Magnetic Circular Dichroism Studies of Hepatic Microsomal Cytochrome P-450†

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ABSTRACT: Magnetic circular dichroism (MCD) spectra have been measured for cytochrome P-450 (P-450) purified from phenobarbital-induced rabbit liver microsomes. The temperature dependence of some of the MCD spectra has also been determined. The MCD spectrum of oxidized P-450 seems to suggest that it is in a state intermediate between the ferric low-spin and ferrous low-spin states. Model experiments suggest that this anomaly arises from the coordination of a thiolate anion to the heme. Reduced P-450 shows a very peculiar MCD spectrum; the spectrum as well as its temperature dependence suggest that the heme in reduced P-450 is a "mixture" in terms of redox and/or spin states. The MCD spectrum of the CO complex of reduced P-450 exhibits an apparent Faraday *A* term around 450 nm which consists of about 50% *C* term and 50% the other terms, indicating that it is not in a purely ferrous low-spin state. The CO complex of reduced cytochrome P-420 (P-420), on the other hand, shows an MCD spectrum characteristic of a ferrous low-spin heme. It is suggested from model experiments that the thiolate anion coordinates to the heme trans to CO in the P-450·CO complex. The Soret region of the MCD spectrum of the EtNC complex of reduced P-450 is characterized by two apparent *A* terms around 430 and 455 nm, whereas that of the corresponding complex of P-420 has only one apparent *A* term around 434 nm.

A family of protoheme-containing proteins, collectively called cytochrome P-450 (P-450),[1] are widely distributed in nature and are functional in a variety of reactions of metabolic importance. Among these proteins, those found in liver microsomes (Omura and Sato, 1962), adrenal cortex mitochondria (Harding et al., 1964), and camphor-grown *Pseudomonas putida* (Katagiri et al., 1968) have been most extensively studied. These studies have shown that the absorption spectra of P-450 are anomalous for protoheme-containing compounds (Sato et al., 1973). For example, the CO complex of reduced P-450 exhibits an intense Soret absorption peak at an unusually long wavelength of about 450 nm. It has also been shown that such anomalies disappear when the cytochrome is modified to a form called cytochrome P-420 (P-420) (Sato et al., 1973). No satisfactory explanations have so far been offered for these atypical properties of this class of hemoproteins, although the hydrophobic nature of the vicinity of the heme (Imai and Sato, 1967a; Ichikawa and Yamano, 1967) and the coordination of a thiolate anion to the heme (Mason, 1965; Murakami and Mason, 1967; Hill et al., 1970; Peisach et al., 1973) have been suggested to be responsible for the unusual properties.

Although P-450 of camphor-grown *P. putida* ($P\text{-}450_{cam}$) has been highly purified and crystallized (Yu and Gunsalus, 1974), purification of P-450's of mammalian origin has been difficult because of their membrane-bound nature. Most physical measurements on mammalian P-450's have, therefore, been performed using membranous or crude solubilized preparations containing hemoproteins other than P-450, and this has hampered studies of the electronic structure of the heme in mammalian P-450's. Recently, however, Imai and Sato (1974a) have described a method for purification of hepatic microsomal P-450 free from other hemoproteins, and more recently it has been brought to homogeneity (Imai and Sato, 1974b; van der Hoeven et al., 1974). Thus, detailed physical studies of this mammalian cytochrome are now feasible.

Like absorption spectra, magnetic circular dichroism (MCD) spectra are very sensitive to changes in the redox and spin states of heme chromophores. MCD can also resolve many components of an absorption band, since the intensity of MCD for an absorption band, i.e., molar ellipticity per gauss, $[\theta]_M$, can be expressed by the equation

$$[\theta]_M = -21.3458[f_1 A + f_2(B + C/kT)] \qquad (1)$$

where f_1 and f_2 are functions describing the shape of the band; k, Boltzmann constant; T, absolute temperature. This equation indicates that the Faraday effect can have three different origins. They are conventionally called the Faraday *A*, *B*, and *C* terms. The *A* term is related to a Zeeman splitting of a degenerate ground or excited state. The *B* term is derived from mixing of the ground or excited state with other excited states by the magnetic field. The *C* term is the only temperature-dependent term and is induced by a temperature-dependent population difference in ground states whose degeneracy has been removed by the magnetic field (Buckingham and Stephens, 1966; Schatz and McCaffery, 1969; Caldwell et al., 1971; Stephens, 1974).

Dolinger et al. (1974) and Dawson et al. (1974) have recently reported the MCD spectra of purified $P\text{-}450_{cam}$ and suspensions of rat liver microsomes. They have, however, not measured the MCD spectra of sufficiently purified microsomal P-450. In this paper, we report the MCD spectra of P-450 purified free from other hemoproteins such as cytochrome P-420 (P-420) and cytochrome b_5 from a phenobarbital-pretreated rabbit. The temperature dependence of some of the MCD spectra is also presented.

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[1] Abbreviations used are: P-450, cytochrome P-450; $P\text{-}450_{cam}$, cytochrome P-450 from camphor-grown *Pseudomonas putida;* MCD, magnetic circular dichroism; P-420, cytochrome P-420; EtNC, ethyl isocyanide; Mb·CO, carbonmonoxy myoglobin; metMb·CN, cyanide metmyoglobin; metMb·N_3, azide metmyoglobin; metMb, metmyoglobin; deoxyMb, deoxymyoglobin.

Experimental Procedure

A male rabbit, weighing 2.5 kg, was injected intraperitoneally with phenobarbital sodium (dissolved in saline) for 5 days at a daily dose of 50 mg/kg of body weight. The animal was killed 24 hr after the last injection. Liver microsomes were prepared and P-450 was purified therefrom as described by Imai and Sato (1974a). The final preparation obtained in 100 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol and 0.2% (w/v) Emulgen 913, a nonionic detergent, was dialyzed at 0° for 35 hr against the same buffer from which the detergent was omitted. This preparation contained 11.2 nmol of P-450/mg of protein and was completely free from P-420 and cytochrome b_5. It was transparent and could be stored for months at −25° without any signs of its conversion to P-420. When the cytochrome in 20% glycerol was cooled below −40°, it formed a milky precipitate. However, in the presence of 50% glycerol, the solution was converted to a good, glassy state at low temperature down to −120°. Low temperature MCD and absorption spectra were, therefore, measured in the presence of 50% glycerol.

Sperm whale myoglobin was purchased from Miles-Seravac Ltd. (England). Ethyl isocyanide (EtNC) was synthesized by the method of Jackson and McKusick (1955). Protohemin chloride was obtained from Daiichi Chemical Co. (Tokyo). The other chemicals used were of guaranteed grade and used without further purification.

A model compound for the P-450·CO complex was synthesized by a modification of the method of Stern and Peisach (1974). A solution containing 228 mg of tetramethylammonium chloride in 10 ml of water was mixed with 30 ml of 1 M NaOH and the mixture was evaporated at 80° to dryness under reduced pressure. The residue was dissolved in a mixture of 1.5 ml of ethanol and 0.5 ml of 2-mercaptoethanol (ethanol solution). A freshly prepared solution of 50 μM protohemin chloride in dimethyl sulfoxide (2.0 ml) was placed in a Thunberg-type cuvet and the ethanol solution obtained above (2.0 ml) was placed in the side arm of the cuvet. After bubbling dried nitrogen gas for 20 sec through both solutions and replacement of the atmosphere with nitrogen, the solutions were mixed. The CO complex was then prepared by gently bubbling CO through the solution for 20 sec and immediately subjected to MCD measurements.

Protein was determined by the method of Lowry et al. (1951). P-450 and P-420 were estimated as described by Omura and Sato (1964). The concentration of myoglobin was determined as described by Hanania et al. (1966).

Absorption spectra were measured at 0° in a Hitachi EPS-3T spectrophotometer, using a cuvet of 5-mm optical path. MCD spectra were recorded in a JASCO J-20A spectropolarimeter equipped with a JASCO electromagnet to produce a longitudinal magnetic field up to 11.4 kG at the sample. The magnet was operated in the normal mode producing the longitudinal magnetic field parallel to the direction of propagation of light. To separate natural circular dichroism (CD) from each MCD spectrum, the spectra were recorded twice; once with magnetic field parallel and then without any magnetic field. The measurements were conducted at 0° unless otherwise indicated. For measurements at 0° was used a cell of 5-mm path and a P-450 concentration of 12.60 μM (in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol); the cell was placed in a quartz Dewar vessel containing ice-water. A cell of 2-mm path containing 40.4 μM P-450 in 100 mM potassium phosphate buffer (pH 7.25) containing 50% glycerol was used for measurements below 0°. The cell equipped with a Cu-(Au-Co alloy) thermocouple was placed in the quartz Dewar vessel and the temperature was regulated by a stream of cold nitrogen from a container of liquid nitrogen. The intensity of MCD was expressed in terms of $[\theta]_M$:

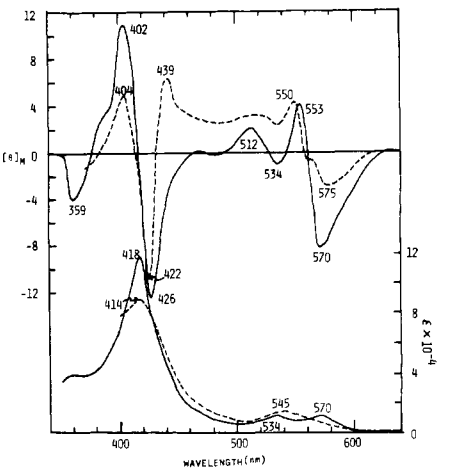

FIGURE 1: MCD (upper) and absorption (lower) spectra of oxidized (——) and reduced (- - -) P-450 at 0°. The concentration of P-450 was 12.6 μM in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol. Reduced P-450 was obtained by adding a few milligrams of solid sodium dithionite immediately before measurements.

$$[\theta]_M = 3300\Delta\epsilon_M \quad (2)$$

where $\Delta\epsilon_M$ is the difference in the molar extinction coefficients for the left circularly polarized light and that for the right per unit magnetic field (gauss). The Faraday parameters were quantitatively determined according to eq 1. Thus, the slope in the $[\theta]_M$ vs. $1/kT$ plot gives the contribution from the Faraday C term and the intercept at $1/kT = 0$ corresponds to the Faraday A and B terms.

Results and Discussion

Oxidized P-450. The MCD spectrum of the oxidized form of hepatic microsomal P-450 is shown in Figure 1 (solid line). The visible region of this spectrum is characterized by two sinusoidal curves seemingly due to Faraday A terms; the larger one consists of a trough at 570 nm and a peak at 553 nm with a crossover at 560 nm and the smaller one has a trough at 534 nm and a peak at 512 nm with a crossover at 525 nm. In the Soret region a larger apparent A term is seen which is composed of a trough at 426 nm and a peak at 402 nm with a crossover at 416 nm; this sinusoidal curve is accompanied by a shoulder around 390 nm. This MCD spectrum is different from those of oxidized P-450$_{cam}$ and an aerobic suspension of rat liver microsomes in that the visible region of the latter spectra is not clearly split into two sinusoidal curves (Dolinger et al., 1974; Dawson et al., 1974).

The MCD spectrum of oxidized P-450 in the visible region is similar to that of carbonmonoxymyoglobin (Mb·CO) which shows two Faraday A terms related to the Q_{0-0} and Q_{0-1} transitions (Vickery et al., 1975). This suggests that the two apparent A terms with crossovers at 560 and 525 nm in the MCD spectrum of oxidized P-450 are also due to Q_{0-0} and Q_{0-1} transitions, respectively. It is, therefore, likely that a ferrous low-spin species is present in the

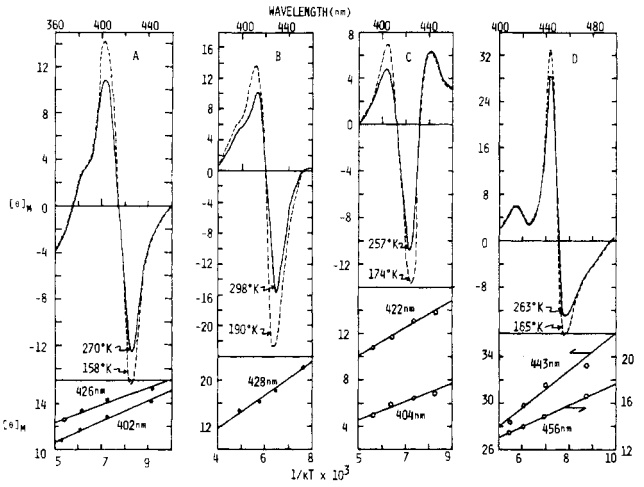

FIGURE 2: Effects of temperature on the Soret MCD spectra of oxidized P-450 (A), metMb·N_3 (B), reduced P-450 (C), and CO complex of reduced P-450 (D). The concentration of P-450 was 20.2 μM in 100 mM potassium phosphate buffer (pH 7.25) containing 50% glycerol. Reduced P-450 was obtained by adding sodium dithionite, and the CO complex by gently bubbling CO through the reduced P-450 solution for 20 sec. metMb·N_3 was prepared by mixing NaN_3 (10 mM) and metMb (8.8 μM) in 65 mM potassium phosphate buffer (pH 6.8). The spectra were measured at indicated temperatures. In the diagrams shown below, the MCD intensities of indicated troughs and peaks are plotted against $1/kT$.

oxidized cytochrome. The absence of an absorption band around 620 nm further suggests that the oxidized cytochrome is devoid of a ferric high-spin species.

Since MCD spectra in the Soret region for ferric low-spin and ferrous low-spin hemes are fairly similar to each other (Vickery et al., 1975), it is difficult to distinguish the two types of low-spin heme based on the Soret MCD spectrum at one temperature. Nevertheless, it may be said that the Soret MCD of oxidized P-450 resembles those of ferric low-spin complexes such as cyanide metmyoglobin (metMb·CN) (Vickery et al., 1975). This is consistent with electron spin resonance (ESR) and Mössbauer studies indicating the ferric low-spin nature of oxidized P-450 (Bayer et al., 1969; Hill et al., 1970; Blumberg and Peisach, 1971; Peisach et al., 1973; Sharrock et al., 1973; Collman et al., 1975). It should be noted that the magnitude of Soret MCD of oxidized P-450 is comparable to that of azide metmyoglobin (metMb·N_3) which is about 80% low spin (Beetlestone and George, 1964; Smith and Williams, 1970).

The intensity ratio of Soret MCD to visible MCD divided by respective absorption intensities is of value in assessing the redox and spin states of hemoproteins (Schatz and McCaffery, 1969). The ratio determined for oxidized P-450 is about 0.22, whereas the values obtained for metMb·CN and Mb·CO are about 1 and 0.14, respectively. The ratio for oxidized P-450 is, therefore, similar to that of Mb·CO which is a typical ferrous low-spin complex rather than that of ferric low-spin species such as metMb·CN.

Although the Soret MCD spectra for low-spin ferrous and ferric hemes are similar to each other as stated above, they are quite different in the Faraday parameter involved. Thus, ferrous low-spin species give an A term, whereas ferric species give a C term (Briat et al., 1972; Vickery et al., 1975). To determine the Faraday parameter involved, we examined the temperature dependence of the Soret MCD and absorption spectra of oxidized P-450. As shown in Figure 2A, both the 402-nm peak and 426-nm trough in the MCD spectrum increased linearly as a function of $1/kT$. The absorption spectrum, on the other hand, remained unchanged even at low temperatures.

Although the observed temperature dependence of MCD should theoretically include a contribution of the change in spin state, this contribution may be neglected because the spin state of oxidized P-450 appears to be as low as that of metMb·N_3 whose spin state is not significantly dependent on the temperature (Iizuka et al., 1968; Iizuka and Kotani, 1969a,b). In fact, the intensity of Soret MCD of metMb·N_3, like that of oxidized P-450, is linearly dependent on $1/kT$ (Figure 2B) but can be estimated as almost C term.

Neglecting the contribution of the change in spin state, it should be pointed out that the two straight lines in Figure 2A, when extrapolated to infinitive temperature, do not cross the vicinity of $[\theta]_M = 0$. This would indicate that the MCD of oxidized P-450 in the Soret region must be composed of several Faraday components. It could be estimated that the 426-nm trough is composed of 30% C term and 70% A or B term and the 402-nm peak of 40% C term and 60% A and/or B terms at 0°. Based on these results, it may be concluded that oxidized P-450 contains both a ferric low-spin species giving rise to the temperature-dependent C term and a ferrous low-spin species which is independent of the temperature. Besides, the contribution of some charge transfer transitions associated with thiolate anion to this peculiar MCD may not be ruled out.

Another characteristic of the MCD of oxidized P-450 is the presence of a shoulder around 390 nm on the positive side. Although the origin of this shoulder is not known, it is of interest to note that it disappears when the cytochrome is converted to P-420 in 1 M KSCN (unpublished observation).

Taking all the observations described above into consideration, it can be concluded that the state of the heme chromophore in oxidized P-450 is unusual in that it represents a kind of mixture of both ferrous and ferric low-spin species. Of course, this does not mean that the cytochrome is a simple mixture of two different forms. It appears that the heme in oxidized P-450 is in a state which is intermediate between the ferric and ferrous low-spin states. In any way, this anomaly seems to be explicable by assuming the coordination of the thiolate anion of a cysteinyl residue to the heme iron in oxidized P-450 as has been suggested by previous investigators (Mason, 1965; Murakami and Mason, 1967; Hill et al., 1970; Blumberg and Peisach, 1971; Peisach et al., 1973). Since thiolate anions can reduce ferric ions in aqueous solution (Bayer et al., 1969; Yang and Huennekens, 1970; Berzofsky et al., 1971; Stern and Peisach, 1974), it may be visualized that in oxidized P-450 the thiolate anion as an axial ligand is contributing to the apparent ferrous low-spin nature of the heme.

This hypothesis seems to be supported by model experiments in which the MCD spectra of metMb complexed with cysteine methyl ester (at pH 9.18) and with 2-mercaptoethanol (at pH 6.86) were measured. As shown in Figure 3, the intensity ratio of the Soret to visible MCD for the cysteinyl complex is 2:3 and the visible MCD has two apparent A terms due to the Q_{0-0} and Q_{0-1} transitions. The general shape of the MCD spectrum for this complex is, therefore, similar to that for oxidized P-450, though the intensity of the MCD for the metMb complex is only about 50% of that for oxidized P-450. In the MCD spectrum for the mercaptoethanol complex, too, two fairly strong A terms associated with the Q_{0-0} and Q_{0-1} transitions can be observed. The shape of visible MCD is strikingly similar to that of oxi-

dized P-450, though the shape of Soret MCD for the mercaptoethanol complex is somewhat peculiar for low-spin hemichromes.

Finally, the low-spin character of the heme in oxidized P-450 suggests that the two axial ligands for the heme iron should have strong ligand fields. It is likely that the axial ligand trans to the thiolate anion may be either nitrogen of histidine or lysine, or sulfur of methionine (Koch et al., 1975).

Reduced P-450. Figure 1 (dotted line) shows the MCD spectrum of dithionite-reduced P-450. It exhibits a wide trough at 575 nm and a sharp one at 422 nm; the latter is much more intense than the former. Three peaks are also seen at 550, 439, and 404 nm, together with a plateau on the positive side ranging from about 530 to 450 nm. The width of the 575-nm trough and the asymmetry of the band shape in the visible region suggest that the spectrum possesses another trough around 590 nm. This spectrum, particularly in the Soret region, is quite peculiar and differs from those so far measured for other hemoproteins. Although it is not yet possible to explain the origins of this anomalous spectrum, it seems that the heme in reduced P-450 is in a state which can be described as a "mixture" of various redox and spin states, as has been suggested by Hill et al. (1970) from its Soret absorption spectrum. From a comparison of this MCD spectrum with those of ferrous high-spin hemes such as deoxyMb (a peak at 430 nm and a trough at 420 nm) and ferric low-spin hemes such as metMb·CN (a peak at 415 nm and a trough at 428 nm) reported by Vickery et al. (1975), it might be assumed that reduced P-450 is a "mixture" of ferrous high-spin and ferric low-spin species. The contribution of a ferrous low-spin species, if any, seems to be very small, since the presence of this species should increase the intensity of the visible MCD spectrum considerably.

As shown in Figure 2C, the 439-nm peak in the Soret MCD spectrum of reduced P-450 is not detectably temperature dependent. The 422-nm trough and the 404-nm peak, on the other hand, are both clearly dependent on the temperature. From this dependence, it can be estimated that the 422-nm trough is composed of 60% *C* term and 40% the other Faraday terms, and the 404-nm peak 80% *C* term and 20% the other terms at 0°. The Soret absorption spectrum is also temperature dependent, but only to a very slight degree. These data suggest that the peak and trough of the Soret transition have different origins and thus reinforce the view that the heme in reduced P-450 is a "mixture" in terms of redox and/or spin states.

However, other explanations are also possible to account for the temperature dependence of the Soret MCD spectrum. It is possible to assume the contribution to the Faraday *C* term of a charge-transfer transition in a ferrous high-spin complex possessing a degenerated ground state (Zerner et al., 1966). Another possibility is to assume a temperature-dependent change in the spin state (Iizuka et al., 1968; Iizuka and Kotani, 1969a,b). However, this possibility may be excluded, because the shape and the positions of the trough and peak in the visible MCD spectrum are clearly different from those of a ferrous low-spin complex. A third explanation is to ascribe the peculiar MCD of reduced P-450 to a serious distortion of the periphery of the heme, but it is at present difficult to discuss this possibility because of the lack of pertinent information.

CO Complex of Reduced P-450. While the CO complex of many protoheme compounds shows a Soret absorption

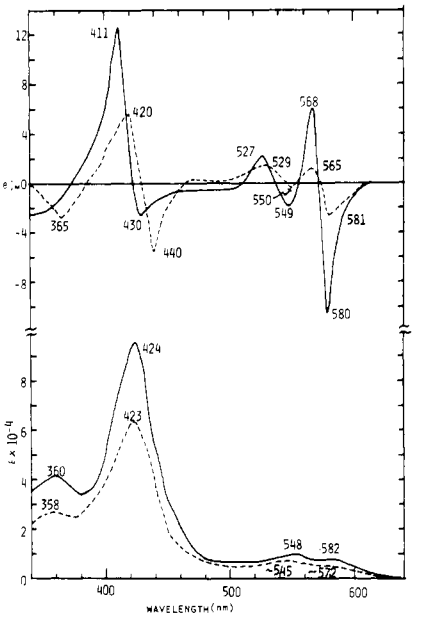

FIGURE 3: MCD (upper) and absorption (lower) spectra of metMb complexes with 2-mercaptoethanol (10 m*M*) (——) and with cysteine methyl ester (1 m*M*) (- - -). The concentration of metMb was 16.8 μM in 65 m*M* potassium phosphate buffer (pH 6.86) for the mercaptoethanol complex and in 65 m*M* potassium phosphate buffer (pH 9.18) for the cysteine ester complex. Before the formation of the complex, the solution of metMb and that of the ligand were bubbled with dried nitrogen gas for 20 sec, and both solutions were then mixed anaerobically just prior to spectral measurements.

peak around 420 nm, the CO complex of reduced P-450 is unusual in that it exhibits a Soret peak in the region of 450 nm. Although the hydrophobic nature of the vicinity of the heme (Imai and Sato, 1967a; Ichikawa and Yamano, 1967) or the coordination of a sulfur anion to the heme (Mason, 1965; Murakami and Mason, 1967; Hill et al., 1970; Peisach et al., 1973) has been suggested to be responsible for this anomaly, no satisfactory explanations are as yet available. To gain further information about the electronic structure of the heme in the CO complex of reduced P-450, we have measured the MCD spectrum of this complex.

As shown in Figure 4 (solid line), the Soret region of the MCD spectrum obtained is characterized by a large peak at 443 nm, a small peak at 416 nm, and a distinct trough at 456 nm. The visible region of the spectrum has a small trough at 573 nm and a small plateau ranging from about 550 to 500 nm. The 443-nm peak and 456-nm trough with a crossover point at 450 nm, clearly associated with the 450-nm absorption peak, seem to compose a Faraday *A* term, though the 443-nm peak is about twice more intense than the 456-nm trough. The visible MCD spectrum, unlike those of typically ferrous low-spin hemoproteins, is not clearly split into two components arising from the Q_{0-0} and Q_{0-1} transitions, and the magnitudes of the spectrum on both the plus and minus sides are smaller than those for ferrous low-spin complexes. This MCD spectrum is as a whole similar to that of the CO complex of reduced camphor-free P-450$_{cam}$ (Dolinger et al., 1974), except that the crevice around 425 nm on the plus side is deeper in the case of P-450$_{cam}$.

When the CO complex of reduced P-450 in the glycerol-containing buffer is allowed to stand at room temperature, it is gradually converted to the CO complex of reduced P-420 as evidenced by the shift of the Soret absorption peak from 450 to 420 nm; this conversion is almost complete

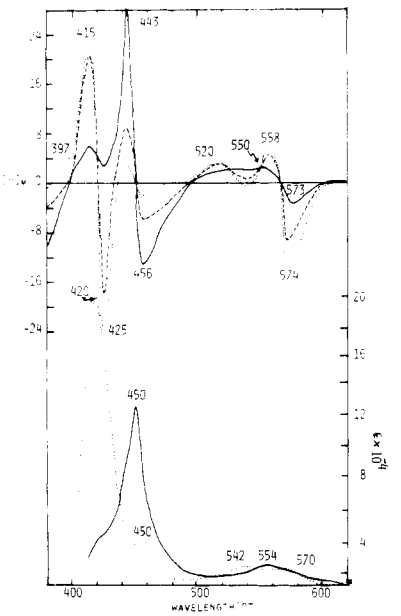

FIGURE 4: MCD (upper) and absorption (lower) spectra of the CO complex of reduced P-450 at 0°. The concentration of P-450 was 12.6 μM in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol. The CO complex was prepared as described in Figure 2. MCD spectra were recorded at 0° immediately after the complex formation at 0° (——), after incubation for 5 hr at room temperature (– –), and after incubation for 15 hr (· · ·).

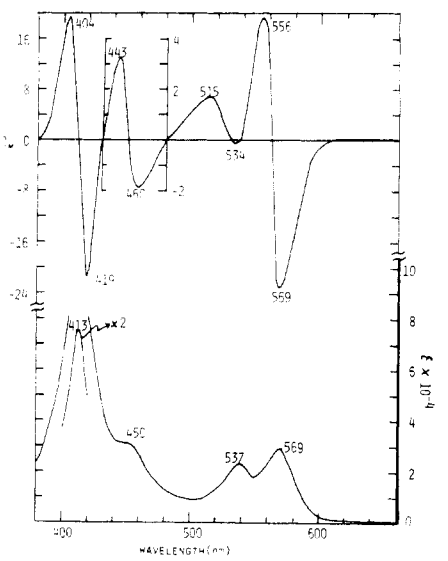

FIGURE 5: MCD (upper) and absorption (lower) spectra of the CO complex of reduced protoheme in the presence of 2-mercaptoethanol and tetramethylammonium hydroxide. The protoheme concentration was 16.9 μM in dimethyl sulfoxide–ethanol solution. The model CO complex was prepared as described in Experimental Procedure. A Thunberg-type cuvet with 1-cm optical path was used.

after 15-hr incubation. Concomitant with this conversion, the MCD spectrum undergoes a time-dependent change as shown in Figure 4. Thus, the 573-nm trough is intensified about fourfold after 15-hr incubation, concomitant with the appearance and intensification of clear peaks at 558 and 520 nm in the visible region. In the Soret region, the apparent A term around 450 nm is seriously attenuated and at the same time another apparent A term appears around 420 nm. The resultant MCD is now similar to that of Mb·CO which has a Soret absorption peak at 420 nm and contains a typically ferrous low-spin heme (Vickery et al., 1975). It is suggested that the CO complex of reduced P-450 does not contain a typically ferrous low-spin heme, but is converted to this state only when it is modified to the P-420 form.

As has been shown for zinc porphyrins (Gale et al., 1972), ferrous low-spin hemes should give A terms for both the Q and Soret transitions and the MCD spectra should not be temperature dependent. However, as shown in Figure 2D, the Soret MCD spectrum of the CO complex of reduced P-450 is clearly temperature dependent, indicating the involvement of a Faraday C term. The Soret absorption spectrum, on the other hand, is not temperature dependent. It can be estimated from the results shown in Figure 2D that the MCD trough at 456 nm is composed of 40% C term and 60% the other terms and the peak at 443 nm 50% C term and 50% the other terms at 0°. These findings also support the view that the CO complex is not in a purely ferrous low-spin state, but contains a high-spin component (Nicholls, 1973) or involves corresponding charge transfer transitions having C terms.

As to the origin of this anomaly in the electronic structure of the heme in the CO complex, Stern and Peisach (1974) have recently reported an elegant model experiment in which they demonstrated that in dimethyl sulfoxide–ethanol reduced protoheme can exhibit a 450-nm absorption peak provided that CO, mercaptoethanol, and a strong base such as tetramethylammonium hydroxide are present in the system. This observation has led them to conclude that the axial ligand in this model system as well as in the CO complex of reduced P-450 is a thiolate anion. We have also found that the absorption peak (shoulder) at 450 nm in their model system gives an MCD which has an apparent A term around 450 nm and is fairly similar to that of the CO complex of reduced P-450.

As shown in Figure 5, this apparent A term has a trough at 460 nm and a peak at 443 nm and the intensity of the peak is about twice as high as that of the trough. It is evident that this MCD pattern is almost the same as that seen in the MCD spectra of the CO complex of hepatic P-450 (cf. Figure 4; Dolinger et al., 1974; Dawson et al., 1974) and that of P-450$_{cam}$ (Dolinger et al., 1974).

When Mb, instead of hemin chloride, was used to construct the model system of Stern and Peisach (1974), we could observe neither the 450-nm absorption peak nor the MCD A term around 450 nm; the MCD spectrum of this Mb-containing model system was characteristic of a typically low-spin hemochrome (data are not shown). This would indicate that the 450-nm peak cannot result even in the presence of both thiolate anions and CO, unless the imidazole nitrogen is released from the heme iron of Mb. These findings lend a further support to the view that a thiolate anion ($-S^-$) is the ligand trans to CO in the CO complex of P-450, and thus the unusual Soret band of this complex may be related to some charge transfer transitions attributable to $-S^-$ and/or CO. In addition, the contribution of bending or distortion of the porphyrin ring to the unusual Soret transition may not be excluded.

EtNC Complexes of Reduced P-450. It has been reported that reduced P-450, both in the microsomal bound form (Imai and Sato, 1966, 1967b) and in the purified form (Imai and Sato, 1974a), exhibits two Soret absorption peaks at 430 and 455 nm upon addition of EtNC. The relative intensities of these two peaks undergo reversible change depending on pH (Imai and Sato, 1966, 1967b). It has been postulated from these facts that the EtNC complex of re-

duced P-450 exists in two forms, i.e., the 430 and 455 forms, which are in a pH-dependent equilibrium (Imai and Sato, 1966, 1967b). Figure 6 (broken line) shows the MCD spectrum of the EtNC complex of reduced P-450. It possesses two apparent *A* terms around 430 and 455 nm, corresponding to the two Soret absorption peaks, in the Soret region, and two other *A* terms around 533 and 560 nm in the visible region. It appears from this spectrum that both the 430 and 455 forms of the complex are mainly in the ferrous low-spin state. The small peak around 400 nm, which is also observable in the MCD spectrum of Mb·CO (Vickery et al., 1975), may be ascribed to a vibrational band of the Soret transition. The similarity of the apparent *A* term around 455 nm of the EtNC complex to that around 450 nm in the corresponding CO complex (Figure 4) suggests that the heme in the 455 form is in a state resembling that in the reduced P-450·CO complex. It might be argued that in the 455 form the heme is coordinated with the thiolate anion and EtNC. On the other hand, the 430 form of the EtNC complex may represent a state in which the thiolate anion is replaced by EtNC which coordinates to the heme trans to a strong ligand such as the imidazole nitrogen. Since the 430 form is in a pH-dependent equilibrium with the 455 form, it is likely that the thiolate anion of the cysteine residue still remains in the vicinity of the heme in the 430 form.

P-450 is known to be convertible by a variety of treatments to a modified form called P-420, the CO complex of which shows a Soret absorption peak at 420 nm like most protoheme-containing compounds (Omura and Sato, 1964; Imai and Sato, 1967a). Figure 6 (solid line) shows the MCD spectrum of the EtNC complex of reduced P-420 prepared from P-450 by treatment with 1 *M* KSCN (Imai and Sato, 1967a). It exhibits Faraday *A* terms around 434, 530, and 560 nm which may be assigned to the Soret, Q_{0-1} and Q_{0-0} transitions, respectively, and is typical of ferrous low-spin hemes such as Mb·CO (Vickery et al., 1975). Since the positions of the Soret and visible MCD of the P-420 complex are clearly different from those of the 430 form of the P-450 complex, it is evident that the states of the heme in these two forms differ from each other. An explanation for this difference might be to assume that the structural alteration of the protein accompanying the conversion of P-450 to P-420 has displaced the thiolate group to a location far distant from the heme, so that the thiolate anion can no more exert any influence on the heme.

Conclusion

In this paper, we have measured the MCD spectra of P-450 purified from phenobarbital-induced rabbit liver microsomes and determined the temperature dependence of some of the spectra obtained. The results, which are as a whole consistent with those reported by Dolinger et al. (1974) and Dawson et al. (1974) on purified $P\text{-}450_{cam}$ and rat liver microsomes, further emphasized that P-450 is a very unusual hemoprotein.

Although we are aware of the fact that no theoretical framework for interpretation of the magneto-optical effects observed for heme compounds is as yet available, we have attempted in this paper to obtain some preliminary information concerning the structure of the heme environment in P-450 by comparing the spectra obtained with those of myoglobin and its derivatives (Vickery et al., 1975). This attempt, together with the results of model experiments, have led us to a tentative idea that many of the anomalies of P-450 and its derivatives are explicable by assuming that one of the axial ligands to the heme in oxidized P-450 is the thiolate anion of a cysteinyl residue. It should, however, be noted that this conclusion is by no means proven, but may serve as a useful working hypothesis in further pursuits of the structure of P-450. To reach a definite conclusion, it is highly desirable to study this unusual hemoprotein by several other physical methodologies including direct magnetic measurements.

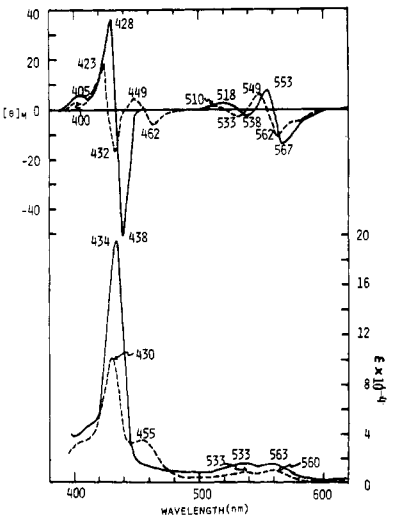

FIGURE 6: MCD (upper) and absorption (lower) spectra of the EtNC complexes of reduced P-450 (- - -) and reduced P-420 (——) at 0°. The concentrations of P-450 and P-420 were 10.1 and 20.2 μM, respectively, in 100 m*M* potassium phosphate buffer (pH 7.5) containing 20% glycerol. P-420 was produced by incubating P-450 with 1 *M* KSCN at room temperature for 1 hr. EtNC was added to the dithionite-reduced sample to a concentration of 1 m*M*.

Addendum

After submission of this paper, we became aware of a report (Vickery et al. (1975)) describing the MCD spectra of purified $P\text{-}450_{cam}$ and liver microsomes from untreated and dimethylbenzanthracene-treated rats. Their results are also consistent with those reported in this paper.

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