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Magnetic Circular Dichroism Studies XXXV. A Comparison of Cytochromes *P*-450 and *P*-448*

(hemoproteins/hepatic microsomes/drug metabolism)

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ABSTRACT **Cytochromes *P*-450 and *P*-448 in microsomal suspensions have been shown to be spectrally distinct by magnetic circular dichroism spectroscopy. Furthermore, this technique can be used to measure induction of these two cytochromes by phenobarbital and 3-methylcholanthrene. Magnetic circular dichroism spectroscopy is thus at least as useful as difference spectroscopy for the investigation of *P*-450 and *P*-448 and more informative because the presence of cytochrome b_5 and hemoglobin can be detected concurrently. We have also shown that the molar magnetic ellipticity for reduced + CO treated cytochrome *P*-450 of *Pseudomonas* grown on camphor is of similar value to that of reduced + CO treated microsomal *P*-450 and *P*-448.**

Cytochrome *P*-450 from rat liver microsomes has been implicated in the biological oxidation of a wide variety of endogenous and foreign natural products and synthetic molecules. Absorption spectroscopy has revealed two types of *P*-450: one, induced by phenobarbital (PB), shows a peak at 450 nm for the reduced + CO minus reduced difference spectrum; another one, induced by polycyclic hydrocarbons such as 3-methylcholanthrene (3MC), shows a maximum at 446 or 448 nm and is known as *P*-448¶.

We have previously shown the applicability of magnetic circular dichroism (MCD) spectroscopy to the investigation of cytochrome *P*-450, thereby permitting more direct spectral studies (1), and now wish to report a comparison of cytochromes *P*-450 and *P*-448 using MCD. Cytochrome b_5, the other major cytochrome component of rat liver microsomes, can also be identified by MCD (1). This has enabled us to measure changes in the *P*-450 or *P*-448 to b_5 ratio resulting from the administration of PB or 3MC. The present study was done on an instrument of higher sensitivity than the one employed in the previous study (1), thereby allowing for the use of an electromagnet (12.0–15.0 kG) rather than a superconducting magnet (49.5 kG) requiring liquid helium. The use of this instrument has been described elsewhere*.

Abbreviations: MCD, magnetic circular dichroism; CD, circular dichroism; *P*-450, *P*-448, and *P*-420, microsomal cytochromes *P*-450, *P*-448, and *P*-420; b_5, cytochrome b_5; *P*-450$_{cam}$, cytochrome *P*-450 from camphor-grown *Pseudomonas putida*; PB, phenobarbital; 3MC, 3-methylcholanthrene.

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¶ For general reviews see refs. 3–8 of ref. 1.

EXPERIMENTAL

Twenty male, 30-day-old, Sprague–Dawley rats (Simonsen, Inc., Gilroy, Calif.), born on the same day and ranging in weight from 103–120 g were used. Cytochromes *P*-450 and *P*-448 were induced by three daily intraperitoneal injections with PB (80 mg/kg per day) in propylene glycol (5 rats) and 3MC (20 mg/kg per day) in corn oil (5 rats). Ten rats were used as controls and received equivalent injections of propylene glycol (5 rats) and corn oil (5 rats). Injection amounts were based on the average weight of 110 g. Food was removed on the fourth day and the rat was sacrificed by a blow to the head on the fifth day. The injection schedule was designed so that two rats were sacrificed per day, control rats 1 day, induced rats the next, etc. The weights of the rats at sacrifice were less than 200 g.

After sacrifice, liver microsomes were obtained as follows: the livers were exposed, perfused with isotonic saline, removed, rinsed, weighed, and minced. Their weights ranged from 4.2 to 13.5 g (all but three were less than 10 g). The livers were then homogenized with 3 volumes of pH 7.4 buffer (0.01 M potassium phosphate, 1.15% KCl) with a Potter-Elvehjem homogenizer and the homogenates were centrifuged at 1000 × *g* for 20 min. The resulting supernatant was centrifuged twice at 9000 × *g* for 30 min and then at 97,000 × *g* for 60 min. The pellet was resuspended in 1.15% KCl (unbuffered) by a 10-sec application of a sonic probe (Heat Systems-Ultrasonics, Inc.) and centrifuged at 97,000 × *g* for 30 min. Tissues were maintained at 0–4° throughout. The final pellet was covered with argon, sealed, and stored overnight at −12°. The entire isolation procedure took approximately 6 hr.

A Japan Spectroscopic Co. spectropolarimeter (JASCO model J-40) was used for all MCD and CD measurements and a Cary 14M spectrophotometer for all absorption spectra. MCD measurements using a 12.0 kG magnet were performed with the field direction parallel and then antiparallel to the direction of light propagation. The MCD spectral data were corrected for natural CD by running a spectrum with no field (MCD_{obs} = CD + MCD for parallel magnetic fields, MCD_{obs} = CD − MCD for antiparallel fields) and it is these corrected data which are reported in the tables. The MCD parameters were: scale, 1 or 2 mdeg/cm; pathlength, 1 cm; scan speed, 2 cm/min; wavelength expansion, 5 nm/cm; slit, 2 nm; time constant, 4 sec. The instrument was calibrated daily for both intensity, using camphorsulfonic acid*, and wavelength, using a holmium perchlorate solution (2).

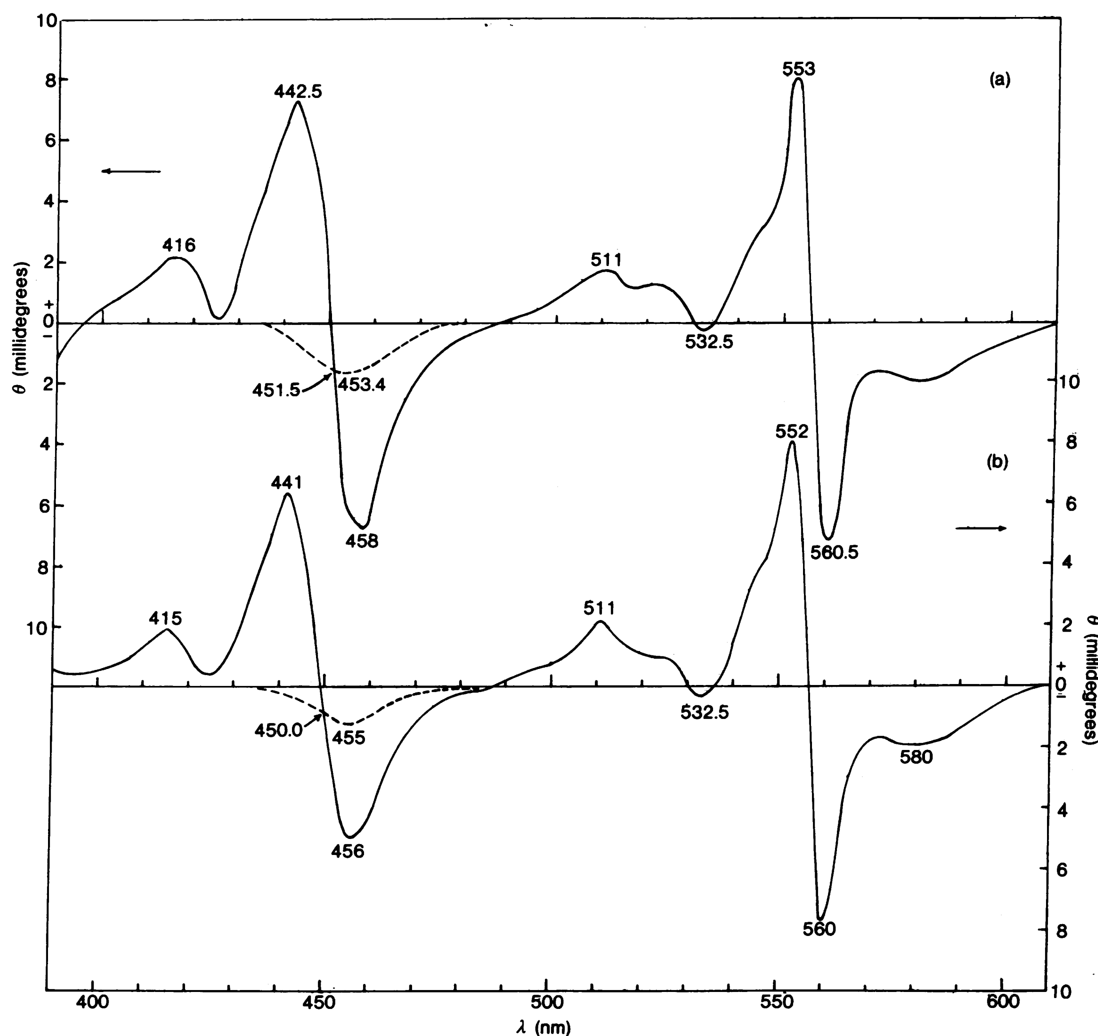

FIG. 1. (a) MCD (——) and CD(-----) spectra of a reduced + CO microsomal suspension from a PB induced rat (no. 11); (b) MCD (——) and CD (-----) spectra of a reduced + CO microsomal suspension from a 3MC induced rat (no. 12). The MCD spectra shown here were obtained with the magnetic field parallel to the direction of light propagation and have not been corrected for CD.

The suspensions were prepared by dilution with deaerated buffer to an OD of approximately 1.5 at 400 nm and placed in a stoppered cuvette, thermostated to 7°. For each microsomal sample, first the untreated (oxidized) spectra (two MCD and one CD) were run and then the reduced + CO spectra (two MCD and one CD). A difference absorption

TABLE 1. *Ellipticities and concentrations of cytochromes P-450 and P-448*

Group	Ellipticity, millidegrees[a]		Concentration, μM	
	θ_{457}	θ_{443}	MCD[b]	Difference spectrum[c]
P-450 Induction by PB (5 rats)	−6.7 ± 2.1 at 457.8 ± 0.6 nm	9.5 ± 3.0 at 443.2 ± 0.8 nm	4.2 ± 1.3	3.0 ± 1.0
PB Control (5 rats)	−2.3 ± 0.4 at 458.0 ± 0.8 nm	3.6 ± 0.8 at 442.8 ± 0.8 nm	1.6 ± 0.3	1.2 ± 0.2
P-448 Induction by 3MC (5 rats)	−3.5 ± 1.2 at 456.8 ± 1.0 nm	5.3 ± 2.3 at 442.1 ± 1.0 nm	2.3 ± 0.9	2.1 ± 0.8
3MC Control (5 rats)	−2.7 ± 0.3 at 457.5 ± 0.5 nm	3.4 ± 0.3 at 443.0 ± 0.9 nm	1.6 ± 0.1	1.4 ± 0.1

[a] The average and standard deviation of the wavelengths and ellipticities of 10 scans, five with the magnetic field parallel to the direction of light propagation (MCD_{obs} = CD + MCD) and five antiparallel (MCD_{obs} = CD − MCD).

[b] Using a molar magnetic ellipticity of 32.0 deg·cm^2·dmol^{-1}·G^{-1} for $[\theta]_{457} - [\theta]_{443}$ determined for solubilized, camphor-free, reduced + CO *P*-450$_{cam}$ from *Pseudomonas putida* (1).

[c] From the method of Omura and Sato (5).

TABLE 2. *Ellipticities and concentrations of cytochrome b_5*

Group	Ellipticity, millidegrees[a]		Concentration, from MCD[b]
	θ_{560}	θ_{552}	
PB Induction (5 rats)	−8.5 ± 1.0 at 560.4 ± 0.3 nm	9.6 ± 1.5 at 552.6 ± 0.3 nm	1.8 ± 0.2 μM
PB Control (5 rats)	−5.5 ± 0.7 at 560.8 ± 0.5 nm	5.8 ± 0.8 at 552.7 ± 0.3 nm	1.1 ± 0.1 μM
3MC Induction (5 rats)	−7.1 ± 1.9 at 560.6 ± 0.3 nm	7.4 ± 2.2 at 552.7 ± 0.5 nm	1.4 ± 0.4 μM
3MC Control (5 rats)	−6.2 ± 0.5 at 560.5 ± 0.6 nm	6.3 ± 0.4 at 552.4 ± 0.3 nm	1.2 ± 0.1 μM

[a] The average and standard deviation of the wavelengths and ellipticities of 10 scans, five with the magnetic field parallel to the direction of light propagation (MCD_{obs} = CD + MCD) and five antiparallel (MCD_{obs} = CD − MCD).

[b] Using a molar magnetic ellipticity of 84.4 $deg \cdot cm^2 \cdot dmol^{-1} \cdot G^{-1}$ for $[\theta]_{560} - [\theta]_{552}$ (1).

spectrum (reduced + CO minus reduced) was also obtained. The suspensions were reduced with iron-free $Na_2S_2O_4$ (Fisher Chemical Co.) and saturated with carbon monoxide (Liquid Carbonic, Inc.).

The protein concentrations of all the microsomal suspensions were determined by the Hartree modification of the Lowry method (3), with bovine serum albumin as a standard. All MCD data presented have been normalized to a 1 mg/ml protein concentration for easy comparison.

RESULTS AND DISCUSSION

The MCD and CD spectra of reduced + CO microsomal suspensions from rats induced with PB and with 3MC are shown in Fig. 1a and b, respectively. The important features of these spectra are the MCD A terms (4) centered at about 450–452 nm from cytochrome *P*-450 (or *P*-448) and those centered at about 556 nm from cytochrome b_5 (1). Examination of Fig. 1 shows that there is a great similarity in the shapes of the MCD spectra of both microsomal preparations. The spectra shown are representative of those found for all the samples examined.

The data in Tables 1 and 3 include a comparison of *P*-450 and *P*-448 concentrations as determined from the molar magnetic ellipticity for solubilized, camphor-free, reduced + CO P-450_{cam} from camphor-grown *Pseudomonas putida* (1) and as determined by the difference spectral method of Omura and Sato (5). The individual ratios of these concentrations (the means and standard deviations are listed in Table 3) range from 1.0 to 2.0, with all but two being 1.3 or

TABLE 3. *Concentration comparisons*

Group	Ratio of P-450 or P-448 concentrations, MCD/difference spectroscopy[a]	Ratio of P-450 or P-448 to b_5 concentrations by MCD[b]
P-450 Induction by PB (5 rats)	1.4 ± 0.4	2.3 ± 0.5
PB Control (5 rats)	1.2 ± 0.1	1.4 ± 0.2
P-448 Induction by 3MC (5 rats)	1.2 ± 0.1	1.6 ± 0.2
3MC Control (5 rats)	1.2 ± 0.1	1.3 ± 0.2
Overall statistics	1.3 ± 0.2	

[a] The individual *P*-450 or *P*-448 concentrations determined by MCD (the means and standard deviations are listed in Table 1, column 3) have been divided by their respective concentrations determined by difference spectroscopy (Table 1, column 4). The average and standard deviation of these ratios are reported for each group.

[b] The individual *P*-450 or *P*-448 concentrations (the means and standard deviations are listed in Table 1, column 3) have been divided by their respective b_5 concentrations (Table 2, column 3). The average and standard deviation of these ratios are reported for each group. All concentrations were determined by MCD.

TABLE 4. *Comparison of P-450 characterization by MCD and difference spectroscopy*

Group	Rat no.	MCD Crossover point[a], nm	Difference spectral peak, nm
P-450 Induction by PB	3	451.0	452.0
	7	451.0	450.0
	11	451.5	450.0
	15	451.5	450.0
	19	451.5	450.0
	(mean)	451.3 ± 0.3	450.5 ± 0.9
PB Control	2	452.0	450.0
	6	452.0	450.0
	10	450.5	450.0
	14	452.0	450.0
	18	452.0	450.0
	(mean)	451.7 ± 0.7	450.0 ± 0.0
P-448 Induction by 3MC	4	449.5	448.0
	8	449.5	448.0
	12	450.0	448.5
	16	450.5	450.0
	20	450.8	449.0
	(mean)	450.1 ± 0.6	448.7 ± 0.9
3MC Control	1	451.5	451.0
	5	451.0	450.0
	9	451.0	450.0
	13	451.5	450.0
	17	451.5	450.0
	(mean)	451.3 ± 0.3	450.2 ± 0.5

[a] The crossover point is the point where the MCD_{obs} and CD curves intersect (see 451.5 nm in Fig. 1a and 450.0 nm in Fig 1b).

less. This is consistent with the results of Vickery *et al.* (6) and indicates that the molar magnetic ellipticity of bacterial reduced + CO P-450_{cam} is similar to that of microsomal reduced + CO *P*-450 (and *P*-448). Vickery *et al.*, however, find a value of less than 1 for this ratio, whereas we find a value of greater than 1 (bottom of Table 3, 1.3 ± 0.2). As noted by Vickery *et al.*, determination of the concentration of cytochrome *P*-420, the denatured form of *P*-450, by MCD is complicated by the overlap of its A term at about 420 nm with the Soret MCD band of *P*-450 and b_5. In addition, the molar magnetic ellipticity for pure *P*-420 has not yet been determined.

Cytochrome b_5 concentrations determined by MCD can be found in Table 2. Tables 1 and 3 show the use of MCD to demonstrate successful induction of *P*-450 and *P*-448 by PB and 3MC. This can be seen by looking at the absolute amounts of *P*-450 or *P*-448 as determined by (*1*) MCD or (*2*) difference spectroscopy (Table 1, columns 3 and 4), or (*3*) by looking at the ratio of *P*-450 or *P*-448 to b_5 (Table 3, column 2). A Student *t* test analysis of the induction as measured by any of these three methods shows there to be approximately a 99% probability that the *P*-450 concentration has been increased by PB administration and greater than an 80% probability than the *P*-448 concentration has increased because of 3MC injections. When measuring the induction by use of the *P*-450 (or *P*-448) to b_5 ratio (Table 3, column 2), one must keep in mind that the b_5 concentration is being increased by PB (significantly) and 3MC (slightly). Despite the b_5 concentration changes, there is still a definite increase in the *P*-450 to b_5 ratio as well as a slight increase in the *P*-448 to b_5 ratio compared to their respective controls. Close analysis of the induction of *P*-450 and *P*-448 for individual rats as seen by the three methods reveals that the induction became less effective as the study progressed. This may be due to the fact that the injection amounts were based on the original average weight and were not increased as the animals got heavier; furthermore, induction may be less effective in older animals.

A comparison of the use of MCD and difference spectroscopy to characterize *P*-450 and *P*-448 is shown in Table 4. Because the crossover point of an MCD A term corresponds to the absorption maximum (4), one would expect that if *P*-450 and *P*-448 are spectrally distinct, their MCD crossover points would be different. This was indeed found to be the case, since the mean of the crossover points for *P*-450 is at 451.5 ± 0.3 nm, whereas that from *P*-448 is at 450.1 ± 0.6 nm. A Student *t* test analysis of the results in Table 4 shows a comparable level of confidence in the MCD and difference spectral characterizations that these two cytochromes are spectrally distinct. Both methods demonstrate the distinction to approximately a 99% confidence level. The MCD crossover points are consistently 1.0–1.5 nm to the red of the corresponding difference spectral peaks.

The spectral distinction between *P*-450 and *P*-448 has already been demonstrated by Fujita *et al.* (7), for the solubilized forms of these microsomal cytochromes by direct absorption spectroscopy. We have now demonstrated this distinction for unsolubilized *P*-450 and *P*-448 for the first time by a more direct method than difference spectroscopy.

An additional point which should be made is that MCD can be used to detect the presence of hemoglobin in the microsomal samples. Oxyhemoglobin shows a strong MCD A term with a crossover point at 576 nm (4). If appreciable amounts of hemolgobin are present, they would, therefore, be easily detected in the oxidized spectra. By this criterion, no significant amounts of hemoglobin were found in any of the samples.

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1. Dolinger, P. M., Kielczewski, M., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E. & Djerassi, C. (1974) *Proc. Nat. Acad. Sci. USA*, **71**, 399–403.
2. McNeirney, J. & Slavin, W. (1962) *Appl. Opt.* **1**, 365.
3. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427.
4. Djerassi, C., Bunnenberg, E. & Elder, D. L. (1971) *Pure Appl. Chem.* **25**, 57–90.
5. Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2379–2385.
6. Vickery, L., Salmon, A., & Sauer, K. (1974) *Biochim. Biophys. Acta*, in press.
7. Fujita, T., Shoeman, D. W. & Mannering, G. J. (1973) *J. Biol. Chem.* **248**, 2192–2201.