Hydration of Folded and Unfolded Gas-Phase Proteins: Saturation of Cytochrome *c* and Apomyoglobin

James L. Fye, Jürgen Woenckhaus, and Martin F. Jarrold*

Department of Chemistry, Northwestern University
2145 Sheridan Road, Evanston, Illinois 60208

Received November 3, 1997

We have examined the hydration of gas-phase cytochrome *c* and apomyoglobin and found that the average number of water molecules adsorbed under near-saturation conditions by unfolded conformations is substantially *less* than by folded ones. This suggests that cooperative effects, where water molecules interact strongly with more than one site on the protein, are very important in the solvation of the folded conformation. Solvent interactions play a critical role in determining the structures and properties of proteins in solution. For example, hydrophobic interactions are believed to provide the dominant force driving protein folding.[1,2] Studies of the hydration of gas-phase proteins and peptides can provide information about solvent interactions that is not available from solution studies, such as thermodynamic information for the adsorption of specific water molecules. So far there have only been a few studies of hydrated proteins and peptides in the gas phase. Chait[3] and Williams[4] and their collaborators have recorded mass spectra for partially dehydrated peptide and protein ions generated by electrospray ionization. Kebarle and collaborators[5] have measured $\Delta G°$ at 293 K for the addition of the first few water molecules to several small peptides, the largest being Gly_4H^+. We have recently reported measurements of $\Delta H°$ and $\Delta S°$ for the first few water molecules adsorbed by bovine pancreactic trypsin inhibitor (BPTI), a 58-residue protein.[6] In this communication, we describe studies of the hydration of gas-phase cytochrome *c* and apomyoglobin in a broad range of $(M + nH)^{n+}$ charge states. Previous studies have indicated that these proteins undergo unfolding transitions, somewhat analogous to acid denaturation in solution, as the charge increases.[7−11] Therefore, studies of folded and unfolded conformations are possible.

The measurements were performed using an injected ion drift tube apparatus equipped with an electrospray source.[8] The number of water molecules adsorbed by the protein ions in the drift tube was determined from mass spectra of the ions exiting the drift tube. The drift tube was cooled to −20 °C to promote the hydration reactions. Drift time distributions were recorded by injecting 50-μs pulses of ions into the drift tube and recording their arrival time at the detector.

The filled circles in the upper half of Figure 1 show the average number of water molecules adsorbed by the $(M + 4H)^{4+}$ to $(M + 13H)^{13+}$ charge states of equine cytochrome *c* with 0.73 Torr of water vapor. The results shown in the figure were obtained with a helium buffer gas plus water vapor pressure of around 5 Torr and a drift field of 13.3 V cm^{-1}. If the drift field is lowered by a factor of 2 so that the ions spend twice as long in the drift tube, the average numbers of adsorbed water molecules are not significantly different from those obtained with the higher drift field. This suggests that the hydration reactions

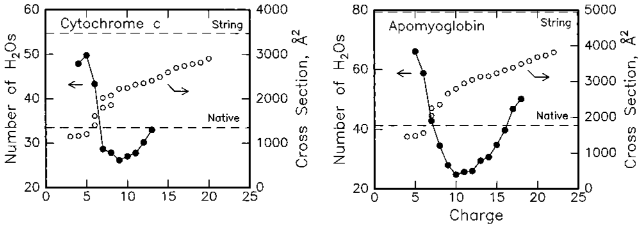

Figure 1. Plot of the average number of water molecules adsorbed on cytochrome *c* and apomyoglobin $(M + nH)^{n+}$ ions with 0.73 Torr of water vapor at −20 °C (filled circles and left-hand scale). The average cross sections, determined from mobility measurements,[8,9] are also shown (open circles and right-hand scale). The dashed lines show cross sections calculated for the native conformations (crystal structure coordinates) and extended strings (see text).

$$(M + nH)^{n+}(H_2O)^m + H_2O \rightleftarrows (M + nH)^{n+}(H_2O)_{m+1} \quad (1)$$

are in equilibrium. As can be seen from Figure 1, around 30 water molecules adsorb on the 7+ to 13+ charge states while around 50 water molecules adsorb on the 4+ and 5+ charge states. The distribution of water molecules adsorbed on each charge state is roughly Gaussian with a full width at half-maximum that is generally around one-half the average number of adsorbed water molecules. In addition to the results shown in Figure 1, measurements were performed with water vapor pressures of 0.19, 0.39, and 0.58 Torr. The results at these lower pressures showed the same general behavior as observed with 0.73 Torr of water vapor: Substantially more water molecules adsorbed on the 4+ and 5+ charge states than on the 7+ to 13+ charge states, although the averages show an overall decrease as the water vapor pressure was decreased. At −20 °C the equilibrium vapor pressure of water (ice) is 0.776 Torr. The highest water vapor pressure employed in the experiments, 0.73 Torr, is close to the equilibrium vapor pressure, so the average number of water molecules adsorbed at 0.73 Torr is close the maximum average number that can be adsorbed under equilibrium conditions.

The unfilled circles in the upper half of Figure 1 show the average cross sections of the cytochrome *c* $(M + nH)^{n+}$ ions. The cross sections were derived from drift time measurements as described elsewhere.[8] The dashed lines in the figure show cross sections calculated for the native conformation (crystal structure coordinates[12]) of cytochrome *c* and an extended string generated by setting all Φ and Ψ angles (except those between Cys14 and Cys17) to 180°.[8,13] Cross sections determined for the 3+ to 5+ charge states of cytochrome *c* are close to the value estimated for the native conformation. Between the 5+ and 7+ charge states there is an unfolding transition, and for the 7+ to 20+ charge states, the cross sections increase and approach the cross section calculated for the extended string. Clearly, the sharp decrease in the number of adsorbed water molecules that occurs between the 5+ and 7+ charge states is correlated with the unfolding transition.

The lower half of Figure 1 shows analogous results for apomyoglobin. Apomyoglobin is around 50% larger than cytochrome *c*, and the unfolding transition (open circles) is broader

---

[1] Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1.

[2] Dill, K. A. *Biochemistry* **1990**, *29*, 7133.

[3] Chowdhury, S. K.; Katta, V.; Chait, B. T. *Rapid. Commun. Mass Spectrom.* **1990**, *4*, 81.

[4] Rodriguez-Cruz, S. E.; Klassen, J. S.; Williams, E. R. *J. Mass Spectrom.* **1997**, *8*, 565.

[5] Klassen, J. S.; Blades, A. T.; Kebarle, P. *J. Phys. Chem.* **1995**, *99*, 15509.

[6] Woenckhaus, J.; Hudgins, R. R.; Jarrold, M. F. *J. Am. Chem. Soc.* **1997**, *119*, 9586.

[7] Clemmer, D. E.; Hudgins, R. R.; Jarrold, M. F. *J. Am. Chem. Soc.* **1995**, *117*, 10141. Shelimov, K. B.; Jarrold, M. F. *J. Am. Chem. Soc.* **1996**, *118*, 10313.

[8] Shelimov, K. B.; Clemmer, D. E.; Hudgins, R. R.; Jarrold, M. F. *J. Am. Chem. Soc.* **1997**, *119*, 2240.

[9] Shelimov, K. B.; Jarrold, M. F. *J. Am. Chem. Soc.* **1997**, *119*, 2987.

[10] Covey, T. R.; Douglas, D. J. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 616.

[11] Collings, B. A.; Douglas, D. J. *J. Am. Chem. Soc.* **1996**, *118*, 4488.

[12] Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585.

[13] Clemmer, D. E.; Jarrold, M. F. *J. Mass Spectrom.* **1997**, *32*, 577.

S0002-7863(97)03778-5 CCC: $15.00

and shifted to slightly higher charge.[9] Dashed lines show the cross sections calculated for the native conformation of myoglobin[14] and for an extended string. The average number of water molecules adsorbed by the 5+ and 6+ charge states is around 60. The average decreases to around 25 on the $(M + 10H)^{10+}$ ion and then gradually increases again. The sharp decrease in the number of adsorbed molecules is clearly correlated with the unfolding transition evident in the cross section measurements.

We have previously reported that the free energy changes at 273 K for adsorbing the first few water molecules on the folded 5+ charge state of cytochrome *c* were larger than on the unfolded 7+ charge state.[15] We suggested that this behavior could result from some of the water molecules entering the folded 5+ charge state as structural water molecules. The new results presented here cannot be rationalized in this way. Only six structural water molecules have been found for cytochrome *c* in recent NMR studies.[16] The low free energy changes found in our previous studies of the first few steps in the hydration of cytochrome *c* indicate that the charge is effectively self-solvated. However, an increase in the charge should result in an increase in the number of adsorbed water molecules. A systematic increase in the average number of adsorbed water molecules is seen for cytochrome *c* $(M + nH)^{n+}$ ions with $n > 9$ and apomyoglobin $(M + nH)^{n+}$ ions with $n > 10$. This increase probably results both directly from the increased charge and indirectly from the charge causing the protein to unfold further and expose more-favorable hydration sites.

Solvation energies of proteins are usually estimated from an accessible surface area (ASA) model where the solvation energy is given by[17,18]

$$\Delta G_{\text{solv}} = \sum_i \text{ASA}_i C_i \tag{2}$$

In this expression ASA_i is the accessible surface area for group *i* and C_i is a coefficient determined by fitting solvation data for model compounds. There is evidence that this group additivity approach fails for molecules with several polar groups. For example, the free energy changes for transferring *p*-hydroxybenzyl alcohol and *m*-hydroxybenzyl alcohol between toluene and water differ by 13.2 kJ mol^{-1}. This large difference has been attributed to the two hydroxy groups in *m*-hydroxybenzyl alcohol being at almost an ideal distance to form a hydrogen-bonded chain with a water molecule.[19] That the number of water molecules adsorbed by the unfolded charge states of cytochrome *c* and apomyoglobin is substantially *less* than by the folded ones is in striking contrast to the predictions of the ASA approximation. This suggests that cooperative effects, where the water molecules interact strongly with more than one site on the protein, are very important in the hydration of the folded conformation. These results indicate that the ASA approximation overestimates the solvation energy difference between the native and unfolded conformations, as suggested by the recent theoretical analysis of Lazaridis et al.[20] Cooperative effects analogous to those discussed above have been observed in the binding of H_3O^+ to polyethers in the gas phase.[21,22]

The unfolding transition that occurs between the 5+ and 7+ charge states of cytochrome *c* is believed to be driven by Coulomb repulsion. In solution, $(M + nH)^{n+}$ charge states up to around 11+ remain folded.[23] This difference between the gas phase and solution phase is at least partly a result of the high dielectric constant of water. If water molecules are added to the unfolded $(M + 7H)^{7+}$ charge state in the gas phase, at some point it should fold. Figure 2 shows drift time distributions recorded for the folded $(M + 5H)^{5+}$ and the unfolded $(M + 7H)^{7+}$ charge states of cytochrome *c* as a function of water vapor pressure. These measurements were performed with a helium plus water vapor pressure of around 5 Torr. The drift time scales have been approximately corrected for the varying water vapor pressure using Blanc's Law[24] and multiplied by the charge to remove its effect on the drift time. The drift time depends on the average collision cross section. For the folded 5+ charge state, the peak at around 9 ms in Figure 2 is close to the drift time expected for the native conformation. This peak remains in approximately the same position as the number of adsorbed water molecules increases. For the unfolded 7+ charge state, there are two peaks present in the drift time distribution in the absence of the water, and as the number of adsorbed water molecules increases, the distribution shifts over to the more folded conformation at shorter time. The addition of only 29 water molecules is apparently enough to promote the folding of the 7+ charge state to a more compact conformation. However, more water molecules are needed to drive the 7+ charge state to a completely folded conformation which would be at ~9 ms in Figure 2. Similar water-induced folding transitions have been observed for the 7+ charge state of apomyoglobin.

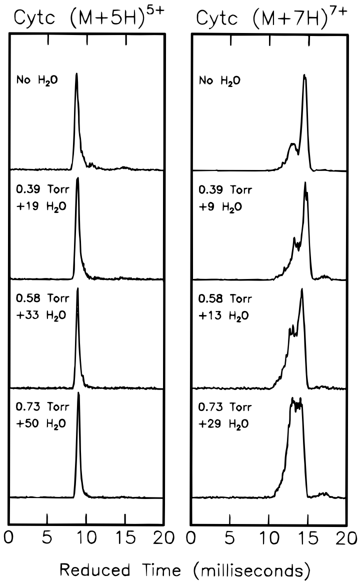

Figure 2. Drift time distributions measured for the $(M + 5H)^{5+}$ and $(M + 7H)^{7+}$ charge states of cytochrome *c* with 0.00, 0.39, 0.58, and 0.73 Torr of water vapor. The water vapor plus helium buffer gas pressure was around 5.0 Torr, and the drift tube was at −20 °C. The average number of adsorbed water molecules is shown for the different water vapor pressures for both charge states. The drift time scale has been approximately corrected using Blanc's Law to account for the different water vapor pressures, and the time scale has been multiplied by the charge to remove its effect on the drift time.

Acknowledgment. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. These studies were performed on an apparatus supported by the National Science Foundation (CHE-9618643). J.W. acknowledges the Alexander von Humboldt Stiftung for a fellowship.

JA9737782

---

(14) Evans, S. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *213*, 885.

(15) Woenckhaus, J.; Mao, Y.; Jarrold, M. F. *J. Phys. Chem. B* **1997**, *101*, 847.

(16) Qi, P. X.; Urbauer, J. L.; Fuentes, E. J.; Leopold, M. F.; Wand, A. J. *Struct. Biol.* **1994**, *1*, 378.

(17) Lee, B.; Richards, F. M. *J. Mol. Biol.* **1971**, *55*, 379.

(18) Makhatadze, G. I.; Privalov, P. L. *Adv. Prot. Chem.* **1995**, *47*, 307.

(19) Ben-Naim, A. *Biopolymers* **1990**, *29*, 567.

(20) Lazaridis, T.; Archontis, G.; Karplus, M. *Adv. Prot. Chem.* **1995**, *47*, 231.

(21) Sharma, R. B.; Kebarle, P. *J. Am. Chem. Soc.* **1984**, *106*, 3913.

(22) Meot-Ner, M.; Sieck, L. W.; Scheiner, S.; Duan, X. *J. Am. Chem. Soc.* **1994**, *116*, 7848.

(23) Theorell, H.; Åkesson, Å. *J. Am. Chem. Soc.* **1941**, *63*, 1818.

(24) Mason, E. A.; McDaniel, E. W. *Transport Properties of Ions in Gases*; Wiley: New York, 1988.