



RESEARCH ARTICLE

Mass Spectra and Ion Collision Cross Sections of Hemoglobin

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Abstract

Mass spectra of commercially obtained hemoglobin (Hb) show higher levels of monomer and dimer ions, heme-deficient dimer ions, and apo-monomer ions than hemoglobin freshly prepared from blood. This has previously been attributed to oxidation of commercial Hb. Further, it has been reported that that dimer ions from commercial bovine Hb have lower collision cross sections than low charge state monomer ions. To investigate these effects further, we have recorded mass spectra of fresh human Hb, commercial human and bovine Hb, fresh human Hb oxidized with H₂O₂, lyophilized fresh human Hb, fresh human Hb both lyophilized and chemically oxidized, and commercial human Hb oxidized with H₂O₂. Masses of α -monomer ions of all hemoglobins agree with the masses expected from the sequences within 3 Da or better. Mass spectra of the β chains of commercial Hb and oxidized fresh human Hb show a peak or shoulder on the high mass side, consistent with oxidation of the protein. Both commercial proteins and oxidized fresh human Hb produce heme-deficient dimers with masses 32 Da greater than expected and higher levels of monomer and dimer ions than fresh Hb. Lyophilization or oxidation of Hb both produce higher levels of monomer and dimer ions in mass spectra. Fresh human Hb, commercial human Hb, commercial bovine Hb, and oxidized commercial human Hb all give dimer ions with cross sections greater than monomer ions. Thus, neither oxidation of Hb or the difference in sequence between human and bovine Hb make substantial differences to cross sections of ions.

Key words: Hemoglobin, Mass spectrum, Monomers, Dimers, Tetramers, Oxidation, Collision cross section

Introduction

Hemoglobin (Hb) carries oxygen in mammalian red blood cells [1, 2] and serves as a model multimeric protein system. Adult human hemoglobin (Hb A) is assembled from two α and two β chains ($\alpha_2\beta_2$), with 141 and 146 amino acid residues, respectively, each folded similarly to myoglobin [1, 3]. Each chain binds a prosthetic heme group with a central iron. Ferric (Fe³⁺) Hb (also referred to as ^{met}Hb) does not bind oxygen, while ferrous (Fe²⁺) Hb can bind oxygen to form ^{oxy}Hb. Each subunit can be in the heme-bound form (holo-, α^h , and β^h) or heme-free

form (apo-, α^a , and β^a). The subunits bind noncovalently to form a tetrameric structure [4].

Electrospray ionization (ESI) mass spectrometry (MS) has been widely used to study Hb [5, 6]. In ESI MS, ions of monomers (α , β), dimers ($\alpha\beta$), and tetramers ($\alpha_2\beta_2$) are observed [7–11]. The conformations of these gas-phase ions and the relation of these conformations to solution conformations of the same species are of interest [12, 13]. Collision cross sections [13–16] provide a measure of an ion's size. Cross sections of ions formed from Hb have come from measurements of migration time with ion mobility mass spectrometry (IMS) [7, 11] and from measurements of ion axial kinetic energy losses with a triple quadrupole MS system [8].

The mass spectra and properties of Hb ions formed from commercial lyophilized Hb [6, 8, 10, 17, 18] differ

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significantly from the same species of Hb freshly prepared from mammalian red blood cells [7, 9]. Boys et al. found heme-deficient dimer and apo-monomer ions were formed from commercial bovine Hb, but not from fresh bovine Hb [9]. The acid-induced dissociation of freshly prepared bovine $^{\text{met}}\text{Hb}$, showed a highly symmetric unfolding mechanism, $(\alpha^{\text{h}}\beta^{\text{h}})_2 \rightarrow 2\alpha^{\text{h}}\beta^{\text{h}}$ [9], while the commercial protein produced a heme-deficient dimer ($\alpha^{\text{h}}\beta^{\text{a}}$) as an intermediate in the disassembly process [10]. With commercially obtained bovine $^{\text{met}}\text{Hb}$, Wright and Douglas reported surprisingly that gas-phase dimer ions ($\alpha^{\text{h}}\beta^{\text{h}}$) (+11, +12) had smaller cross sections than monomer ions (α^{h}) in low charge states (+7, +8) [8]. However, with freshly extracted human Hb, Scarff et al. found that cross sections of gas-phase dimer ions ($\alpha^{\text{h}}\beta^{\text{h}}$) (+12-+14) were greater than those of monomer ions (α^{h}) (+6-+10) [7]. It had been noted earlier that in commercial $^{\text{met}}\text{Hb}$, there can be oxidative modifications [9, 17, 18], mainly sulfoxide formation on methionine residues [9]. It was proposed that this oxidation might be responsible for the unusual properties of commercial Hb. This led us to ask whether the unusually low cross sections of dimer ions reported by Wright and Douglas could also be attributed to oxidation or some other chemical change in commercial Hb, or possibly to the sequence difference between bovine and human Hb.

This work, therefore, was undertaken to further elucidate the differences between hemoglobins from different sources and hemoglobins prepared in different ways, and to determine if there are differences in the cross sections of ions from these various hemoglobins. Physiologically inactive $^{\text{met}}\text{Hb}$ was used to allow comparison with previous studies. We have recorded ESI mass spectra of fresh human Hb, commercial human Hb, commercial bovine Hb, fresh human Hb chemically oxidized with H_2O_2 , lyophilized fresh human Hb, fresh human Hb both lyophilized and chemically oxidized, and commercial human Hb chemically oxidized. With the same solution and mass spectrometer conditions, commercial human Hb and fresh human Hb give significantly different spectra, with commercial human Hb forming higher levels of dimers and monomers. Oxidation, lyophilization, and lyophilization followed by oxidation of human Hb all give higher levels of monomer and dimer ions in the mass spectrum. Oxidation of fresh Hb also produces heme-deficient dimers in the mass spectrum. Both the commercial human Hb and commercial bovine Hb give α -chain monomer masses within 3 Da or better of the masses expected from the sequences, and, thus do not show evidence of oxidation. However, the β chains do show evidence of oxidation. Heme-deficient dimers with the commercial hemoglobins show masses 32 Da higher than expected, indicating that the commercial proteins were partially oxidized. Further oxidation of commercial human Hb did not produce major changes to the spectrum. Thus, differences in the spectra between fresh and commercial human Hb are partially due to oxidation, as proposed by Boys et al. [9], but also due to lyophilization of the proteins. Cross

sections of monomer, dimer, and tetramer ions of fresh human Hb, commercial human Hb, commercial bovine Hb, and commercial human Hb further oxidized with H_2O_2 have been measured. With all four hemoglobins, cross sections of dimer ions were similar, and greater than cross sections of monomer ions. Thus, we cannot reproduce the result of Wright and Douglas [8]. However it appears that neither oxidation of the protein nor the difference in sequence between bovine and human Hb make substantial differences to the collision cross sections.

Experimental

Triple Quadrupole Mass Spectrometer System

A home-built ESI triple quadrupole mass spectrometer described previously [16, 19, 20], but modified to increase sensitivity, was used. Protonated ions, formed by pneumatically-assisted electrospray (sprayer voltage=4 kV), pass through a 2.4 mm diameter aperture in a curtain plate (1150 V), a dry nitrogen curtain gas (~ 2 L/min), an ion sampling orifice (250 V) into a region with a background pressure of ca. 0.7 Torr. Ions then pass through a skimmer (150 V), into a quadrupole ion guide Q0 ($\sim 9 \times 10^{-3}$ Torr, DC offset=120 V). To increase the sensitivity, the sampling orifice diameter was increased to 0.25 mm, and the skimmer aperture diameter to 2.90 mm. The 50 L s^{-1} turbo pump on the Q0 region was replaced with a 360 L s^{-1} pump. These changes increased the gas flow into Q0 by a factor of about 7, and the sensitivity by about the same amount. The ion optics from Q0 through to the detector were not changed. In Q0, ions are cooled to translational energies and energy spreads of about 1–2 eV per charge [21]. After passing through a short radio frequency (rf) quadrupole (DC offset=108 V) [22], ions enter a quadrupole, Q1, (DC offset=95 V), a collision cell with a quadrupole ion guide, Q2, (DC offset=105 V), and a quadrupole, Q3, (DC offset=90 V). Pulse counting was used for ion detection. To record mass spectra, Q1 was operated as a mass filter. The pressures in Q2 were measured with a precision capacitance manometer (model 120AA; MKS Instruments, Boulder, CO, USA).

Collision Cross Section Measurements

Cross sections were measured with axial kinetic energy loss experiments, as described previously [15, 16, 23]. In Q2, ions lose kinetic energy through collisions with a low density gas. The energy losses can be related to the collision cross sections with an aerodynamic drag model [24] through

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

where E is the ion kinetic energy at the exit of Q2, E_0 is the ion kinetic energy at the entrance of Q2, C_d is a drag coefficient for diffuse scattering [16], n is the gas number density, m_1 is the mass of the protein ion, m_2 is the mass of

the collision gas (Ar), l is the length of Q2, and σ is the collision cross section. The pressure of Ar in the collision cell was varied between 0 and 1.2 mTorr and the stopping potential was obtained with the rod offset of Q3 at each pressure. Cross sections were then calculated by plotting $-1 \ln \frac{E}{E_0}$ versus $-\frac{C_d m m_2 l}{m_1}$. For cross section measurements, Q1 was operated as an rf-only ion guide, and Q3 was operated as a mass filter. Uncertainties in cross sections are the standard deviations of three measurements.

Ion Masses

Masses of dimers and heme-deficient dimers from unoxidized or oxidized hemoglobins, 20 μ M in 10% MeOH/90% H₂O, 10 mM NH₄Ac, were measured with a linear quadrupole ion trap time-of-flight mass spectrometer system (LIT-TOF) [8]. To record spectra of apo-monomers with the LIT-TOF, proteins were denatured in 50% MeOH, 0.5% acetic acid. ES Tuning mix for LC/MSD Ion Trap (Agilent, Santa Clara, CA, USA) was used for mass calibration.

Solutions and Reagents

Human ^{oxy}Hb (“fresh Hb”) was prepared via standard procedures [4, 9]. Fresh human blood was centrifuged (Sorvall RC-5B Plus; Mandel Scientific, Newton, CT, USA) at 3000 g for 10 min at 10 °C, and the clear supernatant, plasma, and buffy coat were discarded by suction. The collected red blood cell pellets were then resuspended in a 5-fold (vol/vol) excess of 0.9% (wt/vol) sodium chloride solution and washed four times with centrifugation at 5500 g for 20 min at 4 °C. After mixing with an equal volume of cold water/toluene 90/10 (vol/vol) to extract stromal cell impurities, centrifugation of packed red blood cells at 15,000 g for 30 min at 10 °C gave an aqueous layer of purified hemolysate, which was then dialyzed at 4 °C with 10 mM NH₄Ac for 42 h with four buffer exchanges, using a cellulose membrane (MWCO 3500; Spectra/Por, Rancho Dominguez, CA, USA). The concentration of ^{oxy}Hb (as tetramer) was determined to be 0.4 mM with UV-Vis spectrometry (Cary 5000 UV spectrophotometer, Varian, Inc., Palo Alto, CA, USA), with the standard pyridine hemochromogen method [25, 26]. Desalted ^{oxy}Hb was oxidized to ^{met}Hb with a 1.5-fold stoichiometric excess of potassium ferricyanide (K₃Fe(CN)₆) for 5 min at 25 °C [27]. The ^{met}Hb solution was then frozen with liquid nitrogen and stored at -80 °C. Before MS analysis, the solution was quickly thawed and desalted on a 3 \times 25 cm G-25 Sephadex column (GE Healthcare, Buckinghamshire, UK).

Freshly prepared human Hb stock solution (50 μ L, ca. 0.4 mM) was freeze dried in a Manifold Freeze-Dryer (Flexi-Dry™ MP; FTS Systems, Bohemia, NY, USA) at 5 \times 10⁻² Torr at -80 °C for 2 h. The lyophilized powders were then dissolved in 50 μ L water with 10 mM NH₄Ac to give the concentration prior to freeze drying (ca. 0.4 mM).

Commercial human and bovine ^{met}Hb were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared by dissolving the lyophilized powder in 10 mM NH₄Ac with the same concentration as fresh Hb. Dialysis and storage of commercial Hb were as with fresh Hb.

Commercial and fresh human Hb were oxidized by adding 30% highly purified hydrogen peroxide (H₂O₂, TraceSelectUltra; Sigma-Aldrich, St. Louis, MO, USA) to produce a solution of 20 μ M Hb in 200 μ M to 300 μ M peroxide, and the oxidation was allowed to proceed for 1 h. Excess H₂O₂ was removed by buffer exchange with equal volumes of 10 mM NH₄Ac [28] with centrifugation (MSE MicroCentaur; London, UK) at 13,000 rpm for 10 min, with five repeats.

For MS of native proteins, the fresh and commercial Hb solutions were diluted to 20 μ M or 5 μ M with 10% methanol (MeOH) or 10% acetonitrile (ACN) in 10 mM NH₄Ac at pH 6.8 (measured with a Accumet model 15 pH meter (Fisher Scientific, Fairlawn, NJ, USA), as in [8]). Samples were infused into the ESI source with a syringe pump (Harvard Apparatus, St. Laurent, PQ, Canada) at 1 μ L/min.

Acetic acid (99.99%), pyridine (99.9%), and K₃Fe(CN)₆ (ACS grade) were from Sigma-Aldrich, St. Louis, MO, USA. Methanol, ACN, toluene (all HPLC grade), and NH₄Ac (ACS grade) were from Fisher Scientific, Fairlawn, NJ, USA.

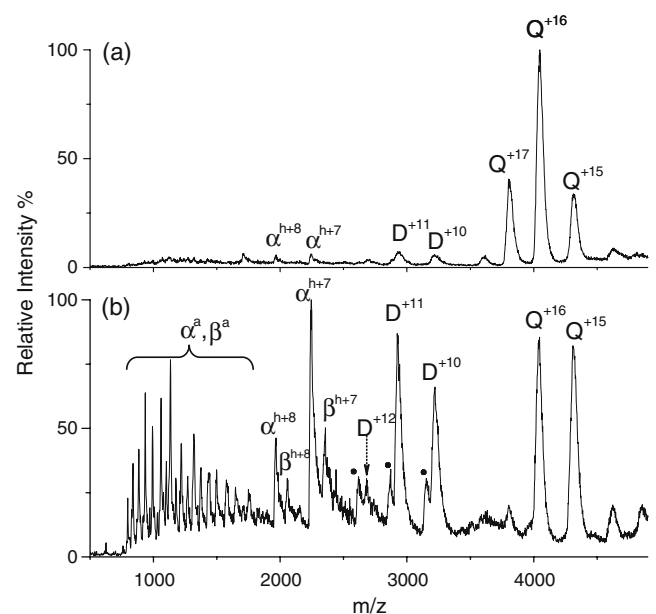


Figure 1. ESI mass spectra of fresh human Hb, (a) 20 μ M Hb in 10% MeOH, and (b) 5 μ M Hb in 10% ACN. Notation: α^a , β^a , apo-monomers; α^h , β^h , holo-monomers; D, dimers, $\alpha^h\beta^h$; Q, tetramers, $(\alpha^h\beta^h)_2$. Peaks labelled with a filled circle correspond to heme-deficient dimers

Results and Discussion

Mass Spectra of Hemoglobin

Figure 1 shows ESI mass spectra of freshly prepared human Hb in different solvents recorded with the triple quadrupole system. With a 20 μM Hb solution in 10% MeOH (Figure 1a), tetramer ions ($\alpha^h\beta^h$)₂ with charge states from +15 to +17 dominate the spectrum. Only low levels of monomer and dimer ions are observed. With a 5 μM fresh Hb solution containing 10% ACN (Figure 1b), more intense ions of holo-monomers, α^h , β^h (+7, +8), and dimers, $\alpha^h\beta^h$ (+10 to +12), are seen. Apo-monomers, α^a , β^a , in high charge states, and some heme-deficient dimers ($\alpha^h\beta^a$ or $\alpha^a\beta^h$) are also observed. These were previously shown by others to be present with commercial Hb [11, 17, 18] but not with fresh Hb [7, 9]. Masses of the heme-deficient dimers from fresh Hb, measured with the TOF system, are as expected from the sequence. Thus, the formation of heme-deficient dimers is likely due to the addition of ACN which partially destabilizes the protein. The solution with ACN (5 μM Hb) was used to produce dimer and monomer ions for cross section measurements. Dilution of the 20 μM solution of Hb in MeOH gave only slight increases in intensities of the monomer and dimer ions (spectrum not shown). These results indicate that addition of ACN apparently leads to dissociation of tetramers to monomers and dimers.

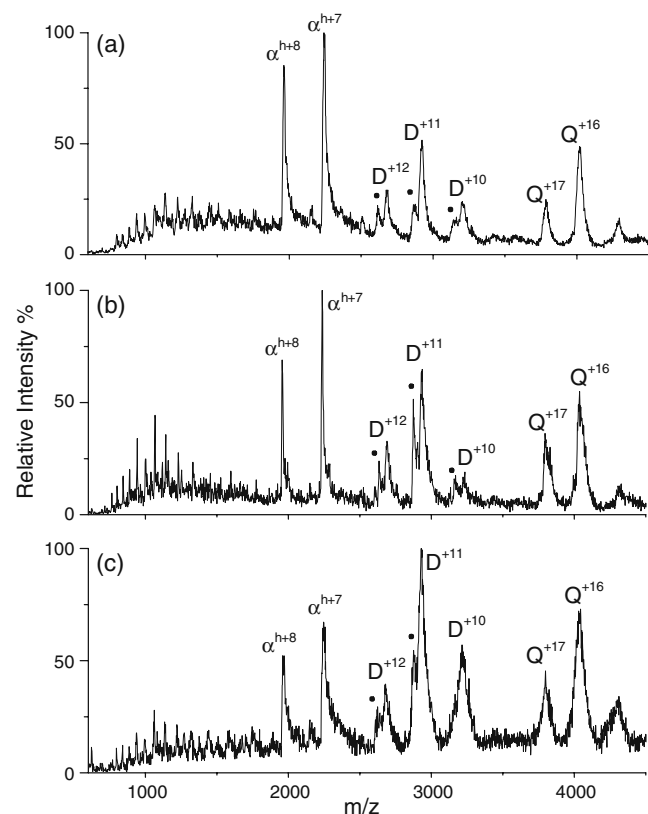


Figure 2. ESI mass spectra of (a) commercial human Hb, (b) commercial bovine Hb, and (c) oxidized commercial human Hb. Notation as in Figure 1

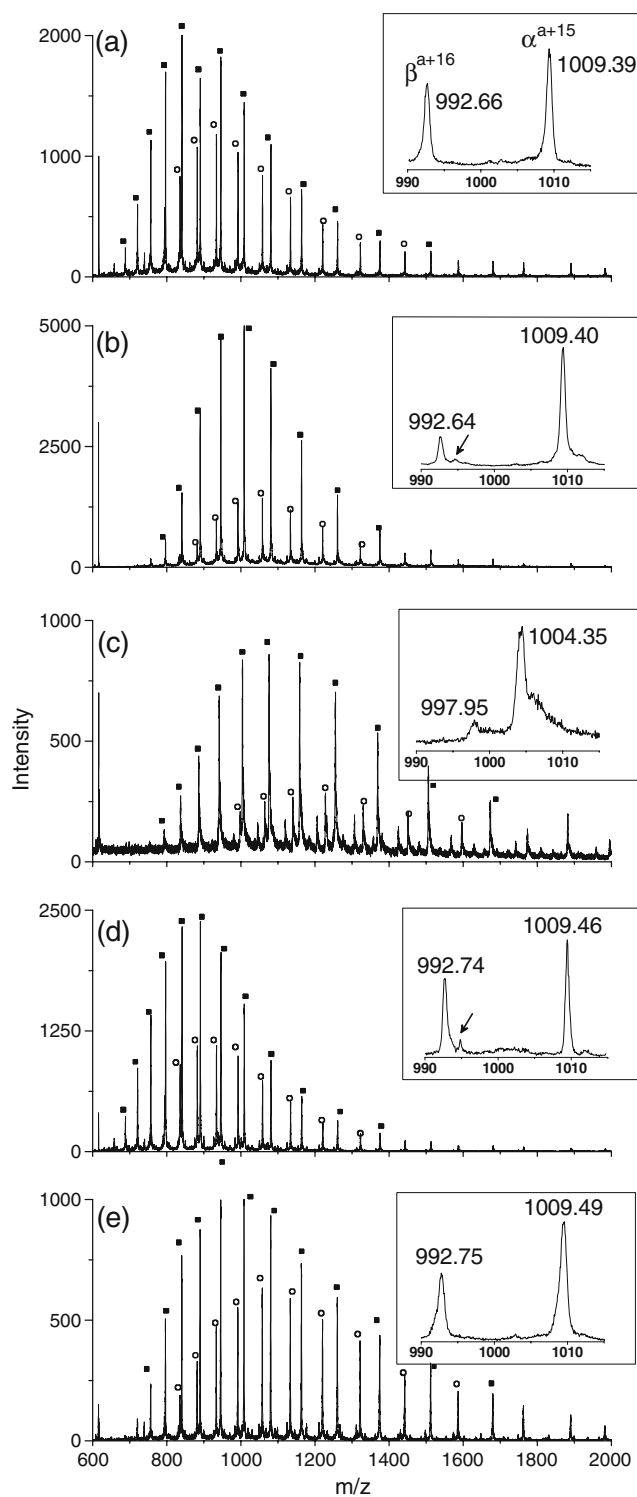


Figure 3. Mass spectra of apo-monomer ions, α^a (filled squares), β^a (open circles), from (a) fresh human Hb, (b) commercial human Hb, (c) commercial bovine Hb, (d) oxidized fresh human Hb, and (e) lyophilized fresh human Hb, in denaturing solutions. The insets show peaks of the β^{a+16} and α^{a+15} ions with the measured mass to charge ratios (m/z) labelled. Arrows in (b) and (d) indicate β^{a+16} ions with masses 32 Da higher than expected. Minor peaks adjacent to β^a peaks in (c) are from holo-alpha ions

Table 1. Molecular weights of human and bovine Hb species (Da)

Species	α^a	α^h	β^a	β^h	$\alpha^h\beta^h$	$(\alpha^h\beta^h)_2$
Human Hb	15126	15743	15867	16484	32227	64453
Bovine Hb	15053	15669	15954	16571	32240	64480

Figure 2 shows ESI mass spectra of Hb obtained commercially, recorded with the triple quadrupole system. With the same solution (10% MeOH) and mass spectrometer operating conditions, commercial human Hb, bovine Hb, and oxidized human Hb gave similar mass spectra and produced higher levels of monomers (α^h , α^a , β^a) and dimers ($\alpha^h\beta^h$) than fresh Hb (Figure 1a). Heme-deficient dimers were also observed with all the commercial proteins, consistent with previous reports [9, 11, 17, 18]. The masses of these dimers, assigned $\alpha^h\beta^a$ structures [9, 11], are 32 Da greater than expected, indicating that the commercial proteins were partially oxidized. These observations agree partially with those of Boys et al. [9], who also found that dimer and monomer ions are more abundant with

commercial Hb than with fresh Hb in spectra with their TOF mass spectrometer. However they found that tetramer ions still dominated the spectra with both fresh and commercial bovine Hb, whereas we find much higher levels of monomers and dimers with commercial Hb. In aqueous solution, the tetramer-dimer dissociation constant K_D for met Hb (H_2O) was reported to be 4.0×10^{-6} M at neutral pH (0.1 M KCl, 10 mM bis-Tris) [29]. With this K_D and 20 μ M Hb, ca. 20% of the tetramers should dissociate to dimers, so some dimers might be expected in the mass spectrum. The K_D value for dissociation of $\alpha\beta$ dimers to α and β monomers (carbonmonoxy Hb) is much lower, approximately 10^{-12} M [30, 31], so minimal dissociation of dimers to monomers is expected. The solvent composition can strongly affect K_D . For

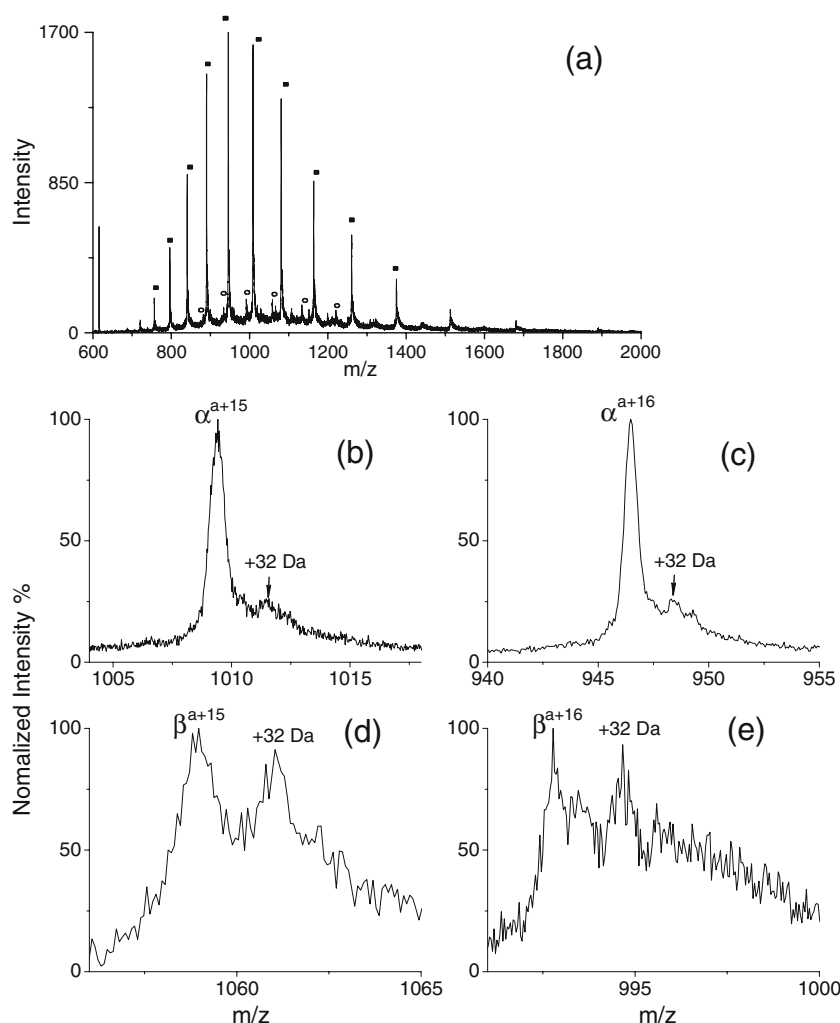


Figure 4. (a) Mass spectrum of commercial human Hb further oxidized with H_2O_2 and denatured in solution (α^a , filled squares, β^a , open circles) and detailed views of the (b) α^{a+15} , (c) α^{a+16} , (d) β^{a+15} , (e) β^{a+16} peaks. The main peaks observed correspond to unmodified α - and β - globins. Mass shifts from the oxidative modifications are indicated

deoxyHb, in 0.1 M phosphate buffer alone a $K_D \approx 10^{-7}$ M was found [32] for dissociation of tetramers, while in a different solution (0.1 M Tris, 0.1 M NaCl, 1 mM Na₂EDTA), K_D decreased to ca. 10^{-11} M [33]. Addition of 10% organic solvent, MeOH or ACN (Figure 1a and b) may also change the degree of dissociation in solution. Note that Boys et al. did not use 10% MeOH in their Hb solutions. Mass spectra also depend strongly on MS system operating conditions. With a higher pressure in Q0 or a lower voltage difference between the orifice and skimmer (ΔV_{os}), more tetramers are seen [8]. If the mass spectrometer is optimized for maximum transmission of the tetramers, as was done by Boys et al. [9], monomer and dimer ions might be expected to decrease in relative intensity [8]. Thus, it is difficult to determine the levels of dissociation in solution by ESI-MS, and spectra with different instruments cannot be compared directly.

Figure 3 shows ESI mass spectra of Hb denatured in solution, recorded with the TOF system. Peaks from apo- α and apo- β ions can be seen. In Figure 3c, peaks from holo- α ions from bovine Hb can also be seen. The insets show the β^{a+16} and α^{a+15} peaks in detail. Here, the TOF mass analyzer was used to give higher resolution and higher mass accuracy (the FWHM of monomer peaks is ca. 0.7 Th; the expected width from the isotopic distribution ca. 0.6 Th). Measured masses of the α subunits of fresh human Hb and commercial human Hb agree within 0.8 Da (0.005% or better) with masses calculated from the protein sequences (Table 1); of commercial bovine Hb within 3 Da (0.02%). Thus the α chains of the proteins were not oxidized. Additional peaks on the high mass side of the apo- β peaks of the commercial proteins (Figure 3b) are consistent with partial oxidation of the proteins. Thus, partial oxidation of the Hb leads to the observation of heme-deficient dimers in the spectrum as noted by Boys et al. [9]. High mass tails of the peaks of the apo- α ions with commercial Hb may be attributed to some impurity adducts or chemical decomposition occurring during the freeze drying process or storage of the freeze-dried solid [34, 35]. Figure 3d shows that oxidation of fresh human Hb with H₂O₂ gives β^a ions with a 32 Da increase in mass. Figure 3e show that lyophilization of fresh human Hb does not give β^a ions with a 32 Da increase in mass, and thus does not oxidize the protein.

The sulphur-containing residues, methionine (Met) and cysteine (Cys), are easily oxidized [36]. Human Hb contains two Met and one Cys in the α chain, and one Met and two Cys in the β chain [1]. Initial oxidation of Met generates Met sulfoxide ($-(CH_2)_2-(S=O)-CH_3$), which, with more intense chemical attack [36], can then form the irreversible product Met sulfone ($-(CH_2)_2-(O=S=O)-CH_3$). Cys residues are more susceptible to oxidation than Met residues, and the possible irreversible oxidation products are the sulfenic acid ($-CH_2-SOH$), the sulfinic acid ($-CH_2-SO_2H$), and the sulfonic acid ($-CH_2-SO_3H$) [28, 37]. Hydrogen peroxide is a biologically relevant oxidant [28, 38]. At low concentrations of H₂O₂, hydroxyl radicals are required for oxidation, catalyzed by

trace transition metal ions [39]. Here we used a relatively high concentration of H₂O₂, in which the oxidation is a direct effect, independent of intermediate hydroxyl radicals [28, 40]. Extensive exposure to H₂O₂ can substantially increase a protein's hydrophobicity, which can lead to irreversible conformational changes [41]. With Hb, β -subunits are more susceptible to oxidation [28]. The ratio $[H_2O_2]/[Hb]$ and reaction time were carefully controlled to minimize conformational changes to Hb. In our experiments, ratios of $[H_2O_2]/[Hb]$ in the range 10 to 15 were chosen. Greater than a 15-fold excess of H₂O₂ was found to cause irreversible collapse of the tetrameric structures, so that only monomers appeared in the mass spectra.

Commercial human Hb was chosen for further oxidation for comparison to the cross section results of Wright and Douglas who used commercial Hb. Although the total ion intensity decreased (by about half) after oxidation, the mass spectrum (Figure 2c) still showed abundant tetramer ions, indicating that the folded structure in solution was well preserved. Mass spectra of Hb that was oxidized with H₂O₂ and denatured in solution, recorded with the TOF mass analyzer, showing peaks from the α^a and β^a monomers, are presented in Figure 4. An adduct corresponding to an increase in mass of 32 Da is seen with both monomers. The β chain shows a greater degree of oxidation. Chemically oxidized commercial human Hb showed a spectrum (Figure 2c) similar to human Hb without reaction with H₂O₂ (Figure 2a), indicating that this further oxidation does not cause significant changes to the mass spectra of human Hb.

The effects of oxidation and lyophilization on the spectra of fresh human Hb folded in solution were then investigated. Figure 5 shows mass spectra of fresh human Hb before and after oxidation, lyophilization, and both lyophilization and oxidation, recorded with the TOF system. Also shown is the spectrum of commercial Hb recorded with the same instrument. The proteins were in 10% MeOH/90% water to avoid destabilizing the hemoglobins, which can lead to the formation of heme-deficient dimers (such as with ACN, Figure 1b). The insets show the regions of the spectra near the peaks of the +11 dimer ions. Comparison of Figure 5a and b shows that oxidation of human Hb increases the intensities of the dimers $\alpha^h\beta^h$ (+11,+12) and monomer α^h (+7,+8) peaks, and causes the appearance of heme-deficient dimer ions with masses 32 Da higher than expected in the mass spectrum. Comparison of Figure 5a and c shows that lyophilization also increases the intensities of monomers and dimers, but does not in itself lead to formation of a heme-deficient dimer. Comparison of Figure 5a and d shows that lyophilization of the protein, followed by oxidation causes the appearance of an oxidized heme-deficient dimer. This provides direct evidence that oxidation, not lyophilization, leads to the appearance of the heme-deficient dimer ions. Lyophilization, however, seems to produce some subtle changes to the protein that increase the relative intensities of monomer and dimer ions, but are not readily detected by mass spectrometry. Finally, comparison of

Figure 5. Mass spectra of (a) fresh human Hb, (b) oxidized fresh human Hb, (c) lyophilized fresh human Hb, (d) fresh human Hb oxidized after lyophilization, and (e) commercial human Hb. The insets show peaks from the +11 dimer ions in detail. Spectra (b), (d), and (e) show heme-deficient dimer ions (indicated with the arrow) with a 32 Da mass shift, while (a) and (e) do not show these peaks. Solutions were 20 μ M Hb in 10% MeOH and 10 mM NH_4Ac . Notation is the same as in Figure 1

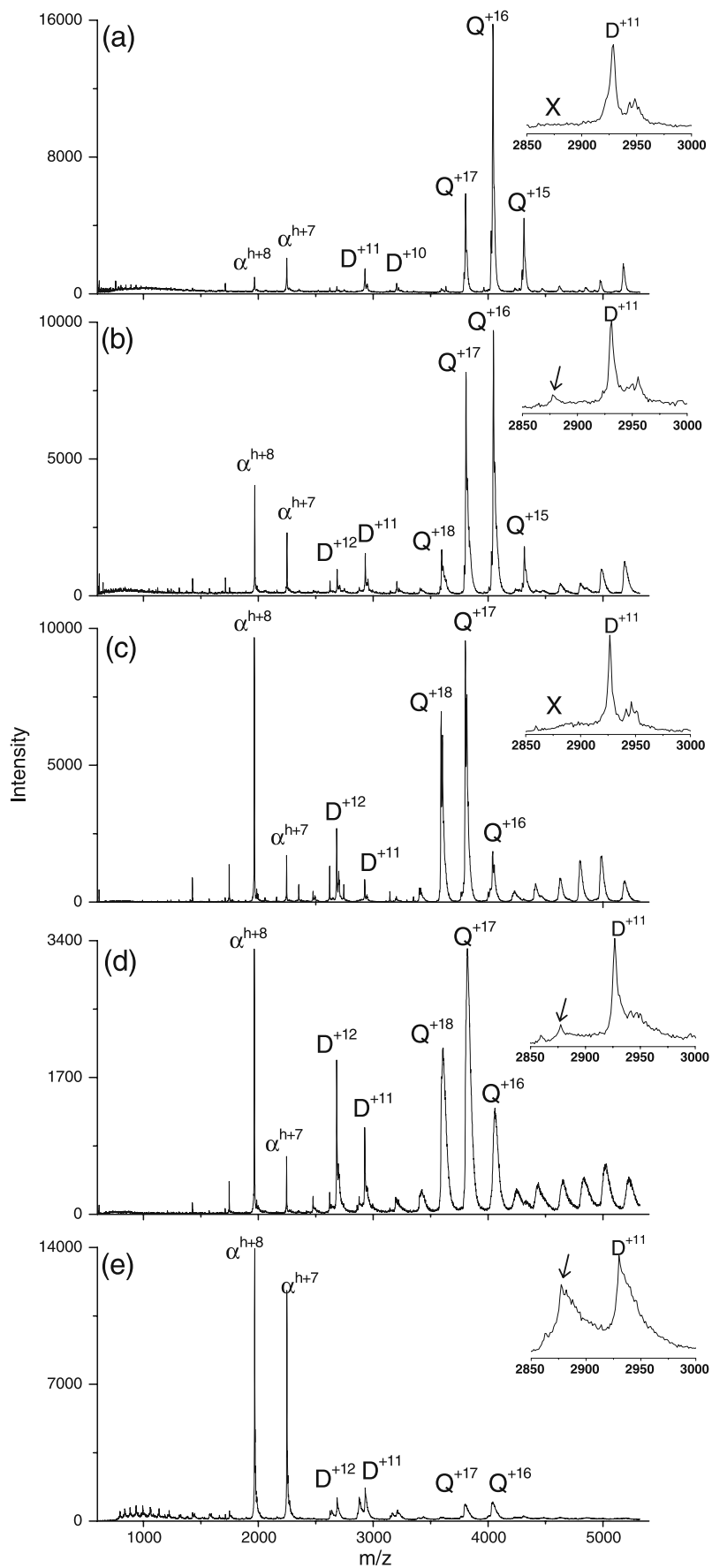


Figure 5e with Figure 5a–d shows that the commercial protein has the highest levels of dimers, monomers and heme-deficient dimers.

Collision Cross Sections

Collision cross sections provide information on the overall “size” of ions in the gas phase. Table 2 lists cross sections of monomer (α^h , +7, +8), dimer ($\alpha^h\beta^h$, +11, +12) and tetramer ($(\alpha^h\beta^h)_2$, +16, +17) ions formed from fresh human Hb, commercial human Hb, commercial bovine Hb, and human Hb further oxidized with H_2O_2 . Also shown are cross sections measured by Douglas and Wright [8] and Faull et al. [11]. In all cases, cross sections of gas-phase dimer ions measured here are intermediate between cross sections of monomer and tetramer ions.

To compare with sizes of the same species of human Hb in solution, the radius of gyration, r_g , measured by small angle X-ray scattering [42, 43], and the Stokes radius, r_s , measured by size exclusion chromatography [44–46], are used [8]. Areas $A_s = \pi r_s^2$ and $A_g = \left(\frac{5}{3}\right)\pi r_g^2$, calculated from r_s and r_g are shown in Figure 6. Average cross sections of monomer ions are similar (within 10%) to A_s . Average cross sections of dimer ions fall between A_g and A_s . Average cross sections of tetramers are similar to A_g (less than 5% difference), and are only 1.2 times larger than A_s . Thus gas-phase monomer, dimer, and tetramer ions from fresh Hb and commercial Hb have compact structures, similar to the folded native conformations in solution.

Cross sections of human Hb ions have been reported by Faull et al. [11] and Scarff et al. [7]. With fresh human Hb, our cross sections for monomers dimers and tetramers generally agree with those of Faull et al. [11] within 8% or better. Both Faull et al. and Scarff et al. found that dimer cross sections are intermediate between monomer and tetramer cross sections. The cross sections estimated by Scarff et al. from traveling wave ion mobility measurements [7] are somewhat larger than those reported here and by Faull et al. (35% larger for monomers, 20% for dimers, and 17% for tetramers). The difference may be due to the calibration procedure used by Scarff et al. in which only relative cross sections are estimated. With commercial human Hb, our monomer ion cross sections are within 6% of the cross sections of commercial bovine Hb reported by Wright and Douglas [8] (Table 2). The major difference is

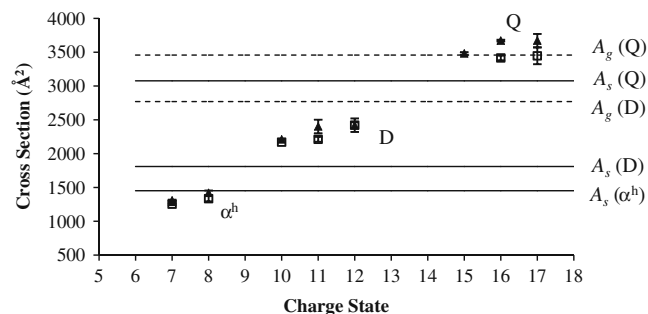


Figure 6. Collision cross sections as a function of charge state for fresh human Hb (filled triangles) and commercial human Hb (open squares). Notation is the same as in Figure 1. Solid lines show cross sections, A_s , of tetramer, dimer, and monomer ions calculated from the Stokes radii. Dashed lines show cross sections, A_g , of dimer and tetramer ions calculated from the radii of gyration

with the dimer ions; cross sections of dimer ions measured here are consistently greater than cross sections of monomer ions. We also find cross sections of tetramer ions about 25% larger than reported by Wright and Douglas. Variations in sequences of Hb from diverse species [47] may give different cross sections. Tetramer ions from HbS, with the point mutation $\beta\text{Glu6Val}$, have slightly larger (6%) cross sections than those of HbA [7]. For comparison, we also re-measured cross sections of ions from commercial bovine Hb (Table 2). Slightly larger cross sections were observed for bovine Hb (ca. 12% for monomers, 8% for dimers, and 16% for tetramer ions) than Hb A. These small differences may be attributed to sequence differences. Bovine and human Hb share 88% sequence homology in the α chain and 83% in the β chain. However, the differences in the sequences do not substantially change the cross sections of these protein ions and do not make dimer ions more compact than monomer ions.

The larger cross sections of tetramer ions measured here, in comparison to cross sections reported by Wright and Douglas [8], may be the result of the different degree of solvation or adduct formation of the tetramer ions [8]. When ions pass through the orifice-skimmer region ($\Delta V_{os} = 100$ V), ion activation mainly causes desolvation and removal of adducts from ions. Following this, internal energy added to the ions can cause the ions to unfold. Thus, highly desolvated ions, or ions with fewer adducts, tend to have

Table 2. Collision cross sections of Hb ions (\AA^2)

Species	Charges	Douglas and Wright commercial bovine Hb [8]	Faull et al. commercial human Hb ^a [11]	Fresh human Hb	Commercial human Hb	Commercial bovine Hb	Oxidized commercial human Hb
α^h	+7	1339±76	1420	1310±10	1258±14	1415±30	1317±17
	+8	1530±127	1525	1420±34	1339±38	1510±42	1367±10
$\alpha^h\beta^h$	+11	1225±101	2355	2402±100	2232±36	2498±86	2137±113
	+12	1225±191	2484	2420±44	2421±100	2548±54	
$(\alpha^h\beta^h)_2$	+16	2826±292	3460	3672±13	3414±34	4113±143	3476±88
	+17	2763±330	3649	3672±98	3445±123	3854±72	

^aAt pH 9.5 in buffered ammonium acetate

greater cross sections. In our experiments, when the flow rate of the nitrogen curtain gas was approximate 2 L/min, tetramer ions from fresh and commercial Hb were found to be relatively highly desolvated or have fewer adducts. The peak widths (FWHM) of +16, +17 ions were ~ 15 Th and mass shifts, if attributed to solvation, corresponded to only ~ 4 water molecules (Q1 scan), much less than seen in the study of Wright and Douglas (FWHM ≈ 50 Th; if attributed to solvation, ca. 17 attached water molecules). Monomer and dimer ions do not show solvation and so this effect only applies to the tetramer ions. The lower degree of solvation here may be a result of the changes to the ion sampling interface.

Because some commercial Hb has been found to be oxidized, the effect of further oxidation on cross sections was investigated. Table 2 shows cross sections of human Hb that was chemically oxidized. Only cross sections of ions with the highest intensity (+7, +8 α^h , +11 $\alpha^h\beta^h$, and +16 ($\alpha^h\beta^h$)₂) are shown. Oxidized human Hb and fresh human Hb produce ions with nearly the same cross sections, within the combined uncertainties. Thus, the unusual dimer ion cross sections of Wright and Douglas cannot be attributed to oxidation of the protein.

Conclusions

With the same solution and mass spectrometer conditions, fresh human Hb, oxidized fresh human Hb, lyophilized fresh human Hb, lyophilized and oxidized fresh human Hb, and commercial human Hb all give somewhat different mass spectra. More dimer and monomer ions are seen with oxidized fresh human Hb, lyophilized fresh human Hb, lyophilized and oxidized fresh human Hb, and the commercial Hb used here. All commercial Hb samples showed similar spectra, independent of protein sequence or oxidation. The different spectra with fresh and commercial Hb are partially due to oxidation and freeze drying of the commercial proteins, but may also be due to some other subtle change to the commercial proteins.

Fresh and commercial human Hb give cross sections of dimer ions intermediate between tetramer and holo- α ions, and cross sections similar to the same species in solution. Commercial bovine Hb had cross sections 10% to 20% larger than commercial human Hb, possibly a result of the differences in the sequences. Further oxidation of commercial lyophilized Hb does not have a substantial effect on cross sections as long as the protein remains folded. Both human and bovine Hb give dimer ions with cross sections greater than monomer ions. We have not been able to reproduce the unusual result of Wright and Douglas. Neither sequence difference between bovine Hb and human Hb nor additional oxidation of Hb gives dimer ions with cross sections less than those of monomer ions. To some extent, the use of commercial Hb is an uncontrolled experiment because the history of the sample is unknown and may vary from sample to sample. As we show here, lyophilization of the proteins produces changes to the mass spectra. Thus,

there may be some other subtle change to the protein that accounts for the unusual result of Wright and Douglas.

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