RESEARCH ARTICLE

Gas-Phase Ions of Human Hemoglobin A, F, and S

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Abstract

Hemoglobin (Hb) ($\alpha_2\beta_2$) is a tetrameric protein–protein complex. Collision cross sections, hydrogen exchange levels, and tandem mass spectrometry have been used to investigate the properties of gas-phase monomer, dimer, and tetramer ions of adult human hemoglobin (Hb A, $\alpha_2\beta_2$), and two variant hemoglobins: fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) and sickle hemoglobin (Hb S, $\alpha_2\beta_2$, E6V[β]). All three proteins give similar mass spectra. Monomers of Hb S and Hb F have similar cross sections, ca. 10% greater than those of Hb A. Cross sections of dimer ions of Hb S are 11% greater than those of Hb A and 6% greater than those of Hb F. Tetramers of Hb S are 13% larger than tetramers of Hb A or Hb F. Monomers and dimers of all three Hb have similar hydrogen-deuterium exchange (HDX) levels. Tetramers of Hb S exchange 16% more hydrogens than Hb A and Hb F. In tandem mass spectrometry, monomers of Hb S and Hb F require ca. 10% greater internal energy for heme loss than Hb A. Dimers (+11) of Hb A and Hb S dissociate to monomers with asymmetrical charge division; dimers of Hb F (+11) dissociate with nearly equal charge division. Tetramer ions dissociate to monomers and trimers, unlike solution Hb, which dissociates to dimers. The most stable dimers are from Hb S; the most stable tetramers from Hb F. The results with Hb S show that a single mutation in the β chain can change the physical properties of this gas-phase protein–protein complex.

Key words: Hemoglobin, Conformation, Cross sections, Hydrogen/deuterium exchange, MS/MS, Protein–protein complex

Introduction

Hemoglobin (Hb) carries oxygen in mammalian red blood cells and has been well studied as a model tetrameric protein. In adult humans, the predominant Hb, hemoglobin A (Hb A, $\alpha_2\beta_2$), consists of two α -chains and two β -chains, each noncovalently bound to an iron-containing heme group. In newborns, 80% of human Hb is fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) [1], which is formed from two α -chains and two γ -chains, also bound noncovalently. The Hb F concentration decreases after birth to less than ca. 10% after 20 wk [1]. The normal γ -chains are of two types, γ^G and γ^A , which differ only in one amino acid residue at position 136 (Gly in γ^G and Ala in γ^A). The ratio of γ^G to γ^A is 3:1. The α chains of Hb F and Hb A are identical, while the γ chains differ

from the β chains in 39 amino acid residues, with 22 on the exterior [1]. This difference in sequence results in several significant functional differences between Hb A and Hb F. For example, Hb F shows lower responses to 2,3-diphosphoglycerate (DPG) binding and thus increased oxygen affinity in the presence of DPG [2] and, in solution, Hb F dimers and tetramers are more strongly bound than the same species of Hb A [3–5].

Over 1000 Hb variants have been discovered. The variant Sickle hemoglobin, Hb S ($\alpha_2\beta_2$, E6V[β]), causes anemia. In fully or partially deoxygenated blood, this substitution changes the conformation of Hb to allow tetramers to pack into polymers and thus alter the shape of red blood cells [6]. This molecular stacking also decreases the solubility of the protein and consequently impairs oxygen delivery to tissues [7]. However, in vitro, Hb S has entirely normal functional properties in dilute solution [8]. In contrast, Hb F has been tested to treat sickle cell anemia [9] because it has an anti-

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sickling effect due to unfavorable contacts for polymerization [10]. The molecular weights of species of Hb A, S, and F are listed in Table 1.

Electrospray ionization mass spectrometry (ESI-MS) has been widely employed to study Hb A and its variants, from peptide sequencing, to the analysis of higher-order assembly, and interactions with other large molecules [11]. In the analysis of primary structure, Hb variants can be identified with direct mass analysis of globin chains [12], measurements of tryptic peptide masses [13], or tandem mass spectrometry of Hb chains or peptides [14, 15].

Hemoglobin is also of interest as a model protein–protein complex. Studies of the structure of Hb tetramers or subunits with ESI-MS provide information on the physical properties of Hb ions in the gas phase. Collision cross sections and hydrogen/deuterium exchange (HDX) levels provide insights into the conformations of ions. Recently, studies of Hb cross sections have come from measurements of migration time with ion mobility mass spectrometry (IMS). Using traveling-wave IMS, Scarff et al. have shown that gas-phase tetramer ions of Hb S +15 to +18 have greater cross sections than those of Hb A. The ratio of Hb S cross section to Hb A cross section increased with the charge on the ion from ca. 1.02 for +15 ions to 1.07 for +18 ions [16]. An alternative approach to determining cross sections is to measure ion axial kinetic energy losses with a triple quadrupole mass spectrometer [17, 18]. In a recent study of human Hb A, we showed that oxidation of methionine and cysteine residues on the β -chain, and lyophilization of Hb, change the appearance of Hb mass spectra but do not influence the cross sections of gas-phase Hb ions [17].

Hydrogen/deuterium exchange can be combined with MS to measure the number of hydrogens in O-H, N-H, and S-H groups that are exposed on a protein's surface. With Hb, HDX can be done in solution prior to injection of Hb into an ESI source [19] or directly in the gas phase with trapped ions exposed to D₂O vapor [18]. The mechanism of solution-phase HDX is well established [20]. For the same protein, greater exchange indicates that more hydrogens are accessible because the protein has a more flexible or more unfolded conformation. In contrast the mechanism of gas-phase HDX is less well understood [21]. To exchange in the gas phase, a hydrogen must be accessible on the surface of the protein. However it must also be near a charged site [21–25]. Thus, ions with different cross sections may

not show different exchange levels [23–25] and interpretation of gas-phase HDX experiments is more difficult.

In MS/MS, ions of large noncovalently bound complexes such as Hb dissociate into smaller constituents. The gas-phase binding strength and stability of ions from different Hb variants can be examined and compared. The binding strength is related to the noncovalent interactions of the subunits' interface. Two types of interface exist in solution Hb tetramers. The first, between the dimer pairs that form the tetramers is referred to as the $\alpha_1\beta_2$ ($\alpha_1\gamma_2$) or $\alpha_2\beta_1$ ($\alpha_2\gamma_1$) interface; the second, between the two individual α - and β (γ)-subunits to form dimers is referred to as the $\alpha_1\beta_1$ ($\alpha_1\gamma_1$) or $\alpha_2\beta_2$ ($\alpha_2\gamma_2$) interface [10]. In MS, the noncovalent binding at interfaces can be broken in a controlled way, either during the ion-sampling process without precursor ion mass selection [26] or in a collision cell after mass selection of a precursor ion [27]. With non-selective fragmentation, Apostol showed that the stability of intact tetramer ions can be affected by single mutations in the β -globins [26], indicating that a small change in sequence may greatly influence the stability of the entire protein complex in the gas phase.

Because Hb has been extensively studied with MS, the physical properties of gas-phase Hb ions and any relation of these properties to the solution-phase properties of Hb are potentially informative. The availability of natural Hb variants allows studying the effects of changing the individual chains on the properties of this model gas-phase protein–protein complex. In this study, we have measured cross sections, HDX levels and MS/MS spectra of gas-phase ions of Hb S and Hb F, and have compared these to the same properties of Hb A. Physiologically inactive ^{met}Hb (ferriHb with Fe³⁺ in the heme) was used since it is more stable than ^{oxy}Hb (oxygen-bound ferroHb with Fe²⁺ in the heme) [28]. The proteins were freshly prepared from blood rather than purchased commercially, to allow more reproducible results [17]. We show that although Hb A, S, and F give similar MS spectra, the gas-phase ions of the different hemoglobins can have different properties. Tetramer ions of Hb S have greater cross sections and greater HDX levels than tetramer ions of Hb A and Hb F. In MS/MS, dimer ions of Hb F dissociate to two monomers with a nearly equal division of the charges of the precursor ions, while Hb A and Hb S dissociate with asymmetrical charge distributions. Gas-phase tetramers of Hb F are more stable than tetramers of the other two proteins. The order of the binding strengths of the gas-

Table 1. Molecular Weights (MW) of Human Hb Species (Da)

Species ^a	α^a	α^h	β^a or γ^a	β^h or γ^h	$\alpha^h\beta^h$ or $\alpha^h\gamma^h$	$(\alpha^h\beta^h)_2$ or $(\alpha^h\gamma^h)_2$
Hb A	15126	15743	15867	16484	32227	64453
Hb F ^b	15126	15743	15995	16612	32354	64709
Hb S	15126	15743	15837	16454	32196	64392

^a α^a , apo- α , heme-free state; α^h , holo- α , heme-bound state.

^b Calculated with γ^G .

phase dimer ions is: Hb S > Hb A > Hb F. The results with Hb S show that a single mutation in a protein can significantly change the properties of a gas-phase protein complex.

Experimental Methods

LIT-TOF System and Gas-Phase HDX

HDX experiments were performed with a home-made linear quadrupole ion trap reflectron time-of-flight mass spectrometer system (LIT-TOF), as described previously [18, 29–32]. Protonated ions generated by pneumatically assisted ESI (5 kV), pass through an aperture (5 mm diameter) in a curtain plate (1000 V), a dry nitrogen curtain gas (~2 L/min), an orifice (0.25 mm diameter, 220 V), a skimmer (0.75 mm diameter, 20 V), and enter a chamber with two consecutive radio frequency only quadrupoles, Q0 (DC offset=15 V) and Q1 (DC offset=10 V). For control experiments, the chamber pressure was kept at 10 mTorr of N₂ by partially closing a gate valve between the chamber and a turbomolecular pump. For exchange experiments, the chamber contained 5 mTorr N₂ and 5 mTorr D₂O vapor, set with a needle valve (SS-SS4-A1 Swagelok, Solon, OH, USA), to keep the total pressure 10 mTorr. For HDX, ions are trapped in Q1, and confined axially by timed DC stopping potentials on the entrance lens (Q0/Q1), and exit lens (L1). Typical trapping conditions: 50 ms injection (Q0/Q1=-5 V, L1=60 V), 0–10,000 ms trap (Q0/Q1=40 V, L1=60 V), 20 ms detection (Q0/Q1=40 V, L1=-15 V), and 50 ms drain (Q0/Q1=-5 V, L1=-15 V). After trapping, ions pass through a stack of four focusing lenses (L1–L4), then are mass analyzed with a reflectron-TOF and detected by a dual microchannel plate [18]. A solution of CsI was used for mass calibration.

Triple Quadrupole Mass Spectrometer System

For cross section measurements and MS/MS experiments, a home-built ESI triple quadrupole mass spectrometer described previously [17, 33, 34], but modified to increase sensitivity [17] was used. Positive protein ions, formed by pneumatically assisted ESI (4 kV), pass through a 2.4 mm diameter aperture in a curtain plate (1150 V), a dry nitrogen curtain gas (~2 L/min), and an ion sampling orifice (230 V) into a region with ca. 0.7 Torr background gas. Ions then pass through a skimmer (130 V), and enter a quadrupole ion guide Q0 (~9 mTorr, DC offset=120 V), where ions are cooled to translational energies and energy spreads of about 1–2 eV per charge [35]. After passing through a short radio frequency quadrupole (DC offset=108 V), ions enter a quadrupole, Q1, a collision cell with a quadrupole ion guide, Q2 (20 cm), and a quadrupole, Q3, and are then detected with pulse counting. For cross section measurements, the Q2 rod offset was fixed at 105 V and the Q3 rod offset was increased systematically to give ion stopping curves. For MS/MS experiments, both the Q2 and Q3 rod offsets were varied. In all experiments the collision gas was argon.

Collision Cross Sections

Cross sections were measured with axial kinetic energy loss experiments, as described previously [32–34]. In Q2, ions lose kinetic energy through multiple collisions with low-density Ar. The energy losses are related to the collision cross sections with an aerodynamic drag model [36] 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 to Q2, C_d is a drag coefficient for diffuse scattering [33], n is the collision 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 the collision cell (20.6 cm) and σ is the collision cross section. The pressure of Ar in Q2 was varied between 0 and 1.2 mTorr and stopping curves were obtained at different pressures. Cross sections were then calculated by plotting $-\ln \frac{E}{E_0}$ versus $\frac{C_d n m_2 l}{m_1}$.

MS/MS of Gas-Phase Ions

In MS/MS experiments, a precursor ion is mass selected in Q1, and then injected into Q2 where multiple collisions with Ar cause dissociation. Fragment ions are then mass analyzed in Q3. The Q0-Q2 rod offset difference (ΔV_{Q0-Q2}) and ion charge determine the initial kinetic energy of an ion, E_0 , at the cell entrance. The total internal energy added to ions in the collision cell, ΔE_{int} , can be calculated with a collision model that considers the different numbers of collisions of ions with different cross sections, and the losses of kinetic energies of ions as they pass through the cell [37, 38] by

$$\Delta E_{int} = \Phi \frac{m_2}{M} E_0 \frac{m_1}{m_2} \frac{1}{C_d} \left[1 - \exp\left(-\frac{C_d n m_2 \sigma l}{m_1}\right) \right] \quad (2)$$

where Φ is the average fraction of centre-of-mass kinetic energy transferred to internal energy in a collision (taken to be 1.0), and $M=m_1+m_2$. During experiments ΔV_{Q0-Q2} was systematically increased and the initial energy, E_0 , was calculated from the ΔV_{Q0-Q2} that gave 50% precursor ion loss. The corresponding ΔV_{Q0-Q2} is called the “dissociation voltage.” The pressure of Ar in Q2 was 1.5 mTorr for dissociation of monomers, 2.0 mTorr for dimers and 3.0 mTorr for tetramers. In all experiments the pressures were measured with a precision capacitance manometer (model 120AA; MKS Instruments, Boulder, CO, USA).

Protein Purification

Approximately 3 mL of fresh human sickle blood (Hb S, 80%) and 3 mL of cord blood (Hb F, 80%) were provided by BC Children’s Hospital, and immediately extracted following standard procedures [39, 40], as described in detail previously [17]. After dialysis of ~1 mL of purified hemolysate, the concentration of ^{oxy}Hb (as tetramer) was

determined to be 1.0 mM of Hb S and 1.8 mM of Hb F, with a UV–Vis spectrometer (Nanodrop 1000; Thermal Scientific, Wilmington, DE, USA) and the standard pyridine hemochromogen method [41, 42]. The $^{\text{oxy}}\text{Hb}$ was then oxidized to $^{\text{met}}\text{Hb}$ with a 1.5-fold stoichiometric excess of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) for 5 min at 20 °C [43]. To minimize salt content for electrospray ionization, the Hb was further desalted on a 3×25 cm G-25 Sephadex column (GE Healthcare, Buckinghamshire, UK) to remove excess $\text{K}_3\text{Fe}(\text{CN})_6$. The $^{\text{met}}\text{Hb}$ solution was then frozen with liquid nitrogen and stored at -80 °C. Before MS analysis, the solution was quickly thawed. Fresh Hb A was prepared as described previously [17] and oxidized to $^{\text{met}}\text{Hb}$ prior to MS analysis.

Solutions and Reagents

For MS of native or near-native proteins, the Hb solutions were diluted to 20 μM or 10 μM with 10% methanol (MeOH) or 10% acetonitrile (ACN) in 10 mM ammonium acetate (NH_4Ac) at pH 6.8 (measured with a Accumet model 15 pH meter [Fisher Scientific, Fairlawn, NJ, USA]). For MS of denatured Hb to measure monomer masses, proteins were prepared in 50/50 (vol/vol) MeOH/ H_2O with 0.5% acetic acid. Samples were infused into the ESI sources with a syringe pump (Harvard Apparatus, St. Laurent, QC, Canada) at 1 $\mu\text{L}/\text{min}$.

Acetic acid (99.99%), pyridine (99.9%), CsI (99.99%), and $\text{K}_3\text{Fe}(\text{CN})_6$ (ACS grade) were from Sigma-Aldrich, St. Louis, MO. Methanol (HPLC grade), ACN (HPLC grade), and NH_4Ac (ACS grade) were from Fisher Scientific, Fairlawn, NJ, USA. Nitrogen and argon (99.999% manufacturer's stated purity) were from Praxair, Mississauga, ON, Canada.

Results and Discussion

Mass Spectra of Hemoglobins

With the same operating and solution conditions (10% MeOH, 10 mM NH_4Ac), 20 μM Hb F, and Hb S gave similar MS spectra with the triple quadrupole system as shown in Figure 1a and b. Protein tetramers with charge +16 dominate the MS spectra with lower intensities of +15, +17 tetramer ions and +10, +11 dimer ions. With 10% MeOH solutions nearly no monomer ions are seen with Hb F, and only low-abundance $\alpha^{\text{h}+7}$ ions are observed with Hb S. The β and γ chains are not seen due to the low abundance of monomers and the lower sensitivity of these chains in ESI (see below). As well, oxidation of methionine and cysteine residues in the β chains of Hb A, which is sometimes found with commercial proteins, leads to the formation of heme-deficient dimer ions in mass spectra [17, 40]. We do not observe heme-deficient dimers, which is consistent with previous studies of fresh Hb S [16] and Hb F [44]. Furthermore, measurements of the masses of the apo-monomer ions from denaturing solutions with our TOF system do not show α or β monomers with mass shifts of +16 or +32. Thus,

Figure 1. ESI mass spectra of (a), Hb F, 20 μM with 10% MeOH; (b), Hb S, 20 μM with 10% MeOH; (c), Hb F, 10 μM with 10% ACN; and (d), Hb S, 10 μM with 10% ACN. Notation: D, dimers; Q, tetramers. Holo- α ions, α^{h} , are labeled; apo- α ions, α^{a} , are marked with filled triangles. Black arrows indicate low-intensity γ^{a} or β^{a} ions

we conclude that our fresh proteins do not contain oxidative modifications. Lowering the Hb concentration to 10 μM and changing the solvent to 10% ACN, which slightly destabilizes the protein, increases the levels of monomer ions (apo- and holo-) and dimer ions (Figure 1c and d), as in our previous study [17], making measurement of the properties of monomer and dimer ions possible. In this case, the variant β chains of Hb S and the γ chains of Hb F can be seen but with much lower intensity than the α chains, similar to Hb A [18, 40, 45].

In aqueous solution, usually liganded Hb, carboxy-Hb ($^{\text{CO}}\text{Hb}$) or $^{\text{oxy}}\text{Hb}$, is used to measure the dissociation equilibrium constant of the tetramer, K_{d} [10]. For the tetramer-dimer equilibrium at neutral pH, K_{d} of $^{\text{CO}}\text{Hb}$ S is reported to be 0.196 μM [46], or 0.4 μM [47]. With these K_{d} , ca. 5% and 7% of the tetramers initially at 20 μM dissociate to dimers, respectively. The K_{d} for dissociation

of $^{\text{CO}}$ Hb F is reported to be $0.66 \mu\text{M}$ (100 mM tris-HCl, 0.1 M NaCl, 5 mM EDTA at pH 7.4) [5] and $0.01 \mu\text{M}$ (150 mM tris-Ac buffer at pH 7.5) [3], which cause ca. 9% and 1% tetramer dissociation, respectively. The 70× difference in K_d of Hb F between these two studies was not explained.

The estimated K_d for dissociation of dimers to monomers in solution of $^{\text{CO}}$ Hb F is much smaller (1.63×10^{-13} M) [4]. As expected from the small dissociation constant, we find low levels of monomer ions in MS spectra. However, we generally find higher levels of dimer ions in mass spectra than calculated from the solution K_d values. Solution conditions, including buffers and organic solvents, as discussed above, have an effect on the degree of dissociation in solution. As well, mass spectra strongly depend on MS system operating conditions [17, 18, 40], making it difficult to compare ion abundances in mass spectra directly with calculated levels of species in solution.

Collision Cross Sections

Table 2 lists cross sections of holo-monomer (α^{h} , +7, +8), dimer (+11, +12) and tetramer (+16, +17) ions formed from fresh human Hb F and Hb S, and compares these with our previous results with Hb A [17]. Generally, cross sections of dimer ions are intermediate between cross sections of monomer and tetramer ions (Figure 2) and as found in solution (see below). Monomers of Hb F and Hb S have similar cross sections, roughly 10% larger than Hb A. For dimers, the order of the cross sections is Hb S > Hb F > Hb A. Dimers of Hb S have cross sections 11% larger than dimers of Hb A, and 6% larger than dimers of Hb F. Cross sections of Hb S tetramer ions are ca. 13% greater than cross sections of Hb F and Hb A tetramer ions, which have the same cross sections within the combined uncertainties. The single localized mutation $\beta\text{Glu6Val}$ in Hb S evidently changes the conformation of monomer, dimer, and tetramer ions in the gas phase. Previously, Scarff et al., using IMS, showed cross sections of gas-phase Hb S tetramer ions (+15 to +18) are ca. 4% larger (averaged over charge states) than Hb A tetramer ions [16]. The absolute cross sections of Hb S and Hb A measured by Scarff et al. are ca. 7% and 17% greater than ours, respectively. Cross sections of the monomer and dimer ions of Hb S and Hb F have not been reported previously.

Table 2. Collision Cross Section ($\text{\AA}^2$) of Variant Hb Ions^a

Species	Charge	Hb F	Hb S	Hb A [17]
α^{h}	+7	1478±34	1510±38	1310±10
	+8	1531±18	1546±65	1420±34
Dimer	+11	2504±46	2664±87	2402±100
	+12	2580±70	2686±100	2420±44
Tetramer	+16	3512±24	4044±92	3672±13
	+17	3774±21	4220±83	3672±98

^a Uncertainties are the standard deviations of three measurements.

Figure 2. Collision cross sections as a function of charge state for Hb A (triangles), Hb F (open circles), and Hb S (diamonds). Notation as in Figure 1

One issue involved in studies of gas-phase conformations of proteins is how they compare with the native conformations in solution. The Stokes radius (r_s), measured by size exclusion chromatography, and radius of gyration (r_g), measured by small angle X-ray or multi-angle light scattering, can be used to estimate “sizes” of proteins in solution. The Stokes radius of a protein correlates with the molecular weight [48]. The Hb A dimer has r_s (24 Å) [49], intermediate between that of the tetramer (31.3 Å) [50] and holo-monomer (21.5 Å) [48]. Because Hb A, F, and S tetramers have nearly the same molecular weight (within 0.5%) (Table 1), they might be expected to show similar r_s values. With light scattering, the r_g values of $^{\text{CO}}$ Hb in 50 mM sodium phosphate buffer were determined to be 29 Å for Hb A and Hb S, and 30 Å for Hb F [51]. Calculated “cross sections” $A_g = (5/3)\pi r_g^2$ [52] are $4403 \text{ \AA}^2$ and $4712 \text{ \AA}^2$, respectively. Thus in these solution measurements Hb S does not show larger “cross sections” than the other two proteins. However Scarff et al. [16] have estimated cross sections of Hb S and Hb A from the crystal structures and found that the Hb S tetramers have cross sections ca. 10% greater than Hb A tetramers, similar to the differences in cross sections seen here.

Gas-Phase H/D Exchange

Table 3 lists the numbers of hydrogens exchanged, the number of exchangeable hydrogens calculated from the sequences, and the percent of the exchangeable hydrogens exchanged, of monomer (α^{h} , +7, +8), dimer (+11, +12) and tetramer (+16, +17) ions from fresh Hb A, S, and F. The β and γ ions show HDX levels similar to the α ions (data not shown). In theory, increasing charge can give an increasing degree of structural perturbation and thus a different HDX level [23–25, 33, 34, 53]. For the ions studied, increasing the charge by one does not cause significant changes in the HDX levels, indicating that the conformations do not greatly change for ions differing by only one charge. This is consistent with our cross section results. The holo-monomer and dimer ions of variant hemoglobins show similar HDX levels within the combined uncertainties, although they have different cross sections. As noted, ions with different cross

Table 3. Numbers of Hydrogens Exchanged (HDX), Numbers of Exchangeable Hydrogens (HDX^{max}) and Percent of the Hydrogens Exchanged (%HDX) for Variant Hb Ions at Δ*V*_{os}=200 V after 10 s Exchange with 5.0 mTorr of D₂O

Species	Charge	Hb F			Hb S			Hb A		
		HDX	HDX ^{max}	%HDX	HDX	HDX ^{max}	%HDX	HDX	HDX ^{max}	%HDX
α ^h	+7	75±2	233	32.2±0.8	83±5	233	35.6±2.1	79±5	233	33.9±2.1
	+8	79±5	234	33.8±2.1	86±3	234	36.7±1.3	80±5	234	34.2±2.1
Dimer	+11	149±2	484	30.8±0.4	147±4	472	31.1±0.8	146±13	472	30.9±2.7
	+12	149±6	485	30.7±1.2	148±5	473	31.3±1.0	146±10	473	30.9±2.1
Tetramer	+16	331±19	962	34.4±2.0	376±6	938	40.1±0.6	321±21	938	34.2±2.2
	+17	339±9	963	35.2±0.9	388±2	939	41.3±0.2	330±29	939	35.1±3.1

sections may not show different gas-phase H/D exchange levels [23–25]. With tetramer ions, Hb S shows a ca. 16% higher HDX level than Hb A and Hb F. Thus, in the gas phase, Hb S tetramer ions have both larger cross sections and greater HDX levels.

The cross section measurements and the HDX measurements occur on different time scales. After ion formation, cross sections are measured in less than the ca. 2 ms required for ions to pass through the triple quadrupole system, while the H/D exchange requires up to 10⁴ ms in the trap before ion detection. Monomers α^h from Hb F and Hb S show larger cross sections than monomers α^h from Hb A, but all α^h monomers show the same exchange levels within the combined uncertainties. It is conceivable that the monomers retain some memory of their conformation when formed, but lose this memory on the time scale of seconds. Cross sections show that Hb S tetramers are larger than Hb A and Hb F tetramers at times of a few ms. The HDX experiments show they retain different and perhaps more unfolded conformations than Hb A and Hb F, for up to 10 s.

When comparing HDX levels among monomer, dimer, and tetramer ions, relative exchange levels, %HDX, are more informative. The order of the relative exchange levels might be expected to be monomer > dimer > tetramer, because when monomers bind to form dimers or tetramers, some of the exchangeable amino acid residues are buried in the interface. This has been observed in solution HDX with commercial bovine Hb, with an exchange time of 53 ms [19]. However in the gas phase, this trend is not seen (Figure 3). Dimer ions give 31% HDX on average, slightly less than that of α^h and tetramer ions. With Hb A and F, the relative exchange levels of tetramer ions (ca. 34.7%) are similar to α^h ions (ca. 33.5%). With Hb S, tetramer ions show slightly higher % HDX than α^h ions (40.7% and 36.2%, respectively). The difference is not as large as gas-phase HDX results reported previously with commercial bovine Hb, where 60% of hydrogens on tetramers and 40% on monomers were exchanged [18].

MS/MS and Binding Strengths

Tandem mass spectrometry of monomer (α^h, +7, +8), dimer (+11, +12), and tetramer (+16, +17) ions from Hb A, F, and

S was used to determine relative binding strengths in the gas-phase ions. Holo-α ions dissociate to apo-α ions with the release of a charged heme, for example, α^{h+7} → α^{a+6} + h⁺ (spectra not shown). Less than 5% neutral heme loss was observed. This is expected because heme is overall singly charged in ^{met}Hb and neutral in ^{oxy}Hb. The results are consistent with previous MS/MS studies of myoglobin, which has a globular structure similar to the Hb α monomer [54, 55].

Dimer ions dissociate into two monomers, as in solution. One clear difference between the hemoglobins is shown in the MS/MS spectra of Figure 4. Under conditions where nearly half of the +11 charged Hb A and Hb S dimer ions dissociate, +7 α ions and +4 β ions, both holo- and apo-, are preferentially formed. With Hb F, two holo-monomers with charges +5 and +6 are the main product ions. This effect also is also seen with the +12 dimers; Hb F +12 ions mainly dissociate to two +6 holo-monomer ions, whereas Hb A and Hb S +12 ions preferentially form +8 α and +4 β ions. Apo-monomers can be formed by further dissociation of holo-monomers. More highly charged holo-monomers require less energy for dissociation because of Coulomb repulsion in the ions [55]. Thus, the +7 holo-monomers show a greater degree of dissociation to apo-monomers than the +4 holo-monomers (Figure 4a and b). The asymmetric dimer dissociation of Hb A and Hb S is similar to the asymmetric charge

Figure 3. Percent of hydrogens exchanged (% HDX) after 10 s trapping as a function of charge state for Hb A (triangles), Hb F (open circles), and Hb S (diamonds). Notation as in Figure 1

Figure 4. MS/MS spectra of +11 dimer ions from (a) Hb A, (b) Hb S, and (c) Hb F with a pressure in Q2 of 2 mTorr of Ar, and ΔV_{Q0-Q2} of 70 V for Hb A and S, of 65 V for Hb F, and with ca 50% dissociation of precursors. Fragments, α ions (red) and β (or γ) ions (blue) are shown; α -apo, h-holo

partitioning of dimers from commercial Hb A seen in a previous study [27]. Asymmetric charge division has also been observed with other dimeric protein assemblies [56]. That Hb F dimers form monomers with nearly equal charges is somewhat unusual. Charge partitioning is influenced by a number of factors, including the number of charges, the gas-phase conformation of the precursor ion, and the conformational flexibility of the monomer in the protein complex [57, 58]. Of 39 different residues between the β - and γ -subunits, three occur at the $\alpha_1\gamma_1$ or $\alpha_1\beta_1$ dimer interface: $\beta 112\text{Cys}$, 116His , and 125Pro are replaced by $\gamma 112\text{Thr}$, 116Ile , and $\text{Glu}125$ [1]. These sequence alternations may be the reason for the different dissociation patterns. The internal energy added to the ion is another factor [59]. With relatively low internal energy (ca. 50% dimer dissociation), holo-monomers dominate in MS/MS spectra and Hb F shows symmetric charge division (Figure 4c); when ΔE_{int} is increased so that there is about 80% dissociation, all dimers, including Hb F, show fragments with asymmetric charge partitioning and mostly apo-monomers since holo-monomers can have additional collisions and gain enough internal energy to lose heme.

Tetramer ions dissociate to highly charged monomers (apo- or holo-) and trimers with 0–4 hemes (spectra not

shown), consistent with previous studies with commercial human Hb A [27], and in contrast to the known solution-phase dissociation where two dimers are formed [19, 40]. Trimers with four hemes have also been reported by Versluis and Heck [27], indicating that an apo- α ion may be directly expelled from the intact holo-tetramer. Versluis and Heck showed that +17 tetramer ions of Hb A predominantly dissociate to single α ions (+10+6) and an $\alpha\beta_2$ trimer. However we find that tetramers of Hb A, +16 and +17, dissociate to $\alpha_2\beta$ trimer ions as well as $\alpha\beta_2$ trimers (+8, +9). The +7 trimers are outside the mass range of our triple quadrupole system and so cannot be seen. However the complementary α and β (+8, +9) ions are seen in the MS/MS spectrum of Hb A (Figure 5a). Here, only the monomer regions of the MS/MS spectra are shown for clear comparison of the different hemoglobins. Hossain and Konermann also observed apo- α and apo- β ions as MS/MS products in their study on bovine Hb [19]. Hemoglobin S shows dissociation pathways similar to Hb A (Figure 5b) in that both α and β monomer ions are produced. In contrast, MS/MS of Hb F shows more intense

Figure 5. Monomer regions of MS/MS spectra of +16 tetramer ions from (a) Hb A, (b) Hb S, and (c) Hb F, with a pressure in Q2 of 3.0 mTorr of Ar. The MS/MS spectra are obtained with nearly 50% dissociation of tetramer precursor ions, at $\Delta V_{Q0-Q2}=40$ V with Hb A, $\Delta V_{Q0-Q2}=45$ V with Hb S, and $\Delta V_{Q0-Q2}=30$ V with Hb F. Notation as in Figure 4

Table 4. Dissociation Voltages (ΔV) and ΔE_{int} values (eV)^a of Ions from Variant Hb with 50% Precursor Ion Dissociation

Species	Charge	ΔV_{os} (V)	Hb F		Hb S		Hb A	
			ΔV	ΔE_{int}	ΔV	ΔE_{int}	ΔV	ΔE_{int}
α^h	+7	100	79±1	140±2	76±2	137±4	77±4	126±7
	+8	100	64±1	132±1	63±2	130±5	62±3	122±6
Dimer	+11	100	64±1	186±3	72±2	221±8	70±2	201±8
	+12	100	45±1	146±4	52±3	172±10	52±2	162±6
Tetramer	+16	100	87±3	382±13	72±4	341±18	78±2	351±9
		150	78±1	340±5	63±4	295±19	68±1	305±5
	+17	100	73±3	352±14	62±2	315±11	66±2	314±7
		150	62±1	298±5	51±1	259±6	56±1	265±6

^a Uncertainties of ΔE_{int} are obtained by propagation of the errors of ΔV and cross sections.

α ions than γ ions (Figure 5c), suggesting the preferred pathway is $\alpha_2\gamma_2 \rightarrow \alpha + \alpha\gamma_2$.

Table 4 lists the dissociation voltages (ΔV_{Q0-Q2} , shown as ΔV for short) and calculated ΔE_{int} values for α^h , dimer and tetramer ions from Hb A, S, and F. The ΔE_{int} results are compared with an orifice-skimmer voltage ΔV_{os} of 100 V, to match the conditions used for cross section measurements. Higher ΔE_{int} values indicate ions must acquire greater additional internal energy in Q2 to dissociate, and thus are more stable in the gas phase. Comparison of ΔE_{int} values for complexes that differ substantially in binding energy requires correction for the different reaction times in Q2 [38, 55, 60]. Under the conditions that give 50% fragment yield, the dissociation occurs over a length of ca. 5 cm near the cell exit [38]. The corresponding reaction times for monomer, dimer, and tetramer ions of the different hemoglobins with various charges are calculated to be 28 ± 1 μ s, 37 ± 3 μ s, and 42 ± 4 μ s on average, respectively. This small variation in reaction times between the different hemoglobins will not influence ΔE_{int} significantly [55, 60] and no correction for different reaction times is necessary. Heme loss from holo- α ions of Hb S and Hb F requires similar ΔE_{int} . The dissociation voltages are nearly the same for Hb S and Hb A, but Hb A has a slightly lower (ca. 10%) ΔE_{int} (Figure 6), because of the smaller α^h cross section of Hb A. The smaller cross section means the ions have fewer collisions and thus slightly less

kinetic energy is transferred into internal energy. The order of the ΔE_{int} values of the dimer ions is, Hb S > Hb A > Hb F. Dimers of Hb S require 6% greater ΔE_{int} than Hb A, and 11% greater ΔE_{int} than Hb F. With tetramers, Hb F requires approximately 11% higher ΔE_{int} than Hb A or Hb S. For the same species, ions with only one more charge clearly show lower ΔE_{int} (Table 4), indicating increased Coulombic repulsion forces destabilize the ions in MS/MS. In these experiments, ΔV_{os} was set to 100 V. When ions pass through the orifice-skimmer region, ion activation first causes desolvation and then increases internal energies of the ions [18]. A nearly 10 V decrease in dissociation voltage was found with tetramer ions when ΔV_{os} was increased from 100 V to 150 V (Table 4) because at ΔV_{os} of 150 V the tetramer ions have greater internal energies before they enter the collision cell. However, the trend in ΔE_{int} values among the different Hb variants remains the same. This effect was not seen with dimer and monomer ions; ΔV_{os} of 100 V and 150 V give similar ΔE_{int} values.

Correlations between solution and gas-phase stability can be investigated by comparing free energy changes ΔG_{sol}^0 and ΔE_{int} for similar dissociation pathways. In solution, dimers of Hb F are more stable than dimers of Hb A because three amino acid replacements increase hydrophobicity on the $\alpha_1\gamma_1$ interface [61], leading to a stronger interface interaction. The dissociation of $\alpha\gamma$ dimers into α and γ is three times slower than the dissociation of $\alpha\beta$ dimers into α and β [4]. The free energy changes (ΔG_{sol}^0) of dimer-monomer dissociation of ^{CO}Hb A and ^{CO}Hb F are 16.7 and 17.3 kcal/mol, respectively, calculated from reported K_d values (4.7×10^{-13} M and 1.63×10^{-13} M [4]). Thus, in the gas-phase, where Hb F dimers are slightly less stable than Hb A dimers, the dimer binding differs from the binding in solution. This suggests that a more complex mechanism may be involved in gas-phase dissociation than just the immediate contacts at the subunit interface. This may be due to the symmetric charge partitioning with Hb F. Less internal energy is required to form monomers with equal charges than with asymmetrical charge partitioning [59], giving a lower ΔE_{int} for Hb F dimers to dissociate than Hb S and Hb A.

Tetramers of Hb F are more strongly bound in solution than tetramers of Hb A and Hb S. In 150 mM tris-Ac buffer at pH 7.5, K_d for the tetramer-dimer dissociation of ^{CO}Hb A

Figure 6. Values of ΔE_{int} as a function of charge state for Hb A (triangles), Hb F (open circles), and Hb S (diamonds). Notation as in Figure 1. The pressure in Q2 was 1.5 mTorr for α^h , 2.0 mTorr for dimers, and 3.0 mTorr for tetramers

is 0.68 μM , of $^{\text{CO}}\text{Hb F}$ 0.01 μM [3], and of $^{\text{CO}}\text{Hb S}$ 0.42 μM [47], and the resultant $\Delta G^{\circ}_{\text{sol}}$ are 8.34 kcal/mol, 10.8 kcal/mol is 8.61 kcal/mol, respectively. In the gas phase, though Hb F also shows greater stability than Hb A and Hb S, ΔE_{int} cannot be compared with $\Delta G^{\circ}_{\text{sol}}$ values because gas-phase tetramers dissociate to monomers and trimers, not two dimers.

Summary

With the same solution and mass spectrometer conditions, fresh Hb F and Hb S give similar mass spectra. The organic solvent used in the solution affects the relative abundances of monomer, dimer and tetramer ions in mass spectra. For all proteins studied, Hb A, Hb S, and Hb F, dimers give cross sections intermediate between monomer and tetramer ions. Gas-phase dimer ions exchange a slightly smaller fraction of their hydrogens than monomer or tetramer ions. Hemoglobin S shows larger cross sections of monomer, dimer, and tetramer ions and greater HDX levels of tetramer ions than Hb A and Hb F. Comparison of ΔE_{int} and $\Delta G^{\circ}_{\text{sol}}$ values of dimers suggests that the binding in gas-phase dimers may differ from that in solution. In solution, $\alpha^{\text{h}}\gamma^{\text{h}}$ is more strongly bound but, in the gas phase, requires less energy to dissociate in MS/MS. In addition, $\alpha^{\text{h}}\gamma^{\text{h}}$ dimers show MS/MS dissociation pathways distinct from $\alpha^{\text{h}}\beta^{\text{h}}$ dimers, with symmetrical versus asymmetrical charge division of monomers. Tetramer ions of Hb F require greater internal energy to dissociate to monomers and trimers. Thus, we do not find consistent relationships between solution-phase stability and gas-phase dissociation energies. The different sequence of the γ chain compared with the β chain of Hb A, of Hb F might be expected to change the physical properties of the gas-phase ions, as is observed. The results with Hb S show that substitution of a single residue in the β chain can also change the physical properties in this gas-phase protein-protein complex.

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References

- Bunn, H.F., Forget, B.G.: Hemoglobin: Molecular, Genetic, and Clinical Aspects, pp. 63–68. W.B. Saunders Company, Philadelphia (1986)
- Bunn, H.F., Briehl, R.W.: Interaction of 2,3-Diphosphoglycerate with Various Human Hemoglobins. *J. Clin. Invest.* **49**, 1088–1095 (1970)
- Dumoulin, A., Manning, L.R., Jenkins, W.T., Winslow, R.M., Manning, J.M.: Exchange of Subunit Interfaces Between Recombinant Adult and Fetal Hemoglobins—Evidence for a Functional Inter-Relationship Among Regions of the Tetramer. *J. Biol. Chem.* **272**, 31326–31332 (1997)
- Mrabet, N.T., Shaeffer, J.R., McDonald, M.J., Bunn, H.F.: Dissociation of Dimers of Human Hemoglobins A and F into Monomers. *J. Biol. Chem.* **261**, 1111–1115 (1986)
- Yamaguchi, T., Adachi, K.: Hemoglobin Equilibrium Analysis by the Multiangle Laser Light-Scattering Method. *Biochem. Biophys. Res. Commun.* **290**, 1382–1387 (2002)
- Murayama, M.: Structure of Sickle Cell Hemoglobin and Molecular Mechanism of Sickling Phenomenon. *Clin. Chem.* **13**, 578–588 (1967)
- Sunshine, H.R., Hofrichter, J., Ferrone, F.A., Eaton, W.A.: Oxygen Binding by Sickle-Cell Hemoglobin Polymers. *J. Mol. Biol.* **158**, 251–273 (1982)
- Caughey, W.S.: Biochemical and Clinical Aspects of Hemoglobin Abnormalities, p. 401. Academic Press, New York (1978)
- Platt, O.S.: Hydroxyurea for the Treatment of Sickle Cell Anemia. *N. Engl. J. Med.* **358**, 1362–1369 (2008)
- Manning, J.M., Dumoulin, A., Li, X.F., Manning, L.R.: Normal and Abnormal Protein Subunit Interactions in Hemoglobins. *J. Biol. Chem.* **273**, 19359–19362 (1998)
- Griffith, W.P., Kaltashov, I.A.: Mass Spectrometry in the Study of Hemoglobin: From Covalent Structure to Higher Order Assembly. *Curr. Org. Chem.* **10**, 535–553 (2006)
- Davison, A.S., Green, B.N., Roberts, N.B.: Fetal Hemoglobin: Assessment of Glycation and Acetylation Status by Electrospray Ionization Mass Spectrometry. *Clin. Chem. Lab. Med.* **46**, 1230–1238 (2008)
- Mandal, A.K., Bisht, S., Bhat, V.S., Krishnaswamy, P.R., Balaram, P.: Electrospray Mass Spectrometric Characterization of Hemoglobin Q (Hb Q-India) and a Double Mutant Hemoglobin S/D in Clinical Samples. *Clin. Biochem.* **41**, 75–81 (2008)
- Wild, B.J., Green, B.N., Cooper, E.K., Lalloz, M.R.A., Erten, S., Stephens, A.D., Layton, D.M.: Rapid Identification of Hemoglobin Variants by Electrospray Ionization Mass Spectrometry. *Blood Cells Mol. Dis.* **27**, 691–704 (2001)
- Williams, J.P., Giles, K., Green, B.N., Scrivens, J.H., Bateman, R.H.: Ion Mobility Augments the Utility of Mass Spectrometry in the Identification of Human Hemoglobin Variants. *Rapid Commun. Mass Spectrom.* **22**, 3179–3186 (2008)
- Scarff, C.A., Patel, V.J., Thalassinou, K., Scrivens, J.H.: Probing Hemoglobin Structure by Means of Traveling-Wave Ion Mobility Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **20**, 625–631 (2009)
- Kang, Y., Douglas, D.J.: Mass Spectra and Ion Collision Cross Sections of Hemoglobin. *J. Am. Soc. Mass Spectrom.* **20**, 290–299 (2011)
- Wright, P.J., Douglas, D.J.: Gas-Phase H/D Exchange and Collision Cross Sections of Hemoglobin Monomers, Dimers, and Tetramers. *J. Am. Soc. Mass Spectrom.* **20**, 484–495 (2009)
- Hossain, B.M., Konermann, L.: Pulsed Hydrogen/Deuterium Exchange MS/MS for Studying the Relationship Between Noncovalent Protein Complexes in Solution and in the Gas Phase after Electrospray Ionization. *Anal. Chem.* **78**, 1613–1619 (2006)
- Kaltashov, I.A., Eyles, S.J.: Studies of Biomolecular Conformations and Conformational Dynamics by Mass Spectrometry. *Mass Spectrom. Rev.* **21**, 37–71 (2002)
- Wytenbach, T., Bowers, M.T.: Gas Phase Conformations of Biological Molecules: The Hydrogen/Deuterium Exchange Mechanism. *J. Am. Soc. Mass Spectrom.* **10**, 9–14 (1999)
- Cox, H.A., Julian, R.R., Lee, S.W., Beauchamp, J.L.: Gas-Phase H/D Exchange of Sodiated Glycine Oligomers with ND₃: Exchange Kinetics Do Not Reflect Parent Ion Structures. *J. Am. Chem. Soc.* **126**, 6485–6490 (2004)
- Evans, S.E., Lueck, N., Marzluff, E.M.: Gas Phase Hydrogen/Deuterium Exchange of Proteins in an Ion Trap Mass Spectrometer. *Int. J. Mass Spectrom.* **222**, 175–187 (2003)
- Valentine, S.J., Clemmer, D.E.: H/D Exchange Levels of Shape-Resolved Cytochrome *c* Conformers in the Gas Phase. *J. Am. Chem. Soc.* **119**, 3558–3566 (1997)
- Valentine, S.J., Clemmer, D.E.: Temperature-Dependent H/D Exchange of Compact and Elongated Cytochrome *c* Ions in the Gas Phase. *J. Am. Soc. Mass Spectrom.* **13**, 506–517 (2002)
- Apostol, I.: Assessing the Relative Stabilities of Engineered Hemoglobins Using Electrospray Mass Spectrometry. *Anal. Biochem.* **272**, 8–18 (1999)
- Versluis, C., Heck, A.J.R.: Gas-Phase Dissociation of Hemoglobin. *Int. J. Mass Spectrom.* **210**, 637–649 (2001)

28. Tomoda, A., Yoneyama, Y., Tsuji, A.: Changes in Intermediate Hemoglobins During Autoxidation of Hemoglobin. *Biochem. J.* **195**, 485–492 (1981)
29. Campbell, J.M., Collings, B.A., Douglas, D.J.: A New Linear Ion Trap Time-of-Flight System with Tandem Mass Spectrometry Capabilities. *Rapid Commun. Mass Spectrom.* **12**, 1463–1474 (1998)
30. Collings, B.A., Campbell, J.M., Mao, D.M., Douglas, D.J.: A Combined Linear Ion Trap Time-of-Flight System with Improved Performance and MSⁿ Capabilities. *Rapid Commun. Mass Spectrom.* **15**, 1777–1795 (2001)
31. Mao, D.M., Ding, C.F., Douglas, D.J.: Hydrogen/Deuterium Exchange of Myoglobin Ions in a Linear Quadrupole Ion Trap. *Rapid Commun. Mass Spectrom.* **16**, 1941–1945 (2002)
32. Mao, D.M., Babu, K.R., Chen, Y.L., Douglas, D.J.: Conformations of Gas-Phase Lysozyme Ions Produced from Two Different Solution Conformations. *Anal. Chem.* **75**, 1325–1330 (2003)
33. Chen, Y.L., Collings, B.A., Douglas, D.J.: Collision Cross Sections of Myoglobin and Cytochrome *c* Ions with Ne, Ar, and Kr. *J. Am. Soc. Mass Spectrom.* **8**, 681–687 (1997)
34. Covey, T., Douglas, D.J.: Collision Cross Sections for Protein Ions. *J. Am. Soc. Mass Spectrom.* **4**, 616–623 (1993)
35. Douglas, D.J., French, J.B.: Collisional Focusing Effects in Radio-Frequency Quadrupoles. *J. Am. Soc. Mass Spectrom.* **3**, 398–408 (1992)
36. Douglas, D.J.: An Aerodynamic Drag Model for Protein Ions. *J. Am. Soc. Mass Spectrom.* **5**, 17–18 (1994)
37. Chen, Y.L., Campbell, J.M., Collings, B.A., Konermann, L., Douglas, D.J.: Stability of a Highly Charged Noncovalent Complex in the Gas Phase: Holomyoglobin. *Rapid Commun. Mass Spectrom.* **12**, 1003–1010 (1998)
38. Mauk, M.R., Mauk, A.G., Chen, Y.L., Douglas, D.J.: Tandem Mass Spectrometry of Protein–Protein Complexes: Cytochrome *c*–Cytochrome *b₅*. *J. Am. Soc. Mass Spectrom.* **13**, 59–71 (2002)
39. Antonini, E., Brunori, M.: Hemoglobin and Myoglobin in Their Reactions with Ligands, pp. 2–6. North-Holland Publishing Company, Amsterdam (1972)
40. Boys, B.L., Kuprowski, M.C., Konermann, L.: Symmetric Behavior of Hemoglobin α - and β -subunits During Acid-Induced Denaturation Observed by Electrospray Mass Spectrometry. *Biochemistry* **46**, 10675–10684 (2007)
41. Deduve, C.: A Spectrophotometric Method for the Simultaneous Determination of Myoglobin and Hemoglobin in Extracts of Human Muscle. *Acta Chem. Scand.* **2**, 264–289 (1948)
42. Paul, K.G., Theorell, H., Akeson, A.: The Molar Light Absorption of Pyridine Ferroporphyrin (Pyridine Haemochromogen). *Acta Chem. Scand.* **7**, 1284–1287 (1953)
43. Antonini, E., Brunori, M., Wyman, J.: Studies on the Oxidation-Reduction Potentials of Heme Proteins. IV. The Kinetics of Oxidation of Hemoglobin and Myoglobin by Ferricyanide. *Biochemistry* **4**, 545–551 (1965)
44. Ofori-Acquah, S.F., Green, B.N., Davies, S.C., Nicolaides, K.H., Serjeant, G.R., Layton, D.M.: Mass Spectral Analysis of Asymmetric Hemoglobin Hybrids: Demonstration of Hb FS [$\alpha(2)\gamma$ $\beta(s)$] in Sickle Cell Disease. *Anal. Biochem.* **298**, 76–82 (2001)
45. Kuprowski, M.C., Boys, B.L., Konermann, L.: Analysis of Protein Mixtures by Electrospray Mass Spectrometry: Effects of Conformation and Desolvation Behavior on the Signal Intensities of Hemoglobin Subunits. *J. Am. Soc. Mass Spectrom.* **18**, 1279–1285 (2007)
46. Williams, R.C., Kim, H.: Carbamoylated Hemoglobin-A and Hemoglobin-S - Physical Properties. *Biochemistry* **15**, 2207–2211 (1976)
47. Manning, L.R., Jenkins, W.T., Hess, J.R., Vandegriff, K., Winslow, R.M., Manning, J.M.: Subunit Dissociations in Natural and Recombinant Hemoglobins. *Protein Sci.* **5**, 775–781 (1996)
48. Fish, W.W., Reynolds, J.A., Tanford, C.: Gel Chromatography of Proteins in Denaturing Solvents—Comparison between Sodium Dodecyl Sulfate and Guanidine Hydrochloride as Denaturants. *J. Biol. Chem.* **245**, 5166–5168 (1970)
49. Ogasawara, N., Yoshino, M., Asai, J.P.: Amp Nucleosidase from *Azotobacter-Vinelandii*. II. Association and Dissociation. *J. Biochem.* **68**, 331–340 (1970)
50. Tseng, Y.C.L., Latham, K.R.: Iodothyronines: Oxidative Deiodination by Hemoglobin and Inhibition of Lipid Peroxidation. *Lipids* **19**, 96–102 (1984)
51. Tam, M.F., Chen, J., Tam, T.C.S., Tsai, C.H., Shen, T.J., Simplaceanu, V., Feinstein, T.N., Barrick, D., Ho, C.: Enhanced Inhibition of Polymerization of Sickle Cell Hemoglobin in the Presence of Recombinant Mutants of Human Fetal Hemoglobin with Substitutions at Position 43 in the γ Chain. *Biochemistry* **44**, 12188–12195 (2005)
52. Glatter, O., Kratzky, O.: Small Angle X-Ray Scattering, p. 156. Academic Press, New York (1982)
53. Hoaglund-Hyzer, C.S., Counterman, A.E., Clemmer, D.E.: Anhydrous Protein Ions. *Chem. Rev.* **99**, 3037–3079 (1999)
54. Chrisman, P.A., Newton, K.A., Reid, G.E., Wells, J.M., McLuckey, S.A.: Loss of Charged Versus Neutral Heme from Gaseous Holomyoglobin Ions. *Rapid Commun. Mass Spectrom.* **15**, 2334–2340 (2001)
55. Mark, K.J., Douglas, D.J.: Coulomb Effects in Binding of Heme in Gas-Phase Ions of Myoglobin. *Rapid Commun. Mass Spectrom.* **20**, 111–117 (2006)
56. Versluis, C., van der Staaij, A., Stokvis, E., Heck, A.J.R., de Craene, B.: Metastable Ion Formation and Disparate Charge Separation in the Gas-Phase Dissection of Protein Assemblies Studied by Orthogonal Time-of-Flight Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **12**, 329–336 (2001)
57. Jurchen, J.C., Garcia, D.E., Williams, E.R.: Further Studies on the Origins of Asymmetric Charge Partitioning in Protein Homodimers. *J. Am. Soc. Mass Spectrom.* **15**, 1408–1415 (2004)
58. Jurchen, J.C., Williams, E.R.: Origin of Asymmetric Charge Partitioning in the Dissociation of Gas-phase Protein Homodimers. *J. Am. Chem. Soc.* **125**, 2817–2826 (2003)
59. Wanasundara, S.N., Thachuk, M.: Theoretical Investigations of the Dissociation of Charged Protein Complexes in the Gas Phase. *J. Am. Soc. Mass Spectrom.* **18**, 2242–2253 (2007)
60. Tesic, M., Wicki, J., Poon, D.K.Y., Withers, S.G., Douglas, D.J.: Gas Phase Noncovalent Protein Complexes that Retain Solution Binding Properties: Binding of Xylobiose Inhibitors to the β -1,4 Exoglucanase from *Cellulomonas fimi*. *J. Am. Soc. Mass Spectrom.* **18**, 64–73 (2007)
61. Adachi, K., Zhao, Y., Yamaguchi, T., Surrey, S.: Assembly of γ - with α -Globin Chains to Form Human Fetal Hemoglobin in Vitro and in Vivo. *J. Biol. Chem.* **275**, 12424–12429 (2000)