Camphor Binding by *Pseudomonas putida* Cytochrome P-450. Kinetics and Thermodynamics of the Reaction†

Brenda Walker Griffin‡ and Julian A. Peterson†

ABSTRACT: At 4°, the association reaction between bacterial ferric cytochrome P-450 and its substrate *d*-camphor is second order with a rate constant of 4.1×10^6 M^{-1} sec^{-1}, while the reverse, camphor–dissociation reaction exhibits first-order kinetics with a rate constant of 6.0 sec^{-1}. Over the temperature range 4–40°, the rate constant for dissociation shows the normal exponential dependence on reciprocal temperature. The equilibrium association constant for camphor binding depends exponentially on reciprocal temperature from 4° to about 13°, where it becomes temperature independent. The thermodynamic parameters for the camphor-binding reaction have been calculated from these data. Pyridine binds strongly to both ferric and ferrous cytochrome P-450 with association constants of 4.9×10^4 and 5.8×10^5 M^{-1}, respectively. The effect of camphor on pyridine binding by reduced cytochrome P-450 was utilized to evaluate the equilibrium association constant of reduced cytochrome P-450 for camphor; the value determined, 2.2×10^8 M^{-1}, is about 1000 times larger than the association constant for camphor binding to the ferric cytochrome.

The classical experiments in which substrate interaction with mammalian cytochrome P-450 was observed by means of difference spectrophotometry (Narasimhulu *et al.*, 1965; Remmer *et al.*, 1966; Imai and Sato, 1966) have served as the genesis for many studies of the properties of cytochrome P-450. The large number of compounds which have been found to alter the absorbance spectrum of mammalian cytochrome P-450 has been divided into two classes by virtue of the characteristic spectral changes which they elicit (Schenkman *et al.*, 1967). Type I substrates cause an increase in absorbance around 385 nm and a decrease in absorbance near 420 nm, which has been presumed to result from a conversion of the spin state of the heme iron from low to high spin (Hildebrandt *et al.*, 1968). Type II substrates, usually nitrogenous bases, cause a decrease in absorbance around 390–400 nm and an increase near 426 nm (Hildebrandt *et al.*, 1968).

The complexes between reduced liver microsomal cytochrome P-450 and hydrophobic ligands, such as ethyl isocyanide and pyridine, exhibit absorbance spectra with two Soret maxima (Imai and Sato, 1967a). However, with cyanide (Miyake *et al.*, 1968) and imidazole (Imai and Sato, 1967a), ligands considered to be hydrophilic, only a single Soret absorbance maximum is observed. From these and other experimental observations (Imai and Sato, 1967b), it has been concluded that the heme group of cytochrome P-450 is located in a hydrophobic "pocket" (Imai and Sato, 1967a). Such an environment would promote the binding of substrates for hydroxylation, since these substances tend to be lipid soluble. The kinetics and thermodynamics of the reaction of ligands and substrates with mammalian cytochrome P-450 have been difficult to investigate owing to the particulate nature of this membrane-bound hemoprotein.

The isolation of a highly purified, soluble cytochrome P-450 from *Pseudomonas putida* cells grown on *d*-camphor (Katagiri *et al.*, 1968; Peterson, 1971) has permitted detailed studies on the properties of this bacterial cytochrome (Dus *et al.*, 1970; Yu and Gunsalus, 1970; Tsai *et al.*, 1970; Peterson, 1971; Griffin and Peterson, 1971; Peterson *et al.*, 1971, 1972a,b). Although there are important differences between the bacterial and mammalian cytochromes P-450, their similarities are numerous enough that the bacterial monooxygenase system is currently being investigated as a model for the reactions catalyzed by the mammalian systems (Peterson *et al.*, 1972b; Gunsalus *et al.*, 1972a).

This report describes some kinetic and equilibrium properties of the reaction of camphor, a type I substrate, and pyridine, a type II substrate, with bacterial cytochrome P-450.

Materials and Methods

Materials. Cytochrome P-450 was prepared by published procedures (Peterson, 1971; Peterson *et al.*, 1972a) from *P. putida* (ATCC 17453) which had been grown on camphor in a 750-l. fermentor by Truett Laboratories, Inc., Dallas, Texas. Reagent grade *d*-camphor was a product of J. T. Baker Chemical Co. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was supplied by Dr. J. J. Chart, Ciba Pharmaceutical Co., Inc. All other reagents were of the highest quality commercially available and were used without further purification.

Methods. Cytochrome P-450 concentration was usually determined by measuring the absorbance difference between the absorbance band maximum of the carbon monoxide complex of ferrous cytochrome P-450 and the ferrous cytochrome at 446 nm relative to 490 nm ($\Delta\epsilon_M = 95 \times 10^3$ M^{-1} cm^{-1}) (Peterson, 1971). Various other spectrophotometric methods for the determination of cytochrome P-450 which have been described (Peterson, 1971) all give agreement to within 10%. Pyridine solutions were standardized by measurement of the absorbance at 257 nm, for which $\epsilon_M = 2.85 \times 10^3$ M^{-1} cm^{-1} (Krumholz, 1951). The absolute absorbance spectra were recorded with either a Cary 14R spectrophotometer or an Aminco-Chance dual-wavelength split-beam spectrophotometer, both of which were equipped with thermo-

† From the Department of Biochemistry, The University of Texas Southwestern Medical School at Dallas, Dallas, Texas 75235. *Received March 20, 1972.* Supported, in part, by U. S. Public Health Service Research Grants AM 13366 and GM 16488 and Research Grant I-405 from The Robert A. Welch Foundation. After the publication of a preliminary communication describing these results (Peterson and Griffin, 1971) similar results were reported by Gunsalus and coworkers (1972a,b)

‡ Postdoctoral fellow of The Robert A. Welch Foundation.

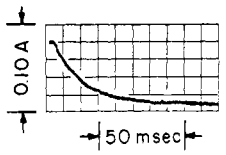

FIGURE 1: Oscilloscope trace of the absorbance decrease at 418 nm during the interaction of ferric cytochrome P-450 and camphor. One driving syringe of the stopped-flow apparatus was filled with a 5.0 μM solution of low-spin cytochrome P-450 in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 M potassium chloride, and the other syringe was filled with a 20 μM solution of camphor in the same buffer. After mixing the two solutions, the absorbance change at 418 nm was monitored as a function of time. The temperature was maintained at 4° throughout the experiment.

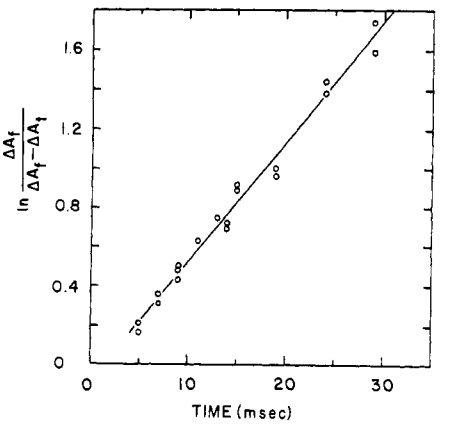

FIGURE 2: Kinetics of the camphor-association reaction of ferric cytochrome P-450: first-order plot of the absorbance change at 418 nm in the presence of excess camphor. The experimental data of Figure 1 are plotted as a first-order reaction with respect to $\Delta A_f - \Delta A_t$, as described in Results. The first-order reaction rate constant determined from the slope of the plot is 47 sec^{-1}.

stated cell holders. For measurement of the temperature, an iron-constantan thermocouple was inserted into the solution in the spectrophotometer cell, and the electromagnetic force (emf) was measured with reference to an ice water bath by means of a precision potentiometer. The pH of buffers was measured with a Radiometer PHM26 pH meter with expanded scale.

The rapid reaction spectrophotometric experiments were performed with an Aminco-Chance dual-wavelength spectrophotometer in the single wavelength mode. Equal volumes of the reaction solutions were mixed with an Aminco-Morrow stopped-flow attachment which has a mixing time of between 3 and 4 msec. The output of the photomultiplier was converted to absorbance by a rapid response log amplifier circuit designed by D. Mock of this department. The response time of this amplifier was determined to be less than 0.1 msec.

Electron paramagnetic resonance (epr) spectroscopy (Tsai *et al.*, 1970) and magnetic susceptibility measurements (Peterson, 1971) have established that camphor binding converts ferric bacterial cytochrome P-450 from a low- to high-spin state. Concomitant with this spin-state conversion, camphor binding by the enzyme results in a shift of the Soret absorbance band maximum from 418 to 392 nm, with $\epsilon_M \simeq 1.0 \times 10^5$ M^{-1} cm^{-1} for both of these states (Peterson, 1971).

The spectral changes in the Soret region which occur when compounds interact with mammalian cytochrome P-450 have been utilized to construct double-reciprocal plots from which spectral dissociation constants, K_s, are extrapolated (Schenkman *et al.*, 1967). However, because bacterial cytochrome P-450 binds camphor so strongly, this method is inapplicable (Peterson, 1971). Therefore, the equilibrium constant for the camphor-binding reaction has been determined by direct calculation

$$\text{P-450} + \text{cam} \rightleftharpoons \text{P-450}\cdot\text{cam}$$

The concentration of P-450·cam was calculated from the decrease in absorbance at 418 nm ($\Delta\epsilon_M = 5.1 \times 10^4$ M^{-1} cm^{-1}) (Peterson, 1971) on adding a known concentration of camphor to camphor-free ferric cytochrome P-450. For these calculations, the concentration of "free," or unbound, cytochrome P-450 was determined by subtracting P-450·cam from the total concentration of enzyme capable of binding camphor, which represented at least 90% of the total cytochrome P-450 for the preparations used in this study. In a publication from this laboratory, the association constant for the camphor-binding reaction of ferric cytochrome P-450 was reported to be 4.7×10^5 M^{-1} at 25° in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 M potassium chloride (Peterson, 1971).

Results

The kinetics of camphor binding were studied at 4° by measuring the rate of change in absorbance at 418 nm which occurs when camphor is mixed with ferric cytochrome P-450 in the stopped-flow apparatus. It would be desirable to have camphor present in excess to drive the reaction to completion and to observe pseudo-first-order kinetics; however, because the rate constant for camphor binding is very large even at this low temperature, the upper limit on the camphor concentration which could be used in these experiments was determined by the time constant of our instrument. As a result of these limitations, the ratio of total camphor to total cytochrome P-450 concentrations was varied from 4 to 16 in a series of experiments in which the total final (after mixing) concentration of cytochrome P-450 was held constant at 2.5 μM.

An example of the experimental kinetic traces recorded in these experiments is shown in Figure 1 for $[cam]_T:[P\text{-}450]_T = 4$, a ratio for which a major part of the reaction is observable. The data are plotted (Figure 2) as a first-order reaction with respect to $\Delta A_f - \Delta A_t$, *i.e.*, the difference between the final absorbance change and that at time t. Since, under these conditions not all of the cytochrome P-450 is converted to the camphor complex, $\Delta A_f - \Delta A_t$ is not directly proportional to the total unbound cytochrome P-450 at time t, but only that part which remains to be bound to camphor at equilibrium. Analysis of a first-order reaction that proceeds to equilibrium and for which the reverse reaction is also first order yields a rate constant that is the sum of those for the forward and reverse reactions (Jencks, 1969). Therefore, the first-order reaction rate constant determined from Figure 2 was corrected by substracting the rate constant of the camphor dissociation reaction, which, as described below, has a value of 6.0 sec^{-1}. The corrected rate constant (41 sec^{-1}) was then divided by the concentration of camphor to give the second-order rate constant (4.1×10^6 M^{-1} sec^{-1}) for the binding of camphor to ferric cytochrome P-450.

It should be noted that in this experiment 72% of the total cytochrome P-450 or 1.8 μM is camphor bound at equilibrium and that the free camphor concentration changes from 10 to 8.2 μM during the course of the reaction. Under these conditions, first-order kinetics were observed over approximately

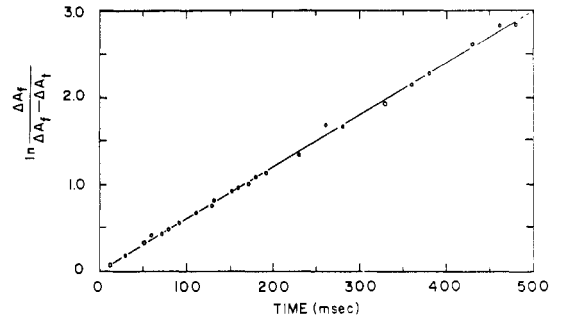

FIGURE 3: Kinetics of the camphor-dissociation reaction: first-order plot of the absorbance change at 418 nm in the presence of excess metyrapone. The two driving syringes of the stopped-flow apparatus contained, respectively, a 1.0 mM solution of metyrapone in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 M potassium chloride and a 2.5 μM solution of high-spin ferric cytochrome P-450 in the same buffer with 0.1 mM camphor also present. After mixing the two solutions, the absorbance increase at 418 nm was measured as a function of time. The temperature was maintained at 4°. A plot of the data as a first-order reaction with respect to high-spin cytochrome P-450 is linear with a rate constant of 6.0 sec^{-1}.

80% of the reaction. At the higher camphor concentrations used (with the same concentration of cytochrome P-450), first-order kinetics were also observed and second-order rate constants calculated as described above were in good agreement.

The rate of dissociation of camphor from the camphor–ferric cytochrome P-450 complex was measured by trapping the free ferric cytochrome P-450 with metyrapone to form the metyrapone–ferric cytochrome P-450 complex, which has a characteristic absorbance band maximum at 422 nm (Peterson *et al.*, 1971). The reaction sequence, which is strongly supported by experiments described below, consists of the slow camphor-dissociation step resulting in formation of the unbound ferric enzyme, followed by very rapid combination of this form of the enzyme with metyrapone

$$\text{P-450·cam} \xrightarrow{k_{-1}} \text{P-450} + \text{cam}$$

$$\text{P-450} + \text{MP} \xrightarrow{k_2} \text{P-450·MP}$$

Such a technique (Warburg, 1949) has been used to determine the rate constant for oxygen dissociation from oxyhemoglobin by trapping free hemoglobin as the carbon monoxide complex (Gibson and Roughton, 1955). Previous studies demonstrated the competitive nature of metyrapone and camphor binding to ferric cytochrome P-450 (Peterson *et al.*, 1971). The equilibrium association constant for the metyrapone complex of the ferric cytochrome has a value of 4.3×10^8 M^{-1} (Peterson *et al.*, 1971) which satisfies the condition that the metyrapone binding reaction of cytochrome P-450 be essentially irreversible. In addition, as will be described below, the rate of the metyrapone binding reaction is much faster than the rate of the camphor dissociation reaction.

The camphor–ferric cytochrome P-450 complex was mixed in the stopped-flow apparatus with a large excess of metyrapone, as described in the legend to Figure 3, and the rate of increase in absorbance at 422 nm due to formation of the metyrapone–ferric cytochrome P-450 complex was recorded. This reaction exhibits first-order kinetics (Figure 3) with a first-order reaction rate constant of 6.0 sec^{-1}. The final (after mixing) concentration of metyrapone was varied over a range of concentrations from 0.5 to 5.0 mM to verify that the reaction order and rate constant are independent of metyrapone concentration. This result indicates that metyrapone binding is not the rate-limiting step. Since metyrapone and camphor are competitors with respect to binding cytochrome P-450, we conclude that the first-order rate constant determined in this experiment is that for the rate-limiting step of camphor dissociation.

TABLE I: Comparison of the Observed and Calculated Values of the Equilibrium Association Constant for Camphor Binding to Ferric Cytochrome P-450 at 4°.

Constant	Value
k_1 (camphor association) (M^{-1} sec^{-1})	4.1×10^6
k_{-1} (camphor dissociation) (sec^{-1})	6.0
$K_{eq,cam}$ (calcd) = k_1/k_{-1} (M^{-1})	6.8×10^5
$K_{eq,cam}$ (exptl) (M^{-1})	4.7×10^5

In another experiment, analogous to the camphor-binding experiments described above, the rate of metyrapone binding to camphor-free ferric cytochrome P-450 was measured. At 4° the experimentally determined second-order rate constant is 9.5×10^5 sec^{-1} M^{-1}. Thus, in the metyrapone displacement experiments for which the metyrapone concentration was 0.5 mM (Figure 3), the pseudo-first-order rate constant for formation of the metyrapone–cytochrome P-450 complex was calculated to be 480 sec^{-1}, which is much larger than the observed rate constant of 6.0 sec^{-1}. Finally, it should be noted that the equilibrium constant calculated from the experimental forward and reverse reaction rate constants agrees well with the observed equilibrium constant at the same temperature (Table I). These results taken together strongly support the validity of the techniques employed to determine the rate constants for both the association and dissociation reactions of camphor with ferric cytochrome P-450.

Thermodynamics of Camphor Binding. During the determination of the equilibrium association constant for the reaction of camphor with ferric cytochrome P-450, a rather unusual temperature dependence of the equilibrium constant was observed. The equilibrium constant for camphor binding was found to be exponentially dependent on reciprocal temperature from 4° to about 13°. However, there is a definite break in the van't Hoff plot (Figure 4) near 13°, above which temperature the equilibrium constant appears to be temperature independent.

Because of this temperature dependence of the equilibrium constant, the following experiments were designed to determine if the association and dissociation reactions are affected similarly by a change of temperature. The association reaction of camphor with cytochrome P-450 is very rapid even at 4°, so the association reaction rate constant could not be measured at higher temperatures with our instrumentation. However, since the rate of dissociation of camphor from the camphor–ferric–cytochrome P-450 complex is relatively slow, it was easily measured as a function of temperature. The log of the reaction rate constant for the dissociation of camphor from ferric cytochrome P-450 was found to be linearly dependent on the reciprocal of the absolute temperature, shown in Figure 5. This is in contrast to the corresponding plot of the equilibrium constant in Figure 4, which exhibits a break in the plot at 13°.

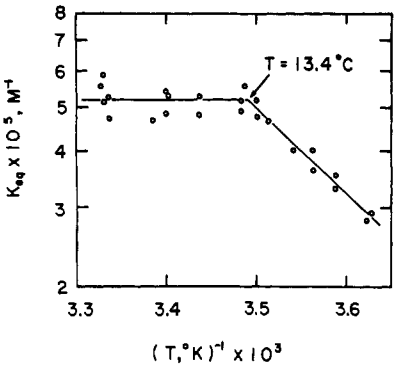

FIGURE 4: Temperature dependence of the equilibrium association constant for camphor binding to ferric cytochrome P-450. Aliquots of a solution of known camphor concentration were added to a 1.0 μM solution of low-spin ferric cytochrome P-450 in 0.1 M morpholinopropanesulfonate buffer (pH 7.4) and 0.1 M in potassium chloride. After each camphor addition, the decrease in absorbance at 418 nm was recorded, and the association constant was calculated as described in Methods. The spectrophotometer cells were thermostated to the desired temperature, which was measured with an iron-constantan thermocouple.

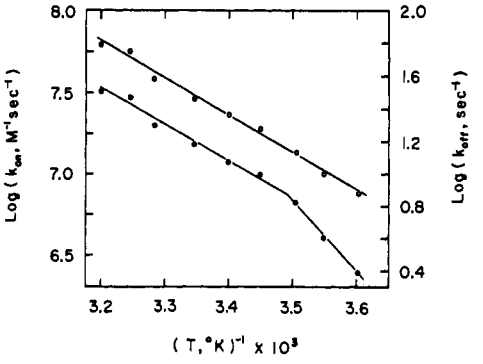

FIGURE 5: Temperature dependence of the experimental rate constant for camphor dissociation and the computed rate constant for camphor association to ferric cytochrome P-450. Experimental conditions for the measurement of the dissociation rate constant k_{-1} (○) are given in the legend to Figure 3, except that the temperature was varied between 4 and 40°. The association rate constant k_1 (●) was computed at temperatures higher than 4° from the experimentally determined temperature dependences of k_{-1} and K_{eq} (Figure 4) as $k_1 = k_{-1}K_{eq}$.

Pyridine Binding to Ferric and Ferrous Cytochrome P-450. The absorbance spectra of the pyridine complexes of ferric and ferrous cytochrome P-450 are essentially identical to those of the respective metyrapone complexes of the cytochrome (Peterson *et al.*, 1971) and are shown in Figure 6. It should be noted that the absorbance spectrum of the pyridine complex of ferrous cytochrome P-450, which has an intense maximum at 444 nm, does not depend on pH over the pH range 6.0–8.0. In contrast, it has been reported that the difference absorbance spectrum of the pyridine complex of reduced microsomal cytochrome P-450 exhibits absorbance maxima near 423 and 446 nm, and that the relative intensity of these maxima depends on pH (Imai and Sato, 1967a). The pyridine complex of ferric cytochrome P-450 exhibits a low-spin-type epr spectrum with g values of 2.50, 2.27, and 1.90.

A direct titration of low-spin ferric cytochrome P-450 with pyridine, in order to determine the association constant, $K_{eq,py}$, of the pyridine complex, was not undertaken because of the similarity of the absorbance spectra of these two forms of the hemoprotein. However, since pyridine, like metyrapone, can effectively displace camphor from ferric cytochrome P-450, resulting in a shift of the Soret band from 392 to 422 nm, the value of $K_{eq,py}$ could be determined indirectly, as Peterson *et al.* (1971), have done in the case of metyrapone. The basic assumption is that pyridine binding and camphor binding by the ferric cytochrome are mutually exclusive, so that the following equilibrium is established when both pyridine and camphor are added to the enzyme

$$K_{eq} = \frac{[\text{P-450}\cdot\text{py}][\text{cam}]}{[\text{P-450}\cdot\text{cam}][\text{py}]} = \frac{K_{eq,py}}{K_{eq,cam}}$$

Data for the calculation of K_{eq} were obtained from a spectrophotometric titration experiment in which a given amount of pyridine was added to a solution containing known concentrations of ferric cytochrome P-450 and camphor. The concentration of the pyridine complex was calculated from the increase in absorbance at 422 nm, for which $\Delta\epsilon_M$ was determined to be 4.7×10^4 M^{-1} cm^{-1}. $K_{eq,py}$ was determined to be 4.9×10^4 M^{-1} at room temperature (Table II).

In experiments with ferrous cytochrome P-450 in the absence of camphor, it was found that this form of the enzyme binds pyridine very strongly in a 1:1 molar ratio (Figure 7), with $K_{eq,py}$ for this complex equal to 5.8×10^5 M^{-1} (Table III). In our study, the observation that the presence of camphor greatly affected both the rate and extent of reaction between pyridine and reduced cytochrome P-450 suggested an experiment similar to that described above by which the association constant for camphor binding, $K_{eq,cam}$, by reduced cytochrome P-450 could be determined. A direct spectrophotometric determination of this equilibrium constant is not possible since the absorbance spectrum of the reduced cytochrome is virtually unchanged by the addition of camphor (Peterson, 1971). By monitoring the increase in absorbance at 444 nm on adding pyridine to reduced cytochrome P-450 in the presence of a known concentration of camphor, the concentrations of the various species necessary for a calculation

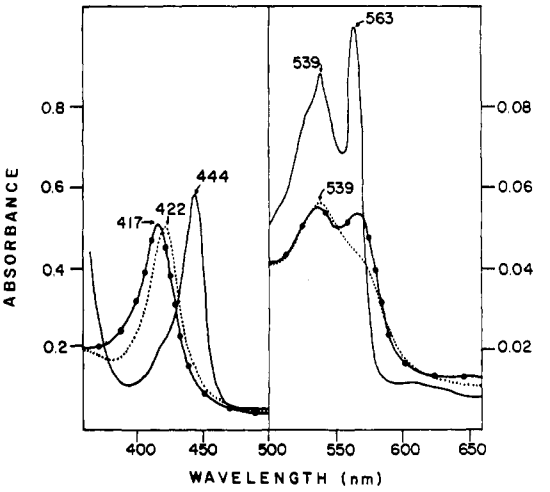

FIGURE 6: Absorption spectra of the pyridine complexes of ferric and ferrous cytochrome P-450. The sample cuvet contained a 4.9 μM solution of low-spin ferric cytochrome P-450 in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 M potassium chloride (●); to this was added pyridine to give a final concentration of 50 mM (.....). Then sodium dithionite was added (———). The spectra were recorded at room temperature.

TABLE II: Determination of $K_{eq,py}$ for the Binding of Pyridine to Oxidized Cytochrome P-450.[a]

Addition	[E^{3+}·py], μM	[E^{3+}·cam], μM	[py], μM	[cam], μM	K_{eq}	$K_{eq,py} \times 10^{-4}$, M^{-1}
1	0.78	1.08	85.4	10.5	11.3	4.2
2	0.89	0.97	104	10.6	10.7	4.4
3	1.01	0.85	123	10.8	9.6	4.9
4	1.06	0.80	142	10.8	9.8	4.8
5	1.14	0.70	160	10.9	9.2	5.1
6	1.20	0.66	179	10.9	9.0	5.2
7	1.29	0.57	215	11.0	8.7	5.4
				Average	9.8	4.9

[a] The sample cuvet contained a 1.86 μM solution of cytochrome P-450 (designated E) in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 M potassium chloride and 11.6 μM total camphor. [E^{3+}·py] was calculated from the increase in absorbance at 422 nm ($\Delta\epsilon_{mM}$ = 47 mM^{-1} cm^{-1}) on adding pyridine. Then [E^{3+}·cam] and [cam] were determined from a knowledge of their initial concentrations and [E^{3+}·py]. The experiment was carried out at room temperature.

TABLE III: Determination of $K_{eq,py}$ for the Binding of Pyridine to Reduced Cytochrome P-450.[a]

Addn	[E^{2+}·py], μM	[E^{2+}], μM	[py], μM	$K_{eq,py} \times 10^{-5}$, M^{-1}
1	1.06	1.27	1.07	7.9
2	1.24	1.09	1.72	6.7
3	1.35	0.98	2.43	5.7
4	1.42	0.91	3.18	4.9
5	1.56	0.77	5.03	4.0
			Average	5.8

[a] A 2.4 μM solution of camphor-free cytochrome P-450 (designated E) in 20 mM potassium phosphate buffer (pH 7.4) with 0.1 M potassium chloride was reduced with sodium dithionite after which additions of pyridine were made to the solution. [E^{2+}·py] was determined from the increase in absorbance at 444 nm for which $\Delta\epsilon_{mM}$ = 86 mM^{-1} cm^{-1}. The value of [py] was calculated from the total amount of pyridine added and the amount bound to ferrous cytochrome P-450. The absorbance measurements were made at room temperature.

of K_{eq} were determined. Then $K_{eq,py}$ determined for the pyridine complex of reduced cytochrome P-450 in the absence of camphor were used to evaluate $K_{eq,cam}$ for the reduced form of the cytochrome. The average value of $K_{eq,cam}$ determined from such an experiment, carried out at room temperature (Table IV) is 2.2 × 10^8 M^{-1} which, however, must be considered only a good approximation because of the scatter in the calculated values of K_{eq}. This scatter is probably attributable to error in the measured absorbance change, as a result of the extreme slowness of the dissociation of camphor from reduced cytochrome P-450.

Discussion

The results of this study demonstrate the rapid reaction and very tight binding of camphor to the ferric form of bacterial cytochrome P-450. Kinetic studies of the camphor-binding reaction were, of necessity, carried out at 4° because of the response time of our instruments. The observed rate of decrease in absorbance at 418 nm follows first-order kinetics with respect to both camphor and cytochrome P-450. This finding indicates that the rate-limiting step determined spectrophotometrically is the actual binding reaction of a camphor molecule to the ferric form of the enzyme. Several experimental observations support the validity of the metyrapone trapping technique as a means of determining the rate of dissociation of camphor from the enzyme. At 4° the equilibrium constant calculated from our experimental values of k_1 and k_{-1} agrees with the experimental value of this constant. Therefore, the camphor-binding reaction can be described as

$$\text{P-450} + \text{cam} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{P-450·cam}$$

The changes in geometry and/or protein conformation in the vicinity of the heme iron atom of cytochrome P-450 which are reflected in the spin state conversion on camphor binding are not yet understood.

The thermodynamic parameters for the camphor-binding reaction, calculated from Figure 4, are given in Table V for 4.6 and 21°, temperatures where the equilibrium constant does and does not, respectively, depend on temperature. In addition, Table V contains the thermodynamic activation parameters which were determined directly for the dissociation of camphor from cytochrome P-450 (Figure 5) and indirectly for the camphor–association reaction. Although ΔG for the overall reaction is very nearly the same in the two temperature regions, the values of both ΔH and ΔS are considerably different in the two regions. From the data presented, it is not possible to estimate the contributions of various processes, such as ionization reactions and coulombic interactions, to these thermodynamic quantities. The thermodynamic parameters for the spin state equilibria, *i.e.*, low spin ⇆ high spin, of some complexes of metmyoglobin with ligands such as hy-

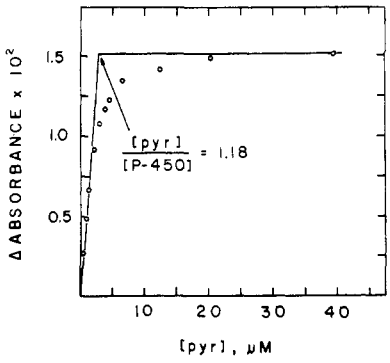

FIGURE 7: Plot of the absorbance changes observed on adding pyridine to ferrous cytochrome P-450 in the absence of camphor. Aliquots of a pyridine solution were added to a 2.4 μM solution of camphor-free cytochrome P-450 in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 M potassium chloride and sodium dithionite. The increase in absorbance was measured at 444 nm after each pyridine addition and corrected for dilution. The titration experiment was carried out at room temperature.

TABLE IV: Determination of $K_{eq,cam}$ for the Binding of Camphor to Reduced Cytochrome P-450.[a]

Addn	$[E^{2+}\cdot py]$, μM	$[E^{2+}\cdot cam]$, μM	[py], mM	$K_{eq} \times 10^3$	$K_{eq,cam} \times 10^{-8}$, M^{-1}
1	0.28	1.39	3.86	2.05	2.83
2	0.51	1.16	5.78	3.06	1.90
3	0.76	0.91	7.69	4.35	1.33
4	1.37	0.30	15.3	2.62	2.21
5	1.51	0.16	19.0	2.10	2.76
			Average	2.84	2.21

[a] A 1.67 μM solution of cytochrome P-450 (designated E) in 20 mM potassium phosphate buffer (pH 7.4) with 0.1 M potassium chloride was made 40 μM in camphor, followed by the addition of sodium dithionite. On adding pyridine, the increase in absorbance at 444 nm was measured and $[E^{2+}\cdot py]$ was calculated as described in Table II. The camphor concentration was held constant at 40 μM throughout the experiment, which was carried out at room temperature.

TABLE V: Thermodynamic Parameters for the Camphor-Binding Reaction of Ferric Cytochrome P-450.[a]

Reaction	T (°C)	ΔH (kcal/mole)	ΔG (kcal/mole)	ΔS, cal/(mole °K)
Overall	4.6	8.7	−7.0	56.4
	21.0	0	−7.7	26.2
Reverse	4.6	10.5	15.1	−16.7
	21.0	10.5	15.4	−16.8
Forward[b]	4.6	19.2	8.1	39.7
	21.0	10.5	7.7	9.4

[a] The values in this table were determined from the plots shown in Figs. 4 and 5. For the reverse and forward reactions, the thermodynamic parameters are ΔH^*, ΔG^*, and ΔS^* for an activated state which lies intermediate between the reactants and products. [b] These values were calculated from the experimentally determined values for the overall and reverse reactions: thus $\Delta H_{over} = \Delta H^*_{for} - \Delta H^*_{rev}$.

droxide, azide, and imidazole have been published (George *et al.*, 1961). While ΔH, ΔS, and generally ΔG have the same signs as do the corresponding values for the camphor binding reaction of cytochrome P-450, the absolute values of these quantities are considerably larger for ferric cytochrome P-450 than for metmyoglobin. However, for reasons cited above, no quantitative comparison of the thermodynamic parameters for the spin-state conversions of these two hemoproteins can be made. It is perhaps not entirely fortuitous that the thermodynamic parameters of camphor binding at 21° are in the same range as those which have been measured for the transfer of hydrocarbons from water to a nonpolar solvent (Kauzmann, 1959). This comparison suggests that the driving force for substrate binding to ferric cytochrome P-450 is the removal of camphor from an aqueous environment to a hydrophobic region of the protein in proximity to the heme group.

Through an analysis of the effect of camphor on the extent of binding of pyridine by ferrous cytochrome P-450, the equilibrium association constant of the camphor complex of ferrous cytochrome P-450 was evaluated as 2.2×10^8 M^{-1}. Thus, although camphor does not alter the absorbance spectrum of the ferrous cytochrome (Peterson, 1971) it appears to bind more strongly to the ferrous than to the ferric state of cytochrome P-450 by about three orders of magnitude. A demonstration of substrate binding to the reduced enzyme has not previously been reported, but has been postulated to occur in the overall hydroxylation pathway (Gunsalus *et al.*, 1972a; Peterson *et al.*, 1972b).

It should be noted that in the normal assay for camphor hydroxylation catalyzed by cytochrome P-450, with the buffer camphor saturated (5 mM), all of the ferric enzyme would be present as the camphor-bound form, a consequence of the large association constant of the cytochrome for its substrate. In addition, the second order rate constant for the camphor-binding reaction is estimated to be 1.5×10^7 M^{-1} sec^{-1} at 25° (Figure 5), which would result in a pseudo-first-order rate constant for camphor binding of 7.5×10^4 sec^{-1} under the assay conditions. Since the turnover number for cytochrome P-450 under similar conditions at 25° is 20 sec^{-1} (Peterson, 1971; Gunsalus *et al.*, 1972a), the camphor-binding reaction cannot be the rate-limiting step in the overall hydroxylation reaction.

Acknowledgments

The authors gratefully acknowledge the advice and encouragement of Dr. Ronald W. Estabrook and also the expert technical assistance provided by Mrs. Marguerite Gunder.

References

Dus, K., Katagiri, M., Yu, C.-A., Erbes, D. L., and Gunsalus, I. C. (1970), *Biochem. Biophys. Research Commun. 40*, 1423.

George, P., Beetlestone, J., and Griffith, J. S. (1961), *in* Haematin Enzymes, International Union of Biochemistry Series, Vol. 19, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., Oxford, Pergamon Press, p 105.

Gibson, Q. H., and Roughton, F. J. W. (1955), *Proc. Roy. Soc., Ser. B 143*, 310.

Griffin, B., and Peterson, J. A. (1971), *Arch. Biochem. Biophys. 145*, 220.

Gunsalus, I. C., Tyson, C. A., and Lipscomb, J. D. (1972a), *in* Second International Symposium on Oxidases and Oxygenases, King, T., Morrison, M., and Mason, H., Ed., New York, N. Y., Academic Press (in press).

Gunsalus, I. C., Tyson, C. A., Tsai, R., and Lipscomb, J. D. (1972b), *Chem.-Biol. Interactions 4*, 75.

Hildebrandt, A. G., Remmer, H., and Estabrook, R. W. (1968), *Biochem. Biophys. Res. Commun. 30*, 607.

Imai, Y., and Sato, R. (1966), *Biochem. Biophys. Res. Commun. 22*, 620.

Imai, Y., and Sato, R. (1967a), *J. Biochem. (Tokyo) 62*, 464.

Imai, Y., and Sato, R. (1967b), *J. Biochem. (Tokyo) 62*, 239.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill Book Co., pp 586–589.

Katagiri, M., Ganguli, B. N., and Gunsalus, I. C. (1968), *J. Biol. Chem. 243*, 3543.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Krumholz, P. (1951), *J. Amer. Chem. Soc. 73*, 3487.

Miyake, Y., Mori, K., and Yamano, T. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1616.

Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. (1965), *Life Sci. 4*, 2101.
Peterson, J. A. (1971), *Arch. Biochem. Biophys. 144*, 678.
Peterson, J. A., and Griffin, B. W. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1143.
Peterson, J. A., Ishimura, Y., Baron, J., and Estabrook, R. W. (1972b), *in* Second International Symposium on Oxidases and Oxygenases, King, T., Morrison, M., and Mason, H., Ed., New York, N. Y., Academic Press (in press).
Peterson, J. A., Ishimura, Y., and Griffin, B. W. (1972a), *Arch. Biochem. Biophys. 149*, 197.
Peterson, J. A., Ullrich, V., and Hildebrandt, A. G. (1971), *Arch. Biochem. Biophys. 145*, 531.
Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillete, J., Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. (1966), *Mol. Pharmacol. 2*, 187.
Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3*, 113.
Tsai, R., Yu, C.-A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., and Beinert, H. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1157.
Warburg, O. (1949), Heavy Metal Prosthetic Groups and Enzyme Action, London, Oxford University Press, p 101.
Yu, C.-A., and Gunsalus, I. C. (1970), *Biochem. Biophys. Res. Commun. 40*, 1431.

Flavine–Protein Interactions in Flavoenzymes. Temperature-Jump and Stopped-Flow Studies of Flavine Analog Binding to the Apoprotein of *Azotobacter* Flavodoxin†

Bruce G. Barman‡ and Gordon Tollin*

ABSTRACT: Equilibrium binding constants, kinetic parameters, and thermodynamic parameters have been determined for the binding of several flavine analogs to the apoprotein of *Azotobacter* flavodoxin (Shethna flavoprotein). The results indicate that the ribityl phosphate side chain and the N-5 position of the isoalloxazine ring act cooperatively during the rate-limiting step of the binding process. Kinetic measurements using temperature-jump and stopped-flow methods provide strong evidence for a phosphate-induced protein conformational change. The values for the individual rate constants and activation entropies are interpreted in terms of the detailed processes which occur during coenzyme binding. Preliminary studies using *Peptostreptococcus elsdenii* flavodoxin suggest that the mechanism of flavine mononucleotide binding is similar to that of the *Azotobacter* protein.

Studies of flavine–protein interactions have been facilitated by the fact that in most flavoenzymes the flavine is not covalently bound to the protein and may be resolved by treatment with acid (Warburg and Christian, 1938; Hinkson, 1968) or KBr dialysis (Massey and Curti, 1966). The resolved apoprotein may then be recombined with modified flavines and the enzymatic activity (when possible), redox behavior, and spectral properties compared to those of the native holoenzyme. In this way, the positions on the isolloxazine ring and ribityl side chain that influence the above properties can be determined.

We have measured the kinetics and thermodynamics of binding of several flavine analogs to the Shethna apoprotein (*Azotobacter* apoflavodoxin).[1] This flavoprotein is particularly useful for this type of study because of its high stability, low molecular weight, and possession of only one flavine binding site. The flavine mononucleotide (FMN) cofactor is easily resolved and can be subsequently recombined with essentially complete renaturation as evidenced by circular dichroism (CD) and redox properties (Edmondson and Tollin, 1971a,b). The apoprotein will efficiently bind other flavine analogs as well. Previous work in our laboratory has demonstrated the importance of the ribityl phosphate side chain and N-5 ring nitrogen of FMN in determining redox properties (Vaish and Tollin, 1971; Edmondson and Tollin, 1971b; Edmondson *et al.*, 1972). It has also been observed that the presence of the 5′-phosphate group increases the flavine–protein association constant for *Azotobacter* flavodoxin (Hinkson, 1968; Edmondson and Tollin, 1971c). To date, all of the studies concerning flavine–protein interactions have used static methods such as absorption, fluorescence, and CD spectroscopy of analog, holo-, and apoflavoproteins, titration methods, equilibrium analog-binding studies, and simple kinetic measurements of rate limiting steps in analog binding. Some of this work has suggested that the phosphate group of FMN induces a protein rearrangement that facilitates the binding process (Edmondson and Tollin, 1971b; D'Anna and Tollin, 1972). However, there is no kinetic evidence to support this mechanistic proposal.

Relaxation methods (Eigen, 1954) provide a powerful tool for the study of rapid biological reactions that are mechanistically complicated. For each independent step in a reaction

† From the Department of Chemistry, University of Arizona, Tucson, Arizona 85721. *Received July 10, 1972.* Supported in part by a grant from the National Institutes of Health (1R01-AM15057) and taken from a dissertation submitted by B. G. B. in partial fulfillment of requirements for a Ph.D. degree, University of Arizona, 1972.

‡ Present address: Department of Chemistry, University of Minnesota, Minneapolis, Minn.

[1] Although earlier work (Hinkson and Bulen, 1967; Benemann *et al.*, 1969; Cusanovich and Edmondson, 1971) had shown that the Shethna flavoprotein would not substitute for ferredoxins in the photosynthetic reduction of $NADP^+$ by spinach chloroplasts, more recent studies by Van Lin and Bothe (1972) have demonstrated that this flavoprotein can indeed substitute for ferredoxin and thus can be classified as a flavodoxin.