Metabolism-based Covalent Bonding of the Heme Prosthetic Group to Its Apoprotein during the Reductive Debromination of BrCCl₃ by Myoglobin*


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The reductive metabolism of BrCCl₃ by ferrous myoglobin leads to the alteration of the prosthetic heme to form products that can be dissociated from the protein and to those that are irreversibly bound to the protein. The major dissociable or soluble heme metabolites have recently been characterized. In this study, the irreversibly bound heme product was characterized by Edman degradation, amino acid analysis, and electronic absorption and mass spectrometry of peptides derived from the altered protein. It was found that the prosthetic heme was modified by a CCl₃ moiety derived from BrCCl₃, and was covalently bound to histidine residue 93, the normal proximal ligand to the hemeiron. The data are consistent with a mechanism by which the trichloromethyl radical reacts with the heme to form an intermediate that either can alkylate the proximal histidine residue or form soluble metabolites. The covalent bonding of the heme prosthetic moiety to the apoprotein likely leads to a change in the tertiary structure of the protein that may be responsible for its altered catalytic activity as well as its enhanced susceptibility to proteolysis. Similar processes may account, at least in part, for the covalent alteration of the heme prosthetic group of other hemoproteins caused by xenobiotics and endogenous substrates.

Alkyl halides, such as BrCCl₃ or CCl₃, can be reduced by various hemoproteins to form alkyl radicals (1–5). This reaction has toxicological importance, since the reduction of CCl₃ and BrCCl₃ to the trichloromethyl radical by liver microsomal cytochrome P-450 is the initial event leading to the hepatotoxicity produced by these xenobiotics (6, 7). During this metabolism the cytochrome P-450 is inactivated and rapidly lost from the endoplasmic reticulum (8, 9). In experiments where the prosthetic heme of P-450 cytochromes was radio-labeled, it was shown that administration of CCl₃ to rats caused approximately 28% of the total P-450 heme to become irreversibly bound to the apoprotein moiety (10). A similar product was formed in vitro, when CCl₃ was incubated with liver microsomal suspensions or a reconstituted system containing purified cytochrome P-450 (11–13). Several other classes of compounds that activate P-450 cytochromes have also been shown to produce irreversibly bound heme-derived products (11, 13–15). It has been speculated that a common feature of each of these reactions is the formation of either a radical or cation radical metabolite (13). Very little is known, however, about the chemical nature of the irreversibly bound heme-derived products due to the complexity of the membrane-bound P-450 enzyme system and the difficulty in obtaining adequate amounts of pure products for structural characterization.

Recently, myoglobin was found to catalyze a similar reaction; the reductive debromination of BrCCl₃ to form heme-derived metabolites that were irreversibly bound to the protein and those that were dissociable from the protein (16). The dissociable or soluble heme metabolites were characterized as β-carboxyvinyl, α-hydroxy-β-trichloromethyl, and α,β-bistrichloromethylketone derivatives of the heme prosthetic group and appeared to result from the initial regiospecific attack of the trichloromethyl radical at the ring I vinyl substituent (16). In the present study the protein-bound heme-derived metabolite has been characterized as an altered heme moiety covalently bound to the proximal histidine residue 93. We believe that the methods employed for isolating and characterizing the heme-protein adduct could be used for the characterization of similar products derived from other hemoproteins, including those of cytochrome P-450.

EXPERIMENTAL PROCEDURES

Materials

BrCCl₃ was from Aldrich and was washed with 5% Na₂CO₃ and distilled prior to use. Elastase was from U. S. Biochemicals. Amino-peptidase M (1 unit/mg) was from Calbiochem. Whale myoglobin was obtained from Sigma and U. S. Biochemicals.

Reaction of Myoglobin with BrCCl₃

The reaction mixture contained 140 μM myoglobin in 50 mM potassium phosphate, pH 7.4, in a total volume of 500 μl. In order to allow continuous spectral measurements under anaerobic conditions, the reaction was carried out in a three-neck round bottom flask fitted with a cuvette. The mixture was made anaerobic by purge and evacuation cycles with the use of argon, which was scrubbed of oxygen by a mixture of Zn amalgam and chlorine dioxide (17). Myoglobin was reduced stoichiometrically to the ferrous state by the addition of sodium dithionite. The reaction was then started by the addition of 3 mM BrCCl₃. After 60 min at room temperature with mixing, K₃Fe(CN)₆ was added to a final concentration of 225 μM to ensure that the hemes were in the oxidized state. The ferricyanide had no
effect on the HPLC profile of the products. The reaction mixture was acidified to a pH value of 2.2 with HCl, and the soluble hemes were extracted from the mixture with an equal volume of methylethylketone (three times) as described by Teale (18). The resulting aqueous phase (Mb-H) was treated with 2-butanone under conditions that had been reversibly modified by a heme-derived product (Mb-H) and apomyoglobin.

**Isolation of Heme-adducts**

**CNBr Treatment**—The Mb-H was partially separated from apomyoglobin in the Mb-H extract by chromatofocusing (PBE 94 and Polybuffer 96 from Pharmacia LKB Biotechnology Inc.) on a column (1 x 15 cm) equilibrated with 0.025 M ethanolamine-Cl, pH 9.5, in the running manner. The column was loaded with the Mb-H extract (12.6 mg of protein in 10 ml of water) and then eluted with polybuffer 96 (diluted 1:10 with water and adjusted with HCl to a pH value of 6.8) at a flow rate of 1.0 ml/min; the column fractions were monitored at 280 and 405 nm. The enriched Mb-H fraction eluted at a pH value of 8.1 and was further purified by HPLC on a C4 Hi-Pore column (0.46 x 25 cm from Bio-Rad) with a linear gradient (1.25%/min) from water (Solvent I) to 75% acetonitrile/2-propanol (1:1) (Solvent II) with 0.1% trifluoroacetic acid throughout, at a flow rate of 1.0 ml/min. The mixtures were dissolved in methanol and applied to a gold FAB probe in a linear gradient from 35% Solvent II to 50% Solvent II (0.6%/min) with an average flow rate of 8.0 ml/min. Yields of heme-peptide ranging from 9 to 14 mg were obtained from an initial 500-ml reaction mixture.

Complete hydrolysis of the elasate heme-peptide (28.8 OD equivalent at 266 nm) was carried out by treatment with cinnamoylpeptidase M (0.9 mg/ml) in 100 mM potassium phosphate, pH 7.4, with subsequent additions of 0.45 mg/ml of cinnamoylpeptidase M every 24 h over a period of 110 h at 37 °C. The resulting heme product was purified from the reaction mixture on a C4 Hi-Pore column (0.46 x 25 cm from Bio-Rad) at a flow rate of 1.0 ml/min and a linear gradient of 1.25%/min. Contaminating peptides, presumably derived from cinnamoylpeptidase M, were removed from the product by further treatment with elasate followed by HPLC purification as described above.

**Characterization of the Heme-adduct**

**Mass Spectrometry**—Positive ion fast atom bombardment (FAB) mass spectra were obtained with a Kratos M850 (Kratos Analytical Ltd., Manchester, United Kingdom [U. K.]) double focusing instrument, equipped with a mass range of 10000 a.m.u. at full accelerating voltage (8 kV). The instrument was fitted with a model B11NF sapphire field fast atom gun (Ion Tech, Ltd., Teddington, U. K.) and a postaccelerator detector. The post-accelerator detector was operated at 14 kV. Xenon was used to bombard the samples at 8 kV. The samples were dissolved in methanol and applied to a gold FAB probe in a matrix of 3-nitro-benzyalcohol. 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.

Experimental conditions for recording mass spectra on the quadrupole time-of-flight mass spectrometer have been described previously (19). Ionization was accomplished by the bombardment of the sample in 0.5 µl of monothioglyceral with 6-8 kV cesium ions (19).

**Amino Acid and Edman Sequence Analyses**—Amino acid analysis was performed on a Beckman System 6000 amino acid analyzer with ninhydrin detection after hydrolysis of heme-peptides with 6 N HCl in evacuated sealed tubes at 110 °C for 20–24 h. Amino acid sequences of peptides were determined with the use of a Beckman model 890 M liquid-phase protein sequencer with the standard program. Prior to running the heme-peptides the sample was treated with 3 µg of polybrene and three pre-cycles were run.

**Electronic absorption spectra** were obtained with a Hewlett-Packard 8450A diode array spectrophotometer. HPLC was performed with the use of a Waters Instrument (Millipore Corp., Milford, Massachusetts).

**RESULTS**

**Formation of Protein-bound Heme**—Fig. 1 (panel A) shows the HPLC profile of apomyoglobin prior to the reaction with Br2CN. The major fraction with absorption at 220 nm (fraction 4) corresponds to apomyoglobin and the fraction with absorption at 405 nm (fraction 2) corresponds to heme, which dissociated from the protein under the acidic conditions of the chromatography. As previously reported (16), the reaction of Br2CN with ferrous apomyoglobin resulted in the alteration of the prosthetic heme (panel B) to form several heme-derived products that could be detected by their absorption at 405 nm. Fractions 1, 3, and 6 were identified as 3-carboxyvinyl, α-hydroxy-β-trichloromethyl, and αβ-bistrichloromethyl heme derivatives of the I vinyl group, respectively (16). Fraction 5 appeared to represent a heme product covalently bound to the protein (Mb-H) because it absorbed light appreciably at both 405 and 220 nm and remained in the aqueous phase, along with apomyoglobin, after extraction of the reaction mixture with 2-butanol under conditions that are commonly used to extract heme from hemoprotein (18).

**Characterization of the Protein-bound Heme Adduct by Peptide Mapping**—Treatment of apomyoglobin with CNBr yielded three peptides, as expected from a protein that contains 2 methionine residues (Fig. 2, panel A, fractions 1, 2, and 3) (20). In contrast the CNBr cleavage of Mb-H after its partial purification from apomyoglobin by chromatofocusing produced in addition to fractions 1–3, a heme-peptide fraction that was detected at both 220 and 405 nm and appeared to be formed at the expense of fraction 2 (panel B). Moreover, comparison of the ratio of peak areas of fractions 2 versus 3 or 1 from the chromatograms of CNBr-treated apomyoglobin and Mb-H, before enrichment of Mb-H by chromatofocusing, revealed that approximately 20% of myoglobin in the reaction mixture was covalently modified by a heme-derived product (MA) consisting of a 600E gradient system controller and a 490E variable wavelength detector. The data were collected with the use of a Waters 760 Series system (PE Nelson, Cupertino, CA). A Waters HPLC system equipped with a Hewlett-Packard 1040A diode array detector was also used. All samples were dried under vacuum in a Speed Vac apparatus (Savant Instruments, Farmingdale, NY).
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FIG. 2. HPLC profiles of peptide fragments resulting from the cleavage of apomyoglobin or Mb-H by CNBr. Panel A, apomyoglobin; panel B, a fraction enriched with Mb-H isolated by chromatofocusing. The peptides were separated on a HiPore C4 column (0.46 x 25 cm from Bio-Rad) at a flow rate of 1.0 ml/min. A linear gradient (1.25%/min) starting 5 min after sample injection, from Solvent I (0.1% trifluoroacetic acid in H2O) to Solvent II (0.1% trifluoroacetic acid in CH3CN/2-propanol (1:1)) was used.

FIG. 3. HPLC profile of fragments resulting from proteolysis of Mb-H with elastase. The HPLC conditions are as described in Fig. 2.

The amino acid analysis of the peptide confirmed the sequence results by showing that the peptide was composed of nearly equimolar amounts of glutamine (or glutamate), serine, histidine, and threonine along with 2 equivalents of alanine (results not shown).

The elastase heme-hexapeptide was further hydrolyzed with aminopeptidase M to give two major heme fractions (Fig. 4 fractions 1 and 2). In experiments on the time course of this reaction, it was found that the initial product formed was fraction 2, that then was slowly converted to fraction 1. Amino acid analysis of fraction 1 showed that the only amino acid in the product was a histidine residue, clearly demonstrating that it was the site of heme attachment and that the histidine-heme bond could be cleaved by the conditions of the acid hydrolysis (Table II).

Characterization of the Heme Adduct by Mass Spectrometry—The positive ion FAB mass spectrum of the elastase heme-hexapeptide, obtained on a double focusing magnetic sector instrument, showed a molecular ion multiplet with an ion of highest intensity in this cluster at m/z 1310.2 (Fig. 5). This spectrum was consistent with a molecule comprised of the hexapeptide Ala-Gln-Ser-His-Ala-Thr and a heme group containing the additional elements of CCl3 (heme-CCl3). The major ion at m/z 1310.2 corresponded to the Fe3+ heme-peptide (M+H)+ ion (calculated m/z 1310.5), containing the most abundant isotopes of carbon (13C), chlorine (35Cl), and iron (56Fe). A similar cluster of ions also appeared at m/z 1208.8 that corresponded to M+ and (M+H)+ ions for the heme-pentapeptide adduct Ala-Gln-Ser-His-Ala and the same heme-CCl3 moiety. Partial cleavage of the protein on the C-terminal side of Ala 94 by elastase explained the appearance of this product as a contaminant in the sample.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino Acid</th>
<th>Amount (pmol)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Ala</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>Gln</td>
<td>190</td>
</tr>
<tr>
<td>3</td>
<td>Ser</td>
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<td>5</td>
<td>Ala</td>
<td>105</td>
</tr>
<tr>
<td>6</td>
<td>Thr</td>
<td>50</td>
</tr>
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* Includes break-down product peak.

The results shown in Table I were obtained by the protocol described in the Materials and Methods section. The data presented in Fig. 3 were obtained from a second experiment, also using the protocol described in the Materials and Methods section.

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Fragmentation observed in the FAB mass spectrum (Fig. 5) provided additional support for the structural assignment of the heme-peptide adduct. The signal at \( m/z \ 697.0 \) corresponded to that expected for the intact heme moiety containing a \( \text{CCl}_2 \) substituent (calculated \( m/z \ 697.2 \)). Losses of one and two chlorine atoms from this fragment accounted for the formation of ions at \( m/z \ 682.0 \) and \( 627.1, \) respectively. The signal at \( m/z \ 614.1 \) was that expected for the \( (M+H)^+ \) ion of the intact hexapeptide Ala-Gln-Ser-His-Ala-Thr (calculated \( m/z \ 614.3 \)), whose sequence was previously deduced by Edman degradation (Table I). When added together, the masses of the heme fragment ion and the neutral hexapeptide accounted for the \( m/z \) value of the \( (M+H)^+ \) ion of the intact heme-\( \text{CCl}_2 \)-hexapeptide adduct.

Further characterization of the heme-hexapeptide adduct was attempted by recording collision-activated dissociation mass spectra of the \( (M+H)^+ \) ion at \( m/z \ 1310 \) on the triple quadrupole mass spectrometer or on a four sector tandem mass spectrometer. Unfortunately, the facile cleavage of the heme-\( \text{CCl}_2 \) moiety from the hexapeptide precluded the assignment of the amino acid residue involved in the covalent bond to the heme group.

Evidence for a covalent bond between the histidine residue and the heme group, however, was obtained by hydrolyzing the heme-hexapeptide adduct with aminopeptidase M and analyzing the resulting heme adduct by mass spectrometry on the quadrupole Fourier transform instrument. The mass spectrum of the heme-hexapeptide (Fig. 6) showed a cluster of ions at \( m/z \ 851.3, 852.3, 853.3, 854.3, \) and \( 855.3 \). The first and second signals in this pattern corresponded to \( M^+ \) (calculated \( m/z \ 851.3 \)) and \( (M+H)^+ \) (calculated \( m/z \ 852.3 \)) ions for the heme-\( \text{CCl}_2 \)-hexapeptide covalent attached to His-93. The remaining signals in the multiplet contained additional isotopes of carbon, chlorine, and iron. Losses of the elements, \( \text{CH}_4\text{Cl}_2, \) \( (84.0 \text{ Da}), \) and \( \text{C}_6\text{H}_5\text{NO}_2 \) (His, \( 155.1 \text{ Da}) \) from both the \( M^+ \) and \( (M+H)^+ \) ions afforded the fragments at \( m/z \ 767.2 - 768.2 \) and \( 696.2 - 697.2, \) respectively. Additional losses of HCl from the heme fragment at \( m/z \ 696.2 - 697.2 \) explained the appearance of ions at \( m/z \ 660.2 - 661.2. \) Loss of a chlorine atom from \( m/z \ 661.2 \) afforded the ion at \( m/z \ 626.2. \)

The remaining ion clusters in the spectrum, at \( m/z \ 712.2, 676.2, \) and \( 641.2, \) appeared to result from loss of His, HCl, and Cl\(_2\), respectively, from a second heme-hexapeptide adduct containing an additional oxygen atom. The \( M^+ \) ion for this molecule occurred in the spectrum at \( m/z \ 867.2. \) This product appeared to be a degradation product formed during the aminopeptidase M treatment, since it was not apparent in the mass spectrum of the heme hexapeptide isolated from the elastase digest (Fig. 5).

**Characterization of the Heme Adduct by Electronic Absorption Spectrometry.—**The absorption spectrum of the elastase heme-hexapeptide (Fig. 7, panel A) was nearly identical to the spectrum of heme (panel B). The elastase heme-hexapeptide exhibited absorbance maxima at 396, 496, and 620 nm, whereas the spectrum of heme had maxima at 397, 497, and 618 nm. The absorbivities at the Soret, however, were quite different with values of 79 and \( 250 \text{ mm}^{-1} \text{ cm}^{-1} \) for heme hexapeptide and heme, respectively. These results showed that the covalent bond between the histidine residue and the prosthetic heme did not disrupt the conjugation of the porphyrin \( \pi \) system and indicated that the position of bonding was at the periphery of the heme group.

**DISCUSSION**

In this study, peptide mapping and mass spectrometry have provided clear evidence that the reduction of BrCCl\(_4\) by ferrous myoglobin results in the covalent bonding of the heme prosthetic group to its proximal ligand, histidine residue 93, and that the bound heme moiety, and not the amino acid, has been altered by the substitution of the elements of \( \text{CCl}_2. \) This finding represents the first demonstration that the irreversibly bound heme-protein product formed during hemoprotein-mediated metabolism involves a covalent bond between the heme and the histidine residue.
heme prosthetic group and an amino acid residue of the protein. The chemical, enzymatic, chromatographic, and mass spectrometric approaches employed to reach this conclusion should be applicable for the characterization of other heme-protein adducts.

The covalent bonding of heme to histidine residue 93 could have conceivably resulted from the initial activation of either heme or protein by the trichloromethyl radical (13). Since this radical is formed by the reductive debromination of BrCCl₃, presumably at the distal side of the heme, where oxygen normally binds and a channel for substrate entry and a pocket for substrate binding has been proposed (21, 22), this would rule out the activation of the proximal histidine ligand and instead indicates that the trichloromethyl radical activates the prosthetic heme.

Although there are several possible pathways that the trichloromethyl radical could activate the prosthetic heme, including initial attack at the meso carbon, the mechanism most consistent with the current findings as well as with the formation of the previously characterized soluble heme products, is incorporated in Scheme I and II. Scheme I depicts the proposed mechanism for the formation of the soluble products (16). The first step in this pathway is the activation of BrCCl₃ to the trichloromethyl radical. This species then selectively adds to the β carbon of the I vinyl to form a heme centered π-radical, which upon delocalization of the electron to the iron forms a cationic species and the ferrous iron. Addition of water to the cationic site gives the trichloromethyl alcohol adduct (compound 3) that was isolated previously (16). The cationic species may also deacetylate to produce the trichloromethyl vinyl adduct, which may react in either one of two pathways. One pathway is the reductive debromination of another equivalent of BrCCl₃; the trichloromethyl radical can then add to the same vinyl group forming a radical that subsequently abstracts a hydrogen atom, presumably from the protein (4). This accounts for the formation of the histidinyl trichloromethyl adduct (compound 6) that was previously characterized (16). The other pathway leads to the formation of the acrylic acid heme product (compound 1). The first step is envisioned as the peripheral reductive dechlorination of the trichloromethyl vinyl adduct to give a delocalized π-radical heme species. The electron can delocalize to the iron to form a ferrous iron and a cationic species (A), which upon subsequent addition of water would yield an unstable dichloroalcohol compound that would be expected to form the acyl chloride and undergo subsequent hydrolysis to the corresponding acrylic acid adduct (compound 1).

The regiospecificity of formation of the soluble heme products is likely due to the steric inaccessibility of the region near the II vinyl group. This is apparent from the crystal structure of myoglobin and that of myoglobin reconstituted with a heme derivative that contains an isopropyl moiety substituted for this vinyl group (23). This substitution caused a flipped orientation of the heme with the bulky isopropyl moiety in the region normally restricted to the I vinyl group. Thus, it appears that spatial constraints preclude attack by the bulky trichloromethyl group at the II vinyl site.

Scheme II illustrates a proposed mechanism for the formation of the protein-bound heme adduct that is consistent with the regiospecific formation of the soluble products and the electronic absorption spectra and mass spectrum of the histidine-bound heme adduct. The cationic intermediate A proposed in Scheme I may be attacked by an imidazole nitrogen of histidine 93, at either one of two electrophilic centers on the vinyl group leading to species B or C. Of the two possible structures proposed species B is favored. The mass spectrum of the histidine heme adduct showed a fragment ion at m/z 767.2 that was formed by the loss of CH₂Cl₂ from the M⁺ ion (Fig. 6). Unless the M⁺ ion suffers extensive bond reorganization prior to dissociation, elimination of CH₂Cl₂ would seem to be much more facile from structure B, particularly its potential enantiomeric form D, than from structure C. The nucleophilic reactivity of the imidazole nitrogens of histidine residues in proteins is well known. For example, diethylpyrocarbonate has been employed as a histidine selective acylating agent and is known to form an acid labile N-carboxyhistidyl adduct (24). In studies on the affinity labeling by 16α-bromoacetoxyprogesterone of 20β-hydroxy-steroid dehydrogenase, the histidyl imidazole nitrogens were sites of alkylation (25). Furthermore, studies with phospholipase A₂ have shown that a single nitrogen of the imidazole ring was alkylated exclusively by various bromoketones and methyl p-nitrosobenzenesulfonates (26).

The covalent bonding of the proximal histidine residue to the heme prosthetic group may cause significant movement of residues in the active site, which might perturb the entire three-dimensional structure of the hemoprotein. In support of this contention, it was recently found that the covalently altered myoglobin was more rapidly hydrolyzed by trypsin than was the native protein (13). This result may help to explain how administration of CCl₃, as well as other xenobiotics that cause the formation of heme-protein adducts leads to the loss of the protein moiety of select P-450 cytochromes from the endoplasmic reticulum in vivo (8–10, 27, 28). Perturbations of the structure of myoglobin may also help to explain the enhanced reducing activity associated with the altered hemoprotein (13). For example, the reduced altered myoglobin was rapidly oxidized by molecular oxygen, presumably leading to the formation of reduced oxygen species such as superoxide anion radical and hydrogen peroxide (13). Such an activation phenomenon may have significant toxicological implications.
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Scheme I. Proposed mechanism for the formation of the soluble heme products. Compounds 1, 2, 3, and 6 correspond to the fractions in Fig. 1.

Scheme II. The proposed mechanism for the formation of the histidine heme-adduct. Species A is the same in both Scheme I and II. Species B and C represent two possible histidine-heme adducts. Species D is a tautomeric form of species B.

Since the reaction described in this investigation is selective and occurs while the heme prosthetic group is in the active site of the protein, it might be useful for characterization of amino acids at the active-site of other hemoproteins, such as heme oxygenase, prostaglandin H synthetase, as well as mammalian P-450 cytochromes.

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