ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 195, No. 1, June, pp. 178–187, 1979

Comparative Study of Two Highly Purified Forms of Liver Microsomal Cytochrome *P*-450: Circular Dichroism and Other Properties[1]

YOUNG-LING CHIANG[2] AND MINOR J. COON

Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109

Received September 25, 1978; revised January 24, 1979

The two major forms of rabbit liver microsomal cytochrome *P*-450, *P*-450$_{LM2}$ and *P*-450$_{LM4}$, which were previously shown to differ in their absorption spectra, electrophoretic and immunochemical properties, and substrate specificities, have been further characterized by several methods. (a) The two cytochromes have different CD spectra in the ferric state but similar spectra when reduced. Upon conversion of *P*-450$_{LM2}$ to *P*-420 by treatment with sodium dodecyl sulfate, the CD spectrum is greatly diminished except in the far ultraviolet region, whereas the conversion of *P*-450$_{LM4}$ to *P*-420 with this detergent results in a spectrum with a new positive band in the visible region. (b) Although *P*-450$_{LM4}$ has a much higher tryptophan content than *P*-450$_{LM2}$, the fluorescence spectra of these proteins are similar in magnitude. Upon denaturation, the fluorescence of *P*-450$_{LM4}$ increases, thereby indicating a large quenching effect in the native protein. (c) Studies on the interaction of dilauroyl-glyceryl-3-phosphorylcholine with the cytochromes showed that *P*-450$_{LM2}$ gives a much stronger Type I difference spectrum than does *P*-450$_{LM4}$. This phospholipid has no significant effect on the state of aggregation of these cytochromes as judged by calibrated gel filtration. The CD spectra of *P*-450$_{LM2}$ and *P*-450$_{LM4}$ are unchanged in the visible region but are enhanced in the far ultraviolet region upon the addition of phosphatidylcholine. The results appear to indicate an increase in α-helical content, particularly with *P*-450$_{LM4}$, in the presence of the phospholipid.

The occurrence of multiple forms of *P*-450$_{LM}$[3] has been well documented, as reviewed elsewhere (3, 4), and several forms of the cytochrome have been purified to electrophoretic homogeneity from liver microsomes in this laboratory (3, 5–7) as well as by other investigators (8–12). The phenobarbital- and β-napthoflavone-inducible cytochromes from rabbit liver microsomes, designated as *P*-450$_{LM2}$ and *P*-450$_{LM4}$ by their relative electrophoretic mobilities, have been partially characterized and shown to differ in their absorption spectra and subunit molecular weights (7), immunochemical properties (13), and substrate specificities (4). Such data, as well as preliminary findings on the amino-terminal amino acid sequence (14), strongly indicate that these are distinct proteins.

In the present paper, we have extended our studies on the characterization of *P*-450$_{LM2}$ and *P*-450$_{LM4}$ with the use of several methods, including CD and fluorescence spectrophotometry. The results confirm the earlier conclusion that these cytochromes are quite different proteins and provide information about the heme crevice structure and interactions with phosphatidylcholine. CD and ORD spectra of cytochrome *P*-450 in microsomal suspensions from phenobarbital-treated rabbits (15, 16) and partially purified cytochrome *P*-450

[1] This research was supported by Grant PCM76-14947 from the National Science Foundation and Grant AM-10339 from the United States Public Health Service. Preliminary reports of this investigation have been presented (1, 2).

[2] Supported by a National Research Service Award from the National Institutes of Health, No. 1 F32 GM06107.

[3] Abbreviations used: *P*-450$_{LM}$, liver microsomal cytochrome *P*-450; *P*-450$_{LM2}$ and *P*-450$_{LM4}$, phenobarbital- and β-naphthoflavone-inducible forms, respectively, of *P*-450$_{LM}$, so designated according to their electrophoretic properties (3).

0003-9861/79/070178-10$02.00/0

from such microsomes (17) have been described, and more recently magnetic CD spectra of purified P-450$_{LM2}$ and P-450$_{LM4}$ have been reported (9, 18–20). Such studies have been useful in examining the spin state of this cytochrome and the redox state-dependent conformational changes. However, CD studies have not been carried out previously with P-450$_{LM4}$. Tryptophan fluorescence spectrophotometry has been used with partially purified adrenal mitochondrial cytochrome P-450 (21), but we are unaware of previous studies on tryptophan fluorescence with P-450$_{LM}$.

EXPERIMENTAL PROCEDURES

Enzyme preparations. P-450$_{LM2}$ and P-450$_{LM4}$ were isolated from liver microsomes of phenobarbital-treated rabbits, and P-450$_{LM4}$ was also isolated from liver microsomes of β-naphthoflavone-treated rabbits according to procedures described elsewhere (7, 22). Unless stated otherwise, the P-450$_{LM4}$ used in the various experiments was from phenobarbital-induced animals. The enzyme preparations were homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The cytochrome P-450 specific content, expressed as nanomoles per milligram of protein, ranged from 16 to 19 for P-450$_{LM2}$ and from 13 to 15 for P-450$_{LM4}$; the range of values for each cytochrome is apparently due to variable heme loss during purification. Protein concentrations were determined by the method of Lowry *et al.* (23) as slightly modified in this laboratory, and the values were corrected by the factors previously obtained from amino acid analysis of the purified cytochromes (7). P-450$_{LM}$ was determined from the CO difference spectrum of the reduced protein using an extinction coefficient of 91 mM^{-1} cm^{-1} (24) for the difference between A_{max} (in the 450-nm region) and A_{490}. For tryptophan determinations, the molar concentration of polypeptide chains was calculated from the protein concentration using the minimal molecular weights of 47,600 and 54,000 for P-450$_{LM2}$ and P-450$_{LM4}$, respectively (7).

CD spectra. CD spectra were recorded at ambient temperature using a Model ORD/UV-5 or Model J40C spectropolarimeter (Japan Spectroscopic Co.) with a path length of 1.0 cm in the visible region and 0.1 cm in the far uv region. Molar ellipticity, $[\theta]$, is expressed as degree · centimeter squared per decimole of P-450$_{LM}$. A mean amino acid residue weight of 113 was used in the computation of mean residue ellipticities, $[\theta]_R$, for the far uv region of the CD spectra. Base lines were determined with all components present except P-450$_{LM}$.

Fluorescence emission spectra. Fluorescence emission spectra were determined at ambient temperature with a ratio recording spectrofluorometer constructed by Dr. D. P. Ballou and Mr. G. S. Ford. The excitation wavelength for tryptophan fluorescence was 285 nm. Samples were in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 20% glycerol, in the presence or absence of 6 M guanidine hydrochloride and 30 mM β-mercaptoethanol.

Determination of tryptophan residues. Titration of tryptophan residues in P-450$_{LM}$ by N-bromosuccinimide (25) was performed using an Aminco DW-2 spectrophotometer. Small aliquots of aqueous 10 mM N-bromosuccinimide were added sequentially to a sample of the enzyme in 2.0 ml of 0.1 M sodium acetate buffer, pH 4.0, either with or without 8 M urea present. The number of tryptophan residues modified was calculated from the decrease in absorbance at 280 nm, using a molar extinction coefficient of 5500 (25), with correction for the small volume changes.

Materials. N-Bromosuccinimide was obtained from Sigma; urea and guanidine hydrochloride, both ultrapure grade were from Schwarz/Mann; and dilauroylglyceryl-3-phosphorylcholine was from Serdary Research Laboratories.

RESULTS

CD Spectra of P-450$_{LM2}$ and P-450$_{LM4}$

The CD spectra of the ferric forms of P-450$_{LM2}$ and P-450$_{LM4}$ isolated from phenobarbital-treated rabbit liver microsomes are shown in Fig. 1. Both cytochromes show several negative bands in the region of 330 to 500 nm. The negative Soret Cotton effect seen with P-450$_{LM2}$ at 422 nm is much stronger than that of P-450$_{LM4}$, which is centered at 410 nm. In addition, both cytochromes have weak bands in this region, including one at 350 nm, whereas P-450$_{cam}$

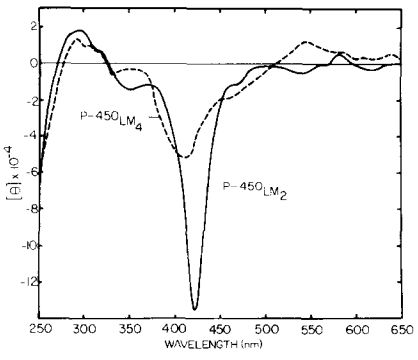

FIG. 1. CD spectra of ferric P-450$_{LM2}$ and P-450$_{LM4}$. The concentrations of P-450$_{LM2}$ and P-450$_{LM4}$ were 10 and 15.6 μM, respectively.

(26) and adrenal mitochondrial P-450_{scc} (21) have a strong band at this wavelength. P-450_{LM2} shows a weak positive band at 580 nm, whereas P-450_{LM4} shows a broader but stronger band centered at 545 nm. Although some of these CD bands are small, they are highly reproducible. In the near uv region, with both cytochromes a positive band is located around 290 to 295 nm with a shoulder at 310 nm. In experiments not shown, purified P-450_{LM4} from β-naphthoflavone-treated animals had an identical CD spectrum to that of P-450_{LM4} from phenobarbital-treated animals.

It has been observed previously that the purified P-450_{LM4} preparation is a mixture of high and low spin forms, as judged by absorption and epr spectra, and that the high spin form is favored at low protein and low detergent concentrations (7). Therefore, the CD spectrum of P-450_{LM4} shown in Fig. 1 was recorded at the highest sensitivity of the instrument and the lowest protein concentration required for obtaining satisfactory spectra. The peak position and shape of the CD spectrum in the Soret region also indicated that the P-450_{LM4} preparation is a mixture of high and low spin forms. It may be noted that, under the same conditions used for the CD experiment, the P-450_{LM4} preparation had an absorption spectrum with A_{395}/A_{418} equal to 1.2, which corresponds to a content of about 80% high spin form. This value was estimated algebraically using simultaneous equations, with the assumption that the P-450_{LM2} and P-450_{LM4} spectra published earlier (7) represent completely low and high spin states, respectively. The effect of detergent on the CD spectrum of P-450_{LM4} is shown in Fig. 2. The addition of Renex at a final concentration of 0.9% caused a shift of the Soret band from 410 to 420 nm without affecting the area covered by this Cotton effect. This red shift apparently indicates an increase in the low spin form of the cytochrome. The appearance of a slight shoulder at 410 nm is believed to be due to the contribution of the remaining high spin form. In addition, a decrease in magnitude and shift of the 545-nm band to 550 nm was observed. No further change in the CD spectrum of P-450_{LM4} was noticed on the addition of more detergent

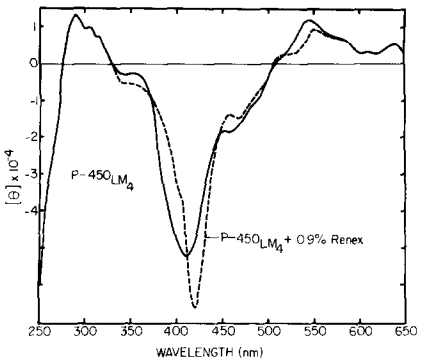

FIG. 2. Effect of Renex 690 on the CD spectrum of ferric P-450_{LM4}. The conditions were as in Fig. 1, but with the addition of the detergent at a final concentration of 0.9%.

to the sample. The absorption spectrum of the Renex-treated sample was examined, and it was found that A_{395}/A_{418} had decreased to 0.9, which corresponds to a high spin content of about 55%. In other experiments not shown, the effect of aniline at a saturating concentration on the CD spectra of the cytochromes was studied. As indicated in the report by Ruckpaul *et al.* (17) concerning the CD spectrum of partially purified cytochrome P-450 from phenobarbital-induced rabbit liver microsomes, 17 mM aniline decreased the intensity of the Soret band of P-450_{LM2} by about 20% without a shift in the maximum. The CD spectrum of aniline-treated P-450_{LM4} was very similar to that of the Renex-treated sample, indicating the extensive conversion of high to low spin, which may arise from the ligation of aniline to the sixth coordination position of the iron. The addition of a saturating level of benzphetamine to P-450_{LM2} or P-450_{LM4} did not change the Soret CD spectra; this finding was unexpected in view of the effect of this substrate on the absorption spectrum of P-450_{LM2} (20).

When P-450_{LM2} was reduced with dithionite, the intensity of the Soret CD band decreased strikingly to about half of the original, and the maximum shifted to 393 nm (Fig. 3). In contrast, the Soret CD band of ferrous P-450_{LM4} was located at 396 nm, with only a slight decrease in intensity as compared with the oxidized form. Thus, the overall spectra of P-450_{LM2} and P-450_{LM4} in

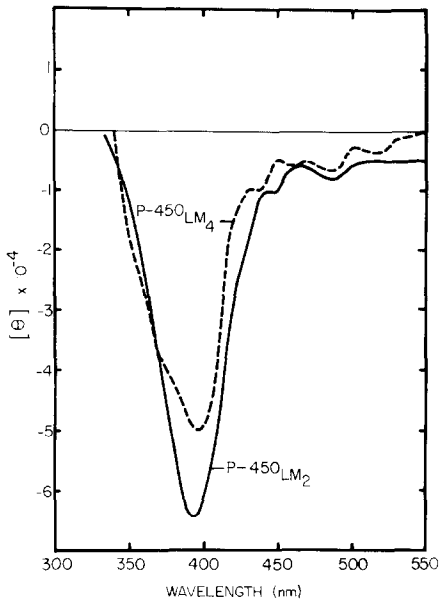

FIG. 3. CD spectra of ferrous P-450_{LM2} and P-450_{LM4}. The conditions were as in Fig. 1, but a few crystals of sodium dithionite were added to the samples.

the reduced state are very similar. The results of Ruckpaul *et al.* (17) with a mixture of cytochromes are in general accord with these findings. Differences in the dichroic properties of these two cytochromes were also observed with the carbon monoxide complex of the reduced form, as shown in Fig. 4. The spectra are characterized by two negative CD bands with different intensities: P-450_{LM2} shows such bands at 363 and 456 nm, and those of P-450_{LM4} are at 361 and 454 nm, respectively. This splitting of the Soret band has its counterpart in the absorption spectra, which are typical for the "hyperporphyrin" compound (27). In addition, a unique positive band at 427 nm was observed in the CD spectrum of the CO complex of P-450_{LM4}.

CD Spectra of Cytochrome P-420 Derived from P-450$_{LM2}$ and P-450$_{LM4}$

It has been reported by several laboratories that cytochrome P-450 is converted to an inactive form, termed P-420, by a variety of agents, such as bile salts, sodium dodecyl sulfate, urea, and KSCN (28, 29). Therefore, we examined the changes in CD spectra upon the conversion of P-450_{LM} to P-420 by treatment of the native cytochromes with such reagents. Figure 5 shows the effect of sodium dodecyl sulfate at several concentrations on the CD spectrum of ferric P-450_{LM2}. The intensity of the CD spectrum was substantially decreased by the addition of 0.008% detergent, and the spectrum was completely lost when the concentration was increased to 0.015%. At the latter concentration, P-450_{LM2} was completely converted to P-420. The loss of the CD spectrum was evidently not due to the release of the heme group, since the typical carbon monoxide complex of reduced cytochrome P-420 was formed as detected by the absorption spectrum. Furthermore, neither the ferrous P-420 nor its carbon monoxide complex showed a CD spectrum. In addition, the effect of sodium dodecyl sulfate on the secondary structure of the cytochrome was studied by the change of the CD spectrum in the far uv region. Whereas the native P-450_{LM2} has an α-helical content of about 33%, as calculated from its ellipticity at 208 nm according to the method of Greenfield and Fasman (30), after addition of 0.015% sodium dodecyl sulfate the value was found to be 30%. Apparently, therefore, the secondary structure of this protein is virtually unchanged on conversion to P-420.

In contrast, the addition of sodium dodecyl sulfate to P-450_{LM4} decreased the negative ellipticity at 410 nm with the concomi-

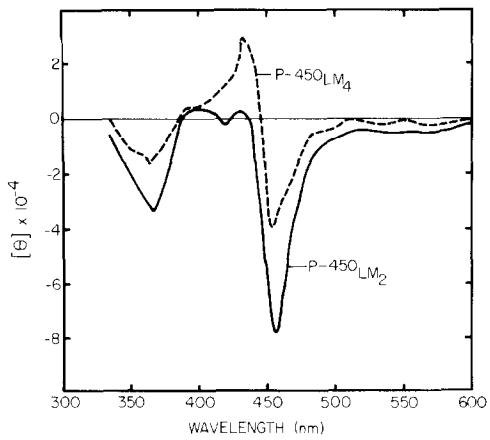

FIG. 4. CD spectra of CO complexes of ferrous P-450_{LM2} and P-450_{LM4}. Samples as in Fig. 1 were bubbled with CO for 30 s, and a few crystals of sodium dithionite were then added.

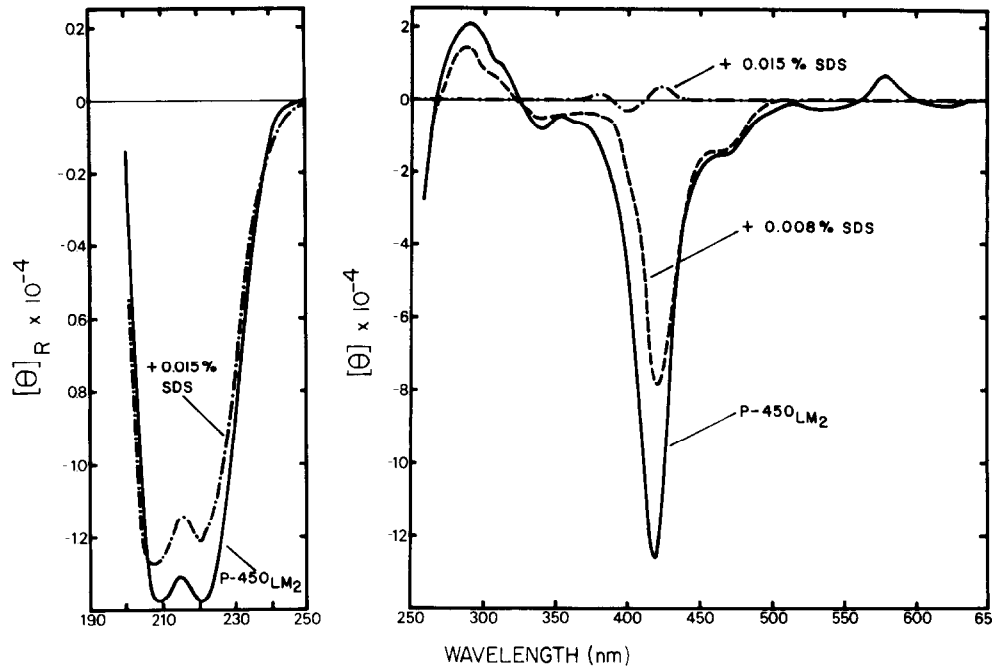

FIG. 5. CD spectrum of P-420 formed from P-450_{LM2}. The conditions were as in Fig. 1, but with conversion to P-420 by the addition of sodium dodecyl sulfate at the final concentrations indicated.

tant appearance of a positive band at 430 nm (Fig. 6). The increase at 430 nm was complete at 0.042% detergent. The positive band at 290 nm was greatly diminished, but only a small change was observed in the 550-nm region. At the highest sodium dodecyl sulfate concentration the absorption spectrum of the CO complex had a Soret maximum at 420 nm, thus indicating that heme remained bound to the protein, although not necessarily at the original site. However, dithionite reduction of the P-420 derived from P-450_{LM4}, with or without CO present, completely eliminated the CD spectrum. The CD spectrum of native P-450_{LM4} in the far uv region indicated a helical content of about 25%, which is lower than that of P-450_{LM2}. An apparent increase of α-helical content to about 34% was observed, however, upon conversion of P-450_{LM4} to P-420. On the other hand, in experiments not shown, cytochrome P-420 prepared by treatment of P-450_{LM2} or P-450_{LM4} with 1.2 M KSCN by the method of Imai and Sato (28) exhibited no CD spectrum in the visible region in both the oxidized and reduced states.

Tryptophan Fluorescence Spectra

The amino acid analysis reported previously (7) indicated the presence of one tryptophan residue per polypeptide chain of P-450_{LM2} and seven residues in P-450_{LM4}. In studies not presented, titrations with N-bromosuccinimide indicated that the numbers are about 3 and 7, respectively. The reason for the discrepancy with P-450_{LM2} is not known at this time. As shown in Fig. 7, the tryptophan fluorescence emission of P-450_{LM2} was maximal at 320 nm, whereas that of P-450_{LM4} was maximal at 330 nm when the samples were excited at 285 nm; at these wavelengths the relative fluorescence intensities of the two proteins were found to be quite similar. On the other hand, in the presence of 6 M guanidine and 30 mM β-mercaptoethanol, the maximum for P-450_{LM4} was shifted to 350 nm, and what appears to be a shoulder centered at about the same wavelength was observed for P-450_{LM2}. Of particular interest, the magnitude of tryptophan emission was greatly increased upon denaturation only with P-450_{LM4}. Presumably all tryptophan resi-

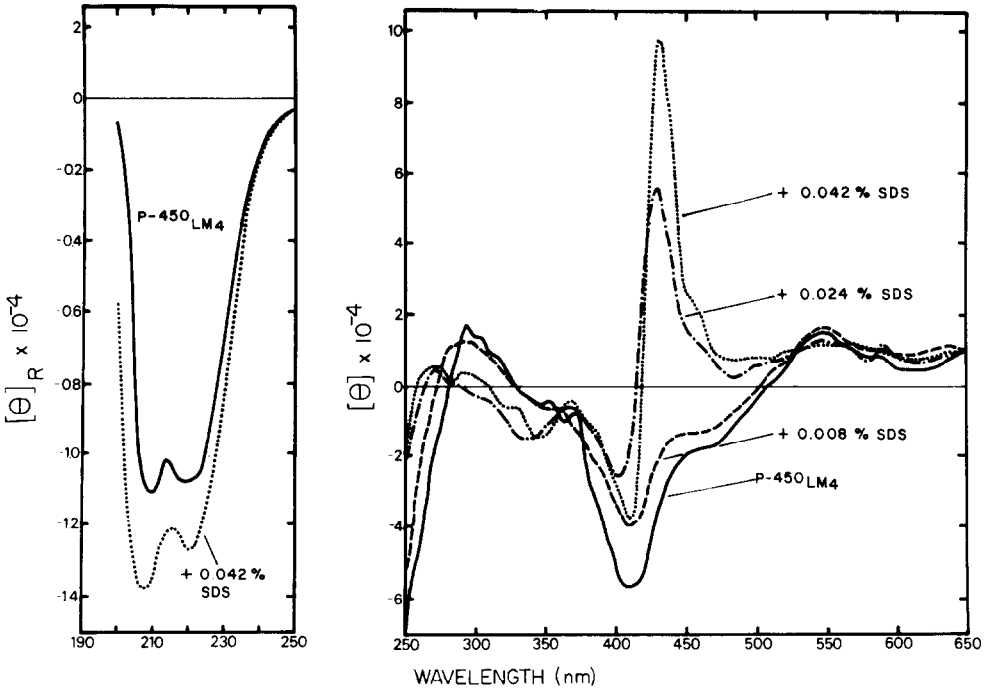

FIG. 6. CD spectrum of *P*-420 formed from $P\text{-}450_{LM4}$. The conditions were as in Fig. 1 but with conversion to *P*-420 by the addition of sodium dodecyl sulfate at the final concentrations indicated.

dues are exposed to the solvent in the denatured proteins, which then exhibit the normal emission maximum of this amino acid in aqueous solution.

Interaction of Phospholipid with Cytochromes

This laboratory has previously shown that phosphatidylcholine is required for maximal activity of cytochrome *P*-450 in the reconstituted liver microsomal enzyme system (31, 32). Since highly purified $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$ contain essentially no phospholipid (less than one molecule per polypeptide chain) and only low levels of Renex 690 (7), such preparations are suitable for determining whether the combination of phospholipid with the cytochrome causes spectral changes. As shown in Fig. 8, the addition of dilauroylglyceryl-3-phosphorylcholine to $P\text{-}450_{LM2}$ gave a typical Type I difference spectrum with a peak at 390 nm and a trough at 423 nm. In other experiments the concentration of the phospholipid was varied, and from an inverse plot of the data the K_s was determined to be about 0.45 mM. In contrast, $P\text{-}450_{LM4}$ gave only slight spectral changes suggestive of a Type I difference spectrum. Since such spectra are believed to be indicative of a low to high spin state conversion, and $P\text{-}450_{LM4}$ as isolated

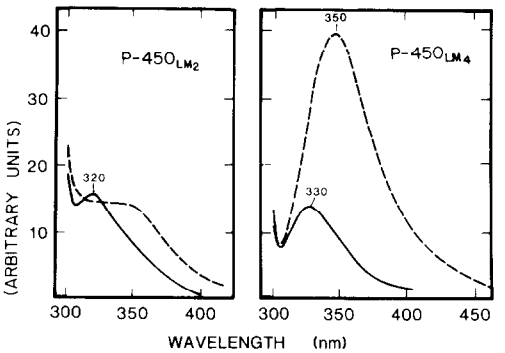

FIG. 7. Fluorescence emission spectra of $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$. The solid line indicates the results obtained with the native cytochromes (3.1 μM $P\text{-}450_{LM2}$ or 2.9 μM $P\text{-}450_{LM4}$), and the dashed line indicates the change when the cytochromes were denatured in 6.0 M guanidine hydrochloride in the presence of 30 mM β-mercaptoethanol.

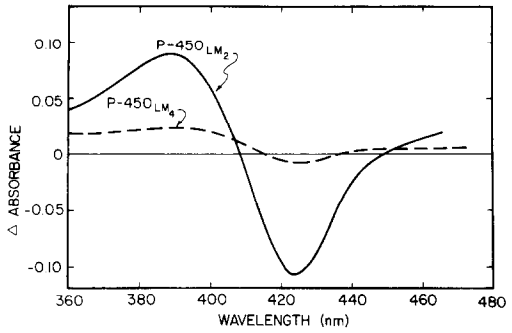

FIG. 8. Phosphatidylcholine difference spectrum with P-450$_{LM2}$ and P-450$_{LM4}$. In each experiment, 1.0 ml of 17.2 μM cytochrome in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol and 1.0 mM EDTA, was placed in one compartment of a pair of tandem cells. The other compartment contained 2.0 mg of dilauroylglyceryl-3-phosphorylcholine sonicated in 1.0 ml of the same buffer mixture. The total light path was 0.875 cm in each cell. After the baseline was recorded, the solutions in one of the tandem cells were mixed, and the difference spectrum was recorded.

is already largely in the high spin state, a large spectral change due to the addition of phospholipid would not be expected.

In earlier experiments, solubilized but unpurified P-450$_{LM}$ was shown to have an apparent molecular weight of about 350,000 by several techniques, but this value was considered provisional in view of the heterogeneity of the preparation (33). As indicated in Table I, the apparent molecular weights of purified P-450$_{LM2}$ and P-450$_{LM4}$ determined by calibrated Sepharose 6B column chromatography in the absence of added detergent or lipid were 300,000 and 500,000, respectively. Clearly, therefore, the purified proteins are in an aggregated state, and from the known minimal molecular weights the aggregates would appear to contain about six and nine heme polypeptides in P-450$_{LM2}$ and P-450$_{LM4}$, respectively. Recently, a sedimentation coefficient of 13.5 S and molecular weight of 330,000 were reported for P-450$_{LM2}$ (34), in good agreement with our results. Upon the addition of dilauroylglyceryl-3-phosphorylcholine the apparent molecular weights determined by gel filtration increased somewhat. Since at least 20 molecules of this phospholipid are known to be bound per polypeptide chain of P-450$_{LM2}$ (32) and since similar data are not yet available for P-450$_{LM4}$, the most reasonable interpretation of the data is that the changes shown in the table may be largely due to lipid binding rather than an increase in the aggregation state of the cytochromes.

The effect of phospholipid on the cytochromes was also examined by CD spectrophotometry. In experiments not shown, the CD spectra for P-450$_{LM2}$ and P-450$_{LM4}$ were unchanged in the visible region upon the addition of dilauroylglyceryl-3-phosphorylcholine at a final concentration of 1.0 mg/ml. The result with P-450$_{LM2}$ was somewhat un-

TABLE I

MOLECULAR WEIGHTS OF P-450$_{LM_2}$ AND P-450$_{LM_4}$[a]

Cytochrome	Estimated molecular weights		
	Apparent, phospholipid absent	Apparent, phospholipid present	Minimal[b]
P-450$_{LM_2}$	300,000	500,000	49,000
P-450$_{LM_4}$	500,000	650,000	55,000

[a] The apparent molecular weights were determined by gel filtration chromatography at 4°C on a Sepharose 6B column (1.6 × 58 cm) previously equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol and 1.0 mM EDTA. The column was saturated with P-450$_{LM_2}$ or P-450$_{LM_4}$, and 1 ml of equilibrating buffer mixture containing 40 nmol of cytochrome with or without 1.0 mg of dilauroylglyceryl-3-phosphorylcholine was then added, followed by more of the equilibrating buffer mixture. The standards used for molecular weight calibration were IgG (160,000), pyruvate kinase (237,000), apoferritin (480,000), β-galactosidase (520,000), and thyroglobulin (670,000).

[b] The minimal molecular weights are based on amino acid analysis and also include heme and carbohydrate, as reported previously (7).

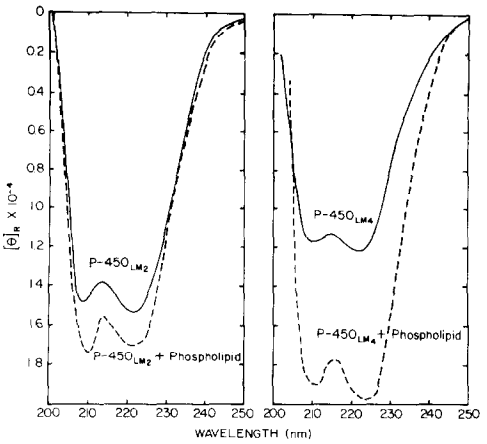

FIG. 9. Effect of phospholipid on CD spectra of cytochromes in far uv region. The ellipticity was expressed as mean residue ellipticity using a mean residue weight of 113. The spectra of P-450$_{LM2}$ (0.17 mg protein/ml, including apoenzyme) and P-450$_{LM4}$ (0.10 mg protein/ml, including apoenzyme) in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol and 1.0 mM EDTA, were determined in the presence and absence of dilauroylglyceryl-3-phosphorylcholine. The final concentration of the phospholipid was 50 μg/ml.

expected in view of the phospholipid binding spectrum reported above. However, a change in the CD spectra of the cytochromes was observed in the far uv region, as shown in Fig. 9. The data were examined by the method of Greenfield and Fasman (30) for determining α-helical content, and it was calculated that P-450$_{LM2}$ had undergone an apparent increase from 37 to 44% upon the addition of phospholipid. With P-450$_{LM4}$ the effect was more pronounced, with an apparent increase from 25 to 49%.

DISCUSSION

The data presented in this paper clearly demonstrate that P-450$_{LM2}$ and P-450$_{LM4}$ differ in their CD spectra. Ferric P-450$_{LM2}$, which is isolated in the low spin state, has a negative Soret CD band which is twofold stronger and at a longer wavelength than the corresponding band of ferric P-450$_{LM4}$, which is isolated as an apparent equilibrium mixture of low and high spin states. The addition of Renex, which changes P-450$_{LM4}$ from the state characterized by a Soret absorption maximum at 395 nm to that at 418 nm, causes a similar red shift of the Soret band in the CD spectrum. Furthermore, increasing the P-450$_{LM4}$ concentration (data not presented) or the addition of aniline has similar effects on both the absorption and CD spectra. In this connection, much remains to be learned about the heme environment in these two cytochromes, including the identity of the sixth ligand. In the ferrous state, P-450$_{LM2}$ and P-450$_{LM4}$ have highly similar CD spectra, with Soret bands at about 395 nm, suggesting that significant conformational changes had occurred, particularly upon reduction of ferric P-450$_{LM2}$. A shift of the Soret CD peak similar to that observed upon the reduction of P-450$_{LM4}$ was found for P-450$_{cam}$ by Peterson (26). Dawson *et al.* (20) have reported that the magnetic CD spectra of ferric P-450$_{LM2}$ and P-450$_{LM4}$ are different due to differences in spin state, whereas the spectra of the ferrous cytochromes and their CO complexes are similar. Their interpretation of these changes is similar to that presented above.

Ruckpaul *et al.* (17) presented evidence earlier that the negative CD Soret band of partially purified P-450$_{LM}$ from phenobarbital-induced rabbit liver microsomes is very strong and calculated the rotational strength, dipole strength, and anisotropy from resolved Gaussian curves of CD and absorption spectra. They interpreted the low anisotropy value as indication of a relatively open heme pocket. Using the same reasoning, our results indicate that the heme pocket of ferric P-450$_{LM4}$ is probably less open than that of ferric P-450$_{LM2}$. This interpretation is in accord with a solvent perturbation difference spectral study reported previously (1). The high accessibility of the heme iron of partially purified P-450$_{LM}$ and of purified P-450$_{cam}$ has also been shown by nmr studies of the rate of solvent proton relaxation (35, 36). The large decrease in intensity of the CD Soret band upon reduction of P-450$_{LM2}$ may reflect the closing of the heme pocket to give a structure similar to that of reduced P-450$_{LM4}$. The closing of the heme crevice upon reduction has been reported for other hemeproteins as well (37, 38).

The change of the CD Soret band of P-450$_{LM4}$ from negative to positive upon conversion to P-420 by sodium dodecyl sul-

fate treatment (see Fig. 6) is quite striking. The decrease and eventual loss of the CD spectrum of P-450$_{LM2}$ on conversion to P-420 by this detergent, on the other hand, indicates that the heme is now in a symmetrical environment. P-420 made from both P-450$_{LM2}$ and P-450$_{LM4}$ by KSCN treatment is inactive in CD.

The studies on tryptophan fluorescence emission show the quenching effect of heme or of other amino acids in native P-450$_{LM4}$. Presumably energy transfer from tryptophan to heme is possible for suitably positioned tryptophan residues. The nonquenched tryptophan residues may possible be located in highly hydrophobic domains, as indicated by the fluorescence maxima at 320 to 330 nm, according to studies by Teale (39).

Phosphatidylcholine, which greatly enhances the rate of electron transfer from NADPH to cytochrome P-450, catalyzed by NADPH-cytochrome P-450 reductase (31), and also the rate of substrate hydroxylation in the reconstituted enzyme system (4), does not cause the formation of larger aggregates or membrane-like structures. We have previously demonstrated that in the presence of the phospholipid the apparent K_D of the reductase and the K_s of benzphetamine for P-450$_{LM2}$ are significantly decreased (32), but the reason for these changes was not clear. The results presented here showing an apparent effect of phospholipid on the α-helical content of P-450$_{LM4}$ (and to a somewhat lesser extent with P-450$_{LM2}$) suggest that the changes in catalytic activity and binding constants may result from structural changes in the cytochromes. The possibility should also be considered, however, that the CD spectral changes observed in the far uv region may result in part from slight changes in turbidity and therefore in light scattering (40).

ACKNOWLEDGMENTS

The authors are grateful to Sylvia B. Dahl for purifying cytochrome P-450 and to Susan O. Krezoski for electrophoretic examination of the proteins.

REFERENCES

1. VATSIS, K. P., CHIANG, Y. L., AND COON, M. J. (1977) *Fed. Proc.* **36**, 832.
2. CHIANG, Y. L. (1978) *Fed. Proc.* **37**, 1719.
3. HAUGEN, D. A., VAN DER HOEVEN, T. A., AND COON, M. J. (1975) *J. Biol. Chem.* **250**, 3567–3570.
4. COON, M. J., VERMILION, J. L., VATSIS, K. P., FRENCH, J. S., DEAN, W. L., AND HAUGEN, D. A. (1977) *in* Drug Metabolism Concepts (Jerina, D. M., Ed.), American Chemical Society Symposium Series, No. 44, pp. 46–71, American Chemical Society, Washington, D. C.
5. VAN DER HOEVEN, T. A., AND COON, M. J. (1974) *J. Biol. Chem.* **249**, 6302–6310.
6. VAN DER HOEVEN, T. A., HAUGEN, D. A., AND COON, M. J. (1974) *Biochem. Biophys. Res. Commun.* **60**, 569–575.
7. HAUGEN, D. A., AND COON, M. J. (1976) *J. Biol. Chem.* **251**, 7929–7939.
8. IMAI, Y., AND SATO, R. (1974) *Biochem. Biophys. Res. Commun.* **60**, 8–14.
9. HASHIMOTO, C., AND IMAI, Y. (1976) *Biochem. Biophys. Res. Commun.* **68**, 821–827.
10. RYAN, D., LU, A. Y. H., KAWALEK, J., WEST, S. B., AND LEVIN, W. (1975) *Biochem. Biophys. Res. Commun.* **64**, 1134–1141.
11. KAWALEK, J. C., LEVIN, W., RYAN, D., THOMAS, P. E., AND LU, A. Y. H. (1975) *Mol. Pharmacol.* **11**, 874–878.
12. JOHNSON, E. F., AND MULLER-EBERHARD, U. (1977) *J. Biol. Chem.* **252**, 2839–2845.
13. DEAN, W. L., AND COON, M. J. (1977) *J. Biol. Chem.* **252**, 3255–3261.
14. HAUGEN, D. A., ARMES, L. G., YASUNOBU, K. T., AND COON, M. J. (1977) *Biochem. Biophys. Res. Commun.* **77**, 967–973.
15. YONG, F. C., KING, T. E., OLDHAM, S., WATERMAN, M. R., AND MASON, H. S. (1970) *Arch. Biochem. Biophys.* **138**, 96–100.
16. SHICHI, H., KUMAKI, K., AND NEBERT, D. W. (1978) *Chem. Biol. Interact.* **20**, 133–148.
17. RUCKPAUL, K., REIN, H., JÄNIG, G.-R., WINKLER, W., AND RISTAU, O. (1977) *Croat. Chem. Acta* **49**, 339–346.
18. SHIMIZU, T., NOZAWA, T., HATANO, M., IMAI, Y., AND SATO, R. (1975) *Biochemistry* **14**, 4172–4178.
19. COLLMAN, J. P., SORRELL, T. N., DAWSON, J. H., TRUDELL, J. R., BUNNENBERG, E., AND DJERASSI, C. (1976) *Proc. Nat. Acad. Sci. USA* **73**, 6–10.
20. DAWSON, J. H., TRUDELL, J. R., LINDER, R. E., BARTH, G., BUNNENBERG, E., AND DJERASSI, C. (1978) *Biochemistry* **17**, 33–42.
21. WANG, H.-P., AND KIMURA, T. (1976) *J. Biol. Chem.* **251**, 6068–6074.
22. COON, M. J., VAN DER HOEVEN, T. A., DAHL, S. B., AND HAUGEN, D. A. (1978) *in* Methods in Enzymology, Vol. 52, pp. 109–117, Academic Press, New York.
23. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.

24. OMURA, T., AND SATO, R. (1964) *J. Biol.Chem.* **239,** 2370–2378.
25. SPANDE, T. F., AND WITKOP, B. (1967) *in* Methods in Enzymology (Hirs, C. H. W., Ed.), Vol. 11, pp. 498–506, Academic Press, New York.
26. PETERSON, J. A. (1971) *Arch. Biochem. Biophys.* **144,** 678–693.
27. HANSON, L. K., EATON, W. A., SLIGAR, S. G., GUNSALUS, I. C., GOUTERMAN, M., AND CONNELL, C. R. (1976) *J. Amer. Chem. Soc.* **98,** 2672–2674.
28. IMAI, Y., AND SATO, R. (1967) *Eur. J. Biochem.* **1,** 419–426.
29. ICHIKAWA, Y., AND YAMANO, T. (1967) *Biochim. Biophys. Acta* **131,** 490–497.
30. GREENFIELD, N., AND FASMAN, G. D. (1969) *Biochemistry* **8,** 4108–4116.
31. STROBEL, H. W., LU, A. Y. H., HEIDEMA, J., AND COON, M. J. (1970) *J. Biol. Chem.* **245,** 4851–4854.
32. COON, M. J., HAUGEN, D. A., GUENGERICH, F. P., VERMILION, J. L., AND DEAN, W. L. (1976) *in* The Structural Basis of Membrane Function (Hatefi, Y., and Djavadi-Ohaniance, L., Eds.), pp. 409–427, Academic Press, New York.
33. AUTOR, A. P., KASCHNITZ, R. M., HEIDEMA, J. K., AND COON, M. J. (1973) *Mol. Pharmacol.* **9,** 93–104.
34. BEHLKE, J., JÄNIG, G.-R., AND PFEIL, D. (1979) *in* Proceedings of the Scientific Conference on Cytochrome P-450-Structural and Functional Aspects (Coon, M. J., Gunsalus, I. C., Maričić, S., and Ruckpaul, K., Eds.), Acta Biol. Med. Ger., in press.
35. GRIFFIN, B. W., AND PETERSON, J. A. (1975) *J. Biol. Chem.* **250,** 6445–6451.
36. REIN, H., MARIČIĆ, S., JÄNIG, G.-R., VUK-PAVLOVIC, S., BENKO, B., RISTAU, O., AND RUCKPAUL, K. (1976) *Biochim. Biophys. Acta* **446,** 325–330.
37. TAKANO, T., KALLAI, O. B., SWANSON, R., AND DICKERSON, R. E. (1973) *J. Biol. Chem.* **248,** 5234–5255.
38. KAMINSKY, L. S., CHIANG, Y. L., AND KING, T. E. (1975) *J. Biol. Chem.* **250,** 7280–7287.
39. TEALE, F. W. J. (1960) *Biochem. J.* **76,** 381–388.
40. URRY, D. W., MASOTTI, L., AND KRIVACIC, J. (1970) *Biochem. Biophys. Res. Commun.* **41,** 521–524.