Ts'o, P. O. P. (1976), *Biopolymers 15,* 2277.
Govil, G. (1976), *Biopolymers 15,* 2303.
Hingerty, B., Subramanian, E., Stellman, S. D., Broyde, S. B., Sato, T., and Langridge, R. (1975), *Biopolymers 14,* 227.
Johnson, E. D., and Bovey F. A. (1958), *J. Chem. Phys. 29,* 1012.
Kim, S. H., Berman, H. M., Seeman, N. C., and Newton, M. D. (1973), *Acta Crystallogr., Sect. B 29,* 703.
Lee, C. H., and Tinoco, I., Jr. (1977), *Biochemistry 16,* 5403.
Lee, C. H., Ezra, F. S., Kondo, N. S., Sarma, R. H., and Danyluk, S. S. (1976), *Biochemistry 15,* 3627.
Perahia, D., Pullman, B., and Saran, A. (1974), *Biochim. Biophys. Acta 340,* 299.
Remin, M., and Shugar, D. (1972), *Biochem. Biophys. Res. Commun. 48,* 636.
Rubin, J., Brennan, T., and Sundaralingam, M. (1972), *Biochemistry 11,* 3112.
Seeman, N. C., Rosenberg, J. M., Suddath, F. L., Kim, J. J. P., and Rich, A. (1976), *J. Mol. Biol. 104,* 109.
Son, T. D., and Guschlbauer, W. (1975), *Nucleic Acids Res. 2,* 873.
Stout, C. D., Mizuno, H., Rubin, J., Brennan, T., Rao, S. T., and Sundaralingam, M. (1976), *Nucleic Acids Res. 3,* 1111.
Waugh, J. S., and Fessenden, R. W. (1957), *J. Am. Chem. Soc. 79,* 846.

Resonance Raman Studies of Hepatic Microsomal Cytochromes P-450: Evidence for Strong π Basicity of the Fifth Ligand in the Reduced and Carbonyl Complex Forms†

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ABSTRACT: Resonance Raman spectra have been measured for cytochromes P-450 purified from liver microsomes of phenobarbital-treated rabbits (PB P-450 and PB P-448) and of 3-methylcholanthrene-treated rabbits (MC P-448). In the reduced state, all three cytochromes P-450 exhibit Raman spectra of ferrous high-spin type but show the so-called "oxidation state marker" (band IV) at unusually low frequencies, indicating extensive delocalization of electrons from the iron d_π orbital to the porphyrin $\Pi^*(e_g)$ orbital and, consequently, the strong π basicity of the fifth ligand of the heme iron. The reduced CO complexes of the cytochromes P-450 also exhibit band IV at markedly lower frequencies than CO complexes of hemoglobin and myoglobin. These anomalies observed for the reduced form and CO complex disappear upon conversion of the cytochromes to the catalytically inactive form called cytochrome P-420. Oxidized PB P-450 shows a Raman spectrum which is characteristic of typical ferric low-spin heme compounds, whereas those of PB P-448 and MC P-448 are of the ferric high-spin type. PB P-450 is also clearly distinguishable from the two P-448 preparations in the reduced state. The reduced form of cytochrome P-420, produced by laser illumination, exhibits two sets of Raman lines and, therefore, seems to be a mixture of both high- and low-spin species.

Cytochrome P-450 (P-450)[1] is a generic name for a family of protoheme-containing proteins that are widely distributed in nature and catalyze a variety of monooxygenation (hydroxylation) reactions of metabolic importance. The reduced CO complex of this class of hemoproteins exhibits the Soret absorption band at about 450 nm, a wavelength which is approximately 30 nm longer than that observable for usual ferrous protoheme–CO complexes (Sato et al., 1973). This and other spectral anomalies of P-450 disappear when it is converted into the catalytically inactive form called cytochrome P-420 (P-420) by various treatments (Sato et al., 1973). Currently, extensive efforts are being made to elucidate the structural basis of the spectral anomalies of P-450 by both physicochemical and coordination chemical approaches.

It has been shown that synthetic protohemin complexes having a thiolate anion (RS^-) and an arbitrary nitrogenous base as axial ligands to the heme iron display electron paramagnetic resonance (EPR) spectra that are closely similar to that exhibited by P-450 in the ferric low-spin state (Collman et al., 1975; Koch et al., 1975; Tang et al., 1976; Ogoshi et al., 1975). Model experiments have also provided evidence that the heme in the substrate complex of oxidized P-450 is in the pentacoordinated high-spin state possessing a thiolate anion as the fifth ligand (Collman et al., 1975; Tang et al., 1976). These conclusions are being supported by Mössbauer (Koch et al., 1975) and magnetic circular dichroism (MCD) studies (Dawson et al., 1976). Nuclear magnetic resonance (NMR) (Keller et al., 1972) and Mössbauer spectra (Sharrock et al., 1973) of the substrate complex of reduced P-450 have suggested that its heme iron is in the ferrous high-spin state. It has further been reported that synthetic ferrous protoheme complexes having a thiolate anion, but not a nitrogenous base, as a ligand trans to CO exhibit the unusual Soret absorption band characteristic of the CO complex of reduced P-450 (Stern and

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[1] Abbreviation used are: P-450, cytochrome P-450; $P\text{-}450_{cam}$, cytochrome P-450 from camphor-grown *Pseudomonas putida*; P-420, cytochrome P-420; Hb, hemoglobin; Mb, myoglobin; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; MCD, magnetic circular dichroism; CM, carboxymethyl.

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Peisach, 1974; Collman and Sorrell, 1975; Chang and Dolphin, 1975; Chang and Dolphin, 1976). This structure for the CO complex of P-450 has also been suggested by MCD studies (Vickery et al., 1975; Shimizu et al., 1975; Collman et al., 1976).

Resonance Raman spectroscopy of hemoproteins gives vibrational spectra of the iron–porphyrin in situ interacting with its immediate environments (Spiro, 1975). It is possible from the so-called "oxidation state marker" [which will be referred to as band IV in this paper as in Kitagawa et al. (1976)] to distinguish two types (σ and π) of iron–ligand interactions, namely, d_{z2}(Fe)–lone pair (ligand) and d_{π}(Fe)–Π^* (ligand) interactions for ferrous low-spin hemoproteins (Kitagawa et al., 1976). A study of ^{15}N-enriched iron porphyrin has made it possible to assign band IV (Abe et al., 1978) and indicated that the frequency of band IV reflects the amount of electrons delocalized to the porphyrin $\Pi^*(e_g)$ orbital (Kitagawa et al., 1977a); as electrons are delocalized from the iron d_{π} orbital to the porphyrin $\Pi^*(e_g)$ orbital in the ferrous low-spin state, band IV shifts to lower frequency. It is therefore expected that the resonance Raman spectra of the P-450's are characterized by strong π basicity due to the thiolate anion, which is coordinated to the heme iron as can be deduced from the aforementioned studies. It has, in fact, been found that the frequency of band IV observed for the reduced form of P-450 purified from camphor-grown *Pseudomonas putida* ($P\text{-}450_{cam}$) is unusually low (Ozaki et al., 1976), although no such anomaly has been detected in the oxidized form (Champion and Gunsalus, 1977).

Owing to recent progress in purification techniques for membrane-bound P-450's, it is now established that mammalian liver microsomes contain multiple species of P-450 and one or more of these species are specifically induced by the administration of a drug to the animal (Imai and Sato, 1974a,b; Ryan et al., 1975; Haugen and Coon, 1976; Hashimoto and Imai, 1976). It is, therefore, of interest to examine whether or not the above-mentioned anomaly in the Raman spectrum observed for $P\text{-}450_{cam}$ can be also detected in the various species of hepatic microsomal P-450's. In the present study, we measured the resonance Raman spectra of three preparations of liver microsomal P-450's, two of which were purified from phenobarbital-treated rabbits (PB P-450 and PB P-448) and one from 3-methylcholanthrene-treated rabbits (MC P-448). Optical absorption and EPR studies, which will be published elsewhere, have indicated that the heme in oxidized PB P-450 (M_r = 50 000) is in the ferric low-spin state and that in PB P-448 (M_r = 54 000) and MC P-448 (M_r = 54 000) is in the ferric high-spin state.

Experimental Procedure

Gel electrophoretically homogeneous preparations of PB P-450 (Imai and Sato, 1974) and MC P-448 (Hashimoto and Imai, 1976) were isolated from phenobarbital- and 3-methylcholanthrene-induced rabbit liver microsomes, respectively, by the published methods. The method for purification of PB P-448 from phenobarbital-induced rabbit liver microsomes will be reported elsewhere. In brief, PB P-448 was separated from PB P-450 on the basis of the difference in elution behavior from a hydroxylapatite column and purified by subsequent CM-Sephadex C-50 column chromatography. The specific contents of PB P-450, PB P-448, and MC P-448 were 17.7, 17.3, and 17.5 nmol of P-450 chromophore/mg of protein, respectively. PB P-448 and MC P-448 were dissolved in 100 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, whereas the solvent for PB P-450 contained 0.2% (w/v) Emulgen 913 in addition to the phosphate–glycerol. Equine skeletal muscle myoglobin (Mb) (Sigma type I) was further purified by CM-cellulose column chromatography as metmyoglobin (metMb). $MetMbSCH_3$ (pH 8.2) and $metMbSC_2H_5$ were formed by adding an excess amount of $NaSCH_3$ and $NaSC_2H_5$, respectively, to 0.7 mM metMb solution in 10 mM potassium phosphate buffer (pH 7.2). $MetMbN_3$ was obtained by adding a 50-fold excess of NaN_3 to the metMb solution.

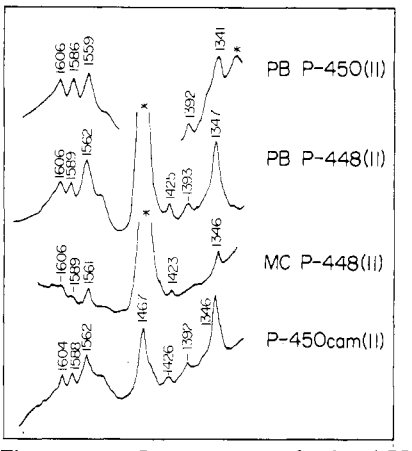

FIGURE 1: The resonance Raman spectra of reduced PB P-450 (100 μM), PB P-448 (100 μM), MC P-448 (50 μM), and $P\text{-}450_{cam}$. The last one was reproduced with permission from Ozaki et al. (1976). Copyright 1976 Japanese Biochemical Society. Solvent: 100 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol for PB P-448 and MC P-448, and 0.2% (w/v) Emulgen 913 was also included for PB P-450. Instrumental conditions: excitation, 488.0 nm; laser power at sample point, 60 mW for PB P-450, 180 mW for PB P-448 and MC P-448; slit width, 3 cm^{-1}; time constant, 8 s; scan speed, 10 cm^{-1}/min.

For measurement of resonance Raman spectra, 100 μL of 0.02 to 0.2 mM P-450 solution was placed in a cylindrical cell, and the laser light was illuminated from the bottom of the cell. The scattered light was collected at right angles from the incident light. The temperature of the sample was maintained below 10 °C by flushing cold nitrogen gas to the front edge of the cell, where the laser light entered. All P-450's were reduced by a small amount of solid dithionite. Before and after reduction nitrogen gas was flushed into the cell solution without bubbling. After each measurement, CO was bubbled into the reduced P-450 solution in the Raman cell, and the absorption spectrum of the reduced CO complex was measured in a micro cell (1 × 3 × 30 mm^3) by using a Hitachi 124 spectrophotometer to determine the P-450 remaining. Resonance Raman spectra were measured with the 488.0-nm line of an argon ion laser (Spectra Physics, Model 164-02) and were recorded in a JEOL-400D Raman spectrometer equipped with a HTV-R649 photomultiplier. The frequency calibration of the spectrometer was performed with indene as a standard (Hendra and Loader, 1968) within an accuracy of ±1 cm^{-1}.

Results

The resonance Raman spectra of reduced PB P-450, PB P-448, and MC P-448 are shown in Figure 1, which also includes that of reduced $P\text{-}450_{cam}$ reported previously (Ozaki et al., 1976) for comparison. The intense Raman line seen around 1465 cm^{-1} was due to glycerol present in the solvent. Since PB P-450, but not the two PB P-448 preparations, was relatively unstable, one solution of reduced PB P-450 was used for recording the spectrum between 1300 and 1400 cm^{-1} and another fresh solution from the same batch for recording the rest of the spectrum to minimize its conversion to the P-420

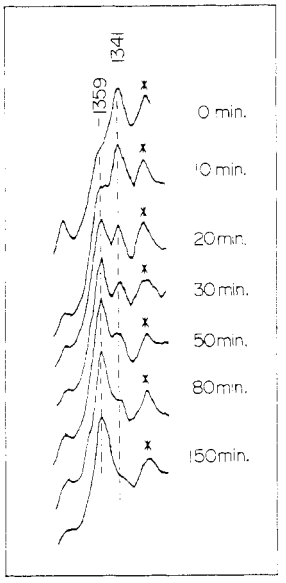

FIGURE 2: Time-dependent change in the Raman spectrum of reduced PB P-450 during the laser illumination. Time specified beside each spectrum shows the scan starting time measured from the initiation of laser illumination. Laser power was 180 mW, and other conditions were the same as those in Figure 1.

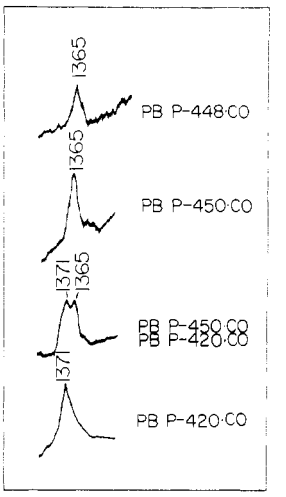

FIGURE 3: Band IV of the CO complexes of PB P-448 (100 μM), PB P-450 (100 μM), and PB P-420. The spectrum designated by PB P-450·CO + PB P-420·CO was measured 14 min after the initiation of laser illumination (50 mW). PB P-420·CO was produced by laser illumination for 30 min. Instrumental conditions were the same as those in Figure 1, except a laser power of 50 mW was used in this measurement.

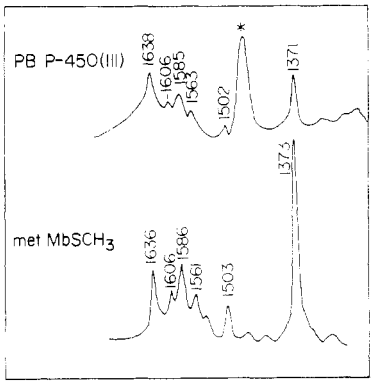

FIGURE 4: The resonance Raman spectra of oxidized PB P-450 (100 μM) and $MbSCH_3$ (500 μM). Laser power: 60 mW for PB P-450 and 180 mW for $MbSCH_3$. Other instrumental conditions were the same as those in Figure 1.

form. As can be seen, all three preparations in the reduced state showed the spectra which were essentially identical with that of reduced $P\text{-}450_{cam}$ and were characteristic of ferrous high-spin hemes (Kitagawa et al., 1976). Reduced PB P-450, PB P-448, and MC P-448 exhibited band IV at 1341, 1347, and 1346 cm^{-1}, respectively, as polarized lines. When 1-pentanol (final concentration 1%) was added to oxidized MC P-448 (107 μM) and then reduced, there was a shift of band IV to 1342 cm^{-1}. This shift of band IV in the reduced state was presumably related to the change of absorption spectrum in the oxidized state from the high- to low-spin type upon the addition of 1-pentanol (Yoshida and Kumaoka, 1975). It therefore appears that reduced P-450's which are in the high-spin state in the oxidized form exhibit band IV at 1346–1347 cm^{-1}, whereas those which are in the low-spin state in the oxidized form display band IV at 1341–1342 cm^{-1}. In any way, these two sets of frequencies are markedly lower than the standard frequencies of band IV so far measured for ferrous high-spin hemoproteins, i.e., 1356 cm^{-1} for dHb (Kitagawa et al., 1976), 1356 cm^{-1} for dMb (Kitagawa et al., 1976), and 1355 cm^{-1} for type *a* of reduced cytochrome c′ (Kitagawa et al., 1977b).

When reduced PB P-450 was subjected to the laser illumination (~180 mW) at 10 °C, there occurred gradual conversion into the P-420 form, as evidenced by the appearance of Soret band at about 420 nm on addition of CO. This conversion was accompanied by a change in the band IV region of the Raman spectrum, as illustrated in Figure 2. It can be seen that the laser illumination caused a gradual intensity decrease in the Raman line at 1341 cm^{-1} and the appearance of a new line at 1359 cm^{-1}. The 1341-cm^{-1} line disappeared completely 150 min after the initiation of illumination, at which time the complete conversion to the P-420 form could be confirmed by the absorption spectrum of its CO complex. It is, therefore, reasonable to assign the 1341- and 1359-cm^{-1} lines to band IV of P-450 and P-420, respectively. If so, the decrease in intensity of the 1341-cm^{-1} line should parallel the increase in intensity of the 1359-cm^{-1} line, but estimation of their precise intensities relative to the standard line (at 1317 cm^{-1}, due to solvent) was impossible because of a continuous change of the base line with time. A rough estimate of the peak height from a reasonable base line indicated that 50% conversion into P-420 took place ca. 20 min after the initiation of illumination at ~180 mW and ca. 40 min at ~ 60 mW. Both reduced PB P-448 and MC P-448 did not show any tendency for conversion to P-420 under the same illumination conditions.

Figure 3 illustrates the Raman spectra in the band IV region for the CO complexes of reduced PB P-448 and PB P-450. Both CO complexes exhibited band IV at 1365 cm^{-1} in spite of the significant difference observed in band IV of their ferrous high-spin state. It should be noted that in these measurements the laser power was set as low as 50 mW to minimize the photodissociation of the CO complexes. As shown in Figure 3, two Raman lines were observable at 1365 and 1371 cm^{-1} for the PB P-450·CO complex only 14 min after the initiation of illumination, and within 30 min the 1365-cm^{-1} line disappeared completely, accompanied by intensification of the 1371-cm^{-1} line. The CO complex of PB P-448 did not show such a spectral change. The absorption spectrum showed that the CO complex of PB P-450, but not that of PB P-448, was converted to the P-420·CO form during the laser illumination. Therefore, it could be concluded that the reduced CO complex of PB P-420 shows band IV at 1371 cm^{-1} and that the conversion of PB P-450 to the P-420 form occurs more rapidly in

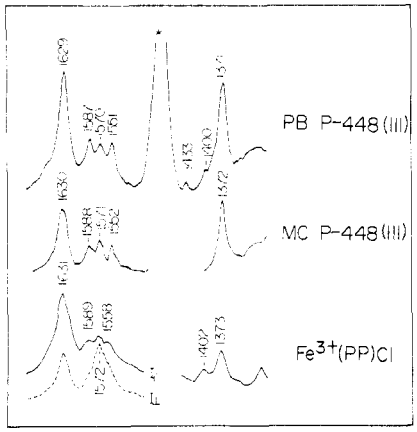

FIGURE 5: The resonance Raman spectra of oxidized PB P-448 (100 μM), MC P-448 (30 μM), and hemin in 1% sodium dodecyl sulfate solution [Fe^{3+} (PP)Cl]. $I_{\parallel}$ and $I_{\perp}$ in the 1500–1650 cm^{-1} region for Fe^{3+} (PP)Cl denote the electric vector of scattered radiation parallel and perpendicular to that of the incident radiation, respectively. Instrumental condtions were the same as those in Figure 1.

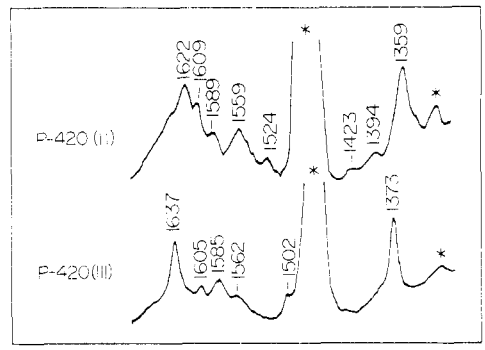

FIGURE 6: The resonance Raman spectra of reduced and oxidized PB P-420. Reduced PB P-420 was produced from reduced PB P-450 by laser illumination for 150 min (180 mW). Oxidized PB P-420 was obtained by adding 1 M KSCN to oxidized PB P-450. Complete conversion to the P-420 form in both cases was confirmed by measuring the absorption spectrum of their heme-CO complex later.

the reduced CO complex than in the reduced form.

The Raman spectra of oxidized PB P-450 and metMbSCH$_3$ are shown in Figure 4. Although data are not shown, MbSC$_2$H$_5$ exhibited a Raman spectrum essentially similar to that of MbSCH$_3$. The frequencies of the Raman lines in the spectrum of oxidized PB P-450 were almost identical with those of MbSCH$_3$, though their relative intensities were somewhat different from each other. However, this coincidence did not seem to provide evidence for the coordination of a thiolate anion to the heme of oxidized PB P-450, because MbN$_3$ also showed a Raman spectrum which was very similar to that of MbSCH$_3$ (data not shown). These results seem to imply that the heme in oxidized PB P-450 is in the ferric low-spin state and free from strains caused by the protein moiety. In Figure 5 are compared the Raman spectra of oxidized PB P-448 and MC P-448 with that of ferric iron protoporphyrin chloride reported previously (Kitagawa et al., 1977b). It is evident that the three spectra are similar to one another. This suggested that the heme in both oxidized PB P-448 and MC P-448 is in the ferric high-spin state free from structural distortions, although Spiro and Burke (1976) have pointed out that the effect of the protein moiety on the heme structure appears most sensitively in the ferric high-spin state. The results shown in Figures 4 and 5 also indicated that the frequencies of band IV of oxidized P-450's (1371–1372 cm^{-1}) are not atypical, unlike the case of their reduced form.

In the frequency region below 800 cm^{-1}, oxidized PB P-448 showed two prominent Raman lines at 349 and 755 cm^{-1} (data not shown). These two lines clearly correspond to those of oxidized P-450$_{cam}$ at 351 and 754 cm^{-1} reported by Champion and Gunsalus (1977), who also detected an additional line at 691 cm^{-1} and suggested the appearance of C–S stretching mode of the fifth ligand. Because of the presence of an intense Raman line due to glycerol in this frequency region, it was not possible for us to confirm this Raman line in oxidized PB P-448. We attempted to measure the Raman spectrum in the presence of 5% glycerol, but the solution became turbid under this condition during the measurement.

Figure 6 shows the Raman spectra of reduced and oxidized P-420. The reduced P-420 employed in these measurements was produced from reduced PB P-450 by prolonged illumination with the laser light (150 min, at 180 mW). The Raman spectrum of reduced P-420 is characterized by four prominent Raman lines in the 1550–1600-cm^{-1} region (1622, 1609, 1589, and 1559 cm^{-1}), where only two lines are usually observable for typical ferrous low-spin hemoproteins such as reduced cytochrome b_5 (1617 and 1585 cm^{-1}) (Kitagawa et al., 1976). Therefore, little doubt remains that the lines at 1622 and 1589 cm^{-1} in the spectrum of reduced P-420 were due to the ferrous low-spin heme species. On the other hand, the Raman lines at 1609 and 1559 cm^{-1} are usually intense in the ferrous high-spin hemoproteins such as dMb (Kitagawa et al., 1976), suggesting that these two lines in reduced P-420 were due to the ferrous high-spin heme species. The presence of two sets of Raman lines strongly suggests the coexistence of two molecular species, i.e., high- and low-spin species, in the reduced P-420 preparation employed. An attempt was made to prove the temperature-dependent equilibrium between the high- and low-spin species by measuring the Raman spectrum under a wide range of temperatures, but Raman measurements in the frozen state were unsuccessful.

The oxidized P-420 preparation employed in Figure 6 was prepared by adding 1 M KSCN to oxidized PB P-450 (Imai and Sato, 1967). Two other oxidized P-420 preparations, produced from oxidized PB P-450 by the laser illumination (240 mW) for 4 h and by adding NaOH to pH 7.8, were also used (data not shown). All three preparations exhibited almost the same Raman spectra, which were also closely similar to that of the oxidized PB P-450 (Figure 4), indicating the ferric low-spin nature of the heme in oxidized P-420. Circumstantial evidence suggests that oxidized P-450 has a thiolate anion as the fifth ligand (Collman et al., 1975; Koch et al., 1975; Tang et al., 1976; Ogoshi et al., 1975) and that the thiolate anion is either protonated or replaced by some other ligand upon conversion into P-420. If this is the case, then the similarity of the Raman spectrum of oxidized P-420 to that of oxidized PB P-450 implies that the Fe^{3+}–S^- interaction in oxidized PB P-450 is not strong enough to cause an unusual Raman spectrum.

To facilitate the following discussion, the band IV frequencies measured in this and other studies for the reduced form, reduced CO complex, and oxidized form of various P-450's, P-420, hemoglobin, and myoglobin are summarized in Table I.

Discussion

Reduced P-450 and P-420. The general feature of Raman spectra of reduced PB P-450, PB P-448, and MC P-448 resemble those of dMb (Kitagawa et al., 1976) and type *a* of reduced cytochrome *c'* (Kitagawa et al., 1977b), indicating the presence of ferrous high-spin heme in these reduced P-

TABLE I: The Frequencies of Band IV of P-450 and Some Related Hemoproteins (cm^{-1}).

	reduced	reduced·CO	oxidized
PB P-450	1341	1365	1371
PB P-448	1347	1365	1371
MC P-448	1346		1371
$P\text{-}450_{cam}$ [a]	1346		1371
$P\text{-}450_{cam}$·MP [b]	1358		1371
PB P-420	1359	1371	1372
Hb	1356	1372 [c]	1371 [d]
Mb	1355	1370 [c]	1371 [d]

[a] Ozaki et al. (1976). [b] Mp, metyrapone; Ozaki et al. (1976). [c] Rimai et al. (1975). [d] Kitagawa et al. (1976).

450's. This conclusion is in agreement with that drawn from NMR (Keller et al., 1972) and Mössbauer studies (Sharrock et al., 1973). However, as shown in Table I, the frequencies of band IV for the reduced P-450's are notably lower than those for usual ferrous high-spin hemoproteins. The unusually low frequencies of band IV are probably caused by strong π basicity of the fifth ligand in these reduced P-450's.

Although band IV for all the reduced P-450's examined is located at lower frequencies than that for usual ferrous high-spin hemoproteins, the reduced P-450's can be classified into two groups, i.e., those exhibiting band IV at 1341–1342 cm^{-1} (MC P-448 in presence of 1-pentanol and PB P-450) and those showing band IV at 1346–1347 cm^{-1} (PB P-448 and MC P-448). The reduced form of the camphor complex of $P\text{-}450_{cam}$ also belongs to the latter group (1346 cm^{-1}) (Ozaki et al., 1976). It is interesting to note that the P-450's of the former and latter groups are in the low- and high-spin states, respectively, when they are in the oxidized form. This suggests that an appreciable difference exists in the basicity of the fifth ligand between the two groups of reduced P-450's. This difference may be explained by assuming a shorter Fe^{2+}–S^- distance or a smaller displacement of the Fe^{2+} ion from the porphyrin plane in the former than in the latter, because both structural features are expected to cause more pronounced delocalization of electrons to the porphyrin $\Pi^*(e_g)$ orbital and, consequently, a lower frequency of band IV. The similarity of frequencies of the other Raman lines suggests that the rest of the heme structure is similar in the reduced form of the two groups of P-450's.

When reduced PB P-450 is converted into reduced P-420, band IV shifts to the normal frequency of 1359 cm^{-1} (Figure 2), as in the case of the conversion of reduced $P\text{-}450_{cam}$ into P-420 (Ozaki et al., 1976). An explanation for this shift is to assume that protonation of the RS^- ligand takes place during the conversion. Since the coordination of neutral sulfur to ferrous heme does not cause any anomaly in the Raman spectrum, as seen for reduced cytochrome *c* (Kitagawa et al., 1975), protonation to the RS^- ligand would lead to the shift of band IV to the normal frequency. However, from the frequencies of the porphyrin ring vibrations, we cannot distinguish the heme to which RSH is bound from the heme to which an arbitrary nitrogenous base having only one lone pair is coordinated. In fact, Collman et al. (1976) have reported that the MCD spectrum of reduced P-420 resembles those of reduced heme model complexes having either a thiol or a nitrogenous base as the fifth ligand. Detection of an Fe–ligand stretching mode seems to be necessary to distinguish the two alternative possibilities.

It is well known that, depending upon conditions, P-420 may be observed in a high- or low-spin state or a state indetectable by EPR spectroscopy (Murakami and Mason, 1967). Only the EPR active P-420's are supposed to be reconvertible to P-450 through treatment with polyols or glutathione (Ichikawa and Yamano, 1967). Although, in the absorption spectra no multiplicity was recognized in the reduced state of the P-420's (Murakami and Mason, 1967), the Raman spectrum of reduced P-420 (Figure 6) provided clear evidence for the coexistence of high- and low-spin species.

CO Complex of Reduced P-450. Upon binding of CO to reduced hemoproteins, there occurs back-donation of electrons from the $d_\pi(Fe^{2+})$ to $\Pi^*(CO)$ orbital and, therefore, results in a shift of band IV to a higher frequency (Kitagawa et al., 1976). The back-donated electrons, which occupy the antibonding orbital of CO[$\Pi^*(CO)$], would weaken the C–O bond and thus lower the C–O stretching frequency (ν_{CO}). It is, therefore, expected that the back-donation of more electrons will lead to a higher frequency of band IV as well as to a lower frequency of ν_{CO}, unless any particular interaction exists between CO and the apoprotein.

As summarized in Table I, the band IV frequencies for the CO complexes of reduced PB P-450 and PB P-448 are clearly lower than those reported for HbCO and MbCO (Rimai et al., 1975), indicating that the strong π basicity of the fifth ligand is still causing considerable delocalization of electrons to the porphyrin $\Pi^*(e_g)$ orbital even in the CO complexes of these reduced P-450's. Band IV of the CO complex of reduced P-420 is, however, located at 1371 cm^{-1}, as in the case of HbCO and MbCO, indicating that P-420·CO has lost the characteristic feature of P-450·CO.

The finding that the C–O stretching frequency of PB P-450·CO (ν_{CO} 1949 cm^{-1}) (Rein et al., 1977) is as high as that of HbCO (ν_{CO} 1951 cm^{-1}) (Alben and Caughey, 1968) is unexpected, because the change in band IV frequency upon the formation of CO complex [$\Delta = \nu(Fe^{2+}\text{·CO}) - \nu(Fe^{2+})$] is fairly larger for P-450·CO (Δ = 24 cm^{-1}) than for HbCO (Δ = 17 cm^{-1}). This apparent inconsistency between Δ and ν_{CO} might be interpreted reasonably if the CO–histidine (distal) interaction in HbCO (Ikeda-Saito et al., 1977) is taken into account. The fact that the ν_{CO} of abnormal HbCO which lacks distal histidine is higher by more than 10 cm^{-1} than that of normal HbCO (Caughey et al., 1969) implies that the interaction lowers the ν_{CO} of HbCO more than expectable from simple back-donation. An alternative explanation assumes that the fifth ligand donates π electrons to porphyrin significantly

TABLE II: Comparison of the Frequencies of Several Raman Lines Sensitive to Distortion of Heme in the Ferric High-Spin State (cm^{-1}).

Fe^{3+} (PP)Cl [a]	Fe^{3+} (MP)Cl [b]	PB P-448	MC P-448	$P\text{-}450_{cam}$ [c]	CPO [d]	cyt *c'* [e]	H_2O·Mb [f]
1631 (dp)	1632	1629	1630	1623	1627	1631	1613
1589 (p)	1588	1587	1588	1584	1588	1583	1581
1572 (ap)	1572	1570	1571	1570	1566	1570	1562

[a] Iron protoporphyrin in 1% sodium dodecyl sulfate aqueous solution; Kitagawa et al. (1977b). [b] Iron mesoporphyrin dimethyl ester in CH_2Cl_2; Spiro and Burke (1976). [c] Ozaki et al. (1976). [d] Chloroperoxidase; Champion et al. (1976). [e] *R. rubrum* cytochrome *c'* (type II); Kitagawa et al. (1977b). [f] Sperm whale myoglobin at pH 7.0; Kitagawa et al. (1976).

but less to CO ligand trans to it.

Oxidized P-450. Unlike the case of reduced P-450's, no anomaly can be seen in the frequency of band IV for oxidized P-450's (see Table I). This suggests that the Fe^{3+}-S^- interaction, characterizing the EPR spectrum of oxidized P-450 (Tsai et al., 1970), is not so strong to exert any significant influence on the stretching frequencies of the C-C and C-N bonds of the porphyrin ring. In such a situation, it is unlikely that the C-S stretching mode of the fifth ligand appears in the resonance Raman spectrum, although Champion and Gunsalus (1977) have taken the C-S stretching vibration into consideration as a possible origin of the 691-cm^{-1} line of oxidized P-450_{cam}.

Spiro and Burke (1976) have pointed out that distortion of the heme by the apoprotein is most prominent in the ferric high-spin state. In fact, the frequencies of Raman lines for H_2O·metMb differ considerably from those for ferric high-spin iron porphyrin, particularly in the frequency region of 1500–1650 cm^{-1} (Kitagawa et al., 1976). The frequencies of several Raman lines in this frequency region determined for the oxidized forms of PB P-448 and MC P-448 are compared with those for some other ferric high-spin hemoproteins and iron porphyrins in Table II. As can be seen, ferric protoporphyrin chloride in 1% sodium dodecyl sulfate (Kitagawa et al., 1977b) gives the Raman frequencies which are most similar to those of PB P-448 and MC P-448. Therefore, it may be concluded that the structure of the ferric high-spin heme in PB P-448 and MC P-448 is affected by the apoprotein only to an insignificant extent.

In conclusion, the frequency of band IV can be used as a practical indicator of the amount of electrons delocalized to the porphyrin $\Pi^*(e_g)$ orbital and that of all the reduced P-450's (PB P-450, PB P-448, MC P-448, and P-450_{cam}) and their CO complexes are found to be unusually low, indicating a strong π basicity of the fifth ligand of the heme iron. This anomaly disappears upon conversion to the P-420 form. Therefore, the strong π basicity of the fifth ligand seems essentially important for the catalytic function of cytochrome P-450's.

References

Abe, M., Kitagawa, T., and Kyogoku, Y. (1978), *J. Chem. Phys.* (in press).

Alben, J. O., and Caughey, W. S. (1968), *Biochemistry 7,* 175.

Caughey, W. S., Alben, J. O., McCoy, S., Boyer, S. H., Charache, S., and Hathaway, P. (1969), *Biochemistry 8,* 59.

Champion, P. M., and Gunsalus, I. C. (1977), *J. Am. Chem. Soc. 99,* 2001.

Champion, P. M., Remba, R. D., Chiang, R., Fitchen, D. B., and Hager, L. P. (1976), *Biochim. Biophys. Acta 446,* 486.

Chang, C. K., and Dolphin, D. (1975), *J. Am. Chem. Soc. 97,* 5948.

Chang, C. K., and Dolphin, D. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3338.

Collman, J. P., and Sorrell, T. N. (1975), *J. Am. Chem. Soc. 97,* 4133.

Collman, J. P., Sorrell, T. N., and Hoffman, B. M. (1975), *J. Am. Chem. Soc. 97,* 913.

Collman, J. P., Sorrell, T. N., Dawson, J. H., Trudell, J. R., Bunnenberg, E., and Djerassi, C. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 6.

Dawson, J. H., Holm. R. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C., and Tang, S. C. (1976), *J. Am. Chem. Soc. 98,* 3707.

Hashimoto, C., and Imai, Y. (1976), *Biochem. Biophys. Res. Commun. 68,* 821.

Haugen, D. A., and Coon, M. J. (1976), *J. Biol. Chem. 251,* 7929.

Hendra, P. J., and Loader, E. J. (1968), *Chem. Ind. (London)* 718.

Ichikawa, Y., and Yamano, T. (1967), *Biochim. Biophys. Acta 131,* 490.

Ikeda-Saito, M., Iizuka, T., Yamamoto, H., Kayne, F. J., and Yonetani, T. (1977), *J. Biol. Chem. 252,* 4882.

Imai, Y., and Sato, R. (1967), *Eur. J. Biochem. 1,* 419.

Imai, Y., and Sato, R. (1974a), *J. Biochem. (Tokyo) 75,* 689.

Imai, Y., and Sato, R. (1974b), *Biochem. Biophys. Res. Commun. 60,* 8.

Keller, R. M., Wüthrich, K., and Debrunner, P. G. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2073.

Kitagawa, T., Kyogoku, Y., Iizuka, T., Ikeda-Saito, M., and Yamanaka, T. (1975), *J. Biochem. (Tokyo) 78,* 719.

Kitagawa, T., Kyogoku, Y., Iizuka, T., and Saito, M. (1976), *J. Am. Chem. Soc. 98,* 5169.

Kitagawa, T., Abe, M., Kyogoku, Y., Ogoshi, H., Sugimoto, H., and Yoshida, Z. (1977a), *Chem. Phys. Lett. 48,* 55.

Kitagawa, T., Ozaki, Y., Kyogoku, Y., and Horio, T. (1977b), *Biochim. Biophys. Acta 495,* 1.

Koch, S., Tang, S. C., Holm, R. H., Frenkel, R. B., and Ibers, J. A. (1975), *J. Am. Chem. Soc. 97,* 916.

Murakami, K., and Mason, H. S. (1967), *J. Biol. Chem. 242,* 1102.

Ogoshi, H., Sugimoto, H., and Yoshida, Z. (1975), *Tetrahedron Lett.,* 2289.

Ozaki, Y., Kitagawa, T., Kyogoku, Y., Shimada, H., Iizuka, T., and Ishimura, Y. (1976), *J. Biochem. (Tokyo) 80,* 1447.

Rein, H., Böhm, S., Jänig, G.-R., and Ruckpaul, K. (1977), *Croat. Chem. Acta 49,* 333.

Rimai, L., Salmeen, I., and Petering, D. H. (1975), *Biochemistry 14,* 378.

Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B., and Levin, W. (1975), *Biochem. Biophys. Res. Commun. 64,* 1134.

Sato, R., Satake, H., and Imai, Y. (1973), *Drug Metab. Dispos. 1,* 6.

Sharrock, M., Münck, E., Debrunner, P. G., Marshall, V., Lipscomb, J. D., and Gunsalus, I. C. (1973), *Biochemistry 12,* 258.

Shimizu, T., Nozawa, T., Hatano, M., Imai, Y., and Sato, R. (1975), *Biochemistry 14,* 4172.

Spiro, T. G. (1975), *Biochim. Biophys. Acta 416,* 169.

Spiro, T. G., and Burke, J. M. (1976), *J. Am. Chem. Soc. 98,* 5482.

Stern, J. O., and Peisach, J. (1974), *J. Biol. Chem. 249,* 7495.

Tang, S. C., Koch, S., Papaethymiou, G. C., Foner, S., Frankel, R. B., Ibers, J. A., and Holm. R. H. (1976), *J. Am. Chem. Soc. 98,* 2414.

Tsai, R., Yu, C.-A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., and Beinert, H. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 1157.

Vickery, L., Salmon, A., and Sauer, K. (1975), *Biochim. Biophys. Acta 386,* 87.

Yoshida, Y., and Kumaoka, H. (1975), *J. Biochem. (Tokyo) 78,* 455.