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NMR STUDIES OF P-450 MODEL SYSTEMS:

NEW STRUCTURAL PROBES FOR SULFUR-CONTAINING HEMOPROTEINS

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SUMMARY

A simple model for ferrous cytochrome P-450 has been investigated by proton and carbon-13 Fourier transform NMR. In the proton spectrum of the β-phenethyl mercaptan-protoheme-CO complex, the protons α and β to mercaptide sulfur are observed 2.62 and 0.62 ppm upfield of tetramethylsilane. The ^{13}CO spectra show characteristic shifts at 204.7 and 197.0 δ for neutral and deprotonated mercaptan complexes, respectively.

INTRODUCTION

Spectroscopic comparisons of model systems (1) with cytochrome P-450 have provided rather strong evidence for a mercaptide-iron linkage in these hemoproteins (2). However, there still remains some disagreement concerning the nature of the proximal and distal ligands (3) of these and other hemoproteins, such as chloroperoxidase (4), which have similar spectroscopic properties.

We present here NMR studies of model compounds as new probes to provide more definitive characterization of the mercaptide-heme bond.

MATERIALS AND METHODS

Protoheme IX bis(dioctylamide) and bis(dimethylamide) were used for neutral and deprotonated conditions, respectively. The longer alkyl chain afforded high solubility in the aromatic thiol solvent. The protohemin bis-(dialkylamides) were synthesized from protohemin IX (Calbiochem) and the

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appropriate diamines (Aldrich), using the pivaloyl anhydride coupling procedure and purification by column chromatography as previously described (5). These new compounds exhibited ferrous-CO NMR spectra consistent with their structures.

Deuterated solvents were obtained from Stohler Isotope Chemicals, and 90% enriched ^{13}CO from Merck, Sharp and Dohme. β-Phenethyl mercaptan (Pfaltz and Bauer) was distilled *in vacuo*. Sodium dithionite (Baker) and 18-crown-6 (Aldrich) were used as received.

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 using 10 mm tubes, internal deuterium lock, a 5 KHz sweep width and 8K data points. Chemical shifts are reported in ppm downfield of internal TMS, except for ^{13}C spectra in DMSO-d_6, where the solvent peak at 39.6 δ was used (6).

Solutions were prepared 0.03 M in heme in NMR tubes equipped with a spacer for visible spectroscopy and closed by a silicone septum (7). Solutions for mercaptide NMR were prepared by dissolution of the heme and 18-crown-6 (~0.1 M) in DMSO-d_6. After degassing, ^{12}CO or 90% ^{13}CO was admitted, and the heme reduced by a few microliters of D_2O saturated with sodium dithionite. Phenethyl mercaptan was added to give a threefold excess over heme. The resultant DMSO-heme-CO complex was then titrated with approximately 1 equivalent of 3 M deuterated dimsyl anion, prepared from DMSO-d_6 and NaH (8), to yield the mercaptide-heme-CO complex, as judged by changes in the NMR spectrum.

For mercaptan-heme-CO NMR, the heme was dissolved in pure phenethyl mercaptan under CO, shaken over a D_2O-dithionite solution, and transferred to a CO-filled NMR tube. The D_2O exchanged with ϕCH_2CH_2SH to provide the internal deuterium lock.

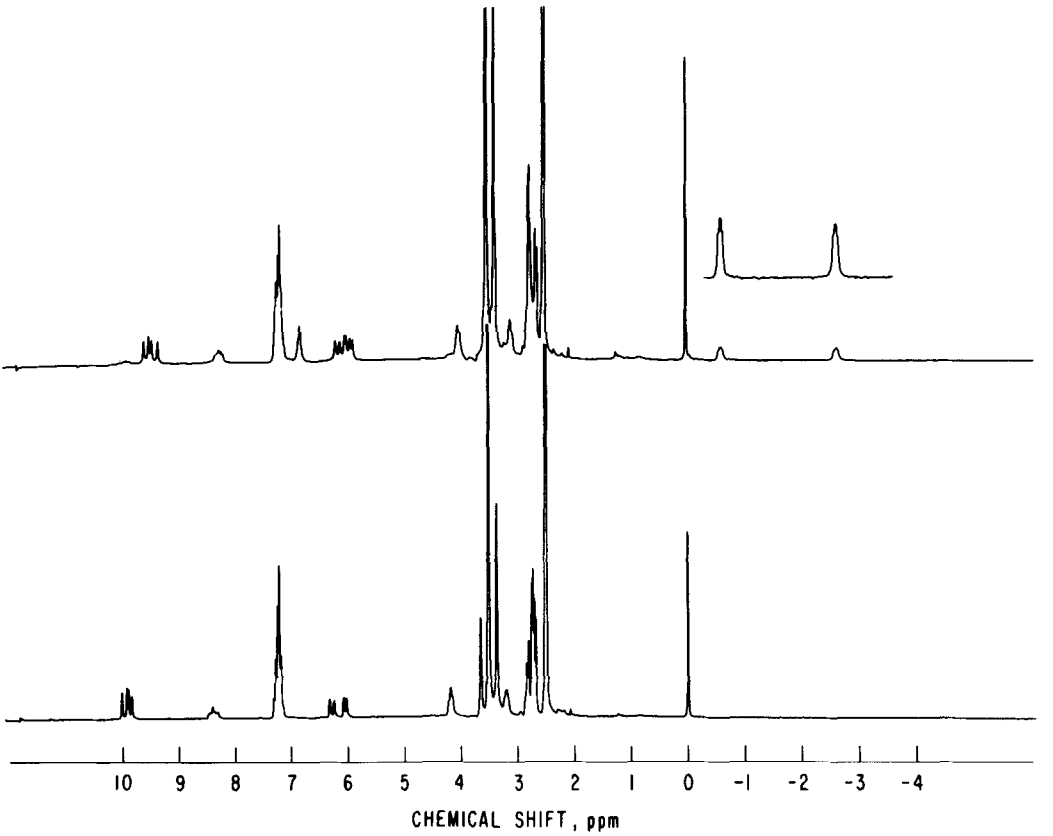


Figure 1. NMR spectra of ferroprotoheme bis(dimethylamide) as the DMSO-heme-CO complex (lower trace) and the mercaptide-heme-CO complex (upper trace).

RESULTS

The proton NMR spectrum of protoheme IX bis(dimethylamide), as a ferrous DMSO-heme-CO complex, is shown in Figure 1 (lower trace). The resonances of the threefold excess of phenethyl mercaptan are also present. No evidence for mercaptan binding is seen in this spectrum, which is confirmed by the visible spectrum showing bands at 563 and 533 nm, typical of a DMSO-heme-CO complex (7). Titration with dimsyl anion solution gave a spectrum (upper trace) in which >80% of the heme was converted to the mercaptide-CO complex, which showed a single, broad visible band at ~555 nm and a Soret band at ~460 nm. A number of heme resonances shift upon formation of the mercaptide complex, but the most striking features are the bound mercaptide methylenes

at -0.62 and -2.62 δ. Shifts for the aromatic protons of the bound ligand are also seen upfield of their free solution positions.

The proton NMR spectrum of a neutral mercaptan-heme-CO complex was obtained in neat, undeuterated phenethyl mercaptan. This complex gave visible bands at 567 and 539 nm and a Soret band at 420 nm, in agreement with previously reported thiol complexes (9), and in contrast to the water-heme-CO complexes which have a Soret band at 414 nm. Dilution with an equal volume of benzene, or warming or cooling the neat mercaptan-heme-CO solutions, caused no change in the sharp 420 nm Soret band, demonstrating complete RSH binding. We attribute our inability to observe bound phenethyl mercaptan NMR resonances to rapid exchange (10). The proton chemical shifts of these three complexes are shown in Table 1.

The chemical shift values of ^{13}CO bound to these complexes were also obtained. The same visible spectral characteristics were seen as in proton NMR. Titration of the DMSO-heme-^{13}CO complex at 207.7 δ with dimsyl anion led to the mercaptide-heme-^{13}CO complex, which showed a large upfield shift to 197.0 δ. This is in contrast to the value obtained in neat mercaptan for mercaptan-heme-^{13}CO of 204.7 δ. Thus, a chemical shift difference of 8 ppm is seen in the deprotonation of mercaptan-heme-CO complexes.

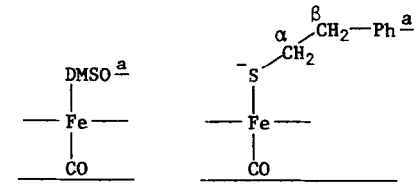
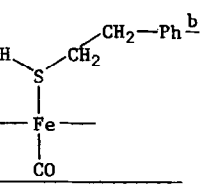
DISCUSSION

Proton NMR studies of hemoproteins are generally carried out on paramagnetic complexes, in which certain heme substituents are hyperfine-shifted to positions outside the usual 0-10 δ diamagnetic region (11). Studies of biologically interesting <u>diamagnetic</u> hemoproteins, such as oxy or carbonmonoxy hemoglobin, have been hampered by the fact that proton resonances for all heme-related groups, including the bound histidine, occur in the 0-10 δ region and are hence obscured by the envelope of the protein protons (12).

It appeared that ^{13}CO would be an ideal probe for the diamagnetic complexes, inasmuch as the ligand is readily available, has a high affinity, and

Table 1

Proton Chemical Shifts of the Protoheme Model in ppm Downfield of TMS

Heme Substituent	DMSO [a]		
Meso-H	10.04-9.87	9.61-9.36	10.24-9.24
-CH=C	8.42	8.27	8.30
-C=CH_2	6.34,6.26 6.08,6.03	6.21,6.13 5.94,5.89	6.30,6.22 6.00,5.95
-CH_2CH_2-C(=O)	4.19,3.21	4.02,3.09	4.50 [c]
Ring methyls	3.65-3.37	3.38	3.59-3.53
-NR_2	2.74	2.76	1.3 -0.8 [c]
Sulfur Ligand			
1) free			
Ph	7.24	7.20	7.14-6.99
-CH_2CH_2-	2.84-2.68	2.58-2.44	2.67-2.49
2) bound			
Ph	—	6.58,6.02	—
-CH_2CH_2-	—	-0.62(β),-2.62(α)	—

[a] Protoheme IX bis(dimethylamide) in DMSO-d_6.

[b] Protoheme IX bis(dioctylamide) in neat phenethyl mercaptan.

[c] Other resonances obscured by solvent.

is easily observable. However, studies of a variety of hemoglobins and myoglobins have revealed a rather small variation in the ^{13}C shifts obtained. For example, hemoglobins from sources as disparate as human, rabbit, carp, and bloodworm all occur within 206-208 δ (13). Larger ^{13}C-enriched ligands such as isonitriles show a greater protein-to-protein variation, but this seems to be more a function of the distal pocket than of the proximal ligand (14).

This communication presents new data for the use of ^{13}C and ^{1}H NMR of diamagnetic proteins as diagnostic probes of electronic and structure features. The ^{13}CO chemical shift of 197.0 δ obtained for the P-450 mercaptide model is substantially different from values of both histidine-containing proteins and model imidazole-heme complexes (15). The value of 204.7 δ for the neutral mercaptan complex is shifted much less, but is still outside the region where imidazole hemoproteins such as hemoglobin occur. Thus, observation of the ^{13}CO resonances in proteins postulated to have cysteinyl ligands, in conjunction with the characteristic visible spectra, should confirm assignment of mercaptide or mercaptan as a ligand. The sensitivity of these shifts to deprotonation of the sulfur might also allow probing of hydrogen-bonding effects.

Perhaps even more promising are the prospects for proton NMR study of sulfur-containing hemoproteins. The chemical shifts of the protons α to sulfur in cysteine are expected to be nearly the same as those in phenethyl mercaptan (16). The fact that the large, ring current-induced shielding (17) of the porphyrin macrocycle moves the resonance of these protons in phenethyl mercaptide to a position ~3 ppm upfield of TMS leads us to predict that a bound cysteinyl mercaptide residue will be directly observable. While we have not been able to observe bound neutral mercaptan in the ^{1}H NMR, the fact that the ring current of the heme dominates the position of the shifts means that a neutral cysteine ligand should also appear upfield of TMS. While more difficult to model, a diamagnetic sulfur-heme-oxygen complex would be expected to behave similarly, and hence be observable in the protein.

In conclusion, ^{1}H and ^{13}C NMR studies of sulfur-containing hemoproteins offer the possibility of not only conclusively demonstrating the nature of the ligand in diamagnetic complexes, but also of yielding detailed stereochemical information (18). Such studies are underway in these laboratories.

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11. For example, see La Mar, G. N., Overkamp, M., Sick, H., and Gersonde, K. (1978) Biochemistry 17, 352-361.
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18. After this paper was submitted we learned[19] of a ^{13}CO NMR study of bacterial cytochrome P-450-^{13}CO and myoglobin-^{13}CO by N. A. Matwiyoff and S. B. Philson (1972, unpublished). They reported δ values of 200.3 ppm and 207.7 ppm for the P-450 and myoglobin-^{13}CO, compared to our values of 197.0 and 205.5 for the corresponding mercaptide-heme-^{13}CO and imidazole-protoheme-^{13}CO model compounds. The similar shifts of 8.5 ppm for imidazole to mercaptide and 7.4 ppm for myoglobin to P-450 provide additional evidence for the mercaptide group in P-450$-$CO.
19. We are grateful to Professors I. C. Gunsalus and P. R. Debrunner for bringing this work to our attention.