The amount of structural and kinetic detail which EPR has supplied for this system involving six different radical-pair intermediates shows why this approach is particularly appealing for studying the nature of reactions in organic solids. One might think that the substantial lattice strain which is generated during radical-pair formation would make this type of reaction a poor model for solid-state reactions in general, but it could equally well be that hypothetical reaction in an unstrained lattice is in fact the poor model. Only the first molecule to react in a perfect crystal could react in a truly strain-free environment. All molecules reacting subsequently should be more or less sensitive to strain generated by earlier events, and the type of strain effects revealed in the present work may be widespread in solid-state chemistry.

There are a number of directions in which this work could be profitably extended. Molecular mechanics could yield insight on the nature of intermolecular interactions in these crowded cages. ENDOR spectroscopy could give more geometric detail on the structure of the rearranged radical pairs. Analysis of the ultimate products is obviously necessary, especially since some of the pair-pair conversions are not quantitative. We have made preliminary attempts to study other solvates of TPPP, but the crystals we could grow from chloroform, chlorobenzene, acetone, ethyl acetate, and ether were either microcrystalline or needles too fine for single-crystal EPR investigation.

Experimental Section

Preparation of the compounds and operation of the EPR spectrometer was described in the preceding paper.[4] The method for determining hfs and zfs tensors has been previously described.[19]

X-ray Data were collected on a crystal 0.3 mm on each edge mounted in a sealed quartz capillary. The Enraf-Nonius CAD-4 diffractometer used Mo Kα irradiation with a graphite monochromator and pulse height discrimination. Lattice parameters were refined on the basis of 25 reflections with $6° < \theta < 19°$. Intensity data were collected using ω-2θ scans over an ω range of $(0.75 + 0.35°) \tan(\theta)$, slow enough to give $\sigma(I)/I = 0.02$ but not exceeding 60 s. Three standard reflections were monitored after each 10^4 s of X-ray exposure and showed less than 8% variation during data collection. Structure factors were calculated for the 2434 unique reflections after Lorentz-polarization and background corrections. Reflections were weighted according to a standard scheme.[20] The 2243 reflections with $I > 2\sigma(I)$ were used in structural refinement.

The structure was solved by direct methods and refined using the 1977 version of the Enraf-Nonius SDP package. Positional parameters of all atoms, isotropic thermal parameters for the hydrogens, and anisotropic thermal parameters for all other atoms were varied in the final refinement cycles to minimize $\sum w(|F_o| - |F_c|)^2$. The final values of R and of $R_w = [\sum w(|F_o| - |F_c|)^2/\sum wF_o^2]^{1/2}$ were 0.051 and 0.057, respectively.

Acknowledgment. This work was supported by the National Science Foundation (DMR 76-01996) and in its early stages by a Camille and Henry Dreyfus Teacher-Scholar Grant. The EPR spectrometer and the X-ray diffractometer were obtained with the aid of NSF departmental instrument grants. J.M.M. is grateful for the hospitality of Professor J. M. Thomas, University of Cambridge, and for a Senior Visiting Fellowship from the Science Research Council of Great Britain during preparation of the manuscript.

Supplementary Material Available: Thermal parameters and rms vibrational amplitudes, observed and calculated structure factors, bond lengths, bond angles, least-squares planes, torsional angles, nonbonded distances from O1 and O2 (14 pages). Ordering information is given on any current masthead page.

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Mercaptide-Chelated Protoheme. A Synthetic Model Compound for Cytochrome P-450[1]

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Contribution from the Department of Chemistry, University of California, San Diego, La Jolla, California 92093. *Received January 30, 1981*

Abstract: A synthetic protohemin compound having a covalently attached and protected mercaptide function has been prepared. Reduction of the hemin and deprotection of the mercaptide in alkaline aqueous suspension (cetyltrimethylammonium bromide) or in dimethyl sulfoxide solution afforded mercaptide-chelated protoheme which displays the spectroscopic properties of cytochrome P-450. Nuclear magnetic resonance spectra of the carbon monoxide complex of this model compound display new resonances in both proton NMR and ^{13}CO NMR which serve to confirm the structure and to afford new probes for studies of the proteins themselves. Kinetics and equilibria of CO binding of this model compound in aqueous suspension indicate that the model has lower affinity for CO than does cytochrome P-450, suggesting that the charge on the sulfur may be reduced in the protein.

Comparisons of UV-visible, Mössbauer, ESR, and other spectra of cytochromes P-450 with those of model systems have provided strong evidence for the presence of a mercaptide-iron bond in at least some forms of the P-450 enzyme.[2,3] Except for the crystal structure study of protohemin dimethyl ester *p*-nitrothiophenolate,[4] all these model systems have consisted of solutions of heme (or hemin) in the presence of rather large concentrations (0.001-0.1 M) of excess mercaptide ion.

Although static, spectroscopic properties of such model systems are not affected by this excess base (RS^-), dynamic properties could be greatly affected as they are with other bases such as

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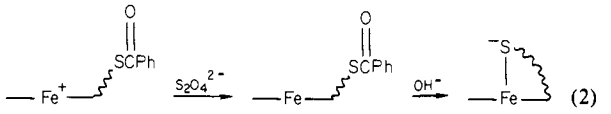
imidazole,[5] pyridine,[5] or hydroxide.[6] We therefore sought to prepare models in which no excess base is present and in which the four-coordinated heme is also absent. Having successfully accomplished this with a chelated heme compound whose dynamic properties duplicate those of *R* state hemoglobin,[7] we began a program to apply this "chelated heme" approach to cytochrome P-450 and have prepared heme compounds containing covalently attached mercaptide functions. A complication, not present in imidazole-chelated hemes, arises as a result of the facile oxidation–reduction processes in iron(III)–mercaptan systems illustrated in eq 1.[8] To avoid this problem, we have prepared hemin com-

$$2\text{hemin}^+ + 2\text{RSH} \rightleftharpoons 2\text{heme} + \text{RSSR} + 2\text{H}^+ \quad (1)$$

pounds from a protected mercaptan and accomplished deprotection in situ only after reduction of the iron(III).



(2)

Experimental Section

The 220-MHz proton NMR spectra were recorded on a Varian HR-220/Nicolet TT-100 pulse/Fourier transform spectrometer, using 4-KHz sweep width and 8K data points. Proton-noise-decoupled ^{13}C spectra were recorded at 20 MHz on a Varian CFT-20 pulse/Fourier transform spectrometer. Spectra were obtained by using 10-mm tubes, internal deuterium lock, a 5-KHz sweep width, and 8K data points. Chemical shifts are reported in parts per million downfield of Me_4Si, internal except for ^{13}C spectra in Me_2SO-d_6, where the solvent peak at δ 39.6 was used.

Solutions were prepared 0.03 M in heme in NMR tubes equipped with a spacer for visible spectroscopy and closed by a silicone septum.[7a] Solutions for mercaptide NMR were prepared by dissolution of the hemin in 1 mL of Me_2SO-d_6. After the solution was degassed, ^{12}CO or 90% ^{13}CO was admitted, and the heme was reduced with a few microliters of D_2O saturated with sodium dithionite. The resultant Me_2SO–heme–CO complex was then titrated with approximately 1 equiv of 3 M deuterated dimsyl anion, prepared from Me_2SO-d_6 and NaH, to yield the mercaptide–heme–CO complex, as judged by changes in the NMR spectrum.

Visible spectra were recorded on a Cary 15 spectrophotometer.

Cetyltrimethylammonium bromide (Aldrich) was recrystallized twice from water. Deuterated solvents were obtained from Stohler Isotope Chemicals, sodium dithionite from Baker Chemical Co., 90% enriched ^{13}CO from Merck, Sharp and Dohme, and O_2 and CO from Matheson Chemical Co. Other chemicals, identified below, were used as received.

1-Amino-3-mercaptopropane Benzoyl Ester Hydrochloride (1). This protected thiolamine was synthesized by the method of Felder, Fumagalli, and Pitre[9] by converting 3-bromopropylamine hydrobromide (Aldrich) to 2-mercaptodihydrothiazine, mp 128–131 °C (lit.[9] 129–131 °C), and then through 1-amino-3-mercaptopropane hydrochloride, mp 216–217 °C (lit.[9] 217–218 °C), to the final product **1**, mp 122 °C (lit.[9] 126 °C).

Protohemin Chloride Monodimethylamide Monoacid (2^+Cl^-). Protohemin chloride (Calbiochem), 1.0 g (1.53 mM) in 60 mL of dry pyridine, was treated with small aliquots of pivalyl chloride (Aldrich) until a small sample of the reaction mixture, when added to dry methanol and chromatographed on thin-layer silica gel plates, revealed that about 50% of the hemin was converted to mixed hemin, pivalyl anhydride. Then 0.32 g (4 mM) of dry dimethylamine hydrochloride, converted to the 18-crown-6 (Aldrich) complex in pyridine, was added. After the reaction mixture was stirred 2 h and poured into 250 mL of 3 N hydrochloric acid, the precipitated product mixture was collected by filtration. Drying and chromatographing the product on silica gel (Davison Grade 62), eluting with methanol–chloroform, 1:4, afforded the pure protohemin chloride monodimethylamide monoacid (2^+Cl^-).

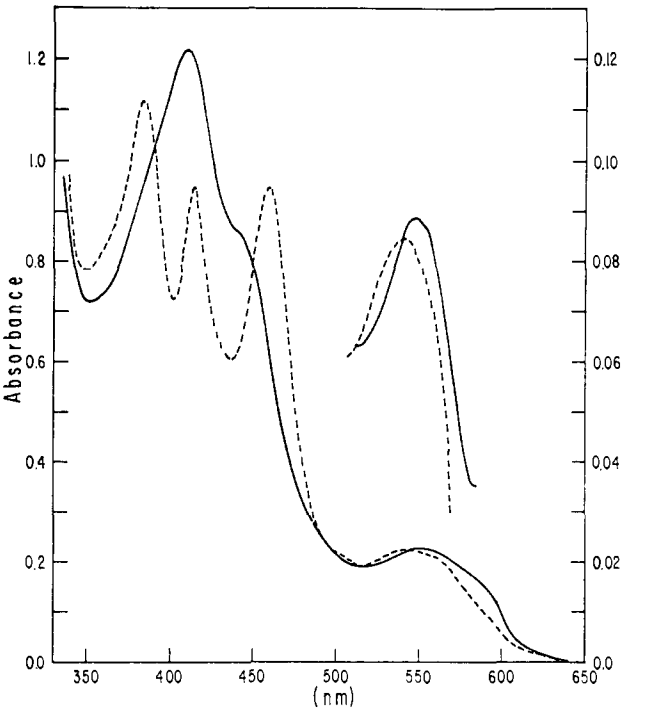


Figure 1. Spectra of mercaptide-chelated protoheme complexes in Me_2SO. The spectrum of the mercaptide–heme complex **4** (–) was obtained following reduction of the hemin (3^+-Cl^-) by 18-crown-6 DT and addition of sodium hydride: (---) **4**-CO, addition of 1 atm of CO. This spectrum is similar to that reported[2] for the dimercaptide compound (**6**-CO) prepared by a different method.

Protohemin Chloride Dimethylamide (3-(Benzoylthio)propyl)amide (3^+-Cl^-). The monoacid, 2^+-Cl^-, 0.5 g (0.74 mM) in 30 mL dry pyridine, was treated with 0.24 g (2 mM) of trimethylacetyl chloride at 0 °C. After complete conversion to the anhydride (confirmed by the methanol method, above) the solution was treated with 2.3 g (1 mM) of the amine **1** and the mixture stirred 1 h at room temperature. The solution was poured into 200 mL of 2 N hydrochloric acid and the product isolated and chromatographed as above, eluting with 8% methanol in chloroform; a second chromatography on Mallinckrodt silica gel S-7, eluting with 2% methanol in chloroform, afforded a center fraction of 150 mg (18%) of protohemin chloride dimethylamide (3-(benzoylthio)propyl)amide (3^+-Cl^-). The NMR which confirms this structure is discussed below.

Protohemin Chloride Di(3-(benzoylthio)propyl)amide (5^+-Cl^-). Protohemin chloride (Calbiochem) was activated with a slight excess of pivalyl chloride as described above and treated with an excess of the amine **1** to produce, after chromatography on Davison silica gel Grade 62, the diamide (5^+-Cl^-). The NMR of the (reduced) carbon monoxide complex of this compound in Me_2SO confirms the structure.

Conversion of Protected Mercaptide Hemin (3^+-Cl^-) to Chelated Mercaptide Heme Derivatives. These transformations are shown in Scheme I.

A. In Me_2SO. A solution of 5 mL of dimethyl sulfoxide (Burdick and Jackson) containing 1 mg of an 18-crown-6 complex of sodium dithionite[10] and 25 mg of 18-crown-6 ether was degassed with argon in a septum-sealed tonometer. About 25 nM hemin 3^+-Cl^- in a few microliters of Me_2SO was added with a syringe. The immediate reduction to the iron(II) compound **3**-$(Me_2SO)_2$ was indicated by the appearance of typical hexacoordinated type bands at 424, 522, and 553 nm.[7a] This solution was either treated with carbon monoxide to produce **3**-CO or with about 2 mg of sodium hydride. Warming the latter solution about 5 min resulted in displacement on the benzoyl group to release mercaptide and allow the formation of mercaptide-chelated heme **4**, λ_{max} at 408 and 548 nm (Figure 1). Addition of carbon monoxide afforded **4**-CO, λ_{max} at 384 nm (ϵ = 46 mM^{-1}), 460 (ϵ = 66 mM^{-1}), and 549 (ϵ = 12.5 mM^{-1}) (Figure 1). The extinction coefficients (mM) are based upon the assumption that **3**-CO has the same extinction coefficient as that determined for protoheme dimethyl ester in Me_2SO, λ_{max} at 413 (ϵ = 72 mM^{-1}) and 546 nm (ϵ = 13.7 mM^{-1}). The **3**-CO peak at 414 nm also appeared, indicating the presence of some of **3**-CO. The amount of the

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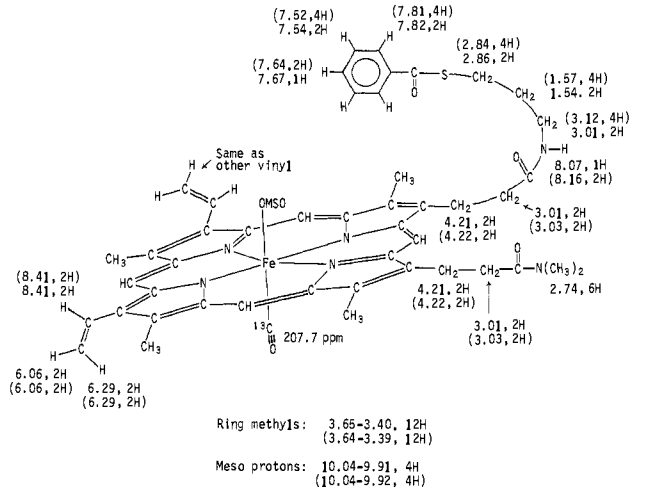


Figure 2. Protohemin chloride dimethylamide, (3-(benzoylthio)-propyl)amide carbon monoxide complex (**3**-CO) in Me_2SO-d_6. The NMR peak positions are indicated at the appropriate protons in parts per million downfield from Me_4Si. The ^{13}C position is in ppm downfield from Me_4Si, referenced to Me_2SO-d_6. Values in parentheses refer to the corresponding dithiobenzoylpropyldiamide complex, **8**-CO, obtained from 5^+-Cl^-.

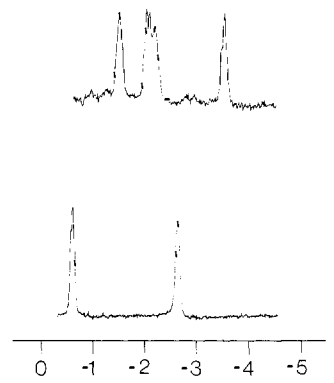


Figure 3. NMR spectra of mercaptide chelated heme–CO (**4**-CO), upper spectrum, and β-phenethyl mercaptide heme–CO, lower spectrum, in Me_2SO-d_6. Only the upfield resonances are shown.

414-nm species varied from 10 to 30% with method of preparation, possibly due to oxidation of the low concentration mercaptide. However, at the higher concentrations used for NMR, very little **3**-CO was apparent. Exposure of the solution of **4**-CO to oxygen resulted in a rapid change to the spectrum of **3**-CO. A solution of the deoxy form **4** of chelated mercaptide heme in Me_2SO–CH_2Cl_2 was cooled to −80 °C and exposed to dioxygen. Immediate oxidation to Fe(III) was evidenced by color and spectra changes.

NMR Characterization of the Benzoyl Protected and Deprotected Mercaptide Heme Compounds 3-CO and 4-CO. A carefully degassed solution of the hemin compound 3^+-Cl^-, 0.03 M in Me_2SO-d_6, was saturated with CO and treated with excess sodium dithionite in Me_2SO-d_6 as described above and the proton NMR and visible spectra determined. The visible spectrum was identical with that described above for **3**-CO. The NMR spectral positions, identified by decoupling procedures and by analogy with other heme compounds, are indicated at the appropriate positions in Figure 2. The use of ^{13}CO afforded the spectrum of the bound ^{13}CO at 207.7 ppm.

After treatment of this solution with about 1.1 equiv of dimsyl anion in Me_2SO-d_6 the NMR spectrum was redetermined. The upfield portion of the spectrum is compared with that of the protoheme complex with β-phenethyl mercaptide and CO in Figure 3. Peak positions are indicated on the structure in Figure 4, and a visible spectrum of the NMR solution was identified with **4**-CO. These spectra afford definitive evidence for the structures of **3**-CO and **4**-CO and, by inference, the structure of 3^+-Cl^-. The use of ^{13}CO afforded a spectrum of the bound ^{13}CO at 197.4 ppm in **4**-^{13}CO.

B. Spectroscopic Studies in Cetyltrimethylammonium Bromide ($CetMe_3NBr$). A 10-mL solution of 2% aqueous $CetMe_3NBr$ containing 0.2 g of potassium hydroxide was degassed with argon in the cuvette described above and treated with 10 μL of saturated aqueous sodium

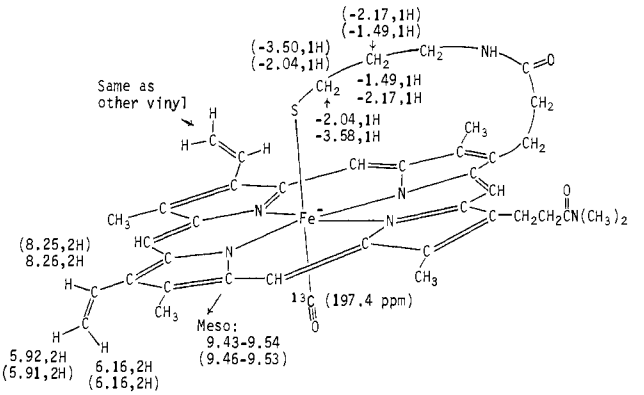


Figure 4. Mercaptide-chelated protoheme–CO (**4**-CO). The NMR positions in parentheses refer to the dimercaptide compound, **6**-CO.

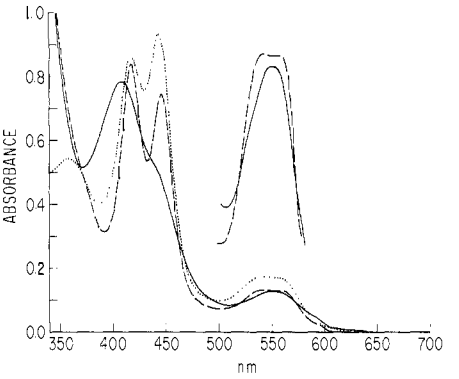


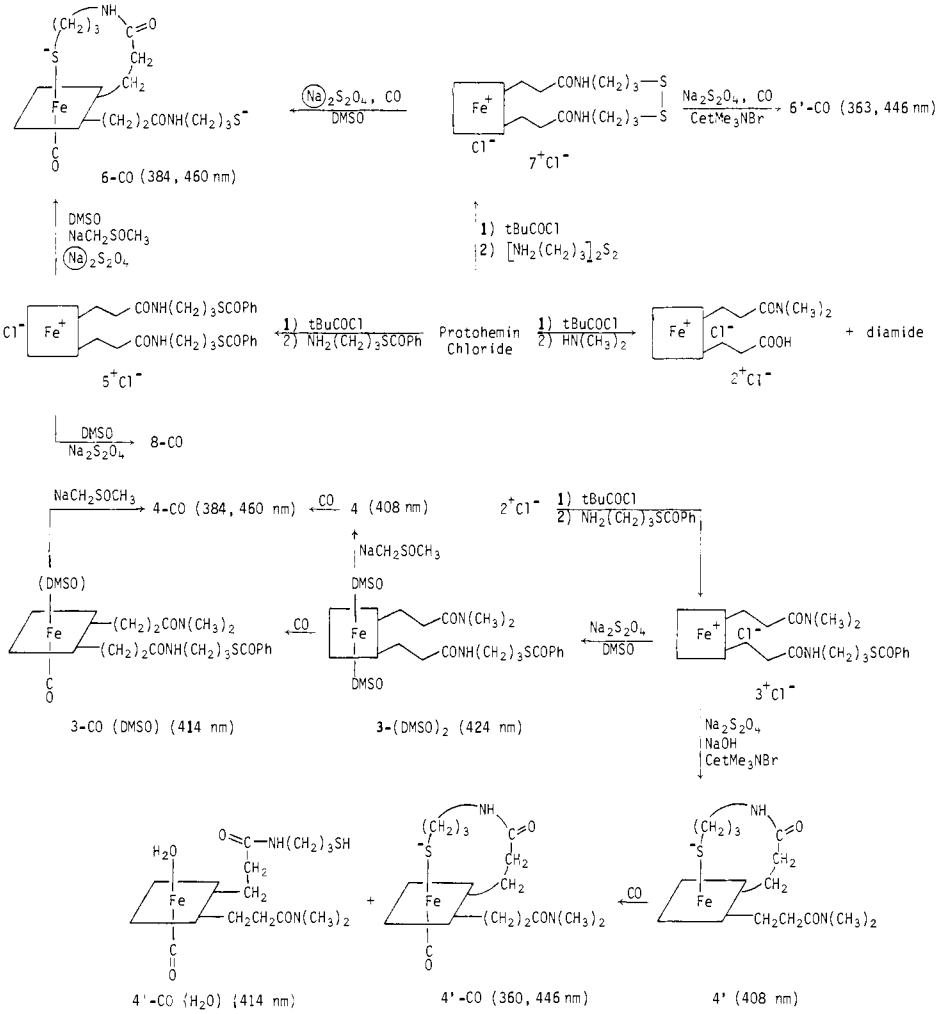
Figure 5. Visible spectra of the mercaptide-chelated hemes **4′**, **4′**-CO, and **6′**-CO in 2% cetyltrimethylammonium bromide micelles prepared from 3^+-Cl^- and 7^+-Cl^- as described in the text: (—) reduced thiolate **4′**; (---) CO complex **4′**-CO obtained upon addition of CO to **4′**; (···) CO complex **6′**-CO.

dithionite. Enough of the protected merceptan heme 3^+-Cl^- to make this suspension ~5 μM in heme was added in 10 mL of methanol. The spectrum of this solution showed a strong Soret band at 400 nm (heme–OH_2).[7a] Warming the suspension to ~40 °C for a few minutes resulted to hydrolysis of the thioester indicated by a change of the spectrum to one having λ_{max} = 408 and ~440 nm, shown in Figure 5. Addition of carbon monoxide caused this spectrum to change to one having λ_{max} at 414 and 446 nm with a slight shoulder around 360 nm. This hyperporphyrin band is obscured by the dithionite absorption. Very similar spectra were obtained in this medium when the dithioester 5^+-Cl^- or disulfide 7^+-Cl^- was employed. In the case of 7^+-Cl^- less dithionite was required, and the 363-nm band could be clearly seen in the spectrum of **6′**-CO shown in Figure 5. The ratios of the 446-nm absorption to that at 363 nm in the micellar suspension is much closer to that found in P-450 preparations than are the ratios of these absorbances observed in most organic solvents (compare Figures 1 and 5). The ratios of absorbances at 414 and 446 nm varied somewhat from one preparation to the next but were generally near one-to-one, indicating about 50% conversion to the mercaptide-chelated hemes **4′**-CO or **6′**-CO. This indicates a lower mercaptide affinity in the aqueous $CetMe_3NBr$ micellar suspension than in Me_2SO. Further evidence for lower RS^- affinity in this solvent was obtained by reducing protohemin dimethyl ester under the same conditions used for 3^+-Cl^- but in the presence of CO and 0.05 M mercaptoethanol, dithiothreitol, or *n*-butanethiol. Only traces (<5%) of the 446-nm peak were observed.

Carbon Monoxide Affinities. Sequentially decreasing the carbon monoxide pressure over the solution of the chelated mercaptide heme–CO complex (**4′**-CO above) in $CetMe_3NBr$ suspension resulted in a change from the 446-nm absorbance to the 408-nm absorbance of the deoxy form of this heme **4** with isosbestic points at 435 and 460 nm. At 6.1 torr the 414-nm band of **3**-CO had not decreased. Further decrease to zero pressure of CO resulted in a shift from 414 to 408 nm. The equilibrium constant, calculated for changes at 446 nm, was 6×10^4 M^{-1} ($P_{1/2}$ = 13 torr) at 25 °C, 2×10^4 M^{-1} ($P_{1/2}$ = 50 torr) at 35 °C, and 8×10^3 M^{-1} ($P_{1/2}$ = 120 torr) at 44.8 °C. A similar titration of the dimercaptide compound (**6′** ⇌ **6′**-CO) revealed a $P_{1/2}$ = 20 torr at 25 °C.

Kinetics of Carbon Monoxide Reaction with 4 and 6. A solution of mercaptide-chelated heme–CO complex (the mixture of **4′**-CO (H_2O)

Scheme I



*Primed and unprimed numbers refer to the same chemical structure in $CetMe_3NBr$ solution and DMSO solution, respectively.

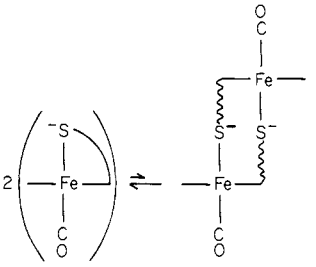
and 4'-CO) in 2% $CetMe_3NBr$ was prepared as described above. Various amounts of carbon monoxide were added to the tonometer and flash photolysis kinetic studies carried out as previously described.[7a,c] At 58.7 torr of CO pressure the return following flash photolysis was in excess of 10^4 s^{-1} at 414 nm and 107 s^{-1} at 446 nm. A plot of the observed rate of return of the 446-nm peak observed in several such determinations against CO concentration revealed a second-order rate constant, $l' = (1.1 \pm 0.1) \times 10^6$ M^{-1} s^{-1}. A similar study of the dimercaptide heme 6'-CO revealed a second-order rate constant $l' = 5 \times 10^5$ M^{-1} s^{-1}.

Results and Discussion

Syntheses. Inspection of molecular models indicated that the simplest chelation arm which could bring about binding of the mercaptide to iron without severe strain is the propyl mercaptide group shown in Figure 4. However, the preparation of a pure hemin–mercaptan compound is complicated by the facile oxidation–reduction process of eq 1. To avoid this process we prepared two kinds of mercaptide compounds protected from this reaction as disulfide (compound 7^+-Cl^-) or benzoyl derivatives (3^+-Cl^- and 5^+-Cl^-) as outlined in Scheme I. In the cases of the previously described disulfide (7^+-Cl^-), addition of sodium dithionite reduced the iron quickly and the disulfide more slowly.[2] With the benzoyl derivatives 3^+-Cl^- and 5^+-Cl^- the iron was first reduced by addition of sodium dithionite to produce **3**, **3**-CO, or similar heme–CO complexes having a proximal oxygen ligand (either H_2O or Me_2SO). Subsequent addition of base cleaved the thioester to produce **4**-CO or **6**-CO in Me_2SO or **4'**-CO or **6'**-CO in aqueous micelles. These techniques avoid exposing RS^- to Fe(III).

Characterization. A solution of the carbonmonoxyprotoheme dimethylamide (3-(benzoylthio)propyl)amide **3**-CO (0.02 M) was prepared in Me_2SO-d_6 by reduction of 3^+-Cl^- with 18-crown-6 sodium dithionite complex, and the visible and NMR spectra were determined. The visible spectrum is consistent with Me_2SO–Hm–CO complex, and the NMR spectrum shows that the benzoyl-protecting group of **3**-CO is still intact (see Figure 2). Addition of excess dimsylsodium in Me_2SO-d_6 to this solution resulted in a visible spectrum identical with that of a dilute solution of **4**-CO, and the NMR spectrum is tabulated in Figure 4.

These two NMR spectra, determined on solutions whose visible spectra correspond to those observed in dilute solution, serve to define the structures of both the precursor hemin 3^+-Cl^- and the mercaptide-chelated heme **4**-CO. In particular, the positions of the protons α and β to the S^- upfield of tetramethylsilane are explicable only in terms of these protons experiencing the strong ring current of the porphyrin system. These upfield shifts have also been observed in imidazole-chelated hemes.[7a] The observation that both the α- and β-protons are differentiated by this ring current affords evidence for chelation of the mercaptide as shown in Figure 4 rather than dimerization. Therefore even at this high concentration there is no appreciable dimer formation.



(3)

Table I. Rate Constants for Reaction of Carbon Monoxide with Cytochromes P-450 and Model Mercaptide–Heme Complexes

heme compd	solvent	temp, °C	k_{on}^{CO}, M^{-1} s^{-1}	k_{off}^{CO}, s^{-1}	K^{CO}	ref
Fe(II) protoporphyrin *n*-Bus K	DMA	23	1×10^5	19	10^4	12
4	$CetMe_3NBr$–H_2O	25	1×10^6	18	6×10^4	this work
6	$CetMe_3NBr$–H_2O	25	5×10^5			this work
bacterial P-450 (camphor-free)	H_2O	4	5×10^6		2×10^6	14
bacterial P-450 (camphor)	H_2O	4	4×10^4	0.14	2.6×10^5	14
LM P-450	H_2O	4	4.5×10^5	0.63	7×10^5	15
	H_2O	20	10^6	1.4	7×10^5	
LM P-450	H_2O	22	6.6×10^5			16
LM P-450	H_2O	23	9×10^6 1.6×10^6 2×10^5	0.4, 0.05		17

The dimer would have the simpler NMR previously observed for phenethyl mercaptide–heme–CO in which the NMR peaks of the α- and β-protons are observed as single resonances at δ −2.62 and −0.62, respectively.[11]

This characterization of chelated mercaptide protoheme–CO (**4**-CO) by NMR definitively identifies the RS^-–Fe–CO structure with the 450–460-nm Soret band and 360–380-nm hyperporphyrin band. The related spectrum of the deoxy form **4**, having a Soret band at 408 nm, can be definitely assigned the five-coordinated RS^-–Fe structure since only one RS^- is available and the spectrum of **4** is essentially identical with that of reduced P-450_{cam}. These findings confirm the conclusions of Change and Dolphin[12] that external mercaptide ion does not form a bis adduct $(RS^-)_2$heme. The identical spectra obtained with the monomercaptide heme **4** and the dimercaptide heme **6** further document this conclusion.

The NMR of the mercaptide chelated heme reveals four highly upfield shifted resonances (of one proton each) at δ −3.5, −2.17, −2.04, and −1.49. We assign these as shown in Figure 4 by using the center of gravity of each pair as being near those observed with the β-phenethyl mercaptide complex. A smaller but significant upfield shift compared to neutral CO complexes is also seen in the meso and vinyl proton resonances.

We attribute the large upfield shift of the protons α and β to the S^- to the large porphyrin ring current along with a smaller effect of the negatively charged sulfur. Based upon the NMR shifts and the sensitivity of ring current effects to distance, we conclude that one of each of the α- and β-protons is closer to the heme ring than is its geminal partner. Molecular models indicate this to be a stable conformation which is difficult to change without breaking the S–Fe bond. Similar separation of geminal proton resonances were observed in chelated protoheme.[7a]

These significant chemical shift changes, resulting from slight changes in the position of these α-hydrogens above the heme plane, may afford a means of determining not only the presence of the $-CH_2$–S–Fe linkage in proteins but also the geometry of the cysteine as well. The positions of these NMR peaks at −2 to −3 ppm are well outside of the protein absorptions. The *S*-methyl resonance of the axial ligand of ferrocytochrome *c* was identified in the proton NMR spectrum of this hemoprotein over a decade ago.[13] Therefore, it seems quite feasible to detect the analogous resonances from the axial ligand of P-450, given the recent advances in high-field FT NMR spectrometers. Although these upfield ^1H peaks have not yet been identified in P-450, the upfield-shifted ^{13}CO resonance reported here for the chelated heme and previously for the benzylmercaptide–protoheme ^{13}CO has been observed in cytochrome P-450_{cam}.[11]

Carbon Monoxide Affinities and Kinetics. Table I lists association and dissociation rates and titration affinities for P-450 and two model systems, BuS^- K* + protohemin dimethyl ester and mercaptide chelated hemes **4′** and **6′**. Comparison of the affinities for the various forms of cytochrome P-450 reveal a range of K^{CO} = (2–20) × 10^6 M^{-1}. Mercaptide-chelated heme in the aqueous $CetMe_3NBr$ suspension has an affinity outside this range, 6 × 10^4 M^{-1}, and the external mercaptide heme has an even lower affinity, 10^4 M^{-1} in toluene or dimethylacetamide.[12]

Even more striking is the large CO dissociation rate constants for the model compounds (18 s^{-1} for the external mercaptide[12] heme and 6–10 s^{-1} for the chelated forms compared to 0.05–1.4 for LM P-450-CO). While our measured association rates for **6** (5 × 10^5 M^{-1} s^{-1}) are in good agreement with those for LM P-450,[16,17] the dissociation rates we have determined for 4′-CO or 6′CO are faster than those reported for cytochromes P-450 except for one report of a "fast-reacting form" of P-450.[15b]

A possible explanation for our faster dissociation rates as well as variations within the P-450 enzymes is the base-elimination mechanism which we have documented for model compounds having proximal strain, i.e., weak Fe–base bonding. Because RS^- binds poorly to Fe(II) or to the Fe–CO complex, this mechanism seems highly likely, both in the models and the proteins.

$$RS^-\text{–Fe–CO} \overset{fast}{\rightleftharpoons} RS^- + \text{Fe–CO} \xrightarrow{1} RS^- + \text{Fe} + \text{CO} \rightarrow RS^-\text{–Fe} \quad (4)$$

The observation that two attached bases (as in **6′**-CO) results in about half the dissociation rate of that having one base is consistent with this mechanism.

However, Chang and Dolphin[12] found that both association and dissociation rates are independent of BuS^- concentration in dimethylacetamide. This seems inconsistent with base elimination for the fast dissociation rate which they observed. In their solvent, RS^- could be less dissociated. An alternative explanation is the destabilization of the Fe–CO bond by σ donation from proximal anions.[6]

In $CetMe_3NBr$ suspension the mercaptide-chelated heme–CO complex is about 50% in the chelated form and 50% in aquo form although when CO is removed the mixture reverts to **4′** (Soret band at 408 nm) rather than to a mixture of **4′** and the open form **3**.[2] Therefore, in contrast to neutral ligands such as imidazole, which greatly increase CO affinity, RS^- seems to compete with CO for binding to iron. The observed affinity of RS^-–Fe for CO (K = 6 × 10^4 M^{-1}) is not appreciably higher than that of four-coordinated heme.[18] Imidazolate–heme and hydroxyheme also have greatly reduced CO affinities compared to their protonated forms.[6] Clearly, deprotonation of the proximal base drastically reduces the affinity of the base–Hm for carbon monoxide whether the base is nitrogen, oxygen, or sulfur. As we have noted elsewhere,[6] such deprotonation increases the equilibrium constant for

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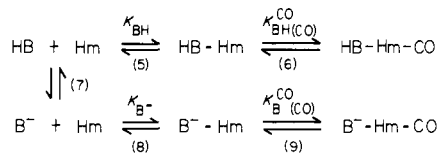
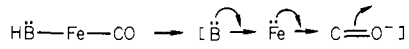
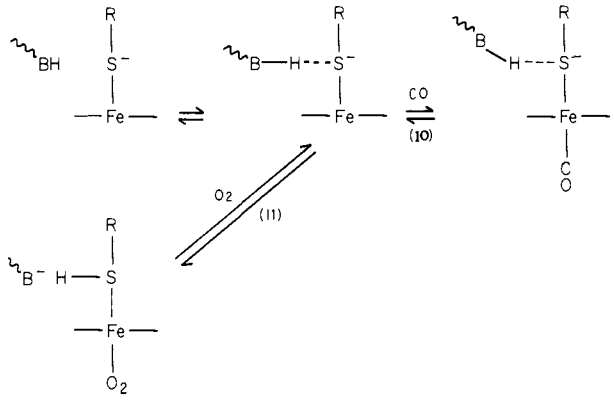
formation of the high-spin five-coordinated heme.



That is, $K_{B^-} > K_{BH}$ whereas $K_{B^-}{}^{CO} < K_{BH}{}^{CO}$. In the case of protoheme in wet Me_2SO containing 0.1 M NaOH, the high-spin heme–OH^- is unaffected by 1 atm of CO![6,12] Reduced CO affinities of anion heme complexes is probably a result of repulsion through the Fe–CO σ bond since π back-bonding to CO should be increased with increased electron density. This is consistent with the decrease in ν_{CO} from 1951 to 1935 cm^{-1} upon imidazole deprotonation.[6]



The large decrease in CO affinity with increase in proximal base charge suggests that this change could be the source of low CO affinity in cytochrome P-450 as compared to hemoglobin or myoglobin. The CO affinity of cytochrome P-450 is reported to be around 10^6 M^{-1}, considerably higher than that of mercaptide-chelated protoheme but lower than the affinity of myoglobin. We suggest that some proton source is located near the thiolate group and that this group either hydrogen bonds to the mercaptide or transfers a proton.

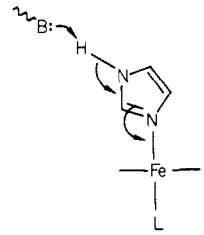


Such a mechanism also provides a means of stabilizing the oxygen complex against iron oxidation relative to model systems. The cytochrome P-450 model systems RS^-–Hm as well as other anion complexes such as Im^-–Hm are much more sensitive toward oxidation relative to the enzyme than are the hemoglobin models. For example, chelated protoheme–O_2 in the ionizing solvent dimethylformamide–water is stable for hours at −45 °C whereas RS^-–Hm or Im^-–Hm are oxidized as rapidly as they are mixed with dioxygen at −80 °C. Anion heme dioxygen complexes probably dissociate into superoxide ion.

$$O_2 + X^-\text{–Fe} \rightleftharpoons X^-\text{–Fe–}O_2 \xrightarrow{b} X^-\text{–}Fe^+ + O_2^-\cdot \quad (12)$$

The protonation or hydrogen-bonding scheme shown in eq 10 would serve both to stabilize the O_2 complex against reaction 12b and to shift its spectrum toward that of oxymyoglobin.

Because the dioxygen affinity increases rapidly with increasing proximal basicity[7c] and carbon monoxide affinity decreases with deprotonation of the proximal base, a very sensitive method of modulating O_2 vs. CO affnities is available to proteins through hydrogen bonding the proximal imidazole hydrogen.[7d] Thus increasing the negative charge on the imidazole by hydrogen bonding or proton removal as shown below would shift both eq 12a and 12b to the right. Since 12b is very small in heme proteins, such a shift could easily result in an increase in the ratio of $XHmO_2$ relative to both dissociated forms or an increased stability of $XHmO_2$. In the extreme of complete anion formation, superoxide dissociation is to be expected. Therefore, in a given environment (e.g., polar solvent), the total Fe–O_2 bond stability should go through a maximum as the anionic character of the proximal base is increased.



for L = O_2, triplet dioxygen, affinity increases, superoxide affinity decreases;
for L = CO, affinity decreases

This provides an alternative to the much discussed steric differentiation between the stabilities of the carbon monoxide and dioxygen complexes of low affinity hemoproteins.[19,20] The effect of this deprotonation or hydrogen bonding on dioxygen affinities has not been demonstrated, but the sensitivity of dioxygen affinity to basicity of the proximal base[7c] suggests that such hydrogen bonding should greatly increase O_2 affinity.

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