Effects of Bacterial Endotoxin on Human Cross-Linked and Native Hemoglobins[†]

Wieslaw Kaca,[‡,§] Robert I. Roth,[*,‡,||] Kim D. Vandegriff,[⊥] G. Chi Chen,[▽] Frans A. Kuypers,[#] Robert M. Winslow,[⊥] and Jack Levin[‡,||]

Department of Laboratory Medicine and Cardiovascular Research Institute, University of California School of Medicine, and Veterans Administration Medical Center, San Francisco, California 94121, Department of Medicine, University of California School of Medicine, San Diego, California 92161, and Children's Hospital, Oakland Research Institute, Oakland, California 94609

Received March 31, 1995; Revised Manuscript Received June 12, 1995[⊗]

ABSTRACT: Previous investigations have demonstrated that hemoglobin (Hb) is a binding protein for bacterial endotoxin (lipopolysaccharide, LPS) and that the structure and biological activity of LPS are altered in the presence of Hb. In the present study, the influence of LPS on the structure of native human HbA_0 and covalently cross-linked Hb (ααHb) was studied by analyzing the absorption and circular dichroic spectra of Hb in the wavelength region of 200–650 nm. Incubation of oxyHb with each of several LPSs resulted in a decrease in the intensity of the major Soret band at 414 nm with a shift in the maximum peak to 410 nm, decreases in the intensities of the major visible region peaks at 541 and 577 nm, and the appearance of increased absorbance in the visible region in the range of 630 nm. The resultant spectra are characteristic of methemoglobin formation. These spectral changes were time-dependent and LPS-concentration-dependent. Production of methemoglobin was prominent with chemically modified, partially deacetylated rough LPS, and was observed to a lesser extent both with native, complete rough and with native smooth LPSs. The influence of LPS on the absorption spectrum of methemoglobin also was directly tested. The conversion of methemoglobin to hemichrome in the presence of LPS was demonstrated and was shown to be reversible. Analysis of circular dichroic spectra of Hb demonstrated LPS-induced spectral changes in the visible and Soret regions consistent with the production of a substantial quantity of metHb, but did not demonstrate any alteration in the far-UV region (210–240 nm). Moreover, Hb oxygen affinity was only slightly altered after incubation with any of several LPSs. In conclusion, analyses of absorption and circular dichroic spectra reveal the potential of LPS to produce a facilitated oxidation of both αα-cross-linked human Hb and native human HbA_0, without substantial changes in the secondary structure of the globin.

Purified cell-free human hemoglobin (Hb)[1] is being evaluated as a substitute for the transfusion of red cells. However, in vivo toxicities remain a major limitation for the clinical use of Hb, and a role for bacterial endotoxin (lipopolysaccharide, LPS) in the observed toxicity has been proposed (Roth & Kaca, 1994). We recently have shown that purified, native human hemoglobin (HbA_0) or a chemically cross-linked human hemoglobin (ααHb) forms complexes with bacterial endotoxin (LPS) (Kaca et al., 1994a), and that complex formation significantly enhances deleterious biological activities of LPS in vitro, including activation of coagulation (Kaca et al., 1994b), stimulation of mononuclear cell tissue factor production (Roth et al., 1993), and transformation of endothelial cells into a procoagulant surface (Roth, 1994). In vivo, Hb and LPS have been demonstrated by others to synergistically stimulate coagulation, complement proteolytic cascades, and increase lethality (White et al., 1986; Feola et al., 1988a,b). Comparisons of several chemically distinct LPSs, including chemically modified species that allow detailed investigation of LPS structure/function relationships, have identified LPS chemical moieties that are critical for this interaction (Kaca et al., 1994b). Hb/LPS complex formation also was associated with a decrease in the aggregation state of LPS, i.e., a decrease in size and density of the LPS macromolecular structure (Kaca et al., 1994a), and we proposed that this alteration in LPS structure may constitute a physiologically significant process when Hb and LPS coexist intravascularly. The affinity of Hb for LPS has been shown to be high (Kaca et al., 1994a), and unexpectedly high levels of LPS contamination of Hb solutions have been demonstrated (Roth et al., 1993), thus making the in vivo interaction of Hb and LPS of considerable concern for the development of this potential red cell substitute.

[†] Supported, in part, by U.S. Army Medical Research, Development, Acquisition and Logistics Command Research Contracts, MIPR 90 MM0535 and MM4585HL7. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. This work was also supported, in part, by Research Grant DK 43102 from the NIDDKD, National Institutes of Health; Research Grant HL 14237 (Arteriosclerosis Specialized Center of Research); and the Veterans Administration.

* Direct correspondence to this author at Veterans Administration Medical Center (111 H2), 4150 Clement St., San Francisco, CA 94121. Telephone: (415) 221-4810, ext. 3679. Fax: (415) 221-7542.

[‡] Department of Laboratory Medicine, University of California School of Medicine, San Francisco.

[§] Present address: Institute of Microbiology and Immunology, University of Lodz, Lodz, Poland.

[||] Veterans Administration Medical Center.

[⊥] Department of Medicine, University of California School of Medicine, San Diego.

[▽] Cardiovascular Research Institute, University of California School of Medicine, San Francisco.

[#] Children's Hospital.

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: hemoglobin, Hb; bis(3,5-dibromosalicyl) fumarate αα-cross-linked hemoglobin, ααHb; αα-cross-linked methemoglobin, metααHb; bacterial lipopolysaccharide, LPS.

0006-2960/95/0434-11176$09.00/0 © 1995 American Chemical Society

Cell-free Hb is particularly susceptible to oxidation and denaturation, and Hb or its degradation products, heme or iron, can act as Fenton reagents to promote hydroxyl radical formation (Sadrzadeh et al., 1984). In animals, Hb infusion has been shown to result in the generation of reactive oxygen compounds (Feola et al., 1988b) and has been associated with lipid peroxidation and with organ failure (Paller, 1988). These deleterious consequences of Hb infusion can be reduced by iron chelation, suggesting that the instability of cell-free Hb, the release of iron, and the resultant production of toxic oxygen- and lipid-derived free radicals may be responsible for some of the observed in vivo deleterious consequences of Hb infusion (Feola et al., 1988a; Paller, 1988; Faasen et al., 1988; Simoni et al., 1990). In addition, the presence of Hb, or free iron which is released from Hb during the process of Hb oxidation and denaturation, enhances mortality associated with Gram-negative infection (Eaton et al., 1982), and both free iron and free heme potentiate oxidant-mediated cell damage (Faasen et al., 1988; Balla et al., 1991). Therefore, an important aspect of the development of cell-free Hb solutions has been the evaluation of various derivatized Hb preparations for enhanced stability.

Although our previous studies have demonstrated that Hb binding has a major influence on LPS structure and function, it was not known whether this interaction reciprocally changes the conformation or stability of Hb. However, it has been reported that methemoglobin and oxyhemoglobin are converted to hemichrome in the presence of fatty acids (Akherm et al., 1989), and since fatty acids are one of the main components of LPS (Rietschel et al., 1991), we thought it possible that LPS could similarly alter Hb. In order to assess whether LPS can induce protein structural changes in Hb leading to its oxidation, we studied the abilities of several purified and chemically characterized LPSs to alter the absorption and circular dichroic spectra of both native and chemically modified human Hb.

MATERIALS AND METHODS

Reagents. Human serum albumin (HSA) (25%, for injection) was purchased from Nybcen (New York, NY). Sterile, endotoxin-free 0.9% NaCl was purchased from Travenol Laboratories (Deerfield, IL). Xylenol orange (*o*-cresolsulfonephthalein-3′,3″-bis(methyliminodiacetic acid), sodium salt) was obtained from the Aldrich Chemical Co. (Milwaukee, WI); butylated hydroxytoluene and ammonium ferrous sulfate were obtained from Sigma (St. Louis, MO).

Hemoglobin. Human hemoglobin was prepared and purified, as described previously (Winslow et al., 1995), by the Blood Research Division at the Letterman Army Institute of Research, San Francisco, CA. Human hemoglobin was covalently cross-linked between Lys 99 residues of the α subunits (ααHb) with bis(3,5-dibromosalicyl) fumarate (DBBF). The ααHb stock solution was 9.6 g/dL, pH 7.4, in Ringers acetate and contained less than 0.4 EU/mL endotoxin (referenced to *Escherichia coli* lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test (Levin & Bang, 1968). The ααHb stock solution was stored at −70 °C and then diluted with sterile, pyrogen-free 0.9% NaCl prior to use. Purified non-cross-linked human hemoglobin A_0 (HbA_0), 8.4 g/dL, was prepared from Hb by ion-exchange HPLC (Christensen et al., 1988). All hemoglobin concentrations are given on a per heme basis.

Methemoglobin (metααHb) was prepared by oxidation of ααHb with potassium ferricyanide (Di Iorio, 1981). The reaction was carried out at 4 °C in the dark for 30 min with occasional mixing. The molar ratio of $K_3Fe(CN)_3$ to ααHb was 1.2:1. MetααHb was separated from ferrocyanide and residual ferricyanide by Sephadex G-25 M (PD-10, Pharmacia, Piscataway, NJ) column chromatography. The methemoglobin stock solution (3 mg/ml) was stored at 4 °C in 0.05 M potassium phosphate (pH 7.4) and was used within 2 weeks.

Bacterial Endotoxin (Lipopolysaccharide, LPS). Smooth LPSs were extracted with phenol−water according to the Westphal method (Westphal & Jann, 1965), whereas rough LPSs were extracted by the phenol−chloroform−petroleum ether (PCP) method of Galanos (Galanos et al., 1969). Crude LPSs were further purified by sequential treatment with RNAse and DNAse, followed by ultracentrifugation at 100000*g* for 3 h, as described previously (Kaca et al., 1987). All LPSs were essentially free of nucleic acids (indicated by a lack of absorbance at 260 nm), and contamination by proteins was less than 2% on the basis of BCA protein assays.

Crude *E. coli* 026 (smooth) LPS was purchased from Difco and then further purified as described above. *Proteus mirabilis* O3 smooth strain LPS was provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland, and was purified as described above. The deep rough *Salmonella minnesota* 595 LPS, Re type, was extracted by the PCP method (Galanos et al., 1969) and then further purified. *S. minnesota* 595 lipid A was prepared from 134 mg of Re 595 LPS by hydrolysis of the ester-bound ketodeoxyoctulosonic (KDO) residue with sodium acetate buffer (pH 4.4) for 1 h at 100 °C (Brade et al., 1983). The hydrolysate was dialyzed to obtain purified lipid A (67% recovery from the Re 595 starting material) and then lyophilized and stored at 4 °C. KDO (83.5%) from LPS 595 was released by this procedure, as determined colorimetrically using thiobarbituric acid. Singly deacetylated *S. minnesota* 595 LPS (OH37 LPS) was produced from 380 mg of Re 595 LPS by hydrolysis of a single ester-bound 3-hydroxytetradecanoyl fatty acid from the reducing glucosamine of LPS with 0.2 N NaOH for 30 min at 37 °C (Myers et al., 1990). The hydrolysate then was cooled to 4 °C and neutralized to pH 6.5 with 0.1 N HCl. Released fatty acids were extracted by $CHCl_3$/MeOH (2:1) followed by precipitation of OH37 LPS by EtOH/acetone (2:1) at 4 °C. The OH37 LPS sediment was centrifuged at 10000*g* for 30 min, washed twice with cold EtOH, resuspended in water, and lyophilized (61% recovery of the starting material was obtained). One hundred percent removal of 3-hydroxytetradecanoyl fatty acid was demonstrated by gas−liquid chromatography (GLC) analysis of methyl esters of fatty acids isolated from OH37 LPS.

Absorption Spectra of Hemoglobin in the Presence of Endotoxin. Solutions of LPS and Hb were prepared in 0.05 M Tris buffer, pH 7.4, in phosphate-buffered saline (PBS), pH 7.4, or in endotoxin-free 0.9% NaCl. The Hb/LPS mixtures were incubated at 37 °C for various periods of time, and Hb absorption spectra (between 350 and 730 nm) were then recorded using either a Lambda 3B (Perkin-Elmer, Norwalk, CT) or an upgraded Cary-14 (On-Line Instrument Systems, Bogart, GA) UV/vis spectrophotometer. The percentages of oxyHb, metHb, and hemichromes were

determined by the method described by Winterbourn (Winterbourn, 1990). Each experiment was performed at least three times, and representative data are presented.

Circular Dichroism. Circular dichroic (CD) spectra of Hb and Hb/LPS complexes were measured with a Jasco J-500A spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). Samples were measured in 50 mM potassium phosphate, pH 7.4. The data are expressed as molar ellipticity on the basis of heme concentration. α-Helical content was estimated from the mean residue ellipticity at 222 nm on the basis of amino acid residues according to the reference value of $[\theta]_{222} = -30\,300 f_H - 2340$ (Chen et al., 1972), where f_H represents the fraction of α-helix.

Oxygen Affinity Measurements. P_{50} values were determined for Hb and Hb/LPS complexes from O_2 equilibrium curves obtained with a Hemox-Analyzer (TCS Medical Products, Philadelphia, PA). Samples were diluted in Hemox buffer (pH 7.4) provided by the manufacturer.

Measurement of Peroxides and Oxidants. Peroxides or other oxidizing agents were determined with the ferrous oxidation/xylenol orange (FOX) reagent, prepared as described previously (Jiang et al., 1992). Briefly, ammonium iron (Fe^{2+}) sulfate, xylenol orange, and butylated hydroxytoluene were dissolved in 90% methanol/10% sulfuric acid to produce the FOX reagent. Various concentrations of ααHb alone or ααHb and OH37 LPS in endotoxin-free 0.9% NaCl were incubated at 37 °C for 60 min, and then 2 mL of FOX reagent was added to 0.2 mL of each sample. Following an additional 30-min incubation at room temperature, absorbances of Fe^{3+}−xylenol orange complexes were measured at 570 nm with a Lambda 3B UV/vis spectrophotometer (Perkin-Elmer, Norwalk, CT). Samples were assayed in duplicate or triplicate, and mean values are presented.

RESULTS

Absorption Spectroscopy. Changes in the absorption spectra of ααHb and HbA_0 resulting from their interaction with LPS were examined with three chemically distinct enterobacterial LPSs (Figure 1). In the presence of LPSs, HbA_0 (Figure 1A) and ααHb (Figure 1B) each underwent substantial spectral alterations, characterized by decreases in the major visible region peaks at 541 and 577 nm and the appearance of an increased absorbance in the range of 630 nm. Also noted with each LPS was a decrease in the intensity of the major Soret peak of oxyHb at 414 nm (data not shown in Figure 1; see Figure 2). These spectral changes are characteristic of ferric Hb species (Winterbourn, 1990). The spectral changes were most prominent with deacetylated OH37 LPS (curve 4), with lesser changes induced by 595 LPS (curve 3) and 03 LPS (curve 2). The spectral changes in HbA_0 induced by OH37 LPS were slower in Tris buffer, pH 7.4 (Figure 1C, curve 4) than in 0.9% NaCl at pH 6.5 (Figure 1A), although similar spectral changes were eventually observed (after 18 h) at pH 7.4 (Figure 1D, curve 4). The pH of buffered incubations (pH 7.4) remained constant during the study; incubations performed in 0.9% NaCl showed slight pH differences between Hb alone (pH 6.5) and Hb/LPS mixtures (pH 6.5−6.8). The reduction of absorbance in the range of 630 nm to less than control (Hb alone, line 1), observed with 595 LPS/HbA_0 or 595 LPS/ααHb (Figure 1A−D, line 3), suggests that both Hbs increased the solubility of 595 LPS and therefore decreased its light scattering, in comparison to the light scattering by the less soluble 595 LPS sample in the reference cuvette.

Because the most prominent Hb spectral alterations were observed after incubation with OH37 LPS, we further characterized this interaction by investigating the time course (Figure 2 and 3) and LPS concentration dependence (Figure 4) of these spectral changes. These experiments were done in PBS (pH 7.4) to maintain constant pH and maximize Hb stability during the course of the reaction. In the Soret region, a time-dependent progressive loss of intensity of the major Soret peak at 414 nm was associated with a shift in the peak maximum to 410 nm (Figure 2A). Progressive decreases in the major visible region peaks at 541 and 577 nm (Figure 2B) also were observed over a 120-min time period and were accompanied by the appearance of a gradual increase in absorbance in the range of 630 nm.

From mathematical evaluation of these changing spectra (as described in Materials and Methods), the percentage of oxyααHb was shown to be substantially decreased (from 90% initially to 30%) after incubation with OH37 LPS for 120 min, and the calculated percentages of metααHb and hemichromes each were increased from 4% initially to 37% and 30%, respectively, after incubation for 120 min with OH37 LPS (Figure 3A, closed symbols). Time-dependent changes of the spectrum of native HbA_0 in the presence of OH37 LPS also were recorded, demonstrating alterations similar to those observed with ααHb. Increased proportions of $MetHbA_0$ and hemichromes to 35% and 39%, respectively, after a 60-min incubation at 37 °C accompanied a decrease in $oxyHbA_0$ to 26% (Figure 3B, closed symbols). Production of metHb preceded the production of hemichromes. In contrast to the prominent Hb oxidation forms induced by OH37 LPS, incubation of ααHb or HbA_0 in the absence of LPS resulted in minimal Hb oxidation (Figure 3A,B, open symbols).

The rate of conversion of oxyHb to metHb and hemichromes was shown to be dependent on LPS concentration (Figure 4). After incubation of ααHb with OH37 LPS for 1 h, spectral changes in the Soret and visible regions similar to those shown in Figure 2 were observed to be greatest at 1 mg/mL LPS, but were also produced by 0.5 mg/mL and 0.05 mg/mL LPS (spectra not shown for Figure 4). Apparent isosbestic points were present at 381 and 437 nm in the Soret region, and at 523 and 589 nm in the visible portion of the spectrum (spectra not shown). On the basis of mathematical evaluation of these spectra, LPS concentration-dependent conversion of ααHb to metααHb and hemichromes was demonstrated (Figure 4A). At the highest concentration of OH37 LPS studied (1 mg/ml), nearly all of the starting oxyααHb (89%) was converted to metααHb and hemichromes (46% and 41%, respectively) after 1 h. Similar to ααHb, LPS dose-dependent spectral changes were recorded for HbA_0 (data not shown), and nearly all of the $oxyHbA_0$ was converted to $metHbA_0$ (41%) and hemichromes (32%) after 1 h (Figure 4B). The spectral changes of ααHb and HbA_0 in the presence of various concentrations of OH37 LPS were also tested in unbuffered 0.9% NaCl, and similar extents of denaturation were observed (data not shown).

Since the time-dependent production of metHb from oxyHb in the presence of LPS preceded the production of hemichromes (Figure 3), we directly examined the ability of metHb to form hemichromes. MetααHb, produced as

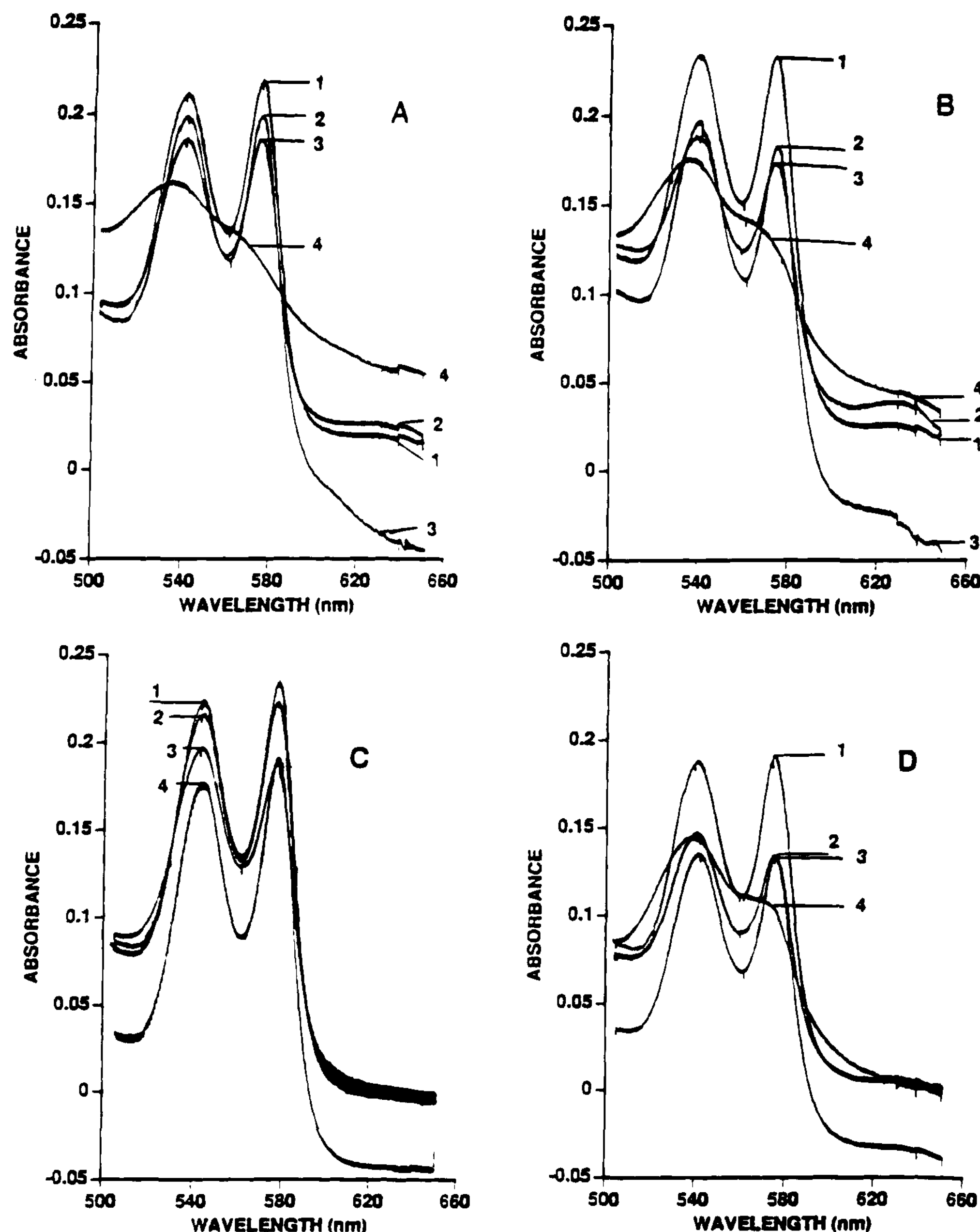

FIGURE 1: Hemoglobin absorption spectra in the absence and presence of LPS. Native oxyhemoglobin (HbA_0, 15 μM) and cross-linked oxyhemoglobin ($\alpha\alpha$Hb, 16 μM) preparations were incubated with each of several LPSs (each 1 mg/mL) at 37 °C, and Hb absorption spectra in the visible region were recorded. Hb in the absence of LPS is shown as line 1 in each graph. LPSs examined included *P. mirabilis* O3 LPS (line 2), *S. minnesota* 595 LPS (line 3), and *S. minnesota* 595 OH37 LPS (line 4). (A) Incubations with HbA_0 for 1 h in 0.9% NaCl. (B) Incubations with $\alpha\alpha$Hb for 1 h in 0.9% NaCl. (C) Incubations with HbA_0 for 1 h in 0.05 mM Tris buffer, pH 7.4. (D) Incubations with HbA_0 for 18 h in 0.05 mM Tris buffer, pH 7.4. In each experiment, the sample cuvette contained Hb with or without LPS, and the reference cuvette contained 0.9% NaCl (for Hb spectra alone) or LPS alone (1 mg/mL in 0.9% NaCl) (for Hb/LPS mixture spectra).

described in Materials and Methods, was incubated with OH37 LPS, and spectra were obtained (Figure 5). After the reaction was completed, the mixture of met$\alpha\alpha$Hb plus OH37 LPS (1 mg/mL) had a pH of 6.6; the pH of met$\alpha\alpha$Hb alone was 6.3. A time-dependent (Figure 5B) and LPS concentration-dependent (Figure 5C) increase in absorbance at 537 nm and decrease in absorbance of the prominent met$\alpha\alpha$Hb peak at 630 nm were observed, indicating transformation from high-spin met$\alpha\alpha$Hb to low-spin hemichromes. After a 10-min incubation with OH37 LPS (which generated curve 3 in Figure 5B), the Soret peak was not appreciably altered (Figure 5A). However, more prolonged incubation demonstrated a decrease in the intensity of the major Soret peak and a shift of the peak maximum from 405 to 411 nm (Figure 6, described below).

Since production of hemichromes from oxyHb can be either reversible or irreversible, we examined whether the addition of albumin, which forms complexes with LPS, allowed re-formation of met$\alpha\alpha$Hb from the hemichrome/LPS mixture (Figure 6). Human albumin was used because albumin is a well-characterized LPS binding protein (Galanos et al., 1972) and therefore had the potential ability to bind LPS already bound to hemichromes. Met$\alpha\alpha$Hb (Soret peak absorbance at 405 nm, Figure 6, spectrum 1) was first

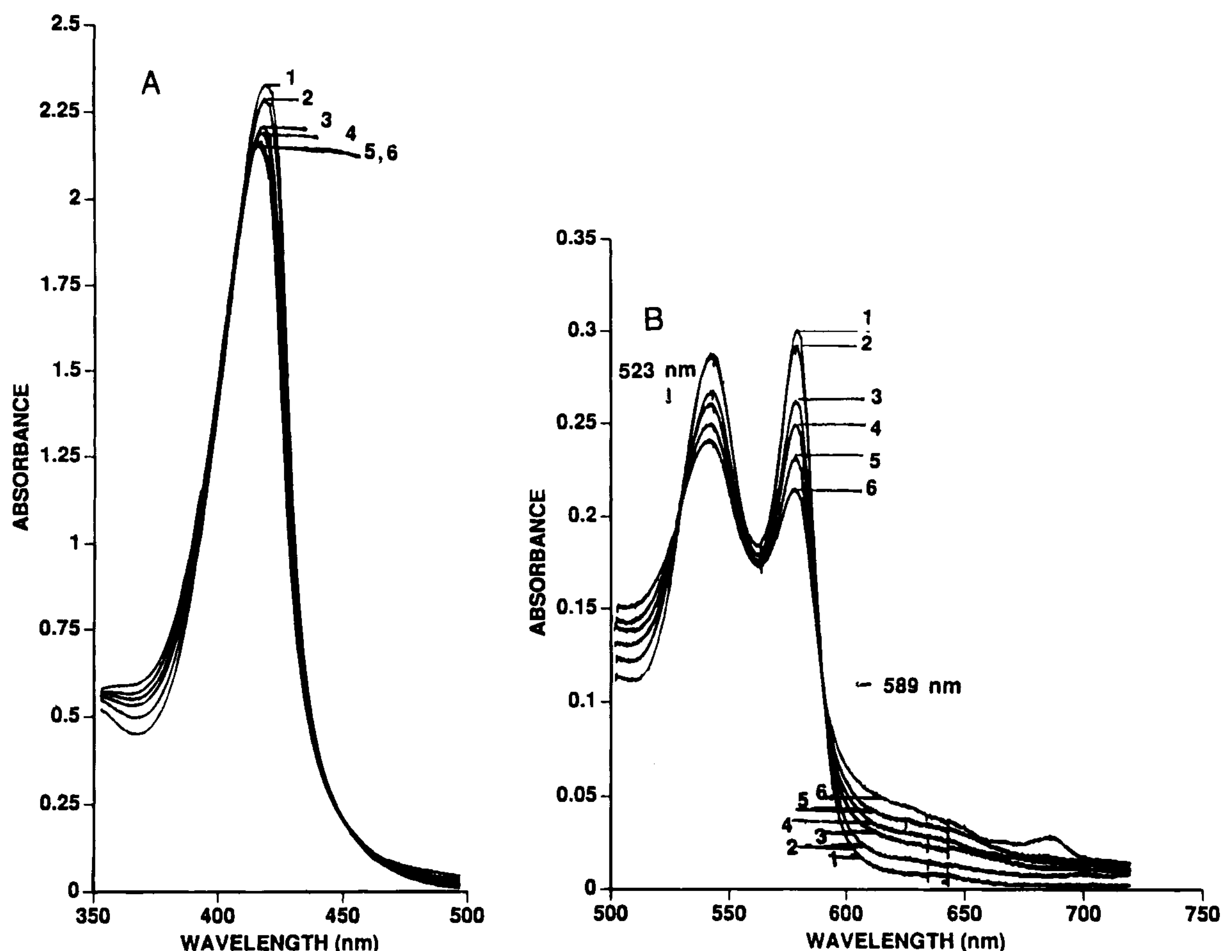

FIGURE 2: Time course of changes in the hemoglobin absorption spectrum in the presence of LPS. ααHb (21 μM in PBS, pH 7.4) was incubated at 37 °C in the presence of 0.3 mg/mL *S. minnesota* 595 OH37 LPS, and absorbance spectra in the Soret (A) and visible (B) regions of the Hb spectrum were obtained at various times of incubation. Line 1, initial spectrum of ααHb alone; line 2, 10 min; line 3, 20 min; line 4, 40 min; line 5, 90 min; line 6, 120 min. The sample cuvette contained Hb in PBS with or without LPS, and the reference cuvette contained PBS (for ααHb spectra alone) or LPS alone (0.3 mg/mL in PBS) (for ααHb/LPS mixture spectra). The arrows indicate the apparent isosbestic points.

incubated with OH37 LPS to produce hemichromes (Soret peak absorbance at 411 nm, Figure 6, spectrum 2). Human albumin then was added to the hemichrome/LPS mixture, and a shift of the Soret peak maximum from 411 nm back to 405 nm was subsequently observed (Figure 6, spectrum 3). These results are consistent with transformation of low-spin hemichromes back to high-spin metααHb, suggesting that the process of hemichrome formation in the presence of LPS was reversible. The transformation of hemichromes to metααHb was completed within 5 min at room temperature and was associated with a decrease in the amplitude of the peak absorbance at 405 nm in the final solution, as compared to the intensity of the initial metααHb absorbance peak before hemichrome formation. The decreased intensity of the 405-nm peak in the final metααHb/LPS mixture, after addition of albumin solution, suggested either that the LPS-induced formation of hemichromes was only partially reversible when LPS was removed (although no shoulder could be detected at 411 nm in Figure 6, spectrum 3) or that the process of reversing hemichromes back to metααHb was associated with a loss of heme from hemoglobin. Heme loss may have been associated with its binding to albumin, a well-known phenomenon (Vandegriff & Le Tellier, 1994). However, in the absence of LPS, heme loss from metααHb to albumin, as indicated by a decrease in absorbance at 405 nm, was not detectable before 15 min, and there was no shift of the peak maximum absorbance from 405 to 411 nm (data not shown).

Circular Dichroic Spectroscopy. The demonstration that Hb binding of LPS resulted in heme oxidation suggested that LPS binding could initially alter globin structure and secondarily destabilize the heme pocket. To evaluate this possibility, we used circular dichroic (CD) analysis of Hb to assess the ability of LPS to affect Hb secondary structure. Initially, we studied *S. minnesota* 595 OH37 LPS because Hb oxidation by this LPS was prominent and rapid. ααHb was incubated with OH37 LPS for 2 h, after which absorbance measurements of the starting Hb and the Hb/OH37 LPS mixture indicated that oxyHb had decreased from 97% to 50% with the production of 42% metHb and 8% hemichrome. Following incubation of Hb with LPS, we observed a decrease in the intensity of the CD peak at 579 nm, a decrease in intensity and a shift to lower wavelength of the Soret peak (from 420 nm to 418 nm), and a decrease in the intensity of the near-UV peaks at 259 and 265 nm (Figure 7). These changes in the Hb CD spectrum can be accounted for by the loss of a substantial fraction of oxyHb and the concomitant production of MetHb. Compared to Hb/LPS, MetHb by itself demonstrated more prominent changes from Hb alone in this wavelength region (i.e., 250–

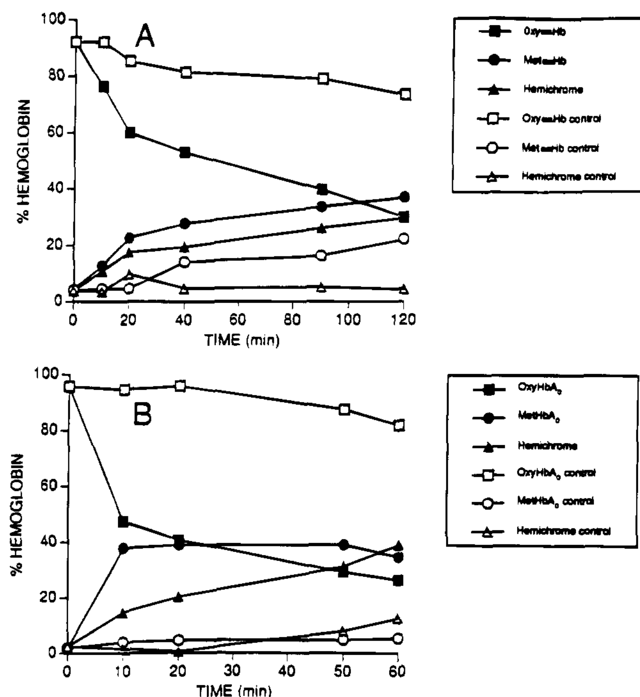

FIGURE 3: Time-dependent conversion of ααHb (A) and HbA_0 (B) to metHb and hemichromes in the presence of *S. minnesota* 595 OH37 LPS (0.3 and 0.8 mg/mL LPS incubated with ααHb and HbA_0, respectively). Percentages of oxyHb, metHb, and hemichromes were determined as described in Materials and Methods. Open symbols, Hb alone; closed symbols, Hb + LPS.

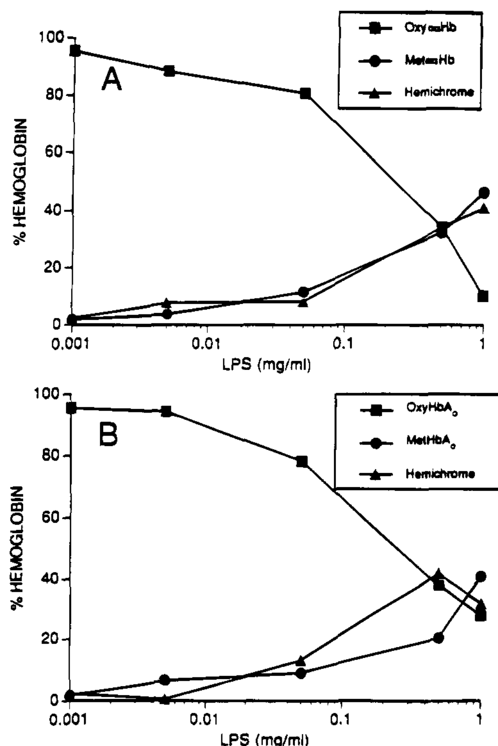

FIGURE 4: Conversion of ααHb (A) and HbA_0 (B) to metHb and hemichromes in the presence of various concentrations of *S. minnesota* 595 OH37 LPS after a 1-h incubation. Percentages of oxyHb, metHb, and hemichromes were determined as described in Materials and Methods.

600 nm), and also showed a considerable decrease in CD intensity in the far-UV region (Figure 7). In contrast, there was no important spectral change in the wavelength range of 210–240 nm after a 2-h incubation of Hb with OH37 LPS. α-Helical content was estimated to be 53% for the globin in either the absence or the presence of LPS. Therefore, although the visible and near-UV spectral data were consistent with destabilization of the heme, there was no evidence for alteration of globin structure. CD spectral changes induced by a smooth LPS (*E. coli* 026) (data not shown) were similar to those observed with *S. minnesota* 595 OH37 LPS, although an overnight incubation was required with *E. coli* 026 LPS in order to generate the altered CD spectra observed with OH37 LPS after 2 h.

P_{50} Measurements. We measured the oxygen affinity of Hb in the absence and presence of LPS in order to evaluate the possible influence of LPS binding on Hb function. These measurements were made after a 2-h incubation period, a time sufficient to result in Hb/LPS complex formation (Kaca et al., 1994a), but prior to the formation of substantial quantities of oxidized Hb species unable to bind oxygen. Hb at 1 mg/mL (16 μM) and 1 mg/mL of each LPS were utilized because the two components of Hb/LPS complexes are of approximately equal concentration by weight (Kaca et al., 1994a), and little unbound Hb is calculated to be present. P_{50} values for ααHb (26.6 mmHg) and HbA_0 (9.6 mmHg) were slightly decreased by both smooth and rough LPSs (Table 1). Non-cross-linked cell-free HbA_0, which exhibited high oxygen affinity (P_{50} = 9.6 mmHg) similar to that measured with lysed whole blood (P_{50} = 10.0 mmHg; data not shown), best demonstrated the trend toward higher oxygen affinity when in the presence of LPS (P_{50} = 7.3 mmHg in the presence of OH37 LPS).

FOX Assays for Oxidants. Because Hb oxidation is known to result in production of the free radical peroxide, and because our data indicated that LPS was capable of stimulating the production of metααHb and hemichromes, we used the FOX assay to investigate whether detectable Hb-derived oxidants were increased in the presence of LPS. The FOX assay is based on rapid peroxide-mediated oxidation of Fe^{2+} from ammonium ferrous sulfate to Fe^{3+}; the latter forms Fe^{3+}/xylenol orange complexes which are measured at 570 nm (Jiang et al., 1992). ααHb alone, at a concentration higher than 0.2 mg/mL, generated an agent that oxidized Fe^{2+} to Fe^{3+} in a concentration-dependent manner (Figure 8). The addition of 1 mg/mL OH37 LPS to Hb did not alter the concentration of detectable oxidizing products, and LPS alone did not generate oxidizing products (Figure 8, △). In confirmatory experiments, concentrations of other LPSs as great as 1 mg/mL similarly did not increase the concentration of detectable oxidizing products derived from Hb (data not shown). Addition of Hb alone or Hb plus LPS to FOX reagent, prepared without ammonium ferrous sulfate, did not produce Fe^{3+}/xylenol orange complexes; i.e., there was no detectable absorbance at 570 nm. This indicated that Fe^{3+} from Hb was not the source of the Fe^{3+} that bound to xylenol orange.

DISCUSSION

Cross-linked hemoglobin is being developed as a potential red blood cell substitute (DeVenuto & Zegna, 1982; Sehgal et al., 1984; Winslow, 1992; Vandegriff, 1993). To date, clinical use of Hb solutions has been restricted because of their toxicity, which in some instances may be related to contamination by bacterial endotoxin (Roth et al., 1993; White et al., 1986; Litwin et al., 1963). We have shown

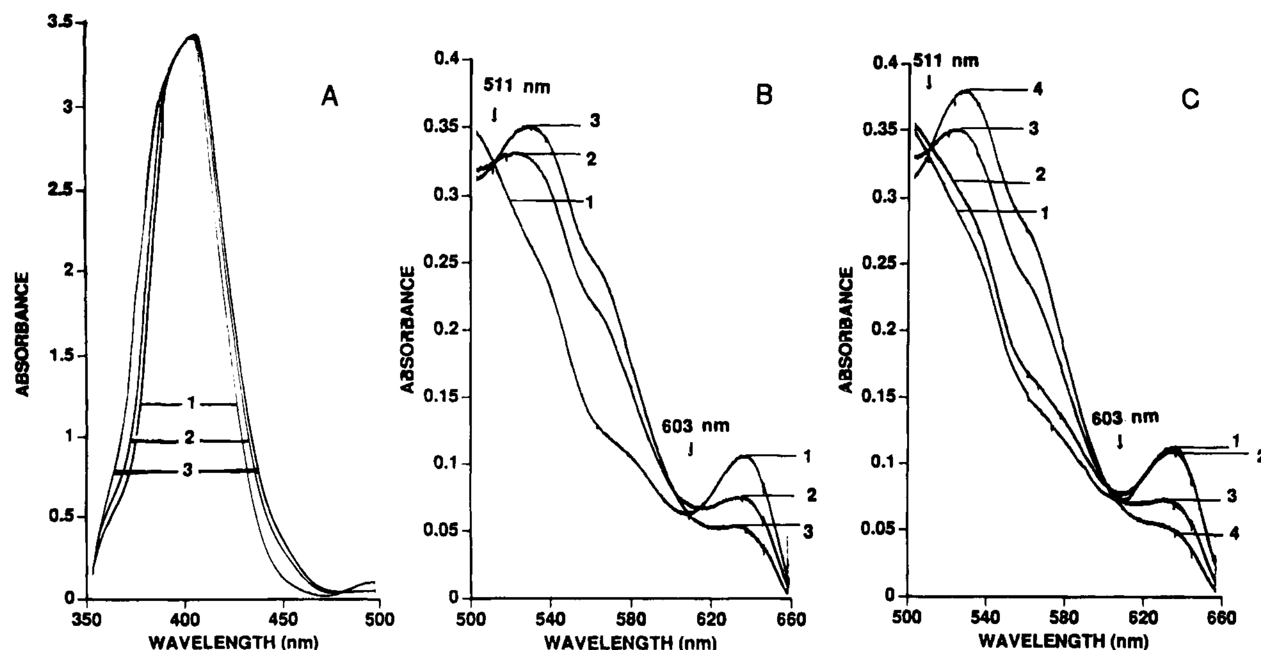

FIGURE 5: Methemoglobin absorption spectra in the absence and presence of LPS. MetααHb (24 μM), prepared from ααHb as described in Materials and Methods, was incubated at 37 °C in the absence or presence of OH37 LPS, and metααHb absorbance spectra were recorded as a function of time of incubation (A and B) or LPS concentration (C). The arrows indicate the apparent isosbestic points. Panels A (Soret) and B (visible region): line 1, initial spectra of metααHb alone; lines 2 and 3, metααHb with 1 mg/mL OH37 LPS incubated for 5 and 10 min, respectively. Panel C (visible region): line 1, metααHb alone; line 2, metααHb and 0.05 mg/mL LPS; line 3, metααHb and 0.5 mg/mL LPS; line 4, metααHb and 1 mg/mL LPS. Samples were incubated for 15 min. The sample cuvette contained metααHb with or without LPS, and the reference cuvette contained 0.9% NaCl (for metααHb spectra alone) or LPS alone at the appropriate concentration in 0.9% NaCl (for metααHb/LPS mixture spectra).

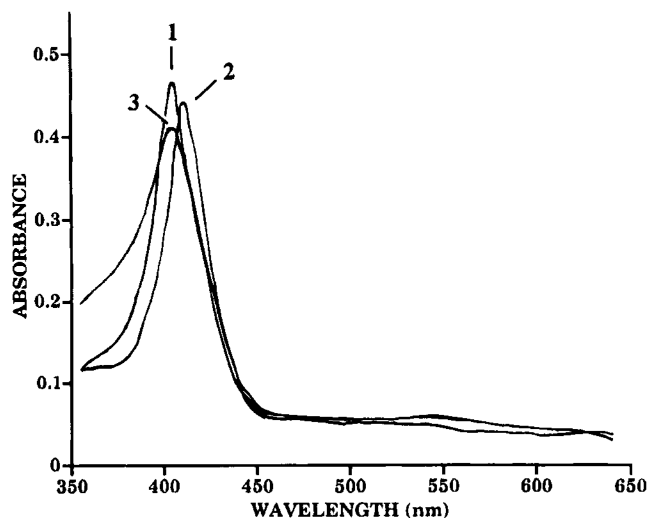

FIGURE 6: Reversibility of hemichrome formation from methemoglobin in the presence of LPS and albumin. MetααHb alone (prepared as described in Materials and Methods) (line 1) was incubated with 1 mg/mL OH37 LPS for 30 min at 37 °C (line 2). Human albumin (1 mg/mL) subsequently was added, and after 5 min at 37 °C incubation, the resulting absorbance spectrum in the Soret region was recorded (line 3). The sample cuvette contained metααHb with or without LPS or with LPS and albumin; the reference cuvette contained 0.9% NaCl (for metααHb spectra alone) or LPS alone in 0.9% NaCl (for metααHb/LPS and metααHb/LPS/albumin mixture spectra).

previously that a variety of Gram-negative bacterial endotoxins form complexes with hemoglobin (Kaca et al., 1994a,b). Furthermore, in the presence of hemoglobin, LPS is more reactive in some biological models, i.e., Limulus amebocyte activation and tissue factor production by human endothelial cells and mononuclear cells (Kaca et al., 1994a,b; Roth et al., 1993; Roth, 1994). These results indicate that hemoglobin can significantly alter the physicochemical features of LPS.

In the present study, we have shown that oxyhemoglobin may be less stable in the presence of LPS. The observed spectral changes in the Soret and visible regions of the absorption spectra of cross-linked ααHb and native HbA_0, produced by a variety of chemically different LPSs, are indicative of methemoglobin and hemichrome formation. Time- and concentration-dependent methemoglobin and hemichrome formation were most prominently demonstrated with partially deacetylated OH37 LPS. The lowest concentration of OH37 LPS demonstrating this effect was 0.05 mg/mL (18 μM on the basis of the known molecular weight of this LPS derivative). The concentration of ααHb in these experiments was 21 μM, suggesting that about 1 mol of OH37 LPS was bound per mole of Hb. The smooth *P. mirabilis* O3 and deep rough mutant *S. minnesota* 595 LPSs also were shown to oxidize hemoglobins, although less effectively than partially deacetylated LPS. The mechanism by which LPS causes Hb oxidation is not known. However, similar spectral changes in Hb have been described previously after addition of carbon-centered radicals (Minetti et al., 1993). Since fatty acids are known to be a common source of carbon-centered radicals (Akherm et al., 1989; Minetti et al., 1993; Buege & Aust, 1978), it is possible that the fatty acyl components of LPS may be a source of free radicals that in turn produce Hb oxidation.

Our data indicate that once methemoglobin is present, further incubation with LPS results in its fast conversion to hemichromes. The conversion of methemoglobin to hemichrome by LPSs was at least partially reversible with the addition of human albumin, suggesting that the binding of

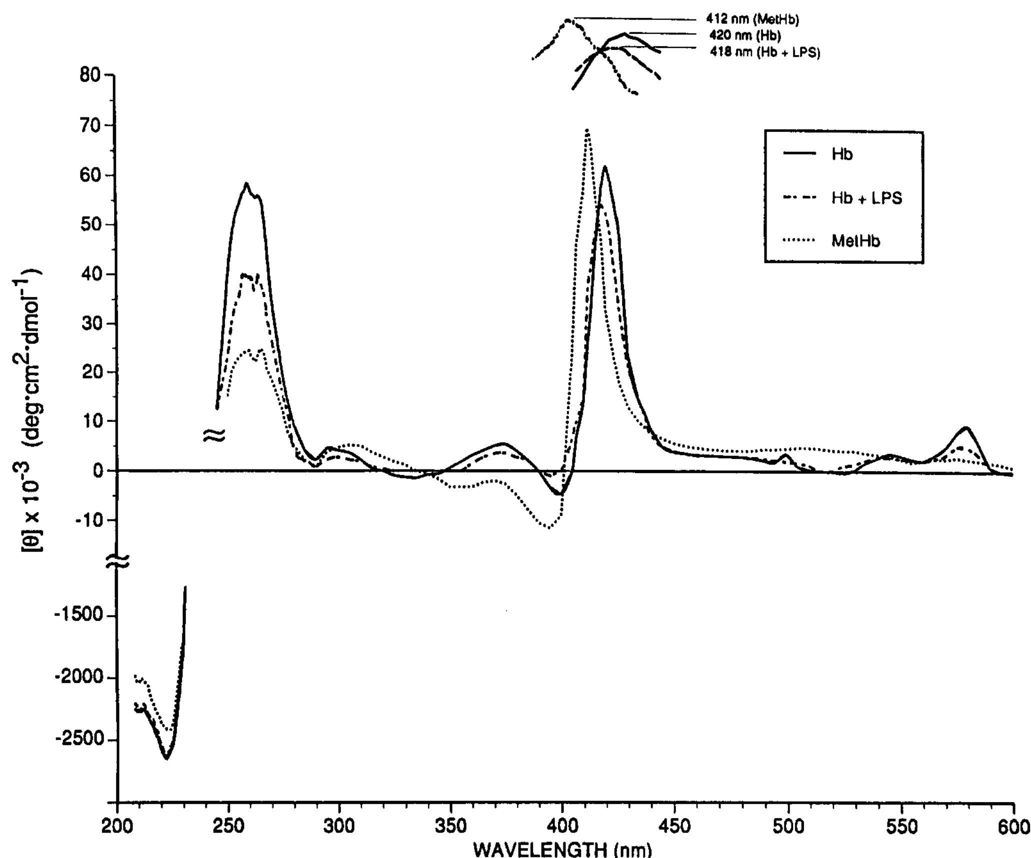

FIGURE 7: Circular dichroic (CD) spectra of Hb in the absence and presence of LPS. CD spectra were measured at room temperature between 200 and 600 nm for ααHb alone (13.8 μM heme, ambient oxygenation), ααHb/LPS (13.8 μM heme, 0.5 mg/mL *S. minnesota* 595 OH37 LPS, after a 2-h incubation at 37 °C), and metααHb (31.5 μM heme). Measurements in the far-UV region were made with samples diluted 5−10-fold. A 1-cm path length cell was utilized for measurements between 250−400 and 430−600 nm, and a 0.2-cm path length cell was utilized for measurements of the major Soret (400−440 nm) and far-UV (210−250 nm) regions. Ellipticities, [θ], are expressed on a molar heme basis. Wavelengths for the Soret peak maxima are identified on inset tracings presented with an expanded x-axis.

Table 1: P_{50} Values for Hb and Hb/LPS Complexes[a]

	P_{50}
ααHb alone	26.6
ααHb + LPS[b]	25.1
ααHb + LPS[c]	25.6
HbA_0 alone	9.6
HbA_0 + LPS[d]	8.7
HbA_0 + LPS[e]	7.3

[a] Oxygen affinity measurements were obtained for cross-linked (ααHb) and native (HbA_0) hemoglobins alone or in the presence of LPS after a 2-h incubation at 37 °C. Measurements were obtained prior to the production of oxidized Hb species. P_{50} was determined by utilizing both smooth and rough LPSs. Equal concentrations of Hb and LPS were utilized (each 1 mg/mL prior to dilution in Hemox buffer). [b] *P. Mirabilis* 03 (smooth) LPS. [c] *S. minnesota* Re 595 (rough) LPS. [d] *E. coli* 026 (smooth) LPS. [e] *S. minnesota* 595 OH37 (rough) LPS.

LPS to albumin is stronger than LPS binding to hemoglobin. Since hemichrome reversibility was associated with loss of magnitude of the resultant metααHb Soret peak compared to the initial spectrum, there may have been only partial reconversion of metααHb to hemichrome; alternatively, some of the heme in hemichrome may have been lost to albumin.

The superior ability of partially deacetylated OH37 LPS to facilitate methemoglobin and hemichrome formation, compared to that of the intact parent rough 595 LPS, suggests a possible biochemical mechanism for the Hb−LPS interaction. Rough LPS, suspended in aqueous solution at physi-

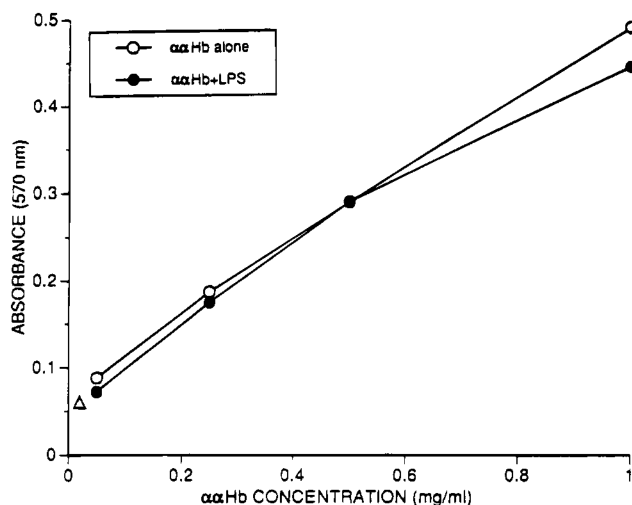

FIGURE 8: Production of free radicals in the absence and presence of LPS. OxyααHb (concentration, 0.05−1 mg/mL) was incubated at 37 °C for 60 min in the absence (○) or presence (●) of 1 mg/mL OH37 LPS. FOX reagent was then added, and oxidizing products were detected by absorbance at 570 nm. LPS alone (1 mg/mL) (△) did not produce detectable oxidants. Each point is the mean of two determinations. The data are representative of three independent experiments.

ologic conditions of temperature and divalent cation concentration, is aggregated to form nonlamellar cubic structures, with its hydrophobic fatty acids facing inward and the hydrophilic carbohydrate components exposed to the external

environment (Rietschel et al., 1991). Partial deacetylation may disturb the supramolecular structure of LPS and expose the fatty acids of the lipid A component of LPS (Myers et al., 1990). One may speculate that the hydrophobic fatty acids of LPS interact with some hydrophobic domain of hemoglobin and subsequently facilitate iron oxidation and degradative processes that affect the globin protein. Detoxification of bacterial lipopolysaccharides in vivo occurs via enzymatic release of secondary acyl chains from lipid A (Munford & Hall, 1986) in a process similar to the deacetylation reaction that generates OH37 LPS. Therefore, it is a concern that enzymatically deacetylated LPSs in vivo may facilitate hemoglobin degradation.

Our CD experiments to further describe Hb–LPS interactions identified structural changes consistent with the formation of substantial quantities of metHb, a conclusion similar to that determined from the absorbance spectral analyses. The major CD changes in the visible and Soret regions were consistent with heme oxidation, although the CD spectrum in these regions is also sensitive to the overall quaternary structure of the protein (Sugita et al., 1971; Geraci & Parkhurst, 1981). The lack of CD alteration in the far-UV region of the spectrum (e.g., 210–240 nm) provided evidence that there were no substantial changes in the globin secondary structure (Chen et al., 1972). In general, the overall secondary conformation of Hb is considered to be insensitive to changes in iron valence state and the binding of extrinsic ligands (Myer & Pande, 1978). However, the CD spectrum in the near-UV region (250–300 nm), which also was altered in the presence of LPS, has been suggested previously to be sensitive to the environments of aromatic amino acids at the $\alpha_1\beta_2$ interface (Geraci & Parkhurst, 1981; Zentz et al., 1994) as well as to disulfide chromophores (Zentz et al., 1994), suggesting that there may have been some localized globin conformational changes induced by LPS. Interestingly, there were only minor changes in oxygen affinity associated with LPS binding to Hb. With the conditions of our experiments (i.e., the relative Hb and LPS concentrations and the time of incubation), we previously have demonstrated that Hb and LPS form complexes with approximately equal weights of the two components. Accordingly, each chain of the Hb tetramer might bind a single smooth LPS, or a small number of rough LPSs. On the basis of our previous observations that Hb can interact with rough LPSs and partial lipid A structures (Kaca et al., 1994b) and the known ability of Hb to intercalate into lipid bilayers (Szebeni et al., 1988), it is likely that binding occurs predominantly via interactions between Hb and lipid A. Heme is unlikely to be directly involved in this binding process since the oxygen affinity of Hb is only minimally affected by LPS, although as a result of the binding of LPS there apparently are changes in globin conformation sufficient to destabilize the heme pocket such that the rate of heme oxidation is increased.

LPS-mediated oxidation of Hb to methemoglobin and hemichromes is likely to facilitate iron release and the generation of free radicals, conditions known to contribute to Hb-related toxicity (Sadrzadeh et al., 1984; Simoni et al., 1990; Gutteridge, 1986), although we detected no increase in the production of oxidizing species in Hb/LPS mixtures, compared with Hb alone. We postulate the LPS-induced Hb oxidation may be an important component of Hb toxicity in vivo by a two-step mechanism for Hb toxicity which involves biochemical changes in both LPS and Hb as a result of Hb/LPS complex formation. First, binding of Hb to LPS partially disaggregates the high molecular weight (> 10^6 Da) LPS micelles and enhances LPS biological activity, as we have shown previously (Kaca et al., 1994a,b; Roth et al., 1993; Roth, 1994). Second, Hb/LPS complexes exhibiting high LPS biological activity may then result in a series of secondary reactions which can play important roles in the pathophysiology of endotoxic shock. These reactions include the activation of polymorphonuclear leukocytes with subsequent generation of oxygen free radicals (Yoshikawa, 1990), the enhanced production of procoagulant activity from both mononuclear cells (Roth et al., 1993) and endothelial cells (Roth, 1994), and the facilitation of Hb oxidation with heme or iron release and production of oxygen free radicals (Gutteridge, 1986). The formation of hydroxyl radicals can cause activation of the complement cascade (Shingu et al., 1989, 1992) and other harmful reactions, leading to organ dysfunction, that have previously been described following the administration of LPS-containing or stromal lipid-containing hemoglobin solutions (Feola et al., 1988a).

In conclusion, by analysis of absorption and circular dichroic spectra of Hb, we have shown that enterobacterial endotoxins can facilitate degradation of the Hb molecule through mechanisms that potentially involve heme loss, increased rate of iron oxidation, and conversion of Hb to hemichromes and methemoglobin. All of these changes are along the pathway to Hb denaturation and may contribute to in vivo toxicity of cell-free hemoglobin. On the basis of an approximately equal weight ratio of Hb and LPS in Hb/LPS complexes and the typical LPS plasma concentrations encountered during endotoxemia (picograms to nanograms per milliliter), only a small fraction of infused Hb would be expected to initially undergo LPS-facilitated degradation. However, endotoxemia can persist during sepsis despite detoxification mechanisms that normally result in rapid LPS clearance, indicating continuous influx into the circulation of new LPS from an infectious focus or the gastrointestinal tract. The continuous or recurrent entry of LPS into the circulating blood would be expected to result in an increasing fraction of denatured Hb during the prolonged circulation of the infused Hb.

ACKNOWLEDGMENT

The assistance and advice of Dr. John P. Kane, Departments of Medicine, Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California School of Medicine, San Francisco, contributed importantly to these investigations.

REFERENCES

Akherm, A. A., Anreyuk, G. M., Kisel, M. A., & Kiselev, P. A. (1989) *Biochim. Biophys. Acta 992*, 191–194.

Balla, G., Vercellotti, G. M., Muller-Eberhard, U., Eaton, J., & Jacob, H. S. (1991) *Lab. Invest. 65*, 648–655.

Brade, H., Galanos, C., & Lüderitz, O. (1983) *Eur. J. Biochem. 131*, 195–200.

Buege, J. A., & Aust, S. D. (1978) *Methods Enzymol. 52*, 302–310.

Chen, Y.-H., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131.

Christensen, S. M., Medina, F., Winslow, R. M., Snell, S. M., Zegna, A., & Marini, M. A. (1988) *J. Biochem. Biophys. Methods 17*, 145–154.

DeVenuto, F., & Zegna, A. (1982) *Surg., Gynecol. Obstet. 155*, 342–346.

Di Iorio, E. E. (1981) *Methods Enzymol. 76*, 57–72.

Eaton, J. W., Brandt, P., & Mahoney, J. R. (1982) *Science 215*, 691–693.

Faasen, A. E., Sundby, S. R., Panter, S. S., Condie, R. M., & Hedlund, B. E. (1988) *Biomater., Artif. Cells, Artif. Organs 16*, 93–104.

Feola, M., Simoni, J., Canizaro, P. C., Tran, R., & Raschbaum, G. (1988a) *Surg., Gynecol., Obstet. 166*, 211–222.

Feola, M., Simoni, J., Tran, R., & Canizaro, P. C. (1988b) *Biomater., Artif. Cells, Artif. Organs 16*, 217–226.

Galanos, C., Lüderitz, O., & Westphal, O. (1969) *Eur. J. Biochem. 9*, 245–249.

Galanos, C., Rietschel, E. T., Luderitz, O., & Westphal, O. (1972) *Eur. J. Biochem. 31*, 230–233.

Geraci, G., & Parkhurst, L. J. (1981) *Methods Enzymol. 76*, 262–275.

Gutteridge, J. M. C. (1986) *FEBS Lett. 201*, 291–295.

Jiang, Z.-Y., Hunt J, V., & Wolff, S. P. (1992) *Anal. Biochem. 202*, 384–389.

Kaca, W., Knirel, Y. A., Vinogradov, E. V., & Kotelko, K. (1987) *Arch. Immunol. Ther. Exp. 35*, 431–437.

Kaca, W., Roth, R. I., & Levin, J. (1994a) *J. Biol. Chem. 269*, 25078–25084.

Kaca, W., Roth, R. I., Ziolkowski, A., & Levin, J. (1994b) *J. Endotoxin Res. 1*, 243–252.

Levin, J., & Bang, F. B. (1968) *Thromb. Diath. Haemorrh. 19*, 186–197.

Litwin, M. S., Walter, C. W., Ejarque, P., & Reynolds, E. S. (1963) *Ann. Surg. 157*, 485–493.

Minetti, M., Mallozzi, C., Scorza, G., Scott, M. D., Kuypers, F. A., & Lubin, B. H. (1993) *Arch. Biochem. Biophys. 302*, 233–244.

Munford, R. S., & Hall, C. L. (1986) *Science 234*, 203–205.

Myer, Y. P., & Pande, A. (1978) *The Porphyrins III* (Dolphin, D., Ed.) pp 271–322, Academic Press, New York.

Myers, K. R., Truchot, A. T., Ward, J., Hudson, Y., & Ulrich, J. T. (1990) Cellular and Molecular Aspects of Endotoxin Reactions (Nowotny, A., Spitzer, J. J., & Ziegler, E. J., Eds.) pp 145–156, Excerpta Medica, Amsterdam.

Paller, M. S. (1988) *Am. J. Physiol. 255*, F539–F544.

Rietschel, E. Th., Seydel, U., Zähringer, U., Schade, U. F., Brade, L., Loppnow, H., Feist, W., Wang, M.-H., Ulmer, A. J., Flad, H.-D., Brandenburg, K., Kirikae, T., Grimmecke, D., Holst, O., & Brade, H. (1991) *Infectious Disease Clinics of North America* (Young, L. S., Glauser, M. B., Eds.) Vol. 5, pp 753–779, W. B. Saunders, Philadelphia.

Roth, R. I. (1994) *Blood 83*, 2860–2865.

Roth, R. I., & Kaca, W. (1994) *Art. Cells, Blood Substitutes, and Immobilization Biotechnol. 22*, 287–398.

Roth, R. I., Levin, J., Chapman, K. W., Schmeizl, M., & Rickles, F. R. (1993) *Transfusion 35*, 919–924.

Sadrzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E., & Eaton, J. W. (1984) *J. Biol. Chem. 259*, 14354–14356.

Sehgal, L. R., Gould, S. A., Rosen, A. L., Sehgal, H. L., & Moss, G. S. (1984) *Surgery 95*, 433–438.

Shingu, M., Nonaka, S., Nobunaga, M., & Ahmadzadeh, N. (1989) *Dermatologica 179* (Suppl. 1), 107–112.

Shingu, M., Nonaka, S., Nishimukai, H., Nobunaga, M., Kitamura, H., & Tomo-Oka, K. (1992) *Clin. Exp. Immunol. 90*, 72–78.

Simoni, J., Feola, M., & Canizaro, P. C. (1990) *Biomater., Artif. Cells, Artif. Organs 18*, 189–202.

Sugita, Y., Nagai, M., & Yoneyama, Y. (1971) *J. Biol. Chem. 246*, 383–388.

Szebeni, J., Hauser, H., Eskelson, C. D., Watson, R. R., & Winterhalter, K. H. (1988) *Biochemistry 27*, 6425–6434.

Vandegriff, K. D. (1993) *Biotechnol. Genet. Eng. Rev. 10*, 403–453.

Vandegriff, K. D., & Le Tellier, Y. C. (1994) *Biomater., Artif. Cells, Immobilization Biotechnol. 22*, 443–455.

Westphal, O., & Jann, K. (1965) *Methods Carbohydr. Res. 5*, 91–93.

White, C. T., Murray, A. J., Smith, D. J., Greene, J. R., & Bolin, R. B. (1986) *J. Lab. Clin. Med. 108*, 132–137.

Winslow, R. M. (1992) *Hemoglobin-based Red Cell Substitutes*, pp 143–150 and 162–163, Johns Hopkins University Press, Baltimore, MD.

Winslow, R. M., & Chapman, K. W. (1994) *Methods Enzymol. 231*, 3–16.

Winterbourn, C. C. (1990) *Methods Enzymol. 186*, 256–274.

Yoshikawa, T. (1990) *Methods Enzymol. 186*, 660–665.

Zentz, C., Pin, S., & Alpert, B. (1994) *Methods Enzymol. 232*, 247–267.

BI950729Y