Gas-Phase H/D Exchange and Collision Cross Sections of Hemoglobin Monomers, Dimers, and Tetramers

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The conformations of gas-phase ions of hemoglobin, and its dimer and monomer subunits have been studied with H/D exchange and cross section measurements. During the H/D exchange measurements, tetramers undergo slow dissociation to dimers, and dimers to monomers, but this did not prevent drawing conclusions about the relative exchange levels of monomers, dimers, and tetramers. Assembly of the monomers into tetramers, hexamers, and octamers causes the monomers to exchange a greater fraction of their hydrogens. Dimer ions, however, exchange a lower fraction of their hydrogens than monomers or tetramers. Solvation of tetramers affects the exchange kinetics. Solvation molecules do not appear to exchange, and solvation lowers the overall exchange level of the tetramers. Cross section measurements show that monomer ions in low charge states, and tetramer ions have compact structures, comparable in size to the native conformations in solution. Dimers have remarkably compact structures, considerably smaller than the native conformation in solution and smaller than might be expected from the monomer or tetramer cross sections. This is consistent with the relatively low level of exchange of the dimers. (J Am Soc Mass Spectrom 2009, 20, 484–495)

Water is believed to play an important role in stabilizing protein structure in solution, with the largest effect credited to the burial of hydrophobic groups within the core of the native protein [1]. Electrospray ionization has allowed the study of protein ions in the gas phase, free of water. In the absence of water, monomeric proteins can adopt compact conformations similar to the solution conformations, but also extended conformations far removed from the native state [2, 3]. Much of the current understanding of the "size" of protein ions in the gas phase comes from physical methods of measuring collision cross sections using ion mobility spectrometry at pressures of a few Torr [4–7], at atmospheric pressure [8], or at pressures of ca. 1×10^{-3} Torr [7, 9, 10]. Measurements of ion kinetic energy loss in triple quadrupole systems have also been used to determine collision cross sections of proteins [11–13] and protein–ligand complexes [11, 14, 15]. Chemical probes of protein conformation have been used as well, with much of the work using gas-phase hydrogen deuterium exchange (H/Dx) of trapped ions, either in linear [16–19] or 3D [20] quadrupole ion traps, or ion cyclotron resonance (ICR) mass spectrometers [21–25].

The study of the structure of monomeric proteins in the gas phase has been extended to assemblies containing more than one protein. Ion mobility measurements of complex macromolecular protein complexes at atmospheric pressure [8] or at a pressures of ca. 1×10^{-3} Torr have shown some success [9, 10]. The resolution of these experiments, ca. 5–20, is lower than more conventional mobility measurements at pressures of a few Torr.

Protein–protein and protein–ligand complexes can be studied by gas-phase H/Dx of trapped ions. The pressures of deuterating reagent in linear trap experiments can be $\sim 5 \times 10^{-3}$ Torr, ca. 10^3 times greater than used in 3D traps [20], 10^1 to 10^2 times greater than used in mobility experiments [26, 27], and 10^3 to 10^4 times greater than in ICR experiments [21–25]. The higher pressures allow faster exchange experiments. The conformations of gas-phase apo- and holomyoglobin ions confined in a linear trap were studied with H/D exchange by Mao et al. [16]. The apomyoglobin ions were formed by dissociating the heme from holomyoglobin ions in the ion sampling interface of the trap system [16]. The holo- and apomyoglobin ions with charge states between +8 and +14 showed comparable exchange levels after 4 s.

Protein–protein complexes have not been studied with collision cross sections determined from measurements of axial kinetic energy losses in triple quadrupole experiments. These experiments are relatively low-resolution, and cannot easily distinguish different coexisting conformations of ions of a given charge state. However, such measurements are relatively precise (±few %) and are sufficiently gentle that they can be applied to

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noncovalently bound complexes [11, 14, 15]. For instance, Collings et al. showed that the cross sections of apomyoglobin ions, formed by dissociation of holomyoglobin in the interface region of a triple quadrupole mass spectrometer, can be significantly greater than the cross sections of holomyoglobin ions [11]. It was proposed that when activated, holomyoglobin ions first unfold, and then release the heme group to give unfolded apomyoglobin ions [11]. Thus, the mechanism of the gas-phase formation of apomyoglobin from holomyoglobin parallels that of solution myoglobin [28].

In this study, we examine the gas-phase properties of the multimeric protein complex, hemoglobin, along with its monomeric and dimeric subunits, and how the gas-phase properties compare with the solution properties. In solution, mammalian hemoglobins are tetramers. The monomeric subunits include two pairs of heme-containing α- and β-subunits in a tetrahedral arrangement. Monomers (α or β), dimers ($\alpha\beta$), and tetramers ($\alpha_2\beta_2$) are in equilibrium [29]. Each of the subunits can be in the hologlobin (heme-bound) form (α^h and β^h) or apoglobin form (α^o and β^o) [30]. ESI can form multiply protonated gas-phase ions of these noncovalently bound hemoglobin complexes, provided that the solvent composition and instrument conditions are appropriately chosen [31–34]. Hemoglobin provides a model case where the properties of the gas-phase dimer subunits and tetramers can be compared directly with their monomeric subunits.

Although commercial lyophilizate of bovine hemoglobin should not be used to infer any significant biological relevance [35], it is used here as a well established model for protein–protein interactions in the gas phase. We have measured the H/D exchange levels and collision cross sections of ions of the monomers (α^h, β^0) dimers ($\alpha^h\beta^h$) and tetramers $(\alpha^h\beta^h)_2$. Slow dissociation of the tetramers to dimers, and dimers to monomers during the H/D exchange experiment did not prevent drawing conclusions about the exchange levels of the ions. Assembly of the monomers into tetramers and higher order multimers (artifacts of ESI) causes the monomers to exchange a greater fraction of their hydrogens. However, the dimer ions exchange a lesser fraction (~30%) than the monomer ions (~40%). Cross sections show that the monomer ions in low charge states and tetramer ions have compact structures, similar in size to the native conformations in solution. Dimers have very compact structures, considerably smaller than the solution conformation, and smaller than might be expected from the monomer or tetramer cross sections. This is consistent with the relatively low H/D exchange levels of the dimers.

Experimental

Solutions, Reagents

Bovine hemoglobin (H2625) was from Sigma Chemical Co. (St. Louis, MO), methanol (HPLC grade) and ammonium acetate (A637-500) were from Fisher Scientific (Fair Lawn, NJ). D_2O (99.9% D) was from Cambridge Isotope Laboratories (Andover, MA).

Sample Preparation

Stock hemoglobin solutions were dialyzed overnight in 10 mM ammonium acetate (pH 6.8) using a cellulose membrane (MWCO 8000; Spectra/Por, Rancho Dominguez, CA). For gas-phase H/D exchange and collision cross section measurements, before MS analysis, 10% methanol was added to the hemoglobin solutions to reduce the surface tension, for final solutions containing 10 mM ammonium acetate at pH 6.8 and water/methanol in a 9/1 ratio. The addition of 10% methanol is not expected to cause changes to the protein conformation. For example, addition of 10% methanol to solutions of Mb does not appreciably change the UV and CD spectra, Soret absorption, tryptophan fluorescence, charge state distributions, or H/D exchange levels of the protein [36].

H/D Exchange of Gas-Phase Ions

H/D exchange experiments were performed with a linear quadrupole ion trap reflectron time-of-flight mass spectrometer system (LIT-TOF) described previously [37, 38]. Ions are formed by electrospray of solutions infused at 2 μL/min through a 2 cm long fused silica capillary sprayer [39]. Ions then pass through an aperture (5 mm diameter) in a curtain plate (1000 V), pass through a dry nitrogen "curtain" gas (~1 L/min), an orifice (0.25 mm diameter, 60–320 V), a skimmer (0.75 mm diameter aperture, 20 V) (orifice skimmer voltage difference ΔV_{OS} = 40–300 V), and enter a chamber containing two radio frequency (rf) only quadrupoles, Q_0 (length 5 cm, field radius r_0 = 4.17 mm, DC offset = 15 V, and Q_1 (length 20 cm, field radius r_0 = 4.17 mm, DC offset = 10 V) pumped by a turbomolecular pump to a base pressure of 2 mTorr. The region between the orifice and skimmer is evacuated to a background pressure of ~2 Torr by a rotary pump. The trap chamber pressures were measured with a precision capacitance manometer (model 120AA; MKS Instruments, Andover, MA). For control experiments, the quadrupole chamber pressure was raised to 10 mTorr of N_2 by partially closing a gate valve between the chamber and the turbo pump. For exchange experiments, the turbo pump gate valve was adjusted to obtain a trap chamber pressure of 5 mTorr N_2, and then D_2O vapor was flowed through the trap chamber at a pressure of 5 mTorr, set with a needle valve (SS-SS4-Al Swagelok, Solon, OH). Thus the final trap pressure was 10 mTorr (5 mTorr N_2 + 5 mTorr D_2O). Quadrupole Q_1 is operated as a linear ion trap (LIT) with a DC offset of 10 V. Ions are confined radially by the rf potentials (frequency 768 kHz) of the quadrupole, and axially by timed DC stopping potentials applied to the entrance lens (Q_0/Q_1, 4.0 mm aperture covered with a 90% transmitting 50 mesh grid) and exit lens (L_1, 0.7 mm

aperture, which also acts as the differential pumping orifice). All data presented here were done with a quadrupole radio frequency voltage of 500 $V_{0\text{-}p}$ pole to ground, which gives the +18 tetramer ion and the free heme q-values of 0.14 and 0.77, respectively. Increasing the trapping mass command voltage to increase the q-values of the ions did not affect the relative (or absolute) intensity ratios of dimer and tetramer peaks or the extent of desolvation, but destabilized the lower m/z ions.

For trapping, the entrance (Q_0/Q_1) and exit (L_1) lens voltages are +40 V. After the trapping period, the exit lens voltage is lowered to −15 V, and ions leave the trap through a stack of four focusing lenses (L_1–L_4) and enter the source region of a reflectron-TOF (SCIEX, Concord, ON, Canada) used for mass analysis. Ions are extracted into the flight tube with a series of acceleration pulses and detected by a dual microchannel plate (MCP) (MCP 40/12/8 D EDR 46:1 MS CH None (PN 31,345); BURLE Electro-Optics, Inc., Sturbridge, MA). The timing sequence for the ion storage and TOF acquisition is controlled by an arbitrary waveform generator (AWG-344; PC Instruments Inc., Akron, OH) which triggers home-made power supplies for the trap entrance and exit plates, as well as a pulse generator (model 500; Berkeley Nucleonics, San Rafael, CA) to control the TOF-MS detection. Typical trapping conditions: 50 ms drain, 50 ms injection, 1–10,000 ms trap, 20 ms detection. A five point adjacent average smoothing function was applied to each spectrum before converting time of flight to m/z. ES Tuning mix for LC/MSD Ion Trap (G2431A; Agilent, Santa Clara, CA) was used for mass calibration.

Collision Cross Sections

Collision cross sections of ions were measured with axial kinetic energy loss experiments with a triple quadrupole mass spectrometer [13, 39, 40]. Protonated protein ions, generated by ESI, pass through an orifice in a curtain plate (880 V), an orifice (220 V), a skimmer (120 V), (orifice-skimmer voltage difference ΔV_{OS} = 100 V), and enter an rf-only quadrupole ion guide (Q_0) with a DC offset of 117.5 V. The pressure in the ion guide is ~4 mTorr, measured with a precision capacitance manometer (model 120AA; MKS Instruments, Andover, MA). In Q_0, ions cool to ion translational energies and energy spreads of about 1–2 eV per charge. Ions then pass through a quadrupole (Q_1, DC offset = 110 V) operated in rf-only mode and are injected into a quadrupole collision cell (Q_2, DC offset = 105 V) with an initial kinetic energy, E_0, of 12.5 eV per charge, determined by the potential difference between the rod offsets of the first rf quadrupole (Q_0) and the collision cell (Q_2). The collision cell contains argon at pressures between 0 and 1 mTorr. The kinetic energies, E, of ions leaving Q_2 are determined from stopping curves generated with the rod-offset potential of Q_3, operated as a mass filter. Cross sections, σ, are calculated by fitting E to

$$\frac{E}{E_0} = \exp\left(\frac{-C_d n m_2 l \sigma}{m_1}\right) \quad (1)$$

where C_d is a drag coefficient for diffuse scattering [12, 13], n is the gas number density, m_1 is the mass of the protein ion, m_2 is the mass of the collision gas, and l is the length of the collision cell (20.6 cm).

Results and Discussion

Mass Spectra of Hemoglobin

In solution, hemoglobin tetramers are in equilibrium with dimers and monomers. The free-energy of dissociation of deoxy hemoglobin tetramers is $\Delta G^0 = -14$ kcal/mol [41], which gives an equilibrium constant for tetramer dimer dissociation of $K_D = 4.2 \times 10^{-11}$ M. The calculated dimer concentration for a 100 μM hemoglobin solution then is 6.4×10^{-8} M or <0.1% of the tetramer concentration. Similarly for dissociation of carboxydimers to monomers, $\Delta G^0 = -17$ kcal/mol [41], so $K_D = 2.5 \times 10^{-13}$ M and the ratio of concentrations monomer/dimer is calculated to be 2×10^{-3}. Thus the levels of dimers and monomers in solution are expected to be very low. Nevertheless, all ESI MS studies of Hb show substantial levels of dimers and monomers in the spectrum [32, 35, 42–44].

In some cases, our spectra of hemoglobin are similar to those observed by others, but differ in detail. The spectra depend strongly on instrument operating conditions. At near-neutral pH, the ESI mass spectrum of hemoglobin can be dominated by ions of the intact tetrameric quaternary structure, and this has been attributed to the solution composition [32, 35, 42–44]. Figure 1 shows the mass spectra of 100 μM hemoglobin in 10 mM ammonium acetate and 10% methanol recorded here with the LIT-TOF apparatus operated at different quadrupole pressures with ΔV_{OS} = 200 V and with no trapping. For these experiments the voltages of all the ion optic elements were kept constant. When the pressure in the quadrupoles is low (2 mTorr), the mass spectrum is dominated by peaks corresponding to both apo- and holo-α and β monomers (Figure 1a). Tetramer and dimer ions centered at ~ m/z 3500 and m/z 2500, respectively, are observed, but with very low relative intensities (less than 5% normalized relative intensity). Increasing the pressure to 5.0 (Figure 1b) and then 7.5 mTorr (Figure 1c), increases the relative intensities of the dimer and tetramer peaks. When the pressure in the trap is further increased to 12.5 mTorr, the spectrum is dominated by peaks corresponding to the tetramer and higher order multimers; hexamers, $Hb+\alpha^h\beta^h$, near m/z 4500, and octamers Hb_2, near m/z 5000, as described by others [32, 33, 43] (Figure 1d). The low charge states and narrow charge state distributions of the multimers are as expected from proteins folded into their native

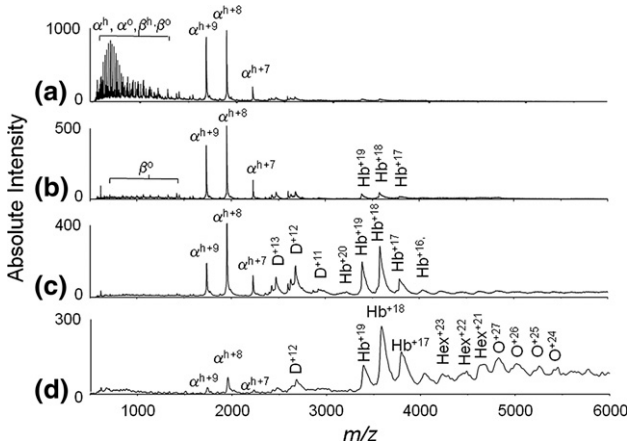

Figure 1. Mass spectra (without trapping) of 100 μM hemoglobin (as a tetramer) in 10 mM ammonium acetate/10% methanol (pH 6.8) obtained at an orifice-skimmer voltage difference of 200 V, and quadrupole pressures of (**a**) 2.0, (**b**) 5.0, (**c**) 7.5, and (**d**) 12.5 mTorr of N_2. Absolute intensities are based on 1×10^6 TOF cycles at 3333.3 Hz (5 min acquisition period).

conformations in solution. The high charge states of the monomers may be a result of asymmetric dissociation of multimers in the orifice skimmer region of the mass spectrometer [45]. The pressure dependence of the abundance of the gas-phase hemoglobin species distribution is not surprising since protein-protein complexes such as the tetramer have relatively small collision cross sections and are therefore cooled less by collisions than the monomer ions [46]. The ratios of kinetic energies of ions that have passed through Q_0 and Q_1 can be calculated from eq 1. For the +18 tetramer, (m_2 = 64,496, σ = 2750 Å^2 the ratio E/E_0 is calculated to be 0.65 with 2.0 mTorr in the chamber, but 0.065 at 12.5 mTorr. For the +14 β^0 monomer (m_2 = 15,608, σ = 2348 Å^2 the ratio E/E_0 at 2.0 mTorr is 0.21 and at 12.5 mTorr, 7.5×10^{-5}. It has been argued that the observation of ions of dimers, tetramers, and higher order multimers such as hexamers and octamers with ESI is most likely a reflection of the presence of these species in solution [32, 35, 42–44]. However hydrogen deuterium exchange experiments provide evidence that the monomers, dimers, and tetramers are present in solution, but that hexamers and octamers are artifacts of ESI [44]. Higher order multimers are not necessarily observed using other methods such as SDS-PAGE [47]. The tetramers and dimers cannot be separated by chromatography because they interchange on a time scale of seconds [48]. However, careful fitting of chromatographic peak shapes allows detection of the dimers and determination of the dissociation equilibrium constant. The monomer concentration is too low to contribute to chromatographic peak shapes [49, 50]. Because the relative abundances of these species depend strongly on the pressure in the ion guide, it is difficult to draw conclusions about the relative abundances of these species in solution from the mass spectra, but it seems the levels of dimers and monomers are greater than expected from the equilibrium solution dissociation of hemoglobin. Recent studies using freshly prepared hemoglobin have shown that the presence of β-monomers in the ESI spectra (Figure 1a–c) are likely artifacts caused by the lyophilization process used to produce commercial hemoglobin [35, 51]. Possibly in these experiments we see higher levels of dimers and monomers than expected because the hemoglobin is oxidized [35], the solutions contain 10% methanol, and multimers can dissociate in the electrospray and ion sampling processes.

CID of Gas-Phase Hemoglobin

Hemoglobin provides a model to compare monomer, dimer, tetramer, and even higher order multimer hologlobin conformations. Apoglobin proteins can be formed by collision induced dissociation (CID) of the hologlobin species by increasing the orifice to skimmer voltage difference. Figure 2 shows the mass spectra of 100 μM hemoglobin in 10 mM ammonium acetate and 10% methanol, recorded with a quadrupole pressure of 10 mTorr at different ΔV_{OS} values with no trapping. A quadrupole pressure of 10 mTorr was used for the experiments of Figure 2 because at ΔV_{OS} = 200 V and below, the spectrum has approximately equal intensities of the α^h-monomers, dimers, and tetramers. The sensitivity of the system increases with increasing ΔV_{OS}. At the lower ΔV_{OS} values of 40 and 100 V (Figure 2a and b) the holo-dimer +11 and +12 species are the most prominent, while the hemoglobin tetramer (+16 to +19) charge states and the α^h monomers (+7 to +9) are also major peaks. Peaks corresponding to oxidized

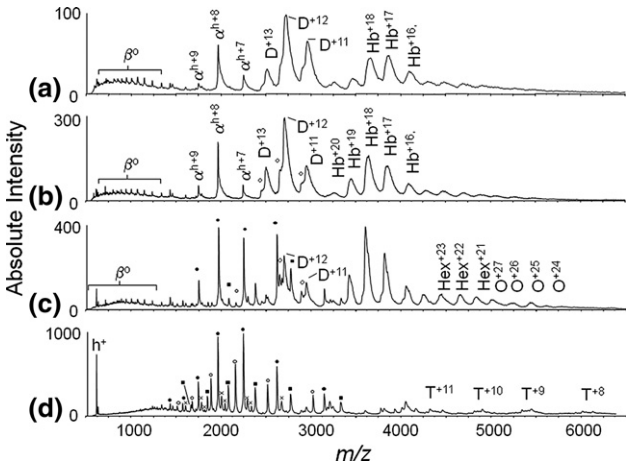

Figure 2. Mass spectra (without trapping) of 100 μM hemoglobin in 10 mM ammonium acetate/10% methanol at 10 mTorr trap pressure (N_2) and orifice-skimmer voltage differences of (**a**) 40 V, (**b**) 100 V, (**c**) 200 V, and (**d**) 300 V. Holo-species are denoted with a superscript h, and apo-species with a superscript o. The intact tetramer is labeled Hb while the low m/z shoulder (open diamond) denotes $\alpha^h\beta^o$ ions. Dimeric ions are D, trimers T, hexamers Hex, and octamers, O. The monomer ions (filled square) α^h, (open square) α^o, (filled circle) β^h and (open circle) β^o are shown in the ΔV_{OS} = 200 and 300 V spectra. Singly charged heme is labeled h+. Absolute intensities are based on 1×10^6 TOF cycles at 3333.3 Hz.

β^o in high charge states were observed at all the ΔV_{OS} voltages used, and were less than 10% relative intensity of the entire spectrum.

The tetramer peaks show higher *m/z* ratios than expected from their calculated masses. This is commonly seen with ESI-MS of protein-protein complexes and has been attributed to clustering with solvent molecules or other species [52–55]. In an attempt to reduce clustering of the tetramer peaks, 100 μM hemoglobin was run in 25% methanol, as described by Simmons et al. [32]. With our instrument no reduction in solvation was seen at ΔV_{OS} = 200 V. When the methanol concentration was increased to 40% the relative intensity of the multimeric peaks decreased to less than 5%, but, at lower values of ΔV_{OS} (40 or 100 V), did show tetrameric desolvation similar to that observed at ΔV_{OS} = 200 V in 10% methanol (Figure 2c.)

At ΔV_{OS} = 200 V, the tetramer ions are the most abundant, while the dimers dissociate by losing heme to form $\alpha^o\beta^h$ or $\alpha^h\beta^o$ dimers, or by dissociating to monomeric species (α^h, α^o, β^h, β^o). (Dimers such $\alpha^o\beta^h$ can be formed in solution during assembly of hemoglobin [56] and dissociation of oxidized hemoglobin [42].) No peaks from $\alpha^o\beta^o$ are observed, consistent with previous results [31, 33, 35, 42, 51]. When ΔV_{OS} is increased to 200 V, the widths of the multimeric peaks decrease significantly. The peak width (FWHM) of the +11 dimer at ΔV_{OS} = 100 V is over 150 Th, compared to a width of 50 Th at ΔV_{OS} = 200 V. Both these peak widths are much greater than expected from the isotopic distribution of a +11 holo-dimer, 1.3 Th FWHM. (The resolution of the TOF is ca. 2000, which gives a peak width of ~1 Th for a monoisotopic peak at this mass). The increased desolvation at higher ΔV_{OS} is more evident in Figure 3, which shows in detail the region from *m/z* 3500 to 4250 at the same four ΔV_{OS} voltages as used in Figure 2, and at 10 mTorr trap pressure. The solvation of the ions may be due to ammonium acetate or water. If the higher than expected *m/z* values in Figure 3 are attributed to clustering with water, the number of water molecules attached to the Hb ions can be calculated. These are shown in Table 1. The numbers of water molecules decrease with increasing ΔV_{OS}, and range from ~70 at 40 V to ~20 at 300 V. The effects of these water molecules on folding of hemoglobin can be considered.

Experiments [57] and molecular dynamics calculations [58] show that the full hydration of carboxymyoglobin, which is analogous to the hemoglobin β-monomer, requires ~350 water molecules. Other estimates give 400 to 600 water molecules. Breaks in the NMR spectra are seen with as little as ~70 and 240 water molecules (for a review see [59]). Thus, the hemoglobin tetramer might be expected to require ~4 × 350 = 1400 water molecules for full solvation and at least 4 × 70 = 280 water molecules to induce a partial folding transition. However in hemoglobin, about 20% of the surface area of the monomers is buried in intramolecular contacts [60], so these numbers might be

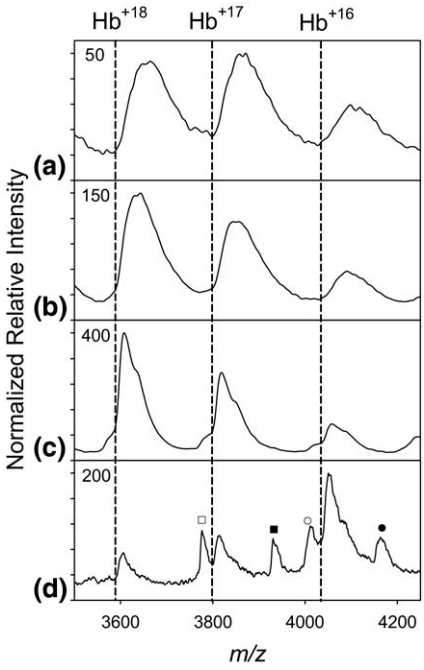

Figure 3. Normalized high-*m/z* region mass spectra of 100 μM hemoglobin (as a tetramer) in 10 mM ammonium acetate/10% methanol obtained at 10 mTorr trap pressure (N_2) and orifice-skimmer voltage differences of (**a**) 40 V, (**b**) 100 V, (**c**) 200 V, and (**d**) 300 V. The dashed lines show the *m/z* ratios of the tetramer ions calculated from the amino acid sequence. The symbols at ΔV_{OS} = 300 V correspond to (open square) $\alpha_2^{h,h}\beta_2^{h,o+17}$, (filled square) $\alpha_2^{o,h}\beta_2^{o,o+16}$, (open circle) $\alpha_2^{h,h}\beta_2^{h,o+16}$, (filled circle) $\alpha_2^{o,h}\beta_2^{o,o+15}$. The intensity scale has been normalized to the most intense peak in each spectrum. The absolute intensity of the most intense peak, based on 1 × 10^6 TOF cycles, is shown in the top left hand corner of each spectrum.

reduced to ca. 1120 and 224, respectively. Thus the 20 to 70 water molecules attached to hemoglobin tetramer ions in these experiments are only about 1.8% to 6.3% of the water molecules needed to fully solvate the hemoglobin tetramer. These water molecules represent about 5 to 18 water molecules per α or β subunit on average. Molecular dynamics calculations of Steinbach and Brooks [58] show that myoglobin with up to 65 water molecules has essentially the same properties as myoglobin with no water molecules (i.e., remains completely unsolvated). Thus, the hemoglobin tetramer ions must still be considered largely "unsolvated." Increasing the curtain gas flow of the LIT-TOF did not significantly affect the desolvation of any of the ions. However, tetramer desolvation was very sensitive to the curtain gas flow rate of the triple quadrupole mass spectrometer used to measure collision cross sections (vide infra).

When ΔV_{OS} is increased to 300 V, the majority of the higher order complexes dissociate to monomeric and trimeric species. The dissociation of hemoglobin tetramers, hexamers, and octamers at high orifice skimmer voltage differences to produce apo- and holo-α- and β-monomers has been described for hemoglobin, sev-

Table 1. Numbers of water molecules bound to hemoglobin ions

ΔV_{OS} (V)	Hb^{+18}	Hb^{+17}	Hb^{+16}
40	76	67	69
100	55	54	53
200	25	23	26
300	22	18	19

eral mutants and covalently linked hemoglobin dimers [31]. Increasing ΔV_{OS} above 200 V causes the complete loss of the hemoglobin octamers and hexamers and the appearance of peaks corresponding to α- and β-globin heterotrimers, as seen by others [31, 33]. Hemoglobin dimers have also been shown to dissociate into holo- and apoglobin monomers [31, 33]. These earlier studies showed that the pathways of the gas-phase disassembly of hemoglobin differ markedly from the well-characterized solution-phase routes [32, 35, 43]. The apparent hemoglobin dissociation pathways observed here are consistent with those reported by Apostol [31] and again by Versluis and Heck [33]. Interestingly, the peaks in Figure 3d assigned to $\alpha_2^{h,h}\beta_2^{h,o+17}$, $\alpha_2^{o,h}\beta_2^{o,o+16}$, $\alpha_2^{h,h}\beta_2^{h,o+16}$, and $\alpha_2^{o,h}\beta_2^{o,o+15}$ are much narrower than the corresponding holo-tetramer peaks. If these peaks are indeed apo-species of the holo-tetramer formed by its dissociation in the gas phase, it would indicate that the loss of even a single heme group from the tetramer is accompanied by a significant loss of solvation (i.e., heme is more strongly bound than solvation molecules). However, MS/MS analysis of these peaks at ΔV_{OS} = 300 V is needed for definite identification. A similar loss of solvation was also observed by Hossain and Konermann who dissociated deuterium labeled hemoglobin tetramers by MS/MS [44]. After pulsed H/D exchange for 53 ms, a highly solvated +17 tetramer peak showed a mass corresponding to the exchange of 500 hydrogens. However, the dissociated holo-α and β monomers of this same peak revealed a total exchange of only 300 hydrogens. The missing 200 mass units were attributed to clustering artifacts [44]. Spectra obtained here with ΔV_{OS} above 300 V showed no evidence of higher order species and were excessively complicated because of extensive in-source fragmentation.

Gas-phase H/D Exchange of Hemoglobin

H/D exchange levels. Figure 4 shows mass spectra of the α^{h+8} monomer, the $\alpha^h\beta^{h+12}$ dimer, and the Hb^{+17} tetramer ions stored for up to 5 s in the presence of 5 mTorr of D_2O and 5 mTorr of N_2 (total pressure 10 mTorr) at values of ΔV_{OS} of 100 V and 200 V. For the control experiments in Figure 4a, e, and i, a gate valve between the chamber and the turbo pump was closed to keep the trap chamber at a constant N_2 pressure of 10 mTorr. As observed previously with ions of peptides and protein monomers [16–19], the peaks broaden and shift to higher mass even without trapping because some H/D exchange occurs during the 50 ms injection step. As the ion storage time increases, the peaks broaden and shift to higher mass, and, after ~1 s, begin to narrow. Figure 5 shows the number of hydrogens exchanged versus trapping time for (a) the α^{h+8}, the $\alpha^h\beta^{h+12}$ and (b) the Hb^{+17} ions at ΔV_{OS} = 100 V and also the Hb^{+17} ions at ΔV_{OS} = 200 V. For all species investigated, the maximum number of hydrogens exchanged was observed within 2–5 s of trapping. Table 2 shows the number of hydrogens exchanged for the different ions.

No evidence for multiple conformations with different exchange levels was found for any of the ions. Both the α^h and β^h monomeric peaks showed exchange levels and behavior similar to those observed for ions of holomyoglobin [16]. Furthermore, when the apo-monomers were formed by CID in the interface region, no significant differences in H/D exchange levels were observed between the apo- and holo-monomers, again as seen with myoglobin [16]. Both the monomer and dimer ions show levels of hydrogen exchange that are largely

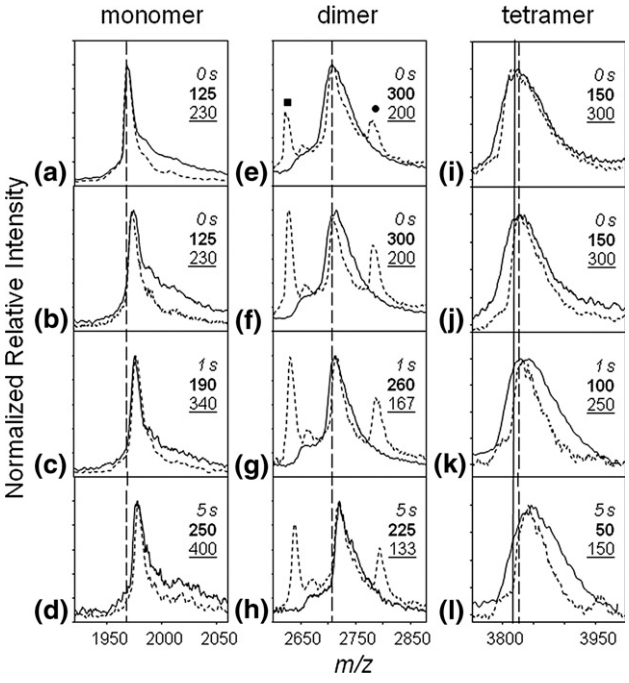

Figure 4. Gas-phase H/D exchange of hemoglobin ions in the linear ion trap at different trap times. Three different *m/z* ranges are shown to demonstrate the H/D exchange of (a–d) monomer α^{h+8}, (e–h) dimer $\alpha^h\beta^{h+12}$, and (i–l) tetramer Hb^{+17}, hemoglobin ions at (solid line) ΔV_{OS} = 100 V, and (dashed line) 200 V. The vertical lines represent the *m/z* in N_2, with no exchange at (dashed line) ΔV_{OS} = 100 V and (solid line) ΔV_{OS} = 200 V. For the monomer and dimer peaks, the *m/z* ratios with no exchange overlap at both ΔV_{OS}. Gas-phase dissociated monomeric globin ions are also observed at ΔV_{OS} = 200 V and are assigned (filled square) α^{h+6}, and (filled circle) β^{h+6}, The pressures in the LIT chamber are (**a**), (**e**), (**i**) 10 mTorr N_2, and (**b**)–(**d**), (**f**)–(**h**), (**j**)–(**l**) 5 mTorr N_2 + 5 mTorr D_2O. The trap time (*italics*) and the absolute intensities based on 1 × 10^6 TOF cycles at ΔV_{OS} = 100 V (**bold**) and 200 V (underline) are shown in the top right corner of each spectrum.

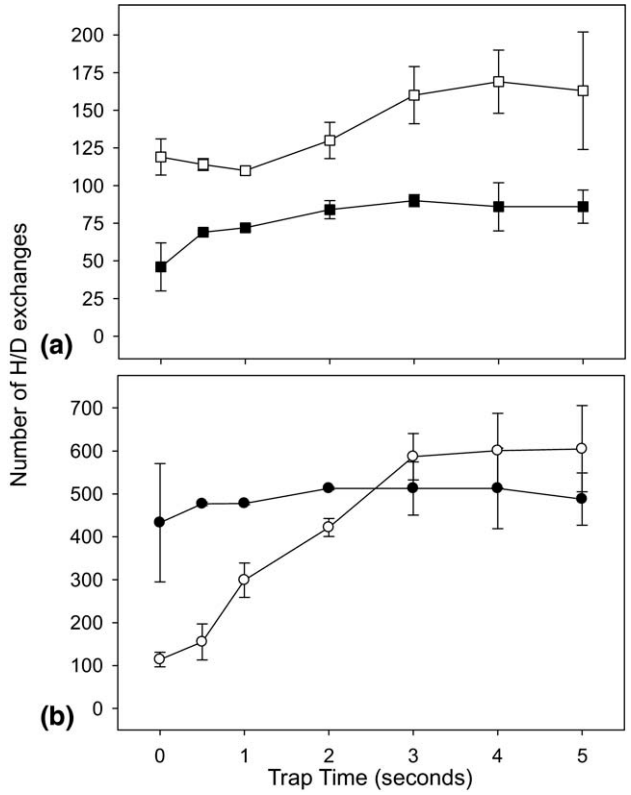

Figure 5. Numbers of hydrogens exchanged versus trapping time for ions of (**a**) α^{h+8} at ΔV_{OS} = 100 V (filled squares), $\alpha^{h}\beta^{h+12}$ at ΔV_{OS} = 100 V (open squares) and (**b**) Hb^{+17} at ΔV_{OS} = 100 V (filled circles) and Hb^{+17} at ΔV_{OS} = 200 V (open circles). The errors are the standard deviations of three trials.

independent of ΔV_{OS}. However, when ΔV_{OS} is increased to 200–300 V, the tetramers show increases in the number of hydrogens exchanged compared with the numbers at lower ΔV_{OS} voltages.

Dissociation of Tetramer and Dimer Ions

When the hemoglobin monomers, dimers and tetramers are confined in the LIT, there are slow time-dependent increases in monomer intensities at the expense of both the dimer intensities and the tetramer intensities. For instance, Figure 4 shows the absolute intensity of the +17 tetramer peak at ΔV_{OS} = 100 V decreasing 66% over 5 s trap time, and the +12 dimer decreasing 25%, while an increase in the absolute intensity of the α^{h+8} of 100% is observed. These changes are caused by slow dissociation of tetramers to dimers, and dimers to monomers as follows:

$$T \xrightarrow{k_1} D \xrightarrow{k_2} M$$

with unimolecular rate constants k_1 for tetramer dissociation and k_2 for dimer dissociation. It is conceivable that tetramers dissociate asymmetrically to trimers which then dissociate to dimers. However we see no evidence for this. The trimer ion intensities do not increase with time. If the asymmetric dissociation to trimers is slow, followed by fast dissociation to dimers, the intensities of the intermediate trimers would remain low. The rate limiting step would then be dissociation of tetramers to trimers and the dissociation would appear to follow the scheme above. More likely, the low charge states and low internal energies of the tetramers favor symmetric dissociation to dimers [61]. We model the dissociation approximately as follows. The rate of dissociation of tetramers is taken from an exponential fit to the decrease in the Hb^{+17} intensity with time. The rate of formation of dimers is equal to the rate of dissociation of tetramers, and the rate of dissociation of dimers is to take from an approximate fit to the intensity of the +12 dimer versus time. During the H/D exchange experiments the levels of hexamer and octamers and trimers are low, and are estimated to contribute less than 10% to the tetramer intensity. Thus they are not included in the approximate kinetic scheme. Approximate fits to the data give $k_1 \approx 0.23\ s^{-1}$ and $k_2 \approx 0.15\ s^{-1}$ at ΔV_{OS} = 100 V, and $k_1 \approx 0.14\ s^{-1}$ and $k_2 \approx 0.31\ s^{-1}$ at ΔV_{OS} = 200 V. The effects of these reactions on the observed exchange levels are discussed below.

Table 2. Hydrogen exchange levels of hemoglobin α^h monomers, $\alpha^h\beta^h$ dimers, and tetramers (Hb) after 5 s reaction with 5 mTorr of D_2O at different orifice skimmer voltage differences (ΔV_{OS})

		Number of hydrogens exchanged			
Charge state	H/DX_{MAX}[a]	ΔV_{OS} = 40 V	ΔV_{OS} = 100 V	ΔV_{OS} = 200 V	ΔV_{OS} = 300 V
α^{h+7}	237	99 ± 19	81 ± 14	92 ± 7	96 ± 4
α^{h+8}	238	89 ± 16	86 ± 11	103 ± 12	100 ± 11
α^{h+9}	239	81 ± 18	89 ± 9	100 ± 7	96 ± 8
$\alpha^{h}\beta^{h+11}$	487	153 ± 18	169 ± 5	142 ± 4	151 ± 7
$\alpha^{h}\beta^{h+12}$	488	159 ± 34	163 ± 37	148 ± 11	—
$\alpha^{h}\beta^{h+13}$	489	137 ± 29	134 ± 39	160 ± 5	156 ± 10
Hb^{+16}	968	360 ± 46	404 ± 62	562 ± 63	587 ± 84
Hb^{+17}	969	403 ± 45	437 ± 61	605 ± 100	599 ± 97
Hb^{+18}	970	449 ± 73	488 ± 62	619 ± 79	586 ± 91
Hb^{+19}	971	462 ± 50	486 ± 47	576 ± 81	—

[a]Maximum number of exchangeable hydrogens calculated from the amino acid sequence + 2 from the heme group of each monomer. The α chain has 140 amide hydrogens and the β chain 144 amide hydrogens.

Relative Exchange Levels of Monomers, Dimers, and Tetramers

Table 3 show the levels of exchange, expressed as a percent of the total number of exchangeable hydrogens, averaged over the charge states shown in Table 2, for monomer, dimer, and tetramer ions. Table 3 shows that the dimer ions exchange less, and therefore somehow have more protected structures, than the monomer or tetramer ions. The lower exchange level of the dimer ions than the tetramers ions is surprising. Because exchangeable hydrogens are involved in binding in the contact regions between the monomers in the dimers, and between the dimers in the tetramers, the exchange levels might be expected to decrease in the order monomer > dimer > tetramer. This is seen in the solution H/D exchange of hemoglobin. Hossain and Konermann [44] studied MS/MS of monomers dimers and tetramers that had undergone solution H/D exchange for 53 ms. The short exchange time was used to avoid any complications from the interchange of tetramers with dimers, and dimers with monomers, during the experiment. Dissociation of the tetramer and dimer ions showed that compared with the free monomer in solution, the monomers exchanged less when in the dimer, and still less when in the tetramer, as expected.

Effects of the Slow Dissociation Reactions

Because some tetramer and dimer ions dissociate during the 5 s H/D exchange time, some dimer and monomer ions are formed in the trap during the exchange period and do not spend the full 5 s undergoing exchange. The effect of this on the exchange levels can be estimated as follows. If T_0 is the initial number of tetramer ions in the trap, the number of tetramer ions at time t is

$$T(t) = T_0 e^{-k_1 t} \tag{2}$$

The number of dimer ions at time t, $D(t)$, is

$$D(t) = D_0 e^{-k_2 t} + A\left(e^{-k_1 t} - e^{-k_2 t}\right) \tag{3}$$

where

$$A = \frac{k_1 T_0}{k_2 - k_1} \tag{4}$$

The first term on the right of eq 3 describes dimer ions that are injected into the trap at $t = 0$ and remain in the trap for the full 5 s. The second term describes dimers that are formed in the trap by dissociation of tetramers, and that partially dissociate to monomers. The average time that the dimers that are formed from tetramers spend in the trap can be calculated as follows. The number of dimers formed between time t and dt, $D(t)$ is

$$D(t) = k_1 T_0 e^{-k_1 t} dt \tag{5}$$

The time these ions spend in the trap is $5 - t$ s. The number of these ions remaining after 5 s is

$$D(t = 5) = k_1 T_0 e^{-k_1 t} \cdot e^{-k_2 (5-t)} dt \tag{6}$$

The time spent in the trap, averaged over all the dimer ions formed from monomers is

$$\langle t \rangle = \frac{\int_0^5 k_1 T_0 e^{-k_1 t} (5 - t) e^{-k_2 (5-t)} dt}{\int_0^5 k_1 T_0 e^{-k_1 t} e^{-k_2 (5-t)} dt} \tag{7}$$

The denominator is just the number of ions in the trap at 5 s, which can be calculated from eq 3 with $t = 5$ s. This calculation is only for the dimers that are formed from tetramers. A fraction of the dimers which is present initially survives for 5 s and is in the trap for the full 5 s. The calculated average time for the dimers formed from tetramers when $\Delta V_{OS} = 100$ V is 2.7 s, and when $\Delta V_{OS} = 200$ V, 2.3 s. Figure 5 shows that dimers undergo about 90% of their exchange in 2.5 s, so that even though some dimers spend less than the full 5 s in the trap, their exchange goes nearly to completion. The dimers that are formed in the trap come from tetramers, and have spent some of their exchange time as tetramers. Because the tetramers have higher exchange levels, these dimers might have slightly higher exchange levels than dimers that spend the full 5 s in the trap. This would lead us to *underestimate* the difference in exchange levels between the dimers and tetramers.

Similarly, a monomer ion formed in the trap at time t stays in the trap for a time $(5 - t)$ s. The number formed at time t is $k_2 D(t) dt$. The average time in the trap is

Table 3. Percent H/D exchange

	% Hydrogens exchanged[a]			
Oligomer[β]	ΔV_{OS} = 40 V	ΔV_{OS} = 100 V	ΔV_{OS} = 200 V	ΔV_{OS} = 300 V
α^h	38 ± 4	38 ± 3	42 ± 2	41 ± 1
$\alpha^h\beta^h$	31 ± 2	32 ± 4	31 ± 2	32 ± 1
Hb	43 ± 5	45 ± 5	61 ± 3	61 ± 1

[a]Percent maximum exchange averaged across charge states in the gas-phase after 5 s.
[b]α^h used are +7, +8, +9, $\alpha^h\beta^h$ dimers used are +11, +12, +13, and Hb tetramers used are +16 to +19.

$$\langle t \rangle = \frac{\int_0^5 k_2 D(t)(5-t)dt}{\int_0^5 k_2 D(t)} \quad (8)$$

The calculated average exchange times for the monomers formed in the trap when ΔV_{OS} = 100 V is 2.6 s, and when ΔV_{OS} = 200 V, 2.7 s. Figure 5 shows that the monomers complete about 95% of their exchange in 2.5 s. Therefore the monomers formed in the trap also have sufficient time for their exchange to go to completion. Some of the monomers will have exchanged as part of a dimer. Because the dimers have lower exchange levels than the monomers, these ions may show slightly less exchange than monomers that spend the full 5 s in the trap. In summary, both the monomers and dimers formed in the trap have sufficient time for their exchange to go nearly to completion. If anything, the monomers and dimers formed during the H/D exchange will cause us to see slightly *smaller* differences between the monomers, dimers and tetramers, and the conclusion that dimers exchange less than monomers or tetramers remains valid.

Effects of Solvation

The exchange levels of the monomer and dimer ions (expressed as a percent) do not change significantly with changes in ΔV_{OS}. The tetramers however, show an increase from 45% to 61% exchange (an increase of 155 exchanged hydrogens) when ΔV_{OS} is increased from 100 V to 200 V or 300 V. When the experiments were repeated with tetramer ions produced from a solution with 40% methanol at ΔV_{OS} = 100 V, ~60% total hydrogen exchange was observed for the tetramer peaks in the gas-phase i.e., the same exchange level as the tetramer ions at 200 V and 300 V when they are produced from a 10% methanol solution. Figure 5b shows that at 200 V the ions with less solvation initially exchange fewer hydrogens, but after 5 s have exchanged a greater number than the tetramers with more solvation at 100 V. Thus solvation affects the kinetics of the H/D exchange. As discussed above, the addition of ca. 20 to 70 water molecules to hemoglobin is unlikely to cause folding or a conformation change. Increasing the degree of solvation decreases the exchange levels. The solvation molecules do not exchange and solvation partially inhibits the H/D exchange, possibly by simply blocking exchange sites.

Hexamer and Octamer Ions

Exchange of the hexamer and octamer ions was investigated with ΔV_{OS} = 200 V. The number of hydrogens exchanged after 5 s is shown in Table 4. Without H/D exchange, the masses of the ions correspond to the addition of about 55 water molecules to the hexamers and 84 water molecules to the octamers. Both the hexamer and octamer ions have higher exchange levels than monomers, dimers or tetramers. Up to 84% H/D exchange is observed compared with 42% for the monomer, 31% in the dimer, and 61% in the tetramer. It might be expected that as the monomers assemble into higher order multimers, an increasing number of hydrogens would be protected in sites that are involved in binding the multimers. Thus, our observations are counterintuitive. As the monomers assemble into higher order multimers they exchange a *greater* number of hydrogens on average.

Table 4. Hydrogen exchange levels of higher order hemoglobin complexes after 5 s reaction with 5 mTorr of D_2O and with ΔV_{OS} = 200 V

Complex	H/Dx_{MAX}[a]	# H/Dx	% H/Dx
$Hb_2+\alpha^h\beta^{h+21}$	1449	1187 ± 154	82
$Hb_2+\alpha^h\beta^{h+22}$	1450	1210 ± 129	83
$Hb_2+\alpha^h\beta^{h+23}$	1451	1219 ± 141	84
Hb_2^{+24}	1928	1256 ± 312	65
Hb_2^{+25}	1929	1344 ± 277	69
Hb_2^{+26}	1930	1476 ± 301	76
Hb_2^{+27}	1931	1573 ± 278	81
Hb_2^{+28}	1932	1450 ± 255	75

[a]Maximum number of exchangeable hydrogens calculated from the amino acid sequence + 2 (heme per monomer).

Cross Section Measurements

ESI mass spectra of hemoglobin obtained using a triple quadrupole mass spectrometer showed subunit distributions different from those obtained using the LIT-TOF. With a ΔV_{OS} of 100 V, the β^o monomer peaks (charge states +12 to +16) dominated the spectrum, with the tetramer and dimer species accounting for 25% and 15% of the total relative intensity, respectively. This is most likely due to the lower pressure in the Q_0 region of the mass spectrometer (4 mTorr) [46]. At higher ΔV_{OS}, the multimer peaks were eliminated, while at lower ΔV_{OS} the signal intensity decreased dramatically. The solvation of the tetrameric peaks could be controlled by varying the flow rate of the nitrogen curtain gas. At high nitrogen flow rates (~4 L/s) the +15 and +16 hemoglobin tetramer peaks are observed with widths (FWHM) of ~50 Th and with masses corresponding to ~17 attached water molecules, similar to that of the tetramer peaks observed at ΔV_{OS} = 200 V with the LIT-TOF. At low nitrogen flow rates (~1 L/s), the +16 and +17 hemoglobin tetramers were observed with widths of ~150 Th and with masses corresponding to ~52 attached water molecules, similar to that observed at ΔV_{OS} = 100 V with the LIT-TOF. The widths of the dimer and monomer peaks were not noticeably affected by the curtain gas flow rate. The collision cross sections of representative hemoglobin monomer, dimer, and tetramer ions are shown in Figure 6. The cross sections, averaged over charge states, are shown in Table 5. Also shown in Table 5 are cross sections for ions of myoglobin. Not surprisingly, the average collision cross section measured for the +7 and +8 α^h ions

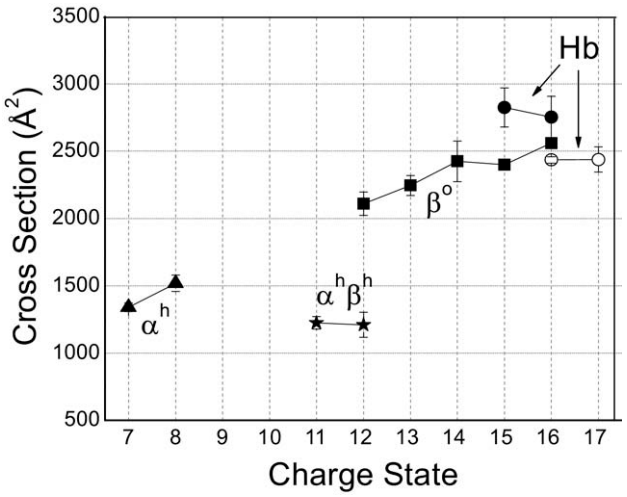

Figure 6. Collision cross sections as a function of charge state for (filled triangle) α^h, (filled star) $\alpha^h\beta^h$, (filled square) β^o, (filled circle) highly desolvated, and (open circle) less desolvated hemoglobin tetramers formed from 100 μM hemoglobin in 10 mM ammonium acetate/10% methanol (pH 6.8). The error bars are the standard deviations of four repeat measurements.

(1430 Å^2) is close to the cross section measured for holomyoglobin ions (1690 Å^2) [11]. The larger cross section in [11] likely derives from the higher charge states (+10 and +11). The cross sections of the β^o monomers (+12 to +16 average = 2348 Å^2) are similar to those measured for apo-myoglobin +12 to +14 (2309 Å^2) [11]. These are considerably greater than the cross sections of α^{h+7} and α^{h+8} ions, most likely because of their greater charges. The +11 and +12 dimer ions, with an average cross section of 1218 Å^2 have lower cross sections than the α^{h+7} and α^{h+8} monomer ions, substantially lower cross sections than +12 to +16 β^0 monomer ions (2000–2500 Å^2) and lower cross sections than the tetramer ions (2789 Å^2), indicating a very tightly folded structure for the dimers.

The tetramers with different degrees of solvation show different cross sections. Table 5 show the average cross sections of the +15 and +16 ions at the high curtain gas flow rate which gives ions with ~17 attached water molecules. At the low curtain gas flow rate, where the ions have masses corresponding to ~50 attached water molecules, the average cross sections are somewhat lower, 2415 Å^2 for the +16 and +17 ions (Figure 5). As discussed above, the solvation is unlikely to cause a folding transition. The difference in cross sections between less and more highly solvated ions may be due to differences in ion activation in the orifice skimmer region. Ion activation here causes the ions to lose solvation and can also cause the protein to unfold. When ions enter the orifice skimmer region with a greater degree of solvation, energy added to the ions mostly causes desolvation. When the ions enter the orifice skimmer region with less solvation, more of the energy added to the ions can cause protein unfolding, instead of removal of solvent, leading to somewhat larger cross sections of the ions.

The measured gas-phase collision cross sections can be compared with "sizes" of species measured in solution by small angle X-ray scattering [65], which gives the radius of gyration, r_g, and by size exclusion chromatography which gives the Stokes radius, r_s. For a sphere of radius r_0, $r_g^2 = (3/5)r_0^2$ [68]. Table 5 compares our cross sections, averaged over charge state, with those calculated from r_g, i.e., $A_g = (5/3)\pi r_g^2$, and from r_s, i.e., $A_s = \pi r_s^2$. The cross section of myoglobin in its native conformation has been estimated to be 1773 Å^2 [69]. Thus the radii of gyration give reasonable estimates of the protein's "size" (1603 Å^2 for holomyoglobin, 2115 Å^2 for apomyoglobin). The low charge state α^h monomers have compact structures, similar to the folded native conformation. The Stokes radii show that, in solution, the dimer has 1.2 times *greater* cross section than the holo-monomers. However, in the gas phase, the dimers have cross sections *lower* than the low charge state α^h monomers. The gas-phase dimers have cross sections that are ~44% of the solution dimer cross section estimated from r_g, and ~67% of the solution cross section estimated from r_s. The tetramer cross sections (2789 Å^2) are similar to the cross sections estimated from the solution Stokes radius (3077 Å^2) and somewhat smaller than that estimated from the radius of gyration (3458 Å^2). The tetramer cross section in the gas phase is ~2.2 times larger than the dimer, while this difference is only 1.7 times in solution (Table 5). In the gas phase, the tetramer cross sections are ca. 2.1 times the cross sections of the α^h monomers, similar to the difference in solution, estimated from the Stokes radii (×2.1). Thus the monomers and tetramers have structures similar in size to the native conformations in solution. In contrast, the cross sections show that dimers have exceptionally compact structures in the gas phase, compared with dimers in solution, and compared to the monomer and tetramer ions.

The lower level of H/D exchange of the dimers is consistent with the compact structure seen in the cross section measurements. While the gas-phase exchange mechanism differs from the solution mechanism, it is plausible that more folded protein ions will have a greater number of hydrogen atoms protected from

Table 5. Comparison of cross sections (Å^2)

Species	r_g	r_s	Cross section[a]	A_g	A_s
Holomyoglobin	17.5 [62]	21.5 [63]	1690[b,d]	1603	1452
Apomyoglobin	20.1 [62]	—	2309[c,d]	2115	
Holo-monomer	—	21.5 [64]	1430	—	1452
Apo-monomer	—	27.7 [64]	2348	—	2410
Dimer	23.0 [65]	24.0 [66]	1218	2770	1810
Tetramer	25.7 [65]	31.3 [67]	2789	3458	3077

[a]Measured collision cross sections averaged across the charge states shown in Figure 6.
[b]For the +10 and +11 ions, data from [11].
[c]For the +12 to +14 ions, data from [11].
[d]Cross sections from [11] reduced by 1/1.2 to correct for diffuse scattering.

gas-phase H/D exchange. For example, compact conformers of cytochrome *c* ions exchange fewer hydrogens than extended diffuse structures [26]. For small peptides, the exchange levels do not necessarily reflect structure [70, 71]. It is not clear how these studies of small peptides apply to proteins or protein–protein complexes. The lower H/D exchange levels of the dimer are at least consistent with the more compact structure seen in the cross section measurements.

Summary

Although the solution equilibrium calculations show there are very low levels of dimers and monomers in solution, these are seen in the mass spectra, even at low orifice-skimmer voltage differences where there should be minimal dissociation of tetramers. However, because the ion intensities depend strongly on operating conditions, especially the quadrupole pressure, it is difficult to draw conclusions about the hemoglobin dissociation in solution from the mass spectra. The gas-phase exchange process is complicated by the slow dissociation of tetramers and dimers, but this did not prevent drawing conclusions about the relative exchange levels of monomers, dimers, and tetramers. Assembly of the monomers into tetramers, hexamers, and octamers causes the monomers to exchange a greater fraction of their hydrogens. Dimer ions have a lower exchange level than monomers or tetramers. Solvation of tetramers by water affects the exchange kinetics. Water molecules of solvation do not appear to exchange and lower the overall exchange level of the tetramers. Cross section measurements show that monomer ions in low charge states, and tetramer ions have compact structures, comparable in size to the native conformations in solution. Dimers have remarkably compact structures, considerably smaller than the native conformation in solution and smaller than might be expected from the monomer or tetramer cross sections. This is consistent with the relatively low level of exchange of the dimers.

Acknowledgments

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