the net heat transfer to the droplets is insufficient for denaturation given the short lifetime of the droplets in the heated tube, and provide a partial explanation as to why the charge distribution characteristic of a protein in equilibrium in solution is to some extent preserved into the gas phase. The present experiment does not provide an indication of the fidelity of this information. Nor do our experiments provide information about the conformational state of the proteins in the gas phase [21].

Our findings lead us to suggest a general guideline for improving the information about solution protein conformation and non-covalent associations obtained by electro spray ionization mass spectrometric measurements. In particular, we suggest the use of methods that decrease the droplet lifetime. Nanospray may be useful in this regard, since the droplets are known to be considerably smaller than those produced during regular electro spray [22].

Acknowledgements

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References

- [1] S.K. Chowdhury, V. Katta and B.T. Chait, *J. Am. Chem. Soc.*, 112 (1990) 9012.
- [2] J.A. Loo, C.G. Edmonds, H.R. Udseth and R.D. Smith, *Anal. Chem.*, 62 (1990) 693.
- [3] J.A. Loo, R.R.O. Loo, H.R. Udseth, C.G. Edmonds and R.D. Smith, *Rapid. Commun. Mass. Spectrom.*, 5 (1991) 101.

- [4] R.D. Smith, J.A. Loo, R.R.O. Loo, M. Busman and H.R. Udseth, *Mass Spectrom. Rev.*, 10 (1990) 693.
- [5] V. Katta and B.T. Chait, *Rapid Commun. Mass Spectrom.*, 5 (1991) 214. V. Katta and B.T. Chait, *J. Am. Chem. Soc.*, 115 (1993) 6317.
- [6] J.C.Y. LeBlanc, D. Beuchemin, K.W. Siu, R. Guevremont and S.S. Berman, *Org. Mass Spectrom.*, 21 (1993) 831.
- [7] U.A. Mirza, S.L. Cohen and B.T. Chait, *Anal. Chem.*, 65 (1993) 1.
- [8] V. Katta and B.T. Chait, *J. Am. Chem. Soc.*, 113 (1991) 8534.
- [9] R. Feng and Y. Konishi, *J. Am. Soc. Mass Spectrom.*, 4 (1990) 638.
- [10] D.L. Smith and Z. Zhang, *Mass Spectrom. Rev.*, 13 (1994) 411.
- [11] M. Dole, L.L. Mach, R.L. Hines, R.C. Mobley, L.P. Ferguson and M.B. Alice, *J. Chem. Phys.*, 49 (1968) 2240.
- [12] J.V. Iribarne and B.A. Thomson, *J. Chem. Phys.*, 64 (1976) 2287.
- [13] J.B. Fenn, *J. Am. Soc. Mass Spectrom.*, 4 (1993) 524.
- [14] P. Kebarle and L. Tang, *Anal. Chem.*, 65 (1993) 972A.
- [15] M.G. Ikonou and P. Kebarle, *J. Am. Soc. Mass Spectrom.*, 5 (1994) 791.
- [16] C.L. Gatlin and F. Turecek, *Anal. Chem.*, 66 (1994) 712.
- [17] S.K. Chowdhury, V. Katta and B.T. Chait, *Rapid Commun. Mass Spectrom.*, 4 (1990) 81.
- [18] M. Pirani and J. Yarwood, *Principles of Vacuum Engineering*, Chapman and Hall, London, 1961.
- [19] T.E. Creighton, *Biochem. J.*, 270 (1990) 1.
- [20] U.A. Mirza and B.T. Chait, *Anal. Chem.*, 66 (1994) 2898.
- [21] D. Suckau, Y. Shi, S.C. Beu, M.W. Senko, J.P. Quinn, F.M. Wampler III and F.W. McLafferty, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 790. B.E. Winger, K.J. Light-Wahl, A.L. Rockwood and R.D. Smith, *J. Am. Chem. Soc.*, 114 (1992) 5897. T.R. Covey and D.J. Douglas, *J. Am. Soc. Mass Spectrom.*, 4 (1993) 616. R.R.O. Loo and R.D. Smith, *J. Am. Soc. Mass Spectrom.*, 5 (1994) 207. Y. Konishi and R. Feng, *Biochemistry*, 33 (1994) 9706. D.E. Clemmer, R.R. Hudgins and M.F. Jarrold, *J. Am. Chem. Soc.*, 117 (1995) 10 141.
- [22] M. Wilm and M. Mann, *Int. J. Mass Spectrom. Ion Processes*, 136 (1994) 167. M. Wilm and M. Mann, *Anal. Chem.*, 68 (1996) 1.