Magnetic Circular Dichroism of Purified Forms of Rabbit Liver Cytochromes P-450 and P-420[†]

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ABSTRACT: Magnetic circular dichroism (MCD) studies of three different forms of rabbit liver cytochrome P-450 are described. The cytochromes were isolated and purified to apparent homogeneity using procedures developed by D. A. Haugen and M. J. Coon [(1976), *J. Biol. Chem. 251,* 7929]. The form of P-450 studied most extensively, PB-P-450-LM2, was shown to be enzymatically active. The major findings are: (1) The three forms of oxidized P-450 (PB-P-450-LM2, PB-P-450-LM3:4, and BNF-P-450-LM4) as isolated show substantially different MCD spectra. These differences are interpreted as being due to changes in the spin states of the heme iron. (2) The MCD spectra of the three forms in the reduced state as well as their carbonmonoxy derivatives are on the whole similar to each other. (3) Conversion to P-420 leads to MCD spectra which are substantially different from the corresponding P-450 spectra. The spectra of the reduced and reduced + CO forms of P-420 are similar to those of deoxy- and carbonmonoxymyoglobin, respectively. (4) Addition of an excess of the type I substrate *d*-benzphetamine to the predominantly low-spin oxidized form of P-450 causes conversion to an approximately equal mixture of high- and low-spin forms. (5) Computer fitting of the MCD spectra of mixed spin-state samples of oxidized P-450 has been performed using library spectra of P-450 in both pure spin states, thereby permitting accurate determinations of the spin-state composition of samples at ambient temperature.

Following the discovery of cytochrome P-450 (Klingenberg, 1958; Garfinkle, 1958) and its subsequent characterization as a heme protein (Omura and Sato, 1964), intensive efforts were begun in several laboratories to purify the protein so that its properties could be studied without interference from other contaminants. The membrane-bound nature of P-450 and its facile conversion to an inactive form called P-420, however, thwarted early attempts at purification. Nonetheless, persistent efforts culminated in the simultaneous publication of two purification procedures leading to a P-450 that was judged to be homogeneous by polyacrylamide gel electrophoresis (van der Hoeven et al., 1974; Imai and Sato, 1974). A third procedure appeared shortly thereafter (Ryan et al., 1975). Continuing efforts by Coon and co-workers subsequently resulted in the isolation of several forms of rabbit liver P-450 (Haugen et al., 1975; Haugen and Coon, 1976a).

Our earlier demonstration of the ability of MCD[1] spectroscopy to distinguish two forms of P-450 in crude preparations (Dawson et al., 1974) led to the desire to isolate purified forms of the proteins and to characterize them as fully as possible with MCD spectroscopy. The MCD spectra of the oxidized, reduced, and reduced + CO states of phenobarbital-induced P-450 have previously been reported by Dawson et al. (1976b) and by Shimizu et al. (1975). Hashimoto and Imai (1976) have reported the corresponding MCD spectra for 3-methylcholanthrene-induced P-450. Bacterially derived P-450_{cam} has been studied with MCD spectroscopy by Dolinger et al. (1974) and by Vickery et al. (1975).

We report here the characterization with MCD spectroscopy of the low-spin oxidized, high-spin oxidized, reduced and reduced + CO states (see Scheme I, states **1**, **2**, **3**, and **5**, respectively) of three different forms of rabbit liver P-450. Unfortunately, the half-life of the oxygen-bound state of mammalian P-450 (**4**, Scheme I) at *ambient* temperatures is too short for it to be examined by MCD.

In addition, a series of experiments designed to monitor substrate binding has been carried out with PB-P-450-LM2 using both type I (*d*-benzphetamine) and type II (aniline) substrates. Finally, the MCD spectra of the oxidized and reduced + CO states of PB-P-420-LM2, PB-P-420-LM3:4, and BNF-P-420-LM4[2] were obtained. The reduced P-420-LM preparations proved to be extremely air sensitive and so were only studied with PB-P-420-LM2. This series of experiments represents the first characterization of P-420 by MCD spectroscopy.

As a consequence of this work a substantial amount of information about the spectroscopic aspects of the active site of several mammalian P-450 heme proteins is available. On the basis of these data more detailed intercomparisons of the MCD spectra of the purified mammalian P-450's with each other, of bacterially derived P-450_{cam} (Dolinger et al., 1974; Vickery et al., 1975), of chloroperoxidase (Dawson et al., 1976b), and

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[1] Abbreviations used: A modification of the nomenclature system of Guengerich et al. (1975) is used to describe liver microsomal cytochromes P-450 (P-450-LM) and P-420 (P-420-LM). Individual P-450-LM proteins are further characterized by number according to their polyacrylamide gel electrophoresis pattern and by the inducing agent used (PB, phenobarbital; BNF, β-naphthoflavone). Thus the purified PB-induced form corresponding to the second electrophoresis band will be called PB-P-450-LM2. P-450_{cam} refers to the protein isolated from camphor-grown *Pseudomonas putida.* Additional abbreviations used are: CD, circular dichroism; MCD, magnetic circular dichroism; DLL, dilauroyllecithin (dilauroylglyceryl-3-phosphorylcholine); EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; 3MC, 3-methylcholanthrene; HA, hydroxylapatite; DEAE, diethylaminoethyl; Hepes,*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

[2] BNF induction is considered to be equivalent to 3MC induction, but is thought to be less dangerous because BNF is not known to be carcinogenic.

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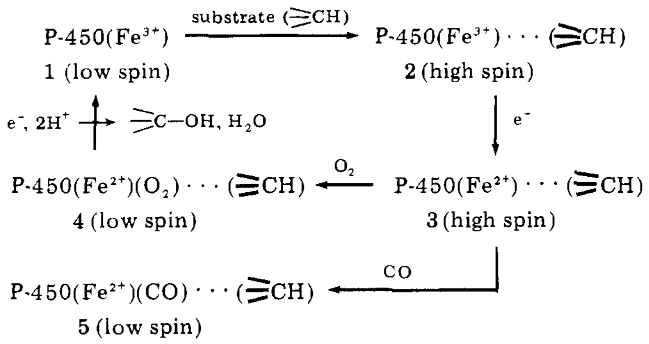
SCHEME I



of synthetic model complexes (Collman et al., 1976; Dawson et al., 1976a) can now be made.

Experimental Procedures

Materials. The following chemicals were obtained from the sources indicated: Sephadex G-25, Triton N-101, phenobarbital, horse heart cytochrome *c* (type III), and steapsin (lipase type II) from Sigma Chemical Co.; poly(ethylene glycol) 6000 and aniline from J. T. Baker Chemical Co.; ammonium sulfate (Ultra Pure) and bovine serum albumin from Schwarz/Mann; glycerol (spectrograde) for P-450-LM isolations from Eastman Chemical Co.; glycerol (reagent grade) for P-450-LM reductase isolations from Mallinckrodt Chemical Works; β-naphthoflavone from Aldrich Chemical Co.; DLL from Calbiochem; HA from Bio-Rad; Whatman standard grade and DEAE-cellulose from H. Reeve Angel and Co.; *d*-benzphetamine hydrochloride was generously provided by Dr. J. W. Hinman of the Upjohn Co., Kalamazoo, Mich.

The poly(ethylene glycol) was recrystallized from acetone–ether before use. The aniline was dried over potassium hydroxide, distilled from zinc dust, and stored over potassium hydroxide and zinc dust before use. The HA used for the P-450-LM isolations was prepared exactly as described by Pakroppa and Müller (1974). It was then mixed with equal parts of underivatized Whatman standard grade cellulose for column chromatography. Commercial HA was used for the reductase isolations. Calcium phosphate gel was prepared by the method of Keilin and Hartree (1938) by careful mixing of solutions of $CaCl_2$ and Na_3PO_4 followed by extensive washing with H_2O. Whatman DEAE-cellulose (DE-52) was used for all DEAE-cellulose chromatography. Fines were removed before use.

Protein Isolations. White, male New Zealand rabbits were used for the isolations and were obtained from L.I.T. (Aptos, Calif.). Induction was delayed until at least 5 days after arrival so as to allow for acclimatization. Phenobarbital induction was accomplished by the use of 0.1% (w/v) PB in the drinking water for 5 days (van der Hoeven et al., 1974). BNF induction was achieved by a single subcutaneous injection of 80 mg/kg of BNF in Mazola corn oil approximately 36 h prior to sacrifice (Nebert et al., 1973). In all cases, the animals were fasted for 24 h prior to sacrifice, at which time their weights ranged from 1.8 to 2.6 kg. All samples were stored in freezers at −20 °C.

Cytochrome P-450-LM Isolations. The procedures of van der Hoeven and Coon (1974) and of van der Hoeven et al. (1974) were followed, with certain modifications, for the isolation of PB-P-450-LM2. These procedures, as extended by Haugen et al. (1975) and by Haugen and Coon (1976a), were also used in the isolation of PB-P-450-LM3:4 and BNF-P-450-LM4. All steps in the protein isolations were carried out at 0–4 °C. The modifications (Dawson, 1976) to the Coon procedure were as follows. The nonionic detergent Triton N-101 was used in place of Renex 690. After poly(ethylene glycol) fractionation, the 8–10% (w/v) fraction was carried on further in the purification in all cases except following BNF induction when the 10–13% fraction was also used. Elution of PB-P-450-LM2 from the HA column was always achieved with 0.1 M potassium phosphate, pH 7.4, buffer (with 20% (w/v) glycerol and 0.1 mM EDTA) rather than with 0.05 M buffer. Likewise, elution of the protein preparations from calcium phosphate gel was achieved with 0.5 M potassium phosphate, pH 7.4, buffer (with 20% (w/v) glycerol and 0.1 mM EDTA) rather than with 0.3 M buffer.

Preparation of P-420-LM. Two methods for preparing P-420-LM were utilized. The first one involved treatment of P-450-LM with steapsin (Maines et al., 1974). For routine spectral measurements, the less complicated method of Imai and Sato (1967) was used. This involved treatment of P-450-LM (with added DLL) with solid KSCN to a concentration of 1.1 M. Complete conversion to P-420 was obtained in 15 min at room temperature. Reduced + CO P-420-LM was prepared by first bubbling the oxidized P-420-LM solution with CO for about 30 s followed by addition of a few grains of solid $Na_2S_2O_4$. Attempts to prepare reduced P-420-LM by simply adding $Na_2S_2O_4$ to oxidized P-420-LM resulted in extensive aggregation of the protein sample. This could be avoided, however, by use of anaerobic techniques. After deaeration under an atmosphere of oxygen-scrubbed high purity N_2 and treatment with KSCN the concentration of the P-420-LM solution was determined from its Soret intensity. Reduction was accomplished by addition of 0.020 mL of an anaerobic $Na_2S_2O_4$ solution (100 mg/mL) by syringe to the septum capped cuvette. The reduced P-420-LM, produced in this manner, was sufficiently stable so that a repeat absorption spectrum measured after completion of the MCD and CD spectra showed only a few percent decrease in the Soret intensity. Reduced BNF-P-420-LM4 was not examined.

Cytochrome P-450-LM Reductase Isolation. The procedure of Vermilion and Coon (1975) was adapted for isolation of rabbit liver P-450-LM reductase. The supernatant resulting from the poly(ethylene glycol) fractionation during the isolation of PB-P-450-LM2 was brought to 25% saturation with ammonium sulfate. Centrifugation resulted in a clear separation of two phases. The upper phase, containing the poly(ethylene glycol), was removed and the lower phase desalted on a Sephadex G-25 column. Partially purified reductase was then prepared by column chromatography on DEAE-cellulose and HA.

Analytical Methods: Protein Assay. Routine protein assays were made using the procedure of Lowry et al. (1951). Protein determinations on the final preparations were done by a modification of the Lowry procedure involving initial precipitation of the protein with trichloroacetic acid in order to avoid colorimetric interference by glycerol and residual detergent. In both cases bovine serum albumin served as the standard. Its concentration was determined from the published extinction coefficient (Kaziro et al., 1961).

PB-P-450-LM2 and Reductase Activity Assay. Only the enzymatic activity of PB-P-450-LM2 was verified because convenient substrates are not known for the other two P-450-LM preparations studied (Haugen et al., 1975). The assay procedure was based on the one described by van der Hoeven and Coon (1974) and involves quantification of the amount of formaldehyde released during the P-450-LM catalyzed N-demethylation of *d*-benzphetamine (Colchin and Axelrod, 1959; Nash, 1953). The modifications in the assay procedure of van der Hoeven and Coon were the use of potassium phosphate buffer in place of Hepes buffer and the omission of

$MgCl_2$ and sodium deoxycholate. In addition, the reaction was quenched with perchloric acid. All activity assays were done in duplicate with PB-P-450-LM2 having a specific content of 17 nmol/mg protein. The reductase activity was measured by the method of Masters et al. (1967) and was found to be 5 μmol per mg protein per min toward cytochrome *c*.

Homogeneity. Homogeneity of preparations was ascertained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with a discontinuous buffer system using the Laemmli procedure (Laemmli, 1970) under the conditions described by van der Hoeven and Coon (1974).

Phospholipid Analysis. An estimate of the amounts of phospholipid present in the final PB-P-450-LM preparations was made by carrying out a phosphate analysis according to the method of Raheja et al. (1973). Because of the large amount of protein required for this analysis, no analysis was done on the BNF-P-450-LM4 preparation.

Spectroscopic Measurements. All MCD spectra were recorded on a Japan Spectroscopic Co. spectropolarimeter (JASCO Model J-40) containing a 15.0 kG electromagnet with the field direction parallel to the direction of light propagation. The MCD spectral data shown have been corrected for natural CD ($MCD_{obsd} = CD + MCD$). All MCD spectra were recorded at 15 °C. The data are reported in terms of molar magnetic ellipticity, $[\theta]_M$, in the units deg $cm^2\ dmol^{-1}\ G^{-1}$. The instrument was calibrated frequently for CD and MCD intensity using camphorsulfonic acid and $CoSO_4$, respectively (Barth et al., 1975), and for wavelength, using a holmium perchlorate solution (McNeirney and Slavin, 1962). The data were recorded, normalized, smoothed (Savitsky and Golay, 1964), and manipulated on a Nova 840 computer and stored on magnetic tape.

Absorption spectra were recorded on Cary 14 or 17 spectrophotometers at ambient temperatures. The data were then digitized by hand, 1 point per 2.5 nm, and inserted into the computer point by point for data manipulation.

MCD spectra of oxidized P-450-LM which were thought to be a mixture of high- and low-spin forms were analyzed by computer to determine the percent of each component present. The MCD spectrum of substrate-bound oxidized $P\text{-}450_{cam}$ (Vickery et al., 1975) was used as a library spectrum for the completely high-spin form. Oxidized PB-P-450-LM2 without added substrate provided the library spectrum for the completely low-spin form. The percentage of low- and high-spin forms was estimated by multiple linear regression using library spectra. The composite spectrum obtained thereby was a close approximation to the observed spectrum. The program which was used for this procedure was based on multiple linear regression procedures described by Bevington (1969).

For spectral determinations, unless otherwise noted, the P-450-LM was stabilized by addition of enough DLL (from a 1 mg/mL stock solution in distilled water) to give a final concentration of about 100 μg/mL (ca. 0.2 mM). The protein solutions were approximately 10 μM. Usually 1.100-mL solution volumes were examined in 1-cm cuvettes. The P-450-LM stock solution concentrations were determined by use of an extinction coefficient of 110 $mM^{-1}\ cm^{-1}$ (Haugen and Coon, 1976a) for the Soret absorption maxim of the reduced + CO PB-P-450-LM2; subsequent dilutions with DLL, $Na_2S_2O_4$, or KSCN were taken into account in order to arrive at the final concentrations. The Soret extinction coefficients of the reduced + CO states of the other two P-450-LM preparations examined were also taken from Haugen and Coon (1976a).

The absorption spectra which are reported were obtained before measurement of the MCD and CD spectra. A second absorption spectrum was always run afterward to check for sample decomposition during the 30 min required to obtain the MCD and CD spectra. With oxidized and reduced + CO samples, essentially no changes were seen; however, for the reduced samples a decrease in intensity of the Soret transition of a few percent was usually seen. Reduced P-450-LM was normally obtained by addition of 0.010–0.020 mL of a freshly prepared 100 mg/mL $Na_2S_2O_4$ solution (approximately a 100-fold excess) to a 1.100-mL sample of the protein prepared as previously described. The reduced + CO state of P-450-LM was obtained by first saturating the sample with CO, followed by treatment with $Na_2S_2O_4$ as above.

Substrate binding was examined by difference absorption and MCD spectroscopy. The normal procedure for difference spectra was to dilute the protein to an optical density of approximately 1.0 at 415 nm and then to add equal volumes of the protein solution to both sample and reference cuvettes. After establishing a baseline, small volumes of the substrate (*d*-benzphetamine or aniline in water) were added to the sample cuvette and equal amounts of water were added to the reference cuvette. In order to look at a wide range of substrate concentrations and not dilute the protein too much, additions were made from two stock solutions, one fairly dilute for the initial additions and one substantially more concentrated for the final additions. The effect of dilution by the added substrate solution was taken into account in calculating the PB-P-450-LM2 concentration used for normalization of the MCD and CD spectra. Difference absorption spectra (see Figure 6) were considered acceptable when the isosbestic points did not change upon addition of more substrate and when the ΔOD at 350 nm was less than half the maximal ΔOD in the 395-nm region. MCD measurements were made on the same solutions as were used for the difference spectra.

Results and Discussion

P-450-LM Isolation. The isolation and purification of the three different preparations of P-450-LM proceeded almost exactly as described by Coon and co-workers (van der Hoeven and Coon, 1974; van der Hoeven et al., 1974; Haugen et al., 1975; Haugen and Coon, 1976a). The specific contents (nmol of P-450-LM per mg of protein) of the preparations examined spectroscopically were: PB-P-450-LM2, 18.6; PB-P-450-LM3:4, 13.6; and BNF-P-450-LM4, 13.0. These values are in excellent agreement with those reported by Haugen and Coon (1976a). The preparation of PB-P-450-LM2 which was examined for enzymatic activity had a slightly lower specific content (17.0) than the above preparation but exhibited similar MCD and absorption spectra. Polyacrylamide slab gel electrophoresis was carried out on all of the preparations examined spectroscopically and indicated a level of purity comparable to that reported by Haugen et al. (1975). It is interesting to note that the PB-P-450-LM3:4 preparation is only resolved by use of slab rather than tube gels. Similar results were obtained in Coon's laboratory upon switching to slab gels (Haugen and Coon, 1976b). Furthermore, it appears that the BNF-P-450-LM4 preparation corresponds to only one of the two bands found for PB-P-450-LM3:4, thus indicating that BNF induces only one of the two proteins present with a molecular weight per heme of about 54 000. The most recent modifications of the PB-P-450-LM isolation procedure (Haugen and Coon, 1976a) yield PB-P-450-LM4 which is free of PB-P-450-LM3.

With apparent molecular weights per heme of 50 000 and 54 000 for P-450-LM2 and P-450-LM4, respectively, the maximum values of the specific contents are 20.0 and 18.5, respectively. The somewhat lower values for the specific con-

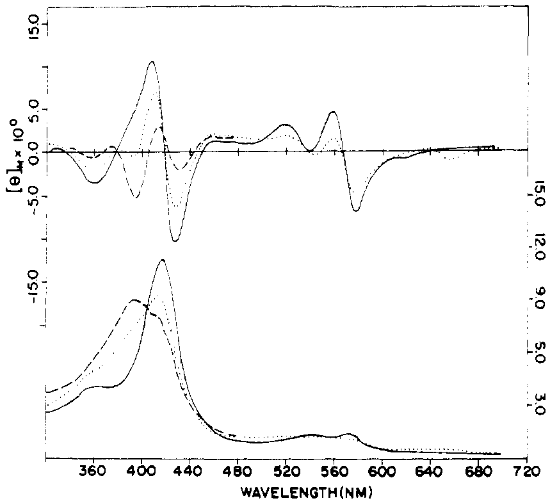


FIGURE 1: MCD (top) and absorption (bottom) spectra of oxidized PB-P-450-LM2 (—), PB-P-450-LM3:4 (···), and BNF-P-450-LM4 (- - -).

tents of these proteins in our preparations, especially the LM4-containing proteins, are probably due to partial conversion to apo-P-450-LM during isolation. This is consistent with the electrophoresis results from this laboratory and from Haugen et al. (1975) and Haugen and Coon (1976a), which do not indicate substantial non-P-450-LM protein contamination in the final preparations (unless, of course, the contaminating protein(s) had the same molecular weight).

Phospholipid analyses were carried out on both of the PB-P-450-LM preparations. The results indicated approximately one molecule of phospholipid per two molecules of PB-P-450-LM2 and essentially no contaminating phospholipid in the PB-P-450-LM3:4 preparation.

The enzymatic activity of PB-P-450-LM2 was quantified by measuring its ability to catalyze the N-demethylation of *d*-benzphetamine. It was found (Dawson, 1976) that the catalytic activity was linear vs. time for up to 10 min, linear vs. added reductase in the presence of excess PB-P-450-LM2, and linear vs. added PB-P-450-LM2 in the presence of excess reductase. A maximal apparent turnover number of 17 nmol of product produced per nmol of PB-P-450-LM2 per min was found. These activity studies clearly demonstrate that the PB-P-450-LM2 used for spectral studies was also capable of functioning enzymatically. The other preparations could not be tested due to the lack of convenient substrates. In any case we believe that the activity and purity of our preparations are such that our MCD spectral results should reasonably reflect the active site situation in the membrane-bound protein.

Spectral Studies on P-450-LM: Oxidized State. It has been observed (Haugen and Coon, 1976a) that the absorption spectrum of the BNF-P-450-LM4 protein is sensitive to its concentration and to the ionic strength of the solution. We investigated these effects on each of the three proteins studied, confirming the observation of Haugen and Coon (1976a) for the BNF-P-450-LM4 but found these effects to be much less evident in the PB-P-450-LM2 and PB-P-450-LM3:4 proteins. For this reason the absorption and MCD spectra of BNF-P-450-LM4 shown in Figure 1 were obtained at a higher dilution and salt concentration (2 μM and 0.78 M KCl) as compared with those of the other two proteins (10 μM and no added KCl). At higher concentrations and lower ionic strengths, the spectra of BNF-P-450-LM4 closely resembled those of PB-P-450-LM3:4. Because of the higher dilution, spectral measurements on BNF-P-450-LM4 were reliable only in the 360–480-nm region.

Substantial differences are seen in the MCD and absorption spectra of the oxidized states of the three P-450-LM preparations examined (Figure 1). Concentrating first on the Soret region, it will be noted that the intensity of the Soret MCD decreases in the order PB-P-450-LM2, PB-P-450-LM3:4, and BNF-P-450-LM4. An explanation for these differences comes from three sources. First, Vickery et al. (1976a) found that the intensity of the Soret MCD of a series of ferrimyoglobin derivatives was directly related to the spin state of the myoglobin complex with low-spin derivatives showing the more intense MCD. Second, this explanation receives cogent support from a comparison of the P-450-LM MCD spectra with those of substrate-free and substrate-bound P-450_{cam} (Dolinger et al., 1974; Vickery et al., 1975), with oxidized chloroperoxidase (Dawson et al., 1976b), and with a high-spin P-450 model complex (Dawson et al., 1976a). The third piece of evidence in support of our arguments concerning the spin states of the oxidized P-450-LM proteins comes from their absorption spectra which show a blue shift in the Soret absorption maxima from about 420 to about 395 nm over the sequence PB-P-450-LM2, PB-P-450-LM3:4, BNF-P-450-LM4. Similar absorption changes have previously been seen on going from a low-spin to a high-spin ferri-heme protein (Smith and Williams, 1968).

The MCD spectrum of PB-P-450-LM2 agrees closely with that of substrate-free P-450_{cam}. On the other hand, the Soret MCD of BNF-P-450-LM4 shows features found in both the substrate-free and substrate-bound P-450_{cam} spectra. In particular, the negative MCD band in the spectrum of BNF-P-450-LM4 at 385 nm has its counterpart in the MCD spectra of substrate-bound P-450_{cam} and oxidized high-spin chloroperoxidase; also seen in the BNF-P-450-LM4 spectrum is the smaller S-shaped feature found at about 418 nm such as is seen in the substrate-free P-450_{cam} and PB-P-450-LM2 spectra. Corresponding pairs of features, but reversed in relative intensity as compared with BNF-P-450-LM4, are observable in the Soret region of PB-P-450-LM3:4. The characterization of substrate-free P-450_{cam} as low-spin and substrate-bound P-450_{cam} as high-spin (Tsai et al., 1970; Peterson, 1971) strongly suggests that the changes seen in the P-450-LM spectra reflect increasing amounts of a high-spin component on going from PB-P-450-LM2 to PB-P-450-LM3:4 to BNF-P-450-LM4. The MCD spectrum of the 3MC-induced P-450-LM protein reported by Hashimoto and Imai (1976) is consistent with the presence of an even higher amount of the high-spin component whereas the spectrum of the PB-P-450-LM protein presented by Shimizu et al. (1975) resembles that of the low-spin protein. The presence of a variable amount of a residual bound substrate could explain the differences in spin states between the oxidized P-450-LM proteins. However, these variations in spin state may also arise from structural differences among the several proteins.

In order to place our qualitative arguments on the spin-state composition of the P-450-LM preparations on a more quantitative basis, we have carried out a computer analysis of the Soret regions of the MCD spectra of PB-P-450-LM3:4 and BNF-P-450-LM4 using a multiple linear regression routine adapted from Bevington (1969). The MCD spectrum of PB-P-450-LM2 is, as already noted, very similar to that of low-spin substrate-free P-450_{cam} (Dolinger et al., 1974; Vickery et al., 1975) and was used as the library spectrum for low-spin P-450. The high-spin library spectrum was the spectrum of substrate-saturated P-450_{cam} which was carefully hand digitized from the data of Vickery et al. (1975) and inserted into the computer point by point.

The first case examined was oxidized PB-P-450-LM3:4

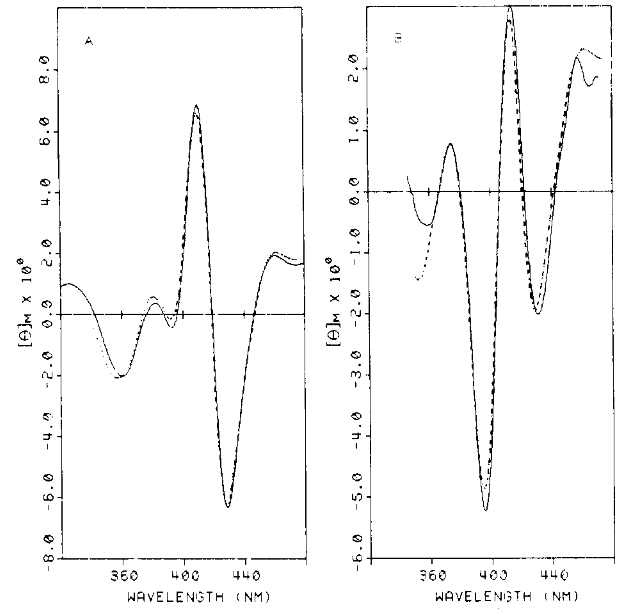


FIGURE 2: (A) Observed (—) and computer fitted (···) MCD spectra of oxidized PB-P-450-LM3:4. (B) Observed (—) and computer fitted (- - -) MCD spectra of oxidized BNF-P-450-LM4.

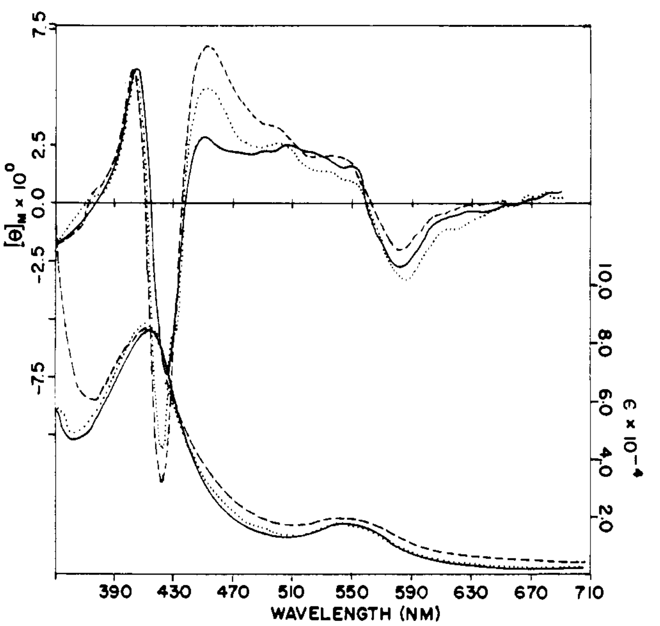


FIGURE 3: MCD (top) and absorption (bottom) spectra of reduced PB-P-450-LM2 (—), PB-P-450-LM3:4 (···), and BNF-P-450-LM4 (- - -).

(Figure 1) and the observed and fitted data over the 480-340-nm Soret region are shown in Figure 2A. The computer fitted spectrum is in remarkably close agreement with the original data. The analysis indicated that the observed spectrum was a mixture of 54% low-spin and 46% high-spin contributors. In an analogous fashion, the oxidized MCD spectrum of BNF-P-450-LM4 (Figure 1) was analyzed and the results are shown in Figure 2B. Again, the observed fit is remarkably good and in this case analysis reveals it to be a mixture of 17% low-spin and 83% high-spin contributors. Haugen and Coon (1976a) have determined that the BNF-P-450-LM4 protein is at least 75% high spin by EPR spectroscopy. It should again be emphasized that the data used for our analyses were obtained at nearly ambient temperatures (15 °C) and thus more accurately represent the physical state of the protein than do the data taken at the much lower temperatures required for EPR measurements of spin state.

Finally, we note that the visible region of the spectra (Figure 1) of the oxidized P-450-LM preparations also reflects the spin states of the proteins. Vickery et al. (1975) have reported that high-spin substrate-bound P-450_{cam} shows a negative MCD band at about 650 nm whereas low-spin substrate-free P-450_{cam} does not exhibit an MCD feature in this region. Furthermore, this band is present in the MCD spectrum of our synthetic five-coordinate hemin-thiolate model complex for high-spin P-450 (Dawson et al., 1976a) as well as in the 3MC-induced high-spin preparation of Hashimoto and Imai (1976) but not in the PB-induced low-spin preparation of Shimizu et al. (1975). Again, measurements at high dilution prevented us from observing this band in the BNF-P-450-LM4 preparation.

Reduced State. The overall similarity in shape of the MCD spectra of the three different forms of P-450-LM in the reduced state is at once apparent on examination of Figure 3. Furthermore, these spectra are substantially different from those of other heme proteins in their reduced states such as deoxyhemoglobin (Djerassi et al., 1971), cytochrome b_5 and deoxymyoglobin (Vickery et al., 1975, 1976a), and cytochrome *c* (Vickery et al., 1976b). Thus the unusual nature of reduced P-450-LM which is seen in their absorption spectra (a blue shift and a decrease in intensity in the Soret region on reduction rather than a red shift and an increase in intensity) is also reflected in their MCD spectra.

The spectra reported here agree fairly well with those reported for reduced P-450_{cam}, both with and without substrate present (Vickery et al., 1975), as well as with the data of Shimizu et al. (1975) and with the data of Hashimoto and Imai (1976). One difference between these spectra and ours is a feature centered at about 560 nm which appears to be a sharp derivative shaped curve superimposed upon a more gentle derivative shaped curve. The sharper feature, which is likely due to reduced P-420-LM (see Figure 5), is especially evident in the spectrum of substrate-free P-450_{cam} measured by Vickery et al. (1975), but is also seen in the spectra presented by Shimizu et al. and Hashimoto and Imai. It is barely seen in the PB-P-450-LM2 spectrum (Figure 3), and not at all in the other two forms of P-450-LM examined here. Thus, we believe that our preparations are substantially free from contamination by P-420-LM.

The differences among the MCD spectra of the reduced forms of the three different P-450-LM preparations (Figure 3) are most pronounced in the positive peak at about 450 nm and the negative peak at about 425 nm. Empirically, these two spectral features are more intense for those proteins whose oxidized spectrum indicates a larger percentage of high-spin character. The peak at about 400 nm does not change very much. This correlation can be extended to chloroperoxidase (Dawson et al., 1976b), which is substantially high-spin at room temperature, and which has even larger intensities at 425 nm and 450 nm than BNF-P-450-LM4. Unfortunately the correlation does not extend to P-450_{cam} where spectra are quite similar in the Soret region whether the reduced chromophore is derived from substrate-free or substrate-bound ferric P-450_{cam} (Vickery et al., 1975). Whether the intensities of the 450 and 425 nm peaks reflect the exact spin state of the reduced protein or subtle change in the local environment of the chromophore will not be clear until well-characterized model complexes are prepared.

Reduced + CO State. The MCD and absorption spectra of the three preparations of P-450-LM in their reduced + CO states are presented in Figure 4. As was first demonstrated with

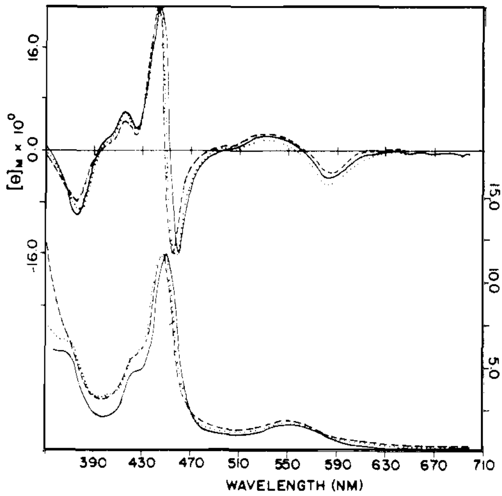


FIGURE 4: MCD (top) and absorption (bottom) spectra of reduced + CO PB-P-450-LM2 (—), PB-P-450-LM3:4 (···), and BNF-P-450-LM4 (- - -).

crude preparations (Dawson et al., 1974), the differences in the positions of the Soret absorption maxima in the spectra of the different preparations are reflected in changes in the crossover points in the Soret region of the MCD spectra. Otherwise, the spectra are remarkably similar in both shape and overall intensity. In particular, all three spectra exhibit a single unresolved feature in the 560-nm region, a sharp derivative-shaped curve crossing the origin at about 450 nm, a resolved shoulder at about 420 nm, and a negative trough at about 375 nm. These bands have been interpreted as being caused by thiolate ligation (Collman et al., 1976). Similar features have been found in the spectrum of this state of chloroperoxidase (Dawson et al., 1976b).

Two aspects of the MCD spectra of the reduced + CO states of the P-450-LM proteins in Figure 4 bear brief comment. First, it might appear after comparison of these spectra with the corresponding spectrum of reduced + CO P-420-LM shown in Figure 5 that the pronounced shoulder at 420 nm in the MCD spectra of the reduced + CO states of the P-450-LM proteins (Figure 4) is evidence of a small amount of P-420-LM in our P-450-LM preparations. Evidence that this is probably not the case comes from the MCD spectrum of the ferrous protoporphyrin IX diethyl ester-carbonyl thiolate complex, a synthetic model for the reduced + CO state of P-450, presented by us in a previous publication (Collman et al., 1976). A pronounced shoulder is also found in its MCD spectrum at 420 nm. Second, the Soret absorption band of reduced + CO P-450 is found at the unusually long wavelength of 450 nm, some 30 nm to the red of those of other heme proteins such as carbonmonoxyhemoglobin and myoglobin. Careful measurements, using minimal amounts of dithionite, reveal a second strong absorption band at about 365 nm (Hanson et al., 1976). In the MCD the corresponding features are the derivative shaped bands centered at 450 nm and the negative band at 375 nm (Figure 4). Corresponding features are also found in the absorption (Hanson et al., 1976) and MCD (Dawson et al., 1977) spectra of the Group VA non-iron "hyper" porphyrins. The iterative extended Hückel calculations of Hanson et al. (1976) on hyperporphyrins and on the thiolate-carbonyl adduct of ferrous protoporphyrin IX provide a common and rational basis for explaining the long wavelength position of the Soret band of reduced + CO P-450.

Spectral results similar to those shown in Figure 4 have previously been reported by Dolinger et al. (1974) and Vickery et al. (1975) for the reduced + CO state of P-450_{cam}. On the other hand curious differences exist between our MCD spectra and those reported by Shimizu et al. (1975) and by Hashimoto and Imai (1976) with regard to the intensity of the Soret region and the ratio of positive to negative bands of the Soret region. Shimizu et al. (1975) report a reduced + CO spectrum in which the sum of the absolute values of the positive and negative peak intensities is comparable to that in our spectra, but in which the positive band is more than twice as intense as the negative band. The spectra shown in Figure 4 are only slightly more intense on the positive side than on the negative side. On the other hand, Hashimoto and Imai report a reduced + CO Soret intensity for their preparation which is over twice as large as that reported here or by any other of the above mentioned authors. The reasons for these discrepancies are not clear.

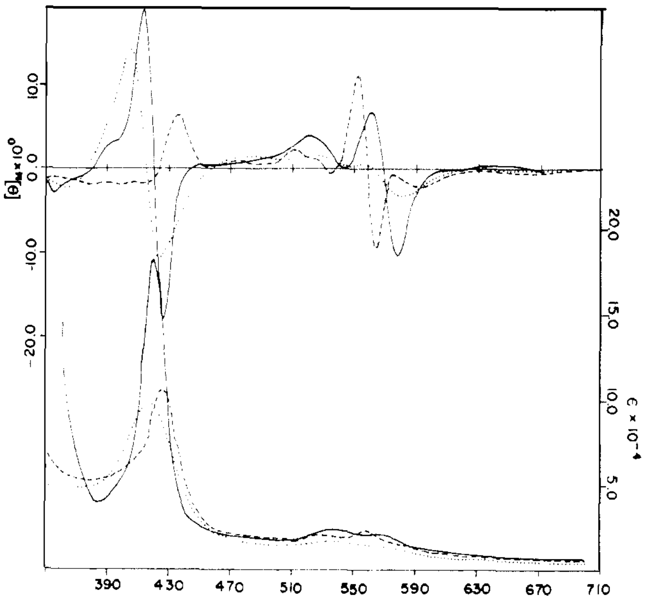


FIGURE 5: MCD (top) and absorption (bottom) spectra of oxidized (···), reduced (- - -), and reduced + CO (—) PB-P-420-LM2.

Spectral Studies on P-420-LM. Each of the three different forms of P-450-LM discussed in the previous section were converted to their respective P-420-LM forms by treatment with KSCN. However, because of the close similarity in their spectra, only the data for PB-P-420-LM are presented. It was initially a concern that the method of converting P-450-LM to P-420-LM did not lead to artifacts. Imai and Sato (1967) had reported that P-420-LM could be prepared by exposure of P-450-LM to high ionic strengths using salts such as KSCN. Other salts were partially effective but KSCN seemed to bring about quantitative conversion. The inherent disadvantage of this method is that SCN^- is a potential ligand for heme iron. Maines et al. (1974) have described another, more involved method for preparing P-420-LM by incubation of P-450-LM with steapsin. An initial experiment was done with PB-P-450-LM2 to verify that the two methods gave similar results; all subsequent experiments were done with the more simple KSCN method.

The absorption and MCD spectra of oxidized, reduced, and reduced + CO PB-P-420-LM2 are shown in Figure 5. Comparison of the MCD spectrum of oxidized PB-P-420-LM2 with that of oxidized PB-P-450-LM2 (Figure 1) reveals that the Soret regions of these two preparations are very similar. The 550-nm regions are somewhat different in that PB-P-450-LM2 shows two clearly resolved MCD effects whereas in PB-P-420-LM2 the MCD bands are very broad and not well resolved. In general, however, the MCD spectrum of oxidized PB-P-420-LM2 is typical of other low-spin heme proteins such as PB-P-450-LM2 (Figure 1), ferricytochrome b_5 (Dolinger et al., 1974; Vickery et al., 1975), ferricytochrome *c* (Vickery et al., 1976b), and cyanometmyoglobin (Vickery et al., 1976a).

In contrast to the similarities in the MCD spectra of the oxidized states of the PB-P-450-LM2 (Figure 1) and PB-P-420-LM2 (Figure 5) proteins, the spectra of the reduced states of the proteins (Figures 3 and 5, respectively) are very disparate. In particular, the dominant MCD feature in the Soret region of reduced PB-P-450-LM2 (Figure 3) is the skewed inverted W-shaped band system centered at about 420 nm whereas the dominant MCD feature in the Soret region of reduced PB-P-420-LM2 (Figure 5) is the positive band at about 440 nm. The visible MCD spectrum of reduced PB-P-420-LM2 shows an intense and sharp S-shaped band whereas this region in the MCD spectrum of PB-P-450-LM2 is broad and poorly defined. In addition, a negative band is seen in the MCD spectrum of reduced PB-P-420-LM2 at about 595 nm. This latter band is probably not due to unreduced PB-P-420-LM2 since the positive lobe of the Soret MCD band found in the spectrum of the oxidized species is not evident in Figure 5. Comparison of the MCD spectrum of reduced PB-P-420-LM2 with those published for the reduced states of other heme proteins shows that the feature at 440 nm has an obvious counterpart only in the spectrum of deoxyhemoglobin (Djerassi et al., 1971; Treu and Hopfield, 1975) and deoxymoglobin (Vickery et al., 1976a). This correlation does not extend to the visible region where deoxyhemoglobin and deoxymyoglobin exhibit relatively weak MCD. On the other hand, several reduced heme proteins, notably cytochrome b_5 (Dolinger et al., 1974; Vickery et al., 1975) and cytochrome *c* (Vickery et al., 1976b; Sutherland and Klein, 1972), exhibit stronger MCD in the visible region than does reduced PB-P-420-LM2 but only weak Soret MCD. Perhaps the most rational explanation for the MCD spectrum of reduced PB-P-420-LM2 in Figure 5 is that it is due to a mixture of variously ligated five- and six-coordinated species.[3]

That the protein-derived ligand in the reduced + CO state of PB-P-450-LM2 and PB-P-420-LM2 must be different is obvious on comparison of their spectra shown in Figures 4 and 5, respectively. Most notably, the Soret absorption and MCD bands in the spectra of PB-P-420-LM2 are found at about 420 nm, some 30 nm to the blue of their position in the PB-P-450-LM proteins. In addition, reduced + CO PB-P-420-LM2 shows MCD bands of higher intensity in the visible region than does reduced + CO PB-P-450-LM2 but does not exhibit the negative band on the blue side of the Soret band which is characteristic of P-450 proteins and of "hyper" porphyrins (Hanson et al., 1976; Dawson et al., 1977). In the case of P-450 we have accumulated further evidence based on the MCD spectra of synthetic model complexes that the protein derived ligand trans to CO is a thiolate anion (Collman et al., 1976). In this study and in the related absorption spectral studies of Collman and Sorrell (1975), Sorrell and Collman (1976), and Chang and Dolphin (1975, 1976), a number of other protein analogue ligands including *N*-methylimidazole, tetrahydrofuran, tetrahydrothiophene, and *n*-propylmercaptan were examined as possible ligands for the reduced + CO state of P-450 and, concomitantly, for that of reduced + CO P-420. The spectra obtained from these model complexes and from carbonmonoxymyoglobin (Vickery et al., 1976a) were all similar to that of reduced + CO PB-P-420-LM2 shown in Figure 5. Thus, a clear assignment of the fifth ligand cannot be made, although histidine or cysteine is the most likely candidate. Although Shimizu et al. (1975) mentioned unpublished work on P-420-LM, the work presented here represents the first full set of MCD spectra to be reported on P-420-LM.

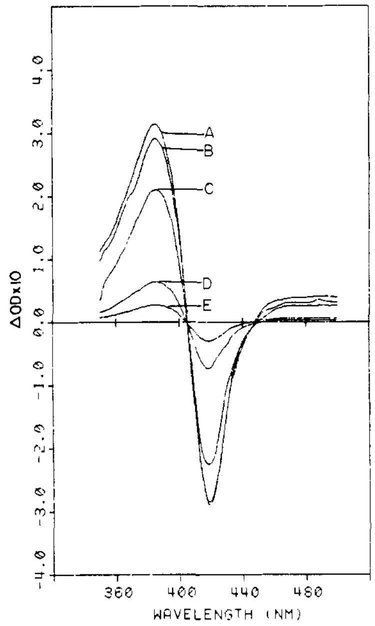


FIGURE 6: Difference absorption spectra resulting from the exposure of oxidized PB-P-450-LM2 (12.45 μM; specific content, 18.6) to *d*-benzphetamine at the following concentrations: (curve A) 1.000 mM; (B) 0.552 mM; (C) 0.228 mM; (D) 0.099 mM; (E) 0.033 mM.

Substrate Binding to PB-P-450-LM2. Type I Interactions. The best known substrate for PB-P-450-LM2, *d*-benzphetamine (Haugen et al., 1975), has been shown by Lu et al. (1969) to give rise to a typical type I difference spectrum (Shenkman, 1970) with partially purified protein. Figure 6 shows the similar result found in this laboratory. More recent work in Coon's laboratory using highly purified PB-P-450-LM2 has shown that *d*-benzphetamine interacts with a spectral dissociation constant of 0.10 ± 0.02 mM (Guengerich and Coon, 1976) in close agreement with the value of 0.11 mM determined by us under similar conditions (Dawson, 1976). During this experiment MCD and absorption spectra were also obtained (Figure 7). As more and more substrate was added, changes were observed throughout the MCD spectrum. The derivative shaped curve centered at 418 nm in the substrate-free spectrum (curve F) decreased in intensity while first a shoulder and then a negative trough increased in the spectrum at 395 nm. Addition of more benzphetamine did not result in further appreciable spectral changes indicating that 1 mM concentration represents nearly saturating conditions. In the absorption spectrum, the intensity of the Soret maximum at about 419 nm decreased while a shoulder developed at about 395 nm. These changes are consistent with a change in the spin state of the iron from low-spin in the substrate-free protein to partially high-spin with substrate present. Comparison with Figure 1 reveals that the substrate-saturated form of PB-P-450-LM2 gives rise to a MCD spectrum (curve A) remarkably similar to that of PB-P-450-LM3:4, which was characterized as an approximately equal mixture of high and low-spin forms. That the substrate-saturated protein does not give rise to an almost completely high-spin preparation is apparent from

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[3] Dr. L. Vickery has suggested to us that a six-coordinate species having one tightly bound imidazole and a second weaker CO-displaceable one could be responsible for the negative MCD band at 595 nm in Figure 5.

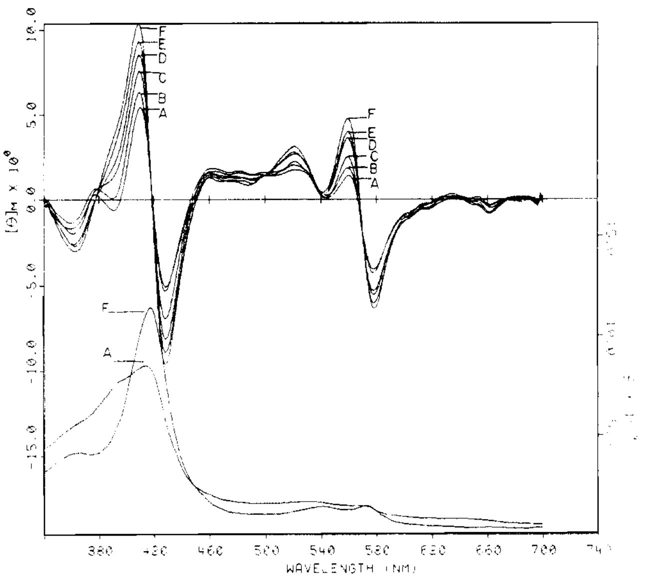

FIGURE 7: MCD (top) and absorption (bottom) spectra of oxidized PB-P-450-LM2 before (curve F) and after (curves A–E) exposure to *d*-benzphetamine at the following concentrations: (curve A) 1.000 mM; (B) 0.552 mM; (C) 0.228 mM; (D) 0.099 mM; (E) 0.033 mM. The sample used was the same as in Figure 6.

TABLE I: Spin-State Composition of PB-P-450-LM2 as a Function of Benzphetamine Concentration.

Curve[a]	[*d*-Benzphetamine] (mM)	High spin (%)
E	0.033	5
D	0.099	19
C	0.228	26
B	0.552	39
A	1.000	46

[a] Spectra are shown in Figure 7.

comparison with the spectrum of BNF-P-450-LM4 (Figure 1). A decrease in intensity of the MCD features in the 500–600-nm region and around 365 nm is also seen on sequential addition of *d*-benzphetamine. Additionally, a negative band at 660 nm, such as is seen in high-spin substrate-bound P-450$_{\mathrm{cam}}$ (Vickery et al., 1975), increases in intensity as substrate is added.

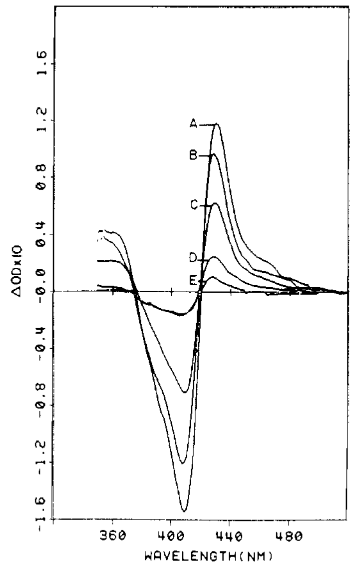

FIGURE 8: Difference absorption spectra resulting from exposure of oxidized PB-P-450-LM2 (9.58 μM; specific content 18.6) to aniline at the following concentrations: (curve A) 17.998 mM; (B) 8.700 mM; (C) 4.018 mM; (D) 1.067 mM; (E) 0.477 mM.

In order to quantify the changes in the PB-P-450-LM2 spectrum brought about by *d*-benzphetamine binding, we have subjected the set of MCD spectra in Figure 7 to the same computer analysis as was done in determining the spin-state compositions of PB-P-450-LM3:4 and BNF-P-450-LM4 (vide supra). Again, the library spectra for the low- and high-spin forms were those of PB-P-450-LM2 without added substrate and substrate-bound P-450$_{\mathrm{cam}}$ (Vickery et al., 1975), respectively. The results of this analysis (Table I) indicate that the high-spin contribution increases from about 5% with 0.033 mM *d*-benzphetamine to about 46% when the *d*-benzphetamine concentration is 1.0 mM. This latter result is of some interest in that it shows that the PB-P-450-LM2 preparation is an approximately equal mixture of high- and low-spin components even in the presence of essentially saturating amounts (1.0 mM is 10 times the spectral dissociation constant) of *d*-benzphetamine. A similar conclusion has been reached by Peterson et al. (1977) from absorption spectral studies. In contrast stands the results of Coon et al. (1976), based on a gel filtration

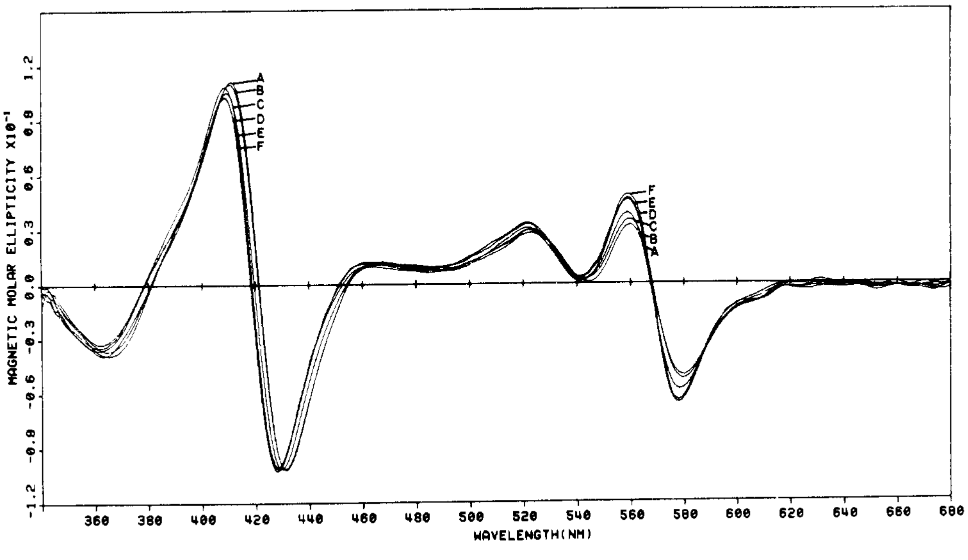

FIGURE 9: MCD spectra of oxidized PB-P-450-LM2 before (curve F) and after (curves A–E) exposure to aniline at the following concentrations: (curve A) 17.988 mM; (B) 8.700 mM; (C) 4.018 mM; (D) 1.067 mM; (E) 0.477 mM. The sample used was the same as in Figure 8.

technique using *d*-benzphetamine, which indicate that 1 mol of *d*-benzphetamine is bound per mol of PB-P-450-LM2. Collectively, the spectroscopic and gel filtration data suggest that only 46% of the substrate bound at near saturation to PB-P-450-LM2 is effective in causing a change to a high-spin state, presumably by displacing (not replacing) the sixth ligand to iron. The partial effectiveness may be due to an equilibrium in the displacement of the sixth ligand by substrate bound at one binding site or may be the result of substrate binding at multiple sites, only one of which allows displacement of the sixth ligand.

In an attempt to resolve this question we examined the effect of temperature on the MCD spectrum of substrate-saturated PB-P-450-LM2 (Dawson, 1976). Changes were seen which were consistent with an increased high-spin content at higher temperatures. However, an alternative explanation for these effects is that they were caused by temperature-dependent MCD C terms. Temperature-dependent changes in the spin state of substrate-bound oxidized P-450_{cam} have been observed by Sligar (1976) and by Paul et al. (1976) for cytochrome P-450_{SCC} of the rat adrenal.

Type II Interactions. The type II difference absorption spectra resulting from sequential additions of aniline to PB-P-450-LM2 are shown in Figure 8. From these data, a spectral dissociation constant of 6.7 mM was determined (Dawson, 1976).This is in fair agreement with the value of 9 mM determined in Coon's laboratory (Nordblom and Coon, 1976b). During the experiment, MCD spectra (Figure 9) were also obtained. The two predominant effects which were seen are the red shift in the Soret MCD crossover point from about 418 nm to about 423 nm and a decrease in MCD intensity in the 500–600-nm region. The spectral changes seen in Figures 8 and 9 are presumably due to replacement by aniline of the sixth ligand to iron in PB-P-450-LM2 (Schenkman, 1970). No change in spin state is expected or seen as a result of this change. The spectra shown in Figure 9 clearly demonstrate that MCD is as sensitive as absorption spectroscopy to such type II interaction, but, contrary to the situation with type I interactions, do not provide any new information beyond that already available from absorption spectroscopy.

Conclusion

In this work we have attempted to integrate our investigation of the MCD spectra of the oxidized, reduced, and reduced + CO states of three highly purified forms of rabbit liver microsomal cytochrome P-450 with the data presented by Shimizu et al. (1975) and Hashimoto and Imai (1976) on individual rabbit liver preparations; with spectra on the corresponding forms of bacterially derived P-450_{cam} (Dolinger et al., 1974; Vickery et al., 1975); with the data for chloroperoxidase, for which there is now strong spectroscopic evidence of thiolate ligation (Dawson et al., 1976b); as well as with the data on the available well-characterized synthetic model complexes for the high-spin oxidized and reduced + CO states of P-450. In addition, we have presented for the first time MCD spectra of the oxidized, reduced, and reduced + CO states of cytochrome P-420. Our data are consistent with thiolate ligation, at least in several states, for each of the P-450 proteins but not for the P-420 proteins. Equally important, we have demonstrated that the spin-state composition of P-450 proteins can be quantified with ease from a computer analysis of digitized MCD spectra obtained at ambient temperatures. In particular, we conclude that *d*-benzphetamine saturated PB-P-450-LM2 is approximately a 1:1 mixture of high- and low-spin components.

Acknowledgments

It is a particular pleasure to acknowledge the important contribution to the work presented here made by Professor Minor J. Coon and his co-workers, Drs. David Haugen and Gerald Nordblom and Mr. John French. Professor Coon's willingness to share up-to-the-minute details concerning the isolation and handling of P-450-LM augmented our efforts immeasurably. We also wish to thank Ruth Records and Nancy Cline for technical assistance, Professor Henry Epstein and Mr. Edward Suddleson for carrying out the electrophoresis, and Professor Robert Simoni and Mr. David Rintoul for doing the phospholipid analysis.

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