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Characterization by NMR of the Heme-Myoglobin Adduct Formed during the Reductive Metabolism of $BrCCl_3$

COVALENT BONDING OF THE PROXIMAL HISTIDINE TO THE RING I VINYL GROUP*

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The reductive debromination of $BrCCl_3$ by ferrous deoxymyoglobin leads to the covalent bonding of the prosthetic heme to the protein. We have previously shown, by the use of peptide mapping and mass spectrometry, that histidine residue 93 is covalently bound to the heme moiety. In the present study the structure of the heme adduct was more completely determined by ^1H and ^{13}C NMR techniques. We have found that the ring I vinyl group of the prosthetic heme was altered by the addition of a histidine imidazole nitrogen to the α-carbon and a CCl_2 moiety to the β-carbon. The electronic absorption spectra of the oxidized and reduced states of the altered heme-protein indicated that the heme-iron exists in a bis-histidine-ligated form. Analysis of the crystal structure of native myoglobin suggested that for the altered heme-protein, histidine residues 97 and 64 are ligated to the heme-iron and that residue 97 has replaced the native proximal histidine residue 93. These movements, in effect a "histidine shuffle" at the active site, may be responsible for the enhanced reducing activity of the altered protein.

Cytochrome P-450, a hemoprotein with monooxygenase activity, is a critical component in the metabolism of various xenobiotics, including drugs and environmental pollutants as well as endogenous compounds. Many of these substrates are metabolized to reactive intermediates that can cause the irreversible inactivation of the enzyme. Three pathways have been described to account for the inactivation of P-450 cytochromes by reactive metabolites (1–3). Two of these pathways have been well characterized and involve the covalent addition of the metabolites to the heme or the protein moiety of P-450 cytochromes (4–11). A third pathway has been recently described and involves the covalent bonding of the heme to the protein moiety (1, 2). For example, when carbon tetrachloride is administered to rats, about 28% of the total P-450 heme in the liver becomes irreversibly bound to the apoprotein moiety (12). Similar adducts are also found when CCl_4 is incubated with liver microsomes (1, 13). Several other xenobiotics, including drugs and endogenous compounds such as hydrogen peroxide and lipid hydroperoxides, can also inactivate P-450 cytochromes by this pathway (1–3, 12–21). The chemical structures of the heme-protein adducts, however, have not been elucidated due to the complexity of the cytochrome P-450 system and lack of sufficient amounts of product for analysis.

Recently, it has been shown that myoglobin and hemoglobin can also form heme-protein adducts (1, 22–24). For example, the reaction of $BrCCl_3$ with sperm whale myoglobin, in a reaction similar to that of CCl_4 with P-450 cytochromes, leads to the formation of a protein-bound adduct (24). When this reaction was performed under single turnover conditions where other reactions that might occur after initial heme adduct formation were minimized, the amount of product formed was sufficient for structural characterization. Three soluble heme products, which could be dissociated from the protein, were also formed in the reaction (22). These soluble heme products have been characterized by the use of mass spectrometry and one- and two-dimensional NMR techniques as derivatives of heme that are modified in a regiospecific manner at the ring I vinyl group (see Fig. 2) (22). The protein-bound adduct was enzymatically hydrolyzed to a heme hexapeptide (Ala-Gln-Ser-His-Ala-Thr) and to heme-histidine fragments. Analysis of these products by the use of mass spectrometry revealed that the heme was altered by a CCl_2 group that was covalently bonded to histidine residue 93 of sperm whale myoglobin (24). We have also discovered that this covalent alteration results in a hemoprotein that can undergo redox cycling, as evidenced by its facile reduction of molecular oxygen and CCl_4 (1). This process has been termed "suicide activation" or "metabolism-based activation."

In the current study, the structure of the heme-myoglobin adduct was more completely defined by NMR and electronic absorption spectroscopy. The results help to define structural features that may contribute to the enhanced reductive activity of the altered myoglobin.

EXPERIMENTAL PROCEDURES

Materials—Pyridine-d_5 and $Br^{13}CCl_3$ (99 atom %) were purchased from Merck. Methanol-d_4 and deuterium oxide were purchased from Cambridge Isotope Laboratories (Woburn, MA). Stannous chloride was from Aldrich. L-Histidine methyl ester was purchased from Fluka A.G. (Switzerland). Sperm whale myoglobin was purchased from U. S. Biochemicals Corp.

Preparation of Heme-Hexapeptide (Ala-Gln-Ser-His-Ala-Thr) and Heme-Histidine Adducts—The reaction of ferrous deoxymyoglobin (140 μM) with $BrCCl_3$ (3 mM) has been previously described (22, 24). The Mb-H[1] extract was prepared from the $BrCCl_3$ reaction mixture by acidification to a pH value of 2.2 with HCl and extraction of the

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[1] The abbreviations used are: Mb-H, myoglobin with covalently bound heme; HPLC, high pressure liquid chromatography; PCOSY, purged COSY.

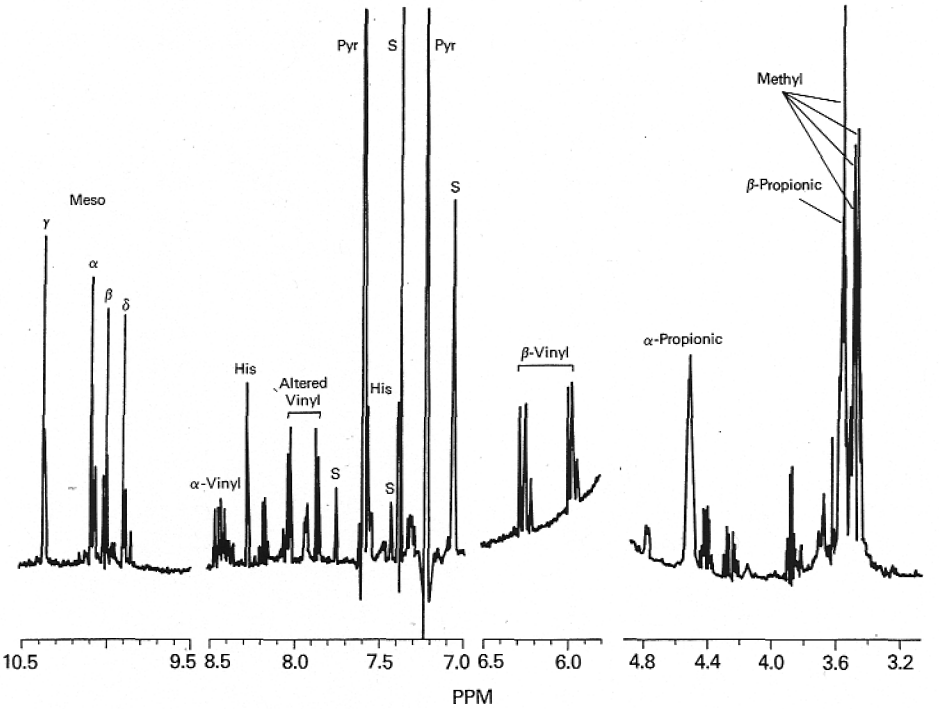


FIG. 1. **500-MHz ^{1}H NMR of the reduced heme-histidine adduct in pyridine.** Signals labeled *pyr* are due to pyridine, and *S* represents signals due to ^{13}C satellites.

soluble hemes with an equal volume of methyl ethyl ketone (three times) (24). The resulting aqueous phase (Mb-H extract) contained a mixture of apomyoglobin that had been irreversibly modified by a heme-derived product (Mb-H) and apomyoglobin. The CNBr fragment (residues 56–131) containing the covalently bound heme was prepared by treatment of Mb-H extract (200 μg of protein/ml) overnight with CNBr (1.0 mg/ml) in 70% formic acid at room temperature (24).

The heme-hexapeptide was prepared by treatment of the Mb-H extract (1 mg/ml) overnight with elastase (0.05 mg/ml) in 67 mM Tris-Cl, pH 8.5 (24). The heme-histidine product was obtained by treatment of the heme-hexapeptide with aminopeptidase M (0.9 mg/ml, starting concentration) in 100 mM potassium phosphate, pH 7.4, with subsequent additions of 0.45 mg/ml peptidase every 24 h over a period of 110 h at 37 °C (24). The heme products were isolated from the reaction mixtures by reverse phase HPLC on C4 columns (24).

NMR Spectrometry—Heme adducts were dissolved in methanol-d_4, and the solutions were evaporated to dryness with a Speed-Vac (Savant Instruments, Inc., Farmingdale, NY) to remove water from the sample. This procedure was repeated with deuterium oxide in the place of methanol-d_4 before use. The heme-hexapeptide residue (2–3 mg) was dissolved in pyridine-d_5 (0.5 ml) under a flow of argon to help exclude moisture. Only a small fraction of the heme-histidine sample (0.5 mg) placed in the NMR tube could be dissolved in pyridine, and thus only a one-dimensional spectrum was obtained. Stannous chloride was added (6–9 mg) to reduce the heme adducts to the diamagnetic pyridine Fe^{2+} complex (22). All spectra were recorded at 20 °C.

^{1}H NMR spectra were obtained on Bruker AM-500 and AM-600 spectrometers, operating at 500 and 600 MHz, respectively. The ^{1}H spectrum of the heme-histidine adduct recorded at 500 MHz resulted from 1024 transients with a 1.5-s delay between transients. The ^{1}H spectrum of the heme-hexapeptide recorded at 600 MHz resulted from 256 transients. For both spectra, resolution enhancement was used by applying a Lorentzian-Gaussian transformation. The phase-sensitive COSY spectrum was recorded with the purged COSY (PCOSY) technique (25), resulting in nearly double the sensitivity of the more commonly used double-quantum filtered COSY experiment. The spectrum is the result of a 700 × 1024 data matrix, recorded with 16 transients for each of the 700 t_1 increments. The spectrum was "purged" (25) with a one-dimensional spectrum, resulting from 128 transients.

The ^{13}C spectrum of the ^{13}C-enriched heme-hexapeptide, prepared from the reaction mixture of myoglobin and $^{13}CBrCl_3$, was obtained on a Varian XL200 at 50 MHz by the collection of 4500 free induction decays at 1.0-s intervals. A ^{1}H-^{13}C spin-echo difference experiment (26) on the ^{13}C-enriched hexapeptide-heme adduct indicated that no protonated ^{13}C nuclei were present. Multiple bond correlations to the enriched ^{13}C site were observed with the use of the heteronuclear multiple bond correlation experiment (27). The heteronuclear multiple bond correlation spectrum was recorded at 500-MHz ^{1}H frequency on a Nicolet NT-500 spectrometer. The two-dimensional spectrum resulted from 140 t_1 increments, with 128 transients/t_1 duration.

RESULTS

Characterization of the Heme Adduct by ^{1}H NMR Spectroscopy

Heme-Histidine Adduct—The proton signals found in the NMR spectrum of the heme-histidine adduct (Fig. 1) were assigned by comparison to the chemical shifts of the protons of heme (Fig. 2, *2*) and the Iβ-carboxyvinyl (*1*), Iα,β-bistrichloromethylethyl (*6*), and Iα-hydroxy-β-trichloromethylethyl (*3*) derivatives of heme that were previously characterized as products of the $BrCCl_3$ reaction (22). Most signals appeared within 0.1 ppm of the Iα,β-bistrichloromethylethyl (*6*) derivative of heme. The downfield signals at 10.39, 10.10, 9.98, and 9.88 ppm corresponded to the four mesoprotons. The four signals in this region at approximately one-third the amplitude of the mesoproton signals probably represented a decomposition product. Other low intensity signals were present in the spectrum and probably corresponded to the same decomposition product. The signals at 4.52 and 3.59 ppm corresponded to the α- and β-methylene protons, respectively, of the propionic acid groups. Although β-methylene protons were not well resolved from the four methyl signals at 3.58, 3.50, 3.49, and 3.47 ppm, the assignments were confirmed by two-dimensional PCOSY studies to be described below.

Of the remaining possibilities, the two vinyl functional groups were probably the sites of covalent modification. In this regard, the doublets at 5.95 and 6.26 ppm and the quadruplet at 8.45 ppm corresponded to two β-carbon protons and one α-carbon proton, respectively, of a single unaltered vinyl moiety. The doublets at 7.88 and 8.05 ppm represented two protons on an altered vinyl moiety. These protons were vicinal rather than geminal protons, because their coupling was 9.2

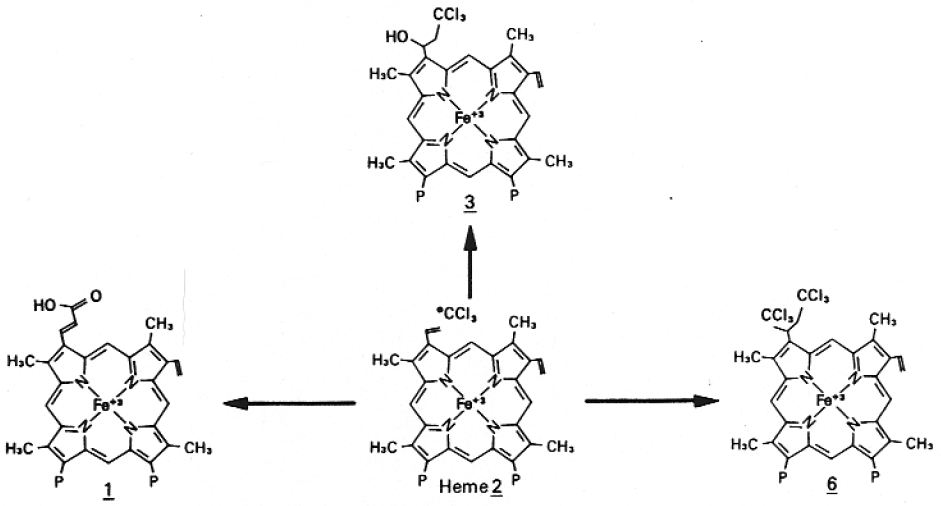


FIG. 2. **Structures of the soluble heme products (22).** The *numbers* refer to the order of elution from the reverse phase HPLC column (22). The products shown are the Iβ-carboxyvinyl (*compound 1*), Iα,β-bistrichloromethylethyl (*compound 6*), and Iα-hydroxy-β-trichloromethylethyl (*compound 3*) derivatives of heme 2.

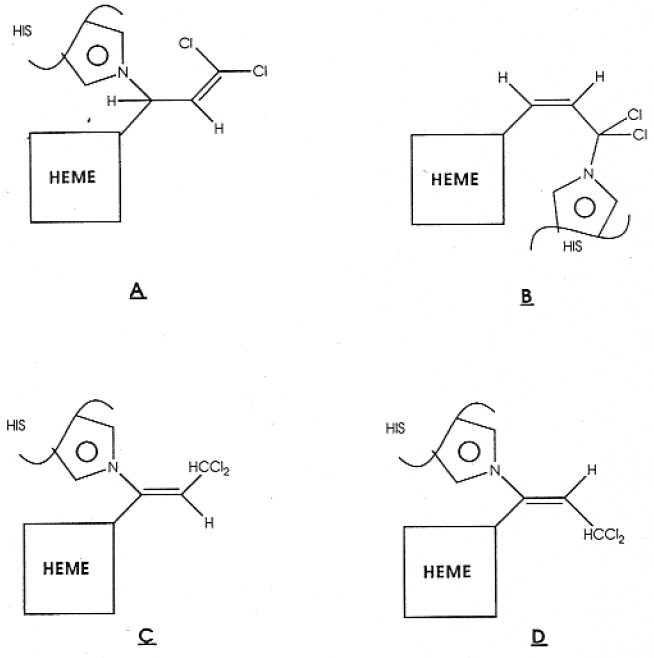


FIG. 3. **The four possible structures of the heme-histidine adduct.** Only a portion of the histidine molecule is shown. The *box* represents the rest of the heme molecule.

Hz. The singlet peaks at 7.57 and 8.28 ppm were assigned to the two protons on the ring-carbon atoms of the histidine imidazole moiety by comparison with similar spectrum of histidine methyl ester in pyridine. These results, in addition to those of the previously reported mass spectrum of this compound (24), are consistent with the heme adduct being one of four possible structures containing a single altered vinyl group (Fig. 3). Moreover, the close correspondence of the resonances found for the heme-histidine product to that of the Iα,β-bistrichloromethylethyl (Fig. 2, *6*) product indicated that the vinyl group of ring I was the site of the alteration (22). Because of the poor solubility of the heme-histidine sample in pyridine, further NMR studies to delineate between the four structures were done on the heme-hexapeptide compound.

Heme-Hexapeptide (Ala-Gln-Ser-His-Ala-Thr) Adduct with ^{13}C Enriched at the Carbon Containing Two Chlorines—The ^{13}C NMR (200 MHz) proton-decoupled spectrum of the heme-hexapeptide isolated from an incubation with 99 atom % $Br^{13}CCl_3$ showed a single peak with a chemical shift of 124.56 ppm. Examination of ^{13}C proton couplings on the Nicolet 500-MHz instrument ruled out the presence of a proton on the ^{13}C-enriched carbon. Long range couplings, however, were observed to protons with chemical shifts between 8.0 and 8.2 ppm and at 7.9 ppm. These signals, which were identified in the PCOSY experiments described below, represented protons on the altered vinyl group. Of the four *structures* proposed for the heme adduct (Fig. 3), *C* and *D* were ruled out because the dichloro-substituted carbon atoms were bonded to protons and would be expected to show a large ^1H-^{13}C coupling. *Structure A* was favored over *structure B*, because the chemical shift was that of an olefinic carbon ($H_2C{=}\underline{C}Cl_2$ has a chemical shift of 125.9 ppm) (28). Moreover, the mass spectrum of the heme-histidine adduct showed a fragment ion corresponding to the loss of CH_2Cl_2, which would more likely arise from *structure A* than from *structure B* (24).

Heme-Hexapeptide (Ala-Gln-Ser-His-Ala-Thr) Adduct with the Natural Abundance of Isotopes—The ^1H NMR spectrum of the heme-hexapeptide (Fig. 4) was also highly similar to that observed for the Iα,β-bistrichloromethylethyl adduct of heme (Fig. 2, *6* (22)), and consequently it was used to assign the following proton signals for the peptide adduct. The signals due to three of the four mesoprotons were evident at 10.35, 10.06, and 9.86 ppm and corresponded to the γ, β, and δ mesoprotons, respectively. The α-mesoproton, however, appeared as two signals at 10.09 and 10.04 ppm in approximately a 3 to 2 ratio, respectively. The signals for the α- and β-methylene protons of the propionic groups appeared at 4.50 and 3.55 ppm, respectively. Resonances at 3.44 and 3.53 ppm corresponded to protons on the methyl groups of rings III and IV. In contrast, the signals for the methyl groups on ring I and ring II each appeared as a pair of signals, in approximately a 3 to 2 ratio. This gave rise to peaks for the ring I methyl group at 3.56 and 3.52 ppm and those for the ring II methyl group at 3.50 and 3.47 ppm. Similar pairs of resonances, in approximately a 3 to 2 ratio, appeared for the protons on the carbon atoms of the histidine imidazole moiety at 8.34 and 8.25 ppm and at 7.42 and 7.40 ppm and for the signals of the altered vinyl group at 8.15 and 8.09 ppm and at 7.89 and 7.87 ppm. Long range couplings to the signals of the altered vinyl group were observed in the ^{13}C experiment described above. The β-protons of an unmodified vinyl moiety gave rise to doublets at 6.25 and 5.96 ppm, while the α-proton of this vinyl group was buried amid signals near 8.40 ppm.

The signals at 8.46, 8.41, 7.80, and 7.78 ppm could not be

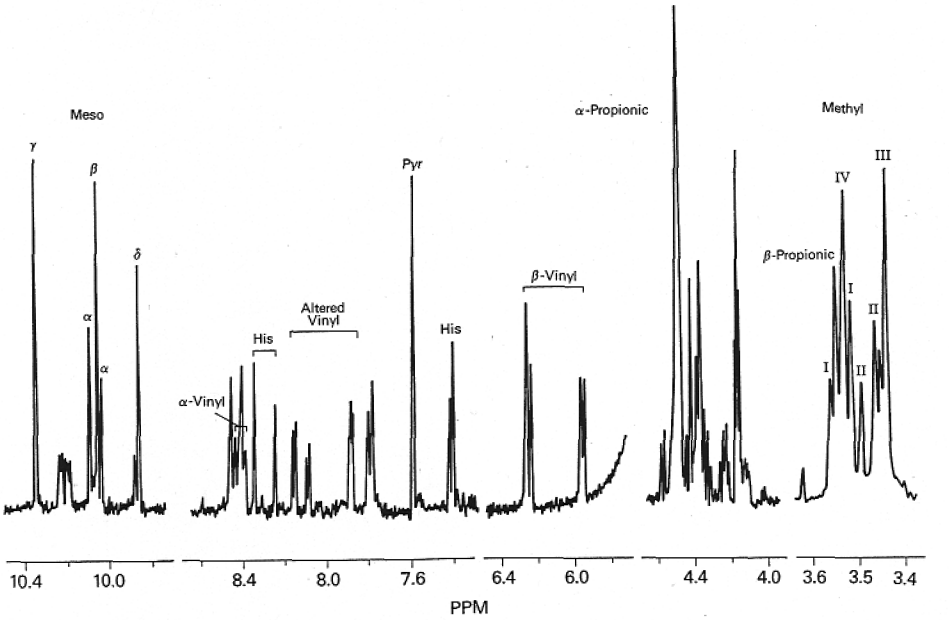


FIG. 4. **600-MHz ^{1}H NMR of the reduced heme-hexapeptide adduct in pyridine.**

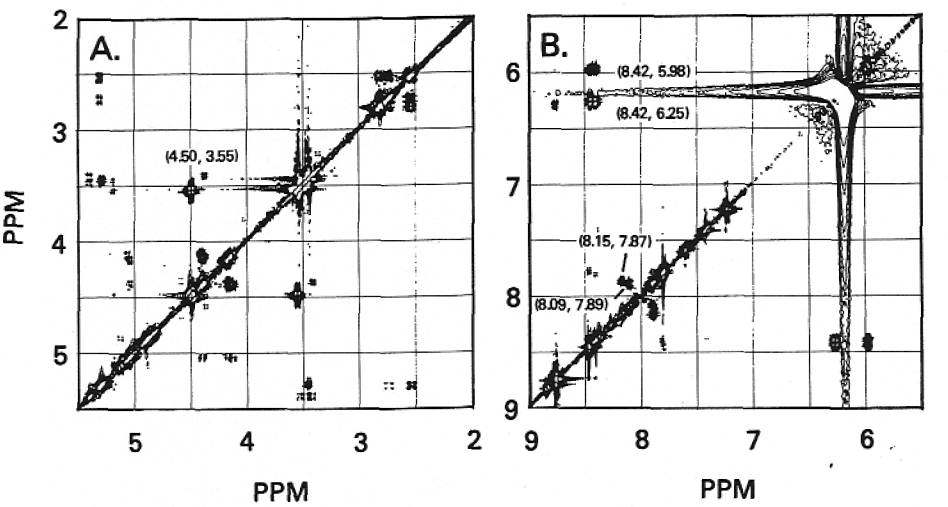


FIG. 5. **Two-dimensional phase-sensitive PCOSY NMR spectrum of the reduced heme-hexapeptide adduct in pyridine.** The spectrum was obtained on a Bruker AM-500 spectrometer as described under "Experimental Procedures."

assigned. Although they appeared as singlets, the PCOSY experiment described below indicated that the signals at 8.46 and 8.41 ppm were coupled, albeit with an extremely small coupling, to the signals at 7.80 and 7.78 ppm, respectively. Although these signals have chemical shifts consistent with protons on an altered vinyl moiety, no long range couplings to the ^{13}C-enriched carbon atom could be detected. These signals thus did not correspond to protons on the altered vinyl group of the heme adduct.

Two-dimensional PCOSY analyses verified the assignments for the β-methylene protons of the propionic groups, the α-proton of the unaltered vinyl group, and the two protons on the altered vinyl group of the heme-hexapeptide (Fig. 5). For example, the spectrum in *panel A* of Fig. 5 revealed that the β-methylene protons of the propionic groups at 3.55 ppm, which were buried among the methyl signals in the one-dimensional spectrum (Fig. 4), were coupled to the α-methylene protons at 4.50 ppm. Similarly, the protons of the β-carbon of the unaltered vinyl moiety at 6.25 and 5.96 ppm were coupled to the adjacent α-proton at 8.42 ppm (Fig. 5, *panel B*). The signals due to the two protons of the altered vinyl moiety were sorted into two sets by their couplings. The proton at 8.15 ppm was coupled to the proton at 7.87 ppm, and the proton at 8.09 ppm was coupled to the proton at 7.89 ppm, both by 8.3 Hz (Fig. 5, *panel B*). Since these four signals represented only two protons, it was concluded that each proton must reside in two different magnetic environments, probably because of restricted rotation about the altered vinyl group, which would give rise to two conformers that interconverted slowly on the NMR time scale.

These conformational effects have confirmed the assignment of the altered vinyl group to ring I. The protons nearest to the altered vinyl group should exhibit a split signal due to the two conformers described above. The ring I vinyl is implicated, since the α-mesoprotons, ring I methyl protons, ring II methyl protons, and the two histidine imidazole protons all exhibited split signals. In contrast, the β-mesoprotons and ring III methyl protons gave rise to only single signals, because they were too far removed from the modified ring I vinyl group. This conclusion is consistent with the finding that the ring I vinyl group is also exclusively modified in each of the three soluble heme products (22). Moreover, it appears that spatial constraints would preclude attack by the bulky trichloromethyl group at the ring II vinyl site. The steric inaccessibility of this region is apparent from the crystal structure of myoglobin and of myoglobin reconstituted with a heme

derivative that contains a bulky isopropyl moiety substituted for the ring II vinyl group (29).

Electronic Absorption Spectra of the Altered Heme Products

The electronic absorption spectrum of the covalently modified myoglobin (Mb-H) in the oxidized state showed a Soret band at 408 nm (Fig. 6, *panel B*) similar to that of the native protein at 406 nm (Fig. 6, *panel A*). The visible region of the spectrum of Mb-H, however, was distinct from that of myoglobin, as only one band (at 530 nm) was observed as opposed to both α and β bands (at 634 and 500 nm, respectively) for the native protein. The spectrum of Mb-H in its reduced state revealed a red shift of the Soret band to 420 nm and the presence of distinct α and β bands at 556 and 526 nm, respectively, with a slight shoulder at approximately 610 nm. In contrast, the spectrum of the reduced native protein showed a Soret band red-shifted to 432 nm and a single maximum in the visible region at 556 nm. Mb-H formed a ferrous carbonyl complex with absorbance maxima at 416, 536, 568, and 620 nm. The ferrous carbon monoxide complex of myoglobin showed absorbance maxima at 423, 542, and 578 nm that were red-shifted as compared with the absorption maximum of Mb-H.

Although the spectrum of the heme-hexapeptide formed during elastase treatment was similar to that of native heme (24), in the presence of 5 mM histidine methyl ester, the absorption spectra in the oxidized, reduced, and reduced CO states (Fig. 6, *panel C*) resembled that of the Mb-H. For example, the oxidized state Soret absorbance was at 406 nm with a visible band at 530 nm. The ferrous state showed absorbance maxima at 414, 552, 522, and 606 nm. The 606-nm band was more distinct in this complex than the shoulder observed at 610 nm in the spectrum of reduced Mb-H (Fig. 6, *panel B*). The ferrous carbonyl complex of the heme-hexapeptide sample showed absorbance maxima at 412, 534, and 560 nm. The spectrum of the heme peptide (Fig. 6, *panel D*) isolated from CNBr cleavage of Mb-H (residues 56–131) even more closely resembled the spectrum of Mb-H, with maxima in the oxidized state at 408 and 530 nm and in the reduced state at 418, 522, and 554 nm with a shoulder at 610 nm. The ferrous carbonyl complex showed absorbance maxima at 416, 532, and 566 nm.

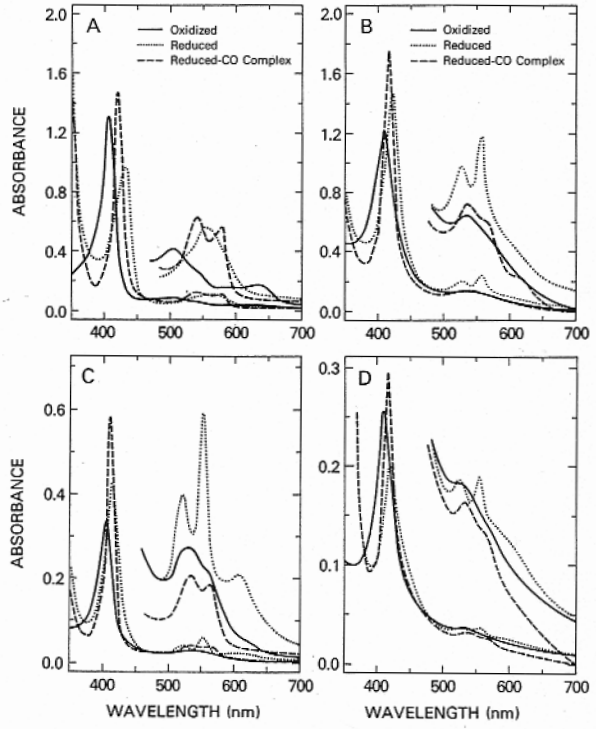


FIG. 6. **Absorption spectra of myoglobin, Mb-H, elastase-heme-hexapeptide, and CNBr-heme-peptide in various states.** Absorption spectra were determined at room temperature with a Hewlett-Packard model 8450A diode array detector. All samples were in 50 mM potassium phosphate (pH 7.4) containing 10 mM EDTA. *Panel A*, native myoglobin was 0.17 mg of protein/ml; *panel B*, Mb-H extract was 1.36 mg of protein/ml; *panel C*, elastase-heme-hexapeptide with 5.0 mM histidine methyl ester; *panel D*, CNBr-heme-peptide (residues 56–131). ——, oxidized; · · · ·, dithionite-reduced; – – –, reduced CO complex. The visible region of the spectra are shown at 5- and 10-fold greater potentiation for *panels A* and *B* and *panels C* and *D*, respectively.

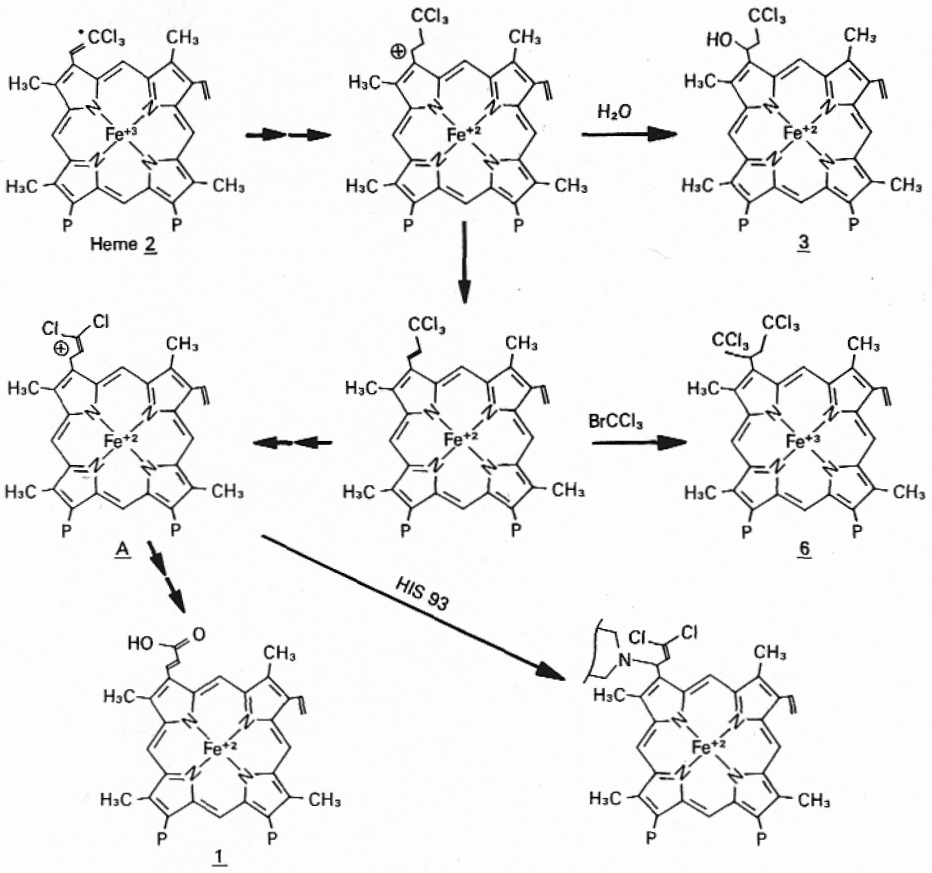
DISCUSSION

In this study, the first complete structural elucidation of a xenobiotic-induced heme-protein adduct has been determined. A mechanism for the formation of the adduct, which is consistent with its structure as well as the structures of the previously characterized soluble heme products (22), is proposed (Scheme I). The first step is the regiospecific reaction of the trichloromethyl radical with the β-carbon of the ring I vinyl moiety. This leads to the formation of a cationic species that can either yield the trichloromethyl alcohol adduct (Scheme I, *3*) or form a trichloromethyl vinyl intermediate. The vinyl intermediate can either form the bistrichloromethyl adduct (*6*) after reduction of another equivalent of $BrCCl_3$ or form another cationic intermediate (*A*) that can eventually result in the acrylic acid adduct (*1*). This intermediate (*A*) also can be attacked at the α-carbon by an imidazole nitrogen of histidine residue 93, leading to the covalently bound adduct characterized in this report.

In contrast, the irreversible binding of the heme of myoglobin to its apoprotein caused by hydrogen peroxide is thought to result from the initial activation of tyrosine residue 103, which subsequently attacks the heme prosthetic group at an as yet unidentified site (23). Therefore, it appears that heme-protein adducts can arise from initial activation of either heme or protein depending on the substrate (1).

The reactivity at the heme mesocarbons and methyl carbons, as well as the pyrrole nitrogens, to xenobiotic metabolites has been well documented (4, 5, 30, 31). In contrast, the reactivity at the vinyl position to xenobiotics is less well known. This reactivity was first reported in the study of the soluble heme products from the $BrCCl_3$ reaction (22). The present report further corroborates this reactivity in the formation of the protein-bound adduct. In a recent study, the reactivity of the ring I vinyl moiety has also been shown in a reaction of myoglobin with nitrite (32).

Although native myoglobin is a storage form of dioxygen, and therefore forms a stable ferrous oxygen complex, the altered heme protein described in this study, as well as that formed by HOOH, rapidly reduces dioxygen, presumably leading to the formation of superoxide anion or hydrogen peroxide (1, 33). The causes of this enhanced autoxidation appear to be due to changes in the active site structure of the altered myoglobin. In the case of myoglobin altered by $BrCCl_3$, there are several indications for a change in its active site structure. First, it is known that the tertiary structure of the entire molecule has been perturbed, as is evident by the ease at which trypsin hydrolyzes the altered myoglobin compared with native myoglobin (1). Second, it is clear from the deduced structure of the protein-bound heme adduct that His-93 cannot remain as the proximal iron ligand after covalent bonding to the α-carbon of the ring I vinyl group due to geometric constraints. Third, the absorption spectrum of the heme



SCHEME I. **Proposed mechanism for the formation of the altered heme products from the reaction of $BrCCl_3$ with myoglobin.**

adduct indicated that in its reduced state it can exist in a bis-histidine-ligated form. This follows from the similarity of the spectral properties of the Mb-H to that of cytochrome b_5, an electron transport protein having such a bis-histidine ligation (34), and that of the heme-histidine adduct in the presence of histidine. Furthermore, the absorption spectrum of the CNBr peptide indicated that the bis-histidine ligands are between residues 56 and 131. Yet unlike cytochrome b_5, Mb-H can form a ferrous carbonyl complex.

Evidently, changes in iron ligation have taken place with replacement of the His-93 by another histidine ligand. Of the 12 histidine residues in native myoglobin, 3 are in proximity to the heme (Fig. 7, *panel A*). These include the proximal ligand, residue 93, the distal histidine residue 64, and residue 97. It can be envisioned that as His-93 covalently binds to the vinyl residue, the heme group moves toward the periphery of the protein. As a first approximation, the heme group was moved 5 Å in this direction while keeping the rest of the molecule constant (Fig. 7, *panel B*). This resulted in a conformation with histidine residues 97 and 64 in position to act as iron ligands. This "histidine shuffle" may help explain the enhanced reduction of dioxygen and CCl_4 associated with this alteration.

Although heme in solution is rapidly oxidized, the stability of the ferrous oxygen complex of heme in myoglobin is thought to be due to H-bonding of the oxygen to the distal histidine (residue 64) and to steric effects by the polypeptide that exclude solvent anions (35, 36). Recently, it has been shown that substitution of the distal histidine with various amino acids by site-directed mutagenesis results in the increased autoxidation of myoglobin, presumably by affecting the above parameters (36). Analogous structural changes probably can be attributed to the histidine shuffle. First, the geometry for hydrogen bonding of the bound dioxygen by the distal histidine would likely not be maintained, thereby decreasing the stability of the ferrous oxygen complex. Second, this histidine shuffle would tend to expose more of the heme to the external environment and allow access to external nucleophiles. Third, since exogenous anions such as cyanide and azide are known to induce autoxidation of hemoproteins (37), an endogenous nucleophile, such as histidine residue 64, should be more effective. It is currently not known which histidine residue (64 or 97) may act as the endogenous nucleophile and which as the proximal iron ligand, since dioxygen plausibly could bind on different faces of the heme for the altered myoglobin and the native form. Furthermore, certain man-made myoglobin mutants (36, 38) that are reported to contain potential endogenous nucleophiles in the active site have been found to autoxidize readily.

The enhanced ability of the altered myoglobin to reduce CCl_4 may be due in part to the loss of the electron-withdrawing effect of the ring I vinyl moiety, which becomes altered as a result of the covalent bonding to the protein. This hypothesis is based on the observation that apomyoglobin reconstituted with mesoheme or deuteroheme, which lacks the electron-withdrawing heme vinyl, has a lower oxidation-reduction potential (39). The lower redox potential may also be due to the stabilization of the ferric state by the apoprotein, possibly by a histidine imidazole group. It has been shown that addition of imidazole to native myoglobin results in a decrease in the redox potential (39).

The formation of activated heme-protein adducts may play a role in tissue injury mediated by radical metabolites of xenobiotics or endogenous compounds. For example, they may be involved in the hepatotoxicity associated with CCl_4 or in the injury to the myocardium due to ischemia and subsequent

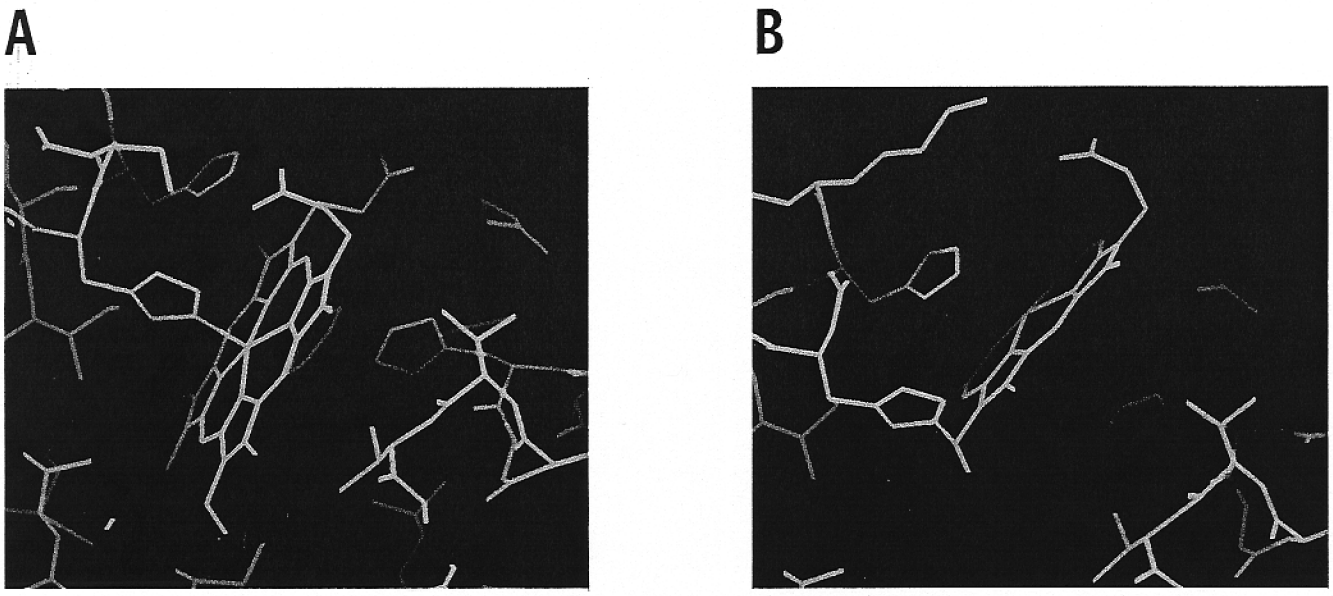


FIG. 7. **Computer-generated depiction of the three-dimensional crystal structure of the heme pocket of myoglobin.** *Panel A,* native myoglobin; *panel B,* altered myoglobin. A covalent bond (1.2 Å) was drawn between the ring Iα-vinyl carbon and the imidazole nitrogen of histidine 93. The heme moiety was moved approximately 5 Å toward the direction of the propionic groups while keeping the rest of the molecule constant. The heme was positioned midway between the imidazole moieties of histidine residue 97 and 64. This represents only an approximation, as large changes in the tertiary structure of the polypeptide occur when the heme covalently attaches to the protein. This figure was plotted on a Silicon Graphics Personal Iris with the use of Polygen Quanta software.

reperfusion (1). The myocardial injury is of special interest, because myoglobin is present in large quantities in this tissue (40), and radical oxygen metabolites are thought to be formed in the heart during reperfusion. This might lead to enhanced production of deleterious oxygen metabolites further promoting tissue injury.

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