Probing Hemoglobin Structure by Means of Traveling-Wave Ion Mobility Mass Spectrometry

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Hemoglobin (Hb) is a tetrameric noncovalent complex consisting of two α - and two β -globin chains each associated with a heme group. Its exact assembly pathway is a matter of debate. Disorders of hemoglobin are the most common inherited disorders and subsequently the molecule has been extensively studied. This work attempts to further elucidate the structural properties of the hemoglobin tetramer and its components. Gas-phase conformations of hemoglobin tetramers and their constituents were investigated by means of traveling-wave ion mobility mass spectrometry. Sick (HbS) and normal (HbA) hemoglobin molecules were analyzed to determine whether conformational differences in their quaternary structure could be observed. Rotationally averaged collision cross sections were estimated for tetramer, dimer, apo-, and holo-monomers with reference to a protein standard with known cross sections. Estimates of cross section obtained for the tetramers were compared to values calculated from X-ray crystallographic structures. HbS was consistently estimated to have a larger cross section than that of HbA, comparable with values obtained from X-ray crystallographic structures. Nontetrameric species observed included apo- and holo- forms of α - and β -monomers and heterodimers; α - and β -monomers in both apo- and holo- forms were found to have similar cross sections, suggesting they maintain a similar fold in the gas phase in both the presence and the absence of heme. Heme-deficient dimer, observed in the spectrum when analyzing commercially prepared Hb, was not observed when analyzing fresh blood. This implies that holo- α -apo- β is not an essential intermediate within the Hb assembly pathway, as previously proposed. (J Am Soc Mass Spectrom 2009, 20, 625–631) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Mass spectrometry (MS) has become an important tool for the study of various aspects of protein structure, including the assembly and disassembly of protein complexes, subunit interactions, and ligand interactions [1]. The study of noncovalent complexes has been facilitated by the use of collisional cooling or dampening of ions by elevating the pressure within the source region or by deceleration [2, 3].

The typical electrospray ionization (ESI) mass spectrum of a protein consists of an envelope of peaks attributed to a series of multiply charged gas-phase ions that can indicate the stability and compactness of its structure in the gas phase [4, 5]. Multiply charged ions are produced by proton attachment, predominantly to exposed basic sites on the protein [6], and those of lowest charge are thought to be most representative of native structure [7]. A highly compact protein would have fewer exposed basic sites than those of an unfolded conformation of the same protein and thus would accept fewer charges [4, 8].

Ion mobility spectrometry is a shape-selective technique, based on the time taken for an ion to traverse a mobility cell containing an inert gas under the influence of a weak electric field [9]. This time is related to the rotationally averaged collision cross section, mass, and charge of the ion. The coupling of ion mobility separation with mass spectrometry has provided a powerful method for the analysis of complex mixtures and for the determination of molecular structure [10].

Ion mobility mass spectrometry has emerged as a complementary technique to the well-established methods of X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy for three-dimensional protein structure analysis [11]. There is now substantial evidence to support the view that the gas-phase protein structure can reflect, under controlled conditions, the native solution-phase structure [1, 6, 12]. Multiple studies have shown good agreement between rotationally averaged cross-sectional measurements obtained from X-ray and NMR structures and those obtained by ion mobility experiments [7, 13–17].

In this study traveling-wave ion mobility mass spectrometry (TWIMS) was used to probe the gas-phase conformations of hemoglobin tetramers and their constituents.

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A traveling-wave ion mobility separator (T-wave) [18] has been incorporated into a quadrupole time-of-flight (TOF) instrument, the Synapt HDMS system (Waters Corp., Milford, MA, USA) [9]. The T-wave allows for samples to be analyzed at biologically relevant concentrations, having speed and sensitivity advantages over the traditional drift-time ion mobility device. The T-wave device does not allow for absolute cross-sectional measurements to be obtained from drift-time information, although these may be estimated by using reference samples of known cross sections [7, 13, 19].

Hemoglobin (Hb) is a tetramer consisting of four globin chains, two α - and two β -, each associated with a heme group. It is the major oxygen-transport protein found in the red blood cells of all vertebrates. Disorders of hemoglobin are the most common of all inherited disorders and, consequently, the molecule has been extensively studied.

The most debilitating Hb variant is that which causes sickle-cell anemia. This disease occurs when a person inherits two particular mutated copies of the β -globin gene. The sickle-cell mutation results in the production of a β -chain with a single amino acid substitution (β^6 Glu $\rightarrow$ Val) and changes the conformation of the assembled tetramer to allow molecular stacking. Polymerization of this sickle-cell hemoglobin molecule (HbS), in deoxygenated blood, causes the characteristic alteration in shape of red blood cells from biconcave discs to crescentic [20].

ESI-MS has been widely used to detect Hb variants in hemoglobin [21–25] and to investigate its structural assembly into a noncovalent complex [26–29] and its corresponding disassembly [30].

The exact assembly pathway for hemoglobin is still under debate. It is known that one α - and one β -monomer come together to form a heterodimer and that two of these dimers associate to form the tetramer. Since α - and β -monomers can exist in heme-free (apo, α^a and β^a) and heme-bound (holo, α^h and β^h) forms [28], it is unclear as to whether the heme groups are attached to both α - and β -monomers before dimer formation or whether association leads to heme recruitment.

Griffith and Kaltashov have suggested that the formation of a heme-deficient dimer intermediate ($\alpha^h\beta^a$) occurs, consisting of a natively folded holo- α -globin (α^h) and a partially unfolded apo- β -globin (β^a), before complete dimer formation, to ensure correct tetramer structure arrangement [26, 27]. The Konermann group, however, reported that the heme-deficient dimer seen consistently when using commercially available samples, in the form of lyophilized powder, was not observed when using freshly prepared samples [31]. They studied the acid-induced denaturation of bovine Hb and concluded that it followed a highly symmetric mechanism: $(\alpha^h\beta^h)_2 \rightarrow 2\alpha^h\beta^h \rightarrow 2\alpha^h_{\text{folded}} + 2\beta^h_{\text{folded}} \rightarrow 2\alpha^a_{\text{unfolded}} + 2\beta^a_{\text{unfolded}} + 4\text{ heme}$.

This work attempts to further elucidate the structural properties of the hemoglobin tetramer and its compo-

nents and to determine whether conformational differences between the HbA and HbS molecules can be observed by TWIMS.

Experimental

Samples and Sample Preparation

Samples of fresh whole blood were supplied by University Hospitals Coventry and Warwickshire NHS Trust. Sample preparation for mass spectral analysis was adapted from that detailed by Ofori-Acquah et al. [29]. Samples (20 μ L) were diluted 10-fold in 10 mM ammonium acetate (pH 6.8) and spun at 3000 g for 15 min in centrifugal filter units with a 10-kDa cutoff (Microcon YM-10, Millipore Corp., Billerica, MA, USA). Sample retained on the filter was diluted a further 20-fold with 10 mM ammonium acetate and desalted by agitating for two 10-min periods with close to 5 mg of ion-exchange mixed bed resin (AG 501-X8, Bio-Rad Laboratories, Hercules, CA, USA) that had been prepared for use by rinsing twice in liquid chromatography MS grade water. The resulting solutions were introduced into the ESI source of a Synapt HDMS System (Waters) by means of fused silica nanospray needles. All solvents and calibration and protein standards were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Mass Spectral Analysis

Data were acquired by means of a Synapt HDMS system in ESI positive mode with a capillary voltage of 1.2 kV from 1000 to 4500 m/z . The TOF mass analyzer was calibrated using 2 mg/mL cesium iodide in 50% aqueous propan-2-ol.

Instrument acquisition parameters were adjusted to provide the optimal ion mobility separation. The cone voltage was 60 V and the collision energy in the trap region was 10 eV. Source temperature and gas flow were 110 °C and 35 mL/min, respectively. Nitrogen was used as the gas in the ion mobility cell and the indicated pressure within the cell was 0.68 mbar, equivalent to a flow rate of 38 mL/min. The backing pressure was increased in increments from 2 to 8 mbar to identify the ideal pressure conditions for transmission of the relevant ionic species. The traveling-wave velocity and wave height were altered in increments from 100 to 600 m/s and 8 to 20 V, respectively, and the conditions that provided the optimal mobility separation were used for all following experiments.

The synchronization of gated release of ions into the ion mobility separator with TOF acquisition allows arrival time distributions of ions to be obtained. For each gate pulse, 200 orthogonal acceleration pushes of the TOF analyzer are recorded to form one ion mobility experiment. The overall mobility recording time is $200 \times t_p$, where t_p is the pusher period [9]. The pusher period depends on the mass acquisition range; for these

experiments, a pusher period of 120 μ s was used, giving a mobility recording time of 24 ms.

Equine myoglobin at a concentration of 10 μ M in 50% aqueous acetonitrile containing 0.2% formic acid was used to provide data that were used to create a calibration curve for cross-sectional measurements.

Data obtained for each hemoglobin tetramer over the m/z range 3000–4500 were deconvoluted onto a true mass scale using maximum entropy modeling (MaxEnt for short) software to provide an estimate of molecular mass. Experiments were carried out in triplicate.

Calibration, Modeling, and Estimation of Cross Section

The equine myoglobin data were used to create a calibration curve for each set of experiments. Absolute cross sections for equine myoglobin were obtained from drift-time ion mobility mass spectrometry (DTIMS) studies (Prof. Michael T. Bowers, personal communication). The calibration was performed using a procedure developed in-house based on previously published work [13, 19, 32, 33]. In brief, normalized cross sections (corrected for charge and reduced mass) were plotted against corrected arrival times (corrected to exclude time spent outside the ion mobility cell) to create a calibration with a power-series fit. The calibration allows one to estimate the cross section of a molecule of interest provided that the mobilities (corrected arrival times) for that molecule lie within the mobilities observed for the calibrant used, irrespective of the size range of cross sections for the calibrant [34, 35]. The calibration was used to estimate rotationally averaged collision cross sections of hemoglobin monomer, dimer, and tetramer for the different charge states observed, based on their arrival time distributions, provided that their corrected arrival times fell along the calibration curve.

To compare the experimental cross sections for the normal and sickle hemoglobin tetramers with accepted values, cross sections were calculated using MOBCAL, a program to calculate mobilities [36, 37], from published X-ray structures held at the RCSB Protein Data Bank [38].

MOBCAL facilitates the use of three approximations to calculate cross sections. The projection approximation (PA) typically results in an underestimation of the cross section of a large ion. It calculates the cross section by averaging the projections produced by every orientation of a molecule and so does not take into account interactions with the buffer gas. The trajectory method (TM) takes into account all interactions, but is computationally intense. The exact hard-sphere scattering model (EHSS) carries out trajectory calculations, while ignoring long-range interactions, but nevertheless gives values within a few percent of the TM approximations [7, 39]. For this work, cross sections were calculated using

the PA and EHSS methods to reduce computational time.

Results and Discussion

Instrument Acquisition Parameters

Considerable optimization of instrument acquisition parameters is required for each individual application of ion mobility separation. This must be tailored to the sample of interest because optimal conditions are dependent on ionic species and mass-to-charge ratio [3]. Controlled optimization of instrument acquisition parameters indicated that a backing pressure of between 6.6 and 6.8 mbar was ideal for intact hemoglobin tetramer analysis. The optimal ion mobility separation of the tetramer was achieved at a traveling-wave velocity and wave height of 400 m/s and 18 V, respectively.

Calibration

A calibration curve was used to allow the estimation of cross sections for different constituents of hemoglobin in different charge states. Cross sections calculated for equine myoglobin were within 2% of absolute values obtained by DTIMS experiments. These results were reproducible across the three datasets acquired.

Hemoglobin Tetramer Analysis

Representative spectra for normal (HbA) and sickle (HbS) hemoglobin analyzed by means of ESI-TOF-MS under nondenaturing conditions are shown in Figures 1a and b, respectively. The data were deconvoluted to give masses of 64,454.7 Da for HbA and 64,395.8 Da for HbS, which were very close to the theoretical masses of 64,453.2 and 64,393.4 Da, respectively [29].

The hemoglobin spectra obtained show the presence of the tetramer $[(\alpha^h\beta^h)_2]$, heterodimer $(\alpha^h\beta^h)$, and apo- and holo-monomer species. The trimer is not seen, as would be expected, because the formation of the hemoglobin tetramer involves the noncovalent association of two $\alpha^h\beta^h$ -dimers. Carefully controlled near-physiological conditions were used in preparing the sample and the absence of any trimer implies that the species observed within the spectra exist naturally in solution. This is consistent with results from isotope labeling studies that showed that nontetrameric ions in the spectrum corresponded to species present in solution [40] rather than products of fragmentation formed during the ESI process [41].

Alpha and beta monomers are observed within the HbA spectrum in both apo- and holo- forms. In a previous study, Griffith and Kaltashov [26] suggested that an α^h monomer first becomes associated with an β^a monomer, to enable the β -chain to incorporate the heme group. This observation was based on the absence of β^h in the spectrum. A subsequent study by Boys and Konermann [31] detected very small quantities of heme-

Figure 1. Mass spectra of (a) normal (HbA) and (b) sickle (HbS) hemoglobin analyzed by ESI-TOF-MS under near-physiological conditions, labeled with charge states of tetramer (Q), heterodimer (D), and apo- and holo-monomers (superscripts “a” and “h” refer to apo- and holo-forms, respectively).

deficient dimer and found that both α - and β -monomers were capable of binding heme. The discrepancies observed are thought to be attributable to differences between the commercially prepared and freshly obtained samples used. In the work reported here, in which fresh blood samples were used, β^h was observed in multiply charged states.

It has been reported [42] that without the attachment of the heme group, α - and β -monomers adopt extensively unfolded conformations. Cross sections for various charge states of α - and β -monomers in both the apo- and holo- forms have been estimated and our observations suggest that the predominant conformations of α - and β -monomers in the gas phase are similar to each other and show little change in the absence or the presence of heme (Figure 2). The cross section of each of the molecules studied increases with an increase in charge, thought to be a result of the effects of Coulomb repulsion [16, 43].

The heme-deficient dimer observed in previous studies is not observed here. The existence of both apo- and holo- forms of α - and β -monomers, all of similar cross sections, does not support the need for a β^a to associate with α^h for the β -monomer to recruit heme. Analysis of commercially prepared human hemoglobin (data not shown) does show the presence of a heme-deficient dimer at a molecular mass 32 Da higher than expected; this is in agreement with previous work conducted by the Konermann group [31] on bovine hemoglobin, who attributed the additional mass to the occurrence of oxidative modifications in the commercial protein.

Figure 2. Average estimated cross sections for charge states of (a) apo- α and holo- α monomers and (b) apo- β and holo- β observed within three datasets.

Figure 3. Average estimated cross sections for holo- α , holo- β , heterodimer, and HbA tetramer, from three datasets.

We observe many more charge states of α^a than of β^a . The number of charges accepted on a protein is related to the number of exposed basic sites on the protein's surface. A more folded protein has fewer of its basic sites exposed than an unfolded conformation and thus cannot accept as many charges. This may suggest that the α -chain adopts more unfolded conformations in the gas phase than is possible for the β -chain but, alternatively, the absence of higher charge states of β^a in the spectra may be ascribed to the different desolvation behavior of α - and β -monomers. The α -chain ionizes preferentially over the β -chain because of its greater nonpolar character, thereby competing more effectively for charge [41].

By estimating the cross sections of monomer, dimer, and tetramer, a picture of the assembly process can be obtained (Figure 3). The $[M + 12H]^{12+}$ charge state of dimer has an estimated cross section of 3001 Å². The $[M + 6H]^{6+}$ charge states of α^h and β^h have estimated average cross sections of 1583 and 1488 Å², respectively. If these two globin monomers come together to form a dimer, one would expect that the cross section of that dimer would be approximately the addition of the cross sections of the two constituent parts and, indeed, that is the case here. One would further expect that the cross section observed would be slightly smaller than the sum of the monomer subunits because the contact area on both of the monomers would be compacted and contribute less to the overall cross section. The data are in agreement with this.

The average estimated collision cross sections for HbA and HbS, for four different charge states, are illustrated in Figure 4. The data indicate a difference in cross section between normal and sickle-cell hemoglobin and a variation in cross section with charge state.

For HbA and HbS for the charge states studied, the cross sections observed for HbS are somewhat larger than those of HbA. Secondary, tertiary, and quaternary structural considerations make it difficult to determine what the charge state of a molecule should be, theoretically, within a particular solvent at a particular pH. It is clear from previous work that the lowest charge states observed under near-physiological conditions are most

Figure 4. Estimated cross sections for HbA and HbS tetramers for three different charge states, showing averaged values from three datasets with corresponding errors.

representative of the native protein structure [7]. The $[M + 18H]^{18+}$ charge state, for HbA and HbS, is suspected to be representative of a tetrameric structure that is beginning to denature.

The reproducibility of the cross sections estimated for the $[M + 15H]^{15+}$, $[M + 16H]^{16+}$, and $[M + 17H]^{17+}$ charge states of HbA, between the three replicate datasets, is $\pm 1\%$, believed to be representative of the reproducibility capabilities of the experiment. The larger deviation in estimation of cross section for the same charge states for HbS, of $\pm 3\%$, may reflect the presence of a broader population of conformations of the HbS molecule of similar cross section.

The rotationally averaged cross sections for HbA and HbS calculated from X-ray crystallographic structures were 3313 and 3733 Å² for the PA and 4343 and 4775 Å² for the EHSS, respectively. Values estimated experimentally for the $[M + 15H]^{15+}$, $[M + 16H]^{16+}$ and $[M + 17H]^{17+}$ charge states of HbA and HbS fall between these two theoretical approximations and agree with the X-ray observation that HbS has a larger cross section than that of HbA. Gas-phase conformations, although illustrative of solution-phase structures under controlled conditions, have been shown previously to be smaller than those predicted by EHSS approximations [6]; a more compact conformation is thought to be adopted in the gas phase because increased intramolecular interactions cause polar side chains to collapse onto the protein's surface [14].

Conclusions

This work demonstrates the use of TWIMS to probe gas-phase conformations of three-dimensional protein structure and noncovalent complexes.

TWIMS has been successfully used to analyze hemoglobin tetramers. Cross sections calculated for intact hemoglobin tetramers are comparable to those estimated from published X-ray crystallography data and conformational differences are observed between the HbA and HbS molecules. Nontetrameric species observed, including apo- and holo- forms of α - and

β -monomers and $\alpha^h\beta^h$ -dimers, are naturally present in equilibrium in solution and are not products of fragmentation during the ESI process.

Both α - and β -monomers have cross sections similar to each other, suggesting that they maintain a similar fold in the gas phase. Apo- and holo- forms of the monomers also have similar cross sections, suggesting that α - and β -monomers can retain a folded structure in the absence and the presence of the heme group. Extensively disordered monomer structures are not observed.

A heme-deficient dimer has not been observed and the results do not suggest the requirement for association of β^a with α^h for the β -monomer to recruit heme. The results, obtained on fresh blood samples rather than commercially prepared samples, do not support the hypothesis that a heme-deficient dimer is an essential intermediate in the tetramer assembly process.

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