Covalent Alteration of the Prosthetic Heme of Human Hemoglobin by BrCCl₃
CROSS-LINKING OF HEME TO CYSTEINE RESIDUE 93*

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Recent studies have shown that a protein-bound heme adduct formed from the reaction of BrCCl₃ with myoglobin was due to bonding of the proximal histidine residue through the ring I vinyl of a heme-CCl₃ moiety. The present study reveals that BrCCl₃ also reacts with the heme of reduced human hemoglobin to form two protein-bound heme adducts. Edman degradation and mass spectrometry provided evidence that these protein-bound heme adducts were addition products in which heme-CCl₃ or heme-CCl₂ were bound to cysteine residue 93 of the β-chain of hemoglobin. It appeared that the cysteine residue was bonded regioselectively to the ring I vinyl group of the altered heme moiety, because the nonprotein-bound products of the reaction included the β-carboxyvinyl and α-hydroxy-β-trichloromethylthethyl derivatives of the ring I vinyl moiety of heme. The absorption spectra of the protein-bound adducts in both the oxidized and reduced states were highly similar to those described for hemichromes, which are thought to be involved in the formation of Heinz bodies and subsequent red cell lysis.

Hemoglobin is one of the most extensively characterized proteins; its crystal structure in various redox states, its mechanism of oxygen transport, and its sensitivity to pH, oxygen tension, and other biological effectors have all been thoroughly described (1–6). Due to its high concentration in red cells and the abundance of these cells (5, 7), hemoglobin is a major target protein for reactive metabolites of drugs (8), environmental chemicals (9–11), and carcinogens (12–16). Hemoglobin’s long half-life and ready availability for sampling have led to the use of altered hemoglobins as indicators for exposure to these toxic chemicals (17, 18). Some alterations of hemoglobin, such as the formation of hemichromes and Heinz bodies caused by various chemicals in a process thought to lead to red cell lysis, can also have important biological consequences (19–24). Thus, it is important to elucidate the mechanisms of formation and biological effects of altered hemoglobins.

The major altered heme products from the reaction of BrCCl₃ with myoglobin, including a protein-bound heme adduct, have been previously characterized (25, 26). The ferrous form of the heme-protein adduct was found to reduce oxygen, and we have suggested a “histidine shuffle” mechanism as the cause of this altered activity (26, 27). We have recently shown that the formation of a protein-bound adduct during the reaction of H₂O₂ with myoglobin transforms the hemoprotein to an oxidase (28).

In the present study we have found that two redox-active protein-bound heme adducts are formed during the reductive cleavage of BrCCl₃ by hemoglobin. These adducts were identified with the use of Edman sequencing and mass spectrometry as a heme-CCl₃ or heme-CCl₂ moiety covalently bound to the β-chain at cysteine residue 93. The marked similarity between the spectral characteristics of these adducts and those described for a hemichrome (24) suggests the possibility that hemichromes are protein-bound hemes capable of producing reactive oxygen species and initiating the process of red cell lysis.

**EXPERIMENTAL PROCEDURES**

**Materials**

BrCCl₃ from Aldrich was washed with 5% Na₂CO₃ and distilled prior to use. Elastase was from U. S. Biochemicals. Aminopeptidase M (11 units/mg) was from Calbiochem.

**Preparation of Hemoglobin**

Samples of human blood were collected in vacutainers containing heparin or EDTA. Red cells were isolated by centrifugation for 10 min at 850 × g to remove plasma and washed three times with an equal volume of a phosphate-buffered saline solution (pH 7.3). Washed cells were lysed with an equal volume of water and frozen in liquid nitrogen. The samples were thawed and centrifuged at 100,000 × g for 50 min to remove cell membranes.

**Reaction of Hemoglobin with BrCCl₃**

The reaction mixture contained 250 μM oxyhemoglobin in 350 ml of 50 mM potassium phosphate (pH 7.4). The mixture was made anaerobic by multiple purge and evacuation cycles with the use of argon scrubbed of oxygen by a mixture of zinc amalgam and chromous chloride (29). The hemoglobin was verified as being in the ferrous form by its electronic absorbance maximum at 555 nm. BrCCl₃ was added (1 mmol) with the use of a gas-tight syringe. The mixture was allowed to incubate with gentle stirring in the dark at room temperature under positive argon pressure. After 14 h, potassium ferricyanide was added to a final concentration of 114 μM in order to oxidize any hemoglobin remaining in the ferrous form.

**Isolation of Heme-Peptides**

**Extraction**—The reaction mixture was acidified to a pH of 2.2 with HCl and extracted with 2-butanone to remove reversibly bound hemes according to the method of Teale (30). The aqueous fraction, which contained native apohemoglobin and the irreversibly bound heme-protein adducts (Hb-heme), was then dialyzed against two changes of at least 20 volumes of distilled water at 4 °C.

**Elastase Digest**—To isolate large quantities of heme-peptides for...
analysis, the aqueous fraction, Hb-heme (concentration equal to 138 mM in protein calculated by assuming complete recovery of hemoglobin), was treated with elastase (0.05 mg/ml) in 600 ml of 67 mM Tris-
Cl (pH 8.5) at room temperature in the dark. At 20 h, more elastase was added (0.05 mg/ml). At 22 h, the reaction mixture was diluted into 100 ml aliquots and frozen in liquid nitrogen and stored at
-20 °C. These aliquots were processed one at a time either as is or after further treatment with elastase (0.05 mg/ml) for 68 h to produce shorter heme-peptides.

The 100-ml aliquots of the reaction mixtures were filtered (5 μm) and loaded on a reverse phase HPLC column (Vydac C4, 22 mm x
25 cm) at a flow rate of 6 ml/min. After 20 min of isocratic flow of solvent A (0.1% trifluoroacetic acid in water) at 8 ml/min, a linear gradient was run from 0 to 75% solvent B (0.1% trifluoroacetic acid in acetonitrile) over 240 min. The major heme-peptide adducts were detected by their absorbance at 530 nm and had retention times of 124 and 128 min for the longer heme-peptides and 127 and 134 min for the shorter heme-peptides. The purity of the heme-peptides were checked by the analytical HPLC procedure described in Fig. 2.

Aminopeptidase M Subdigest—The shorter heme-peptide adducts (concentration equivalent to 3.4 A/ml at 398 nm) were treated with aminopeptidase M (0.2 mg/ml) in 100 mM potassium phosphate (pH 7.4) at 37 °C for 5 days under an atmosphere of argon. The concentration of aminopeptidase M was increased by 0.1 mg/ml every 24 h.

Characterization of Heme-Peptides

Mass Spectrometry—Positive ion mass spectra were obtained on a Bio-Ion Nordic AB (Upsalla, Sweden) model BIN-10K plasma desorption mass spectrometer. Samples were dissolved in solutions of 0.1% trifluoroacetic acid in water/methanol (3:1, v/v). Solutions (2–5 μl) were placed on a nitrocellulose-coated aluminum foil 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.

Edman Sequence Analysis—Amino acid sequences of peptides were determined with the use of a Beckman model 890 M liquid-phase sequenator with the standard program. Prior to running the heme-peptides the cup was treated with 3 mg of Polybrene and three precycles were run.

Other—The HPLC system used consisted of a Waters Instrument (Millipore Corp., Milford, MA) 600E system controller with a Waters 490E Programmable Multiwavelength Detector. Data were collected with the use of a Nelson 760 Series system (PE Nelson, Cupertino, CA). Visible absorption spectra were obtained with a Hewlett-Packard 8450 diode array spectrophotometer. A Speed Vac apparatus (Savant Instruments, Farmingdale, NY) was used to dry all samples under vacuum.

RESULTS

Formation of Protein-bound Heme Adducts—The HPLC profiles of untreated and BrCCl3-treated human hemoglobin from red cell lysates are shown in Fig. 1. Under the acidic conditions of the chromatography, the native protein (Panel A) readily dissociated to heme (fraction 2) and apoprotein, which were detected by their absorption at 405 and 220 nm, respectively. The two major apoprotein fractions corresponded to the α- and β-chains of human hemoglobin.

The reaction of BrCCl3 with ferrous hemoglobin caused a complete alteration of the β-chain (profile at 220 nm in Panel B) and loss of approximately 64% of the prosthetic heme, as indicated by the decrease in peak area of fraction 2. Major altered heme products were formed corresponding to fractions 1, 3, 4, 5, and 6 (Panel B). Heme products corresponding to fractions 1 and 3 could be dissociated from the protein by extraction of the reaction mixture with 2-butanone under acidic conditions. Fractions 1 and 3 were identified by comparison to standards (32) as β-carboxyvinyl and α-hydroxy-β-trichloromethylvinyl heme derivatives of the ring I vinyl group, respectively. The identity of the other acid-dissociable heme product (fraction 4) is currently under investigation. The heme products corresponding to fractions 5 and 6, which had a high absorbance at 220 nm and were not extracted into 2-butanone, were irreversibly bound to the protein moiety. The current study focused on the characterization of these two protein-bound heme products.

Characterization of the Protein-bound Heme Adducts by Peptide Mapping—The aqueous phase, obtained after 2-butanone extraction of the reaction mixture and containing the protein-bound heme products, was treated with elastase. Aliquots were taken at various times and analyzed by the use of HPLC (Fig. 2). After a 32-h treatment with elastase, two prominent 405-nm absorbing fractions were observed (Fig. 2, Panel B, fractions I and II). More prolonged treatment with elastase produced fractions III and IV, apparently derived from fractions I and II.

Analysis of the heme-peptides corresponding to fractions I and II by Edman sequencing methods (Table I) gave the same result for both peptides: an initial cycle with no identifiable amino acid followed by the sequence DKLH. This peptide corresponded to residues 94-97 of the β-chain of human hemoglobin (33). The lack of signal for cysteine residue 93 indicated that this was a potential site of heme attachment. The proximal δistidine residue 92, not appearing in this sequence, was therefore ruled out as the site of heme attachment.

Characterization of the Heme Adducts by Mass Spectrometry—The plasma desorption mass spectrum of fraction I (Fig. 3, Panel A) gave a molecular ion at m/z 1311.9, which corresponded to the mass of the pentapeptide CDKLH (average mass 614.7), whose sequence was determined by Edman methods as described above, plus a heme-CCl3 moiety (average mass 698.4) minus a proton. Fragment ions at m/z 698.9 and 662.8 corresponding to heme-CCl3 and heme-CCl2 moieties, respectively, were also present. The mass spectrum of fraction III, a product of the prolonged digestion with elastase, gave a
Metabolism-based Covalent Bonding of Heme to Human Hemoglobin

I. Starting Material

D. 324 hr

0 20 40 60

TIME (Minutes)

FIG. 2. Time course of elastase digestion of Hb-heme examined by HPLC. Elastase digestion was performed as described under "Experimental Procedures," in a total volume of 2 ml. The elastase concentration was increased to 0.105 mg/ml at 124 h, and to 0.166 mg/ml at 152 h. Aliquots were taken after 32, 152, and 324 h, frozen in liquid nitrogen, stored at -20 °C, and immediately injected upon thawing onto a Vydac C18 0.46 X 25-cm reverse phase column. Each injection contained 215 pg of protein. The gradient used consisted of 5 min of isocratic flow of solvent A followed by a linear gradient to 75% solvent B over 60 min and then to 100% solvent B over 5 min. The same quantity of starting material was run on the same gradient (Panel A).

TABLE I

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino acid</th>
<th>Amount (pmol) Fraction I</th>
<th>Fraction II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Asp</td>
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<td>3</td>
<td>Lys</td>
<td>86</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>Leu</td>
<td>78</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>His</td>
<td>31</td>
<td>48</td>
</tr>
</tbody>
</table>

The site of heme attachment could not be determined from molecular ion at m/z 1061.5 (Fig. 3, Panel B), corresponding to the mass of a tripeptide CDK (364.4) plus a heme-CCL moiety minus a proton. Major fragment ions observed at m/z 698.6 (heme-CCL), 663.2 (heme-CCI), and 616.9 (heme) confirmed that the covalently bound heme was a heme-CCL moiety. Thus, fraction III is derived from fraction I by proteolytic cleavage at the carboxyl side of Lys-95. These results ruled out histidine residue 97 as the site of heme attachment.

The mass spectrum of fraction II (Fig. 4, Panel A) contained an ion at m/z 1349.3, which corresponded to the mass of the pentapeptide CDKLH plus a heme-CCL moiety minus a proton. Consistent with this assignment was a fragment ion at m/z 734.9 corresponding to a heme-CCL moiety, along with the previously observed fragment ions corresponding to heme-CCL (m/z 698.4) and heme-CCI (m/z 662.9) moieties. In addition, an ion (m/z 1097.8) corresponding to the mass of heme-CCL plus the tripeptide CDK was also present. The mass spectrum contained a number of other peaks that could not be explained as fragment ions for the heme-peptide adduct or as other related species, and were most likely unrelated impurities. The analysis of fraction IV, formed from the prolonged digestion with elastase, gave a cleaner spectrum, showing a molecular ion at m/z 1097.8 due to a heme-CCL adduct to the tripeptide CDK (Fig. 4, Panel B). Fragment ions were also observed at 734.8 (heme-CCL), 698.4 (heme-CCL), 662.9 (heme-CCL), and 616.8 (heme).

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these mass spectra since the major fragment ions corresponded to structures of the prosthetic heme without the peptide. Previous work with modified heme-peptide adducts formed with myoglobin (25) has indicated that this problem could not be overcome by collision induced dissociation on tandem mass spectrometers, since similar fragmentation patterns were observed. Thus, we carried out a strategy for locating the position of attachment of the heme prosthetic group by mass spectral analysis of the digestion products of the heme-peptides after aminopeptidase treatment.

The mass spectrum of the digestion of fraction III (CDK-heme-CCl₃) with aminopeptidase M showed an ion corresponding to the starting material at m/z 1060.9, an ion at m/z 818.8 representing the heme-CCl₃ moiety attached to cysteine, and fragment ions at 698.4 (heme-CCl₃), 663.3 (heme-CCl), and 617.3 (heme) (Fig. 5, Panel A). Similarly, the mass spectrum of the digestion of fraction IV (CDK-heme-CCl₃) showed an ion at m/z 855.2, corresponding to the attachment of a heme-CCl₃ moiety to cysteine. In addition, fragment ions were observed at 736.1 (heme-CCl₃) and 616.6 (heme) (Fig. 5, Panel B). These results established that the attachment of the prosthetic heme group was to Cys-93.

**Characterization of the Heme Adducts by Visible Absorption Spectrometry**—The visible absorption spectrum of hemoglobin (Fig. 6, Panel A) and the aequorin fraction from the 2-butanone extract of the BrCCl₃-treated hemoglobin, Hb-heme (Fig. 6, Panel B), which contains only apoprotein and a mixture of heme-CCl₃ and heme-CCl₂-derived protein-bound adducts, were compared under identical conditions. Hb-heme in the oxidized state showed absorbance maxima at 408 and 532 nm in comparison to those of the native protein at 404, 494, and 630 nm. The reduction of Hb-heme caused a red shift of the Soret maximum to 420 nm and the appearance of distinct α and β bands at 554 and 524 nm, respectively. The reduced spectrum of native hemoglobin gave maxima at 428 and 554 nm. The ferrous form of Hb-heme made a complex with carbon monoxide, giving absorbance maxima at 416 and 534, and shoulders at 568 and 620 nm. In comparison, native hemoglobin in the ferrous-CO state gave maxima at 418, 538, and 568 nm.

**Scheme 1. Formation of heme products from hemoglobin.**

**DISCUSSION**

A variety of chemicals are known to cause the formation of heme-protein cross-links in cytochrome P-450, myoglobin, and hemoglobin (27); until now, however, only the structures of the adducts of myoglobin caused by BrCCl₃ (25, 26) or H₂O₂ (34) had been elucidated. In this study, we have found that the reaction of BrCCl₃ with hemoglobin present in red cell lysates causes a heme-CCl₃ or heme-CCl₂ moiety to covalently bond to cysteine residue 93 of the β-chain, resulting in a complete modification of the β-chain and concomitant loss of 64% of the total heme. Comparison of peak areas at 405 nm, assuming similar extinction coefficients for all altered products, indicated that 44% of the original heme was metabolized to the heme-protein adducts and 20% to the soluble altered heme products. Thus the heme-protein adducts appear to be major products that account for the loss of both heme and the β-chain.

A mechanism for formation of the altered hemoglobin adducts is shown in Scheme I. Since two of the dissociable heme products isolated from the hemoglobin reaction are identical to those produced in the myoglobin reaction (32), we have assumed that similar mechanisms are involved in the reactions of these proteins with BrCCl₃. Thus, as described previously for myoglobin (25), the ferrous form reductively debrominates BrCCl₃ to a trichloromethyl radical, which then attacks the ring I vinyl moiety. This results in a heme radical attack of water and subsequent hydrolysis, or may be trapped by Cys-93 to form the heme-CCl₃ adduct. The cationic intermediate can be attacked by either water to form the α-hydroxy-β-trichloromethyllethyl heme derivative (25) or by Cys-93 to form the heme-CCl₃ adduct to the protein. The cationic intermediate may also eliminate a proton to form a CC₃-vinyl product, which can undergo an internal reductive dechlorination to form a CC₂ radical that subsequently gives another cationic species after dechlorination of the electron to the heme iron. This can give rise to the acrylic acid adduct as previously described (25, 26) by attack of water and subsequent hydrolysis, or may be trapped by Cys-93 to form the heme-CCl₂-protein adduct. In the case of myoglobin, but not for hemoglobin, a pathway involving the reductive debromination of BrCCl₃ by the CC₃-vinyl heme product leading to...
a bistrichloromethyl adduct of heme was observed.

The cause for the selective modification of the β-chain Cys-93 is unknown at present, although this residue has been identified previously as a target of electrophilic carcinogen metabolites (12, 18). The specificity for the β-chain may reflect the greater accessibility of the β-subunit heme to large substrates such as alkyl isocyanides (35, 36). The tertiary structure of hemoglobin appeared to be greatly altered by BrCCl₃, as the crystal structure of the native protein revealed the sulfur of Cys-93 is 14.76Å from the heme ring I vinyl group, which is too distant for covalent bond formation in the native form. A Dreiding model of heme bound to the peptide HCD, representing residues 92–94 where His-92 is the proximal histidine, showed that a conformation was possible such that the cysteine sulfur could be bonded to the ring I vinyl site even with the proximal histidine ligated to the heme iron. At this time insufficient amounts of material and the instability of the adducts under the conditions necessary for NMR prohibited the confirmation of the structure. The visible absorption spectrum of the protein-bound adducts supports the structure proposed above, as the presence of a Soret band indicated that the porphyrin π system was intact and that cleavage of the heme ring did not occur.

The visible absorption spectra of the protein-bound adducts were similar to those reported for hemichromes and hemes. The cause for the selective modification of the β-chain may generate from normal hemoglobin after treatment with various agents (17), from abnormal hemoglobin that autoxidize readily (24), or from hemoglobin of thalassemia patients (24). Specifically, the oxidized state of the protein-bound adduct gave an absorbance maximum at 532 nm and the reduced form at 554 and 524 nm, whereas the maxima reported for hemichrome (oxidized state) is 535 nm and for hemochrome (reduced state) is 558 and 529 nm (24). The absorbance maxima are also similar to those described for the protein-bound adduct of myoglobin formed from BrCCl₃, found at 530 nm for the oxidized and at 556 and 526 nm for the reduced state (26). These spectral changes for hemichromes have been ascribed to a bishistidine-ligated heme iron (24). We have postulated a similar heme environment for the protein-bound heme of BrCCl₃-altered myoglobin and proposed a histidine shuffle mechanism by which the proximal histidine residue 93 is replaced by histidine 64 and promotes autoxidation of myoglobin (26).

Hemichrome formation is thought to be the initial event leading to the appearance of Heinz bodies and subsequent cell lysis. Thus, hemichromes may represent hemoglobins with a protein-bound heme and, analogous to the case of myoglobin (26, 28) may generate H₂O₂, which may in turn initiate the process of red cell damage and hemolysis.

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