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Magnetic circular dichroism of ferrous carbonyl adducts of cytochromes *P*-450 and *P*-420 and their synthetic models: Further evidence for mercaptide as the fifth ligand to iron*

[hemoproteins/enzyme model compounds/drug metabolism/iron(I) porphyrin/mixed function oxidase]

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ABSTRACT **Absorption and magnetic circular dichroism (MCD) spectra have been obtained for the ferrous carbonyl adducts of cytochromes *P*-450 and *P*-420 as well as synthetic model systems. Ferrous porphyrins with sodium methyl mercaptide and CO in benzene give MCD and absorption spectra which are almost identical to those of the natural enzyme, indicating that in *P*-450 a mercaptide serves as the fifth ligand in the ferrous carbonyl adduct. MCD spectra of models with either propyl mercaptan or *N*-methylimidazole as the axial ligand are identical with that of *P*-420. Thus, no unambiguous assignment of the axial ligand can be made in this case. The infrared stretching frequencies of ferrous porphyrin carbonyl complexes and the absorption spectrum of the CO adduct of Na[Fe^{I}(*meso*-tetraphenylporphyrin dianion)] are consistent with the concept that in *P*-450 considerable electron density is transferred to the iron by the mercaptide ligand.**

Mammalian cytochrome *P*-450 is a membrane-bound mixed function oxidase which mediates the hydroxylation of a wide variety of substrates including steroids, aromatic compounds, hydrocarbons, and barbiturates (1). Because it has only recently been obtained in electrophoretically homogeneous form (2–5), much of the information concerning *P*-450 has come from studies on the soluble, hence easily purified, bacterial oxidase, *P*-450$_{cam}$ (6). All cytochromes *P*-450 contain iron protoporphyrin IX as the prosthetic group and have a common reaction cycle with four well-characterized states (Fig. 1) beginning with a low-spin ferric resting form, **1**, which is converted upon substrate binding to a high-spin ferric state, **2**. Reduction gives a high-spin ferrous complex, **3**, capable of binding dioxygen (for the subsequent hydroxylation step), **4**, or carbon monoxide, **5**.

Cytochrome *P*-450 is unique among hemoproteins for two reasons. First, its ferrous carbonyl adduct absorbs light at the unusually long wavelength of approximately 450 nm. Additional abnormal spectral properties associated with this complex include the virtual absence of a distinct α-band in the 570 nm region and the appearance of an additional band at about 370 nm (*vide infra*). This very unusual but characteristic absorption spectrum has been the subject of much speculation; for instance Hill *et al.* (7) have implied that the state of the iron could be quite different from that found in other hemoproteins such as myoglobin. Second, only one other

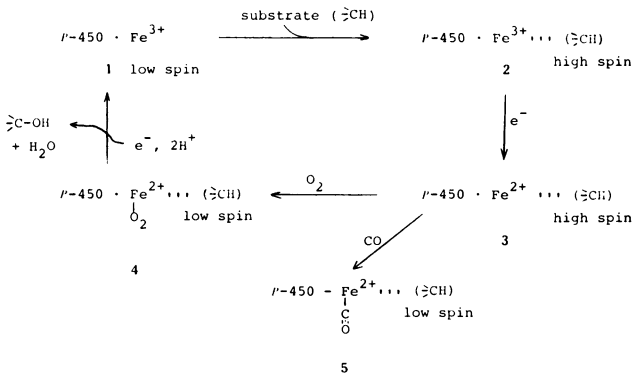

FIG. 1. The enzymatic cycle for *P*-450.

heme protein (8) is capable of activating oxygen for insertion into organic molecules. We feel that an explanation of the unusual spectroscopic properties of *P*-450 may be the key to understanding its unique enzymatic capabilities.

Recently, simple model systems have been prepared and characterized which have shed much light on the nature of the axial ligand in *P*-450 (9–14). All of these studies, and additionally an unpublished MCD comparison of camphor-bound ferric *P*-450$_{cam}$ with the high spin ferric models† have strongly implicated *mercaptide* as the fifth ligand to iron in stages **1**, **2**, and **5** (Fig. 1). Cytochrome *P*-450 loses its catalytic activity upon treatment with various compounds such as organic solvents, detergents, sulfhydryl reagents, and salts which convert it to an inactive form called cytochrome *P*-420, which, like *P*-450, derives its name from the position of the Soret band of its ferrous carbonyl adduct (15). The structure of cytochrome *P*-420 has been debated because it is not clear whether the mercaptide ligand is lost or simply protonated on conversion to *P*-420. The electron paramagnetic resonance (EPR) spectra of *P*-450 and *P*-420 are not significantly different (6), indicating that, at least in the ferric stages, the axial ligands may be the same. As further evidence for the nature of the fifth ligand in both the active and inactive forms of *P*-450, we report here the absorption and magnetic circular dichroism (MCD) spectra for several synthetic model systems and for the highly purified protein.

Magnetic circular dichroism has become an important and potent spectroscopic technique for the elucidation of the molecular and electronic structures of complex molecules as a result of advances in theory and in the development of

Abbreviations: *P*-450 and *P*-420, cytochromes *P*-450 and *P*-420; THF, tetrahydrofuran; TpivPP, *meso*-tetrapivalamidophenylporphyrin dianion; TPP, *meso*-tetraphenylporphyrin dianion; PPIXDEE, protoporphyrin diethyl ester dianion; (Na)SCH$_3$, sodium methyl mercaptide–crown ether complex; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance.

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† J. H. Dawson, S. C. Tang, R. H. Holm, E. Bunnenberg, and C. Djerassi, manuscript to be submitted.

sensitive MCD instruments (16–18) during the last decade. As a result of these advances, the basic concepts governing MCD at the molecular level are generally well understood and are covered in several review papers on the subject (19, 20). This technique has been used by us (21, 22) and others (23, 24) to study crude and partially purified preparations of cytochrome *P*-450. We now report its use to characterize the highly purified protein.

EXPERIMENTAL

All standard reagents were purchased commercially and used without further purification. The glycerol used in the protein purification was Spectrograde (Eastman). Benzene and tetrahydrofuran (THF) were distilled from sodium-benzophenone ketyl under nitrogen; dimethylsulfoxide was distilled from sodium hydride under vacuum. Fe *meso*-tetrapivalamidophenylporphyrin (TpivPP) (25), Fe *meso*-tetraphenylporphyrin (TPP) (26), and dibenzo-18-crown-6 (27) were prepared by literature methods. Fe protoporphyrin diethyl ester (PPIXDEE) was synthesized by reduction of hemin chloride diethyl ester with $Na_2S_2O_4$ in benzene under carbon monoxide. All operations carried out with the ferrous porphyrin complexes were done in a Vacuum/Atmospheres model MO-40-1 inert atmosphere box (<1 ppm O_2) and all solvents and reagents were degassed before use.

Absorption spectra were recorded on a Cary 14 spectrophotometer at 25°. Infrared measurements were obtained on a Perkin-Elmer 621 instrument using CaF_2 cells. EPR data were obtained with a Varian E-line EPR spectrometer system on frozen solutions. All MCD spectra were recorded on a Japan Spectroscopic Co. spectropolarimeter (JASCO model J-40) containing a 15.0 kG (1.5 T) electromagnet with the field direction parallel to the direction of light propagation. For the protein samples, the MCD spectral data were corrected for natural circular dichroism (CD) (MCD_{obs} = CD + MCD) and it is these spectra which are reported in the figures. The protein spectra were recorded at 15° and the spectra of the model compounds, at room temperature. The MCD spectra are reported in terms of molar magnetic ellipticity, $[\theta]_M$, in the units degree $cm^2\ dmol^{-1}\ G^{-1}$. The MCD instrument was calibrated daily for both intensity, using camphor sulfonic acid (18), and wavelength, using a holmium perchlorate solution (28). All MCD data were recorded, normalized, smoothed (29), and manipulated on a Data General-Nova computer model 840 and were stored on magnetic tape. The spectra of the model compounds have been corrected for contamination by the μ-oxo-bridged ferric dimer when necessary by subtraction of its contribution from the observed spectrum.

Cytochrome *P*-450. Highly purified cytochrome *P*-450 was prepared by the method of Coon (2, 3) with only slight modifications which will be reported elsewhere in greater detail. The protein was stored under nitrogen in small aliquots at −20° in a 0.01 M potassium phosphate buffer containing 20% (vol/vol) glycerol, 0.1 mM ethylenediaminetetraacetic acid, and 0.1 mM dithiothreitol at pH 7.4. The purity of the protein was established to be 17 nmol of *P*-450 per mg of protein as determined from the extinction coefficient, 117 $mM^{-1}\ cm^{-1}$ (2), for reduced plus carbon monoxide *P*-450 and by the protein concentration using the Lowry *et al.* method (30). Polyacrylamide gel electrophoresis indicates that the purity of the protein is comparable to that prepared in Coon's laboratory. For all spectral determinations, the protein was stabilized by addition of enough dilauryl lecithin (from a 1 mg/ml of stock solution in water) to give a final concentration of 100 μg/ml.

Cytochrome *P*-420. Cytochrome *P*-450 prepared as above was converted to cytochrome *P*-420 by addition of solid KSCN to give a final concentration of 1.2 M (15). Conversion of *P*-450 to *P*-420 by treatment with steapsin (31) led to an identical reduced plus carbon monoxide MCD spectrum. The concentration of *P*-420 was determined from the initial *P*-450 concentration.

Model Systems. A stock solution of sodium methyl mercaptide–crown ether complex ($\textcircled{Na}SCH_3$) was prepared by stirring the crown ether with excess $NaSCH_3$ (see below) in benzene for 3 days followed by filtration. The mercaptide concentration was determined by addition of $AgNO_3$ and titration of the remaining Ag^+ with NaSCN using $FeCl_3$ as the indicator. Alternatively, an aliquot of the solution was evaporated to dryness and the residue was analyzed for sulfur. Portions of this stock solution and a solution of the porphyrin complex were mixed in the inert atmosphere chamber and sealed in a cuvette or in the infrared cells with rubber septa. Addition of CO was accomplished by purging the solution by means of a syringe needle.

Sodium Methyl Mercaptide, $NaSCH_3$. Sodium (2.3 g) was dissolved in 50 ml of liquid ammonia under nitrogen to give a deep blue solution. This was then treated dropwise with dimethyl disulfide, $(CH_3S)_2$, until the blue color was discharged. The flask was allowed to warm to room temperature during which time the ammonia evaporated, leaving a pure white flocculent powder. The product was extracted with anhydrous ether in a Soxhlet apparatus to remove traces of $(CH_3S)_2$, then dried *in vacuo*.

Na[Fe^I(TPP)]. A solution of sodium anthracenide was prepared by stirring 875 mg.of anthracene with 120 mg of freshly cut sodium in 20 ml of THF followed by dilution to 25.0 ml. FeTPP (340 mg) was dissolved in 50 ml of hot THF and treated with 2.9 ml of the sodium anthracenide solution. After stirring for 20 min, the solution was filtered, diluted with 50 ml of heptane, and concentrated under reduced pressure. The crystals were collected by filtration and recrystallized from THF–heptane as long purple needles, yield 250–300 mg. EPR (−160°), g = 2.30, 1.93 [literature (32, 33): 2.30, 1.93]; UV/visible (THF), λ_{max} = 425, 510, 575, 610, 675 nm [literature (33): 390, 420, 510, 580, 615, 680]; μ (25° under argon), 5.0 Bohr magnetons [literature (32): 5.2 Bohr magnetons]. Because of the extreme oxygen sensitivity of this compound, no satisfactory elemental analysis could be obtained; however, the ratios were determined: *Calculated* C: N: Fe: Na = 44: 4.0: 1.0: 1.0; *Found*, 43: 3.8: 1.0: 1.0.

RESULTS

Addition of a benzene solution of sodium methyl mercaptide, $NaSCH_3$, solubilized by dibenzo-18-crown-6 or 18-crown-6 to FeTpivPP or FePPIXDEE followed by exposure to carbon monoxide results in the absorption spectra shown in Fig. 2 with the Soret maxima at 449 nm. If instead of benzene, dimethylsulfoxide or THF is used as the solvent, the Soret bands of both model complexes are shifted to 462 nm. Solutions of this species are very sensitive to oxygen, presumably because of the redox properties of both the mercaptide and oxygen molecules. It is well known (25) that ferrous porphyrins are oxidized to the ferric state by molecular oxygen in a very rapid bimolecular reaction. Mercaptide ion, available to reduce this ferric complex back to Fe(II), is thus con-

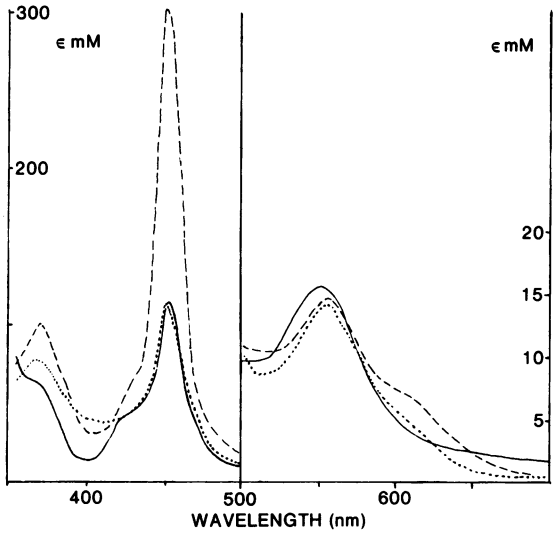

FIG. 2. Absorption spectra for: (——) highly purified *P*-450 (reduced + CO), (- - - - -) FeTpivPP and (Na)SCH_3 + CO in benzene, (· · · · ·) FePPIXDEE and (Na)SCH_3 + CO in benzene.

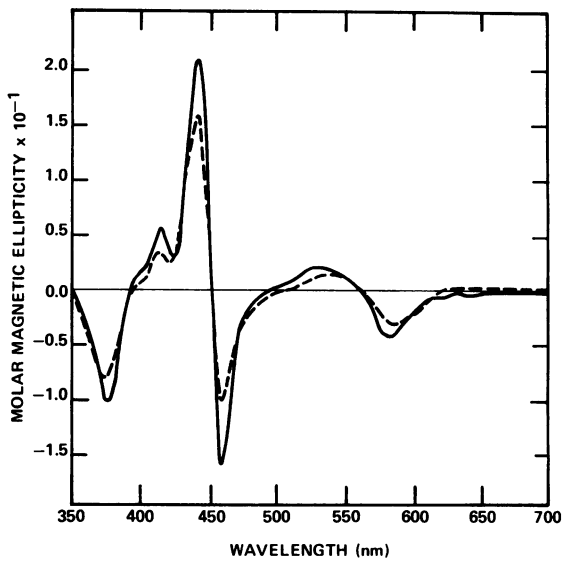

FIG. 3. MCD spectra for: (——) highly purified *P*-450 (reduced + CO), (- - - - -) FePPIXDEE + (Na)SCH_3 + CO in benzene.

verted to disulfide, resulting in a gradual decrease in the thiolate concentration. Because of this extreme O_2 sensitivity, at low porphyrin concentrations (5 μM) a 50- to 100-fold excess of (Na)SCH_3 is necessary to produce the 449 nm chromophore; whereas at higher porphyrin concentrations (1 mM), between a 1- and 2-fold excess is sufficient. The excess thiolate is evidently necessary to eliminate the effects of last traces of oxygen in solution which at the higher concentrations become negligible.

The MCD spectra of FePPIXDEE + (Na)SCH_3 + CO and highly purified *P*-450 (reduced + CO) are displayed in Fig. 3. It is of interest to note the extremely close correspondence of the peak positions and band shapes between the two species. The cross-over point at 450 nm matches the maximum of the absorption band quite closely. The trough at 380 nm in the MCD spectra (Fig. 3) reflects the shoulder‡ in the absorption spectrum of the protein and the distinct peak observed in the model system (Fig. 2). Similar results were obtained with FeTpivPP + (Na)SCH_3 + CO.

Spectra of models for cytochrome *P*-420 as well as the natural system are presented in Fig. 4. Two cases have been examined as possible models to explain the nature of *P*-420: replacement of ligands (mercaptide by imidazole) and protonation (mercaptide → mercaptan). As can be seen, the similarity is so great as to preclude distinction between the two possibilities.

DISCUSSION

The remarkably close similarity between the absorption and MCD spectra of cytochrome *P*-450 and the model system provides compelling evidence that the axial ligand in the enzyme ferrous carbonyl complex is mercaptide, RS^-, presumably the conjugate *S*-base of cysteine. These findings, along with the previous work on the two ferric states (9, 11–14), and our own unpublished work† may imply that *S*-cysteinate is the axial ligand in the remaining ferrous states, 3 and 4 (Fig. 1) as well§. However, none of these spectral similarities sheds light on the detailed structure of the ferrous-CO adduct since such a synthetic complex has not yet been isolated. Nonetheless, it is clear that this species possesses some unusual features not found for other hemoproteins; these properties probably result from the interaction of the RS^- ligand with iron.

The axial mercaptide base is a polarizable electron donor *trans* to carbon monoxide which is a good π-acid. This combination should result in a net drift of electron density through the iron to the carbonyl group. The situation is similar to that in which iron is in a lower formal oxidation state. In this connection, it is of interest that addition of CO to a THF solution of $Na[Fe^I(TPP)]$ gives an absorption spectrum in which the Soret maximum occurs at 455 nm. This band is at a much higher wavelength than that of the corresponding *ferrous* carbonyl $Fe^{II}(TPP)(CO)$ and probably reflects accurately the increased electron density at the iron. However, this is not meant to say that thiolate has reduced the ferrous complex to an iron(I) species, as the spectra of the two complexes are quite different, aside from the abnormally long wavelength position of the Soret band. Further evidence for this idea of high electron density at iron is obtained from the infrared CO stretching frequencies of the model compounds (Table 1). As stated above, there should be increased backbonding into the π^* orbital of the carbonyl, decreasing the vibrational frequency (35). νCO for the mercaptide species is the lowest observed for any of the iron porphyrin carbonyls examined. Thus, it appears that the unusual spectral properties observed for cytochrome *P*-450 can be explained by a model in which there is considerable electron density at the iron as compared with other hemoproteins where the

‡ Under strictly anaerobic conditions, using a minimum of dithionite, this shoulder is resolved into a distinct peak; see Guengerich *et al.* (34).

§ Recent calculations by L. K. Hanson and M. Gouterman (personal communication) suggest that the ligand in reduced *P*-450 may be a mercaptan rather than a mercaptide. Because the results in this paper and others (9–14) strongly indicate that the axial ligand in both the ferric and ferrous-carbonyl states of *P*-450 is a mercaptide, the above calculations, if true, necessitate a proton transfer mechanism between the mercaptide ligand and a suitable donor/acceptor in the protein on conversion from ferric to ferrous to ferrous-carbonyl *P*-450.

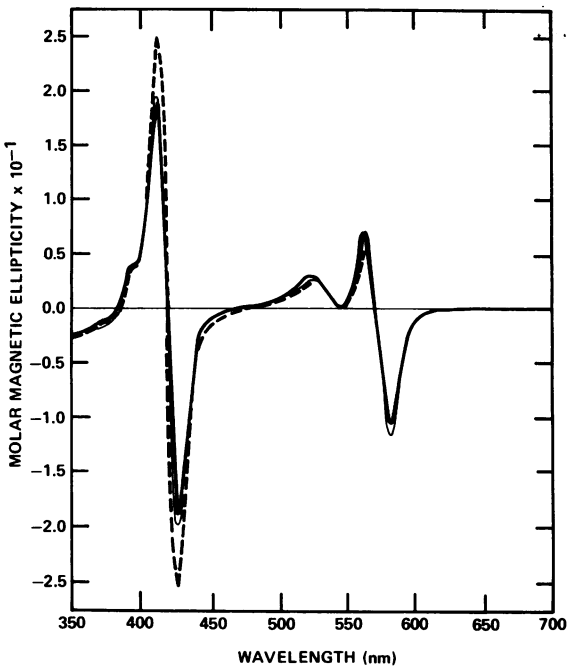

FIG. 4. MCD spectra for: (——) highly purified *P*-420 (reduced + CO), (- - - - - -) FePPIXDEE + *N*-methylimidazole + CO in benzene, (——) FePPIXDEE + C_3H_7SH + CO in benzene.

axial bases (e.g., imidazole, thioether, or thiol) are themselves good π-acids (36).

A related and more detailed explanation for the unusually long wavelength Soret absorption of cytochrome *P*-450 has recently been presented by L. K. Hanson and M. Gouterman (personal communication) and our results are consistent with their interpretation. In the single crystal polarized absorption spectrum of $P\text{-}450_{cam}$, the shoulder at about 370 nm‡ (Fig. 2) is resolved into a separate peak. This suggests the possibility that the Soret transition has been mixed with a charge transfer transition, resulting in a splitting of the Soret into two bands, one at higher wavelength and one at lower wavelength. This additional band is seen in the absorption spectrum of the model compounds as well as in the MCD spectra of both the model and the protein.

The shift in the position of the absorption peak for the mercaptide model complex on changing solvent is worthy of note. As the polarity of the solvent is increased, the peak varies from 449 to 462 nm. Since the peak in the protein itself is at about 450 nm, this may indicate that its active site is non-polar.

The model system described here is a dramatic improvement over the rather ill-defined system of Stern and Peisach (13). The absorption spectrum of our system mirrors that of the protein in both magnitude and structure (maximum at 450 nm, shoulder at 420 nm; see Fig. 2), whereas their system absorbs maximally at 413 nm with a smaller peak at 450 nm. Moreover, our work indicates that in a polar solvent the mercaptide species absorbs at 460 nm, not 450 nm, bringing into question the source of the 450 nm peak in their model system. The nature of the band at 413 nm is also unclear. The very recent MCD comparison of Stern and Peisach's model system and partially purified *P*-450 by Shimizu *et al.* (24) suffers from the same limitations. Although their protein MCD spectrum correlates well with ours, that of Stern and Peisach's model system is markedly dissimilar.

Cytochrome *P*-420, the inactive form of *P*-450, shows many spectral characteristics which are similar to those of normal heme-containing enzymes. However, there is little evidence as to whether the mercaptide ligand is displaced or simply protonated in the transformation *P*-450 → *P*-420. Some reagents used to prepare *P*-420, such as organo-mercurials, obviously could react with the sulfhydryl ligand, rendering it inactive. Other simple compounds used to denature the enzyme, such as acetone or detergents, could serve to disrupt the protein or membrane, allowing the thiolate to be protonated. Examination of these two possibilities by comparison of the MCD spectra of the native cytochrome and models gives no additional evidence for either possibility since the spectra appear to be indistinguishable (Fig. 4).

It is hoped that preparation of a model dioxygen complex having mercaptide as the axial ligand will give more information about the unusual properties of cytochrome *P*-450.¶

Table 1. Spectral data for Fe(TpivPP) + B + CO

B	νCO* (cm^{-1})	λ_{max}* (nm)
N-Methylimidazole†	1964 (1965)	427†
THF†	1961‡ (1955)	417‡,§
Tetrahydrothiophene†	1970 (1972)	428
$n\text{-}C_3H_7SH$	1970	422
(Na) SCH_3	1945 1902¶	449

* Benzene solution; numbers in parentheses refer to spectra obtained in KBr pellets.
† These complexes have been isolated and fully characterized.
‡ THF as solvent.
§ Ref. 25.
¶ Spectrum with ^{13}CO.

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¶ Just prior to submission of this manuscript we learned of related work (37).

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