Structure of the Novel Heme Adduct Formed During the Reaction of Human Hemoglobin with BrCCl₃ in Red Cell Lysates*

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It was previously shown that the reductive debromination of BrCCl₃ to trichloromethyl radical by human hemoglobin leads to formation of dissociable altered heme products, two of which are identical to those formed from myoglobin and one which is novel. In this study, we have elucidated the structure of this novel adduct with the use of mass spectrometry, as well as ¹H and ¹³C NMR as a substitution product of a α-CCl=CCl₂ moiety for a β-hydrogen atom on the prosthetic heme's ring I vinyl group. From studies with the use of ¹³C-enriched BrCCl₃, it was determined that the added carbon atoms were derived from 2 eq of BrCCl₃. A mechanism that involves multiple reductive events and a radical cation heme intermediate is proposed. Consistent with this mechanism, cellular reductants were found to selectively enhance the amount of this novel dissociable heme adduct. These studies reveal fine differences between myoglobin and hemoglobin in the accessibility of reactive intermediates to the ring I vinyl group, as well as the potential importance of cellular reductants on the course of heme alteration.

Hemoglobin is a major target protein for reactive metabolites of drugs (1), environmental toxicants (2–4), and carcinogens (5–9). The high abundance, availability, and long half-life of human hemoglobin have led to the use of altered hemoglobins as indicators of exposure to these toxic agents (10, 11). In addition, some hemoglobin-based red cell substitutes, which are being developed for clinical use (12, 13), are also covalently altered by reactive intermediates with potentially deleterious consequences (14). Furthermore, it has been speculated that covalent alteration of hemoglobin by reactive intermediates is involved in the formation of hemichromes (15), which are thought to play a role in Feinberg body formation and hemolysis (16). Thus, it is important to study the mechanisms involved in the covalent alteration of hemoglobin and the potential biological consequences of such alterations. One model system for such studies involves the covalent alterations of hemoglobin caused by trichloromethyl radicals.

The reductive debromination of BrCCl₃ to trichloromethyl radical by human hemoglobin and myoglobin has been shown previously to lead to the formation of dissociable and protein-bound prothrombinic heme-derived products resulting from the regiospecific attack of a trichloromethyl radical on the ring I vinyl group of heme (15, 17–20). For the most part, the structures of the products formed from these two hemoproteins are highly similar; in fact, two of the dissociable adducts, a α-hydroxy-β-trichloromethylheme derivative (compound 3) and a β-carboxyvinyl heme derivative (compound 1) are formed from both proteins (Scheme I). In contrast, an α,β-bistrichloromethylheme substitution product of the ring I vinyl of heme was unique to the myoglobin reaction, whereas a novel heme adduct of unknown structure was observed as a major product from the hemoglobin reaction (21). To help explain how identical, as well as unique, products could form from the same reaction with two proteins, we set out to determine the structure of this novel heme product with the use of mass spectrometry, as well as one- and two-dimensional ¹H and ¹³C NMR. The product resulted from the substitution of an α-CCl=CCl₂ moiety for a β-hydrogen atom on the ring I vinyl group of heme. Thus, it appears that the major difference between hemoglobin and myoglobin with respect to modification of the vinyl group is the inaccessibility of trichloromethyl radical at the α- but not the β-carbon region of the ring I vinyl moiety of hemoglobin, presumably due to steric constraints. The mechanism of formation of the novel adduct apparently can involve multiple reductive events, which was consistent with the finding that cellular reductants could selectively enhance the amount of this adduct. However, this interpretation is complicated by the finding that reductants also lead to degradation of some of the other altered heme products. This observation, however, may be of importance in studies on alteration of the prosthetic heme of hemoglobin or possibly other hemoproteins by a variety of agents, especially under biological conditions, where a variety of reductants and reductases could be present.

EXPERIMENTAL PROCEDURES

Materials—Pyridine-d₅ and Br³CCl₃ (99 atom %) were purchased from Merck. Methanol-d₅ and deuterium oxide were purchased from Cambridge Isotope Laboratories (Woburn, MA). Stamnose chloride, human hemoglobin, horse heart myoglobin, and 2,5-dihydroxybenzoic acid were from Aldrich, Sigma, U. S. Biochemical Corp., and Fluka, respectively. Red cell lysates were prepared as described previously (15).

Preparation of Altered Heme Adduct—The reaction of human hemoglobin with BrCCl₃ in red cell lysates was performed as previously reported (15). In short, red cell lysates were made anaerobic, and the resulting ferrous deoxyhemoglobin (250 μM) in a total volume of 500 ml of 50 mM potassium phosphate, pH 7.4, was reacted with BrCCl₃ or Br³CCl₃ (1 mmol was added) at room temperature for 1 h. Extraction of the reaction mixture with 2-butanol under acidic conditions gave a mixture of heme adducts in the organic phase (17). This organic phase was washed with water (1:10) and placed on a Büchner funnel containing C18 resin (50 g, Whatman). The heme products were bound to the C18 material, washed with a liter of 0.1% trifluoroacetic acid (solution A), and then eluted by 26 ml of 0.1% trifluoroacetic acid in acetonitrile (solution B). The eluant was dried under vacuum in a Speed Vac...
Covalent Alteration of Hemoglobin

Ascorbate--Red cell lysates were diluted with 50 mM potassium phosphate buffer. Mass spectra were obtained with a Kratos MSBORF (Kranheme adduct isolated from reactions without exogenous reductant. The adduct isolated from these reactions had a molecular ion identical to the mass spectra (23) were obtained with 355 nm radiation (maximum absorbance of 100 µg of purified material was obtained per batch. NMR and mass spectra were obtained on these samples.

Preparation of Altered Heme Adduct in the Presence of Glutathione or Ascorbate--Red cell lysates were diluted with 50 mM potassium phosphate buffer, pH 7.4, so that the hemoglobin concentration was 200 µM as determined by the chromophore at 576 nm (22). Ascorbate or glutathione was added to the reaction mixture and subsequently made anaerobic and treated with BrCCl3 as described above. The altered heme adduct isolated from these reactions had a molecular ion identical to the heme adduct isolated from reactions without exogenous reductant.

Plasma Desorption Mass Spectrometry--Positive ion mass spectra were obtained on a Bio-Ion Nordic AB (Uppsala, Sweden) model BIN-10K plasma desorption mass spectrometer. Samples were dissolved in 0.1% trifluoroacetic acid in water/methanol (3:1, v/v). Solutions (2-5 µl) were placed on a nitrocellulose-coated, aluminum-coated Mylar sample foil, spin-dried after 5 min, and then washed with 10 µl of 0.1% trifluoroacetic acid in water to remove salts. Sample foils were then placed in the mass spectrometer and the spectra recorded at an accelerating voltage of 16 kV.

Fast Atom Bombardment Mass Spectrometry--Positive ion fast atom bombardment mass spectra were obtained with a Kratos M550RF (Kra- tos Analytical, Ltd., Manchester, United Kingdom) double focusing instrument with a mass range of 10000 atomic mass units at full accelerating voltage (8 kV). The instrument was fitted with a model B11NF saddle-field fast atom gun (Ion Tech, Ltd., Teddington, United Kingdom) and a post accelerator detector, which was operated at 14 kV. Xenon was used to bombard the samples at 8 kV. The samples were dissolves in methanol and applied to a gold fast atom bombardment probe in a matrix of 3-nitrobenzyl alcohol. The mass spectra were acquired at a scan rate of 30 s/decade with a resolution of 1 in 2500. All data were acquired and processed with the Kratos DS-90 data system.

Fourier Transform Mass Spectral Measurements--Samples were prepared by dissolving approximately 10-100 µg of analyte in sample foil, spin-dried after 5 min, and then a linear gradient to 100% solution B was run over the next 10 min. A gradient (Waters curve 8) to 50% solution A and 64% solution B was run over 20 min, and then a linear gradient to 100% solution B was run over the next 10 min. One-minute fractions were collected and the product of interest eluted between 172 and 188 min. To check for purity, an aliquot of each fraction of interest was injected on an analytical HPLC column (Vydac C18, 0.46 x 55 cm) equilibrated with a mobile phase of pH 7.4, so that the hemoglobin concentration was 200 µM as determined by absorbance at 576 nm (22). Ascorbate or glutathione was added to the reaction mixture and subsequently made anaerobic and treated with BrCCl3 as described above. The altered heme adduct isolated from these reactions had a molecular ion identical to the heme adduct isolated from reactions without exogenous reductant.

NMR Spectrometry--NMR spectra were obtained on a Varian XL-200 spectrometer in pyridine-d6 solution following reduction by stannous chloride (17). Typically, 256 free induction decays were collected with an accumulation time of 4 s for one-dimensional spectra. COSY spectra were taken with the use of a data matrix 1024 x 1024 with 256 t1 increments of 16 free induction decays each. Prior to NOESY studies the solutions were degassed by three freeze-exhaust-thaw cycles. Phase-sensitive NOESY spectra were obtained by the method of States et al. (25), in overnight runs, with the use of 1024 t1 increments of 16 free induction decays each with 64 free induction decays.

RESULTS

The HPLC profiles at 220 and 405 nm of untreated and BrCCl3-treated human ferrous deoxyhemoglobin from red cell lysates are shown in Fig. 1 (panels A and B, respectively). The untreated sample exhibited two major 220 nm-absorbing peaks corresponding to the a and b subunits of hemoglobin (26). Under the acidic conditions of the chromatography, the heme readily dissociated from the untreated protein as seen by the major peak at 405 nm (peak 2), as previously found (17). After treatment with BrCCl3, the b-chain was altered and several 405 nm-absorbing peaks were observed. The structures of compounds corresponding to peaks 1, 3, 4, and 5 have been determined previously (Scheme I) (15, 17). Peaks 1 and 3 corre-

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1 The abbreviations used are: HPLC, high performance liquid chromatography; NOESY, nuclear Overhauser effect spectroscopy.
Covalent Alteration of Hemoglobin

The structures of the known altered heme adducts of hemoglobin. The numbers correspond to those in Fig. 1.

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responded to the \( \beta \)-carboxyvinyl (compound 1) and a-hydroxy-\( \beta \)-trichloromethyl ethyl (compound 3) derivatives of the 1 vinyl moiety of heme, respectively (17), and peaks 4 and 5 corresponded to two protein-bound heme adducts due to cross-linking of cysteine 93 of the 1 chain to the heme (15). The co-elution of the \( \beta \)-chain with the \( \alpha \)-chain after treatment with BrCCl (Fig. 1, panel B) reflects the nearly complete alteration of the \( \beta \)-chain by heme, as previously described in a report on the characterization of the protein-bound heme adduct (15). In this report, with the use of a different chromatographic condition optimized for the protein-bound heme adducts, the \( \alpha \)-chain and the altered \( \beta \)-chains were clearly resolved and the association of heme chromophore with the altered \( \beta \)-chains was evident (15). The peaks eluting between peaks 5 and 6 were unstable and were not isolated. The compound (compound 6) corresponding to peak 6 is the focus of this study.

The structure of compound 6 was determined (Fig. 2) as described below. The visible absorption spectrum of compound 6 in methanol had maxima at 406, 500, and 620 nm. This spectrum was consistent with an intact porphyrin \( \pi \) system. The bathochromic shift of the Soret maximum for the heme adduct was consistent with an electrophilic substituent on the heme ring (27).

Analysis of the reaction mixtures by plasma desorption mass spectrometry showed a molecular ion at \( m/z \) 744 that increased in intensity when glutathione was included in the reaction mixture (data not shown). A Fourier transform mass spectrum of compound 6 showed a MH+ ion at \( m/z \) 744.1 (Fig. 3, panel B). The cluster of ions found in the molecular ion region appeared to be due predominantly to chlorine-35 and chlorine-37 isotopes in the molecule, consistent with the distribution of ions predicted from the molecular formula of compound 6 (Fig. 3, panel A). Furthermore, the mass spectrum of compound 6 isolated from a reaction mixture treated with \( ^{13} \)C-enriched BrCCl gave molecular ions with an additional 2 mass units, showing that the added carbon atoms in compound 6 were derived from 2 eq of BrCCl (Fig. 3, panel C).

The \(^1\)H NMR spectrum of the altered heme (Fig. 4, panel A) was for the most part similar to native heme (17). The four singlets near 10 ppm corresponded to the meso protons and the four singlets near 3.5 ppm corresponded to the four methyl groups. The peaks of the \( \beta \)-methylene groups of the propionic residues were visible between the sharp methyl peaks and the \( \alpha \)-methylene protons gave a broad triplet at 4.5 ppm with integrated intensity of 4. However, multiplets with chemical shifts near 8.5 ppm for the \( \alpha \)-proton and 6.0 and 6.3 ppm for the \( \beta \)-protons at vinyl substituents corresponded to only a single unaltered vinyl group. Thus one vinyl group was altered, giving rise to protons that exhibited a pair of doublets at 8.84 and 7.96 ppm in the NMR spectrum. The large coupling between these protons (15.5 Hz) indicated a trans stereochemistry.

To determine which vinyl group was modified and the nature of this modification, two-dimensional NOESY and COSY experiments were conducted. In the NOESY experiments, nuclei close enough to each other to allow polarization transfer through space give rise to off-diagonal peaks at their corresponding chemical shifts (28). Thus for the altered heme, as seen in Table I, 8-meso protons showed interaction with two
methyl and vinyl protons. An interaction of the proton on the altered vinyl group at 8.84 ppm with the methyl substituent at 3.70 ppm, which is one of the two methyls interacting with the δ-meso proton, clearly showed that the ring I vinyl was modified.

Further information on the nature of this modification was obtained by analysis of the heme adduct from the reaction with Br\textsuperscript{13}CCl\textsubscript{3}. In the \textsuperscript{1}H NMR spectrum (Fig. 4, panel B), the olefinic signals at 8.84 and 7.95 ppm showed evidence of further coupling. In the \textsuperscript{13}C NMR spectrum of the product (Fig. 5), doublets appeared with chemical shifts (131.5 and 117.8 ppm) characteristic of olefinic carbons and coupled by 105 Hz, a value suitable for one-bond coupling of olefinic carbons with several negative substituents (29). To provide detailed information on this coupling, we performed a FLOCK experiment, which provides a heteronuclear correlation spectrum between protons and carbons coupled to them by smaller couplings (30). When optimized for couplings near 10 Hz, the spectrum showed that only the carbon at 131.5 ppm was coupled to both of these protons by couplings of this size. Smaller couplings are observed of the carbon resonating at 117.8, 3.3 Hz (\textsuperscript{3}J(H,C)) and 1.8 Hz (\textsuperscript{2}J(H,C)) (Table I). The small vicinal coupling implies that the dihedral angle in the center of the diene must be held near 90° to accommodate the bulk of the substituents. The evidence clearly shows that the vinyl group of ring I has been altered by the substitution of a second vinyl group bearing three chlorine atoms (Fig. 2).

It was discovered that the amount of compound 6 increased approximately 3-fold when reduced glutathione was added in excess of 2.5 mM to the reaction mixture (Fig. 6, panel A), whereas addition of oxidized glutathione at a concentration of 10 mM had little effect (Fig. 6, panel B). Glutathione did not appear to be obligatory for formation of compound 6, since red cell lysate passed over a Sephadex G-25 column to remove glutathione gave the altered heme adduct albeit in low yield (Fig. 6, panel B, Treated-srue). Similar results were obtained with purified hemoglobin from Sigma (Fig. 6, panel B). Studies with the use of ascorbate gave a more dramatic rise (approximately 10-fold) in the amount of compound 6 in red cell lysates (Fig. 7). The amount of compound 3 remained constant (Fig. 7), whereas heme and the other altered heme products (compounds 1, 4, and 5) decreased (Fig. 7). Similar results were obtained with glutathione (data not shown), except that the amount of the altered heme products corresponding to compounds 1, 3, 4, and 5 remained fairly constant at low glutathione concentrations (0.5–2.5 mM), but at higher concentrations of glutathione (5–25 mM), degradation of compounds 1, 4, and 5 was observed while compounds 3 and 6 remained relatively
Covalent Alteration of Hemoglobin

FIG. 6. Effect of glutathione on formation of the altered heme adduct (compound 6). Hemoglobin (200 μM) in red cell lysates was treated with BrCCl₃ and analysed by HPLC with the use of an analytical column as described under "Experimental Procedures." Panel A, the concentration of added glutathione was varied. Panel B, oxidized glutathione (GSSG) or reduced glutathione (GSH) was added to a final concentration of 10 mM. The red cell lysate was passed over a Sephadex G-25 column (PD-10, Pharmacia Biotech) to remove free glutathione and subsequently treated with BrCCl₃ (Treated same). Dialyzed and twice crystallized human hemoglobin purchased from Sigma was reduced with excess dithionite, passed over a Sephadex column as described above, and substituted for red cell lysate (Treated Sigma Hb).

FIG. 7. The effect of ascorbate on the amount of heme and altered hemes (compounds 1 and 3–6) formed from the reaction of BrCCl₃ with human hemoglobin. The procedure was as described under "Experimental Procedures." The structures of compounds 1, 3, 4, and 5 are depicted in Scheme 1. The structure of compound 6 is shown in Fig. 2. •, heme; □, compound 1; ●, compound 3; ▲, compounds 4 and 5; +, compound 6. Compounds 1 and 3 are shown with dotted lines, compounds 4/5 and heme are shown with dashed lines, and compound 6 is shown with solid lines.

constant. A marked loss of heme was found over the entire concentration range of glutathione (0.5–25 mM) with approximately 40% loss at a concentration of 0.5 mM.

DISCUSSION

In this study the structure of the novel altered heme adduct formed in the reaction of human hemoglobin with BrCCl₃ in red cell lysates has been shown to be derived from the substitution of a -C(Cl)=CCl₂ moiety for a β-hydrogen atom on the ring I vinyl group of heme. The regiospecificity of this reaction was the same as that found for all of the other altered heme products that have been characterized from the reaction of BrCCl₃ with myoglobin (17, 20) and hemoglobin (15) and suggests that the initial steps involved in the formation of all the heme adducts may be similar. A mechanism that accounts for the formation of the previously characterized heme adducts of hemoglobin (Scheme II, panel A) (15) involves the initial addition of the trichloromethyl radical on the ring I vinyl moiety with subsequent dechlorination of the electron to form a trichloro-heme cationic species. Water or cysteine could add to this cation to form the previously characterized trichloromethyl alcohol (compound 3) or protein-bound heme adduct (compound 5), respectively (15). The trichlorovinyl-heme species could undergo reductive dechlorination and subsequent dechlorination of the electron to form a dichloro-heme cationic species, which could then form the acrylic acid (compound 1) or protein-bound heme adduct (compound 4) (15, 19, 20).

A mechanism for the formation of compound 6 that is consistent with the above mechanism is depicted in Scheme II (panel B). The dichloro-heme cationic species described above catalyzes a reductive debranion of BrCCl₃ to form a second equivalent of CCl₃ radical, which subsequently adds to the dichlorovinyl moiety. The cation radical species could abstract an H⁺ most likely from the protein and not water (31), and subsequently form the pentachloro compound. Another redox cycle, with reducing equivalent derived from ascorbate or glutathione, or possibly other cellular reductants, leads to intramolecular reductive dechlorination (19, 20). Another redox cycle would give the ferric tetrachloro-heme anion, in a manner similar to that described for reductive metabolism of halogenated hydrocarbons by P450 cytochromes (32), with subsequent elimination of Cl⁻ to give the observed trichloro product, compound 6. Alternatively, the tetrachloro-heme radical could abstract another H⁺ with subsequent dehydrochlorination reaction to give compound 6.

Although the addition of exogenous reductants was found to selectively enhance the amount of compound 6, which tends to
favor the multiple reductive mechanism shown above, the interpretation is complicated by the finding that reductants also lead to losses of some of the altered heme products. This loss was most prominent for the protein-bound heme adduct, consistent with previous reports on the susceptibility of this adduct to redox cycling and self destruction (14, 33, 34). In addition to this complication, it is not clear how this product can form from purified preparations of hemoglobin without exog-
Covalent Alteration of Hemoglobin

enous reductants, unless reducing equivalents can somehow transfer between proteins. It is evident from our current findings that multiple catalytic cycles are involved in the covalent alteration of the prosthetic heme moiety to form compound 6. Although the exact mechanism remains to be determined, it is clear that the level of reductants has a dramatic effect on the course of heme alteration, which is reflected in the profile of heme products observed. These findings should be considered in the interpretation of studies on heme alteration of other hemoproteins in biological systems where a variety of cellular reductants and reductases are usually present.

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