Naked Protein Conformations: Cytochrome *c* in the Gas Phase

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Ion mobility measurements[1] have been used to obtain direct information about the conformers present for naked cytochrome *c* ions in the gas phase. The relative abundance of compact native-like conformations is negligible. However, a number of well-defined, partially folded conformers that are considerably more diffuse than the native structure are observed. These may be intermediates in the folding process. The results suggest that the gas phase is an environment that will allow access to complementary information about protein conformations and the dynamics of protein folding.[2]

The development of gentle ionization techniques[3] has facilitated studies of biological molecules using mass spectrometry. Accurate measurements of molecular weights[4,5] as well as sequence information[5,6] are becoming commonplace, and recently there have been several attempts to use mass spectrometry to deduce information about the three-dimensional structures of biological molecules. For example, the charge distribution generated by electrospray ionization[7] (ESI) has been shown to depend on the conformation of the protein in solution.[8,9] Mass spectrometry has been used to monitor $^2H/^1H$ isotope exchange for proteins in solution[10] as well as in the gas phase.[11,12] Finally, ion beam scattering experiments[13,14] have shown that protein ions in different charge states have different collision cross sections. The ion mobility measurements[1] reported here resolve protein conformers on the basis of their different collision cross sections and provide precise cross sections for the conformers that are present. Ion mobility studies have previously been performed for cytochrome *c*, but the resolution was insufficient to deduce structural information.[15] Recently, some preliminary ion mobility results have been reported for the nonapeptide bradykinin.[16]

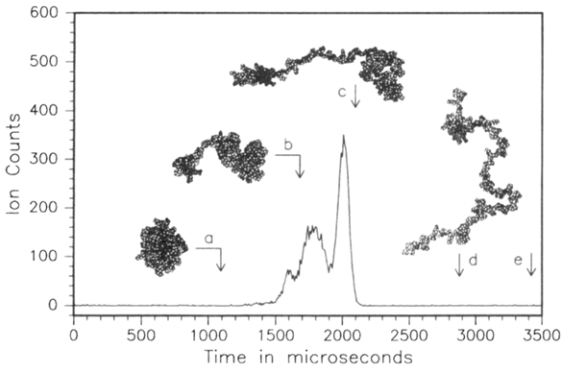

Figure 1. Drift time distribution recorded for the +7 charge state of bovine cytochrome *c* with a nominal injection energy of 130 eV. Arrows show the expected drift times for a variety of cytochrome *c* conformations: (a) the native structure (~1100 μs); (b) a partially unfolded structure with an open heme crevice (1680 μs); (c) an unfolded coil that retains the α-helices (2070 μs); (d) a typical random coil with no secondary or tertiary structure (~2880 μs); and (e) a near-linear conformation (~3425 μs).

Experiments were performed using an injected ion drift tube apparatus with an electrospray ionization[7] (ESI) source similar to that described by Smith et al.[17] The rest of the instrument and the experimental methods have been described previously.[18] The source was operated using electrode voltages ranging from 4100 to 4400 V and solution flow rates of 0.3−0.7 μL/min. Cytochrome *c* ions (bovine and horse heart), generated in a variety of charge states, were focused into a low-energy ion beam and injected into the drift tube. The drift tube is 7.6 cm long and was operated with a drift field of 6.58 V/cm and ~3 Torr of helium buffer gas. After exiting the drift tube, the ions were focused into a quadrupole mass spectrometer set to transmit only the charge state of interest. Drift time distributions were recorded by injecting 50 μs ion pulses into the drift tube and measuring the arrival time distribution at the detector.

Electrospray ionization of cytochrome *c* generates protonated ions in a distribution of charge states that depends on the properties of the solution.[8] The +7 to +9 charge states were produced by spraying a 50:50 mixture of methanol and water containing cytochrome *c* at 4×10^{-5} M. Higher charge states were obtained by adding 0.1−1.0% acetic acid. Figure 1 shows a drift time distribution recorded for the +7 charge state of bovine cytochrome *c*. Three peaks are apparent in this distribution: a small peak at ~1600 μs, a broad one at ~1800 μs, and an intense peak at ~2000 μs. In addition, there is a broad, low-intensity shoulder at drift times of ~1300−1500 μs. The relative abundances of the components present in the distribution were sensitive to the operating conditions of the ESI source and the injection energy. Under some conditions, species with drift times of ~1300−1500 μs were as much as ~20% of the distribution. Drift time distributions recorded for cytochrome *c* in the +8 charge state show two peaks: a broad one which appears to correlate with the broad feature in the +7 distribution at ~1800 μs and a narrower peak at longer times which is apparently the analogue of the peak at ~2000 μs. The drift time distributions for the +9 to +20 charge states are

(1) Hagen, D. F. *Anal. Chem.* **1979**, *51*, 870. Tou, J. C.; Boggs, G. U. *Anal. Chem.* **1976**, *48*, 1351. Carr, T. W. *J. Chromatogr.* **1977**, *15*, 85. Karpas, Z.; Cohen, M. J.; Stimac, R. M.; Wernlund, R. F. *Int. J. Mass Spectrom. Ion Processes* **1986**, *83*, 163. St. Louis, R. H.; Hill, H. H. *Crit. Rev. Anal. Chem.* **1990**, *21*, 321. von Helden, G.; Hsu, M. T.; Kemper, P. R.; Bowers, M. T. *J. Chem. Phys.* **1991**, *95*, 3835. Jarrold, M. F. *J. Phys. Chem.* **1995**, *99*, 11.

(2) Richards, F. M. *Sci. Am.* **1991**, January, 54. Kim, P. S.; Baldwin, R. L. *Annu. Rev. Biochem.* **1982**, *262*, 848. Englander, S. W. *Science* **1993**, *262*, 848. Roder, H.; Elove, G. A.; Englander, S. W. *Nature* **1988**, *355*, 700.

(3) Monagham, J. J.; Barber, M.; Bordolim, R.; Sedgewick, E.; Taylor, A. *Org. Mass Spectrom.* **1992**, *17*, 596. Whitehouse, C. M.; Dreyer, R. N.; Yamashuta, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675. Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299.

(4) Chait, B. T.; Kent, S. B. H. *Science* **1992**, *257*, 1885.

(5) Biemann, K. *Annu. Rev. Biochem.* **1992**, *61*, 997.

(6) For a recent review, see: Burlingame, A. L.; Boyd, R. K.; Gaskell, S. J. *Anal. Chem.* **1994**, *66*, 634R−683R.

(7) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.

(8) Chowdhury, S. K.; Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1990**, *112*, 9012−9013.

(9) Loo, J. A.; Loo, R. R. O.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 101. Mirza, U. A.; Cohen, S. L.; Chait, B. T. *Anal. Chem.* **1993**, *65*, 1. LeBlanc, J. C. Y.; Beuchemin, D.; Siu, K. W. M.; Guevremont, R.; Berman, S. S. *Org. Mass Spectrom.* **1991**, *26*, 831. Rockwood, A. L.; Busman, M.; Udseth, H. R.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 582.

(10) Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1993**, *115*, 6317. Wagner, D. S.; Anderegg, R. J. *Anal. Chem.* **1994**, *66*, 706. Smith, D. L.; Zhang, Z.; Liu, Y. *Pure Appl. Chem.* **1994**, *66*, 89.

(11) Katta, V.; Chait, B. T. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 214. Winger, B. E.; Light-Wahl, K. J.; Rockwood, A. L.; Smith, R. D. *J. Am. Chem. Soc.* **1992**, *114*, 5897. Brown, C.; Camilleri, P.; Haskins, N. J.; Saunders, M. *J. Chem. Soc., Chem. Commun.* **1992**, 761.

(12) Suckau, D.; Shi, Y.; Beu, S. C.; Senko, M. W.; Quinn, J. P.; Wampler, F. M.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 790.

(13) Covey, T. R.; Douglas, D. J. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 616.

(14) Cox, K. A.; Julian, R. K.; Cooks, R. G.; Kaiser, R. E. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 127.

(15) Wittmer, D.; Chen, Y. H.; Luckenbill, B. K.; Hill, H. H., Jr. *Anal. Chem.* **1994**, *66*, 2348.

(16) von Helden, G.; Wyttenbach, T.; Bowers, M. T. *Science* **1995**, *267*, 1483.

(17) Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882−889.

(18) Jarrold, M. F.; Bower, J. E.; Creegan, K. *J. Chem. Phys.* **1989**, *90*, 3615. Jarrold, M. F.; Bower, J. E. *J. Chem. Phys.* **1992**, *96*, 9180.

0002-7863/95/1517-10141$09.00/0

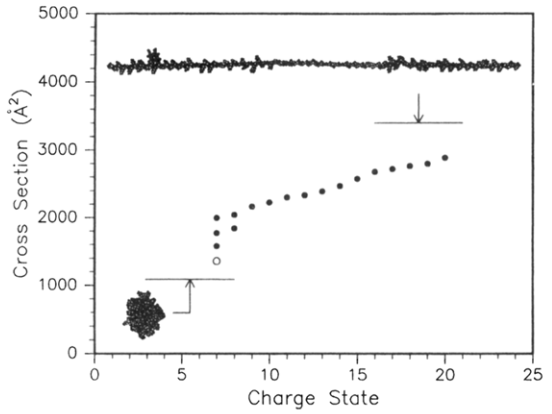

Figure 2. Plot of the collision cross sections for the features observed in the drift time distributions for cytochrome *c* in the +7 to +20 charge states. The horizontal lines show the calculated cross sections for the native and near-linear conformations of cytochrome *c*.

dominated by a single narrow peak that appears to correlate with the ~2000 μs peak.

The peak at ~1800 μs in the drift time distribution shown in Figure 1 is much broader (by a factor of 3) than the distribution calculated from the transport equation for a single isomer.[19] This indicates that at least two structures, which may be interconverting slowly, are present in this region. The peak at ~2000 μs is only slightly broader (by a factor of 1.5) than the calculated distribution, suggesting that the conformers that contribute to this peak are either structurally similar or interconvert rapidly so that structural averaging occurs. The single peaks in the distributions for the +9 to +20 charges states are in good agreement with calculated distributions for a single isomer.

Figure 2 shows a plot of the collision cross sections determined from the drift times[19] for cytochrome *c* in the +7 to +20 charge states. Independent measurements of the cross sections are reproducible to within 2%. The data shown are the averages of several data sets obtained for the +7 to +20 states of bovine (and +8 to +17 charge states of horse heart) cytochrome *c*. There is a small systematic increase in the collision cross sections with charge state, a result that qualitatively agrees with ion beam scattering studies.[13]

Information about the nature of the cytochrome *c* conformers revealed in Figures 1 and 2 can be obtained by comparing the measured collision cross sections to orientationally averaged cross sections calculated for plausible geometries,[20] assuming hard-sphere interactions. The hard-sphere collision distances were taken to be 2.2 Å for He−H collisions and 2.7 Å for He−C, −O, −N, −S, and −Fe collisions. The calculated cross sections are insensitive to the collision distances employed, because cytochrome *c* is much larger than the collision distances.

For native cytochrome *c*, we estimate a collision cross section of 1090 Å², which corresponds to a drift time of ~1100 μs for the +7 charge state (cross sections obtained from crystal structure coordinates[21] and from NMR solution structure coordinates[22] are almost identical). As can be seen from Figure 1, a drift time of ~1100 μs is significantly shorter than any of the features present in the distribution. At the other extreme, the estimated cross section for a near-linear conformation (obtained by straightening out the protein) is 3400 Å². This corresponds to a drift time of 3425 μs for the +7 charge state. Perhaps a more realistic representation of the completely unraveled conformation is a random coil. Using the SYBYL molecular modeling program,[23] we generated 11 independent random coils.[24] A wide variety of random coil geometries, ranging from near-spherical to quite linear, are possible. The cross sections for *most* of these geometries are quite similar. Although cross sections for near spherical and quite linear geometries will be different, these conformations are statistically improbable. The average cross section for the 11 random coil structures is 2860 ± 140 Å², corresponding to an average drift time of ~2880 μs for the +7 charge state. As shown in Figure 1, the random coil occurs at substantially longer drift time than any of the features in the drift time distribution. Thus, the conformers present for the +7 charge state must have geometries between the native and random coil extremes: they are partially folded conformers.

Suckau *et al.*[12] have examined the different charge states of gas phase cytochrome *c* ions using isotope exchange kinetics and concluded that the +6 and +7 charge states are in the native conformation. While our results appear to conflict with this conclusion, this could result from different source conditions or experimental time scales. Our results seem consistent with studies of the denaturing of cytochrome *c* in solution.[25] Nonaqueous solvents (including methanol) depress the temperature where cytochrome *c* denatures, suggesting that hydrophobic interactions play a major role in maintaining the native conformer in solution.[25]

To further understand the features in Figure 1, drift times were estimated for some speculative but plausible partially folded conformers. There have been extensive studies of the folding and denaturing of cytochrome *c* in solution.[26−28] An important early step in denaturing is breaking the Met-80 sulfur−iron linkage which allows the heme crevice to open. Opening cytochrome *c* at the heme crevice leads to many possible structures, including a conformation such as b, which we have randomly picked to show in Figure 1. This partially folded structure has an estimated drift time of ~1700 μs, slightly shorter than the broad feature in Figure 1. Another conformer type that could be near the observed peaks is one in which the secondary structure is retained while the tertiary structure is lost. A conformer of this type (conformer c in Figure 1, again chosen randomly) was formed by retaining all of the original α-helices while randomizing the torsion angles along the remaining parts of the protein backbone. It has an estimated shift time of ~2100 μs, close to the largest feature in the distribution for the +7 charge state. This feature dominates the drift time distributions for the higher charge states. As shown in Figure 2, its cross section systematically increases with increasing charge state and approaches that for the near-linear conformer. This increase may result from a partially folded conformer unraveling sections of α-helix or unfolding further to minimize Coulombic repulsion. Long-range ion-induced dipole interactions may also contribute to the increase in the collision cross section with increasing charge; however, this should be a relatively small effect.[29]

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(19) Mason, E. A.; McDaniel, E. W. *Transport Properties of Ions in Gases*; Wiley: New York, 1988.
(20) See, for example: Jarrold, M. F.; Constant, V. A. *Phys. Rev. Lett.* **1991**, *67*, 2994. von Helden, G.; Hsu, M.-T.; Gotts, N.; Bowers, M. T. *J. Phys. Chem.* **1993**, *97*, 8182. Clemmer, D. E.; Hunter, J. M.; Shelimov, K. B.; Jarrold, M. F. *Nature* **1994**, *372*, 248.
(21) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585.
(22) Qi, P. X.; DiStefano, D. L.; Wand, A. J. *Biochemistry* **1994**, *33*, 6408.
(23) *SYBYL Molecular Modeling Software*, Version 6.1; TRIPOS Inc.: St. Louis, MO, 1994.
(24) The SYBYL software generates different random coils from the primary sequence of amino acids by allowing the torsion angles of the protein backbone to vary randomly within stearic constraints.
(25) Kaminsky, L. S.; Miller, V. J.; Davison, A. J. *Biochemistry* **1973**, *12*, 2215.
(26) Roder, H.; Elove, G. A.; Englander, S. W. *Nature* **1988**, *355*, 700.
(27) Tsong, T. Y. *Biochemistry* **1973**, *12*, 2209.
(28) Myer, Y. P.; Saturno, A. F. *J. Protein Chem.* **1990**, *10*, 379. Myer, Y. P.; Saturno, A. F. *J. Protein Chem.* **1991**, *10*, 481.
(29) Jarrold, M. F.; Bower, J. E. *J. Phys. Chem.* **1993**, *97*, 1746.