An Infrared Spectroscopic Study of Carbon Monoxide Bonding to Ferrous Cytochrome P-450†

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ABSTRACT: Ferrous-carbonyl complexes of the soluble *Pseudomonas putida* cytochrome P-450$_{cam}$ and a denatured form (P-420) of this enzyme, as well as cytochromes P-450 and P-448 in liver microsomes from rats pretreated with phenobarbital and 3-methylcholanthrene, have been studied by infrared spectroscopy. The FeCO bonding was examined in order to probe the spatial relationship between the dioxygen- and substrate-binding sites and to determine whether the presence of a unique axial ligand bound trans to carbon monoxide could be responsible for the red-shifted Soret absorbance band maximum at 450 nm. The *d*-camphor(K^+)-bound cytochrome P-450$_{cam}$ yielded a single infrared absorbance band for the heme-bound carbonyl (ν_{CO} 1940 cm^{-1}) having a bandwidth at half-height ($\Delta\nu_{1/2}$) of 13 cm^{-1}, while the camphor-free enzyme gave rise to two stretching frequencies of equal area at 1963 and 1942 cm^{-1} ($\Delta\nu_{1/2}$ 11–12 and 19–21 cm^{-1}, respectively). Addition of *d*-camphor and a monovalent metal ion (K^+) to the camphor-free ferrous carbonyl-enzyme converted the infrared spectrum back to that of the original camphor-bound enzyme. The area of the 1940-cm^{-1} band was found to equal that of the combined areas of the 1963- and 1942-cm^{-1} bands. Conversion of the native enzyme to a denatured form (P-420) yielded a ν_{CO} 1966 cm^{-1} with $\Delta\nu_{1/2}$ 23 cm^{-1}. The cytochromes in rat liver microsomes yielded ν_{CO} and $\Delta\nu_{1/2}$ different from each other and the bacterial enzyme: cytochrome P-450 (ν_{CO} 1948 cm^{-1}, $\Delta\nu_{1/2}$ ~25 cm^{-1}); cytochrome P-448 (ν_{CO} 1954 cm^{-1}, $\Delta\nu_{1/2}$ ~30 cm^{-1}). These data indicate that (1) the FeCO bonding in each of the cytochromes P-450 is similar to that observed in the case of the ferrous-carbonyl complexes of hemoglobin (HbCO) and myoglobin (MbCO); (2) in the presence of *d*-camphor the heme-carbonyl of the bacterial enzyme exhibits nonlinear bonding, while in its absence both nonlinear and linear bonding exists—evidence for a close spatial relationship between the heme-carbonyl (oxygenyl) and *d*-camphor binding site; (3) FeCO bonding in P-420 is nearly the same as that of denatured HbCO and MbCO, suggesting linear heme-carbonyl bonding and a nitrogenous trans axial ligand; (4) the heme-carbonyl of the liver microsomal enzymes senses a less ordered nearest-neighbor environment than in the bacterial enzyme, perhaps partially the result of these enzymes having larger and more flexible substrate-binding sites; (5) identification of the axial ligand bound trans to the carbonyl is as yet not possible—neither a mercaptide nor nitrogenous ligand may be ruled out on the basis of these measurements.

Infrared spectroscopy is a powerful technique for probing the oxygen binding site of heme proteins. Carbon monoxide has been the ligand of choice for such studies because the C–O stretching frequency is much more easily observed in aqueous media than is that of the O–O stretching frequency (Maxwell and Caughey, 1978) and because its substitution for O_2 is assumed to leave the structure of the protein unaltered. The infrared stretching frequency is related to the C–O bond energy which is very sensitive to differences in both bond type and environment. The present study was initiated to directly probe, by infrared spectroscopy, the dioxygen-binding site of cytochrome P-450 using carbon monoxide as the observable ligand.

A variety of chemical and spectroscopic studies have shown that the binding of substrate dramatically affects the electronic state of the ferric protoporphyrin IX prosthetic group of cytochrome P-450. Concomitant with the binding of *d*-camphor, the hemin iron of the soluble *Pseudomonas putida* cytochrome P-450$_{cam}$[1] undergoes a low- to high-spin transition (Tsai et al., 1970; Peterson, 1971). Metyrapone, a nitrogenous ligand which binds to the hemin iron and which inhibits the cytochrome P-450 catalyzed hydroxylation reaction, will displace the substrate *d*-camphor, suggesting that the substrate-binding site is in close proximity to the hemin iron (Griffin and Peterson, 1972). Additional evidence for this conclusion has been obtained from pulsed nuclear magnetic resonance spectral studies of both the bacterial and liver microsomal cytochromes P-450 (Griffin and Peterson, 1975; Ruckpaul et al., 1976; Rein et al., 1976; Philson, 1977). It was demonstrated that in the absence of substrate, bulk solvent water molecules rapidly

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[1] Abbreviations used are: cytochrome P-450$_{cam}$, cytochrome P-450 isolated from *Pseudomonas putida* grown on *d*-camphor; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; MbCO, ferrous carbonyl-myoglobin; HbCO, ferrous-carbonylhemoglobin; Hb, hemoglobin; Mb, myoglobin; cytochromes P-450 and P-448, liver microsomal cytochromes induced by pretreating rats with phenobarbital and 3-methylcholanthrene, respectively; ν_{CO}, CO stretching frequency in cm^{-1}; $\Delta\nu_{1/2}$, bandwidth at half-height of the CO stretching frequency in reciprocal centimeters; P-420, denatured cytochrome P-450; HRPCO, ferrous-carbonyl horseradish peroxidase; ESR, electron-spin resonance.

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exchange into the hemin iron coordination sphere of the low-spin, substrate-free enzyme. However, substrate binding markedly decreases the access of solvent water molecules to the hemin iron and may completely displace the coordinated water molecule. Binding of substrate to the ferrous form of cytochrome P-450 is less well understood, primarily because the optical absorbance spectrum (Peterson, 1971) and the spin-state (high-spin) (Sharrock et al., 1976) of the heme iron are unaltered in its presence. Presumably, however, the substrate is in close proximity to the heme iron, since binding of the nitrogenous ligand pyridine is competitive with that of camphor (Griffin and Peterson, 1972). Since the ferrous form of cytochrome P-450 binds dioxygen and accepts a second electron, resulting in the activation of the heme-bound oxygenyl and subsequent hydroxylation of substrate (Peterson et al., 1972; Lipscomb et al., 1976), the question of the spatial relationship between the bound camphor and dioxygen molecules is of considerable interest. Although recent studies have shown that a heme-bound oxygenyl can be investigated by infrared spectroscopy (Barlow et al., 1973; Maxwell et al., 1974), we have chosen to initially study the ferrous carbonyl-enzyme for the reasons given above.

Cytochrome P-450 is unique in the class of iron protoporphyrin IX containing heme proteins, since its ferrous-carbonyl complex has a Soret absorbance band maximum at about 450 nm. In general, the ferrous-carbonyl complexes of the other members of this class absorb at about 420 nm. Speculation as to the origin of this 450-nm band has centered on the identity of the axial ligand bound trans to carbon monoxide, and it is now widely accepted that a mercaptide moiety (cysteine?) serves as that ligand. Since such a ligand is considered to be a much better π-electron donor than the usual imidazole (histidine) ligand (Collman and Sorrell, 1975; Chang and Dolphin, 1975, 1976; Collman et al., 1976), it was anticipated that the C-O stretching frequency would be significantly lower for the ferrous-carbonyl complex of cytochrome P-450 than for HbCO (Alben and Caughey, 1968) and MbCO (McCoy and Caughey, 1971; Maxwell et al., 1974).

Materials and Methods

Cytochrome P-450_{cam} was isolated and purified from *Pseudomonas putida* (ATCC 17453) grown on *d*-camphor (J. T. Baker Chemical Co.) as its source of carbon (Peterson, 1971; O'Keeffe et al., 1978). The enzyme isolated in the presence of *d*-camphor was in the high-spin ferric (camphor-bound) state. The purity of the cytochrome P-450_{cam} preparations used was determined by the absorbance ratio at 392 nm (Soret band) relative to 280 nm for the enzyme in 0.1 M potassium phosphate (pH 7.4) containing 0.1 M potassium chloride and 1 mM *d*-camphor; in all cases, this ratio was ≥ 1.2, indicating that the enzyme was at least 85% pure (O'Keeffe et al., 1978). The preparation did not contain measurable amounts of other heme proteins. The concentration of the enzyme was determined from the absorbance at 446 nm for the ferrous-carbonyl complex (ϵ 120 mM^{-1} cm^{-1}) (Peterson, 1971; O'Keeffe et al., 1978).

Liver microsomes were prepared from rats (Charles River®) pretreated with either phenobarbital or 3-methylcholanthrene as previously described (Ebel et al., 1977). Protein was determined by the biuret method (Gornall et al., 1949), and the concentration of cytochromes P-450 and P-448 present was determined from the ferrous-carbonyl minus ferrous optical absorbance difference spectrum using the $\Delta\epsilon = 91$ mM^{-1} cm^{-1} at 450-490 nm (Omura and Sato, 1967; Ebel et al., 1977). The microsomes prepared from phenobarbital- or 3-methylcholanthrene-pretreated rats were stored as pellets under argon at −20 °C until used.

Cytochrome P-450_{cam} was freed of substrate by passing the high-spin camphor-bound ferric enzyme over a Sephadex G-10 column which had been equilibrated with 50 mM morpholinopropanesulfonate neutralized to pH 7.0 with Tris base (Peterson, 1971; O'Keeffe et al., 1978). The low-spin camphor-free ferric enzyme has its Soret absorbance maximum at 418 nm (ϵ 105 $mM^{-1}cm^{-1}$) (Peterson, 1971; O'Keeffe et al., 1978). The absence of substrate was verified by monitoring the ratio of the absorbance at 446 nm (ferrous-carbonyl complex) to that at 418 nm; a ratio of 1.1 was taken to indicate that all of the enzyme was in the substrate-free form (Peterson, 1971; O'Keefe et al., 1978).

Cytochrome P-450_{cam} was converted to P-420 by increasing the pH of a stock solution of the high-spin ferric camphor-bound enzyme to 11 and monitoring the conversion by measuring the absorbance at 420 nm for the ferrous-carbonyl complex (O'Keeffe and Peterson, 1975).

Samples of the camphor-bound and camphor-free ferric cytochrome P-450_{cam} and of P-420 were concentrated in an Amicon concentrator (PM-10 membrane) to 1-2 mM for use in the infrared experiments. The ferrous-carbonyl complexes of cytochrome P-450_{cam} and P-420 were prepared as follows: approximately 1 mL of cytochrome P-450_{cam} (camphor-bound or camphor-free) or P-420 was placed in a 5-mL syringe filled with carbon monoxide. A 10-μL volume of aqueous sodium dithionite (approximately a tenfold molar excess of reducing agent to enzyme) was added and the sample mixed gently for 5 min to ensure complete formation of the ferrous-carbonyl complex. The sample was then transferred directly into the calcium fluoride infrared cell (path length 0.05-0.1 mm). Optical absorbance spectra of these samples in the infrared cell were recorded on a Cary Model 17 spectrophotometer both before and after recording the infrared spectra (Maxwell and Caughey, 1978).

The concentrations of cytochromes P-450 and P-448 in the microsomal fractions prepared from phenobarbital- and 3-methylcholanthrene-pretreated rats were approximately 3.0 and 2.5 nmol/mg of microsomal protein, respectively. In order to prepare ferrous-carbonyl samples at a suitable concentration for infrared experiments, the following procedure was used. A 10-mL volume of 25 mg of microsomal protein/mL (0.1 M potassium phosphate buffer, 0.1 mM EDTA, pH 7.4) was bubbled gently with carbon monoxide for 30 min at room temperature in the presence of 1 mM NADPH. The sample was centrifuged at 100 000*g* for 30 min at 30 °C. The supernatant solution was carefully removed while keeping the microsomal pellet under a carbon monoxide atmosphere. The microsomal pellet, which had a gellike consistency, was transferred directly to the CaF_2 cell.

Difference infrared spectra were recorded in the absorbance mode using a Perkin-Elmer Model 180 infrared spectrometer (Maxwell and Caughey, 1978). A variable path-length cell containing water was used as the reference. Water is a suitable reference, since in the infrared region of interest the background absorbance is due only to water; i.e., there are no sharp or intense broad bands due to protein. The signal to noise ratio of some of the spectra was increased by averaging several individual spectra using a Tektronix Model 4051 graphic computer system (Maxwell and Caughey, 1978). The integrated areas of the infrared absorbance spectra were also obtained with this system and compared to the area of the 1951-cm^{-1} absorbance band of a standard solution of HbCO.

Carbon monoxide was purchased from the Linde Division, Union Carbide Corp. (^{12}CO), and from Prochem Laboratories

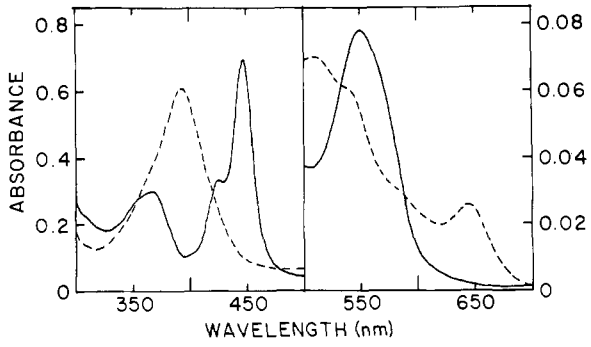

FIGURE 1: Optical absorbance spectra of camphor-bound cytochrome P-450$_{cam}$ [0.92 mM in 0.1 M potassium phosphate (pH 7.4) containing 0.1 M potassium chloride and 1.0 mM *d*-camphor] at 25 °C recorded in an infrared cell having CaF_2 windows and a path length of 0.0633 mm: (- - -) ferric; (—) ferrous-carbonyl complex.

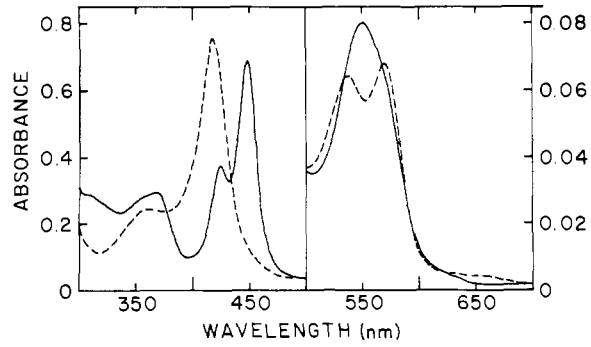

FIGURE 3: Optical absorbance spectra of camphor-free cytochrome P-450$_{cam}$ [1.0 mM in 0.1 M morpholinopropanesulfonate (pH 7.0) with Tris base] at 25 °C, recorded in an infrared cell having CaF_2 windows and a path length of 0.0633 mm: (- - -) ferric; (—) ferrous-carbonyl complex.

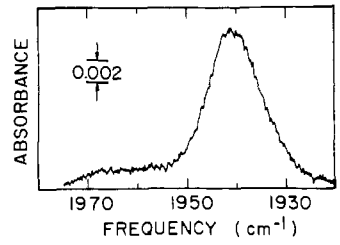

FIGURE 2: Infrared difference spectrum (absorbance mode) of the ferrous-carbonyl complex of camphor-bound cytochrome P-450$_{cam}$ vs. H_2O (concentration, buffer medium, and path length of the infrared cell as in Figure 1).

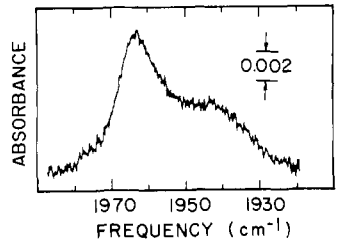

FIGURE 4: Infrared difference spectrum (absorbance mode) of the ferrous-carbonyl complex of camphor-free cytochrome P-450$_{cam}$ vs. H_2O (concentration, buffer medium, and path length of the infrared cell are in Figure 3).

(^{13}CO, 90% enriched). All other chemicals and reagents used in this study were of the highest quality commercially available and were used without further purification.

Results

Camphor-Bound Cytochrome P-450$_{cam}$. Optical absorbance and infrared spectra of camphor-bound cytochrome P-450$_{cam}$ are shown in Figures 1 and 2. The Soret absorbance band maximum for the ferric form is located at 392 nm and is characteristic of the high-spin camphor-bound enzyme (Figure 1) (Peterson, 1971; O'Keeffe et al., 1978). Upon reduction with sodium dithionite and equilibration with carbon monoxide, the Soret absorbance band maximum appears at 446 nm. The sample did not contain an appreciable amount of the denatured form of cytochrome P-450 (P-420), as indicated by the presence of only a small shoulder at 420 nm. Cytochrome P-450$_{cam}$ was stable for the period of time required to record the infrared spectra, as evidenced by the lack of a change in the optical absorbance spectrum. The infrared spectrum of the ferrous-carbonyl complex of camphor-bound cytochrome P-450$_{cam}$ is shown in Figure 2. The absorbance band is at 1940 cm^{-1} and it has a $\Delta\nu_{1/2}$ of 13 cm^{-1}. Since the energy of the C-O bond is related to the mass of the C and O atoms, the identification of this band as being due to the C-O stretch can be confirmed by substitution of $^{13}C^{16}O$ for $^{12}C^{16}O$. The infrared band for the isotopically substituted sample was shifted to 1897 cm^{-1}, which is the frequency predicted (Alben and Caughey, 1968). The intensity of the 1940-cm^{-1} absorbance band is slightly less than that of the comparable band of human HbCO (ν_{CO} 1951 cm^{-1}) (Maxwell and Caughey, 1978) and somewhat broader ($\Delta\nu_{1/2}$ 13 cm^{-1} for cytochrome P-450$_{cam}$ vs. 8 cm^{-1} for Hb). However, the integrated intensity per mole of heme for the respective absorbance bands is identical within the limits of accuracy of this analytical technique (±5%). A much less intense absorbance band centered at about 1966 cm^{-1} representing only a few percent of the total integrated intensity is probably due to the heme-bound carbonyl of P-420 (vide infra) or possibly, at least in part, to a second CO-binding site such as is seen for hemoglobin carbonyls (Caughey et al., 1978).

Camphor-Free Cytochrome P-450$_{cam}$. Optical absorbance and infrared spectra of the camphor-free protein are shown in Figures 3 and 4. The Soret absorbance band maximum at 418 nm is characteristic of the ferric low-spin substrate-free enzyme (Figure 3) (Peterson, 1971; O'Keeffe et al., 1978). Since the spectrum of the ferrous-carbonyl complex is not altered by the presence or absence of substrate, the spectrum shown in Figure 3 suggests that a small portion of the enzyme may have undergone denaturation to P-420 because a definite band at about 420 nm is observed in addition to the Soret absorbance band maximum at 446 nm. The infrared spectrum of the ferrous-carbonyl complex of camphor-free cytochrome P-450$_{cam}$ in the absence of KCl is shown in Figure 4. Two absorbance bands are observed with CO values of 1963 and 1942 cm^{-1}. To substantiate that both bands were due to heme-bound CO, $^{12}C^{16}O$ was again replaced with $^{13}C^{16}O$. The magnitude and direction of shift of both bands (ν_{CO} 1918 and 1896 cm^{-1}) were as predicted for this isotopic substitution (Alben and Caughey, 1968). The absorbance bands were integrated from 1987 to 1920 cm^{-1}, and the total area per mole of heme-carbonyl was identical (±5%) to the area obtained for the 1940-cm^{-1} absorbance band of a comparable sample of the camphor-bound ferrous-carbonyl enzyme. The two curves were manually resolved, and the maxima at about 1963 and 1942 cm^{-1} were determined to have $\Delta\nu_{1/2}$ values of 11-12 and ~21 cm^{-1}, respectively. The absorbance band at 1963 cm^{-1} was integrated separately and found to represent approximately 50% of the total area. The difference in amplitude of these two bands with

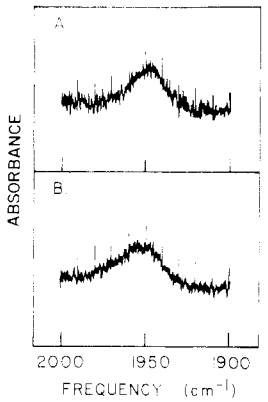

FIGURE 5: Infrared difference spectra (absorbance mode) of the ferrous-carbonyl complexes of (A) cytochrome P-450 and (B) cytochrome P-448 in liver microsomes from rats pretreated with phenobarbital and 3-methylcholanthrene, respectively, vs. H_2O (recorded as described under Materials and Methods).

approximately equal integrated areas is accounted for by different widths at half-height.

Previous studies of substrate binding to cytochrome P-450_{cam} have shown that the presence of KCl significantly increases the K_{eq} for *d*-camphor binding to the ferric enzyme (Peterson, 1971). In order to determine whether KCl has an effect on CO bonding to the reduced enzyme, both the optical absorbance and infrared spectra were recorded in the presence of 0.1 M KCl. The optical absorbance spectra of camphor-free ferric and ferrous-carbonyl cytochrome P-450_{cam} in the presence or absence of 0.1 M KCl are identical to those in its absence. However, KCl does slightly alter the infrared spectrum. The half-bandwidth of the 1942-cm^{-1} band (Figure 4) is narrowed from about 21 to 19 cm^{-1}, while the 1963 cm^{-1} band is unchanged. The total integrated intensity of these carbonyl absorbance bands per mole of heme remains unchanged and the ratio of their integrated intensities is approximately 1.

The ν_{CO} of the enzyme in the presence of substrate (1940 cm^{-1}) is effectively identical with the 1942-cm^{-1} band observed in the absence of substrate; thus, the obvious question arises as to the purity of the camphor-free cytochrome P-450_{cam} sample. The optical absorbance spectrum (Figure 3) of an aliquot of the camphor-free cytochrome P-450_{cam} used for the infrared spectral studies is the same as that previously reported, taking into account that small amount which is either P-420 or unreduced ferric enzyme (Peterson, 1971; O'Keeffe et al., 1978). Electron-spin resonance spectroscopic studies have demonstrated that samples with this optical absorbance spectrum are greater than 90% in the camphor-free low-spin form (Griffin and Peterson, 1975; Ebel et al., 1977; O'Keeffe et al., 1978). Therefore, the bands at 1942 and 1963 cm^{-1} do not result from an equal mixture of camphor-free and camphor-bound enzyme. Also, the band at 1942 cm^{-1} and that at 1940 cm^{-1} in the camphor-free and camphor-bound enzyme, respectively, have different half-bandwidths, indicating that the heme-bound carbonyl senses a different solvent and/or protein environment in each case.

Since the stability of camphor-free cytochrome P-450_{cam} is somewhat limited, several criteria were used to establish that the camphor-free enzyme was stable during the recording of the infrared spectrum and that the new band at 1963 cm^{-1} was not due to a denatured form (P-420) of the heme protein. The optical absorbance spectrum of the carbonyl complex was recorded both before and after the infrared spectra were obtained and was found to be unchanged (Figure 3). Approximately 15% of the camphor-free enzyme was P-420 initially, assuming that the optical absorbance band near 420 nm in the ferrous-carbonyl complex (Figure 3) arises entirely from the denatured form. This amount of contamination could not result in two infrared absorbance bands of equal area. Neither of the two carbonyl-stretching frequencies observed for the camphor-free enzyme match that of the denatured form of the enzyme (P-420), vide infra. Finally, and most importantly, the addition of excess *d*-camphor to the sample already containing 0.1 M KCl yielded an infrared spectrum identical with that of the camphor-bound form (Figure 2); thus, the band at 1963 cm^{-1} cannot be due to the presence of P-420.

Carbonyl Complex of P-420. Denatured forms of cytochrome P-450 (P-420) can be prepared by a variety of techniques and probably represent several different modifications of the protein (Yu and Gunsalus, 1974; O'Keeffe and Peterson, 1975). An essential feature of all P-420 forms of this heme protein is a Soret absorbance band maximum at approximately 420 nm for the ferrous-carbonyl complex as compared to 446 nm for the native enzyme. The infrared spectrum of a sample of the ferrous-carbonyl complex of P-420 prepared by denaturing the camphor-bound ferric enzyme with base (see Materials and Methods) reveals a single absorbance band at 1966 cm^{-1} having a $\Delta\nu_{1/2}$ of about 23 cm^{-1}.

Mammalian Hepatic Cytochrome P-450. The position of the Soret difference absorbance band maximum for the ferrous-carbonyl complex of rat liver microsomal cytochrome P-450 varies and is dependent upon the pretreatment of the animal (Gillette et al., 1972). Pretreatment with phenobarbital results in a band at 450 nm (cytochrome P-450), while pretreatment with 3-methylcholanthrene yields a band at 448 nm (cytochrome P-448). The infrared spectra of the ferrous-carbonyl complexes of these cytochromes in their respective microsomal fractions are shown in Figure 5. A broad infrared absorbance band is observed in each instance. The ν_{CO} values are 1948 and 1954 cm^{-1} with $\Delta\nu_{1/2}$ values of ~25 and ~30 cm^{-1} for the ferrous-carbonyl complexes of cytochromes P-450 and P-448, respectively. The optical absorbance spectrum in the Soret region of each sample was examined before and after the infrared spectrum was recorded, and no significant contribution from P-420 was observed.

Discussion

The results of the present study show that the observed ν_{CO} values for the ferrous-carbonyl complexes of camphor-bound and camphor-free cytochrome P-450_{cam}, as well as of cytochromes P-450 and P-448 in liver microsomes from rats pretreated with phenobarbital and 3-methylcholanthrene, respectively, are in the same frequency range as the values previously obtained for bovine heart MbCO and human HbCO (Table I). These results suggest similar bonding between the carbonyl moiety and the heme iron in all of these heme proteins. This interpretation is strengthened by the fact that the integrated intensities of the 1940-cm^{-1} band of the camphor-bound cytochrome P-450_{cam} and of the combined 1942- and 1963-cm^{-1} bands of the camphor-free cytochrome P-450_{cam} per mole of heme are nearly the same as of the 1951-cm^{-1} band of HbCO, even though their respective $\Delta\nu_{1/2}$ values are somewhat different (Table I).

In the case of MbCO and HbCO, bent end-on bonding between the heme iron and CO was initially suggested on the basis of infrared spectral studies (Caughey et al., 1969; Caughey, 1970, 1971). This proposal was subsequently confirmed by X-ray (Huber et al., 1970; Padlan and Love, 1974; Heidner et al., 1976) and neutron-diffraction (Norvell et al., 1975) studies. Since the stretching frequency for the fer-

TABLE I: Infrared Spectral Parameters for Ferrous-Carbonyl Complexes of Cytochromes P-450 and P-420 and Several Other Heme Proteins.

	ν_{CO} (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1})	reference
cytochrome $P\text{-}450_{cam}$			this work
camphor-bound	1940[a]	13[a]	
camphor-free	1963, 1942	11-12, 19-21[b]	
cytochrome P-450[c]	1948	~25	this work
cytochrome P-448[c]	1954	~30	
cytochrome P-450[d]	1949	~17	Bohm et al. (1976) Rein et al. (1977)
P-420[e]	1966[f]	~23[f]	this work
Mb, bovine heart	1944[g]	12	McCoy and Caughey (1971) Maxwell et al. (1974)
Hb A	1951	8	Alben and Caughey (1968)
Hb Zurich $\alpha_2^A\beta_2^{63\ his\rightarrow arg}$	1951, 1958	8, 8	Caughey et al. (1969)
Hb M_{Emory} $\alpha_2^A\beta_2^{63\ his\rightarrow tyr}$	1951, 1970	7, 8	Caughey et al. (1969)
denatured Hb, Mb[h]	1966[i]	20[i]	O'Toole (1972) Caughey et al. (1973) Yoshikawa et al. (1977)
chloroperoxidase			
pH 3	1942	~30	O'Keeffe et al. (1977)
pH 6	1958	~15	

[a] Similar values have been obtained in the laboratory of Professor J. O. Alben (The Ohio State University) (Bare, 1973) from a sample of the enzyme isolated in the laboratory of Professor I. C. Gunsalus (University of Illinois). [b] $\Delta\nu_{1/2}$ of the band at 1942 cm^{-1} is ~19 cm^{-1} in the presence of 0.1 M KCl and ~21 cm^{-1} in its absence (see Results). [c] Enzymes present in intact microsomes from livers of rats pretreated with phenobarbital (cytochrome P-450) and 3-methylcholanthrene (cytochrome P-448). [d] Partially purified enzyme from livers of rabbits pretreated with phenobarbital. [e] Denatured cytochrome $P\text{-}450_{cam}$ (pH 11). [f] Similar values have been obtained for the thermally or detergent denatured cytochrome P-450 isolated from livers of rabbits pretreated with phenobarbital (Bohm et al., 1976; Rein et al., 1977). [g] A small shoulder of unknown origin is seen at about 1933 cm^{-1}. [h] Denaturation by acid treatment (pH 3). [i] Base treatment yields similar values (pH 11.9) (Bare, 1973).

rous-carbonyl complex of camphor-bound cytochrome $P\text{-}450_{cam}$ is found at 1940 cm^{-1}, it may be argued that the CO is bound to the heme iron in an analogous bent end-on fashion. Implicit in this argument is the notion that interactions with substrate and/or neighboring amino acid residues occur which probably both cause, as well as stabilize, the bent Fe-C-O bonding. Additional evidence in support of bent end-on bonding between the heme iron and CO involves the infrared spectrum of the ferrous-carbonyl complex of the camphor-free enzyme. Two stretching frequencies (1963 and 1942 cm^{-1}) of equal integrated intensity are observed. However, because the band at 1942 cm^{-1} is essentially the same as that observed with the camphor-bound enzyme (1940 cm^{-1}), we initially considered the possibility that only about half of the enzyme had been freed of substrate by the gel-filtration technique (see Materials and Methods) and/or that 50% of the sample denatured to P-420 during this process, resulting in the band at 1963 cm^{-1}. Several criteria (see Results) substantiated the absence of camphor from the enzyme used in this study, as well as the presence of no more than 15% denatured material. The two bands could be due to the presence of two different trans-axial ligands, each of which would result in a single absorbance band in the infrared spectrum. This explanation is unlikely, since the optical absorbance spectra of the ferrous-carbonyl complexes of camphor-bound and camphor-free cytochrome $P\text{-}450_{cam}$ are identical (Figures 1 and 3). If there had been a change in trans ligand, it would be expected, a priori, that there would be at least some change in the optical absorbance spectrum of the complex. Therefore, the presence of two ν_{CO} values in the infrared spectrum of ferrous-carbonyl camphor-free cytochrome $P\text{-}450_{cam}$ is assumed to result from alterations in the immediate environment of the heme-bound carbonyl. We suggest that the band in camphor-free cytochrome $P\text{-}450_{cam}$ at 1963 cm^{-1} may result from the Fe-C-O bonding being less bent in half of the camphor-free form. This proposal is supported by infrared spectral results obtained from a variety of Hb and Mb carbonyls. Shifts in ν_{CO} to larger wavenumbers as a function of structural changes adjacent to the heme-bound carbonyl have been suggested to result from the bent Fe-C-O bonding becoming more linear (Caughey et al., 1969; Caughey, 1970; Caughey et al., 1973; Wallace et al., 1976). The other band in the camphor-free sample has essentially the same wavenumber as in the camphor-bound sample (1942 vs. 1940 cm^{-1});[2] however, the half-bandwidth is larger and altered by the presence of KCl. We believe these differences reflect changes in the solvent and/or protein environment about the bent Fe-C-O in the camphor-bound and camphor-free enzyme.

A direct steric interaction between the bound camphor and carbon monoxide molecules cannot be the only cause of the lower ν_{CO} (Table I) because (1) even in the absence of camphor approximately half of the carbon monoxide assumes the bent Fe-C-O bonding described by the 1940-1942-cm^{-1} stretching frequency, and (2) presumably no stabilizing influence such as a hydrogen-bonding or donor-acceptor interaction is possible between the bound carbonyl and camphor itself. However, binding of camphor to the ferrous enzyme obviously alters the immediate environment of the heme-bound carbonyl so as to preclude the linear Fe-C-O bonding. A supportive observation is that the rate of CO binding to the camphor-bound ferrous enzyme is two orders of magnitude slower than to the ferrous enzyme (Peterson and Griffin, 1972). Thus, the presence of camphor impedes access of CO to the heme-iron binding site. The reason for the large decrease in k_{on} may be that in the

[2] Deconvolution of the overlapping bands at 1963 and 1942 cm^{-1} (camphor-free enzyme) would presumably yield separate bands, one at a slightly higher wavenumber and the other at a slightly lower wavenumber.

presence of camphor, this restriction is partially removed and carbon monoxide may occupy either the 1940–1942- (bent) or 1963-cm^{-1} (linear) site. Since the integrated intensities of these bands are equal, the energy difference between the two environments is assumed to be small.[3]

If the effect of camphor on the heme-bound carbonyl is not just a steric interaction, then what does it do to result in the bent Fe–C–O bonding expressed by the ν_{CO} 1940 cm^{-1}? A simple explanation is that when camphor binds to its site, which is close to the heme iron, it serves to orient the bound carbonyl (1) so as to preclude its bonding in a linear manner and (2) to favor the bent geometry by aligning the heme-bound carbonyl with a specific amino acid residue which stabilizes the polarized C–O bond, possibly via a donor–acceptor or hydrogen-bonding interaction. The proposal of a hydrogen-bonding interaction between the heme-bound carbon monoxide and an amino acid residue in the case of cytochrome P-450_{cam} is strengthened by infrared spectral results obtained from the cytochrome P-450-like heme protein chloroperoxidase. At pH 3 a ν_{CO} at 1942 cm^{-1} with $\Delta\nu_{1/2} \approx 30$ cm^{-1} was obtained, while at pH 6 the ν_{CO} shifted to 1958 cm^{-1} and its $\Delta\nu_{1/2}$ decreased to ~15 cm^{-1} (Table I). Note that in this pH range the optical absorbance spectrum of the ferrous-carbonyl complex of chloroperoxidase, which has its Soret absorbance band maximum at 443 nm (Hollenberg and Hager, 1973), does not change. These data are supportive of a hydrogen-bonding interaction at the lower pH, which is perhaps not present at the higher pH, so that more nearly linear Fe–C–O bonding results. Such an interaction has also been proposed to account for the pH-dependent changes in one of the two ν_{CO} values observed in the case of HRPCO (Barlow et al., 1976). Support for a more linear bond between the heme iron and carbon monoxide in the absence of camphor, at least for that portion of the enzyme giving rise to the ν_{CO} 1962 cm^{-1}, is derived from studies with several Hb carbonyls, including Hb M_{Emory} and Hb Zurich in which structural changes adjacent to the heme iron have been suggested to cause an opening of the crevice, thereby allowing the bent Fe–C–O to become more nearly linear, as expressed by a shift in ν_{CO} to higher wavenumbers (Table I). Denaturation studies of HbCO, MbCO, and ferrous-carbonyl cytochrome P-450 also support this proposal (see below). Regardless of the ultimate explanation at the molecular level, the finding that *d*-camphor affects the ν_{CO} of cytochrome P-450_{cam} provides additional evidence that the substrate binds to the ferrous form of the enzyme and in close proximity to the O_2(CO) binding site.

The ν_{CO} and $\Delta\nu_{1/2}$ values observed for the P-420 species obtained by base denaturation of cytochrome P-450_{cam}, as well as the thermally and detergent denatured cytochrome P-450 purified from livers of phenobarbital-pretreated rabbits (Bohm et al., 1976; Rein et al., 1977), are identical to those of acid-denatured HbCO and MbCO (Table I). Exposure of either HbCO or MbCO to acidic media has been interpreted to indicate a slight protein unfolding which is observable as a shift of the stretching frequency to 1966 cm^{-1} and a broadening of the half-bandwidth to about 20 cm^{-1} (O'Toole, 1972; Caughey et al., 1973; Yoshikawa et al., 1977). The heme is considered to be still attached to the protein and to have a trans-axial histidine ligand, but the restraints previously imposed by the native protein are lost such that the Fe–C–O bonding is more linear. The increase in the half-bandwidth is considered to represent exposure of the bound CO to a less homogeneous environment which may include some external solvent. It is attractive to propose that similar phenomena occur when cytochrome P-450 is converted to P-420. This suggestion is supported by the fact that the first step in the denaturation of the camphor-bound cytochrome P-450_{cam} involves release of camphor from its binding site (O'Keeffe and Peterson, 1975). Thus, the original restricted environment imposed upon the heme-bound carbonyl by the active site is "loosened" or made more random by denaturation. The result is the conversion of the bent Fe–C–O bonding of the camphor-bound form and of half of the camphor-free form to more linear bonding concomitant with an increase in the $\Delta\nu_{1/2}$ indicative of a less-ordered solvent/protein environment. The portion of the camphor-free enzyme already considered to have nearly linear Fe–C–O bonding (1963 cm^{-1}) also senses this less-ordered solvent/protein environment. Because the Soret absorbance band maximum, ν_{CO}, and $\Delta\nu_{1/2}$ values for the ferrous-carbonyl complex of P-420 match those of other denatured heme proteins and of (*N*-methylimidazole) (CO)iron(II) protoporphyrin IX dimethyl ester in chloroform (ν_{CO} 1969; $\Delta\nu_{1/2}$ 29) (Caughey et al., 1973; Maxwell and Caughey, 1978), it seems reasonable to conclude that the trans (5th) axial ligand in P-420 is an imidazole moiety.

Explanations for the differences in ν_{CO} and $\Delta\nu_{1/2}$ values obtained from cytochromes P-450 and P-448 in rat liver microsomes vs. those obtained from cytochrome P-450_{cam} remain to be elucidated. At this time two proposals appear reasonable. Although it has been indicated that multiple forms of the enzyme are present in liver microsomes (Haugen and Coon, 1976; Huang et al., 1976; Atlas et al., 1977), pretreatment of rats with phenobarbital and 3-methylcholanthrene results in the induction of a high percentage of cytochrome P-450 and P-448, respectively (Haugen et al., 1976). Thus, it is perhaps not surprising to find that the heme-bound carbonyl of each class of liver microsomal cytochrome P-450 has a different ν_{CO} and experiences a more flexible solvent and/or protein environment (larger $\Delta\nu_{1/2}$ values) than in the case of bacterial cytochrome P-450 (Table I). This explanation is also consistent with the reported ν_{CO} and $\Delta\nu_{1/2}$ values from partially purified microsomal cytochrome P-450 isolated from the livers of phenobarbital-pretreated rabbits (Table I) (Bohm et al., 1976; Rein et al., 1977) which compare quite favorably with those from the cytochrome P-450 located within intact rat liver microsomes (Table I). On the other hand, it is known that liver microsomal cytochromes P-450 and P-448 are at least partially in the substrate-bound form from optical absorbance and ESR spectral studies (Powis et al., 1977; Kumaki et al., 1978; Ebel et al., 1978). The actual percentage of substrate-bound cytochrome P-450 or P-448 remains to be determined;[4] however, it is generally agreed that the percentage is significantly higher in the case of cytochrome P-448 (Kumaki et al., 1978; Ebel et al., 1978). Thus, it may be that the large $\Delta\nu_{1/2}$ values and the observed ν_{CO} values, which are intermediate between those of the camphor-bound and camphor-free forms of cytochrome P-450_{cam}, reflect the presence of overlapping infrared bands due to the presence of both substrate-bound and -free forms of the liver microsomal cytochromes P-450 and P-448. Obviously, additional experiments are required to determine if either of these explanations is valid.

Since the original proposal of Mason and co-workers that a sulfhydryl moiety may be a ligand to the iron in cytochrome P-450 (Mason et al., 1965; Murikami and Mason, 1967), the presence of a thiol ligand has been assumed to account for the

[3] This point could perhaps be clarified by recording the infrared spectrum as a function of temperature.

[4] Recent experimental data indicates that ferric cytochromes P-450 and P-448 in liver microsomes from rats pretreated with phenobarbital and 3-methylcholanthrene are 35–40% and 65% high-spin, respectively, due to the presence of endogenous substrate (J. Werringloer, S. Kawano, and R. W. Estabrook, unpublished results).

unique ESR spectra (characteristic rhombic distortion) of the ferric enzyme and optical absorbance spectrum (Soret absorbance band maximum at about 450 nm) of the ferrous-carbonyl complex of the enzyme. Recently, several groups have presented investigations of iron(III) and iron(II) porphyrin compounds possessing mercaptide axial ligands as models for cytochrome P-450. They have concluded from their ESR, magnetic circular dichroism, and optical absorbance spectral studies that a mercaptide moiety serves as an axial ligand to the hemin iron in both the low- and high-spin states (Hill et al., 1970; Collman et al., 1975; Koch et al., 1975a,b; Ogoshi et al., 1975; Dawson et al., 1976; Watanabe and Horie, 1976; Tang et al., 1976; Chevion et al., 1977) and to the heme iron of the ferrous-carbonyl complex of the enzyme (Stern and Peisach, 1974; Collman and Sorrell, 1975; Chang and Dolphin, 1975; Chang and Dolphin, 1976; Collman et al., 1976). Two of the alkyl mercaptide heme-carbonyl complexes have been examined by infrared spectroscopy. The ferrous-carbonyl complexes prepared by the addition of $NaSCH_3$ solubilized by dibenzo-18-crown-6 macrocyclic ether to iron 5,10,15,20-tetra(*o*-pivalamidophenyl)porphin in benzene (Collman and Sorrell, 1975; Collman et al., 1976) or by the addition of $KS(CH_2)_3CH_3$ solubilized by dibenzo-18-crown-6 macrocyclic ether to iron protoporphyrin IX dimethyl ester in *N,N*-dimethylacetamide (Chang and Dolphin, 1976) yield stretching frequencies of 1945 and 1923 cm^{-1}, respectively. Direct comparisons between these ν_{CO} values and those of the cytochromes P-450 (Table I) are difficult at this time for several reasons. Depending upon the sources of the enzyme, different ν_{CO} and $\Delta\nu_{1/2}$ values are observed, and in the case of the bacterial enzyme either bent or both bent and linear Fe-C-O bonding is indicated. In addition the two CO values reported from the model compounds are located at quite different frequencies, and only the value at 1945 cm^{-1} falls within the range of frequencies observed for the cytochromes P-450 (Table I). Finally, except for the ν_{CO} 1940 cm^{-1}, all of the ν_{CO} for the cytochromes P-450 have values at higher frequencies than those of the two model compounds but within the range of frequencies observed for other heme-carbonyls having nitrogenous trans-axial ligands (Caughey et al., 1973; Maxwell and Caughey, 1977). Consequently, we conclude that in the case of cytochromes P-450 (1) the identity of the trans-axial ligand cannot be determined from the limited infrared data available and (2) a nitrogenous ligand such as an imidazole moiety remains a viable option.

There is experimental evidence which suggests that the origin of the 450-nm Soret absorbance band maximum for the ferrous-carbonyl complex of cytochromes P-450 may not be due to the presence of a mercaptide trans-axial ligand. The infrared spectral data obtained in this study (Table I), as well as Mossbauer spectral data (Sharrock et al., 1973; Sharrock et al., 1976), suggest that the electron availability from the heme iron to the bound carbonyl in the form of $d\pi$-$p\pi$ back-bonding for the ferrous-carbonyl complexes of cytochrome $P\text{-}450_{cam}$ and P-420 is similar to that in the case of hemoglobins and myoglobins. However, large differences are apparent in their respective optical absorbance spectra (about 420 nm for HbCO, MbCO, and P-420 CO vs. 450 nm for ferrous carbonyl-cytochrome P-450). Denaturation of HbCO, MbCO, and ferrous carbonyl-cytochrome P-450 results in subtle infrared spectral changes consistent with the bent Fe-C-O bonding becoming more nearly linear in each case. However, while denaturation does not result in any significant alteration of the optical absorbance spectra of HbCO and MbCO, conversion of ferrous carbonyl-cytochrome P-450 to P-420 CO involves a dramatic change (~30-nm blue shift). In addition, optical absorbance (Hollenberg and Hager, 1973), Mossbauer (Champion et al., 1973), and infrared spectra (Table I) of chloroperoxidase, the heme protein peroxidase from the mold *Caldariomyces fumago,* are also similar to those of the cytochromes P-450. Furthermore, it has been reported that chloroperoxidase does not have a free mercaptide moiety available to serve as a trans-axial ligand in its ferrous-carbonyl complex (Chiang et al., 1975). These observations may be analyzed in the following manner. Infrared spectroscopy measures energy changes within different vibrational levels, while optical absorbance spectroscopy measures changes in energy between the ground- and excited-state electronic energy levels. Thus, the origin of the red-shifted Soret absorbance band maximum observed in the ferrous-carbonyl complexes of cytochromes P-450 and chloroperoxidase may be linked to a specific alteration (lowering) of the energy of the excited-state electronic level. That is, an as yet uncharacterized molecular interaction(s) between an amino acid residue(s) and the porphyrin ring (cis effect) may exist which lowers the energy of the excited state and gives rise to the long-wavelength absorbance band.

Acknowledgment

The authors thank Drs. B. W. Griffin and J. A. Volpe for their assistance in preliminary infrared spectral studies of the ferrous-carbonyl complex of camphor-bound cytochrome $P\text{-}450_{cam}$.

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