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DIFFERENTIAL SUSCEPTIBILITIES OF THE PROSTHETIC HEME OF HEMOGLOBIN-BASED RED CELL SUBSTITUTES

IMPLICATIONS IN THE DESIGN OF SAFER AGENTS

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Abstract—One approach to the development of an effective red cell substitute has been chemical modification of human hemoglobin to optimize oxygen transport and plasma half-life. Human hemoglobin A_0 and two of these modified hemoglobins, one prepared from the cross-linking of the α-chains at lysine residue 99 by bis(3,5-dibromosalicyl)fumarate (Hb-DBBF) and the other by acylation of lysine residue 82 of the β-chain by mono-(3,5-dibromosalicyl)fumarate (Hb-FMDA), were tested by HPLC for their susceptibility to oxidative damage caused by H_2O_2. Such oxidative insult may occur during ischemia and reperfusion of tissues after transfusion of red cell substitutes to patients with hypovolemic shock and trauma. Hb-DBBF was extremely susceptible to damage of its heme and protein moieties with stoichiometric amounts of H_2O_2, whereas Hb-FMDA was highly resistant, even at 10-fold molar excess and at an acidic pH of 4.7. Hemoglobin A_0 was of intermediate susceptibility, exhibiting alteration of heme and protein moieties at acidic but not neutral pH. Since the degradation of heme can release the potentially toxic agent iron, Hb-FMDA may be a more promising candidate than Hb-DBBF for development as a red cell substitute. A similar approach may be used to assess the susceptibility of other hemoglobin-based red cell substitutes to oxidative damage in order to determine the molecular basis of heme and protein alteration.

Several laboratories have developed chemically or genetically altered human hemoglobins as a substitute for red cells to be used in a number of clinical applications, most notably in the treatment of hypovolemic shock [1, 2]. Although acellular human hemoglobin A_0 (HbA_0)‡ is not a suitable substitute for red cells, due in large part to its high oxygen affinity and instability of its tetrameric state, several chemically modified hemoglobins have been developed to address these shortcomings [2]. The structures of two of these candidates are depicted in Fig. 1. Hb-DBBF is a human hemoglobin derivative resulting from cross-linking of lysine residue 99 of the α-chains by treatment with bis(3,5-dibromosalicyl)fumarate [3, 4]. Hb-FMDA is prepared by the reaction of mono-(3,5-dibromosalicyl)fumarate with human hemoglobin, resulting in a pseudo-cross-link between the β-chains involving a covalent linkage to lysine 82 and an electrostatic interaction with the histidine or valine residue [5]. Both of these products have a tetrameric structure containing either two native α-chains and two modified β-chains, as in the case of Hb-FMDA, or two modified

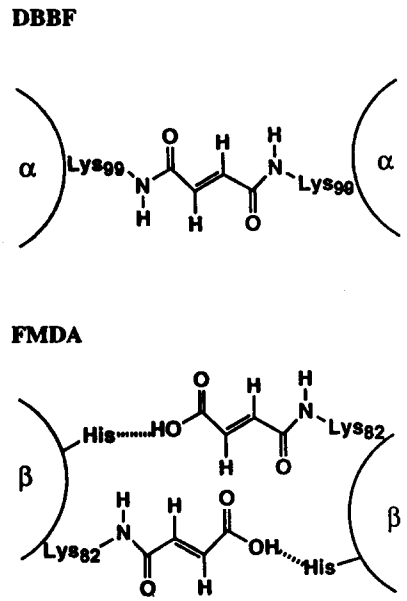

Fig. 1. Structures of cross-linked hemoglobins Hb-DBBF and Hb-FMDA. Hb-FMDA is shown with an electrostatic interaction with a histidine residue of the β-chain; however, the exact residue responsible for the pseudo-cross-link is unknown. A valine residue has also been proposed as a suitable residue for the pseudo-cross-linking [5].

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‡ Abbreviations: HbA_0, human hemoglobin A_0; Hb-DBBF, bis(3,5-dibromosalicyl)fumarate modified human hemoglobin; and Hb-FMDA, mono-(3,5-dibromosalicyl)-fumarate modified human hemoglobin.

α-chains and two native β-chains, as in the case of Hb-DBBF. With the advent of these and other promising candidates for *in vivo* applications [2], the question of safety and mechanisms of potential toxicity has become of great interest, especially in light of the large amounts necessary for transfusion and the compromised state of patients to whom these drugs will be given.

One potential mechanism of toxicity involves the reaction of hemoglobin-based red cell substitutes with oxygen metabolites formed upon reperfusion of ischemic tissues [6]. From previous studies on the oxidative damage of myoglobin [7], the toxicity could occur by at least two pathways. The first pathway involves the formation of protein-bound heme adducts, which have been shown to transform myoglobin from an oxygen storage protein to an oxidase resulting in oxidative stress [7]. The second pathway involves the degradation of heme and release of iron [8] that also could result in oxidative stress [7]. In this respect, desferrioxamine has been shown to reduce some of the toxicities associated with infusion of hemoglobin, presumably by chelating free iron [2, 9, 10] although other mechanisms may also be responsible [11]. In the present study we have found, with the use of a simple HPLC assay, that HbA_0 and Hb-DBBF were susceptible to oxidative heme degradation whereas Hb-FMDA was highly resistant. The information obtained from these studies may be helpful in the design and development of safer agents for use as red cell substitutes.

MATERIALS AND METHODS

Materials. Chromatographically purified HbA_0 and Hb-DBBF prepared as previously described [4, 12] were gifts from the Letterman Army Institute of Research (San Francisco, CA). Hb-FMDA was prepared as previously described [5] and was a gift from Dr. E. Bucci (University of Maryland, Baltimore, MD). Oxyhemoglobin, methemoglobin, and hemichrome concentrations were calculated from absorbance of solutions at 560, 576, and 630 nm with the use of extinction coefficients previously reported for each species at these wavelengths [13]. Multicomponent analysis of the solutions of these hemoglobins revealed the following initial proportions: 92–94% oxyhemoglobin, 3.4–4.4% methemoglobin, and 2–5% hemichrome. H_2O_2 was purchased from Fisher (Pittsburgh, PA) as a 30% solution. Chelex 100 was purchased from BioRad (Melville, NY). Myoglobin from horse heart was purchased from Sigma (St. Louis, MO). Bathophenanthroline disulfonic acid was from ICN Biochemicals (Cleveland, OH).

Auto-oxidation kinetics of hemoglobins. Auto-oxidation experiments for hemoglobin samples (20 μM) were carried out in sealed cuvettes under air at 37°. Absorbance changes due to auto-oxidation in the range of 500–700 nm were recorded with a spectrophotometer (Hitachi SU 2000), interfaced with a computer for multicomponent analysis. First-order auto-oxidation rate constants derived from a plot of percent oxyHb versus time were fitted to a single exponential expression using a non-linear least-squares curve-fitting program [14]. All hemoglobins were converted to the ferrous oxy state prior to each auto-oxidation experiment as described [15].

Treatment of hemoglobins with H_2O_2 and analysis of reaction mixtures by HPLC. The hemoglobins (250 μM) were treated with H_2O_2 (250 or 2500 μM) in a total volume of 1.0 mL of either 50 mM potassium phosphate (pH 7.4) or 50 mM sodium acetate (pH 4.7) for 1 hr at room temperature. These conditions were chosen to reproduce that of a previous study [16] on the formation of heme-protein adducts of myoglobin. In this study, greater amounts of protein-bound heme were formed at a pH of 4.7 relative to that at a pH of 7.4. All buffers were treated with chelex before use. Samples were kept on ice until analysis by HPLC. HPLC was performed with the use of a Waters instrument (Millipore Corp., Milford, MA) consisting of a 600E gradient system controller and a 490E variable wavelength detector. The data were collected with the use of a Nelson 760 Series system (PE Nelson, Cupertino, CA). An aliquot (25 μL) of the reaction mixture was injected onto a reverse-phase HPLC column (C4 Vydac 0.46 × 25 cm) equilibrated with 36% solution A (0.1% trifluoroacetic acid) and 64% solution B (0.1% trifluoroacetic acid in CH_3CN). The flow rate was 1.0 mL/min. A gradient (Waters curve 8) to 51% solution B was run over 20 min and then a linear gradient to 100% solution B was run over the next 10 min. The absorbance at 400 and 220 nm was measured.

Measurement of oxygen consumption. An aliquot (40 μL) of the reaction mixture was placed into a total volume of 1.0 mL of 50 mM potassium phosphate (pH 7.4) and 10 mM ascorbate to give a final hemoprotein concentration of 10 μM. Oxygen consumption of the samples was monitored with a Clark-type electrode on a Yellow Springs 5300 monitor (Yellow Springs, OH).

Measurement of released iron. Iron release was measured on hemoglobin reaction mixtures by a colorimetric method as described previously [17]. In short, an aliquot (100 μL) of the reaction mixture was treated with 50 μL of 11% trichloroacetic acid to precipitate the protein. The sample was spun in a centrifuge (Eppendorf model 5415 C) at 16,000 *g* for 5 min. To a 100-μL aliquot of the supernatant was added solid ascorbate [18] and color reagent [17].

RESULTS

The initial rates of auto-oxidation calculated for Hb-DBBF, Hb-FMDA, and HbA_0 from three separate experiments were 0.34 ± 0.1, 0.12 ± 0.05, and 0.28 ± 0.01 hr^{-1}, respectively. The initial auto-oxidation of Hb-DBBF was found to be 2.8 times as fast as that of Hb-FMDA, in accord with a previous report [19]. Figure 2 shows spectral changes indicative of the differences in the rate of oxidation of HbA_0, Hb-FMDA and Hb-DBBF during exposure to an equimolar concentration of H_2O_2 and a 10-fold molar excess of H_2O_2. Addition of an equimolar concentration of H_2O_2 induced little change in the spectra of oxyform of Hb-FMDA in contrast to HbA_0 and Hb-DBBF. Exposure of the three

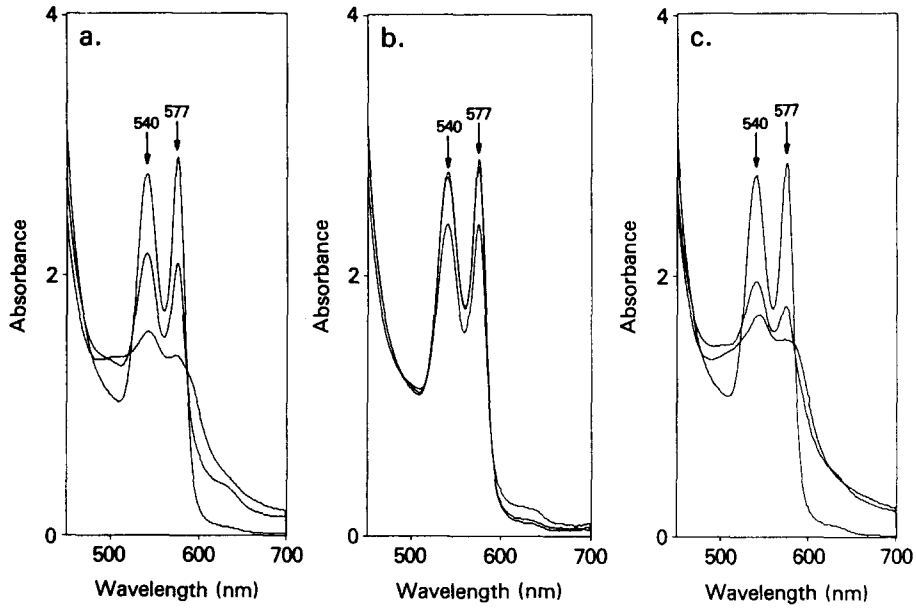

Fig. 2. Spectral changes during the oxidation of HbA_0 (panel a), Hb-FMDA (panel b) and Hb-DBBF (panel c) by H_2O_2. Hemoglobins (20 μM in heme) were incubated with either 20 or 200 μM H_2O_2 for 1 hr at 37° in 50 mM phosphate buffer, pH 7.4. Spectral changes due to oxidation were recorded, and the relative proportions of the oxidation products were calculated as previously described [13]. The spectra at 5 min are shown. No further spectral changes were observed for Hb-DBBF and Hb-FMDA for up to 1 hr, whereas HbA_0 reverted to methemoglobin. Spectra of oxyhemoglobin, were taken (from top to bottom) at time zero, after a 5-min incubation with H_2O_2 (20 μM), and after a 5-min incubation with excess H_2O_2 (200 μM).

hemoglobins to excess H_2O_2 resulted in formation of both ferric and ferryl forms, where the ferryl form was distinguished by peaks at 545 and 580 nm [20]. However, Hb-FMDA appeared to be more stable to oxidative modification and ferrylhemoglobin formation [21].

The effects of H_2O_2 treatment on HbA_0, Hb-DBBF, and Hb-FMDA were examined by the use of HPLC. Protein and heme components were detected by their absorbance at 220 and 400 nm, respectively (Fig. 3) [22]. Figure 3A shows the HPLC profile of human HbA_0. The major fractions with absorption at 220 nm corresponded to the α- and β-chains and the major fraction with absorption at 400 nm corresponded to heme [23]. The fraction containing material with absorbance at 400 nm that co-eluted with the protein corresponded to protein-bound heme [24]. Treatment of HbA_0 with a stoichiometric amount of H_2O_2 at pH 4.7 led to a small loss of heme and alteration of the β-chain with concomitant increase in protein-bound heme (Fig. 3B). The susceptibility of the β-chain to covalent alteration has been noted previously for the $BrCCl_3$ reaction with hemoglobin [23]. Treatment with a 10-fold excess of H_2O_2 under the same conditions led to a greater loss of heme, alteration of both the α- and β-chains, and a greater increase in protein-bound heme (Fig. 3C). Figure 3D shows the HPLC profile of untreated Hb-DBBF. Its α'-chain eluted later relative to the native α-chain because both α-chains were chemically cross-linked (for structure see Fig. 1). Treatment of Hb-DBBF at acidic pH with a stoichiometric amount of H_2O_2 gave a greater loss of heme and destruction of both protein chains in comparison to that of HbA_0 (Fig. 3E). The β'-chain was preferentially degraded, apparently due to destabilization of this chain by the cross-linking of the α-chains. Complete loss of heme and destruction of the protein were found after treatment with a 10-fold excess of H_2O_2 (Fig. 3F). In contrast, Hb-FMDA, whose β-chains had been pseudo-cross-linked and exhibited a greater retention time in comparison to that of native β-chain, was unaltered at all concentrations of H_2O_2 (Fig. 3, G–I).

Figure 4 shows the amounts of heme lost and protein-bound heme formed from HbA_0, Hb-FMDA, and Hb-DBBF after treatment with 1- and 10-fold excesses of H_2O_2 at pH 4.7 (A and B) and pH 7.4 (C and D). Although native hemoglobin was clearly susceptible to oxidative damage at acidic pH, at neutral pH there was no loss of heme (Fig. 4). Hb-DBBF in comparison was highly susceptible to oxidative modification at both acidic and neutral pH. The greater amounts of protein-bound heme formed from Hb-DBBF and HbA_0 under acidic conditions is consistent with that found for the reaction of myoglobin with H_2O_2 [16]. However, formation of protein-bound heme products do not account for all the heme altered. Hb-FMDA was highly resistant to oxidative modification at both acidic and neutral conditions. Furthermore, approximately 50% of the iron (98.6 ± 11.4 nmol iron from

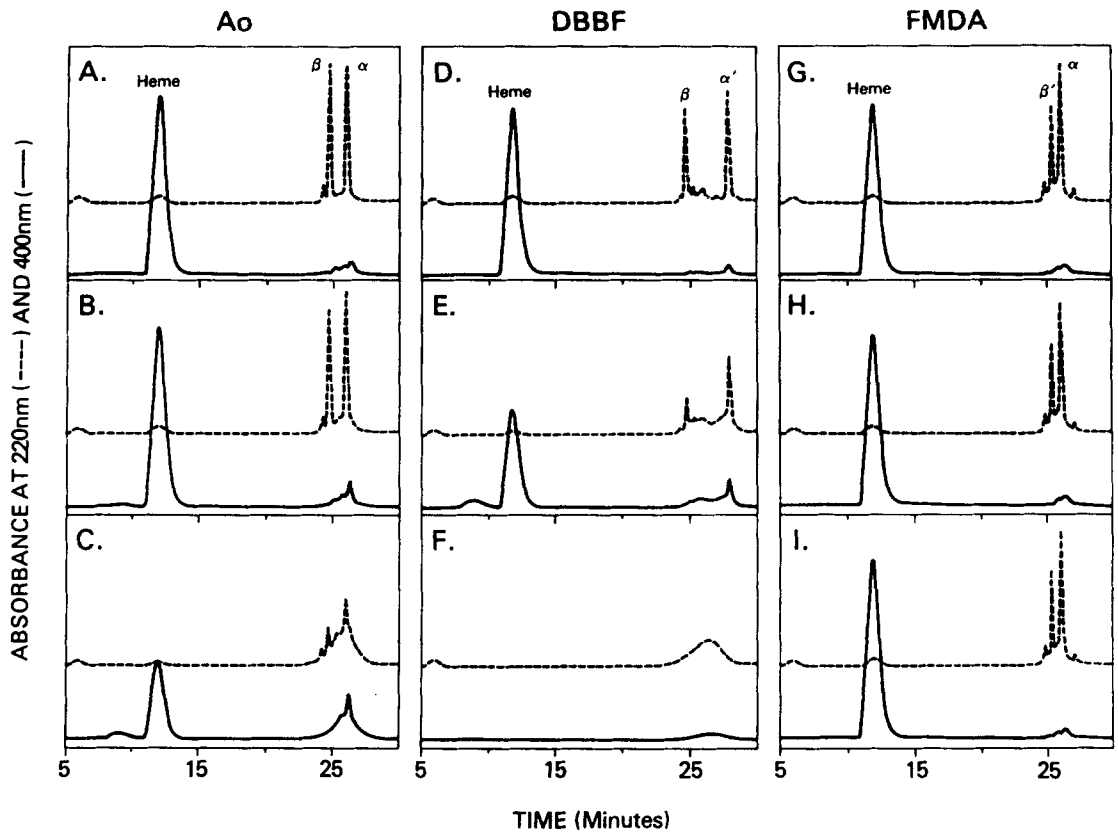

Fig. 3. HPLC profile of untreated and H_2O_2-treated HbA_0, Hb-DBBF, and Hb-FMDA. Panels A, D, and G: untreated; Panels B, E, and H: hemoglobins treated with stoichiometric amounts of H_2O_2 (250 μM); Panels C, F and I: hemoglobins treated with a 10-fold excess of H_2O_2 (2500 μM). The pH of the buffer was 4.7.

a total of 200 nmol of the starting hemoglobin, mean ± SD, N = 3) of Hb-DBBF was released after a 60-min treatment with 10-fold excess H_2O_2 at acidic pH, whereas Hb-FMDA under the same conditions did not release iron by the action of H_2O_2.

Previous studies have shown that treatment of myoglobin with stoichiometric amounts of H_2O_2 leads to formation of the protein-bound heme adduct and transformation to an oxidase [7]. This phenomenon could be clearly seen by treating myoglobin with one equivalent of H_2O_2 for 1 hr at an acid pH, taking an aliquot of this reaction mixture and diluting into an assay mixture containing ascorbate as a reducing agent, and measuring oxygen consumption (Fig. 5). Under the same conditions, however, oxygen consumption from H_2O_2-treated HbA_0, Hb-FMDA, or Hb-DBBF was not detected. Analysis of the Hb-DBBF sample with the use of HPLC as above showed degradation of protein and loss of heme (data not shown).

DISCUSSION

Superoxide radicals are released during the spontaneous oxidation of hemoglobin [25]. Although the mechanism of auto-oxidation is different from that of chemical oxidation of oxyhemoglobins, both follow common pathways in which metHb is initially formed followed by hemichrome formation and precipitation [26]. Although the rate of auto-oxidation of human hemoglobin is generally low, subtle changes in the heme pockets can produce appreciable changes in this rate [21, 27]. The spectral evidence presented here demonstrate clear differences between HbA_0, Hb-FMDA, and Hb-DBBF, not only in the initial rates of auto-oxidation but also in their stability under oxidative challenge by H_2O_2.

We have also shown in this study that the oxidative damage to the protein as well as the heme prosthetic group of native and chemically modified human hemoglobins can be conveniently assessed by an HPLC assay. With the use of this assay, we have found that Hb-FMDA was highly resistant to damage by H_2O_2, whereas under the same conditions Hb-DBBF exhibited significant breakdown of its protein and heme moieties. HbA_0 was of intermediate stability with heme and protein alteration occurring only under acidic conditions.

The oxidative destruction of Hb-DBBF is a potential mechanism of toxicity to be considered in the context of its use as a reperfusion agent. In

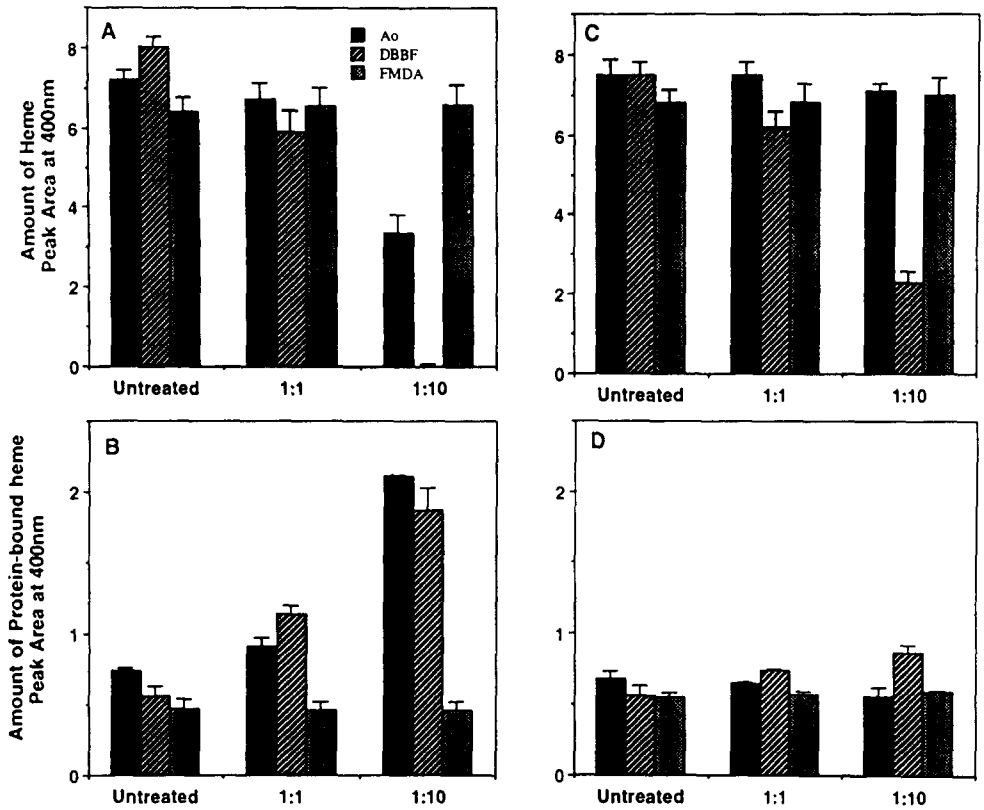

Fig. 4. Relative amount of heme and protein-bound heme after treatment of HbA_0, Hb-DBBF, and Hb-FMDA with H_2O_2. Panels A and B: hemoglobins were treated at pH 4.7; Panels C and D: hemoglobins were treated at pH 7.4. The peak areas at 400 nm were calculated from HPLC profiles. The data for the hemoglobins treated at pH 4.7 were calculated from the profiles shown in Fig. 3. The ratio of H_2O_2 to hemoglobin is indicated. These values are means ± SD, N = 3.

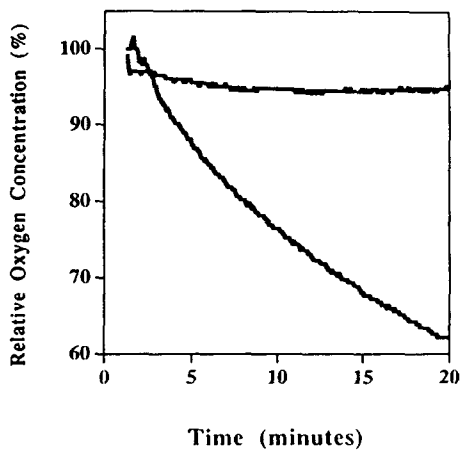

Fig. 5. Oxygen consumption of H_2O_2-treated myoglobin (lower trace) or hemoglobins (upper trace). Myoglobin and HbA_0, Hb-DBBF, and Hb-FMDA were treated with stoichiometric amounts of H_2O_2 at pH 4.7 for 1 hr, and an aliquot of the reaction mixture was taken for measurement of oxygen consumption as described under Materials and Methods. All hemoglobin samples gave the same results; only the Hb-DBBF data were plotted.

analogy to the oxidative modification of the heme of myoglobin [7], the alteration of Hb-DBBF to give protein-bound heme products could be a deleterious pathway leading to oxidase activity and heme degradation. In this regard, we have found in this study that H_2O_2-treated Hb-DBBF was not as efficient in catalyzing oxidase activity as a similarly treated myoglobin sample. This could reflect the lower levels of protein-bound heme formed in the hemoglobin sample, relative to that of the myoglobin sample, as well as the propensity of the protein-bound heme adduct to reduce oxygen to give the oxidase activity. Perhaps of greater significance was the observation that heme of Hb-DBBF was destroyed completely after treatment with excess H_2O_2. This finding clearly indicated that the porphyrin ring of heme was destroyed and iron was released. Free iron is widely thought to be toxic due to its ability to form deleterious oxygen radicals [6, 10, 28–35]. Evidence that desferrioxamine reduces some of the toxicities associated with infusion of hemoglobin [2, 9, 10] also supports this concept, although other toxic mechanisms may also be involved. Furthermore, a recent study has reported liver and kidney damage in swine perfused with Hb-DBBF, which may, in part, be due to iron overload and free radical injury [36]. Consistent with our findings, administration of Hb-FMDA, as well as

analogous derivatives from porcine and bovine hemoglobins, produced no abnormal renal function in rats [37]. Although we could not find any reports of toxicity for Hb-FMDA, it should be noted that, at present, more extensive information is available for Hb-DBBF than for Hb-FMDA. In addition, a considerable amount of Hb-FMDA-derived products have been shown to be excreted in the urine, which is not consistent with the stability of Hb-FMDA observed under *in vitro* conditions [37]. Nonetheless, the potential toxicities from iron overload and free radical injury described above as well as the enhanced likelihood for infection due to iron release [38] clearly indicate the importance of this process.

The exact mechanism responsible for the differences in susceptibilities to oxidative damage among the hemoglobins is not known. The differences on the HPLC profile after a 1-hr incubation with H_2O_2 may reflect the differences in oxidation rates and/or the formation of a stable ferryl species observed with Hb-DBBF. The heme pocket of Hb-FMDA appears to be less accessible to the oxidative attack of peroxide [21]; however, this may not be the only mechanism as HbA_0 is accessible to oxidative attack but its prosthetic heme is not damaged to the extent of that found for Hb-DBBF. The stabilization of the β-chain seemed to be important due to the greater susceptibility of this subunit to oxidative damage. Although at neutral pH both Hb-FMDA and HbA_0 were resistant to oxidative damage, only Hb-FMDA was stable at acidic pH. This may be explained by the stability of the tetrameric structure of Hb-FMDA under acidic conditions [5]. It is possible that Hb-FMDA was locked into a stable conformation, perhaps similar to that of HbA_0 at neutral pH, by cross-linking of the β-chains. The cross-linking of the α-chains, however, appeared to destabilize the conformation with respect to oxidative modification of the β-chain, suggesting that the conformation of the α-chain can affect the entire tetrameric structure.

Acellular hemoglobin, free of blood-borne diseases and compatibility concerns, has been an attractive possibility as a blood substitute. Assuming that chemical and/or genetic manipulations can completely resolve the problems of the short retention of hemoglobin in circulation and its nephrotoxicity, the next step in the design of a viable hemoglobin-based blood substitute is to produce a protein that is resistant to auto-oxidation and to oxidative damage, and will be able to discriminate against physiological mediators such as nitric oxide [39]. With respect to the stability, the susceptibility of other genetically and chemically altered hemoglobin products to oxidative damage could be assessed by the HPLC method described here. The elucidation of the exact molecular determinants involved in this stabilization process may aid in the design of safer hemoglobin-based red cell substitutes.

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