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Structural and Functional Characterization of Glutaraldehyde-Polymerized Bovine Hemoglobin and Its Isolated Fractions

Paul W. Buehler,† Robert A. Boykins,‡ Yiping Jia,† Scott Norris,‡ Darón I. Freedberg,‡ and Abdu I. Alayash*,†

Laboratory of Biochemistry and Vascular Biology, Division of Hematology, and Laboratory of Biophysics, Division of Bacterial, Parasitic and Allergenic Products, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), Bethesda, Maryland 20892

Glutaraldehyde-polymerized bovine hemoglobin (PolyHbBv, trade name Oxyglobin), is a non-site-specific modified hemoglobin-based oxygen-carrying solution, developed for use in veterinary medicine. PolyHbBv was fractionated into four distinct tetrameric and multiple polytetrameric forms ranging in molecular mass (87.2−502.3 kDa) using size exclusion chromatography (SEC) and verified by laser light scattering. We evaluated the structural modification occurring in the fractionated mixture of PolyHbBv and assessed the functionality and redox stability of each fraction in relation to the mixture as a whole. Intramolecular cross-linking evaluation as performed by MALDI-MS and SEC under dissociating conditions revealed no-site-specific tetramer stabilization within the fractions; Intermolecular cross-linking was highly correlated with lysine and histidine modification as determined by amino acid composition analysis. While native unmodified hemoglobin, HbBv, PolyHbBv, and PolyHbBv fractions (F1−F4) revealed significant methionine oxidation, modification, or both, the critical *β*Met55 located in the functionally plastic domains (α1−*β*1 interface) of HbBv was unaltered. Moreover, neither of the two *β*Cys93 located in the highly plastic α1−*β*2 interface were modified in PolyHbBv or in F1−F4. Our structural analysis also revealed that the reported loss in sensitivity to chloride in PolyHbBv could not be attributed to direct alteration of chloride ion binding amino acids. Structural modification imparted by glutaraldehyde resulted in nearly identical functional characteristics of PolyHbBv and its fractions with regard to oxygen equilibrium, ligand binding, and autoxidative kinetics.

Hemoglobin-based oxygen carriers (HBOCs) have been developed using chemical and or recombinant technologies to modify hemoglobin (Hb) with a focus on minimizing toxicity and optimizing oxygen delivery in critical care situations.[1] The so-called early-generation HBOCs are currently being evaluated clinically and typically utilize nonspecific aldehyde-based chemical modifying agents, which function to stabilize, conjugate, or polymerize the noncovalent Hb tetramer.[2,3] Nonspecific chemical modification of Hb with agents such as glutaraldehyde and oxidized sugars (raffinose and dextran) invariably generate heterogeneous mixtures of Hb tetramers, conjugates, and polymers.[4] The broad range of modification to Hb occurring under conditions of nonspecific chemistry raises concerns regarding the uniformity of physicochemical behavior of individual components as they may influence the toxicity and efficacy of the mixture as a whole. In general, human Hb of varying degrees of purity serves as the starting material for most HBOC solutions under clinical development. The efficient regulation of oxygen release from human Hb is controlled within red blood cells (RBCs) via interaction with 2,3-diphosphoglycerate (2,3-DPG);[5] thus, extracellular HBOCs prepared from human Hb are dependent on the nature of chemical modification which ultimately imparts oxygen affinity.

We have recently analyzed another HBOC, oxidized raffinose (O-raffinose) polymerized human HbA_0 (O-*R*-PolyHbA_0), and showed that a non-site-specific modifying agent (O-raffinose) created significant structural alterations to HbA_0 via cysteine modification and that proposed sites of amino acid modification involved in intra- and intermolecular cross-linking were essentially undetectable.[6] Moreover, O-raffinose-based modification induced functional and conformational changes including the stabilization of HbA_0 in the (T) state, lack of cooperativity, and a reduced Bohr effects. These changes may have contributed to abnormal protein function and toxicity.[7] In the current study, a commercially available HBOC approved for veterinary use, Hb glutamer-200 (Oxyglobin, Biopure, Cambridge, MA), was used as a model for the evaluation of another commonly used and nonspecific chemical modifier of proteins, glutaraldehyde.

* To whom correspondence should be addressed. Telephone: (301) 827-3813. Fax: (301) 435-4034. E-mail: alayash@cber.fda.gov.

† Laboratory of Biochemistry and Vascular Biology, Division of Hematology.

‡ Laboratory of Biophysics, Division of Bacterial, Parasitic and Allergenic Products.

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Bovine Hb (HbBv) is preferentially modulated by physiological chloride ion (Cl^-) concentration (100 mM) rather than 2,3-diphosphoglycerate (2,3-DPG).[8−10] However, in the absence of Cl^-, both human Hb and HbBv respond similarly to 2,3-DPG and demonstrate similar oxygen affinity.[11] Substitution of key amino acids within human Hb has been identified in the HbBv amino acid sequence and appears to be responsible for the preferential difference in Cl^--mediated oxygen regulation in HbBv.[9,12−15] In particular, the presence of N-terminal methionine residues in $\beta 1$ and $\beta 2$ globin chain sequence of HbBv replacing Val1 and His2 in human Hb is thought to have major structural influence over the preferential oxygen regulation of HbBv by Cl^- rather than 2,3-DPG.[8] This unique property of HbBv affords the potential for appropriate oxygen release to tissues while circulating outside of the RBC, thus generating interest in its use as an HBOC. The purpose of this study was to first evaluate the physiochemical behavior and structural characteristics of component fractions resulting from the nonspecific polymerization reaction of HbBv with glutaraldehyde. Moreover, previous data from our laboratory indicate a loss of Cl^- oxygen regulatory influence over glutaraldehyde-polymerized BvHb (PolyHbBv).[16] These results suggest either direct modification to one or more key Cl^- binding amino acids or indirect structural constraint of BvHb conferring inaccessibility of Cl^- to sites of binding. Thus, a second purpose of this study was to evaluate structural modifications to key amino acids imparted by glutaraldehyde involved in Cl^- binding.

EXPERIMENTAL SECTION

Hemoglobin Solutions. Highly purified HbBv was a kind gift from Biopure Inc., while PolyHbBv was purchased from Biopure Inc. The purification and preparation of HbBv and PolyHbBv are described in detail elsewhere.[17] DBBF-αα-cross-linked Hb (DBBF-αα-Hb) was a gift of the Walter Reed Army Institute of Research (WAIR). The cross-linking reagent, bis(3,5-dibromosalicyl) fumarate was used in this preparation and specifically links the two α subunits via Lys99 when reacted with deoxyHb. Details of the solutions purity, preparation, and characterization have been previously outlined[18] and are based on the chemistry described by Chatterjee et al.[19] The PolyHbBv mixture fractions were separated and collected via size exclusion chromatography. Each PolyHbBv fraction (F1−F4) was buffer exchanged with five volumes (10 mL each) of 50 mM potassium phosphate, pH 7.4 using 30-kDa cutoff centrifuge tubes (Centricon YM30, Millipore Corp., Bedford, MA). F1−F4 were then concentrated to approximately 5−6 g/dL and stored at −80 °C. Prior to use in experiments, the metHb concentration was reduced to <10% by addition of sodium dithionite (British Drug Houses, BDH) 50 mg/mL of Hb. Excess sodium dithionite was removed from reduced F1−F4 using Sephadex G-25 (Sigma-Aldrich Chemicals, St. Louis, MO) packed columns equilibrated with 50 mM potassium phosphate, pH 7.4.

Size Exclusion Chromatography. PolyHbBv was separated into fractions (F1−F4) on a Bio-Sil-TSK-250 (600 mm × 7.5 mm) size exclusion chromatography (SEC) column (Bio-Rad Laboratories Inc., Hercules, CA) attached to a Waters 626 pump and Waters 2487 dual-wavelength detector and controlled by a Waters 600s controller using Millenium32 software (Waters Corp., Milford, MA). The running buffer consisted of 0.1 M NaH_2PO_4, pH 6.5, pumped at a rate of 0.5 mL/min, and the absorbance was monitored at 214 and 280 nm. F1−F4 were collected on a Spectra/Chrom CF-1 fraction collector (Spectrum Laboratories Inc., Rancho Dominquez, CA). Additionally, HbBv, DBBF-αα-Hb, and PolyHbBv F4 (nonpolymerized) were evaluated using the Bio-Sil-TSK-250 (600 mm × 7.5 mm) column under dissociating nondenaturing conditions with a mobile phase consisting of 0.5 M $MgCl_2$ in 25 mM Tris·HCl, pH 6.8, run at 0.5 mL/min. Under these conditions, dissociation of non-cross-linked Hb tetramer into dimers consisting of $\alpha-\beta$ subunits can be detected; thus, dissociable Hb (~32 kDa) elutes from the SEC column at a later retention time than stabilized tetrameric Hb (~64 kDa).

Size Exclusion Chromatography−Multiangle Laser Light Scattering. Molecular size, polydispersity, and radius of gyration were determined for each Hb fraction by SEC coupled to multiangle laser light scattering (SEC-MALLS). SEC-MALLS data were acquired and analyzed using a Wyatt Technology Corp. Dawn-DSP with Astra version 4.73.04 software. Samples were injected in 100-μL volume and separated on a Shodex OHPAK SB-806HQ column (300 × 8 mm), eluted in 200 mM sodium chloride, pH 6.7, at 0.5 mL/min. The sample passed through a Dawn DSP laser photometer for MALLSand then an Optilab DSP interferometric refractometer for concentration determination. Both detectors operate at a wavelength of 633 nm. All SEC-MALLS measurements were made at ambient temperature.

Oxygen Equilibrium Binding Studies. O_2 equilibrium curves (OECs) of HbBv, PolyHbBv, and F1−F4 were obtained using a Hemox analyzer (TCS Scientific, New Hope, PA). This instrument measures the O_2 tension with a Clark O_2 electrode (model 5331 oxygen probe; Yellow Springs Instruments, Yellow Springs, OH) and simultaneously calculates the Hb saturation using a dual-wavelength photometer. The concentrations of Hb samples were between 60 and 75 μM (heme), and the temperature was maintained at 37 °C. To minimize Hb autoxidation, 4 μL of the Hayashi enzymatic reduction system (208 μM glucose 6-phosphate, 6.8 units/mL Glucose-6-phosphate dehydrogenase, 50 μM NADP+, 6.3 μg/mL ferredoxin, 8 milliunits/mL ferredoxin-NADP+ reductase, and 246 units/mL catalase)[20] was included in the final 4-mL Hemox buffer solution (135 mM NaCl, 5 mM KCl, and 30 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), pH

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7.4. Adair equations were used in the nonlinear least-squares procedure included in the Hemox analyzer software (P_{50} PLUS, version 1.2) to fit each OEC. O_2 equilibrium parameters, P_{50} (pO_2 at which Hb is half saturated), and n_{50} (Hill coefficient) were determined based on the complete OECs generated using Adair constants. This procedure allows for the accurate determination of O_2 binding parameters for modified Hbs that are not fully saturated at atmospheric O_2 partial pressure.[21,22]

Oxygen binding properties of each Hb and F1−F4 were carried out as a function of chloride ion [Cl^-] according to established methods.[23] The effect of Cl^- concentrations ranging from 0 to 750 mM NaCl on oxygen binding was evaluated in Hepes, pH 7.4, at 37 °C.

Rapid Kinetic Measurements. O_2 dissociation rates were measured by rapidly mixing either HbBv, PolyHbBv, or F1−F4 (30 μM heme) with 1.5-mg/mL sodium dithionite in an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer as previously described,[24] and deoxygenation was followed at 437.5 nm in 0.1 M Tris buffer, pH 7.4, at 25 °C. The kinetics of carbon monoxide association with deoxygenated forms of these Hbs and F1−F4 was measured in the stopped-flow apparatus, and the process was monitored at 437.5 nm in 50 mM Tris buffer, pH 7.4, at 25 °C after mixing Hb and CO solutions in the presence of 1.5 mg/mL sodium dithionite.[24]

Autoxidation Experiments. All Hb samples, including F1−F4, were converted to ferrous (Fe^{2+}) or oxyHb immediately prior to autoxidation experiments.[25] Experiments were carried out with 20 μM heme in sealed cuvettes with room air equilibrated 50 mM phosphate buffer at 37 °C. Absorbance changes in the range of 450−700 nm due to spontaneous oxidation of Hb were recorded in a temperature-controlled photodiode array spectrophotometer (Hewlet Packard 8453). Autoxidation reactions were followed to near completion (6 h), at which time, 22 μM potassium ferricyanide ($K_3Fe(CN)_6$) was added to completely oxidize the remaining Fe^{2+} heme. A multicomponent analysis was performed to calculate the oxy, met, and hemichrome species based on their individual extinction coefficients.[26] Autoxidation rates were obtained from plots of $HbFe^{2+}$ to metHb ($HbFe^{3+}$) versus time using nonlinear least-squares curve fitting (single-exponential, two-parameter decay) techniques using Sigma-Plot (SPSS, Chicago IL).

Acid Hydrolysis and Amino Acid Analysis. *5.7 N HCl Hydrolysis.* Approximately 100 μg of each aqueous soluble Hb was placed in glass vials and evaporated to dryness in a Savant vacuum centrifuge. Samples were hydrolyzed in 20 μL of 5.7 N HCl containing 0.2% phenol and flushed with nitrogen prior to heating at 150 °C for 90 min. Following 90 min of heating, glass vials were removed from the heating block, cooled, and evaporated to dryness. The hydrolysate was dissolved in 200 μL of 0.2 N sodium citrate buffer, pH 2.2, syringe filtered, and analyzed with a Hitachi amino acid analyzer (model L-8800). The yields of lysine, histidine, and tyrosine were calculated on the basis of their molar ratios relative to an amino acid standard normalized to alanine.[27] The yields of lysine, histidine, and tyrosine from acid hydrolysis of PolyHbBv and F1−F4 were compared to that of HbBv and theoretical values. Each Hb solution was hydrolyzed and analyzed a minimum of 6 times, and values of each analyses were averaged.

4 N Methanesulfonic Acid Hydrolysis. Determination of the oxidation of methionine as methionine sulfoxide in HbBv was performed as stated above using 4 N methansulfonic acid[28] in place of 5.7 N HCl. Each solution was drawn into 20-μL glass capillary tubes and heat sealed at both ends as described previously[27] to prevent oxidation during the hydrolysis procedure.

Performic Acid Oxidation. Oxidation with performic acid was carried out to accurately identify the number of modified cysteine and methionine residues as cysteic acid and methionine sulfone, respectively, in PolyHbBv and F1−F4 compared to HbBv and theoretical values. Performic acid was prepared by mixing 30% hydrogen peroxide with 88% formic acid in a 1:10 ratio (v/v), and the mixture was left for 1 h at room temperature. A volume of soluble Hb corresponding to ~100 μg of protein was dried using Savant vacuum centrifuge. A total of 100 μL of performic acid was added and allowed to react at 50 °C for 30 min, at which time the sample was dried and prepared for acid hydrolysis as described previously.[27] Amino acid analysis was performed on a Hitachi amino acid analyzer (L-8800), and the yields of cysteic acid and methionine sulfone were calculated on the basis of the molar ratio relative to an amino acid standard normalized to glutamic acid and aspartic acid.[27]

Hemoglobin Digestion. Hb samples (1 nM protein) were denatured with 6 M urea in 0.5 M ammonium bicarbonate buffer, reduced with 10 μL of 100 mM dithiothreitol at 50 °C for 60 min, and alkylated with 10 μL of 500 mM iodoacetamide for 30 min at room temperature. Enzymatic digestion was performed by adding trypsin (modified sequence grade, Roche Diagnostics, Indianapolis, IN) to 0.1 M ammonium bicarbonate diluted (5-fold), reduced, and alkylated Hb in a 1:1 nanomolar ratio. The reaction was performed at 37 °C over a 7-h period at which time digestion was quenched by adding 10 μL of glacial acetic acid. Samples were lyophilized, reconstituted with either 0.1% trifluoroacetic acid (TFA) or 1% acetic acid, and analyzed by MALDI-TOF and LC−MS/MS, respectively, as previously described.[29] Regions of protein modification analysis primarily focused on peptides known to contain amino acids responsible for the binding of Cl^- ions, which allosterically alter O_2 binding to HbBv.

MALDI-TOF Mass Spectrometry Analysis. Hb solutions were desalted using an analytical reversed-phase C18 HPLC column (4.6 × 250 mm, Vydac, Hesperia, CA) and a gradient generated with 0.1% TFA solution and acetonitrile (AcCN) in 0.1% TFA. AcCN was evaporated from the collected material, and samples were pipetted (1 μL) onto a stainless steel MALDI sample plate and mixed with 1 μL of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Sigma Chemical Co., St. Louis, MO) saturated in 50% AcCN/0.1% TFA acid. The sample/matrix were

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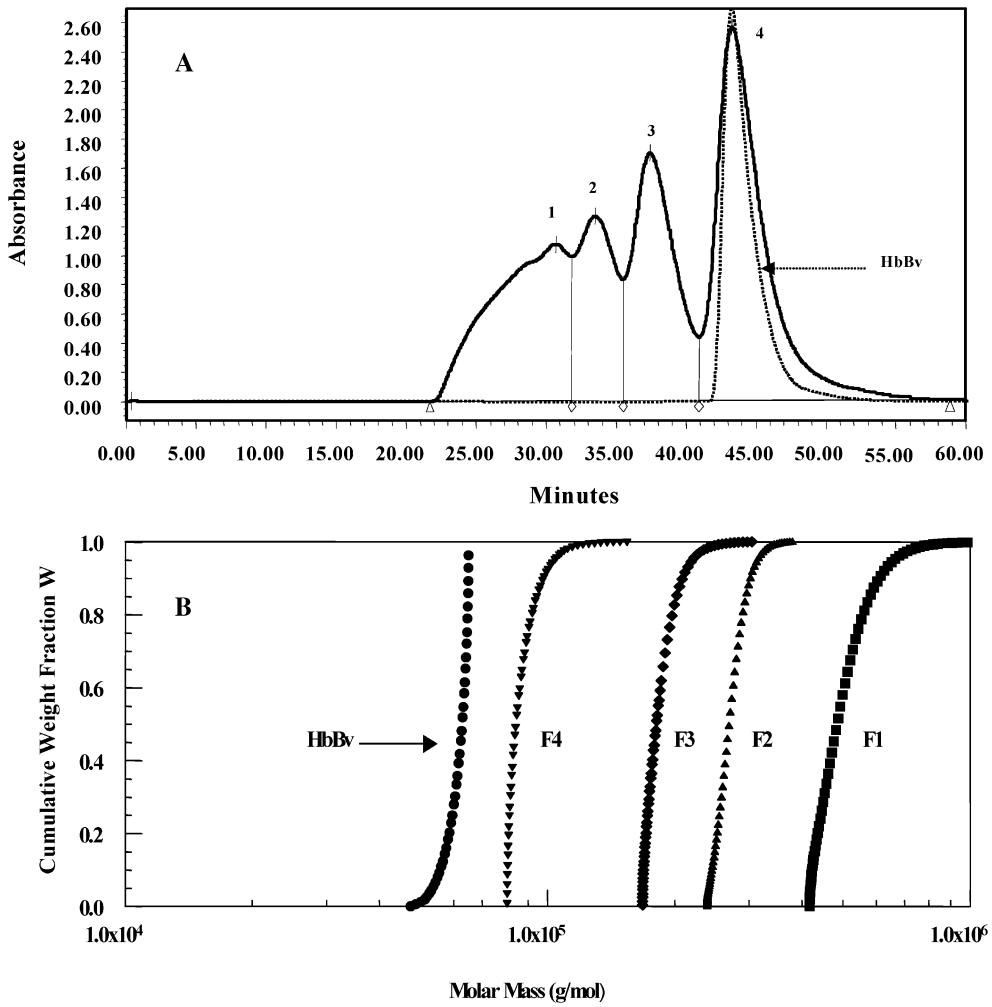

Figure 1. (A) Molecular size distribution of (—) PolyHbBv with fractions 1−4 performed on a Bio-Sil-TSK-250 (600 mm × 7.5 mm) size exclusion column with a running buffer consisting of 0.1 M sodium phosphate, pH 6.5, at a flow rate of 0.5 mL/min. Each fraction was collected from the peak of its elution to its trough; (···) represents the elution of HbBv. (B) Represents the molar mass distribution as determined by SEC-MALLS of HbBv and each collected PolyHbBv fraction (F1−F4) indicated as (●) HbBv, (■) F1, (▲) F2, (◆) F3, and (▼) F4.

air-dried and analyzed on a PerSeptive Biosystems DERP MALDI-TOF mass spectrometer using Data Explorer version 5.0 software (Applied Biosystems, Framingham, MA) operated in the linear mode. Trypsin digests (10 μL of sample) were desalted using C18 ZipTips according to instructions (Millipore Corp.) followed by evaporation to dryness. The desalted tryptic peptides were redissolved in 0.1% TFA and mixed as above with α-cyano-4-hydroxycinnamic acid (Sigma Chemical Co.) as the matrix for optimum ionization of peptides of <5000 kDa and sinapinic acid as the matrix for peptide fragments of >5000 kDa.

LC−ESI Tandem Mass Spectrometry Analysis. NanoLC−MS/MS analysis of Hb peptides was performed using a capillary HPLC coupled directly to a quadrupole ion trap (LCQ Deca) or a quadrupole-TOF (Qstar LCMS/MS) mass spectrometer (ThermoFinnigan, San Jose, CA or Applied Biosystems, Foster City, CA). Peptide digests were dissolved in 0.01% TFA/0.05% acetic acid prior to column injection. A gradient from 0 to 60% (0.01% TFA/0.05% acetic acid and 0.01% TFA/0.05% acetic acid in AcCN) was used for peptide separation using a C18 column (15 cm × 75 nm, Vydac). The ion trap mass spectrometer was optimized with the nanosource with a spray voltage of 2.5 kV and a heated capillary temperature of 180 °C. Ions were isolated with a mass isolation width (m/z) set at 2.0; AGC mode on; activation Q set at 0.25; activation time, 30 ms; and normalized collision energy set to 35% with a default charge set at +2. Scans were generated in both MS/MS and zoom scan mode of analysis (LCQ Deca). The Qstar mass spectrometer was optimized using the nanosource with positive TOF-MS plus three product ion experiments. Information-dependent acquisition (i.e., number of product ion scans) switch criteria was established for ions of >300 m/z and for ions of <700 m/z with a charge state 2−3. All remaining mass spectrometer settings were optimized for the peptide MS/MS experiment utilizing the ABI nanospray ion source. The tandem mass spectra for all peptide analyses were analyzed using both Sequest (ThermoFinnigan) and MASCOT (Matrix Science) and corroborated manually for sequence assignment.

Statistical Analysis. For rapid kinetic experiments, comparisons between HbBv and PolyHbBv were performed by a Student paired t-test followed by an independent comparison between PolyHbBv and F1−F4 using both ANOVA and an a priori test. The same comparisons were performed for autoxidation experiments; in all analyses, $p < 0.05$ was taken as the level of statistical significance. Statistical analyses were performed using SPSS software (Chicago, IL).

Table 1. Hemoglobin Fraction Molecular Size Characterization

Hb sample	M_n (kDa)	M_w (kDa)	M_w/M_n	R_z (nm)
HbBv	60.9	61.2	1.005	24
PolyHbBv (F1)	491.5	502.3	1.022	12
PolyHbBv (F2)	271.3	272.4	1.007	14
PolyHbBv (F3)	184.4	185.9	1.008	16
PolyHbBv (F4)	86.5	87.2	1.008	19

RESULTS AND DISCUSSION

Fractionation and Characterization by SEC and SEC-MALLS. Figure 1A depicts the SEC separation of PolyHbBv and HbBv, including PolyHbBv fractions F1−F4 collected. HbBv eluted from the SEC column at a retention time nearly identical to that of F4, demonstrating the existence of a predominantly nonpolymerized species within the PolyHbBv mixture (37.2%). F1, F2, and F3 represent 25.7, 14.8, and 22.3% of the mixture, respectively. Next, we evaluated each fraction by SEC-MALLS to provide definitive analytical data regarding the molecular weight and homogeneity of each collected fraction.

Data from SEC-MALLS of F1−F4 are depicted in Figure 1B and Table 1, indicating a wide molar mass range. The largest molar mass component, F1 demonstrated a number (M_n) and weight (M_w) average molar mass of 491.5 and 502.3 kDa, respectively, while the smallest molar mass component, F4 demonstrated an M_n and M_w of 86.5 and 87.2 kDa, respectively. Polydispersity (M_w/M_n) values indicate homogeneity within each fraction being comparable to that of highly purified HbBv.

Evaluation of Hemoglobin Oxygen Binding Characteristics. *Oxygen Equilibrium Studies.* The OECs for HbBv, PolyHbBv, and F1−F4 are depicted in Figure 2 with the inset showing the similarity in the P_{50} values of F1−F4 relative to PolyHbBv. Disparity between PolyHbBv and F1−F4 with regard to OEC shape and P_{50} is clearly demonstrated relative to HbBv. Mean ± standard error of the mean (SEM) P_{50} and n (Hill coefficient for oxygen binding) values for PolyHbBv fractions ranged from 33.3 ± 0.4 mmHg and 1.32 ± 0.02 F2 to 36.2 ± 1.0 mmHg and 1.4 ± 0.02 F4, respectively. The mean ± SEM P_{50} and n values for F1−F4 were 35.1 ± 0.4 mmHg and 1.4 ± 0.03, respectively. Mean ± SEM, F1−F4 were comparable to the P_{50} (38.4 ± 0.5 mmHg) and n (1.4 ± 0.02) of PolyHbBv and previously reported PolyHbBv P_{50} and n values.[16] Both nonfractionated and fractionated PolyHbBv demonstrated reduced oxygen affinity (higher P_{50}) and reduced cooperativity (lower n) compared to HbBv, which demonstrated a P_{50} of 27.2 ± 0.33 mmHg and an n of 2.1 ± 0.04. These values were also comparable to previously reported P_{50} and n values for HbBv.[16]

Figure 3 shows the influence of Cl^- on the P_{50} and n of HbBv, PolyHbBv, and F1−F4. As can be seen, the oxygen affinity of HbBv is strongly influenced by [Cl^-] over a range from 0 to 750 mM with the positive slope ($\Delta\log P_{50}/\Delta\log$ [Cl^-]) indicating a net release of Cl^- from the protein. Over the evaluated range of [Cl^-], oxygen affinity is reduced 2-fold (i.e., P_{50} is increased from 21.7 mmHg at a [Cl^-] = 0 mM to 41.0 mmHg at a [Cl^-] = 750 mM) in agreement with earlier reports.[16] In contrast to the reactivity of HbBv in the presence of varying [Cl^-], PolyHbBv and F1−F4 exhibit no change in oxygen affinity as [Cl^-] increases. These data suggest that amino acids involved in Cl^- binding

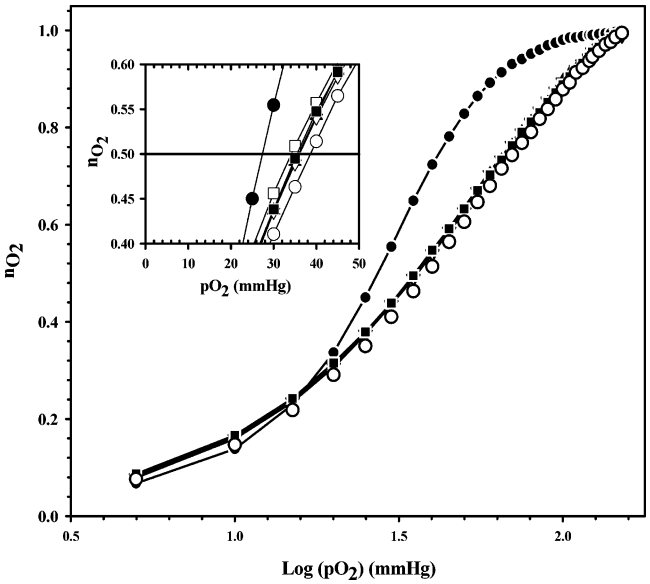

Figure 2. Oxygen equilibrium curves for (●) HbBv, (○) PolyHbBv, (■) PolyHbBv (F1), (□) PolyHbBv (F2), (▽) PolyHbBv (F3), and (▲) PolyHbBv (F4). The results are expressed as fractional oxygen saturation (nO_2) versus log of the partial pressure of oxygen (pO_2). The inset indicates the partial pressure of oxygen at which each Hb solution is half-saturated with oxygen (P_{50}). Oxygen equilibrium curves were determined by the Hemox analyzer using 20 μM heme in 0.01 M phosphate buffer containing 0.1 M NaCl (pH 7.4), antifoaming agent and Hayashi reduction system reagents[20] at 37 °C. Values are representative of the mean of three separate experiments with the SEM being less than 3% for all representations.

(αVal1, αLys99, βMet1, βLys81, βLys103) within HbBv may have been directly or indirectly modified by glutaraldehyde. A primary benefit supporting the use of modified HbBv as a blood substitute is the sensitivity of HbBv reactivity to Cl^-. Normal blood [Cl^-] in humans is narrowly maintained in a range between 100 and 106 mM. The conformational change from oxyHbBv (R state) to deoxyHbBv (T state) and in reverse is in part translated by a narrow tilt in [Cl^-] toward one extreme, thus favoring Cl^- binding and oxygen release in the peripheral tissues, where [Cl^-] is elevated, and conversely Cl^- release and oxygen binding in the lungs, where [Cl^-] is reduced, with the overall result being a net shift in the OEC toward the right in the periphery or toward the left in the lungs.[31] Our data indicate that the influence of Cl^- on PolyHbBv and F1−F4 is essentially lost in a comparable level compared to HbBv; moreover, F1−F4 appear to contribute equally to the overall effect demonstrated by PolyHbBv.

Rapid Kinetic Studies. The kinetics of ligand binding and dissociation were evaluated for HbBv, PolyHbBv, and F1−F4. The dissociation kinetics of oxygen from HbBv was monophasic, whereas the reaction time course for PolyHbBv and PolyHbBv fractions were biphasic. Figure 4A shows the nonlinear least-squares curve fitting of HbBv, PolyHbBv, and F1−F4 and the regression analysis of curve fitting. Figure 4B illustrates the off rate (k_{off}, s^{-1}) of HbBv and the phases in PolyHbBv and F1−F4. A single-exponential process with a $k_{off} = 33.5 \pm 0.1\ s^{-1}$ describes

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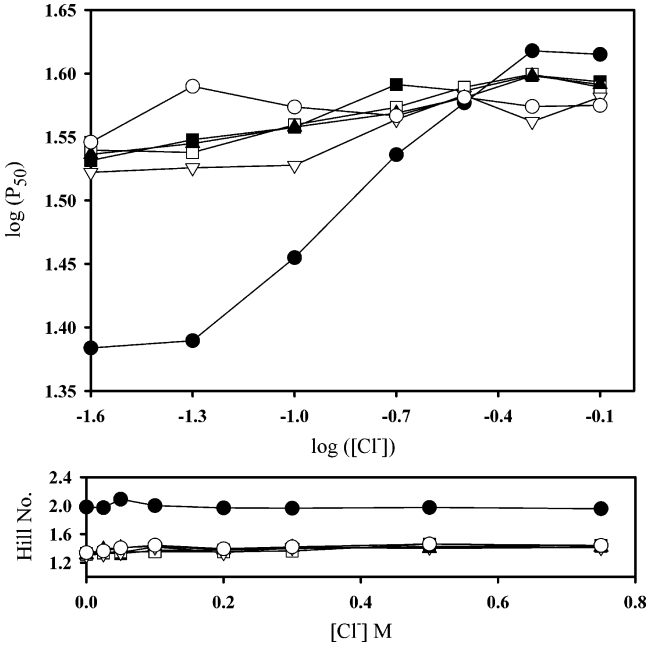

Figure 3. Dependence of P_{50} of (●) HbBv, (○) PolyHbBv, (■) PolyHbBv (F1), (□) PolyHbBv (F2), (▽) PolyHbBv (F3), and (▲) PolyHbBv (F4) on Cl^- ion concentration where each experiment was carried out in 0.1 M Hepes buffer, pH 7.4, at 37 °C under differing Cl^- concentration as indicated. The lower panel shows the plot of the Hill number (n) for each Hb solution as a function of Cl^-. Values are representative of the mean of three separate experiments with the SEM being less than 3% for all representations.

the oxygen dissociation from fully oxygenated HbBv. The fast phase of oxygen dissociation from PolyHbBv comprises ~75% of the reaction ($k_{off} = 60.0 \pm 3.2\ s^{-1}$) and was found to be significantly faster than HbBv ($p < 0.05$). The heterogeneity of kinetics displayed by PolyHbBv may be due to the presence of both faster and slower reacting species in the polymeric mixture. This phenomenon, based on earlier observations, was shown to occur with glutaraldehyde polymerized human Hb.[31] Also shown in Figure 4B are the k_{off} values for F1−F4, which were 63.3 ± 1.2, 57.2 ± 1.0, 63.7 ± 1.4, and 62.8 ± 2.5 s^{-1}, respectively (F1−F4 mean ± SEM = 61.8 ± 1.6 s^{-1}), indicating no difference ($p > 0.05$) between oxygen dissociation behaviors of fractions contained within the PolyHbBv heterogeneous mixture and PolyHbBv itself.

Deoxygenated HbBv, PolyHbBv, and F1−F4 were mixed with CO in the stopped-flow apparatus. Figure 5A shows the nonlinear least-squares fit to the exponential decay and the regression analysis of curve fitting. Figure 5B depicts the second-order rate constants of CO binding (k_{on}, $\mu M^{-1}\ s^{-1}$). The second-order rate for PolyHbBv (0.15 ± 0.01 $\mu M^{-1}\ s^{-1}$) is significantly different from HbBv (0.22 ± 0.02 $\mu M^{-1}\ s^{-1}$) ($p < 0.05$) and similar to previously reported values.[14] The individual k_{on} values for F1−F4 were 0.19 ± 0.01, 0.18 ± 0.01, 0.20 ± 0.02, and 0.19 ± 0.02 $\mu M^{-1}\ s^{-1}$, respectively (mean ± SEM = 0.19 ± 0.02 $\mu M^{-1}\ s^{-1}$), indicating no difference ($p > 0.05$) between CO binding kinetics of fractions contained within the PolyHbBv heterogeneous mixture and PolyHbBv itself. Larger off rates and smaller on rates are expected when native Hb is chemically modified as these characteristics in general represent a desirable property of an oxygen carrier. Unexpectedly, each fraction of the heterogeneous mixture displayed no differences between each other and the mixture itself.

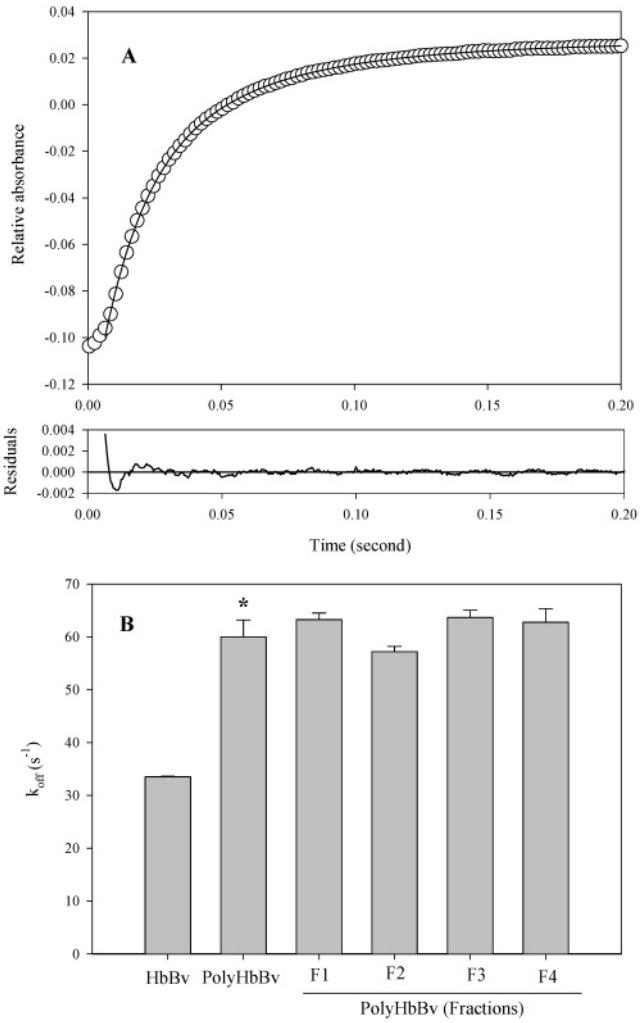

Figure 4. Stopped-flow kinetics of oxygen dissociation from oxyHb. (A) The kinetics of oxygen dissociating from HbBv and PolyHbBv and its fractions were measured in an Applied Photophysics stopped-flow at 25 °C in 0.1 M Tris buffer, pH 7.4. The typical kinetic trace shows the time-dependent increase in absorbance at wavelength of 437.5 nm. Several measurements (between 3 and 6) were carried out by mixing 15 μM Hb and 0.75 mg/mL sodium dithionite (final concentrations). The solid line is the nonlinear least-squares fit to the exponential increase expression. The lower graph of residuals shows the regression analysis of the curve fitting. (B) The bar graph shows the comparison of the off rate of HbBv and the fast rates of PolyHbBv and its fractions. * indicates a significant difference between HbBv and PolyHbBv.

Autoxidation. Figure 6A (PolyHbBv) and Figure 6B (PolyHbBv (F2)) represent a spectrophotometric comparison of spontaneous $HbFe^{2+} \rightarrow HbFe^{3+}$ from 0 to 6 h. Rates of autoxidation (k_{ox}, h^{-1}) were obtained from plots of $HbFe^{2+}$ spontaneous decay to $HbFe^{3+}$ versus time using nonlinear least-squares curve fitting (single-exponential, two-parameter decay) techniques. Reactions were followed to near completion (6 h), at which time 22 μM $K_3Fe(CN)_6$ was added to completely oxidize remaining Fe^{2+} heme. PolyHbBv demonstrated a mean ± SEM k_{ox} of 0.47 ± 0.01 h^{-1}, ~1.3 times that of HbBv (0.36 ± 0.01 h^{-1}). The k_{ox} rates for PolyHbBv (F1−F4) were 0.47 ± 0.02, 0.56 ± 0.01, 0.46 ± 0.01, and 0.40 ± 0.02 h^{-1} (mean ± SEM = 0.47 ± 0.02), respectively.

Analysis of Amino Acid Modification. Modification to amino acids likely to be either involved in Hb polymerization, surface

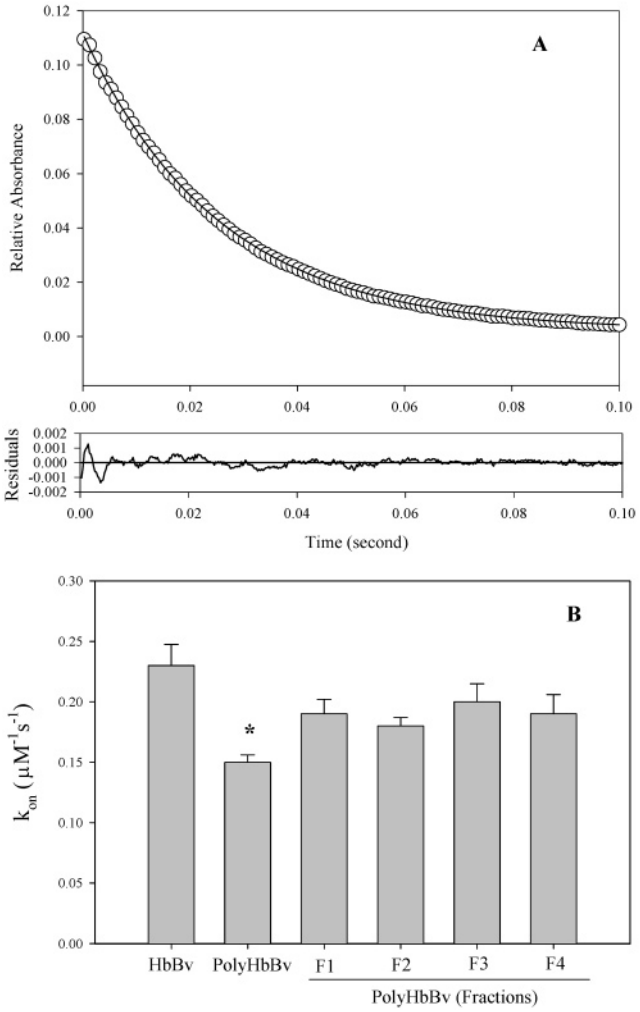

Figure 5. Kinetics of CO binding of HbBv and PolyHbBv fractions. (A) The rapid kinetics of CO binding (k_{on}) with Hb (10 μM) were measured in the stopped-flow with increasing concentrations of CO (0−500 μM) in 50 mM Tris buffer, pH 7.4, 100 mM NaCl at 25 °C. The typical kinetic trace shows the time-dependent decrease measured at 437.5 nm with 10 μM Hb and 250 μM CO (final concentrations). The solid line is the nonlinear least-squares fit to the exponential decay equation. The lower graph of residuals shows the regression analysis of the curve fitting. (B) The bar graph shows the second-order rate constants of CO binding of HbBv. PolyHbBv, and fractions. * indicates a significant difference between HbBv and PolyHbBv.

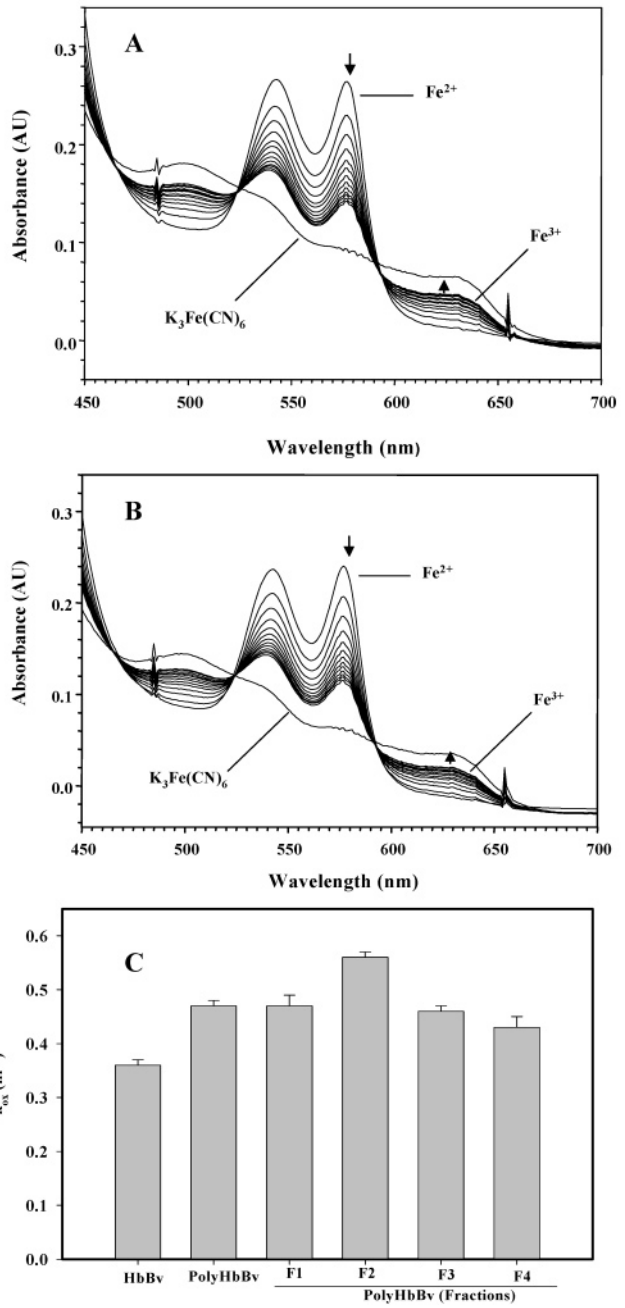

Figure 6. Rates of autoxidation (spontaneous conversion of $HbFe^{2+} \rightarrow HbFe^{3+}$) determined for each Hb solution over a 6-h period in a room air equilibrated cuvette in rapid scanning diode array spectrophotometer at 37 °C. (A, B) Represents the spectral changes recorded during the autoxidation of PolyHbBv and PolyHbBv (F2), respectively. Absorbance spectra are shown for several time points between 0 and 360 min. $K_3Fe(CN)_6$ was added to complete the transition of $HbFe^{2+} \rightarrow HbFe^{3+}$. (C) Rates of autoxidation (k_{ox}, h^{-1}) for HbBv, PolyHbBv, and PolyHbBv (F1−F4) were obtained from the single-exponential, two-parameter decay of the $HbFe^{2+} \rightarrow HbFe^{3+}$ conversion versus time curve.

modification, or both by glutaraldehyde was evaluated. The ϵ-NH_2, heterocyclic NH, OH, and SH functional groups of lysine, histidine, tyrosine, cysteine, and methionine, respectively, have been reported as primary sites of reaction with the two COH functions of glutaraldehyde.[32] Table 2 lists the average number of amino acids from 10 separate analyses on each Hb solution recovered following 5.7 N HCl hydrolysis (lysine, histidine, tyrosine), performic acid oxidation/5.7 N HCl hydrolysis (cysteine as cysteic acid and methionine as methionine sulfone), and 4 N methanesulfonic acid hydrolysis (methionine as methionine sulfoxide). Amino acid recovery for HbBv was identical to theoretical values for HbBv, while the percent recovery from PolyHbBv for listed amino acids was 70%. For F1−F4, recoveries were 63, 65, 68, and 70%, respectively. As demonstrated in Figure 7A, MW correlated ($r^2 = 0.869$) with the sum of modified (i.e., unrecoverable) lysine and histidine residues in F1−F4. Similarly, Figure 7B demonstrates the linear relationship ($r^2 = 0.884$) between F1−F4 MW and modified methionine (as the sulfone) residues. Moreover, oxidation of ~75% of the methionine residues, recoverable as methionine (sulfoxide) from HbBv and depicted in the full scan

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Table 2. Amino Acid Recovery Following Acid Hydrolysis

	theory	experimental					
				PolyHbBv (fractions)			
amino acid	HbBv	HbBv	PolyHbBv	(F1)	(F2)	(F3)	(F4)
lysine	44	43.5 (98.9)	36.5 (82.9)	28.5 (64.8)	30.8 (70.0)	31.5 (71.5)	36.0 (81.8)
histidine	34	37.3 (110)	24.0 (70.5)	25.8 (75.9)	24.8 (72.9)	27.6 (81.1)	25.9 (76.0)
tyrosine	10	9.00 (90.0)	9.00 (90.0)	9.00 (90.0)	9.50 (95.0)	8.40 (84.0)	7.00 (70.0)
cysteine (cysteic acid)	2	1.80 (90.0)	2.00 (100)	1.90 (95.0)	1.80 (90.0)	2.00 (100)	2.10 (105)
methionine (sulfone)		7.73	4.77	2.47	3.66	4.00	5.33
methionine	8	2.00 (25.0)					
methionine (sulfoxide)		5.43					

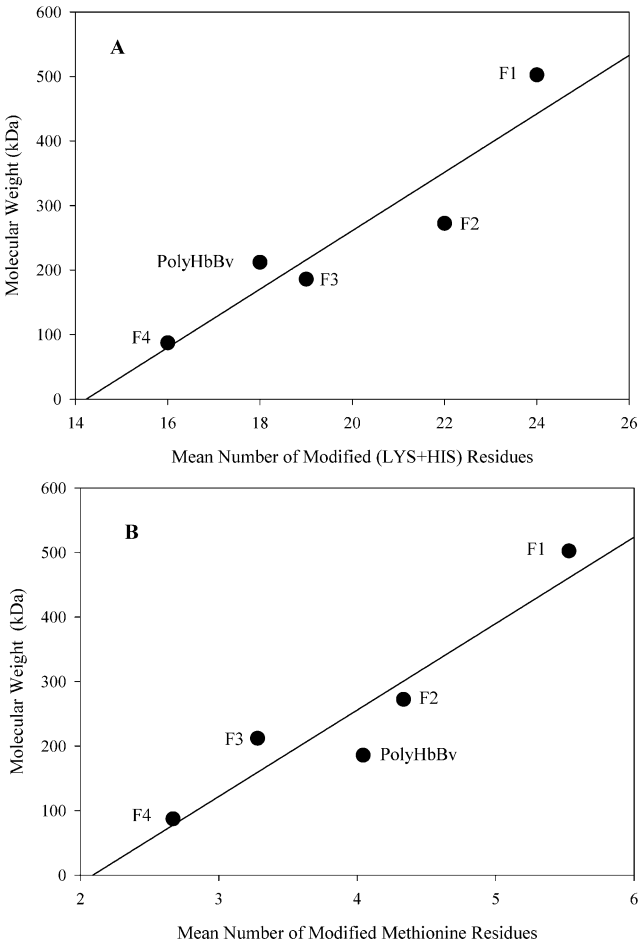

Figure 7. Molecular weight distribution versus modified amino acids in PolyHbBv and PolyHbBv (F1−F4) from values of M_w obtained by SEC-MALLS and amino acid content obtained from acid hydrolysis, respectively. (A) Relationship of modified PolyHbBv fraction MW and extent of modification to lysine + histidine residues within each fraction. A linear relationship with a $r^2 = 0.869$ with PolyHbBv included and $r^2 = 0.980$ without PBv included in the regression analysis was determined. (B) Relationship of modified PolyHbBv fraction MW and extent of modification to methionine residues. A linear relationship with a $r^2 = 0.884$ with PolyHbBv included and $r^2 = 0.942$ without PolyHbBv included in the regression analysis was determined.

MS spectra of tryptic peptides in Figure 8 (A,B) demonstrates extensive oxidation of this amino acid in HbBv prior to reaction with glutaraldehyde. Previous data suggest that oxidation of β55Met located within the $\alpha_1\beta_1$ interface results in T-state (deoxyHb) destabilization.[33] β55Met lies within the tryptic peptide $\beta_{40}-\beta_{58}$ (F_{40}FESFGDLSTADAVM$_{55}$NNPK$_{58}$) with the $[MH]^+$ ion observed at $m/z = 2091.30$. This particular peptide was identifiable and contained the only nonoxidized methionine in HbBv PolyHbBv, and F1−F4. Also of interest is a lack of modification to either of the cysteine residues located at the β-globin chains' 93 positions; interestingly, this key amino acid located in the $\alpha_1\beta_2$ interface plays a highly important role in regulating conformational plasticity of Hb. Taken together, it appears from our data that modification to key amino acids (i.e., β55Met and β93Cys) did not occur in PolyHbBv or F1−F4.

Analysis of Intra- and Intermolecular Cross-Linking. *Intramolecular Cross-Linking*. The MALDI mass spectra of HbBv and F4 (i.e., nonpolymerized fraction) are depicted in Figure 9A and B, respectively. In Figure 9A, the ion observed at $m/z = 15\,076.62$ [M + H] corresponds to the MW of the α-globin chains (theoretical $m/z = 15\,074$) and the ion observed at $m/z = 15\,993.68$ [M + H] corresponds to the MW of β-globin chains (theoretical $m/z = 15\,995$) in HbBv. Glutaraldehyde typically functions as a protein surface polymerizing agent (i.e., external to the central cavity), and to date, no reports in the literature suggest the utility of glutaraldehyde as an intramolecular cross-linking agent (i.e., between globin chains in the central cavity). The MALDI mass spectra in Figure 9B suggest that numerous nonspecific modifications to both α- and β-globin chains in F4 have occurred as a result of reaction with glutaraldehyde. Additionally, the MALDI mass spectra suggest that cross-linking within the central cavity has occurred to some extent based on the presence of a mass species ($m/z > 30\,000$). To further evaluate these findings, we compared a known intramolecularly cross-linked Hb (DBBF-αα-Hb) with F4 and HbBv using SEC under dissociating nondenaturing conditions (mobile phase, 0.5 M $MgCl_2$ in 25 mM Tris HCl, pH 6.8). These conditions allow for dissociation of non-cross-linked Hb tetramers into dimers (MW ~32 000) consisting of $\alpha-\beta$ subunits. In Figure 10, DBBF-αα-Hb is superimposed with F4, while a later column elution time for HbBv is observed indicating Hb tetramer (64 kDa) stabilization and the presence of intramolecular cross-linking in F4. These data substantiate the ability of glutaraldehyde to induce chemical modification within the central cavity.

Intermolecular Cross-Linking (Polymerization). The extent of polymerization as determined by MALDI-MS is shown for F1−F4 in Figure 11A−D, which corresponds to F1−F4, respectively. Given the large molecular mass range (87 kDa (F4)−502 kDa

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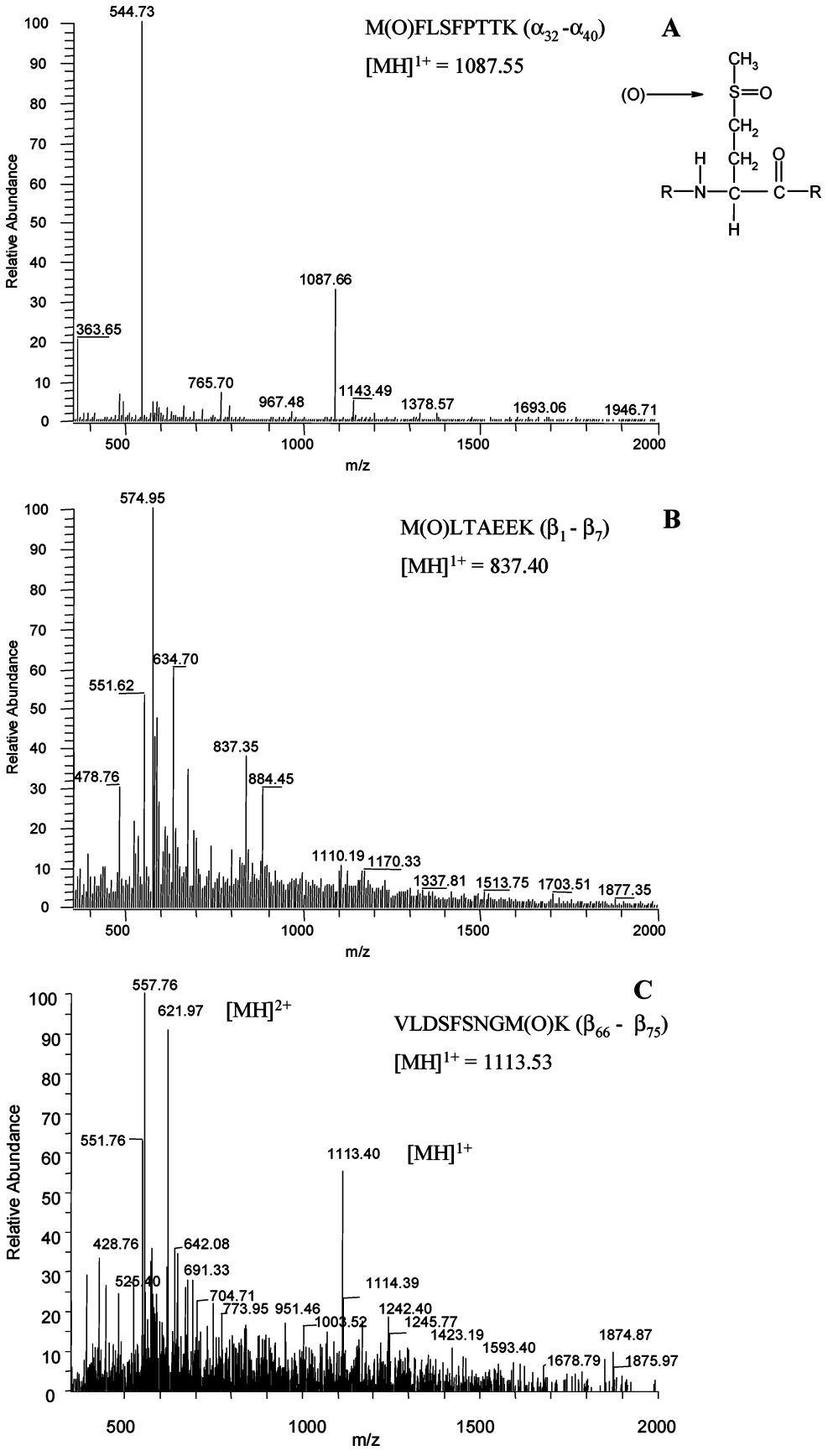

Figure 8. LC−MS of trypsin-digested peptides from HbBv. Three of the four methionine-containing peptides were identified and were consistent with the observed mononisotopic mass of peptide plus the addition of oxygen (O) resulting in methionine sulfoxide peptide (shown as the structure inset in A). (A, B) Represents the peptides containing αMet32 (O), βMet1(O), and βMet74(O), respectively.

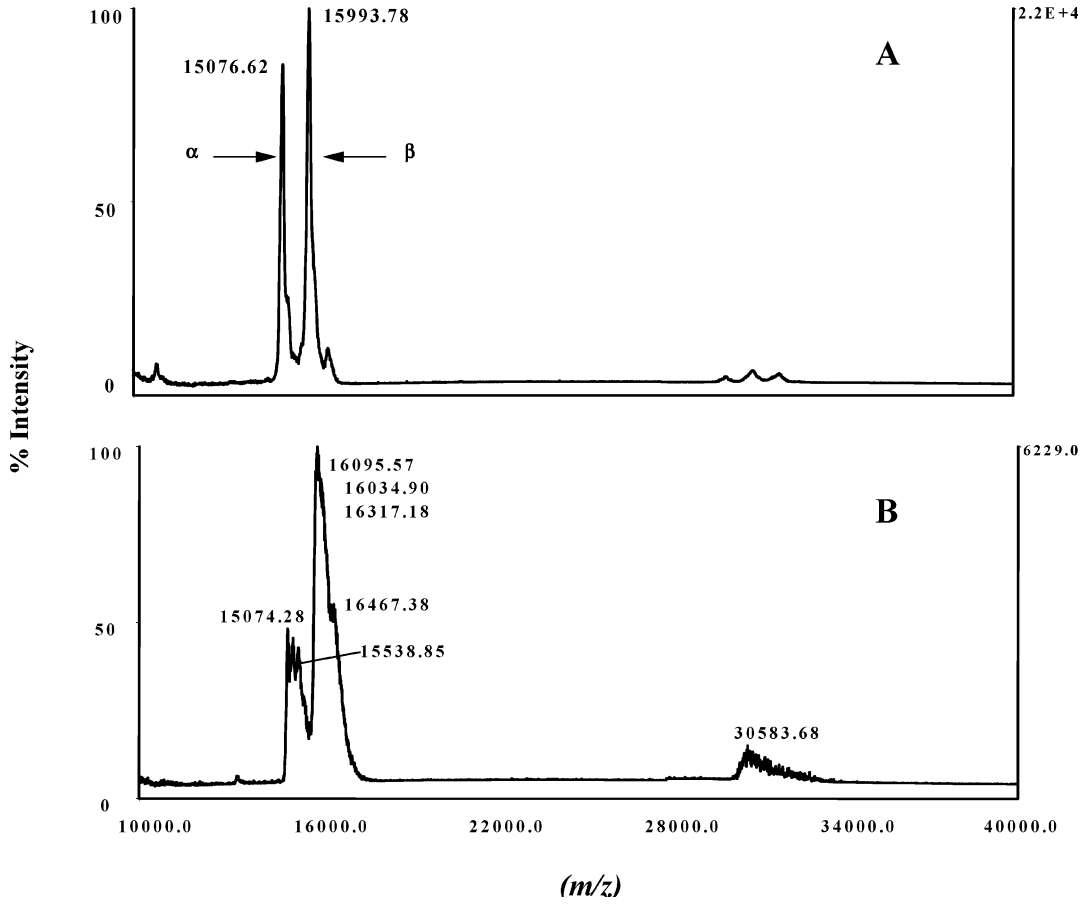

Figure 9. MALDI-MS of (A) HbBv and (B) PolyHbBv (F4) over a *m*/*z* range from 10 000 to 40 000. Mass assignments in (A) are HbBv α chain 15 076.62 [M + H] (theoretical 15 074) and HbBv β chain 15 993.78 [M + H] (theoretical 15 995). (B) PolyHbBv (F4) represents the nonpolymerized fraction in PolyHbBv with the MALDI-MS spectra demonstrating extensive surface modification to α and β chains. The *m*/*z* of 30 583.68 [M + H] indicates the presence of cross-linking between globin chain within PolyHbBv (F4).

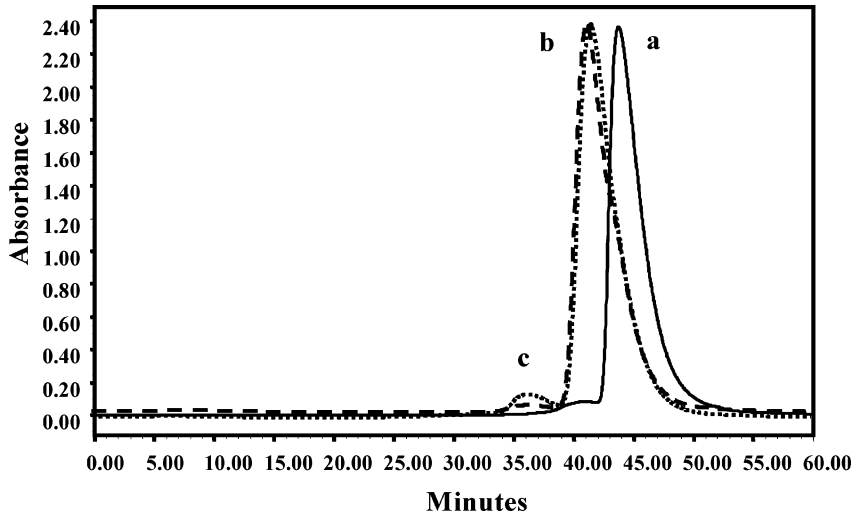

Figure 10. Molecular size distribution of (—) HbBv, (···) PolyHbBv (F4), and (- - -) DBBF-αα-Hb obtained via SEC under dissociating conditions (0.5 M $MgCl_2$ in Tris-HCl, pH 6.8). DBBF-αα-Hb is known to be intramolecularly cross-linked between $\alpha_1-\alpha_2$ chains at the $\alpha_1\alpha_2$ Lys99 positions and thus cannot dissociate; (a) represents the $\alpha-\beta$ dimers (~32 kDa), (b) represents stabilized intact tetratmer (~64 kDa), and (c) represents di-tetramer (~128 kDa).

(F1)) determined by SEC-MALLS, we expected to observe high-mass ranges using mass spectrometry. However, no ions were observed in the *m*/*z* range extending beyond 100 000 kDa, and with the exception of PolyHbBv (F4), PolyHbBv (F1−F3) demonstrated nearly identical spectra in the *m*/*z* range from 10 000 to 100 000 kDa. These data further indicate the nonspecificity of glutaraldehyde in the polymerization processes and suggest that a multitude of differing sites of polymerization occur between HbBv molecules, thus creating numerous species of similar mass within each fraction, yet no single modification results in sufficient

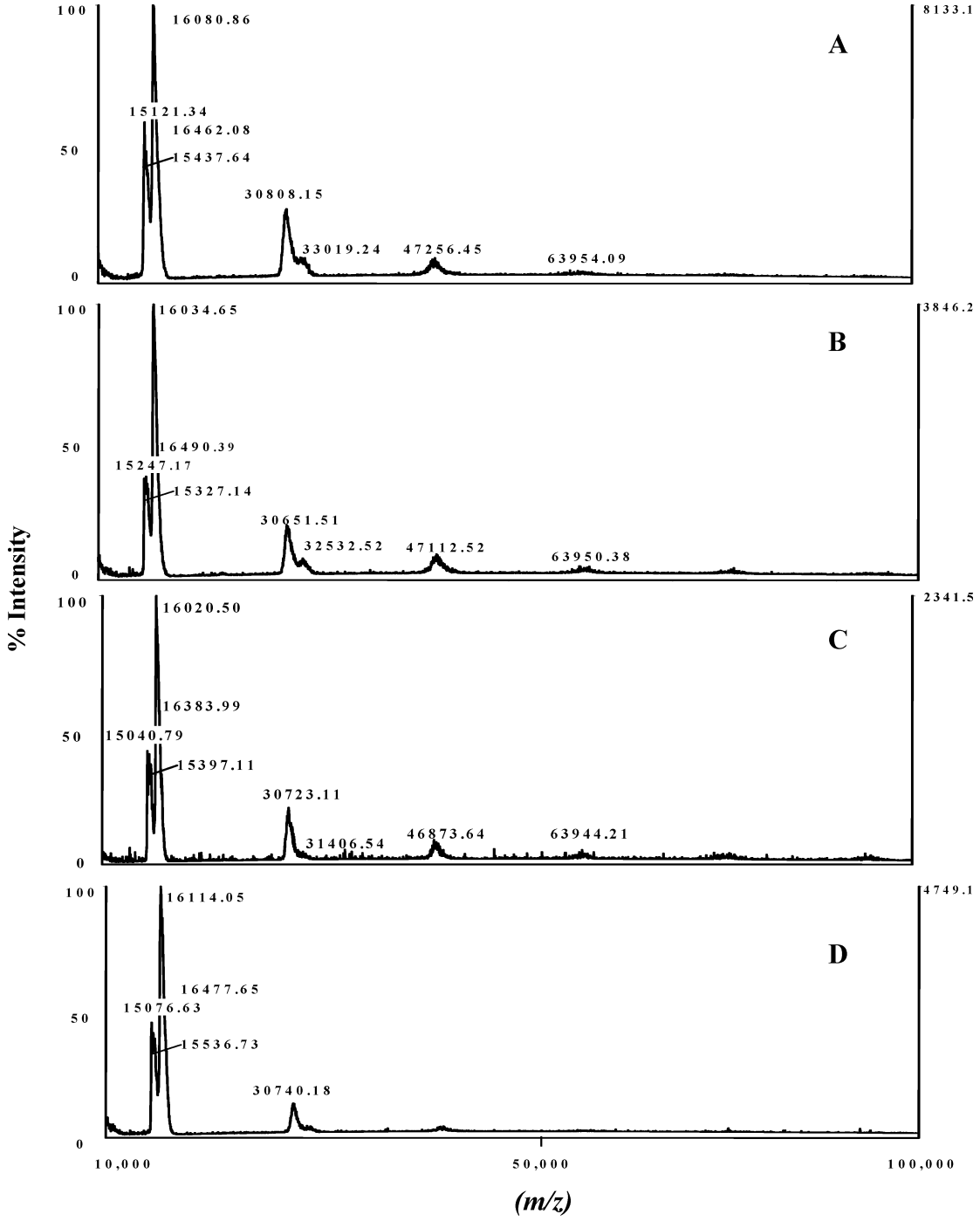

Figure 11. MALDI-MS of (A) PolyHbBv (F1) (largest MW), (B) PolyHbBv (F2), (C) PolyHbBv (F3), and (D) PolyHbBv (F4) (smallest MW) fraction over an *m*/*z* range of 10 000−100 000. MALDI-MS dissociates noncovalently associated globin chains; thus mass ions indicated in (A−C) likely represent singly charged species of dissociated multitetrameric species, while these ions are not observed in (D) F4 (nonpolymeric fraction).

abundance to be detected by MALDI-MS. Additionally, attempts to analyze molecular shape (radius of gyration) by SEC-MALLS support a similar conclusion (data not shown).

Chloride Binding Site Identification. Modification to the five Cl^- binding sites (α1Val, α99Lys, β1Met, β81Lys, β103Lys) was evaluated following trypsin digestion and LC−MS/MS analysis. MS spectra of trypsin-digested HbBv are depicted in Figure 12. Trypsin digestion appears to have yielded a low abundance of the five Cl^- binding site containing peptides as demonstrated by their location within the total ion current, indicating a low relative abundance. Therefore, no definitive MS/MS peptide sequence from the b or y series ions was detected. The ions observed in the full scan MS spectra suggest the presence of four of the five Cl^- binding site peptides as determined from their monoisotopic masses: $\beta_{(76-81)}$ $H_{76}LDDLK_{81}$ ($m/z = 740.39$); $\beta_{(1-7)}$ $M(O)_1TAEEK_7$ ($m/z = 837.40$); $\alpha_{(93-99)}$ $V_{93}LSAADK_{99}$ ($m/z = 818.41$); $\alpha_{(1-7)}$ $V_1LSAADK_7$ ($m/z = 703.40$). The peptide $\beta_{(95-103)}$ $L_{95}HVDPENFK_{103}$ $[MH]^{1+}$ ion ($m/z = 1099.2$) could not be differentiated from the nonoxidized $[MH]^{1+}$ methionine-containing peptide $\beta_{(66-75)}$ $V_{66}LDSFSNGMK_{75}$ ($m/z = 1098.3$) with a high degree of precision

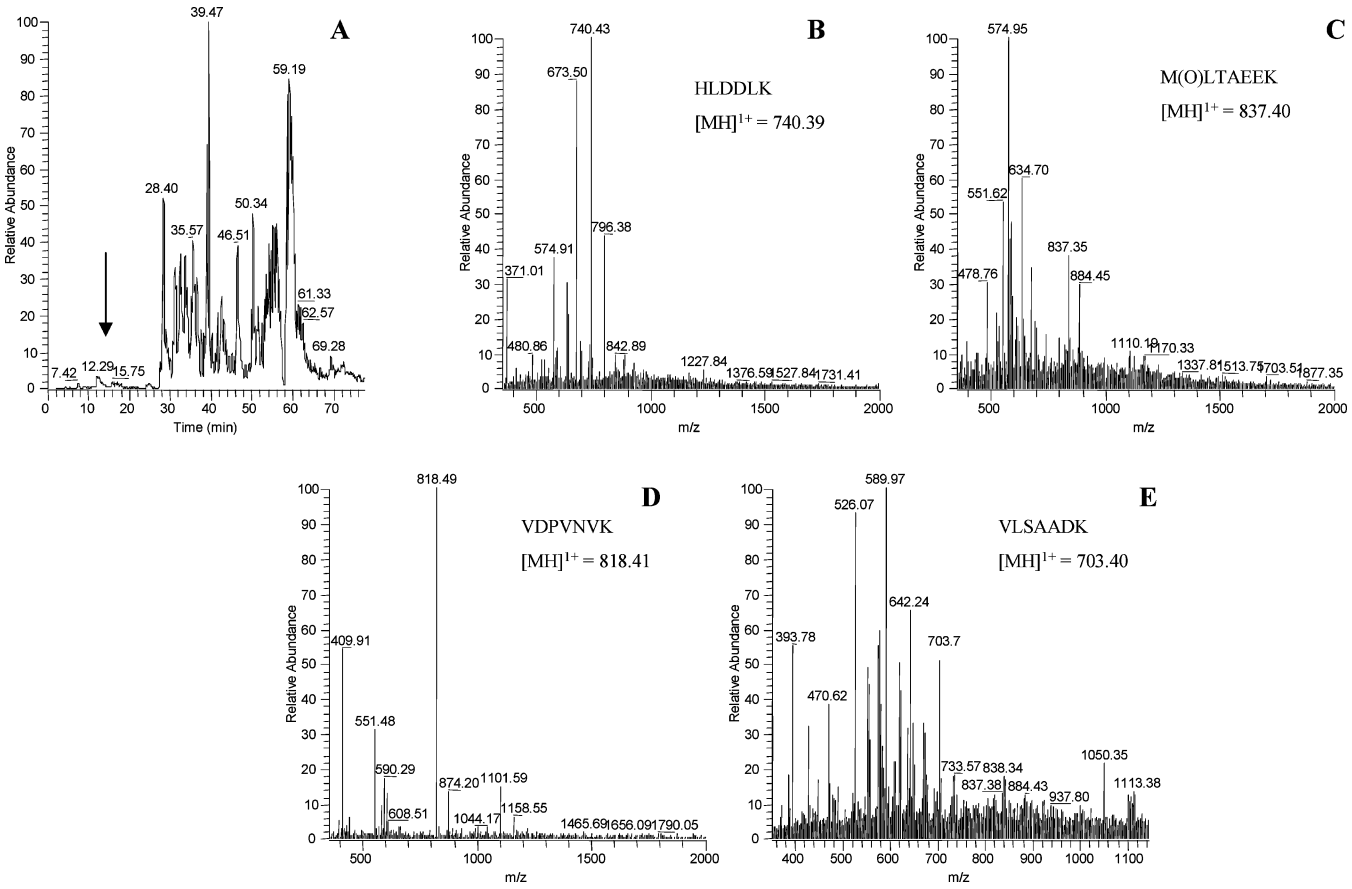

Figure 12. (A) Total ion current of the tryptic peptides from HbBv performed by a capillary HPLC on a quadrupole ion trap mass spectrometer. The arrow indicates the region of the current where the Cl^- binding amino acid peptides are present. All of these peptides were found to be of low abundance from the enzymatic digest and yielded MS data in the full scan MS mode only. Thus, MS/MS analysis of these low-abundance peptides could not be performed adequately to ensure efficient fragmentation of the peptide backbone for sequence identification, (B−E) show the $[MH]^{1+}$ ions of the peptides containing the Cl^- binding amino acids βLys81, βMet1 (sulfoxide), αLys99, and αVal1, respectively. The βLys103-containing peptide could not be differentiated from the unoxidized peptide $\beta_{(66-75)}$ ($[MH]^+$, m/z = 1098.3), as described earlier in the text. The same profile was found in trypsin digested PolyHbBv and PolyHbBv (F1−F4).

due to their low abundance in the tryptic digest. The same ions were identified in the full scan MS of PolyHbBv and its fractions, indicating no significant chemical modification induced by glutaraldehyde. Thus, the reported reduction in Cl^- ion sensitivity in PolyHbBv and F1−F4 relative to HbBv is likely the result of an indirect protein structural alteration induced by glutaraldehyde, which prevents protein mobility upon binding and release of Cl^- ions. Additionally, our data (not shown) indicate a reduction in PolyHbBv and F1−F4 sensitivity to pH relative to HbBv, an effect that we could not attribute directly to Bohr amino acid modification.

CONCLUSION

An extensive evaluation of nonsite specific modification to HbBv using glutaraldehyde is presented for the first time. Significant findings in the functional and structural evaluation of PolyHbBv (Oxyglobin) and the four component fractions F1−F4 are as follows: each fraction contributes nearly identically to the overall functional oxygen binding characteristics of the PolyHbBv mixture; fractions undergo autoxidation kinetics similar to the PolyHbBv mixture; intramolecular cross-linking is present within the fractions, yet nonspecific in nature; the extent of intermolecular cross-linking (polymerization) is highly correlated to modification of lysine and histidine residues; alteration in PolyHbBv and F1−F4 Cl^- ion sensitivity relative to HbBv likely results from a combination of nonspecific cross-linking of HbBv within the central cavity not attributable to direct Cl^- binding amino acid; oxidation of methionine residues is abundant in the HbBv starting material; however, βMet55 residues located in the $\alpha1\beta1$ interface is unaltered in HbBv, PolyHbBv, and F1−F4; finally, the β93Cys residues located in the $\alpha1\beta2$ interface were also found to be unmodified in HbBv, PolyHbBv, and F1−F4. Taken together our data suggest that glutaraldehyde in its reactions with HbBv generates a heterogeneous mixture of components with homogeneous functionality and oxidative stability. Additionally, while lacking site specificity, glutaraldehyde appears not to impose structural modifications to key regions of the Hb molecule.

This can be contrasted with other non-site-specific modifiers such O-raffinose, which alters key amino acids such as β93Cys residues located in the plastic $\alpha1\beta2$ interface.[6] The structural changes imparted by a modifying agent such as O-raffinose may also contribute significantly to abnormal Hb functionality as well as stability.[21,22] Many modified Hbs use various non-site-specific chemistries in their preparation.[4] The nature of chemically induced modifications to Hb intended for use as Hb-based blood substitutes should be understood early in the process of development to rule

out aberrant chemistry as concern for toxicity. Indeed development of second-generation Hb-based blood substitute involving the use of chemical reagents specifically targeting amino acid(s) within key regions of the protein has been an active area of research.[1,34,35] The data from our study likely represent an exception to non-site-specific chemistry in that critical regions of the Hb protein have been spared from glutaraldehyde modification. Overall, however, there is loss of key functions (e.g., chloride regulation of O_2 binding and release and cooperativity) related to normal HbBv functionality.

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