H/D Exchange Levels of Shape-Resolved Cytochrome *c* Conformers in the Gas Phase

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Abstract: The conformations of cytochrome *c* ions (+8 through +18) in the gas phase are examined by simultaneous ion-mobility and hydrogen−deuterium exchange measurements. By varying the voltage used to inject ions into the drift tube it is possible to study H/D exchange of specific conformers observed in the ion-mobility spectra: either very diffuse structures that are favored for all charge states at high injection voltages or compact structures that can be favored for the +8, +9, and +10 charge states at low voltages. The number of exchangeable hydrogens for the diffuse conformer is independent of charge state, with an average value that is quite low: only 63 ± 2 of a possible 198. The compact conformers observed for the +8 through +10 charge states exchange fewer hydrogens (∼46), consistent with the idea that compact structures protect some hydrogens in the gas phase. Many sites that rapidly exchange in solution appear to be restricted for exchange in the gas phase, even for very open conformers.

Introduction

Recent advances in ionization techniques for mass spectrometry (MS) have made it possible to create large bimolecular ions in the gas phase.[1,2] Measurements of accurate molecular weights are becoming commonplace,[3,4] and general sequencing strategies that utilize MS are proliferating.[5] Several recent studies have reported conformational information for proteins in solution based on MS data. For example, the charge state distributions generated by electrospray ionization (ESI) have been shown to depend on the protein's conformation in solution.[6] MS has been used to monitor hydrogen−deuterium exchange for proteins in solution[7−10] and has complemented NMR to give insight into the populations and structures of folding intermediates.[11]

An issue that has emerged is the structure of the protein ion in the gas phase. Structural studies of solvent-free proteins in the gas phase offer a unique opportunity to understand the intrinsic structural nature of proteins and the role of solvent in establishing solution-phase conformations.[12] Strategies for examining the conformations of complex molecular ions in the gas phase can be divided into two categories: chemical reactivity studies,[13−17] which take advantage of reactive differences of different conformations; and non-reactive studies, which utilize collisions with inert partners to infer shape information. By far, reactivity studies are the more prevalent of these approaches, having a rich history that includes many detailed studies of the structures and reaction mechanisms associated with many ion−molecule systems, including small gas-phase peptide ions.[18] McLafferty and co-workers have studied the conformations of multiply charged protein ions by using Fourier transform ion cyclotron resonance (FTICR) techniques to measure the kinetics of hydrogen−deuterium (H/D) exchange in order to infer conformational information.[16,17] These studies have shown evidence that multiple conformations within single charge states produced by ESI can coexist, and correlations were drawn between specific exchange levels observed in the gas phase and conformations that have been characterized in solution.[16]

Measurements of average cross sections for multiply charged protein ions using non-reactive collisions (in triple quadrupole instruments) began with the ion energy loss studies of Douglas,[19,20] and Cooks,[21] and their co-workers. Their results showed that protein ions in different charge states have different collision cross sections, a conclusion that was also drawn from imprints on surfaces that are found after high-energy protein

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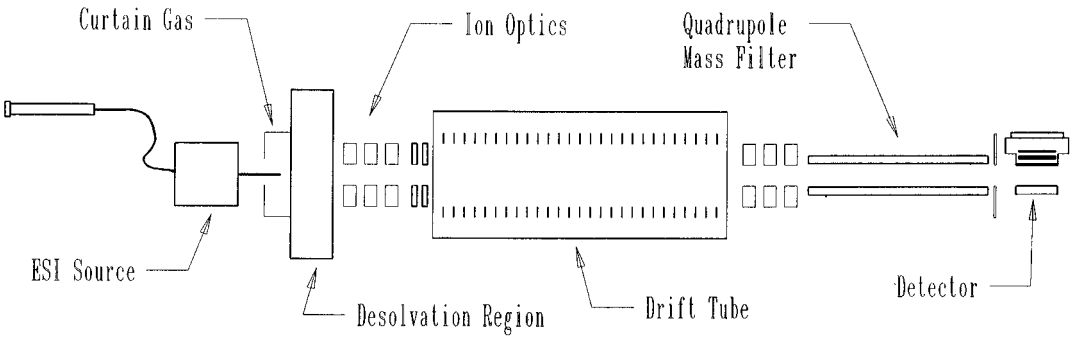

Figure 1. Schematic diagram of the experimental apparatus.

collisions.[22] Jarrold and co-workers combined ESI with ion mobility-MS measurements in order to measure precise cross sections for cytochrome *c* and demonstrated sufficient resolution to separate distinct conformations within single charge states in the gas phase.[23] Bowers and co-workers have reported ion mobility results for singly charged bradykinin formed by MALDI but observed only a single conformation, presumably because bradykinin is a relatively small ion.[24] Several other ion mobility measurements for biological ions have been carried out, although no structural information was deduced from these data.[25]

In this paper we report the first combined physical and chemical approach for studying protein structure in the gas phase. H/D exchange and ion mobility studies of gas-phase cytochrome *c* have been carried out in a drift tube in order to measure the number of exchangeable hydrogens on the protein's surface while simultaneously keeping track of its overall shape. We have chosen to study cytochrome *c* because detailed H/D exchange kinetics and ion mobility studies for this protein have already been reported by McLafferty and co-workers[16,17] and Jarrold and co-workers,[23] respectively. Both of these groups find evidence for multiple conformers within single charge states. Both also find that it is possible to alter conformations by collisions with buffer gasses.[17,26] However, several results of the H/D exchange and ion mobility studies appear disparate. For example, the number of exchangeable hydrogens (within a given exchange level) is found to be independent of charge state,[16,17] while the ion mobility,[23] ion scattering,[19] and surface imprint[22] studies all show evidence that cytochrome *c* systematically opens up with increasing charge state. Furthermore, for the highest charge states, where cross sections corresponding to the most open structures are observed, the number of exchangeable hydrogens in the H/D exchange studies was found to decrease,[16,17] a result that suggests a more compact conformation. Because the H/D exchange and ion mobility results of McLafferty and Jarrold are carried out under different experimental conditions, the results cannot be easily combined to give a more detailed understanding of the intrinsic structure of the protein in the gas phase. It is possible that the two techniques have sampled dissimilar structures, due to variations in electrospray source conditions or other differences in the nature of the chemical and physical measurements. By carrying out these measurements simultaneously we avoid differences that are due to systematic variations between experiments and gain a look at H/D exchange on the surfaces of specific gas-phase conformers.

Experimental Section

General Methods. A schematic diagram of our experimental apparatus is shown in Figure 1. Injected ion drift tube techniques have been described in detail previously.[27] Only a brief description is given here. Multiply charged cytochrome *c* ions are formed in an ESI source that is similar to one described by Smith and co-workers.[28] Electrosprayed droplets enter a variable-temperature, differentially-pumped desolvation region through a 0.1-cm-diameter entrance orifice. The electrospray needle penetrates into a plexiglass case that allows a curtain of dry nitrogen gas to purge the system. This is important in keeping H_2O from ambient laboratory air from interfering with exchange results in which deuterated protein solutions are used.[8] The ESI charge state distributions for cytochrome *c* depend on the properties of the solution.[6] In these experiments the solution was 8.0×10^{-5} M in cytochrome *c* (horse heart, Sigma >99%) in a 1:1 water−acetonitrile solution that also contained from 0.2 to 2.0% acetic acid. Protein ions are extracted from the desolvation region into a high-vacuum region (10^{-4} to 10^{-5} Torr), focussed into a low-energy ion beam, and injected at various energies into the drift tube containing ~2 Torr of helium buffer gas. The buffer gas pressure inside the drift tube is measured using a capacitance manometer. The drift tube is 32.4 cm long with 0.08-cm-diameter entrance and exit apertures and comprises 26 equally spaced electrostatic lenses to ensure a uniform electric field. The drift tube body is made of stainless steel with Teflon spacers at each end, which electrically isolate the entrance and exit plates. In these studies, drift fields ranging from 3 to 15 V cm^{-1} were used. After exiting the drift tube, ions are focussed into a quadrupole mass spectrometer that can be set to transmit a specific mass (for measurements of drift time distributions) or scanned in order to monitor product formation (in the case of H/D exchange studies).

Conformer Separation. Different protein conformations within a given charge state are separated because of differences in their collision cross sections.[23] Ion mobility spectra were obtained by injecting 50-μs ion pulses into the drift tube and recording the arrival time distribution at the detector with a multichannel scalar. These measurements are performed both with and without the buffer gas in the drift tube, and the time that the ions spend traveling across the drift tube is determined from the difference between these two measurements (plus some small corrections that account for the fact that the kinetic energy of the ions exiting the drift tube depends on whether the buffer gas is present). Compact conformers, with small collision cross sections,

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travel across the drift tube more rapidly than more diffuse ones with larger cross sections. Collision cross sections are derived from the experimentally measured drift times using eq 1,[29]

$$\Omega = \frac{(18\pi)^{1/2}}{16}\frac{ze}{(k_B T)^{1/2}}\left[\frac{1}{m_I}+\frac{1}{m_B}\right]^{1/2}\frac{t_D E}{L}\frac{760}{P}\frac{T}{273.2}\frac{1}{N} \qquad (1)$$

where E is the electric field strength, P is the buffer gas pressure, L is the drift tube length, t_D is the drift time, z is the charge state, N is the neutral number density, and m_I and m_B are the masses of the ion and buffer gas, respectively. All of the parameters in eq 1 can be precisely measured. Thus, the reproducibility of our cross sections is excellent, with different measurements usually agreeing to within 1%. The absolute uncertainty of these measurements is a few percent, mainly limited by end effects (e.g., the buffer gas flow near the entrance and exit apertures of the drift tube and penetration of the ions, which reduces the effective length of the drift tube). The variation of the measured mobilities as a function of injection voltage (IV) provides some information about the penetration of the injected ions. In these experiments, variations as a function of IV are approximately the same size as the precision of the mobility measuremnts. Thus, penetration effects are small, a result that is consistent with the predictions of simple hard-sphere collision models of the injection process.[30]

H/D Isotope Exchange Studies. The number of hydrogens that exchange is derived by comparing the mass spectra obtained after the ions have travelled through the drift tube containing pure He with data obtained when the He has been doped with a known fraction of deuterated solvent. The partial pressure of D_2O is determined from the difference between the total pressure and the pressure of the He before adding D_2O. We doublecheck the partial pressure measurements by removing one of the reagent gasses. If either gas is removed, the pressure quickly equilibrates to the pressure of the remaining gas. The number of exchangeable hydrogens is the m/z shift multiplied by the charge state. Most of the data reported here were obtained using D_2O (Cambridge Isotope Laboratories, 99.9%). A smaller number of experiments (which gave essentially the same overall exchange levels) were carried out using CH_3COOD (Sigma, 98 atom % D). In these studies we report the average number of hydrogens that exchange based on differences in the centers of the mass spectral peaks (with and without deuterated solvent). Peak centers can be determined to within 0.3 and 0.4 m/z for the pure He and gas-phase H/D exchange conditions, respectively. [Peaks associated with gas-phase exchange of proteins that were first deuterated in solution (described below) are somewhat broader and m/z values can be measured to within 0.6 m/z.] We estimate the absolute uncertainty of our reported gas-phase H/D exchange values to range from ~4 to 9 for the +8 to +18 charge states, respectively.

All of the present studies were carried using a buffer gas temperature of 300 K. The number of hydrogens that exchange for each conformer depends on the deuterated solvent pressure and the time that the protein ions spend in the drift tube. The drift time of the ions can be measured for each charge state at every pressure by recording the ion mobility distribution. H/D exchange profiles as a function of exchange time of different conformations of cytochrome *c* measured in an FTICR have been shown to follow pseudo-first-order kinetics, and H/D exchange rate constants have been reported.[16] In our present studies, semilogarithmic plots of the number of reactive hydrogens that remain against the deuterated solvent pressure multiplied by the drift time can also be used to derive rate constants for the exchange process. At this point, the only system that we have studied is cytochrome *c*, and so the rate constants that we mention are only estimates which we compare to values reported previously by McLafferty and co-workers.[17] In future studies we will assess the accuracy of our method in detail by studying systems that have been characterized by several methods.

As discussed below our reported maximum H/D exchange levels are significantly lower than those obtained in FTICR experiments.[16,17] This is of concern since it is possible that residual ^1H_2O vapor backexchanges within the drift tube and systematically lowers the exchange level that we observe. Accurate H/D exchange measurements require that the isotopically pure exchange solvent is the dominant reactant gas. We tested the extent that background water vapor interferes with H/D exchange measurements in our drift tube by monitoring H/D exchange of small protonated water clusters [$(H_2O)_nH^+$ ($n = 1-3$)] produced by electrospraying a solution containing purified H_2O and 1% CH_3COOH (EM Science, 99.7%). When the He buffer gas is doped with our smallest measurable pressure of D_2O (~0.001 Torr), the m/z ratio for each cluster increases by $2n + 1$ (the total number of hydrogens in each cluster), indicating complete exchange to form [$(D_2O)_nD^+$ ($n = 1-3$)] has occurred. Immediately after closing the D_2O inlet line, a series of peaks, all differing by 1 amu, is observed. These peaks correspond to partially deuterated clusters. This distribution systematically shifts to lower masses until after ~30 min the mass spectrum is dominated by the parent [$(H_2O)_nH^+$ ($n = 1-3$)] peaks. It should be stressed that when any measurable pressure of D_2O (>0.001 Torr) is added to our system, there is no evidence for contamination due to residual H_2O inside the drift tube. This is an important advantage of high-pressure drift tube techniques compared with H/D exchange studies carried out at very low pressures, where it has been suggested that background water (even at levels as low as 10^{-8} Torr) might influence exchange results.[17,31]

Finally, it is possible that ions undergo proton transfer reactions or change conformations while they are drifting through the tube in the presence of D_2O. To check for this, we recorded drift times at every D_2O partial pressure for every charge state. As discussed below, for the +8 through +17 charge states there is no evidence for changes in either the charge state or the conformation as the ions are drifting through the drift tube. The absence of proton transfer reactions is consistent with the measured gas-phase basicities of protonated cytochrome *c* ions.[15]

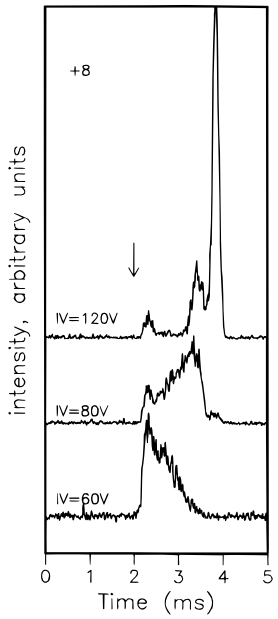

Figure 2. Drift time distributions for the +8 charge state of cytochrome *c* recorded at injection voltages of 60, 80, and 120 V. The arrow near 2 ms is the drift time that is calculated for the native conformation found in solution (see text). For these data, a drift field of 10.03 V cm^{-1} was used and the pressure was scaled to 2.000 Torr.

Results

Favoring Compact or Diffuse Conformers. As ions are injected into the drift tube they are rapidly heated as their kinetic energy is thermalized by collisions with the buffer gas. Further collisions cool the ions to the buffer gas temperature. Figure 2 shows typical drift time distributions we record for the +8 charge state of cytochrome *c*, when the ions are injected into

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Table 1. Summary of Ion Mobility and H/D Results for the +8 through +18 Charge States of Cytochrome *c*[a]

			cytc/D_2O(soln)			
charge state	σ (Å^2)[b]	cytc/H_2O(soln) D_2O(gas)[c]	soln exch[d]	H_2O(gas)[e]	D_2O(gas)[f]	D−H
+8	1260,[g] 2129	~46,[g] 64(3)	152(1)	110	176	66
+9	1352,[g] 2193	~45,[g] 64(3)	150(2)	113(7)	174(3)	61
+10	1511,[g] 2257	~48,[g] 64(3)	149(1)	110(5)	174(2)	64
+11	2300	62(3)	148(4)	112(4)	176(1)	64
+12	2331	63(4)	148(4)	111(4)	180(4)	69
+13	2406	65(2)	148(4)	118(5)	184(4)	66
+14	2486	62(3)	149(4)	121(4)	187(4)	66
+15	2573	62(2)	152(4)	123(4)	184(5)	61
+16	2671	61(4)	153(8)	125(11)	187(8)	62
+17	2740	63(3)	155(6)	120(6)	189(6)	69
+18	2771	60(4)				
av		63(2)	150(2)	116(6)	181(6)	65(5)

[a] Uncertainties correspond to one standard deviation about the mean and are given in parentheses. Unless otherwise noted, results are for the most diffuse conformer observed in the ion mobility spectra. [b] Collision cross sections are derived from ion mobility distributions using eq 1. Values are for the most diffuse conformer observed in drift tube studies, unless otherwise noted. Uncertainties in the cross section measurement are ±1%. [c] Maximum H/D values determined from mass spectra. [d] Exchange level observed after dissolving cytochrome *c* in deuterated solvents (D_2O and CH_3COOD) for ~20 min. [e] Total number of hydrogens exchanged after the partially deuterated protein (see the soln exch column) has been exposed to ^1H_2O in the gas phase. [f] Total number of hydrogens exchanged after the partially deuterated protein (see the soln exch column) has been exposed to D_2O in the gas phase. [g] Value measured for the compact conformer formed at low injection voltages.

the drift tube at low (30−60 V), moderate (60−90 V), and high (90−150 V) injection voltages. At low injection voltages we observe a distribution that is dominated by a peak that arrives near 2.3 ms. The cross section derived from this drift time with eq 1 is 1260 Å^2, a value that is ~17% larger than the cross sections that are calculated for the solution native structure (determined by NMR)[32] or crystal structure[33] (1080 Å^2,[23] for both sets of coordinates). This conformer is the most compact we observe for the +8 charge state and is observed at low injection voltages for all solution conditions employed. At moderate injection voltages (60−90 V), the peak at 2.3 ms decreases and the distribution shifts to longer times. New peaks in the drift time distribution are observed at 3.4 and 3.8 ms. At high injection voltages (90−120 V) the peak at 2.3 ms is substantially diminished and the distribution becomes dominated by the peak arriving at 3.8 ms. No dissociation is observed at these voltages; therefore, the changes must correspond to conformational changes within the protein. Collisional heating of the +8 charge state opens up the protein. From eq 1 and the measured 3.4 and 3.8 ms drift times for the peaks observed in Figure 2, we derive collision cross sections of 1887 and 2129 Å^2, respectively. Cross sections derived for the most compact and most diffuse conformers observed for all charge states studied here are listed in Table 1.

By varying the injection voltage, it is possible to favor the diffuse conformation for all of the charge states studied here. An example is shown in Figure 3 for the +11 charge state that was injected at 120 V into the drift tube containing ~2 Torr of He buffer gas. For all of the charge states studied here, this injection voltage favors the most diffuse conformer observed for cytochrome *c*. The peak displayed in spectrum A (Figure 3), obtained with pure He as the buffer gas, is accurately represented by the distribution that is calculated from the

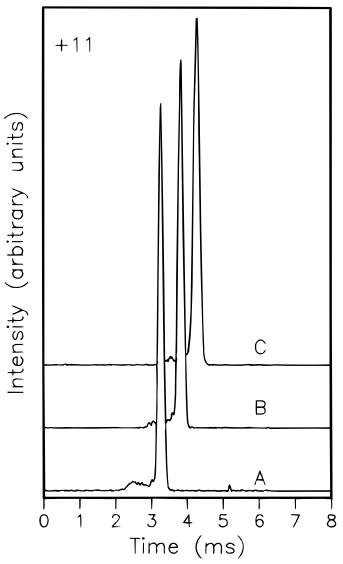

Figure 3. Drift time distributions for the +11 charge state of cytochrome *c* recorded at an injection voltage of 120 V when 2.0 Torr of He gas was doped with different partial pressures of D_2O. Trace A shows the distribution obtained when no D_2O is added. Traces B and C show data recorded with D_2O partial pressures of 0.09 and 0.17 Torr, respectively.

transport equation for a single conformer.[34] This is the case for all of the +9 through +18 charge states and *suggests* that only a single conformation is present under these conditions. The peak corresponding to the most diffuse conformer for the +8 charge state is slightly broader than the calculated distribution, indicating the presence of at least two conformers. As discussed below, cross sections derived from these data are in agreement with the most diffuse conformer observed in previous ion mobility studies.[23] Figure 3 also shows drift time distributions for the +11 charge state when the He buffer gas has been doped with 0.09 and 0.17 Torr of D_2O. As the fraction of D_2O is increased, the peaks in the drift time distributions for all charge states systematically shift to longer times, an indication that the mobility of the ions in the solvent is less than that in pure He. Results with H_2O are identical within our experimental uncertainties. At these low solvent concentrations we observe no evidence for conformational changes or proton transfer reactions resulting from interactions of the protein with the solvents as the protein drifts through the buffer gas. This is especially clear for studies of the +8 state where the relative intensities and positions of the peaks observed remain constant, while the entire distribution shifts to longer times with increasing D_2O pressure.

Gas-Phase H/D Exchange of the Diffuse Conformer. H/D exchange mass spectra for the most diffuse conformers are shown in Figure 4 over the +10 to +11 charge state range. Data for other charge states are similar. The reference spectrum, with no added solvents in the drift tube, displays sharp peaks for each charge state. When the buffer gas is doped with ~0.2 Torr of D_2O or CH_3COOD the peaks shift to higher masses, as shown. Peaks observed upon addition of deuterated solvents are factors of ~1.4 to 2.1 times broader at full width at half maximum (fwhm) than the reference mass spectra, indicating a slight distribution (~10 hydrogens for each charge state) within the exchange level. No mass increase is observed when H_2O is added to the drift tube, an indication that these

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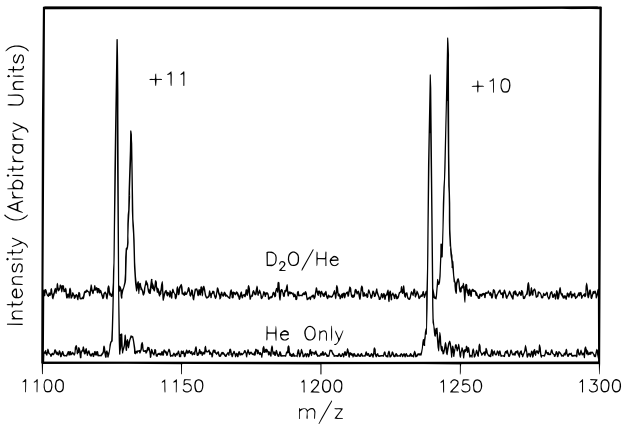

Figure 4. Mass spectra of cytochrome *c* recorded over the 1100 to 1300 *m*/*z* range. The bottom trace shows reference data for the +10 and +11 charge states of cytochrome *c* when the ions are injected into the drift tube containing pure He. The upper trace shows the spectrum obtained when the He buffer gas is doped with ~0.2 Torr of D_2O.

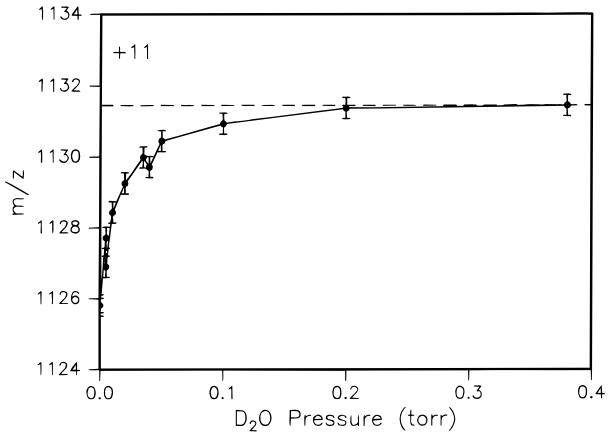

Figure 5. Experimentally measured *m*/*z* values for the +11 charge state of cytochrome *c* as a function of the partial pressure of D_2O. The error bars shown reflect uncertainties in determining peak centers (*m*/*z* ~0.4). The dashed line corresponds to the measured maximum *m*/*z* shift measured for these data.

conformations do not form protein−solvent clusters under these conditions.[35]

Figure 5 shows a plot of the *m*/*z* shift for the +11 state as a function of the partial pressure of the deuterated solvent. The maximum *m*/*z* shift shown in Figure 5 corresponds to exchange of 62 hydrogens. In all of the data we have obtained, all of the rapidly exchanging hydrogens appear to have exchanged when D_2O pressures of ~0.3 Torr or greater are used. The maximum number of rapidly exchanging hydrogens that is measured for each charge state is listed in Table 1. The exchange level observed appears to be independent of charge state and the average of all charge states is 63 ± 2.

Figure 6 shows an example of a semilogarithmic kinetic plot of the number of exchangeable hydrogens that remain [i.e., the difference between the maximum number of hydrogens that exchange and the number that exchange at each pressure], plotted against the deuterated solvent pressure multiplied by the

(35) It is well-known that the voltages used to extract ESI generated ions from high/low pressure interface regions can effectively decluster the ions. We tested this by systematically varying extraction potential between the drift tube and the quadrupole sections of our instrument and find no evidence for clustering of water to the most diffuse conformer. The absence of clusters under our experimental conditions is consistent with recent equilibrium measurements of water binding energies to compact and diffuse forms of cytochrome *c* carried out at low temperatures [Woenckhaus, J.; Mao, Y.; Jarrold, M. F. *J. Phys. Chem.* Submitted for publication].

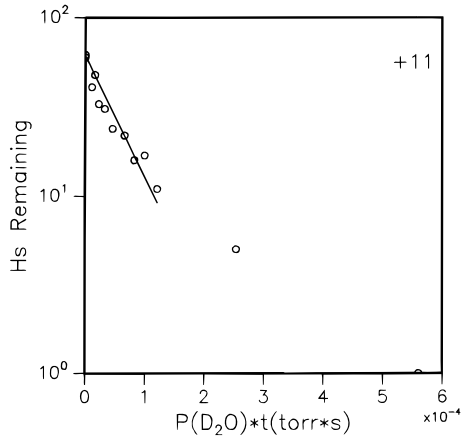

Figure 6. Kinetic plot of the number of exchangeable hydrogens that remain as a function of $P(D_2O)t$ for the diffuse conformer of the +11 charge state.

measured drift time [$P(D_2O)t$] for the +11 charge state. From a least-squares fit of these data we estimate a rate constant of $\sim 5 \times 10^{-13}$ cm^3 s^{-1}. Analysis of other charge states yields values ranging from ~4 to $\sim 7 \times 10^{-13}$ cm^3 s^{-1} for the +8 through +17 charge states, respectively. The overall trend is that as the charge state increases, the rate constants increase within this range.

Gas-Phase H/D Exchange of Compact Conformers. As shown in Figure 2 for the +8 charge state, at low injection voltages relatively compact conformations are favored. Figure 7 shows that similar results can be obtained when the He buffer gas is doped with deuterated solvents (in this case ~0.3 Torr of D_2O). At high injection voltages (120 V), the drift time distribution is dominated by the most diffuse conformer. The corresponding mass spectrum that is also shown is dominated by a single peak that is shifted by ~8 *m*/*z* higher than the parent peak. At low injection voltages (30 V) the compact conformer dominates the ion-mobility distribution. It is important to note that although the compact conformer dominates the distribution, there is a trailing shoulder observed at longer times that must be due to other conformations that are also present at this injection voltage. The corresponding H/D exchange mass spectra in Figure 7 shows that the *m*/*z* shift for the compact conformer is ~2.3 below that observed for the diffuse conformer. The compact conformer exchanges at a lower level (~46).

Mass spectra associated with H/D exchange with gas-phase D_2O display peaks that are broader than the parent peaks. At high injection voltages the mass peak for the diffuse conformer has a full width at half maximum that is ~1.4 times larger than that for the reference peak. When H_2O is substituted for D_2O, the mass spectra are identical (within experimental uncertainty) to the parent mass spectral peaks obtained in pure He. Thus, noncovalent clustering of water molecules is not observed under these conditions for the diffuse conformation. The slightly broader peak indicates a small distribution within the exchange level.

Mass spectral peaks for the compact conformers (such as peak C in Figure 6) for the +8 through +10 charge states are all significantly broader than the parent peaks. This could indicate that additional exchange levels are present, but not resolved. The drift time distribution at low injection voltages is a broad peak that falls off at longer times and shows that multiple compact conformers must be present. Some clustering of solvent molecules could also influence the position of peak C observed for the compact conformer. With 0.3 Torr of H_2O, the +8 through +10 mass peaks for the compact conformers

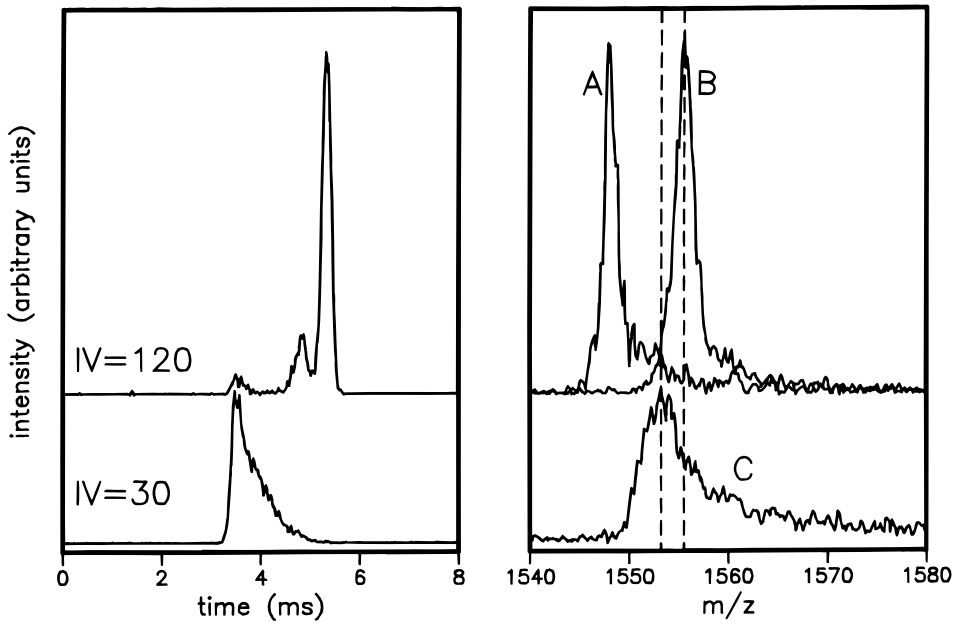

Figure 7. Drift time distributions (left) and mass spectra (right) measured for the +8 charge state of cytochrome *c* in a buffer gas containing ~0.3 Torr of D_2O. The top data in both spectra were recorded with an injection voltage of 120 V. Under these conditions, the drift time distribution is dominated by the most diffuse conformer and the center of the mass spectral peak (B) has shifted by ~8 amu from the parent peak (A) obtained with no added D_2O. The bottom data are obtained at an injection voltage of 30 V. Here the drift time distribution is dominated by a compact conformer and the mass spectrum obtained shows that the peak (C) maximum is shifted by only ~5 amu from the parent peak (A). The dashed lines show the *m*/*z* shift in peak maxima (~2.3) between B and C.

are in the same position as observed for the reference spectrum, but are slightly broadened to higher masses. Although some clustering may occur, the *m*/*z* shift in peak maxima (~5−6) when D_2O is added must be due to H/D substitution in the compact conformer.

Gas-Phase H/D and D/H Exchange of Cytochrome *c* That Has Been Deuterated in Solution. ESI MS studies of proteins dissolved in deuterated solutions are useful for monitoring conformations in solution.[8−11] Our goal in these studies is to provide a doublecheck of the gas-phase exchange levels reported above. Partially deuterated proteins can be exposed to either gas-phase D_2O or H_2O in the drift tube, and the difference in the resulting exchange levels is also a measure of the total number of hydrogens that exchange in the gas phase.

Figure 8 shows a comparison of mass spectra over the +11 charge state region when cytochrome *c* has been dissolved in a 1:1 solution of H_2O/CH_3CN with 2% CH_3COOH (peak A) and a 1:1 solution of D_2O:CH_3CN with 2% CH_3COOD for at least 20 min (peak B). The average *m*/*z* shift of peak B for three separate experiments yields an average exchange level of 148 ± 4. The exchange levels of individual charge states are listed in Table 1, and the average of all charge states is 150 ± 2.

Drift time distributions for each charge state, which are recorded when the partially deuterated protein is injected into the drift tube at 120 V, are dominated by a single narrow peak, and the cross sections derived from these data are identical (within experimental uncertainty) to those derived above for the most diffuse conformer (Table 1). Figure 8 also shows the mass spectra for the +11 charge state obtained after the partially deuterated conformer (from the deuterated solution) is exposed to either D_2O or H_2O in the gas phase. When the diffuse conformer is exposed to additional D_2O in the gas phase the peak in the mass spectrum (peak C in Figure 8) shifts to higher *m*/*z* values, because of further H/D exchange in the drift tube. When exposed to gas-phase H_2O the mass is decreased (peak D), compared with the partially deuterated parent, indicating that hydrogens must have exchanged for deuteriums. The total number of deuterium atoms on the protein after the partially

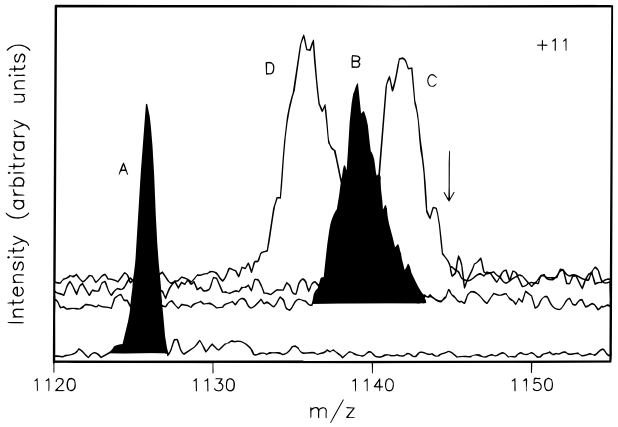

Figure 8. Mass spectral peaks recorded for the +11 charge state of cytochrome *c* using different conditions to deuterate the protein. Peak A is the reference mass spectral peak recorded when the protein is electrosprayed from an isotopically pure hydrogenated solution containing ^1H_2O, CH_3CN, and CH_3COO^1H (see text). Peak B is the data recorded when protein is dissolved in a deuterated solution containing D_2O, CH_3CN, and CH_3COOD and injected into the drift tube containing pure He gas. Peaks C and D show the *m*/*z* shifts observed when the partially deuterated protein is injected into a drift tube containing either D_2O or H_2O, respectively, both at a partial pressure of ~0.3 Torr. The arrow shows the *m*/*z* shift calculated for an exchange level of 198.

deuterated protein (from solution) has been exposed to either H_2O or D_2O in the gas phase can be obtained by comparing the *m*/*z* shifts measured for peaks C and D with peak A. All charge states behave similarly, and the results for the number of exchangeable hydrogens are given in Table 1. The average number of total deuteriums on the diffuse conformer after being exposed to D_2O or H_2O in the drift tube is 181 ± 6 or 116 ± 6, respectively. The measured differences in exchange levels are listed for individual charge states in Table 1, and the average difference is 65 ± 5.

As Chait and co-workers have discussed previously, solution exchange results can be influenced by backexchange of the

deuterated protein with H_2O in ambient laboratory air during the electrospray process.[8] We found that in order to measure the maximum H/D exchange number in solution, high curtain gas flow rates were required. Under these conditions, ion signals were decreased by roughly an order of magnitude and we were unable to carry out detailed studies in our drift tube. In a limited series of studies, cytochrome *c* that had been dissolved in D_2O and heat denatured, we have observed a solution-exchange level of ~200 and backexchange with H_2O in the drift tube yields a D/H exchange level of ~60.

Discussion

Collision Cross Sections. As mentioned above, collision cross sections for cytochrome *c* have been measured previously using ion scattering and ion mobility techniques. Both methods yield cross sections that increase with increasing charge state, but the values are not in quantitative agreement. Collision cross sections measured by ion scattering techniques are expected to have large uncertainties because of difficulties in defining the scattering event.[19,36] The collision cross sections reported in Table 1 agree to within 1% of those measured previously using mobility techniques,[23] indicating that there are no systematic differences between the instruments.

The 2129-Å^2 value measured for the cross section for the most diffuse conformer observed for the +8 charge state is much larger than the 1080-Å^2 value calculated for the native structure and must correspond to a conformer that is very open in nature. As the charge state increases, the collision cross sections for higher charge states systematically increase to 2771 Å^2 for the +18 charge state. A near-linear form of cytochrome *c* (obtained by straightening out the protein) has a calculated cross section of ~3400 Å^2 and provides an upper limit, where no secondary or tertiary structure exists. Although the +18 charge state is highly diffuse it still contains some structure and is significantly more compact than the limiting linear structure.

The cross sections measured for the compact conformers observed for the +8, +9, and +10 charge states (1260, 1352, and 1511 Å^2, respectively) are much closer to the 1080-Å^2 value calculated for the structure of the native protein in solution.[23] These conformers must contain a significant tertiary structure, although as recently discussed,[12] it is unclear if conformations in the gas phase will resemble those in solution.

An issue that arises in the present experiment is the origin of the increases in the collision cross sections that are observed as the charge state increases. The cross section for the diffuse conformer systematically increases by ~3% with each increase in charge such that the value for the +18 charge state (2771 Å^2) is ~30% larger than the 2129 Å^2 cross section measured for the +8 charge state. A straightforward interpretation is that increasing Coulomb repulsion forces the protein to open up. Estimates of the Coulomb energies as a function of charge state show that sufficient energy is available to break numerous hydrogen bonds within the protein, thus forming a more diffuse structure.[26] Long-range ion-induced dipole interactions between the protein ion and the helium buffer gas will also increase with charge state, such that even if the structure remained constant a small increase in the measured cross sections will occur. Detailed modeling of the ion-induced dipole augmentation shows that long-range interactions can influence the cross section by at most only a few percent of the total, even for the highest charge states.[26] Thus, the overall increase observed experimentally must be due to changes in geometry. Recent microscopy studies of the surface imprints left after high-energy collisions of multiply charged proteins with surfaces are consistent with this interpretation.[22]

H/D Exchange Levels of Diffuse and Compact Conformers. The total number of exchangeable hydrogens for neutral N-terminal acetylated horse-heart cytochrome *c* is 198—as reported by McLafferty and co-workers[16,17] by taking into account all N−H and O−H bonds, as well as the imidazole C-2 of histidine. H/D exchange studies of cytochrome *c* in solution by NMR show that in neutral solution the native state rapidly exchanges 144 sites, and under acidic conditions, where the compact A state is favored, 154 exchanges are observed.[37,38] The 63 ± 2 level that we measure for the most diffuse conformer observed in drift tube studies is quite low, about $^1/_3$ of the total. A check of the total exchange value for the diffuse conformer, from gas-phase H/D and D/H exchange data for the solution-deuterated protein, gives an average value of 65 ± 5, in good agreement with the 63 ± 2 level.

When a more compact conformation is favored by the injection process for the +8, +9, and +10 charge states, the number of exchangeable hydrogens decreases to ~46 (Table 1). The decrease in the numbers of exchangeable hydrogens for the compact conformation, compared with the 63 ± 2 level found for the diffuse conformation, is consistent with a structure that protects some hydrogens. These results demonstrate that the level of H/D exchange depends on the gas-phase conformation as proposed previously.[16,17]

At high injection voltages our combined ion mobility and H/D studies for cytochrome *c* suggest that we are examining a single type of conformation, although the slight exchange distribution suggests that minor variations within this conformer type are present. The collision cross sections for this family increase with increasing charge state, while the level of H/D exchange remains constant. The constant level of H/D exchange is surprising. We expected the systematic increase in exposed surface area to lead to greater exchange. Further increase in the level of H/D exchange could also result from the increased number of protons on high charge states compared with low charge states (i.e., the +18 charge state has ten more protons than the +8 state).

Comparison with FTICR Exchange Results. The FTICR studies have shown multiple levels of H/D exchange within single charge states that are also independent of charge state.[16] Although we observe only a single exchange level for the +11 through +18 charge states, the ion mobility data show that we have favored a single conformer by the injection process. For the +8 through +10 charge states, where we have been able to favor either a compact or diffuse conformers, two exchange levels are observed. Thus, H/D exchange studies in a drift tube also show that multiple exchange levels can be observed and the compact conformations exchange fewer hydrogens than the diffuse conformers, consistent with many of the ideas proposed in the FTICR work.

An obvious difference between H/D exchange in a drift tube and that observed by FTICR is that exchange occurs at much higher levels in the latter method. In their first report of coexisting stable conformers for cytochrome *c* McLafferty and co-workers reported four unique exchange states: 53 ± 2 for the +6 and +7 charge states; 82 for the +8 charge state; 113 ± 1 for the +8 through +14; and 74 ± 3, observed for the high charge states, for +12 through +16.[16] The single exchange level that we observe at 63 ± 2 for the diffuse conformer is closest to the 53 ± 2 and 74 ± 3 levels, and it is possible that we have measured a related structure. The exchange level that

(36) Douglas, D. J. *J. Am. Soc. Mass Spectrom* **1994**, *5*, 17.
(37) Jeng, M. F.; Englander, S. W.; Elove, G. A.; Wand, J.; Roder, H. *Biochemistry* **1990**, *46*, 10433.
(38) Wand, A. J.; Roder, H.; Englander, S. W. *Biochemistry* **1986**, *25*, 1107.

we measure for the +8 through +10 compact states is also similar to the 53 ± 2 value measured by FTICR for the +6 and +7 charge states.

In their second report on cytochrome *c*, McLafferty and coworkers reported higher exchange levels, a result that they suggested might be attributed to different electrospray conditions or possibly more complete exchange due to decreased levels of ^1H_2O in their vacuum system.[17] The new exchange levels ranged from 95 ± 3 to 133 ± 2 for ions formed by electrospray that were not modified before the exchange process. The exchange results measured in our drift tube seem unrelated to any of these states. The only value in the second FTICR study that appears similar to our data is the 64 exchanges measured for the +7 charge state that was formed by charge stripping the +15 ion. A result of their second study that is particularly remarkable is that after the protein was exposed to high-energy collisions, an exchange level of 173 ± 2, which corresponds to nearly complete exchange, was observed. Such a high level of exchange would be expected for conformations with little tertiary structure (such as the diffuse conformers that are formed in our experiments at high injection voltages) and where virtually no other restrictions for H/D exchange were imposed. In sharp contrast, H/D exchange of diffuse conformers formed by injection at high energies into the drift tube appears to be a highly restricted process.

There are several differences in the drift tube and FTICR experiments that could account for the differences in the measured H/D exchange levels and are useful to consider in rationalizing the different results produced by these techniques. First, the time scales of these techniques are vastly different. Ion mobility/exchange experiments sample structures over millisecond time periods while FTICR techniques record the exchange process for up to 30 min ($\sim 10^5-10^6$ times longer). The short time scale of the drift tube experiment is counterbalanced by utilizing much higher deuterated solvent pressures (0.005 to 0.4 Torr in these studies compared with $\sim 10^{-7}$ Torr in the FTICR studies). Our estimates of the H/D exchange rate constants (~ 4 to 7×10^{-13} cm^3 s^{-1}) are similar to values reported by FTICR (5 to 46×10^{-13} cm^3 s^{-1}). It is possible that we have sampled the same conformers such as FTICR but have observed much lower exchange levels due to differences in the kinetics associated with the conformational dynamics of the protein. The long time scale of the FTICR experiment makes it potentially more sensitive to reversible unfolding/refolding events that could expose protected regions of the protein to H/D exchange, fluctuations that might not occur significantly during the millisecond time periods that ions spend in the drift tube. If this is the case, then we could be examining the same (or similar) conformations as studied by FTICR and the low levels of exchange would be explained from the short time scales of our measurement, before conformational fluctuations or other mechanisms for increasing exchange (such as H/D transfer within the protein) can occur.

Of course, it is also possible that the structures we have sampled in the drift tube are entirely different than any observed in the FTICR studies. Our H/D exchange results at high injection energies are for what appear to be single conformers for each charge state that are formed after the protein ions are subjected to a rapid heating/cooling cycle. The unique nature of the injection process may favor structures that are not present in the FTICR studies, and this may explain the different exchange levels observed. Differences in the ions's internal or kinetic energies could also result in differences in the exchange levels observed. After initial entry into the drift tube, collisions with the buffer gas ($\sim 10^5$ per cm) should rapidly thermalize internal modes of the protein. The ensuing H/D exchange reactions will occur at a well-defined temperature that is determined by the buffer gas. Collisional excitation in an FTICR clearly leads to a higher exchange level, but it is possible that this is due to differences in the total energy available for exchange, as well as different structures that are favored. Radiative and collisional cooling of ions in an FTICR are expected to occur rapidly, far faster than the time scale of the measurements; however, it is possible that some modes are not quenched entirely and differences in internal energy also contribute to the differences between these experiments. We are currently investigating the temperature dependence of H/D exchange in our drift tube in order to further assess the relationship of the two techniques.

Structural Implications of Combined Measurements. The extremely large cross sections that we measure for the high charge states yield surprisingly low exchange levels, and it is difficult to rationalize a diffuse structure that can protect so many hydrogens. Compact conformers for the +8, +9, and +10 charge states show higher levels of protection than diffuse conformers, a result that suggests that H/D exchange studies in the gas phase can directly probe the three-dimensional conformation. However, the limited exchange levels that we observe for the most diffuse conformer in drift tube studies make it clear any structural assignments made from H/D exchange alone must be interpreted with caution,[18] especially experimental results that probe H/D exchange over short time scales. It is possible that most of the exchangeable hydrogens are exposed in the diffuse conformers, but exchange is restricted for another reason, such as barriers that result from local chemical environments. Detailed studies of the exchange rates and mechanisms for small polypeptide ions show that H/D exchange does not always proceed to completion, even for small polypeptides containing only a few residues.[18,39] Thus, it is not unreasonable that some hydrogens in diffuse gas-phase protein conformers would not exchange even though they might appear sterically accessible.

Although difficult to rationalize, we do not rule out the possibility of a structure that can expand while still protecting the majority of exchangeable hydrogens. Significant hydrogen bonding (which is energetically favorable) can still exist in diffuse conformations and would be expected to restrict exchange. It is interesting that exchange levels do not appear to increase with increasing protonation, and in fact some levels for some proteins appear to decrease above a critical charge state.[16] This suggests that additional protons are protected, perhaps because of intramolecular charge solvation.[15,26] Hydrogens that are expected to undergo rapid H/D exchange are located on moieties that have significant dipole moments, and dipole−dipole or dipole−induced dipole interactions will be energetically favorable and might also restrict exchange. Comparison of the largest cross section that we have measured (2771 Å^2 for the +18 charge state), which corresponds to the most diffuse conformer studied here, to the 3400-Å^2 value that has been calculated as the cross section upper limit (i.e., the value obtained for a near-linear form of cytochrome *c*)[23] shows that even the very diffuse +18 state may retain structure that influences the exchange process.

Conclusions

Combined ion mobility and H/D exchange measurements show that the exchange level for even very diffuse conformers is extremely low ($\sim 1/3$ of the 198 total possible). Although increasing the charge state systematically increases the cross

(39) Gard, E.; Green, M. K.; Gregar, J.; Lebrilla, C. B. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 623.

section (consistent with the protein opening up to minimize Coulomb repulsion[19,22,23]), the H/D exchange level remains independent of charge state (a result that has been observed previously for several proteins[14,16,17]). This suggests either that many exchangeable hydrogens in very diffuse conformers are exposed and exchange in a drift tube is restricted for other reasons or that a structural motif that can expand while still protecting a majority of exchangeable hydrogens is present.

An understanding of the relationship of the diffuse gas-phase conformer to its anteceding solution conformation is beginning to emerge. From the annealing data for the +8 charge state in it appears that electrospray can generate gas-phase conformers that are relatively compact. When these species are injected into the drift tube at high injection voltages, new diffuse conformers are observed. The gas-phase compact conformers can protect some hydrogens that become exposed in the diffuse conformer, a result that is analogous to the protection of hydrogens in solution by compact conformers compared with thermally denatured conformers, although in solution the protein exchanges at a much higher level.

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