Formation of Heme-Derived Products by the Reaction of Ferrous Deoxymyoglobin with $BrCCl_3$

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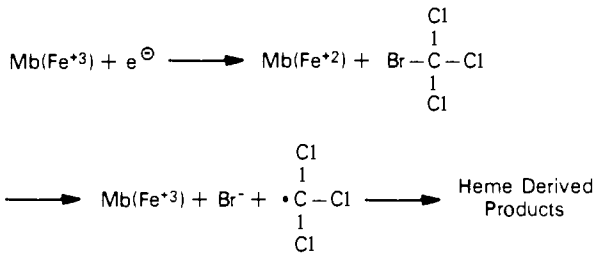
Abstract: The reductive metabolism of $BrCCl_3$ by ferrous deoxymyoglobin leads to the formation of heme-derived products, including those that are covalently bound to the protein. $BrCCl_3$ caused a decrease in the absorption of the oxidized myoglobin in the Soret region, suggesting that the prosthetic heme moiety had been modified. Examination of the total reaction mixture by HPLC revealed the presence of three major soluble heme-derived products and a protein-bound heme adduct. The soluble products were unequivocally identified by electronic absorption, FAB mass spectrometry, and 2D NOESY and COSY NMR as β-carboxyvinyl, α-hydroxy-β-(trichloromethyl)ethyl, and α,β-bis(trichloromethyl)ethyl derivatives of the heme prosthetic group and are believed to result from the initial regiospecific attack of the trichloromethyl radical at the ring I vinyl substituent. As might be expected from the bulk of the trichloromethyl group and the crowded environment of the heme, the α,β-bis-(trichloromethyl)ethyl moiety was held in a rigid conformation about the heme. A similar approach utilizing cytochrome P-450 instead of myoglobin may be used to study the mechanism of inactivation of the P-450 enzyme by the trichloromethyl radical.

Carbon tetrachloride is a hepatotoxic agent that irreversibly inactivates cytochrome P-450 in the liver.[2] This process is thought to be mediated by the trichloromethyl radical metabolite that is produced by the reductive dechlorination of CCl_4 by cytochrome P-450 and results in the accumulation of abnormal heme[3] breakdown products.[2] This increased catabolism of heme is independent of heme oxygenase activity and does not yield CO in the process.[4] Recently, it has been shown that during this reaction at least part of the heme prosthetic group of cytochrome P-450 becomes covalently attached to the protein.[5] The structures or the mechanism of formation of these abnormal heme compounds remains to be elucidated. Due to the complexity of the cytochrome P-450 enzyme-catalyzed reactions, which require NADPH and a flavoprotein reductase in addition to P-450, and the need for large amounts of hemoprotein for product identification, a possible model system for this reaction was employed.

The system used in our experiments is similar to that developed by Castro and co-workers for the study of the reductive dehalogenation of alkyl halides by various hemoproteins including myoglobin, cytochrome P-450, and cytochrome *c*.[6] They have found that the reductive dehalogenation of CCl_4 or $BrCCl_3$ by these hemoproteins results in the stoichiometric production of $CHCl_3$; however, in the case of myoglobin, only 30% yield of $CHCl_3$ was obtained. This lead us to examine whether other metabolites, specifically heme-derived products, were being formed to account for this low yield. Our study describes the isolation and characterization of the major non-protein-associated modified hemes, which are produced when the trichloromethyl radical attacks the heme prosthetic group of myoglobin, and clearly shows that products other than $CHCl_3$ are formed. These modified heme compounds may be similar in structure to the yet uncharacterized abnormal heme compounds produced by the reaction of CCl_4 with P-450 cytochromes.

Scheme I. Reaction of Bromotrichloromethane with Myoglobin



Results

Myoglobin was reduced either by photoreduction or by dithionite to give the Fe^{2+} deoxy form (Scheme I). Since CCl_4 is only slowly reduced by this protein,[6d] the more readily reducible derivative $BrCCl_3$ was used. As previously reported, addition of $BrCCl_3$ resulted in the oxidation of myoglobin as measured by the loss of the absorption due to the ferrous form.[6d] After 60 min, a loss of approximately 30% of the intensity of the Soret chromophore due to the ferric form was apparent, an indication that the prosthetic heme moiety had been altered. This was found to be the case when the total reaction mixture was analyzed with the use of HPLC methods (Figure 1). The products were monitored at 405 and 220 nm to detect heme compounds as well as protein, respectively. Panel A shows the HPLC profile of the control reaction mixture that did not contain $BrCCl_3$. The major fraction with absorption at 220 nm was that of native apomyoglobin (peak 4), and the fraction with absorption at 405 nm was that of the dissociated prosthetic heme group (peak 2). In contrast, when $BrCCl_3$ was added to the reaction mixture, approximately 70% of the heme was lost, and three new fractions, 1, 3, and 6, were detected by their absorption at 405 nm; the weak absorption at 220 nm indicated that they were not associated with protein

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(2) (a) Levin, W.; Jacobson, M.; Kuntzman, R. *Arch. Biochem. Biophys.* **1972**, *148*, 262-269. (b) Noguchi, T.; Fong, K.-L; Lai, E. K.; Alexander, S. S.; King, M. M.; Olson, L.; Poyer, J. L.; McCay, P. B. *Biochem. Pharmacol.* **1982**, *31*, 615-624.

(3) Abbreviation used is heme for iron protoporphyrin IX, regardless of the oxidation state of the iron.

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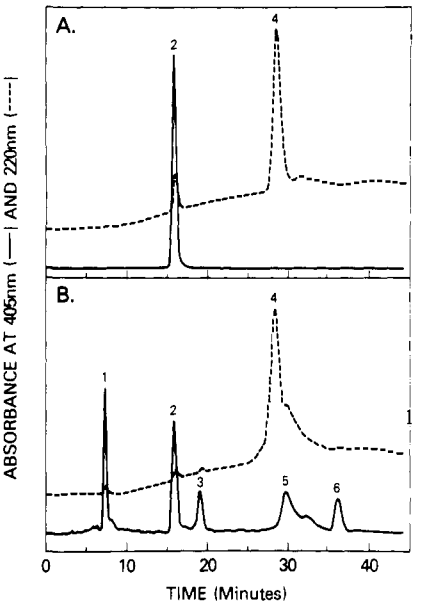

Figure 1. Reverse-phase HPLC of the reaction mixture: (panel A) untreated myoglobin; (panel B) myoglobin treated with $BrCCl_3$. A Bio-Rad C4 Hi-Pore column (0.46 × 25 cm) was used as described under Experimental Section.

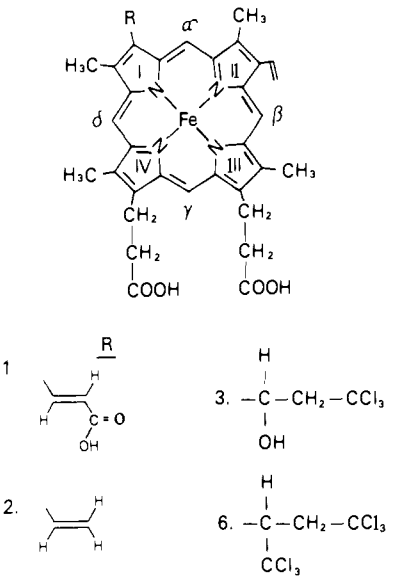

Figure 2. Structures of modified hemes isolated by reverse-phase HPLC. The numbers correspond to those in Figure 1: **1**, β-carboxyvinyl derivative; **2**, heme; **3**, α-hydroxy-β-(trichloromethyl)ethyl derivative; **6**, α,β-bis(trichloromethyl)ethyl derivative.

(panel B). In addition, a fraction (peak 5) was detected with appreciable absorption at both 405 and 220 nm, an indication that this heme derivative was covalently bonded to the protein. In results not shown, the covalent nature was further confirmed by trypsin digestion of the reaction mixtures, resulting in the loss of only fraction 5 on the HPLC profile. Also, a heme chromophore was observed in the acid–acetone precipitates of the $BrCCl_3$-treated myoglobin under conditions that remove the heme moiety from native myoglobin.[5]

The focus of this study was the elucidation of the structures of the soluble non-protein-associated heme compounds corresponding to fractions 1, 3, and 6. Their structures are shown in Figure 2. The visible absorption spectrum of each of the compounds was similar to that of heme and indicated that the heme ring was intact, consistent with modification occurring at the periphery. A bathochromic shift of the Soret maximum for compound **1** was noted and is consistent with the addition of an electrophilic group.[7] It was estimated from the peak area, by

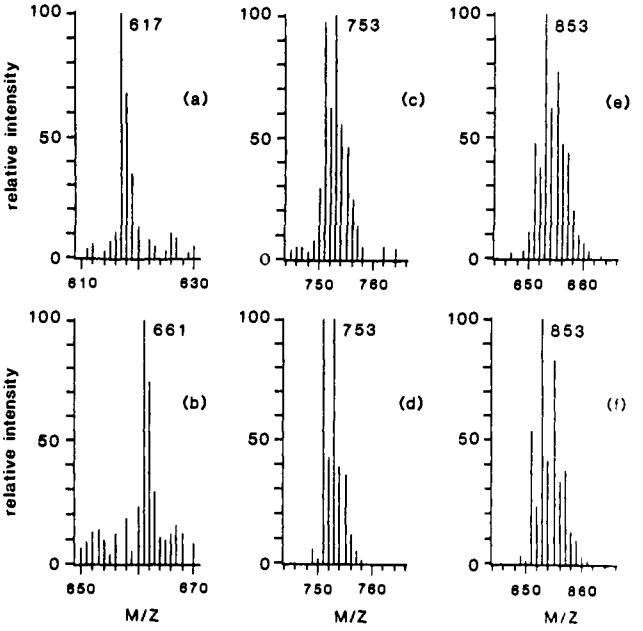

Figure 3. Molecular ion region of the positive ion FAB mass spectra of the isolated hemes. The spectra correspond to the following compounds: (a) heme **2**; (b) β-carboxyvinyl derivative **1**; (c) α-hydroxy-β-(trichloromethyl)ethyl derivative **3**; (d) predicted spectrum for molecular formula corresponding to derivative **3**; (e) α,β-bis(trichloromethyl)ethyl derivative **6**; (f) predicted spectrum for molecular formula corresponding to **6**. The conditions used to obtain these spectra are described under Experimental Section.

assuming that the absorptivities of the modified products were the same, that compound **1** was the major product of the reaction, accounting for approximately 52% of the soluble products. Compounds **3** and **6** each accounted for 24% of the soluble products. The structures of these products were elucidated in the following manner.

The molecular ion regions of the FAB mass spectra of compounds **1**, **3**, and **6** and heme (compound **2**) are shown in Figure 3. The MH^+ ion obtained for compound **1** at m/z 661 (Figure 3b) was 44 mass units greater than that of heme (Figure 3a); this is in accordance with an addition of a CO_2 moiety. The spectra of compounds **3** and **6** showed MH^+ ions at m/z 753 (Figure 3c) and 853 (Figure 3e), respectively. These results indicate that compound **3** is an addition product of heme containing a trichloromethyl and a hydroxyl group, while compound **6** is an addition product containing a bis(trichloromethyl) group. The structural assignments for compounds **3** and **6** are strongly corroborated by the cluster of ions found in the molecular ion region due predominantly to their content of chlorine atoms. Indeed, the ion clusters (Figure 3c,e) are very similar to those simulated from the predicted molecular formulas of these compounds (Figure 3d,f).

To determine whether the carboxyl moiety of the acrylic acid product (compound **1**) was derived from $BrCCl_3$, reactions were repeated with $Br^{13}CCl_3$. In data not shown, the MH^+ ion of compound **1** isolated from this reaction mixture was shifted by 1 mass unit from m/z 661 to m/z 662, showing that the acid moiety is derived from the trichloromethyl group; as expected, an increase of 2 mass units was observed for compound **6**.

The complete structural elucidation of compounds **1**, **3**, and **6** was determined by ^1H NMR techniques. The NMR spectrum of heme (Figure 4a), shows four singlets near 10 ppm that correspond to the meso protons and four singlets near 3.5 ppm that correspond to the four methyl groups. The peaks of the β-methylene groups of the propionic residues are visible between the sharp methyl peaks, and the α-methylene protons give a broad triplet at 4.5 ppm with integrated intensity of 4. Multiplets

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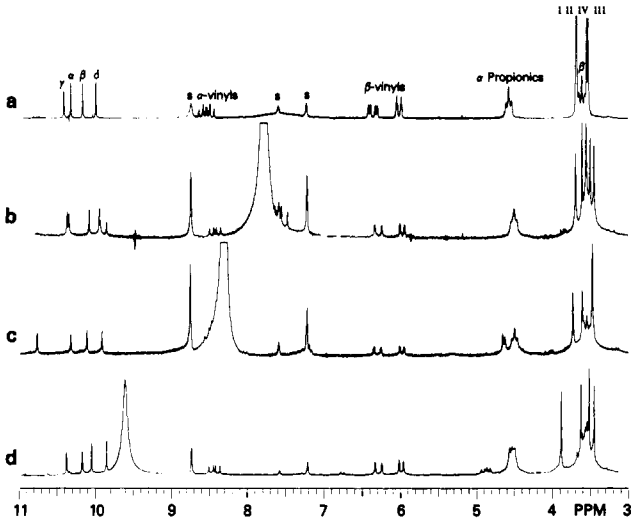

Figure 4. ^{1}H NMR spectra at 200 MHz. The spectra are of the following compounds: (a) heme **2**; (b) β-carboxyvinyl derivative **1**; (c) α-hydroxy-β-(trichloromethyl)ethyl derivative **3**; (d) α,β-bis(trichloromethyl)ethyl derivative **6**.

Table I. NMR Chemical Shifts and NOESY Interaction of Hemin (Compound **2**)

proton	chemical shift (ppm)	interactions		
meso γ	10.38	4.54	3.57	
meso α	10.29	8.53	6.34	3.64
meso β	10.13	8.50	6.30	3.46
meso δ	9.96	3.65	3.48	
vinyl Iα	8.53	6.01	3.65	
vinyl IIα	8.50	5.99	3.64	
vinyl Iβ-trans	6.34	6.01	3.65	
vinyl IIβ-trans	6.30	5.99	3.64	
vinyl Iβ-cis	6.01	8.53		
vinyl IIβ-cis	5.99	8.50		
propionics α	4.54	10.38	3.57	
methyl I	3.65[a]	9.96	8.53	6.34
methyl II	3.64[a]	10.29	8.50	6.30
propionics β	3.57	10.38	4.54	
methyl IV	3.48	9.96		
methyl III	3.46	10.13		

[a] The resolution of the NOESY experiment did not allow differentiation of the interactions of these two methyl groups. They were distinguished by difference nuclear Overhauser experiments, irradiating each of the meso protons.

corresponding to protons on the two vinyl groups appeared near 8.5 ppm for the α-proton and 6.0 and 6.3 ppm for the β-protons. The spectrum of compound **1** (Figure 4b) for the most part is similar to that of heme. Although chemical shifts were slightly altered, the four meso protons, the four methyl groups along with an extra signal at 3.61 ppm due to methanol contamination, and the α- and β-methylene protons of the propionic acid groups were all present. However, multiplets corresponding to only a single native vinyl group were observed, indicating that one vinyl group was altered. A pair of doublets at 9.89 and 7.51 ppm appeared as a result of this alteration. In the spectrum of compound **3** (Figure 4c), although two of the methyl groups were not resolved, the presence of all native functional groups except one vinyl group was evident. The α-vinyl signals were not well resolved from the large signal due to the water contamination but were confirmed in later NOESY and COSY experiments. Signals at 7.17 and 4.63 ppm due to alterations of one vinyl group were detected. The spectrum of compound **6** (Figure 4d) also showed that while the other functional groups were intact, a single vinyl group was modified with resultant new signals at 6.74 and 4.88 ppm.

To determine which vinyl group was modified and the nature of this modification, as well as to confirm assignments of protons, 2D 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.[8] Thus for hemin, as seen in Table I, δ-meso protons showed interaction with two methyl groups, which were thus identified as those on rings I and IV. γ-Meso protons were identified by interaction with the two methylene groups of the propionic acid residues, and the remaining meso protons showed interaction with the methyl and vinyl protons.

Table II. NMR Chemical Shifts and NOESY Interaction of Compound **1**

proton	chemical shift (ppm)	interactions		
meso α	10.36	9.89	7.51	3.54
meso γ	10.34	4.49	3.53	
meso β	10.05	8.42	3.44	
meso δ	9.94	3.68	3.50	
vinyl Iα	9.89	10.36	3.68	
vinyl IIα	8.42	10.05		
vinyl Iβ	7.51	10.36	3.68	
vinyl IIβ-trans	6.28	5.97	3.54	
vinyl IIβ-cis	5.97	6.28		
propionics α	4.49	3.53	10.34	
methyl I	3.68	9.94	9.89	7.51
methyl II	3.54	10.36	6.28	
propionics β	3.53[a]	10.34	4.49	
methyl IV	3.50	9.94		
methyl III	3.44	10.05		

[a] Observed only as off-diagonal interactions in COSY spectra.

Table III. NMR Chemical Shifts and NOESY Interactions of Compound **6**

proton	chemical shift (ppm)	interactions		
meso γ	10.37	4.50		
meso α	10.18	6.74	3.63	
meso β	10.04	8.44	3.44	
meso δ	9.85	3.89	3.51	
vinyl IIα	8.44	10.04	6.00	
ethyl Iα	6.74	10.18	4.88	
vinyl IIβ-trans	6.28	6.00		
vinyl IIβ-cis	6.00	6.28		
ethyl Iβ	4.88	6.74	10.18	3.89[b]
ethyl Iβ′	4.50[a]			
propionics α	4.50	10.37		
methyl I	3.89	9.85		
methyl II	3.63	10.18		
propionics β	3.60[a]			
methyl IV	3.51	9.85		
methyl III	3.44	10.04	4.50	

[a] Observed as off-diagonal interactions of the COSY spectrum. [b] Observed in a difference NOE experiment.

Table II shows the chemical shifts and the NOESY interactions for compound **1**. The protons on the altered vinyl group appeared as a pair of doublets at 9.89 and 7.51 ppm, a downfield shift consistent with an acrylic acid group. The large coupling between these protons (18 Hz) indicates a trans stereochemistry. An interaction of the protons on the altered vinyl group with the methyl substituent at 3.68 ppm, which is one of the two methyls interacting with the δ-meso proton, clearly shows that the ring I vinyl was modified. Therefore, compound **1** is Iβ-carboxyvinyl derivative of heme.

The chemical shifts and NOESY interactions for compound **6** are shown in Table III. Two groups of signals appear in the NMR spectrum (Figure 4d) as the result of the modification, a doublet at 6.74 ppm and a doubled doublet at 4.88 ppm, each representing a single proton. A third set of signals at 4.50 ppm corresponding to one proton was obscured by the α-methylene propionic signal but was revealed by a COSY spectrum. The upfield position of the protons at 4.50 and 4.88 ppm indicates that they are in the β-position. The large coupling (15.2 Hz) between

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Table IV. NMR Chemical Shifts and NOESY Interaction of Compound **3**

proton	chemical shift (ppm)	interactions		
meso α	10.75	7.17	4.63	3.60
meso γ	10.31	4.49	3.54	
meso β	10.10	8.47	3.47	
meso δ	9.90	3.73	3.47	
vinyl IIα	8.47	10.10		
ethyl Iα	7.17	10.75	3.73	
vinyl IIβ-trans	6.30			
vinyl IIβ-cis	5.98			
ethyl Iβ	4.63	10.75	7.17	
propionics α	4.49	10.31	3.54	
methyl I	3.73	9.90	7.17	
methyl II	3.60	10.75		
propionics β	3.54	4.49		
methyl III, IV	3.47	10.10	9.90	

them reveals that they are geminal protons and therefore is consistent with the addition of the trichloromethyl groups across the double bond rather than to a single carbon atom. A difference NOE experiment performed by irradiation of a β-ethyl proton at 4.88 ppm gave an enhanced signal of the ring I methyl group at 3.89 ppm, again showing that the I-vinyl group has been altered and is consistent with compound **6** being a Iα,β-bis(trichloromethyl)ethyl derivative of heme. The irradiation of the Iβ-ethyl protons produced no interaction with the α-meso proton, and irradiation of the I-methyl at 3.89 ppm produced an enhanced signal from the δ-meso proton but none from the Iα-ethyl signal at 6.74 ppm, in contrast to results obtained for compound **3** (Table IV).

These NOESY results suggest that the bis(trichloromethyl)-substituted ethyl group, resulting from the addition of the two trichloromethyl residues to the vinyl group, is held in a rigid conformation in the crowded environment of the porphyrin ring. Moreover, since the vicinal coupling constants of the Iα-ethyl proton with the Iβ-ethyl and Iβ'-ethyl protons is 9.6 Hz and nearly zero, respectively, it appears that the bis(trichloromethyl)ethyl group exists in an antiperiplanar conformation.

Table IV shows the chemical shifts and NOESY interactions for compound **3**. Signals attributed to the altered vinyl group appear as a triplet at 7.17 ppm and a doublet at 4.63 ppm, corresponding to one α-methylene and two β-methylene protons. Since the chemical shift of the β-protons is similar to the chemical shifts of 4.50 and 4.88 ppm found for the β-methylene protons of the bis(trichloromethyl) compound **6** and the chemical shift of the α-proton is 0.4 ppm downfield from the α-proton of compound **6**, the data are consistent with a $-CH(OH)CH_2CCl_3$ substituent. An interaction of the low-field α-proton triplet with the ring I methyl group at 3.73 ppm and the interaction of this methyl group with the δ-meso proton demonstrate that the altered vinyl group is that of the ring I. Therefore, compound **3** is a Iα-hydroxy-β-(trichloromethyl)ethyl derivative of heme.

Discussion

This present study has shown, for the first time, that radical products generated by hemoproteins can attack the vinyl moiety of the prosthetic heme. Other reports have shown that oxidative activation of certain xenobiotics by hemoproteins results in the production of carbon-centered radicals that covalently interact at the pyrrole nitrogen or the meso carbon or abstract a hydrogen atom from a methyl group of the prosthetic heme.[9]

The three major soluble heme products characterized in this study all result from a regiospecific modification of the I-vinyl group. This specificity is likely due to steric constraints at the II-vinyl group preventing the attack. A mechanism for the formation of these products is proposed in Scheme II. The first step involves the reductive debromination of $BrCCl_3$ by ferrous myoglobin to the trichloromethyl radical. Numerous studies with ferrous iron porphyrins,[6] ferrous iron hemoproteins,[2b,6,10a-d,f] γ-radiolysis,[10e,g,h] and molecular orbital calculations[10i] have clearly shown that the one-electron reduction of CCl_4 or $BrCCl_3$ leads to the formation of the trichloromethyl radical. The next step involves the attack of the trichloromethyl radical at the I-vinyl β-carbon with the production of a carbon-centered radical at the α-position (panel A). Delocalization of the electron to the ferric iron would produce a cationic species that may either be attacked by water producing the alcohol product **3** or lose a proton to form A, a (trichloromethyl)vinyl derivative. Alternatively, compound **3** may dehydrate to form the same A derivative.

Since intermediate A is at the ferrous state, another equivalent of $BrCCl_3$ may be reduced to the trichloromethyl radical and added to the substituted vinyl group to form the bis compound **6**. In this process, $H^\bullet$ is probably abstracted from protein and not water.[10d]

The acrylic acid derivative **1** may be formed by a direct internal reduction of the trichloromethyl group to form a dichloromethyl radical that upon delocalization of the electron forms a cationic center (Scheme II, panel B). Nucleophilic attack by water would yield an unstable dichloro alcohol species. After spontaneous dehydrochlorination at this position, the resulting acyl chloride could undergo hydrolysis to form the acid **1** or possibly react with the protein to produce the covalently bound product. Alternatively, direct hydrolysis of A, either during the reaction or under the acid condition of the workup, could result in the formation of compound **1** analogous to the facile hydrolysis of *trans*-(trichloromethyl)-styrene to *trans*-cinnamic acid.[11]

Although the structure of the heme–protein adduct or adducts has yet to be elucidated, the formation of this product indicates that the model reaction described in this paper is similar to the cytochrome P-450 reaction. This possibility can now be studied by characterizing the reaction products of ferrous P-450 with CCl_4 under the conditions described in this paper. Compounds **1**, **3**, and **6** should serve as useful standards for these studies.

Experimental Section

Materials. Whale myoglobin was obtained from Sigma. $BrCCl_3$ was from Aldrich and washed with 5% Na_2CO_3 and distilled prior to use. [$^2H_5$]Pyridine was from ICN Biochemicals. $Br^{13}CCl_3$ (99 atom %) was purchased from Merck.

Production and Isolation of Heme Products. The reaction mixture contained 140 μM myoglobin, 2.5 μM FMN, and 10 mM EDTA in 75 mM potassium phosphate (pH 7.4) in a cuvette stoppered with a rubber septum. The mixture was made anaerobic by purge and evacuation cycles with the use of argon that was scrubbed of oxygen by a mixture of zinc amalgam and chromous chloride.[12] The myoglobin was photoreduced to the ferrous state as previously described.[13] $BrCCl_3$ was added in large excess (3 mM) to the reaction mixture with the use of a gas-tight syringe. Spectra of the reaction mixture were taken periodically on a Hewlett-Packard 8450A diode array spectrophotometer. After 60 min, $K_3Fe(CN)_6$ 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 mixture was directly applied

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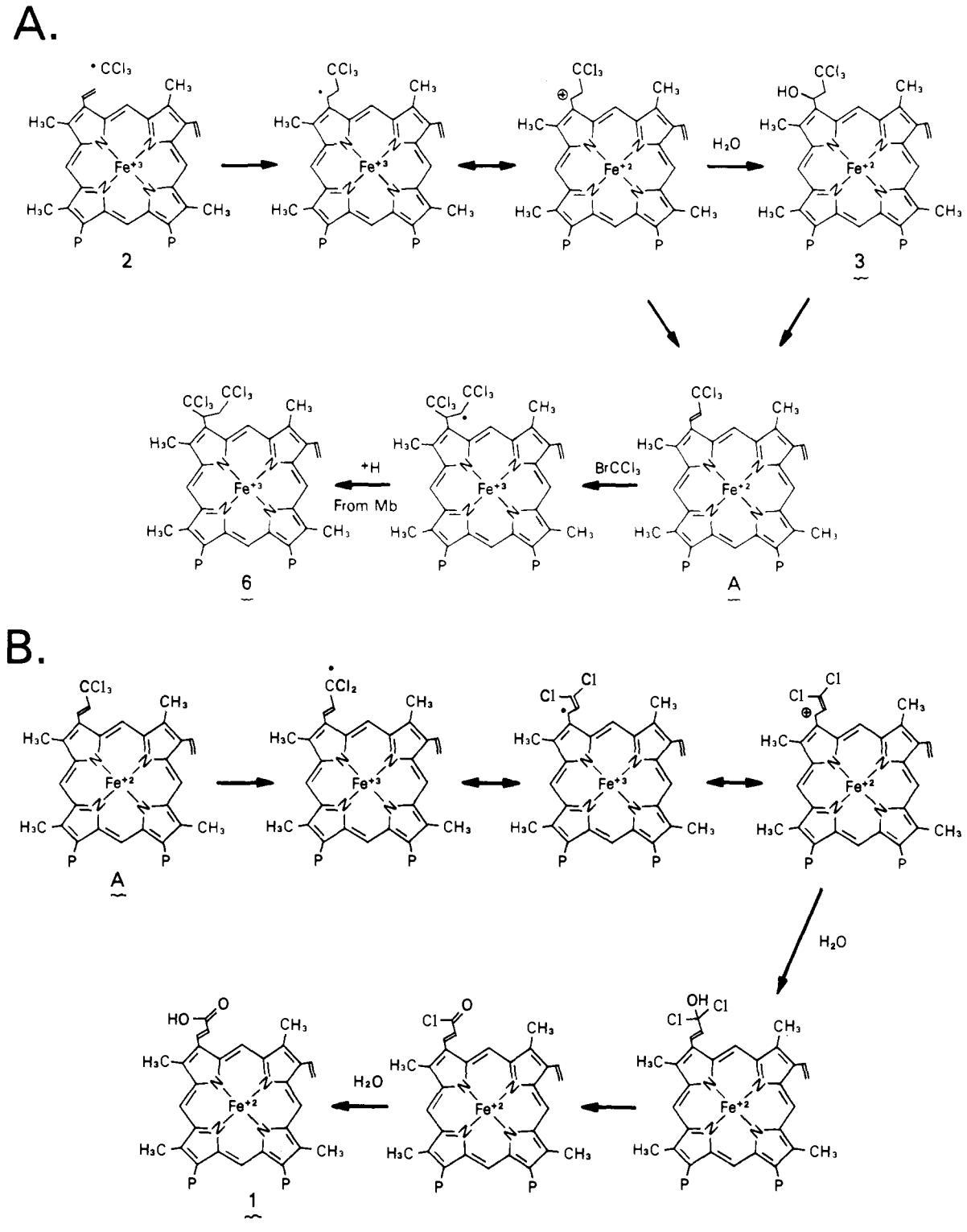
Scheme II



to a C4 column (Bio-Rad Hi-Pore, 0.46 × 25 cm) at a flow rate of 1 mL/min. The mobile phase was water (A) vs acetonitrile/2-propanol (1:1) (B), with 0.1% TFA throughout. The column was washed for 5 min with A and then changed directly to 35% B: a linear gradient was run to 50% B (0.6%/min) and then to 55% B (0.1%/min). The elution products were detected by absorption at 405 and 220 nm. In order to obtain sufficient amounts of modified hemes for NMR analysis, the reaction mixture was scaled up to a volume of 500 mL, and instead of flavin–EDTA, sodium dithionite was used to reduce the myoglobin. Reduction by dithionite gave a similar HPLC profile of the products. The myoglobin was not reduced fully to ensure that excess reducing agent was not present. The reaction mixture was pumped onto a C18 column (Whatmann Partisil M20 10/50 ODS3, 2.2 × 50 cm) and washed (18 mL/min) with A for 35 min and then brought to 35% B: a linear gradient was run to 48% B (0.186%/min), then to 50% B (0.029%/min), and finally to 100% B (1.43%/min). The eluting products were monitored by absorption at 530 nm, and 1-min fractions were collected. All samples were dried down on a Speed-Vac (Savant Instruments Inc., Farmingdale, NY). Since the fraction containing the trichloromethyl alcohol derivative was contaminated with heme and apomyoglobin, further purification on a preparative C4 Hi-Pore column (1.0 × 25 cm) was necessary. A flow rate of 3.5 mL/min was used, starting at 35% B with a linear gradient to 50% B (0.43%/min) and then to 52% B (0.057%/min).

Identification of Heme Derivatives. The following maxima in nanometers were determined on the 8450A diode array spectrophotometer for each compound in methanol: **1**, 402, 506, 626; **2**, 398, 496, 618; **3**, 396, 498, 598; **6**, 398, 498, 618.

Positive ion FAB mass spectra were obtained with a Kratos MS50RF (Kratos Analytical, Ltd., Manchester, U.K.) double-focusing instrument with a mass range of 10000 amu at full accelerating voltage (8 kV). The instrument was fitted with a Model B11NF saddle-field fast atom gun (Ion Tech, Ltd., Teddington, U.K.) and a post accelerator detector (PAD). The PAD was operating 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-benzyl 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.

NMR spectra were obtained on a Varian XL200 spectrometer in [$^2H_5$]pyridine solution following reduction by stannous chloride;[14] 3-5 mg of sample was added to 6-9 mg of $SnCl_2$ in a volume of 0.5 mL. Typically, 256 free induction decays were collected with an accumulation time of 4 s for 1D spectra. COSY spectra were taken with the use of a 1024 by 1024 data matrix with 256 t_1 increments of 16 free induction decays each.[15] Prior to NOESY or difference nuclear Overhauser effect studies, the solutions were degassed by three freeze-exhaust-thaw cycles. Phase-sensitive NOESY spectra were obtained by the method of States et al.,[16] in overnight runs, with the use of 1024 by 1024 data matrices and a mixing time of 0.8 s. This allowed collection of 256 t_1 increments each with 64 free induction decays. To provide difference nuclear Overhauser effect spectra, selected resonances were irradiated for 4 s before the accumulation of 6000 free induction decays for 1 s, which were then subtracted from free induction decays obtained with the irradiating frequency far removed. Fourier transforms of the differences provided spectra in which the irradiated peak and those showing interactions were 180° out of phase.

Conclusions

The reaction of ferrous deoxymyoglobin with $BrCCl_3$ lead to the formation of three major non-protein-associated modified heme products and a protein-bound heme metabolite. The non-protein-associated products were identified as Iβ-carboxyvinyl, Iα-hydroxy-β-(trichloromethyl)ethyl, and Iα,β-bis(trichloromethyl)ethyl derivatives of the prosthetic heme moiety and appear to result from the initial regiospecific attack of the trichloromethyl radical on the I-vinyl group. These findings add to our knowledge of the general reactivity of radicals toward hemoproteins and may aid in understanding the process by which CCl_4 inactivates cytochrome P-450.

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Sulfoximine and Sulfodiimine Transition-State Analogue Inhibitors for Carboxypeptidase A

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Abstract: New substrate analogues [(±)-2-carboxy-3-phenylpropyl]methylsulfoximine and [(-)-2-carboxy-3-phenylpropyl]methylsulfodiimine have been prepared and shown to be potent competitive inhibitors of the zinc enzyme carboxypeptidase A (limiting values of K_i = 2.7 and 0.22 μM, respectively). A complicated pH dependence for K_i is explained by deprotonations occurring on the enzyme, the inhibitor, and the enzyme-inhibitor complex. The mode of inhibitor binding is also characterized by visible absorption and ^1H NMR spectra of the cobalt-substituted enzyme. Mechanistic consequences are considered; no support is found for a concerted mechanism of acyl substitution occurring within the coordination sphere of the active-site metal ion.

Current mechanistic theory suggests that stable substrate analogues which structurally resemble high-energy intermediates along the reaction path for an enzymic reaction should function as potent inhibitors. For the prototypical metalloprotease carboxypeptidase A (ZnCpA), a number of putative illustrations of this principle have been recorded.[1] By now it is apparent that tight-binding, synthetic competitive inhibitors for this enzyme contain the specificity features of a typical substrate (i.e., the terminal carboxylate plus adjacent hydrophobic side chain in proper stereochemical relationship, as in a hydrolytically susceptible *N*-acylphenylalanine peptide, for example), as well as a suitable metal ion ligating group in place of the scissile carboxamide linkage.[1-3] Should this latter moiety bear an appropriate structural similarity to a *carbonyl hydrate*, then an especially favorable match with the active site would be anticipated according to the theory of transition-state mimicry.[4] Enzymic peptide hydrolysis presumably entails a similar intermediate, and the active site ought to attain complementarity to such a metastable species in the course of catalysis, in order that binding energy may be most effectively channeled into kinetic acceleration.[5] Among the more prominent examples of this principle are inhibitors containing a phosphonyl anion at the scissile locus (e.g., CH_3-PO_2^-NHCHBzCO$_2$H);[1a-d] the tetrahedral nature of this functional group appears to provide an excellent steric fit to the active site, while anion coordination satisfies the electron deficiency of the metal ion present therein.

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