The transfer of Br^+ between the two ethylenes requires a transition through the D_{2d} structure (IV),[17] the energy of which was calculated to be 13.47 kcal mol^{-1} above the energy of III (9.20 kcal mol^{-1} above the energy of the isolated systems, II), reflecting sizable distortion of the molecular structure. The geometric parameters that vary most in the transfer are given in Table II.

From the experimental activation parameters, the second-order rate constant for the degenerate transfer of Br^+ from I to Ad=Ad at 25 °C is calculated to be $\sim 2 \times 10^7$ M^{-1} s^{-1}. While it may be argued that I is atypical in that it cannot form normal addition products, we see no reason why a less encumbered bromonium ion would not be prone to transfer at comparable or even greater rates. These results indicate that intermolecular Br^+ transfer from ion to olefin must be considered as competitive with the various product-forming steps during the electrophilic bromination of olefins.

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(17) The energy of the D_{2h} structure is $\sim$0.3 kcal mol^{-1} above the energy of the D_{2d} conformer.

Observation of the Heme–Globin Complex in Native Myoglobin by Electrospray-Ionization Mass Spectrometry

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In their native state, globular proteins are tightly folded, compact structures, certain of which may be characterized by the association of the globin with small, noncovalently bound cofactors and prosthetic groups. These proteins can be denatured and caused to unfold by subjecting them to high temperatures, extremes of pH, detergents, and solutions containing high concentrations of compounds like urea, guanidinium chloride, and organic solvents.[1] As the severity of the denaturing conditions is increased, the interaction between the globin and the cofactor can be weakened, with possible separation of the cofactor from the globin. For example, the oxygen-carrying protein myoglobin contains a noncovalently bound heme group in the hydrophobic pocket of the native globin chain that can be induced to unfold under acidic conditions, thus weakening the heme–globin interaction. Under these conditions, the heme moiety can be readily extracted into an organic phase, and this phenomenon forms the basis of widely used procedures for preparing apomyoglobin from the native proteins.[2,3]

A variety of techniques including acid–base titrations, spectrophotometry, viscometry, circular dichroism, fluorescence, and nuclear magnetic resonance have been applied to monitor these conformational changes in myoglobin.[4] Recently, we have demonstrated[5,6] that conformational changes in proteins can also

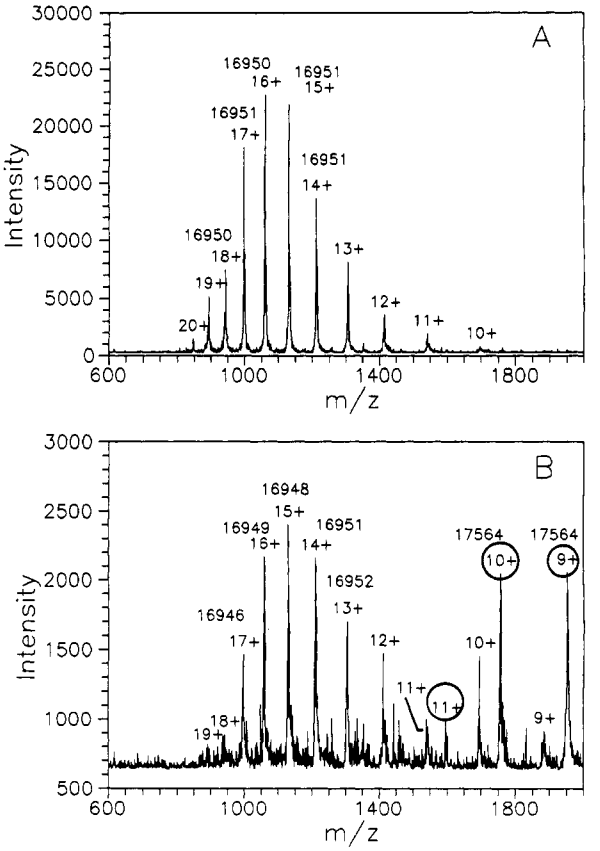

Figure 1. Electrospray-ionization mass spectra of equine skeletal muscle myoglobin obtained from aqueous solutions at different pH values: (A) 3.35; (B) 3.90. The protein concentration is 20–40 μM. The peaks are labeled with the protonation state, $n+$, and the number of protons, n, attached to the protein molecule. The circled protonation states designate the peaks corresponding to the intact heme–globin complex in myoglobin. The most intense peaks are also labeled with the molecular masses determinated from the measured m/z values.

be detected by electrospray-ionization mass spectrometry. Electrospray is a gentle method of ionization that produces intact, multiply protonated gas-phase ions directly from protein molecules in solution.[7-9] 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. Because the availability and the effective pK's 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.[5,6,10]

The acid denaturation of myoglobins has been studied extensively under a variety of conditions.[11-14] In the case of horse[15] and sperm whale myoglobin,[4,14] the onset of denaturation occurs in the pH range 4.5–3.5 and depends strongly on the ionic strength of the solution. It has been shown that some of the histidines are

(1) Creighton, T. E. *Proteins: Structures and Molecular Principles*; W. H. Freeman: New York, 1984. Jaenicke, R.; Rudolph, R. In *Protein Structure A Practical Approach*; Creighton, T. E., Ed.; IRL Press: Oxford, England, 1990.
(2) Rossi-Fanelli, A.; Antonini, E.; Caputo, A. *Biochim. Biophys. Acta* **1958**, *30*, 608–15.
(3) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**, *35*, 543.
(4) Antonini, E.; Brunori, M. *Hemoglobin And Myoglobin In Their Reactions With Ligands*; North-Holland Publishing Company: Amsterdam, 1971; pp 55–134.
(5) Chowdhury, S. K.; Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1990**, *112*, 9012–3.
(6) Katta, V.; Chait, B. T. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 214–7.
(7) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64–71.
(8) Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882–99.
(9) Covey, T.; Bonner, R. F.; Shushan, B. I.; Henion, J. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249.
(10) Loo, J. A.; Loo, R. R. O.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 101–5.
(11) Breslow, E.; Gurd, F. R. N. *J. Biol. Chem.* **1962**, *237*, 371–81.
(12) Flanagan, M. A.; Garcia-Moreno, E. B.; Friend, S. H.; Feldman, R. J.; Scoulondi, H.; Gurd, F. R. N. *Biochemistry* **1983**, *22*, 6027–37.
(13) Matthew, J. B.; Gurd, F. R. N. *Methods Enzymol.* **1986**, *130*, 437–53.
(14) Friend, S. H.; Gurd, F. R. N. *Biochemistry* **1979**, *18*, 4612–9.
(15) Puett, D. *J. Biol. Chem.* **1973**, *13*, 4623–34.

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buried in the native state of myoglobins and do not react with hydrogen ions.[4] The effective pK's of the remaining histidines and those of the glutamic and aspartic acid side chains are lowered considerably, compared to their intrinsic pK values, because of the electrostatic interactions in the native conformation.[14,16] As a result, the net positive charge on the native protein is lower than would be expected from the intrinsic pK values of the side chains. At lower pH values, the unfolding of the protein results in complete protonation of the buried histidines, disruption of the heme-globin interaction, and changes in the effective pK values of the side chains toward the intrinsic pK's.[11,15] Thus, the denatured state may be characterized by the absence of heme and a relatively higher positive charge on the globin. Conversely, the native state of myoglobin may be characterized by the presence of heme in the hydrophobic pocket and by a lower net positive charge on the protein molecule. In the present communication, we use these criteria to differentiate between the electrospray-produced ions of equine myoglobin in a native conformation and those produced from the denatured protein.

Figure 1A shows the spectrum obtained from an aqueous equine myoglobin solution[17] at pH = 3.35 (no salts or buffers added), a pH where the protein is known to be completely denatured.[15] The spectrum shows a single distribution of peaks, with protonation states ranging from 20+ to 10+, with 15+ the most intense. The average molecular mass measured from these peaks is 16 951 ± 1 u and corresponds to the calculated mass of apomyoglobin (16 951.5 u; without heme). These spectral characteristics are similar to those previously reported for the electrospray ionization of myoglobin.[20-23] If we assume that certain solution characteristics of the protein, such as the degree of protonation and the extent of heme-globin complexation, are preserved into the gas phase, then our mass spectrometric results confirm that the protein is in a denatured form in this solution (as evidenced by the high degree of protonation and the total absence of ions corresponding to the heme-globin complex).

Figure 1B shows an electrospray mass spectrum obtained from an equine myoglobin solution at pH 3.9 (no salts or buffers added). This pH is in a range of values where both native and denatured forms of myoglobin can coexist in solution.[14,15] The spectrum is different from that shown in Figure 1A because it exhibits two distinct distributions of charge states. The first ranges from 19+ to 9+, with 15+ the most intense. The average molecular mass calculated from this distribution is 16 949 ± 2 u and corresponds to that of apomyoglobin. The second distribution, in the higher m/z range, shows three peaks with protonation states 11+ to 9+ (shown circled in Figure 1B). The low charge state components (lower than 9+) of this distribution could not be observed because their m/z values lie beyond the range of our instrument (m/z < 2000).[19] Two important features make this distribution distinct from the first one and also from the distribution shown in Figure 1A. The charge states are comparatively lower, and the measured molecular mass is significantly higher (17 564 ± 2 u). This measured mass corresponds to that of the intact heme-globin complex (calculated molecular mass = 17 568.0 u). The correlation between the measured mass and the low charge states of this distribution suggests that these ions arise from the native protein in solution.[24] At a higher pH value (pH = 4.4), where the protein is predicted to be in a wholly native state,[14,15] the first distribution centered around 15+ disappeared completely while the 9+ charge state of the second distribution continued to be observed (data not shown).[26] Similar results were also obtained for dog myoglobin.

Our findings demonstrate that electrospray-ionization mass spectrometry can provide information on the conformation of myoglobin under different solution conditions and that the noncovalently bound heme-globin complex found in the native state can be measured mass spectrometrically. Although the data do not imply that the solution conformation is preserved into the gas phase, the results suggest that native, noncovalent associations of proteins and cofactors in solution can be preserved into the gas phase and observed by mass spectrometry.

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(16) Matthew, J. B.; Gurd, F. R. N. *Methods Enzymol.* **1986**, *130*, 413-35.

(17) Aqueous solutions (acidified with acetic acid, no salts or buffers added) of equine skeletal muscle myoglobin (Sigma Chemical Company, St. Louis, MO) were electrosprayed through a 150 μm i.d. stainless steel syringe needle, whose tip was etched to a conical shape.[5,18] The electrospray mass spectrometer used in the present investigations has been described earlier.[19] The protein concentrations were in the range 20-40 μM, and the flow rates were 0.4-0.8 μL/min.

(18) Chowdhury, S. K.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1660-1664.

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

(20) Mann, M.; Meng, C. K.; Fenn, J. B. *Anal. Chem.* **1989**, *61*, 1702-8.

(21) Berkel, G. J. V.; Glish, G. L.; McLuckey, S. A. *Anal. Chem.* **1990**, *62*, 1284-95.

(22) Henry, K. D.; Williams, E. R.; Wang, B. H.; McLafferty, F. W.; Shabanowitz, J. F.; Hunt, D. F. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9075-8.

(23) Chowdhury, S. K.; Katta V.; Chait, B. T. In *Methods and Mechanisms for Producing Ions from Large Molecules*; Standing, K. G., Ens, W., Eds.; NATO ASI Series; Plenum Press: New York, 1990 (in press).

(24) Peaks corresponding to a complex of heme with apomyoglobin have been observed earlier.[9,25] However, these earlier studies were carried out under solution conditions that favored denaturation, and the charge distribution of the complex was similar to the distribution of apomyoglobin, which centered around 17+ to 19+. The nature and origin of the association complex in the earlier studies are thus uncertain.

(25) Edmonds, C. G.; Loo, J. A.; Barinaga, C. J.; Udseth, H. R.; Smith, R. D. *J. Chromatogr.* **1989**, *474*, 21-34.

(26) The spectra obtained from myoglobin solutions maintained at respectively pH = 3.35, pH = 3.9, and pH = 4.4 were all taken under identical mass spectrometric conditions.

A Synthetic *p*-Nitrophenyl Esterase with Remarkable Substrate Selectivity

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A new linear oligomer, **4** ($n \sim 10$), recently prepared and characterized in our laboratory,[1] has been evaluated as a catalyst for hydrolysis of *p*-nitrophenyl esters of alkanoic acids, **1**. This synthetic material not only exhibits high levels of catalytic efficiency and conforms to the Michaelis-Menten model but also demonstrates enzyme-like specificity for esters derived from acids of moderate chain length ($C_{12} \rightarrow C_{16}$) with **1** ($n = 14$) the optimum substrate ($V_{max} = 7.5 \times 10^{-7}$ M s^{-1}, $K_M = 2.9 \times 10^{-5}$ M, $k_{cat}/K_m = 1.1 \times 10^4$ M^{-1} s^{-1} at 30 °C in 1:1 MeOH-aqueous buffer (pH 8.0) with [cat.] = 2.5×10^{-6} M). Significantly, catalysis is accompanied by rapid release of product from the catalytic site, a 4-(dialkylamino)pyridine.

Duplication of enzymic efficiency and selectivity with synthetic materials has been the goal of much research.[2-15] Studies directed

(1) Rubinsztajn, S.; Zeldin, M.; Fife, W. K. *Macromolecules* **1990**, *23*, 4026. (b) Rubinsztajn, S.; Zeldin, M.; Fife, W. K. *Macromolecules* **1991**, *24*, 2682.

(2) D'Souza, V. T.; Bender, M. L. *Acc. Chem. Res.* **1987**, *20*, 146.

(3) Lehn, J.-M. *Science* **1985**, *227*, 849.

(4) Tabushi, I. *Acc. Chem. Res.* **1982**, *15*, 66.

(5) Breslow, R.; Trainor, G.; Veno, A. *J. Am. Chem. Soc.* **1983**, *105*, 2739.

(6) Cram, D. J.; Lam, P. Y.; Ho, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 839.

(7) Rebek, J.; Askew, B.; Killoran, M.; Demeth, D.; Lin, F.-T. *J. Am. Chem. Soc.* **1987**, *109*, 2426.

(8) Ford, W. T., Ed. *Polymeric Reagents and Catalysts*; ACS Symposium Series 308; American Chemical Society: Washington, DC, 1986.

(9) (a) Kunitaki, T.; Okahata, Y.; Ando, R. *Macromolecules* **1974**, *7*, 140. (b) Kunitaki, T.; Shinkai, S. *Adv. Phys. Org. Chem.* **1980**, *17*, 435.

0002-7863/91/1513-8535$02.50/0