Substrate-Induced Spin-State Transition in Cytochrome $P450_{LM2}$: A Temperature-Jump Relaxation Study†

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Received April 21, 1993; Revised Manuscript Received July 8, 1993®

ABSTRACT: The kinetics of the benzphetamine–$P450_{LM2}$ binding reaction were studied by the T-jump relaxation technique, using the substrate-induced type I spectral change (which reflects transformation of the heme from the low- to the high-spin state) as the criterion for binding. The reciprocal relaxation time (k_{obs}) exhibited a linear dependence on $[E]_{eq} + [S]_{eq}$. The kinetically determined dissociation equilibrium constant (68 ± 10 μM) and that determined by direct titration of the spectral change (61 ± 4 μM) were very similar. These results indicate that the substrate-induced spin-state transition follows a simple bimolecular binding mechanism; that is, the substrate-induced low- to high-spin transition reflects substrate binding.

Most cytochrome P450s are low-spin ($S = {}^1/_2$) in the substrate-free form. Binding of substrate shifts the low-spin state to the high-spin state ($S = {}^5/_2$) and the Soret absorption band from around 417–419 nm to around 390 nm, resulting in the characteristic type I difference spectrum. This shift is associated with transformation of the cytochrome from an "inactive", that is, not reducible, to the active reducible intermediate of the catalytic cycle (Narasimhulu et al., 1966; Narasimhulu, 1971a,b; Narasimhulu & Eddy, 1984). Therefore, substrate effects on spin state are among the major issues in considering the mechanism of regulation of P450 activity.

This topic has been extensively studied by difference spectrophotometry, using the type I spectral change as the criterion for the spin-state transition. Three models have been proposed for the substrate-induced transition. These are (i) the two-state model in which the two states are the not-reducible (low-spin) and reducible (high-spin) (Narasimhulu, 1971a,b) and (ii) the three-state model (Marden & BonHoa, 1987) which has been proposed after reinvestigating camphor binding to $P450_{cam}$. In this model, a second molecule of camphor binds at high concentrations (>0.2 mM) and shifts the high-spin back to low-spin, giving rise to the third state. (iii) The four-state model is the original model proposed for the camphor-induced spin-state transition in $P450_{cam}$ (Sligar, 1976). In the two- and three-state models, there is no spin-state transition independent of substrate binding. Substrate binding to low-spin converts directly to high-spin as in a simple bimolecular reaction. In the four-state model, both substrate-free and substrate-bound P450s are temperature-dependent equilibrium mixtures of high- and low-spin forms. In the absence of substrate, the equilibrium is mostly toward the low-spin. Substrate binds more tightly to the high-spin and shifts the preexisting equilibrium toward the high-spin state. Subsequently, on the basis of temperature-jump relaxation studies, simplified versions of the spin equilibrium model have been proposed (Tsong & Yang, 1978; Fisher & Sligar, 1987). However, stopped-flow measurements show that the substrate-induced spin-state transition in $P450_{cam}$ (Griffin & Peterson, 1972) follows a bimolecular mechanism, which is consistent with the results of Marden and BonHoa (1987) for the bacterial enzyme, and the results of Narasimhulu (1991) for the adrenal $P450_{C21}$.

In order to find out if the bimolecular mechanism is applicable to other P450's molecularity of the benzphetamine-induced spin-state transition in the rabbit liver enzyme, $P450_{LM2}$ was investigated, by the T-jump relaxation method. The results indicate the bimolecular mechanism.

MATERIALS AND METHODS

Glycerol, benzphetamine hydrochloride, dilaurylphosphatidylcholine (DLPC), and most other chemicals were purchased from Sigma Chemical Co. Highly purified phenobarbital-induced rabbit liver $P450_{LM2}$ was a gift from Dr. M. J. Coon.

Assay System. Unless otherwise indicated, the assay system consisted of 0.18 M potassium phosphate buffer, pH 7.25, 5% glycerol, 0.025 mM EDTA, and 50 μg/mL DLPC.

Spectrophotometry. Absorption spectra of purified $P450_{LM2}$ were obtained with a Hitachi split-beam Model U-2000 spectrophotometer equipped with temperature-regulated cuvette holders.

Static Binding Equilibrium Studies. The substrate-produced type I difference spectrum of P450 characterized by a minimum at 420 nm, a maximum around 388–390 nm, and an isosbestic point around 407 nm was used as a criterion for the binding of substrate to the cytochrome. In order to conserve the highly purified enzyme, titrations of the spectral change at high enzyme concentrations (>1 μM) were performed in 0.5-mL microcuvettes, and the Soret spectral region was scanned with the Hitachi spectrophotometer after each addition of the substrate. At lower enzyme concentrations, titrations were performed in 3.0-mL cuvettes by a more precise semi-micro titration procedure. The details of the procedure have been previously described (Narasimhulu, 1977). A cuvette of 12-mm light path containing 3.0 mL of the assay system was placed in a dual-wavelength filter photometer fitted with interference filters of 421 nm (λ_1) and 407 nm (λ_2) of 1-nm half-band-width. The assay system was constantly stirred during the titration with a magnetic stirring attachment. The temperature was regulated by a thermostated circulator and measured with a thermocouple. Microliter volumes (no more than 10 μL total) of an aqueous solution of benzphetamine hydrochloride were added to the assay system, and ΔA(407–420 nm) was recorded with a strip chart recorder.

† This work was supported in part by ONR Contract N00014-75C-0322, by NIH Grant AM18545, and in part by the Harrison Department for Surgical Research.

® Abstract published in *Advance ACS Abstracts,* September 1, 1993.

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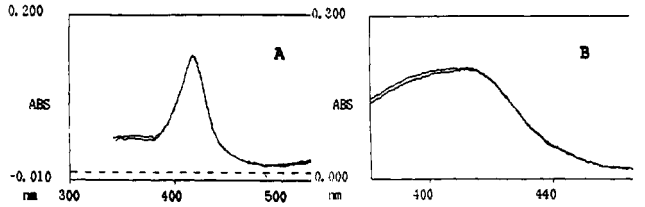

FIGURE 1: Temperature independence of the absorption spectrum of $P450_{LM2}$. The figure shows spectra obtained at 12 °C (upper curves in the slightly separated region) overlayed on those obtained at 27 °C (lower curves). Curve A, in the absence of substrate; curve B, in the presence of saturating concentration (0.8 mM) of benzphetamine. Due to fractional conversion to the high-spin form, curve B represents a mixture of the low- and the high-spin forms.

Table I: Effects of Temperature on the Parameters of the Benzphetamine–$P450_{LM2}$ Binding Reaction

temp (°C)	K_d^{app} (μM)	$[ES]_{max}$ (μM)
13.8	189 ± 5	0.085 ± 0.002
23.8	86 ± 2	0.087 ± 0.004

Temperature-Jump Relaxation Measurements. The T-jump relaxation measurements were made using the apparatus manufactured by Dialog (West Germany), which has been previously described (Drobnies, 1979; Rigler et al., 1974). The temperature-jump was from 23 to 25 °C. The relaxation time at each concentration of the substrate represents an average of 10 jumps at 2500 V.

Data Analysis. The T-jump relaxation data were collected and analyzed by the procedure described by Pasternack et al. (1983). In each case, the best-fitting model (usually two versus three exponentials) for the relaxation data was chosen on the basis of the *F* test for significance. A single exponential did not fit the data in nearly all instances. Static equilibrium binding data as well as the kinetic data [$1/\tau$ vs ($[E]_{eq} + [S]_{eq}$)] were analyzed by a weighted linear least-squares fitting program. The error ranges were calculated by the method of error propogation (Bevington, 1969). In static binding experiments, the concentration of the substrate-induced high-spin formed was calculated from ΔA(390–417 nm) or ΔA(407–420 nm) using extinction coefficients of 128 or 64 mM^{-1} cm^{-1}, respectively.

RESULTS

Effect of Temperature on the Absorption Spectrum of $P450_{LM2}$. Purified substrate-free $P450_{LM2}$ exhibited a Soret absorption maximum at 417 nm, which is characteristic of low-spin P450. Figure 1 shows spectra obtained at 27 °C overlayed on those obtained at 12 °C. Temperature was without significant effect on the absorption spectra of substrate-free enzyme (Figure 1A) as well as in the presence of a saturating concentration (0.8 mM) of benzphetamine (Figure 1B). Accordingly, maximum sub-P450 complex formed was also independent of temperature in the titration experiments (Table I).

In view of the spin equilibrium models reported for $P450_{cam}$ (Sligar, 1976; Fischer & Sligar, 1987), an attempt was made to find out whether or not $P450_{LM2}$ will also exhibit a temperature-dependent spin-state change under experimental conditions (high enzyme and high substrate concentrations) similar to those reported for $P450_{cam}$. The results of an experiment using 12 μM $P450_{LM2}$ are shown in Figure 2. A small decrease in high-spin and an increase in low-spin (amounting to a transition of 2.5–4.6% of the total P450) were detectable when the temperature was decreased from 27 to 12 °C, before (Figure 2A,C) as well as after addition of a high concentration of substrate (2 mM; Figure 2B,C). However, further experiments are needed to find out whether the observed temperature effects are due to high protein concentration and/or the presence of small amounts of residual detergent in the purified preparation used in this experiment (Figure 2). The residual detergent could vary from preparation to preparation, because of difficulties involved in complete removal of detergents used in purification of mammalian P450 enzymes. Detergents may induce a spin-state transition similar to substrates (Denk, 1979; Hashimoto et al., 1980).

Static Equilibrium Binding Experiments. The apparent benzphetamine–$P450_{LM2}$ dissociation constant was dependent on protein concentration as well as temperature. In the concentration range relevant to the experiments shown in Figures 4 and 5, the dissociation constant decreased with an increase in enzyme concentration. The dissociation constant obtained at about 2 μM $P450_{LM2}$ was 61 ± 4 μM at 25 °C. The effects of temperature (13.8 and 23.8 °C) on the parameters of the substrate–$P450_{LM2}$ binding reaction, at 0.3 μM $P450_{LM2}$, are shown in Table I. The decrease in the substrate dissociation constant with an increase in temperature is similar to that reported by others also for $P450_{LM2}$ (Taniguchi & Sato, 1984). The estimated enthalpy of binding was around 13 kcal mol^{-1}. However, the temperature was without significant effect on the $[ES]_{max}$. The calculated $[ES]_{max}$ was very close to that observed at saturating concentrations ($12K_d$) of the substrate. The extent of conversion of $P450_{LM2}$ to the high-spin form was dependent on lipid concentration, reaching a maximum of 50–60% in the presence of 50 μg of DLPC/mL. Regardless of the extent, the maximum converted was independent of temperature.

T-Jump Relaxation Experiments and Data Treatment. T-Jump relaxation traces obtained at the peak wavelengths of the low-spin (417–419 nm) and the high-spin (390 nm) are shown in Figure 3. Downward deflection of the trace indicates an increase in absorption. The absorption at 390 nm increased whereas the absorption at 417 nm decreased, when the temperature was raised from 23 to 25 °C, indicating increased substrate binding, which is consistent with the positive enthalpy observed in the spectral titration experiments (Table I). The $[E]_{eq} + [S]_{eq}$ dependence of the reciprocal relaxation time ($1/\tau = k_{obs}$) is shown in the bottom panel of Figure 4. A representative relaxation trace under the conditions of this experiment is shown in the top panel of Figure 4. There was no observable relaxation in the absence of substrate in this experiment, which is consistent with the absence of a temperature effect on the low-spin absorption spectrum of the substrate-free enzyme (Figure 1A). As would be expected, no significant absorption change was observed around the isosbestic wavelength when the temperature was raised. The benzphetamine–$P450_{LM2}$ binding reaction exhibited two relaxation times under all experimental conditions. Because they were well separated on the time axis, each one was considered separately. The fast relaxation time (τ_f) decreased from 3.1 to 0.4 ms with an increase in substrate concentration from 44 to 736 μM. The linear relationship between the reciprocal relaxation time and the concentration ($[E]_{eq} + [S]_{eq}$) (Figure 4, bottom) indicates that the substrate-induced spin-state transition follows a simple bimolecular binding mechanism:

$$P450_{LM2}{}^{LS} + Benz \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P450_{LM2}{}^{HS}\text{–}Benz \qquad (1)$$

where LS and HS represent low-spin and high-spin P450s

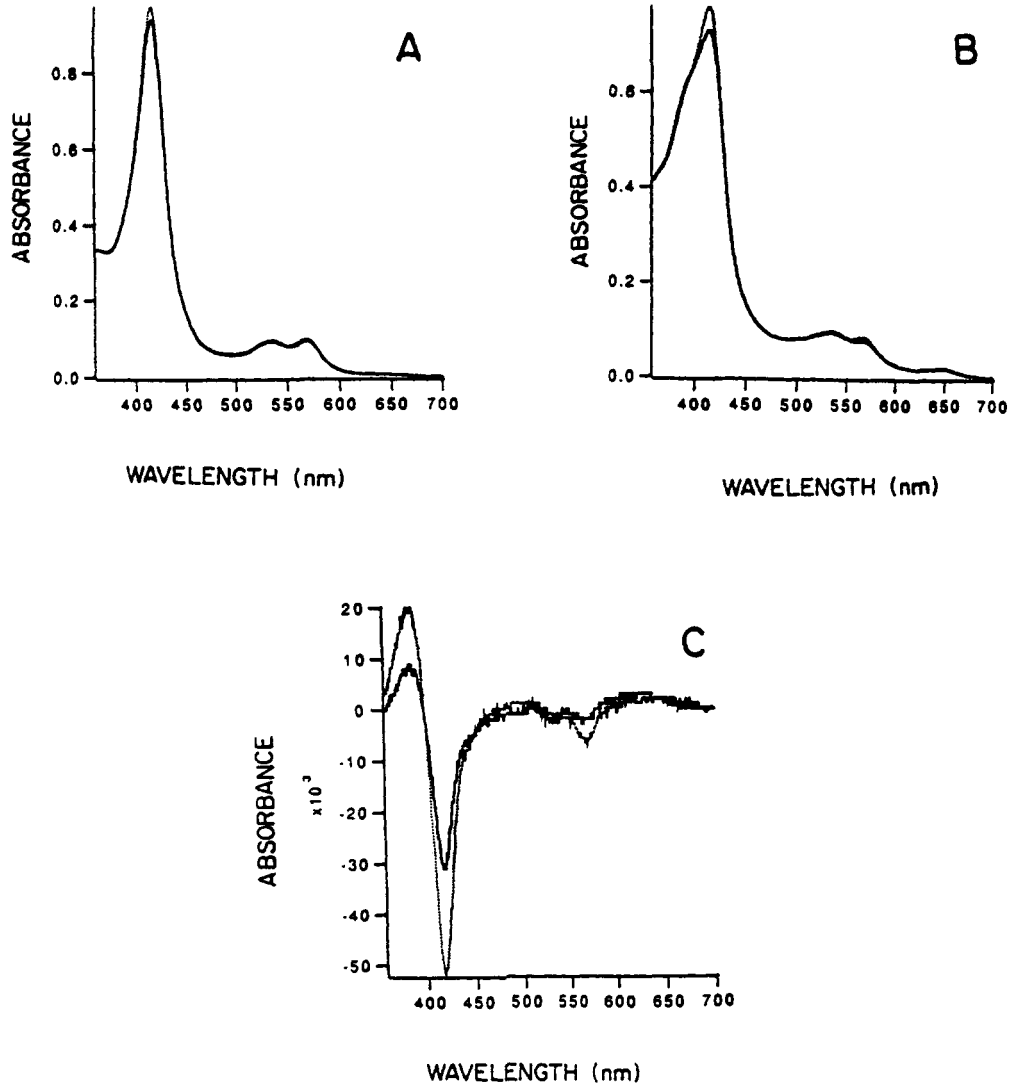

FIGURE 2: Effects of temperature on absorption spectra at high $[P450]_{LM2}$. The figure shows spectra of 12 μM p450 obtained at 12 and 27 °C. In panels A and B, solid lines were at the lower temperature and dotted lines at the higher temperature. (A) In the absence of substrate; (B) in the presence of 2 mM benzphetamine; (C) temperature-induced difference in the absence (solid line) and in the presence (dotted line) of substrate.

and [Benz] represents benzphetamine concentration. The k_{obs} for this model (Hammes & Schimmel, 1970) is

$$k_{obs} = 1/\tau_f = k_1([E]_{eq} + [S]_{eq}) + k_{-1} \quad (2)$$

Applying eq 2 to the bottom panel of Figure 4 (τ_f) gives rate constants $k_1 = (2.9 \pm 0.35) \times 10^6$ mol^{-1} s^{-1} and $k_{-1} = 196.5 \pm 18$ s^{-1}. The kinetic equilibrium dissociation constant (k_{-1}/k_1) was 68 ± 10 μM. This value is very similar to the dissociation constant (61 ± 4 μM) obtained from the spectral titration data. In the experiment shown in the bottom panel of Figure 4, an aliquot of the enzyme was used for no more than three data points (that is, 30 jumps at 2500 V). Points 2, 3, and 4 were obtained with one aliquot, and points 1, 5, and 6 were with another.

The values for k_{obs} for the slow relaxation were considerably smaller, and their standard deviations in some instances were larger than for the fast relaxation. The linear correlation coefficients ranged from 0.9 to 0.988, and the standard errors of estimates were around 20–30% of the median "Y" values. The destribution of residuals was quite good in experiments at the high (not shown) as well as at the low protein concentrations. In addition, the second degree polynomial

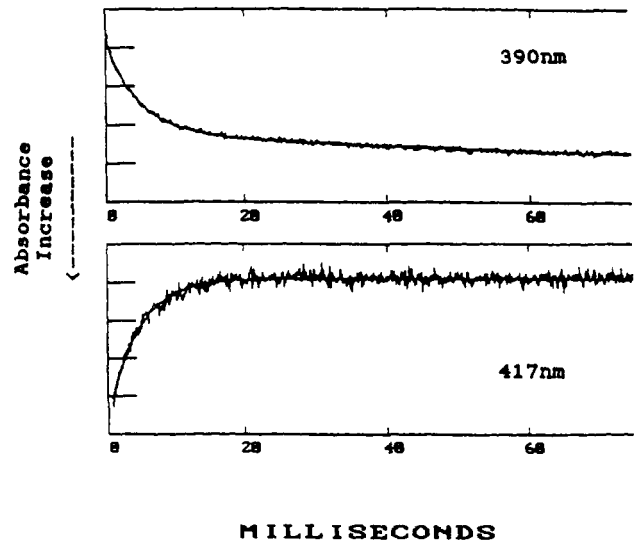

FIGURE 3: Temperature-jump relaxation traces at the peak wavelengths of the low-spin and high-spin forms of $P450_{LM2}$. The figure shows the decrease in absorption at 417 nm (low-spin) and increases at 390 nm (high-spin) when the temperature was raised from 23 to 25 °C.

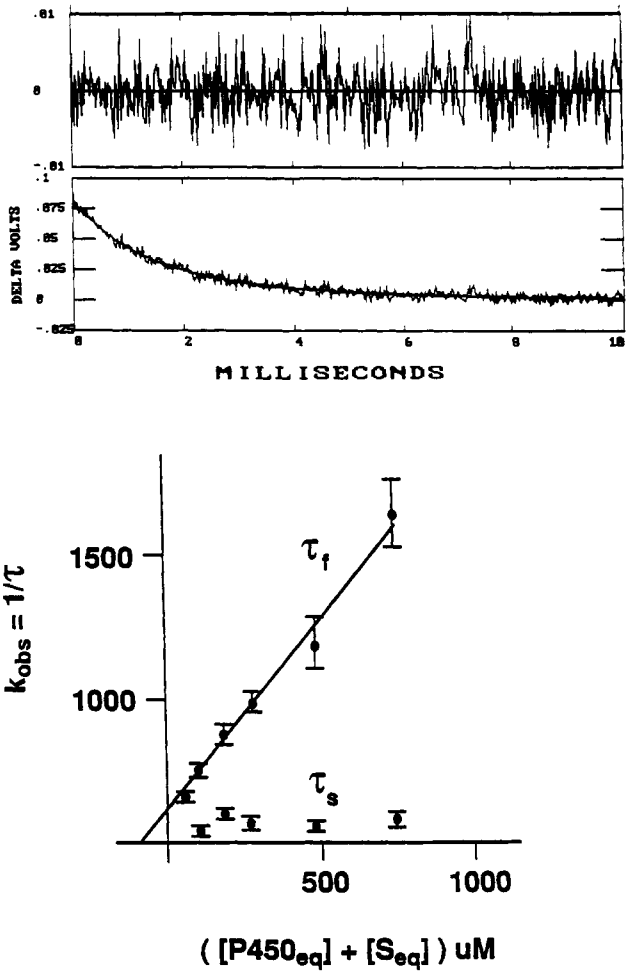

FIGURE 4: Effects of equilibrium concentrations of P450 and substrate on relaxation times. Assay system as described under Materials and Methods. (Top) Representative relaxation trace (at 183 μM benzphetamine) showing the data collection time and goodness-of-fit (SD for the entire curve = 0.0032) of the double-exponential function to the data. (Bottom) Reciprocal relaxation times as a function of $[E]_{eq} + [S]_{eq}$. The concentration of P450 ranged from 1.954 to 1.94 μM. The substrate concentration was increased from 43 to 735 μM. The temperature was raised from 23 to 25 °C. Measurements were made at 390 nm. For the slow relaxation, least-squares fitting was performed excluding point 2 because its residual was >2SEE.

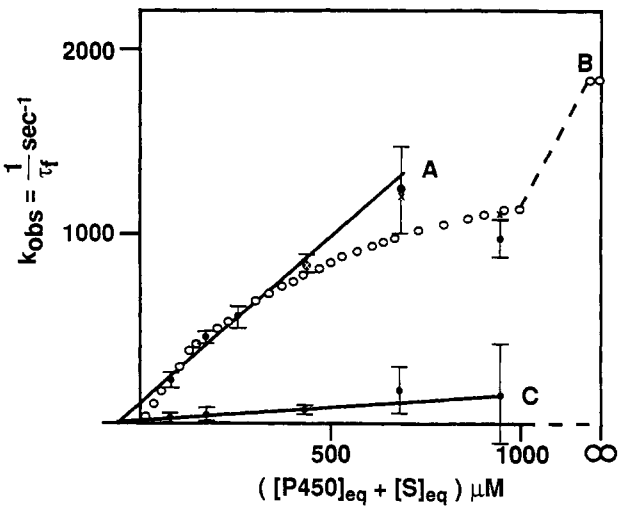

FIGURE 5: Effects of equilibrium concentrations of P450 and substrate on relaxation times. The starting concentration of P450 was 2 μM. Measurements were made at 417 nm. Other experimental conditions were as described under Figure 4. (A) Fast relaxation time; (C) slow relaxation time; (B) theoretical curve (obtained by a curve-fit search program) for the simplified spin equilibrium model (eq 5) of Fisher and Sligar (1987). The dashed line represents a continuation of the theoretical curve to infinite concentration. Crosses show the reproducibility of k_{obs}.

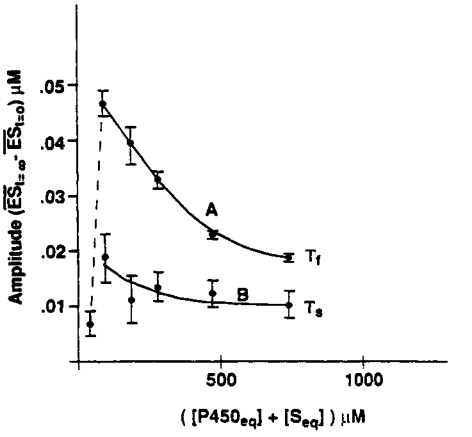

FIGURE 6: Effects of enzyme plus substrate concentration on amplitudes. These amplitudes were from the experiment shown in Figure 4. (A) Fast phase; (B) slow phase.

did not fit better than the first, indicating the absence of significant curvature in the data. Therefore, it is assumed that the slow relaxation is also linearly dependent on $[E]_{eq} + [S]_{eq}$. The kinetic K_d's ranged from 54 to 174 μM. This range overlaps the error ranges for the dissociation constants determined from the fast relaxations. Therefore, it is possible that the dissociation constants are not too different. Judging from amplitudes (Figure 6), the τ_s represents a minor component, less than 20% of the total maximum observed. The maximum observed may be underestimated because the amplitude decreased with an increase in substrate concentration (Figure 6).

The saturation behavior of k_{obs} versus $[E]_{eq} + [S]_{eq}$ has been reported in temperature-jump studies of the binding of benzphetamine to rat liver P450 (Tsong & Yang, 1978) and the binding of camphor and its analogues to $P450_{cam}$ (Fisher & Sligar, 1987). Since these authors used much higher enzyme concentrations (14 and 10 μM) and their results obtained at 417 nm are at variance with the data obtained at 390 nm (Figure 4), the present T-jump study was extended to 12 μM $P450_{LM2}$, and the relaxation measurements were made at 417 nm. The data showed a linear relationship between k_{obs} and $[E]_{eq} + [S]_{eq}$. Substrate concentrations as high as 1.45 mM did not reveal any saturation effect in this experiment using 12 μM P450. Three exponentials were usually needed to obtain a reasonably good fit. The values for k_{obs} for the third exponential were too small to be evaluated. Therefore, only two relaxations (τ_f and τ_s) were considered, which is reasonable because two exponentials usually fit the data at the low protein concentrations (Figures 4 and 5). Overall, it was difficult to work with such high protein concentrations because of protein aggregation. It often needed remixing.

Slow relaxations were observed prior to substrate addition at the high as well as at the low (Figure 5) concentrations of this batch of $P450_{LM2}$ preparation. This relaxation represented less than 6% of the observed maximum after substrate addition, as judged from the amplitudes. As indicated earlier, the maximum is probably underestimated (Figure 6). Since this relaxation was not observed in the experiment using a different batch of the purified enzyme (Figure 4), the possibility that small amounts of residual detergent may be present in the purified preparation was considered. If this is the case, eq 2

becomes

$$k_{obs} = 1/\tau = k_1([E_{eq}] + [R + S]_{eq}) + k_{-1} \quad (3)$$

where R = residual substance. Then the "Y" intercept = $k_{+1}[R] + k_{-1}$, that is, when $[E]_{eq} + [S]_{eq} = 0$, indicating that the "Y" intercept (that is, k_{-1}) would be overestimated by an amount equal to $k_{+1}[R]$. The 2 μM enzyme concentration is considerably less than the substrate concentration (88–950 μM). Assuming that $[R] \ll [E]_T + [S]_T$, subtraction of k_{obs} prior to substrate addition from those after substrate addition is considered as a reasonable approximate correction for the overestimation of k_{-1}.

The results of the T-jump relaxation measurements after such correction are shown in Figure 5. The measurements were made at 417 nm using a low concentration (2 μM) of $P450_{LM2}$. Higher substrate concentration than in the experiment shown in Figure 4 was also tested. The k_{obs} versus $[E]_{eq} + [S]_{eq}$ plot was linear up to 684 μM substrate. However, it deviated from linearity when the substrate concentration was increased to about 940 μM. In order to ascertain that this was not due to some erratic behavior, the last three points were repeated using a fresh enzyme aliquot. They were reproducible within the error range (crosses in Figure 5). This raised a question as to whether or not the near-equality of the k_{obs} at the last two concentrations represents saturation as in the simplified spin equilibirum model of Fisher and Sligar (1987) for the camphor-induced spin-state transition in $P450_{cam}$. Therefore, the applicability of the spin equilibrium model (eq 4 and 5; Hammes & Schimmel, 1970) was tested:

$$E^{LS} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES^{LS} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES^{HS} \quad (4)$$

In this model

$$1/\tau = k_{+2}K_a([E]_{eq} + [S]_{eq})/(1 + K_a[E]_{eq} + [S]_{eq}) + k_{-2} \quad (5)$$

where $K_a = k_1/k_{-1}$. At high $[E]_{eq} + [S]_{eq}$, $1/\tau$ approaches $k_{+2} + k_{-2}$. At low concentrations, it approaches k_{-2}. $K_a(1 + K_{spin}) = 1/$("X" intercept). Attempts were made to fit the data to eq 5. Curve-fitting was performed using a Marquardt search program as described by Bevington (1969). The results are shown in Table II. The best standard error of the estimate was about 20% of the median value of k_{obs}. However, the χ^2 was 6, which is considerably higher than the acceptable value of 2.6 for three degrees of freedom. The kinetic K_d was about 5-fold greater than the steady-state value. The K_{spin} ($=k_{+2}/k_{-2}$) (high-spin/low-spin) was higher than 1000 times the expected value based on 50–60% conversion of low-spin to high-spin at saturating benzphetamine concentration (Table II). Therefore, the curve-fitting procedure was repeated with a fixed value of K_a (obtained from the spectral titration data) and searching for k_{+2} and k_{-2}. The standard error of the estimate for the best fit was even higher (about 52% of the median "Y"). In addition, the second degree polynomial did not fit better than the first, indicating the absence of significant curvature in the data when the last point was excluded. This suggested that the deviation of one data point from linearity may not represent a continuous curved function. Therefore, it is concluded that near-equality of the last two data points (Figure 5) probably does not indicate saturation as in the spin equilibrium model (eq 5).

Since the deviation from linearity may not represent a continuous curved function, it was considered a reasonable approximation to omit the last point for evaluating the data at lower substrate concentration. The linear model (eq 2) gave an acceptable fit to the data. The χ^2 was 2.07, and the

Table II: Summary of Analysis of Data in Figure 5 (τ_f), Based on the Spin Equilibrium Model (eq 5), Showing That the Data Do Not Fit the Model[a]

	T-Jump Data $n = 6$		$n = 5$	
parameters	$P = 3$	$P = 2$	$P = 3$	$P = 2$
K_d (μM)	551 ± 126	61[b] ± 4	576 ± 8	61[c] ± 4
k_{+2} (s^{-1})	1784 ± 275	627 ± 103	1867 ± 565	607 ● 28
k_{-2} (s^{-1})	0.014 ± 57	0.019 ± 84	0.00027 ± 20	0.0003 ● 8
K_{spin} (k_{+2}/k_{-2})	1.2×10^5	3×10^4	7×10^6	2×10^6
SEE (% median Y)	20	50	19	52
χ^2	6.2	66	3.9	68

Spectral Titration Data: K_d Is the Same as in Table III	
parameters	$n = 10$
K_d (μM)	61 ± 4
K_{spin}	1–1.5

[a] The value of K_d shown under spectral titration data is the reciprocal of the negative "X" intercept obtained by least-squares fitting to the Lineweaver–Burk equation. This value is the same, but its meaning is different in the bimolecular and the spin equilibrium models. It represents K_d and $K_d(1 + K_{spin})$, respectively. K_{spin} = H-spin/L-spin = k_{+2}/k_{-2} (Sligar, 1976; Fisher & Sligar, 1987). If the observed 50–60% conversion of $P450_{LM2}$ to high-spin at saturating concentration of benzphetamine (Results) is due to the spin equilibrium of the substrate–P450 complex as in the spin equilibrium models, then K_{spin} would equal 1–1.5. n = number of data points. P = parameters searched. $n = 5$ excluding the last point in Figure 5. [b] Fixed when $P = 2$. [c] Fixed when $P = 2$.

Table III: Summary of Analysis Data Shown in Figure 5 Based on the Bimolecular Mechanism (eq 2)[a]

	T-Jump Data	
parameters	$n = 5$	$n = 6$
K_d (μM)	61.4 ± 20	211 ± 33
k_{+1} (M^{-1} s^{-1})	(1.8 ● 0.09) × 10^6	(1.1 ± 0.05) × 10^6
k_{-1} (s^{-1})	108.5 ● 18.9	229 ± 13.7
SEE (% median Y)	6.4	25
χ^2	2.07	22.8
corr	0.992	0.902

Spectral Titration Data	
parameters	$n = 10$
K_d (μM)	61 ± 4
SEE (% median Y)	2.2
χ^2	0.99
corr	0.998

[a] Weighted linear least-squares fitting. $n = 5$ excluding the last point in Figure 5.

standard error of the estimate was 6% of the median "Y" (Table III). The kinetic K_d (62 ± 22 μM) was consistent with all other results. That is, it was similar to that observed in the experiment shown in Figure 4, which is similar to the steady-state K_d (Table III). Therefore, it is considered that the assumptions made in the treatment of the data shown in Figure 5 are reasonable and that the substrate-induced spin-state transition follows a bimolecular mechanism.

In order to rule out the possibility that the observed linearity of k_{obs} versus $[E]_{eq} + [S]_{eq}$ may represent a limiting case of the two-step spin equilibrium model, equations for the relaxation times for restricted cases of the model (eq 4) were derived from the original differential equations.

Two relaxations are expected. Relaxation for the general case (that is, unrestricted) is

$$1/\tau_1; 1/\tau_2 = \{(k_1C + k_{-1} + k_2 + k_{-2}) \pm [k_1^2C^2 + 2k_1C(k_{-1} - k_2 - k_{-2}) + (k_2 + k_{-2})^2 + k_{-1}^2 + 2k_{-1}(k_2 - k_{-2})]^{1/2}\}/2 \quad (6)$$

where $C = [E]_{eq} + [S]_{eq}$. We have applied this general

equation to the following restricted cases:

(I) k_2's $\ll$ k_1's as in the simplified equilibrium model of Fisher and Sligar (1987):

$$1/\tau_1 = k_1C + k_{-1}$$

$$1/\tau_2 = k_{-2} + \frac{k_2}{1 + K_d/C}$$

These equations are in agreement with the reported equations for this case [eq IV, p 399, in Dixon and Webb (1979)].

$1/\tau_1$ would be linear in C, and $1/\tau_2$ would exhibit saturation as C increases to high values. Saturation was not observed in the present experiments. Only when C is sufficiently less than K_d (so that 1 can be neglected) would the plot be linear. Then bimolecular and spin equilibrium models would become indistinguishable. In the present experiments, C ranged from about K_d to little over $10K_d$.

(II) k_1's $\ll$ k_2's:

$$1/\tau_1 = k_1C$$

$$1/\tau_2 = k_2 + k_{-2}$$

$1/\tau_1$ would be linear in C, passing through the origin, and $1/\tau_2$ would be independent of C. In the present experiments, the linear plots exhibited "Y" intercepts. In addition, relaxation independent of C was not observed.

(III) $k_{-2} \ll k_2$ (k_2 sufficiently small for binomial expansion but not small enough to ignore):

$$1/\tau_1 = k_1(C + K_d) + \frac{k_2(K_d - C)}{K_d + C}$$

$$1/\tau_2 = \frac{k_2(C - K_d)}{C + K_d}$$

Both $1/\tau_1$ and $1/\tau_2$ would be nonlinear with respect to C, which would be inconsistent with the observed linearity.

(IV) $k_{-2} = 0$. This would prevent attainment of equilibrium before and after T-jump. Equilibrium was always reached in the present experiments.

The fastest times of most enzyme reactions have been in the time range of the T-jump method (10^{-6}–1 s). Many examples are tabulated by Fersht (1985). In addition, the bimolecular and the dissociation rate constants for $P450_{cam}$ (Griffin & Peterson, 1972) and $P450_{C21}$ (Narasimhulu, 1991) also lie within the range of the T-jump technique. Therefore, it is likely that any additional relaxations involving spectral changes in $P450_{LM2}$ would have been detectable. Although two relaxations were observed in the present experiments, k_{obs}-$(1/\tau)$ for both were linear in C. Therefore, they probably do not represent any of the limiting cases of the equilibrium model.

Effects of Substrate Concentration on the Amplitude. The amplitude represents the maximum increase in substrate–P450 complex formation after T-jump. Figure 6 shows amplitudes in terms of concentrations of the complex after correcting for spectral overlap of the low- and the high-spin forms at the measuring wavelength. It appears that the amplitude first increases, as would be expected, but then decreases with an increase in substrate concentration. This probably represents a transient effect of substrate because a decrease in the maximum high-spin with an increase in substrate concentration was not observed in direct spectral titration experiments, at the substrate concentrations tested. This indicates that a slower rate-limiting step is probably involved in reaching the static steady-state equilibrium. The present data are not sufficient to propose a reasonable model for this effect of substrate. Any explanation of this effect must take into account the decrease in time available for substrate redistribution, because of the decrease in the relaxation time with the increase in substrate concentration.

It should be noted that relaxation times are independent of amplitudes (eq 2 and 5).

DISCUSSION

The linear dependence of k_{obs} on $[E]_{eq} + [S]_{eq}$ indicates that the substrate-induced spin-state transition in $P450_{LM2}$ follows a bimolecular binding mechanism. The similarity of the kinetic equilibrium dissociation constant (68 ± 10 μM) and the dissociation constant (61 ± 4 μM) determined from direct spectral titration data indicates that the observed rate constants adequately account for the kinetics of the substrate–P450 binding reaction. The significance of the minor component exhibiting slow relaxation is not clear. However, since both fast and slow relaxations follow bimolecular mechanisms, they may represent two different forms of $P450_{LM2}$ exhibiting the same mechanism for the substrate-induced spin-state transition. In view of the high- and low-affinity lipid binding reported for $P450_{LM2}$ (French et al., 1980), it is possible that the two forms are a result of some differences in lipid reconstitution. The deviation from linearity observed at the higher benzphetamine concentration (>900 μM) does not indicate saturation as in the spin equilibrium model (eq 5). This was indicated by the high standard error and χ^2 and unreasonable parameter values (Table II) when attempts were made to fit the data to the model. The fractional (50–60%) conversion of $P450_{LM2}$ to the high-spin state observed in the present study does not indicate temperature-dependent equilibrium between the two spin forms of the substrate–P450 complex, as in the spin equilibrium model (Fisher & Sligar, 1987). This is evidenced by the temperature independence of the maximum spectral change observed in the titration experiments. The temperature independence of the spin state at the low protein concentrations and reasonably strong evidence for the bimolecular mechanism for substrate-induced spin-state transitions in P450's (Griffin & Peterson, 1972; Marden & BonHoa, 1987; Narasimhulu, 1991; this study) indicate that the small temperature effects observed at the high protein and substrate concentrations are probably not due to spin-state transitions as in the spin equilibrium models (Sligar, 1976; Fisher & Sligar, 1987). Other factors such as the presence of residual detergent may be involved (see Results). Thus, the present results are compatible with a simple bimolecular mechanism for the substrate-induced spin-state transition in $P450_{LM2}$.

The bimolecular mechanism is consistent with the two-state model proposed for the adrenal $P450_{C21}$ (Narasimhulu, 1971a,b) and the three-state model proposed for $P450_{cam}$ (Marden & BonHoa, 1987). In these models, substrate binding causes the spin-state transition, and any temperature-dependent changes in spin state are due to changes in substrate binding. This is consistent with the results of the stopped-flow studies showing that substrate-induced spin-state transitions in $P450_{cam}$ (Griffin & Peterson, 1972) and in $P450_{C21}$ (Narasimhulu, 1991) reflect substrate binding. Therefore, the possibility that the bimolecular mechanism is applicable to P450 enzymes in general must be considered.

The bimolecular mechanism is different from the spin equilibrium models (Sligar, 1976; Fisher & Sligar, 1987) which have been extensively used for explaining spin-state transitions in P450's including mammalian enzymes under all experimental conditions (e.g., Ristau et al., 1978; Backes et al., 1982; Tamburini & Gibson, 1983; Tamburini et al., 1984;

Douzou et al., 1989). The question is the following: Does this difference mean $P540_{LM2}$ is different from $P450_{cam}$ and other P450's in the basic mechanism for the substrate-induced spin-state transition? First of all, the assumptions of the spin equilibrium models appear to be inconsistent with the available data. For example, it has been assumed that at high camphor concentrations $P450_{cam}$ is saturated with camphor and any temperature-dependent spin-state change is due to a shift in the spin equilibrium coefficient (K_e = high-spin/low-spin) of the substrate–P450 complex (Sligar, 1976; Fisher & Sligar, 1987). However, a second mole of substrate binds at high concentrations (>0.2 mM), shifting the high-spin back to low-spin (Lange et al., 1977; Marden & Bon Hoa, 1987) even at constant temperature. Therefore, one cannot assume that temperature-induced changes in the spin state are not due to changes in substrate binding. In addition, the kinetic data of Griffin and Peterson (1972) as well as the three-state model proposed by Marden and BonHoa (1987) for camphor binding to $P450_{cam}$ are consistent with the bimolecular mechanism. Furthermore, Fischer and Sligar (1987) do not provide data showing saturation for the best-studied high-affinity substrate camphor in their T-jump relaxation experiments [Figure 5 in Fischer and Sligar (1987)]. If there is no saturation, it would be consistent with the bimolecular mechanism observed by others for camphor binding to $P450_{cam}$. Considering the points discussed above, $P450_{LM2}$, $P450_{cam}$, and $P450_{C21}$ are probably the same in regard to the basic mechanism of the substrate-induced spin-state transition. Marden and BonHoa (1987) have also investigated the binding of linalool to $P450_{lin}$, but they have not drawn any conclusions because maximum low- to high-spin conversion occurred near the solubility limit of the substrate. Thus, it is difficult to interpret the saturation behavior observed for low-affinity poorly soluble camphor analogues (Fischer & Sligar, 1987) in the absence of information on their solubility limitations and their binding to the second site. The saturation behavior observed by Tsong and Yang (1978) in their T-jump experiments has also been interpreted in terms of a spin equilibrium model, similar to that of Fisher and Sligar (1987). Considering the points discussed, an explanation of any deviation from the linear model (eq 2) in T-jump studies on P450 enzymes must take into account the possibility of binding of a second molecule of the substrate, as in $P450_{cam}$, and the solubility limitation of the substrate. These experiments are in progress in the author's laboratory.

ADDED IN PROOF

Experiments after submission of the manuscript have shown that the k_{obs} further decreases with increase in benzphetamine concentration beyond 940 μM, confirming the conclsuion that the deviation from linearity (Figure 5) may not represent saturation.

ACKNOWLEDGMENT

I thank Dr. R. F. Pasternack for making the temperature-jump studies possible, and Drs. Alfin De Vaz and M. J. Coon for their gifts of highly purified cytochrome $P450_{LM2}$, and Dr. C. R. Eddy for assistance in mathematics.

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