Site-Specific Cross-Linking of Human and Bovine Hemoglobins Differentially Alters Oxygen Binding and Redox Side Reactions Producing Rhombic Heme and Heme Degradation†

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ABSTRACT: Chemically modified human or bovine hemoglobins (Hb) have been developed as oxygen-carrying therapeutics and are currently under clinical evaluation. Oxidative processes, which are in many cases enhanced when modifications are introduced that lower the oxygen affinity, can limit the safety of these proteins. We have carried out a systematic evaluation of two modified human Hbs (*O*-R-polyHbA_0 and DBBF−Hb) and one bovine Hb (polyHbBv). We have both measured the oxidative products present in the Hb preparations and followed the oxidative reactions during 37 °C incubations. Autoxidation, the primary oxidative reaction which initiates the oxidative cascade, is highly correlated with P_{50} ($R = 0.987$; $p < 0.002$). However, when the results for the other oxidative processes are compared, two different classes of oxidative reactions are identified. The formation of oxyferrylHb, like the rate of autoxidation, increases for all modified Hbs. However, the subsequent reactions, which lead to heme damage and eventually heme degradation, are enhanced for the modified human Hbs but are actually suppressed for bovine-modified Hbs. The rhombic heme measured by electron paramagnetic resonance, which is the initial step that causes irreversible damage to the heme, is found to be a reliable measure of the stability of ferrylHb and has the tendency to produce degradation products. DBBF−Hb, a Hb-based oxygen carrier (HBOC) for which toxic side effects have been well documented, has the highest level of rhombic heme (41-fold greater than for HbA_0), even though its rate of autoxidation is relatively low. These findings establish the importance of these secondary oxidative reactions over autoxidation in evaluating the toxicity of HBOCs.

Several HBOCs[1] have advanced to phase II/III clinical trials in the United States. The starting material is stroma-free Hb (SFH) or chromatographically purified Hb obtained from sources such as human or bovine outdated blood. Various chemical and/or genetic alterations have been employed to produce a stable and functional HBOC including intratetrameric cross-linked Hb, polymers of Hb tetramers (intra- and intertetrameric cross-linked), and Hb tetramers conjugated to nonprotein macromolecules (*1*). In some cases the protein is fully expressed in a bacterial host system. Chemical modifications are introduced to stabilize the low oxygen affinity state of the Hb tetramer resulting in a low oxygen affinity, which is a desirable property for HBOCs (*1*).

Recent physicochemical studies have shown that some of these chemical reagents induce other changes in the protein that may have adverse physiological consequences. This includes the reported effects of some of these modifications on the normal allosteric modulation of oxygen affinity. Thus, cross-linking and/or polymerization have (has) been shown in some instances to abolish the effect of chloride, induce a decrease in the Bohr effect (pH effect), and in some cases result in total loss of cooperativity (*2*, *3*). Despite these apparent functional limitations some animal studies, as well as recent clinical trials, have reported that these proteins apparently deliver oxygen to tissues (*4*).

The oxidation (autoxidation) of Hb (Fe^{2+}) to the nonfunctional metHb (Fe^{3+}) form is an important concern in the use of Hb as an oxygen-carrying agent. Uncontrolled and spontaneous oxidation of ferrous iron (Fe^{2+}) compromises the function and in some instances the safety of the infused HBOCs. The conversion of up to 40% of glutaraldehyde-polymerized bovine Hb to metHb by 72 h following near-total exchange in animal model has been reported (*5*). The autoxidation of dextran-based modified Hb in the 50%

† The opinions and assertions contained herein are the scientific views of the authors and are not to be construed as the policy of the U.S. Food and Drug Administration.

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[1] Abbreviations: HBOCs, hemoglobin-based oxygen carriers; HPLC, high-performance liquid chromatography; EPR, electron paramagnetic resonance; *O*-R-polyHbA_0, human hemoglobin polymerized with *O*-raffinose; polyHbBv, bovine hemoglobin polymerized with glutaraldehyde; DBBF−Hb, human hemoglobin cross-linked with bis(3,5-dibromosalicyl) fumarate.

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exchange transfusion model was also reported to rise linearly, reaching up to 35% in the first 12 h after transfusion (*6*). Under normal circumstances, however, the overall percent of circulating metHb is the result of a balance between Hb oxidation and metHb reduction, and it remains to be determined to what extent metHb may compromise the ability of a given Hb to unload oxygen at the tissue level. It has been estimated that metHb levels greater than 10% significantly decrease the ability of Hb to oxygenate tissues (*7*).

Considerable indirect and circumstantial evidence suggests that the production and interaction of potentially harmful free radicals can result from the infusion of cell-free Hb preparations (*3*, *8*). Direct cytotoxic effects associated with a number of chemically modified Hbs have been ascribed to redox reactions (involving either one- or two-electron steps) between the heme group and peroxides, particularly, hydrogen peroxide (H_2O_2) (*8*). These cytotoxic effects can be attributed, at least in part, to the ferryl (Fe^{4+}) oxidation state formed when Hb reacts with H_2O_2. This higher oxidation state is a potent oxidant capable of promoting oxidative damage to most classes of biological molecules (*9*).

An additional oxidative pathway, which can contribute to the toxicity of HBOCs, is heme degradation products formed during the autoxidation of oxyHb. Recent studies have identified heme degradation by the characteristic fluorescence of the degradation products formed. The heme moiety has been shown to form these degradation products during the reaction of Hb with H_2O_2 (*10*) and during autoxidation (*11*). Heme degradation fluorescent products were also found in red cells after treatment with H_2O_2 (*12*). The basal level of degradation products found in the red cell was found to increase with cellular age and during in vitro incubation of red cells (unpublished experiments).

Mechanistic studies revealed that heme degradation involves the reaction of two molecules of H_2O_2 with Hb. The first molecule produces ferrylHb ($HbFe^{4+}$) and the second molecule undergoes a one-electron oxidation of H_2O_2 to produce superoxide ($O_2^{\bullet -}$) and/or $^{\bullet}OOH$ in the heme pocket, which attacks the heme resulting in heme degradation (*13*). The formation of these degradation products by the low levels of H_2O_2 generated during autoxidation indicates the sensitivity of Hb to oxidative modification and heme loss.

The clinical usefulness of HBOCs, therefore, requires that the oxygen affinity must be lowered and, in addition, oxidative processes must be either suppressed or controlled. However, the established relationship between low oxygen affinity and increased autoxidation and possibly oxidative modification reactions (*3*) suggests that these oxidative processes may actually be enhanced in some of the chemically or genetically modified HBOCs.

We report here for the first time a complete evaluation of the various oxidative and peroxidative reactions for various chemically modified Hbs currently undergoing clinical evaluation as HBOCs. We measure autoxidation, formation of $HbFe^{4+}$, formation of a rhombic heme (the initial step in the degradation of the heme), and fluorescent heme degradation products. These reactions are correlated with the functional properties of the Hb, and their significance is discussed in the context of safety and efficacy evaluation of HBOCs.

MATERIALS AND METHODS

Hemoglobin Solutions. Ultrapure hemoglobin A_0 (HbA_0) and its *O*-raffinose cross-linked derivative (*O*-R-$polyHbA_0$) (Hemolink) were a kind gift of Hemosol, Inc., Canada. HbA_0 was prepared from outdated human blood and subjected to extensive anionic and cationic chromatographic procedures, resulting in a protein with greater than 99% purity (*14*). *O*-R-$polyHbA_0$ was prepared by reacting HbA_0 with *O*-raffinose, a hexaldehyde obtained by oxidation of the trisaccharide, raffinose. The high level of purity of the Hb preparation was confirmed by the use of SDS−PAGE analysis, isolectric focusing, HPLC, and western blotting (*14*).

Diaspirin cross-linked Hb (DBBF−Hb) was a kind gift from the Walter Reed Army Institute of Research (WRAIR). This Hb is an intramolecularly cross-linked tetramer produced via chemical reaction with bis(3,5-dibromosalicyl) fumarate (DBBF). The production and development of the commercial analogue of this Hb, known as DCLHb (HemAssist), have recently been halted by its manufacturer, Baxter, due to an unfavorable clinical trial outcome that included excessive fatalities in the test groups (*15*).

Bovine hemoglobin (HbBv) was a kind gift from Biopure and was prepared from bovine blood and subjected to extensive anionic and cationic chromatographic procedures, resulting in a highly purified protein. Polymerized hemoglobin (polyHbBv) (oxyglobin) was purchased from Biopure Inc. (Boston). PolyHbBv is inter- and intramolecularly cross-linked with glutaraldehyde and has recently been made commercially available as an oxygen therapeutic for use in dogs with anemia (*16*). Both proteins demonstrated purity to a level greater than 99% (*16*).

PolyHbBv and *O*-R-$polyHbA_0$ were obtained directly from the manufacturers. These solutions, kept in sealed bags, are stable under nitrogen at room temperature or under refrigeration for up to 1−2 years. DBBF−Hb was kept frozen at −80 °C. Prior to use Hb samples were either aliquoted and frozen at −80 °C for future use or used immediately in our experiments after oxygenation.

Oxygen Equilibrium Studies. Oxygen equilibrium curves (OECs) of Hb solutions were obtained using the Hemox analyzer (TCS Scientific, New Hope, PA). This instrument measures the oxygen tension with a Clark oxygen electrode (model 5331 oxygen probe; Yellow Springs Instruments, Yellow Springs, OH) and simultaneously uses a dual-wavelength spectrophotometer to calculate the Hb oxygenation. Oxygen equilibrium experiments were carried out in 0.1 M phosphate buffer, pH 7.4, in the case of human Hbs. For the bovine Hbs 0.1 M NaCl was added to the buffer solutions. The concentration of Hb samples was between 60 and 75 μM (heme), and the temperature was maintained at 37 °C unless otherwise indicated. To maintain the metHb content to a minimum level (<5%), we included 4 μL of the Hayashi enzymatic reduction system in the final solution (4 mL) (*17*). Oxygen equilibrium parameters were derived by fitting the Adair equations to each oxygen equilibrium binding curve by the nonlinear least-squares procedure included in the Hemox analyzer software (p50 PLUS, version 1.2) (*3*). The Adair constants were then used to generate an oxygen-binding curve, which was used to determine the P_{50} and n_{50} (Hill coefficient) for oxygen binding. This procedure made it possible to determine oxygen-binding parameters for

samples of Hb, which were not fully oxygenated at atmospheric oxygen partial pressures.

Rhombic Heme. Rhombic heme was detected by electron paramagnetic resonance at liquid helium temperatures (*18*). An IBM (Bruker) 200D-SRC spectrometer with 100 kHz modulation at 2 mW power was used. The temperature was maintained by an Oxford Instruments ESR-900 continuous flow cryostat. The signal in the region of $g = 4.2-4.3$ was integrated to measure the relative intensity of the rhombic heme.

Autoxidation Experiments. Autoxidation experiments for Hb samples (50 μM) in 50 mM potassium phosphate buffer, pH 7.4, containing 100 μM EDTA at 37 °C were carried out in the dark. Absorbance changes in the range of 490−640 nm due to the spontaneous oxidation of Hbs were recorded on a Perkin-Elmer Lambda 6 spectrophotometer. Multicomponent analysis was used to calculate the time-dependent changes in concentration of the oxy, met, and deoxy, based on known spectra of each species (*19*). First-order autoxidation rate constants were derived from a fit of the data during 10 h incubation to a single exponential expression using a nonlinear least-squares fitting program (Origin 6.1).

Fluorescent Degradation Products. Hb samples (50 μM) in 50 mM potassium phosphate buffer containing 100 μM EDTA, pH 7.4, were incubated in the dark at 37 °C. Fluorescent emission spectra were scanned from 390 to 600 nm at an excitation wavelength (ex) of 321 nm. Spectra were run at 2 h time intervals using a Perkin-Elmer (Model LS 50B) fluorescence spectrophotometer (*10*). These were the same Hb samples used to measure autoxidation. This made it possible to relate the production of fluorescent products to the autoxidation of oxyHb.

Hydrogen Peroxide-Mediated Oxidation Experiments. The reaction of the various metHbs with H_2O_2 under pseudo-first-order conditions was measured in an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer at 25 °C (*3*, *20*). The reactions were carried out in 50 mM Tris-HCl buffer, pH 7.4, at room temperature. The dead time of this instrument is 1.3 ms. Detection at multiple wavelengths was achieved with an Applied Photophysics photodiode array accessory. A minimum of 200 spectra was collected with a maximum time resolution of 2.38 ms per spectrum. Kinetic data were fit to an appropriate fitting function included in the Applied Photophysics software. All rate constants were determined from averages of at least six individual traces.

RESULTS

Oxygen-Binding Studies. Oxygen equilibrium curves (OECs) were obtained for the chemically modified Hbs included in this study under similar experimental conditions as previously reported (*3*, *21*). Chemically modified Hbs exhibited an OEC, which is right shifted relative to that of their native precursor, HbA_0 or HbBv, respectively. The P_{50}s derived from these curves are listed in Table 1, which confirm the oxygen affinity lowering effects of these diverse chemical reagents. The range of P_{50}s calculated for the modified proteins was equal to or greater than that of normal human blood (~30 mmHg). The Hill coefficient at half-saturation (n_{50}) calculated for these Hbs is also reported in Table 1. For DBBF−Hb the Hill coefficient falls within the normal range (2−2.5). However, for the *O*-R-polyHbA_0 and polyHbBv, the Hill coefficient is in the region of 1.0, indicating a loss of cooperativity. In fact, for the polymerized human and bovine Hbs, the combination of low oxygen affinity and non-cooperativity results in the Hb being partially deoxygenated even at atmospheric oxygen pressures (~80%). The oxygen equilibrium curves (OECs) for these Hbs were extrapolated to full saturation by generating oxygenation curves using the Adair constants generated by the Hemox analyzer software. The corrected P_{50}s for *O*-R-polyHbA_0 and polyHbBv are reported in Table 1.

Table 1: Oxygenation Reactions of Human and Bovine Hemoglobins and Their Chemically Modified Derivatives

hemoglobin	P_{50}[a] (mmHg)	n_{50}[a]
HbA_0	15	2.5
O-R-polyHbA_0	52	0.97
DBBF−Hb	32	2.07
HbBv[b]	29	2.1
polyHbBv[b]	46	1.3

[a] The P_{50} (partial pressure of oxygen at which Hb is 50% saturated) and Hill coefficient (n_{50}) are derived from oxygen equilibrium curves. Experimental conditions were 0.1 M sodium phosphate buffer, pH 7.4, and 66−75 μM heme for human Hbs. For bovine Hbs, 0.1 M NaCl was added to the buffer solutions. Each value represents an average of three runs. The standard error (SE) was less than 10%. [b] Alayash et al. (*2*).

Fluorescent Degradation Products and Rhombic Heme in Modified Hemoglobins. The different Hbs undergo oxidative processes during preparation and storage. While Hb oxidation is a reversible process, damage to the heme and the formation of fluorescent degradation products are irreversible. The level of fluorescent degradation products in a 1 mM sample of the various Hb preparations (Table 2) is, therefore, a measure of the irreversible oxidative processes taking place with the different Hbs. Modifications of human Hb are seen to dramatically increase the level of these fluorescent products. On the other hand, modifications of bovine Hb produce a small decrease in the level of degradation products.

The fluorescent degradation products have been attributed to the reaction of superoxide ($O_2^{\bullet-}$) formed when H_2O_2 reacts with ferrylHb (*13*). While the formation of fluorescent products and the release of iron (*10*) are the final steps in this oxidative cascade, the initial irreversible reaction involves damaging the heme with the iron still bound. This damage results in a change in the geometry around the heme iron to produce a high-spin rhombic heme. The normal iron geometry in Hb is the tetragonal geometry. With metHb this gives the high-spin tetragonal complex with $g = 6$ and $g = 2$. Changes in the configuration around the heme do produce low-spin rhombic heme complexes (hemichromes) with the distal histidine coordinating to the heme. The hemichrome EPR spectrum is very sensitive to the heme configuration and the accessibility of solvent to the heme pocket (*18*). A recent paper (*22*) has shown a relationship between the rate of autoxidation and the type of hemichrome formed. However, the high-spin rhombic geometry of the heme is not produced by alterations in the globin of Hb. It is necessary to damage the heme to produce this. It is, therefore, a measure of the initial step, which damages the heme.

Table 2: Oxidation Reactions of Human and Bovine Hemoglobins and Their Chemically Modified Derivatives

hemoglobin	$k_{autoxidation}$ (h^{-1})[a]	$k_{H_2O_2}$ (M^{-1} s^{-1})[b]	fluorescence/ oxidation (AU)[c]	initial MetHb (μM)[d,g]	initial fluorescence (AU)[e,g]	rhombic heme (RI)[f,g]
HbA_0	0.0115 ± 0.0002	8.8 ± 2.3	5.06	71	3.4	0.67
O-R-polyHbA_0	0.0392 ± 0.0014	12 ± 0.55	10.96	128	15.0	13.00
DBBF−Hb	0.0240 ± 0.0004	11.9 ± 20.9	7.81	56	25.5	27.80
HbBv	0.0260 ± 0.0046	13 ± 1.73	5.15	252	4.8	2.07
polyHbBv	0.0359 ± 0.0006	15 ± 1.5	3.16	136	4.3	1.15

[a] First-order rate constants for autoxidation. Experimental conditions are described in Materials and Methods. [b] Second-order rate constants of the metHb reaction with H_2O_2. Experimental conditions are described in the legend of Figure 5. [c] The increase in fluorescence emission was divided by the increase in Hb oxidation during 10 h incubation at 37 °C. [d] Basal metHb levels before incubation. [e] The fluorescence emission at 465 nm prior to incubation in arbitrary units (AU). [f] The rhombic EPR signal was double integrated to give relative intensity (RI). [g] Total hemoglobin in various preparations was corrected to a concentration of 1.0 mM.

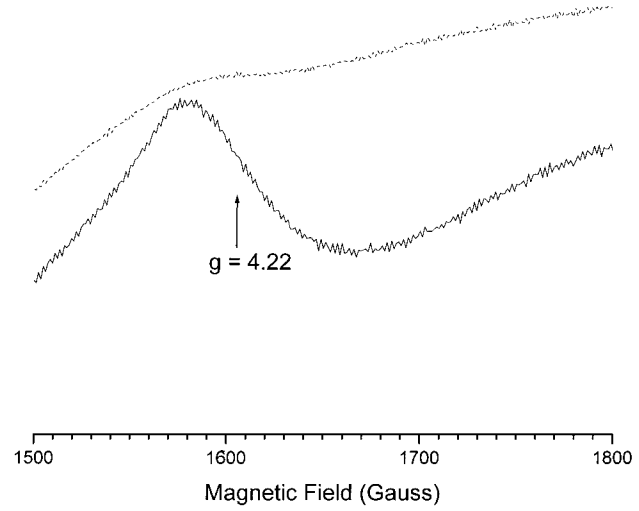

FIGURE 1: Electron paramagnetic resonance (EPR) spectrum in the region from 1500 to 1800 G. Spectra were obtained at 4 K and show the signal from rhombic heme for HbA_0 (---) and *O*-R-polyHbA_0 (—). The concentrated stock solutions of these Hb preparations were used.

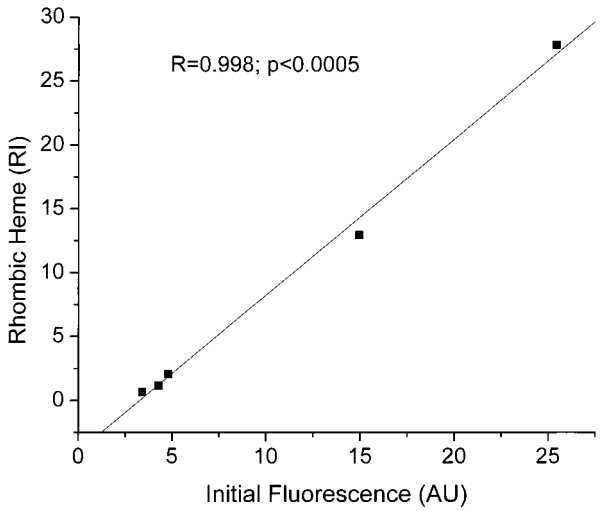

FIGURE 2: Correlation between the intensity of the rhombic heme and the initial fluorescence intensity for an emission wavelength of 465 nm (excitation wavelength 321 nm). The fluorescent measurement was made at a concentration of 50 μM and corrected to the concentration of the stock solution used for the EPR measurements.

Low-temperature electron paramagnetic resonance was used to measure the rhombic heme with a *g*-value in the region of 4.2−4.3. Figure 1 shows the rhombic heme signal for HbA_0 and its polymerized form (*O*-R-polyHbA_0). Included in Table 2 are the double integrated areas of the rhombic heme for all the Hbs studied. Modification of human Hb resulted in a dramatic increase of rhombic heme (about 19-fold for *O*-R-polyHbA_0 and about 41-fold for DBBF−Hb). Interestingly, although nonmodified bovine Hb has three times as much rhombic heme as nonmodified human Hb, modification of bovine Hb results in a 45% decrease in the rhombic heme. A very significant correlation ($R = 0.998$; $p < 0.0005$) between the fluorescent degradation product in these Hb preparations and the rhombic heme (Figure 2) clearly establishes a relationship between the rhombic heme and the production of fluorescent products. Since the rhombic heme can be readily quantitated in any blood or Hb preparation, this determination provides a new and reliable method to measure the tendency to form irreversible oxidative products.

Autoxidation. Table 2 also includes values for the initial levels of metHb in the various Hb preparations. The data in Table 2 show that the levels of metHb vary for the different Hb preparations with some modifications decreasing the met level (DBBF−Hb and polyHbBv) and other modifications increasing the level of metHb (polyHbA_0). This effect is distinct from the heme degradation products and rhombic heme for which both human Hb modifications dramatically increase the level of these products. This comparison is particularly noteworthy for DBBF for which a 41.5-fold increase in rhombic heme and a 7.5-fold increase in fluorescence are associated with a 21% decrease in the level of metHb.

Table 2 also lists the rates of autoxidation of these Hbs carried out at 37 °C in the absence of antioxidant enzymes, superoxide dismutase and catalase. The rates of autoxidation will depend to some extent on the level of the starting metHb with a higher level of initial oxidation resulting in a lower apparent rate constant. Since attempts to reduce the initial oxidized Hb resulted in a protein more susceptible to oxidation, we have used the initial material. Polymerization of HbA_0 with *O*-raffinose produced a 3.4-fold increase in the rate of autoxidation. DBBF−Hb exhibited a 2-fold increase in the rate of autoxidation. The rate of autoxidation for polyHbBv was 1.4-fold higher than that calculated for HbBv (*3*). There was an approximately 20−30% reduction in the rates of autoxidation when antioxidant enzymes were added to the protein solutions (data not shown). This reduction in the rate of autoxidation in the presence of antioxidant enzymes is consistent with earlier reports and confirms the buildup of oxygen free radicals during the autoxidation process (*11*).

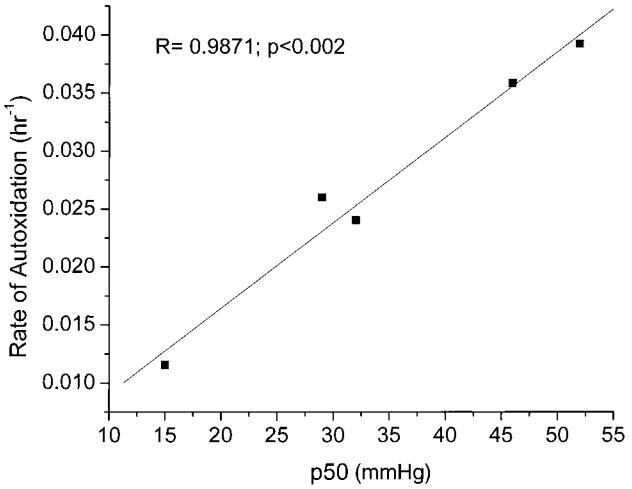

FIGURE 3: Correlation of oxygen affinity of hemoglobins (P_{50}) with the rate of autoxidation of the same modified and unmodified Hbs.

Figure 3 shows the excellent correlation between the rate of autoxidation and the P_{50} ($R = 0.987$; $p < 0.002$). This indicates that a decrease in the oxygen affinity, which increases the amount of Hb deoxygenation, increases the rate of autoxidation. The relationship indicated in Figure 3 implies that the effect of Hb deoxygenation on the autoxidation is greater than any variability in the rates of autoxidation attributed to differences in the initial levels of metHb (Table 2).

Formation of Fluorescent Degradation Products. Figure 4 shows the time-dependent increase in the emission spectra at 37 °C obtained at an excitation wavelength of 321 nm for the various Hbs. Modification of HbA_0 either by intramolecular cross-linking (DBBF−Hb) or by intra- and intermolecular cross-linking (*O*-R-polyHbA_0) increases the formation of the degradation product with an emission maximum at 465 nm by a factor of 3.2 and 5.2, respectively. While degradation of bovine Hb is 1.8 times faster than HbA_0, polymerization of bovine Hb does not have any significant effect on the formation of degradation products.

We have measured the increase in oxidation at the same time that the fluorescence was measured. Consistent with the reported relationship between autoxidation and heme degradation, a linear relationship between the increase in fluorescence and the increase in oxidation was obtained for the different Hbs studied. The slope of these plots was used as a measure of the tendency for heme degradation to occur during the autoxidation process (Table 2). The increase in the slope for modifications of human Hb to lower the oxygen

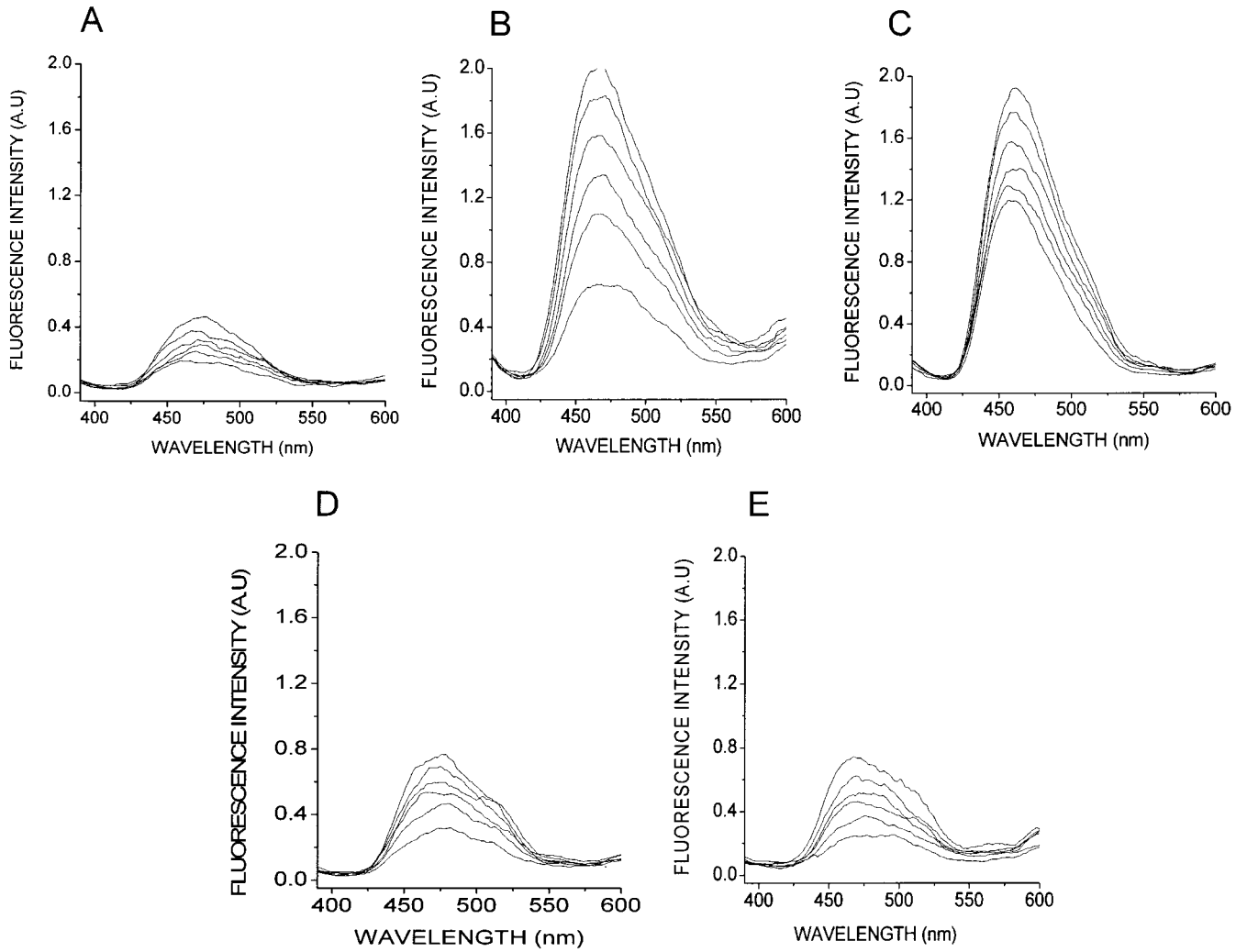

FIGURE 4: Fluorescent emission spectra (excitation wavelength 321 nm) of various hemoglobins in EDTA containing phosphate buffer, pH 7.4, during incubation at 37 °C. Repetitive emission spectra were obtained with a time interval of 2 h for 10 h. Panels: (A) HbA_0, (B) *O*-R-polyHbA_0, (C) DBBF−Hb, (D) HbBv, and (E) polyHbBv.

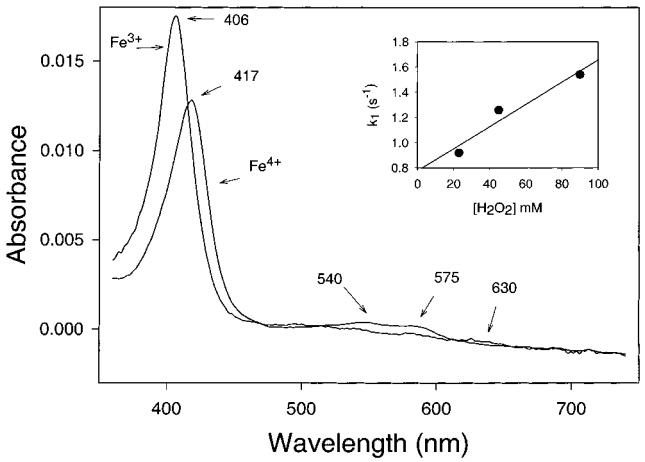

FIGURE 5: Reaction of the ferric (Fe^{3+}) form of HbA_0 (4 μM) with H_2O_2 (90 mM) in the stopped-flow. Experimental conditions were 50 mM Tris-HCl buffer, pH 7.4, at room temperature. Spectral changes in the Soret and visible regions were recorded as a function of time and H_2O_2 concentration. The main figure shows a typical data set of the global fitting routine used to calculate and reconstruct the spectra of the main reaction intermediates when HbA_0 was mixed with a large excess H_2O_2. A single exponential process was monitored at all wavelengths to describe the transition of the ferric (Fe^{3+}) to ferryl (Fe^{4+}) form. The dependence on H_2O_2 concentration of the observed rate constants associated with this kinetic phase is shown in the insert.

affinity (DBBF−Hb and polyHbA_0) indicates that the formation of heme degradation products cannot be attributed solely to an increase in the rate of autoxidation. For bovine Hb, unlike human Hb, polymerization actually decreases the tendency to undergo degradation, and even though the oxidation is more pronounced, there is no increase in the formation of degradation products.

Hydrogen Peroxide Kinetics. The reaction of the Fe^{3+} forms of Hbs with H_2O_2 was monitored in the stopped-flow with a photodiode array detector. Spectral changes occurring in the Soret and visible regions were recorded as function of time and H_2O_2 concentration. Figure 5 shows a typical data set of the global fitting routine used to calculate and reconstruct the spectra of the main reaction intermediates when HbA_0−Fe^{3+} was mixed with H_2O_2. Two spectral components were recorded, the first being that of Fe^{3+} and the second that of Fe^{4+} (*20*, *21*). A single exponential process was monitored at all wavelengths essentially describing the transition from the Fe^{3+} to Fe^{4+} form of HbA_0. The dependence on H_2O_2 concentration of the observed rate constants associated with this kinetic phase is reported in the insert of Figure 5. The apparent rates obtained from the slopes of these plots for all Hbs are reported in Table 2. It is interesting to note that, despite the transient nature of these reactions and the high concentrations of H_2O_2 used in these experiments, the rate of Fe^{3+} to Fe^{4+} is slightly higher with the modified proteins than values obtained for their native proteins. This is consistent with our previous observations, which support the notion that chemical manipulations enhance both the autoxidation and in some instances the formation and stability of Hb in its Fe^{4+} form (*21*).

DISCUSSION

Oxidative Damage Associated with HBOCs. The problems associated with the early crude Hb solutions (i.e., Hb dimerization, renal toxicity, and residual stroma and endotoxin contamination) have been resolved (*1*). The number of new and largely unresolved problems were found during preclinical and clinical development of current generation Hb-based products. These include cardiovascular/hemodynamic effects, gastrointestinal changes, immune cell activation, coagulation changes, oxidative stress, and decreased host resistance to overwhelming infection (*23*).

Numerous in vitro and some in vivo reports suggest that the potential exists for Hb solutions to induce an oxidative stress (*2*). Part of this might be explained by the ability of Hb to serve as a source of toxic oxygen species. Free radical mediated tissue injury is supported by the finding of elevated pancreatic and liver enzymes in the plasma of animals undergoing exchange transfusion with HBOCs (*23*). Increased levels of amylase and lipase after Hb infusions in animals have been frequently observed. Myocardial lesions characterized by mild to moderate focal-to-multifocal myocardial degeneration and/or necrosis in a highly vascularized portion of the myocardium were recently reported in a number of animal models infused with DCLHb, the commercial analogue of DBBF−Hb included in this study (*24*).

Oxidative reactions between Hb and oxidants such as H_2O_2 (generated by Hb autoxidation or from other intracellular sources) may potentially cause a vascular inflammatory cascade of reactions progressing to multiorgan failure (*2*).

To evaluate the potential contribution of Hb-mediated oxidative processes to tissue injury and toxicity, we have measured the various oxidative and peroxidative reactions and attempted to gain insight into the molecular basis for some of the changes in oxidative reactions found to take place.

Oxidative Processes Associated with Hemoglobin. (1) The initial oxidative process in Hb involves autoxidation, which is a source for nonfunctional metHb as well as the reactive oxygen species superoxide ($O_2^{\bullet-}$).

$$HbFe^{2+}O_2 \rightarrow HbFe^{3+} + O_2^{\bullet-} \quad (1)$$

(2) Superoxide can both react with Hb and undergo dismutation to produce hydrogen peroxide (H_2O_2).

$$O_2^{\bullet-} + HbFe^{2+} + 2H^+ \rightarrow HbFe^{3+} + H_2O_2 \quad (2)$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

(3) The H_2O_2 produced during autoxidation also reacts with Hb. These reactions have been extensively characterized and proceed via a two-electron oxidation process (for review, see ref *25*). With either Fe^{2+} or Fe^{3+} Hb, the ferryl species (Fe^{4+}), two oxidation equivalents above Fe^{2+}, is formed. For the reaction with Fe^{3+}, the second electron is taken from the globin producing protein radicals (*26*). The radical represents the second oxidizing equivalent, and both carbon-based radicals and peroxyl are formed simultaneously.

$$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{4+}{=}O + H_2O + O_2 \quad (4)$$

$$HbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}HbFe^{4+}{=}O + H_2O \quad (5)$$

(4) Recent research shows that the formation of the Fe^{4+} form of Hb also results in the formation of heme degradation

products (*13*). It has been shown that heme degradation is produced by the superoxide/perhydroxyl radical formed when a second H_2O_2 molecule reduces the ferrylHb. The initial step in the degradation process involves damage to the heme which results in the loss of the usual tetragonal symmetry around the heme iron to produce rhombic hemes, not normally present in native Hb (Nagababu and Rifkind, unpublished results). The irreversible damage to the heme begins a cascade with the eventual release of the iron and the formation of two fluorescent degradation products (*10*).

$$HbFe^{4+}{=}O + H_2O_2 \rightarrow HbFe^{3+} + O_2^{\bullet -} + H_2O \rightarrow \text{heme degradation} \quad (6)$$

Each of these four oxidative steps results in the formation of reactive products, which may result in tissue and cellular damage. (a) The reactive oxygen species generated during the autoxidation reaction are known to produce cellular damage. (b) The Fe^{4+} associated with the reactions of H_2O_2 can act as a potent oxidant capable of oxidatively damaging most biological substrates, including lipids, nucleic acids, and amino acids (*9*). Formation of Fe^{4+} HBOCs was correlated to cytoxicity in an endothelial cell culture model of ischemia/repurfusion and more recently in cells that lack their antioxidant mechanisms such as glutathione (*27*). (c) The globin radical formed when metHb reacts with H_2O_2 can result in the formation of both intermolecular cross-links and intramolecular cross-links between the heme and amino acids (*28*). (d) Heme degradation results in the release of free iron, which is known to be the source for tissue and cellular damage (*29*). In addition, the hydrophobic porphyrin fragments produced as a result of heme degradation are expected to alter the properties of cellular membranes, thereby perturbing cellular functions (*30*, *31*).

The Hb cross-linking and polymerization involved in producing HBOCs can affect these oxidative processes (*2*). The autoxidation of Hb involves the deoxygenated chains (*19*). It is, therefore, generally found that lowering the oxygen affinity increases the rate of autoxidation. Figure 3 shows the significant correlation obtained between P_{50} and the rate of autoxidation. This is in keeping with earlier studies, which showed that autoxidation rates are inversely proportional to the oxygen affinity of Hb (*32*). Autoxidation as well as the reaction of H_2O_2 with Hb can also depend on the accessibility of the ligand pocket to water (for autoxidation) and H_2O_2 (for the formation of ferrylHb). Both the rate of autoxidation and the rate of the reaction of $HbFe^{3+}$ with H_2O_2 to form the oxyferryl ($HbFe4^{+}$) have been measured (Table 2). The results indicate an appreciable increase in both rate constants for bovine Hb (*3*). This presumably reflects a change in the ligand pocket conformation. In addition, for both Hbs the modifications enhance the rate of autoxidation and the rate constant for the reaction with H_2O_2. The protein modifications used to make HBOCs thus seem to increase the accessibility of the heme pocket increasing autoxidation as well as the initial peroxidative reaction forming $HbFe^{4+}$.

The additional factor necessary to consider in evaluating the oxidative cascade, which Hb undergoes, is the stability or lifetime of the $HbFe^{4+}$. This stability is in large part determined by the decay of the ferrylHb, which is thought to involve the reduction of the Fe^{4+} back to the Fe^{3+} heme (*21*). Thus, even if ferrylHb is formed very rapidly, the damage it can produce depends on how long the ferrylHb persists in solution, as well as the susceptibility of the Hb to damage by $O_2^{\bullet -}$ formed when H_2O_2 reacts with the ferrylHb. It is significant that the Fe^{4+} state has been found to be appreciably more stable for the intramolecularly cross-linked HBOCs such as DBBF−Hb than for HbA_0 (*21*). The longer lifetime may provide additional time for H_2O_2 to react with the ferrylHb and initiate the degradation processes.

The dichotomy between the primary autoxidation reaction and the secondary oxidation reactions is clearly indicated by the behavior of DBBF−Hb. Thus, although the DBBF−Hb preparation contained the lowest level of metHb, this preparation has the highest initial levels of rhombic heme and fluorescent degradation products (Table 2). Even during incubation, DBBF−Hb has an increased tendency to form degradation products as indicated by the increased fluorescence/oxidation (Table 2). However, the increased fluorescence/oxidation is not adequate to explain the initial high level of fluorescent products prior to incubation, suggesting that some of the heme degradation and rhombic heme is formed during the preparation of this modified protein.

With respect to these secondary oxidative products an interesting difference is found between the human and bovine HBOCs. For human Hb modifications we find a dramatic increase in the levels of rhombic heme (20−40-fold) (Table 2, Figure 1) and the fluorescent products initially present (5−8-fold) (Table 2). We also find that during incubation the tendency to form fluorescent products during autoxidation increases (Table 2). Although bovine Hb has more initial rhombic heme than HbA_0, the polymerization to form polyHbBv does not significantly enhance the formation of these products. In fact, as indicated by the 50% reduction in the ratio of fluorescence/oxidation during incubation (Table 2), polymerization of bovine Hb appears to actually protect bovine Hb from these irreversible oxidative processes (Table 2, Figure 4).

Rhombic Heme: A Possible Measure of the Safety of HBOCs. Rhombic heme can be measured in concentrated Hb and even whole blood. Since it involves the measurement of the paramagnetic iron center, there is no interference from oxyHb or deoxyHb and no scattering artifacts. A rhombic geometry around a high-spin iron is not normally present in Hb with a tetragonal geometry. Low levels of rhombic heme have been reported when H_2O_2 is reacted with metHb (*33*). We find that the rhombic heme is much more pronounced when H_2O_2 is reacted with $HbFe^{2+}$ and heme degradation is much more pronounced (*13*). The rhombic heme is the initial product produced when $O_2^{\bullet -}$ formed during the reaction of ferrylHb with H_2O_2 attacks the heme. It then initiates the cascade, which results in the release of iron and the formation of fluorescent degradation products.

The rhombic heme is, therefore, an excellent measure of red cell induced oxidative processes. We have used it as a measure of oxidative processes during cellular aging and in subjects with diabetes and/or cardiovascular disease (Nagababu, Dumswala, and Rifkind, unpublished results).

While all of the Hb oxidative reactions can contribute to cellular and tissue damage, this reaction reflects both the lifetime of ferrylHb as well as the tendency to form degradation products (Figure 2) and the release of free iron. It may therefore provide one of the best measures of the safety of a blood substitute.

On the basis of our results this would suggest that polyHbA_0 and particularly DBBF−Hb may have serious problems, while the bovine-based blood substitutes or other analogues with similar properties may prove to be more useful.

DBBF−Hb is of particular value in assessing the potential use of rhombic heme as a measure of the safety of a blood substitute. DBBF−Hb does not have much of an effect on the rate of autoxidation and the rate for the formation of ferrylHb ($k_{H_2O_2}$); it has a P_{50} similar to that of Hb in the red cell and still retains much of its cooperativity. It would have, therefore, been thought to be an ideal HBOC. However, its rhombic heme is higher than all of the other Hbs studied. It is tempting to speculate that the recent clinical failures in demonstrating the safety of DCLHb, the commercial analogue of DBBF−Hb, may be in part due to the unusual susceptibility of this protein to oxidative modification. Recently published preclinical studies reported the detection of myocardial lesions in a number of animal models infused with DCLHb. These lesions are characterized by a mild to moderate focal-to-multifocal myocardial degeneration and/or necrosis in a highly vascularized portion of the myocardium. In addition, we have recently demonstrated that under mild oxidative stress DBBF−Hb can indeed induce growth arrest in cultured endothelial cells, which ultimately leads to apoptotic and necrotic cell death (*34*).

These results suggest that the safety of HBOCs does not necessarily require that the $O_2^{\bullet-}$ and H_2O_2 formed during autoxidation be eliminated. The difficulties with DBBF−Hb suggest that it is instead the subsequent oxidative and degradation processes and the associated release of free iron, which must be minimized. These products can be toxic and are not efficiently eliminated by the cellular antioxidant systems. This observation is particularly promising for the potential development of safe second generation HBOCs, which require a low oxygen affinity and will be expected to undergo more rapid autoxidation (Figure 3). The results with polymerized bovine Hb (Table 2) indicate that it is possible to separate the autoxidation processes closely coupled to oxygen affinity and the more toxic degradative oxidative processes.

Strategies to combat autoxidation reactions of Hbs are evolving. These include, for example, the cross-linking of antioxidant enzymes, superoxide dismutase and catalase, to Hb (*35*) or antiferryl compounds such as nitroxide (*36*) and trolox (*37*) to control heme-mediated side reactions. Site-directed mutagenesis of the distal pocket of myoglobin (used as a prototype for Hb) and Hb constructs have been produced in which autoxidation and other oxidative side reactions have been substantially inhibited with favorable oxygen affinities maintained (*22*, *38*−*40*).

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