Interactions of Substrate and Product with Cytochrome $P450_{2B4}$†

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ABSTRACT: Interactions of the substrate(s) benzphetamine and the product (P) desmethylbenzphetamine with cytochrome $P450_{2B4}$ were studied by difference spectrophotometry. A two-sites model in which site 1 binding, causing Type I transition (low- to high-spin) must precede site 2 binding, causing Type II transition (high- to low-spin), gave an acceptable fit to the spectral titration data. The equilibrium association constant of substrate for site 1 (K_1) was greater than that for site 2 (K_2), and the K_2 for the product was greater than K_1, indicating that the substrate binds preferentially to site 1 and the product prefers site 2. In addition, competition between P and a strong Type II ligand (1-benzylimidazole) and a noncompetitive type of interaction between S and the same Type II ligand was observed. This indicates that P binds to the same site as the Type II ligand and S binds to a different site. The observed high-spin maxima for both P ($EP_1{}^{HS}{}_{max}$) and S ($ES_1{}^{HS}{}_{max}$) were similar to those calculated using the K_1 and K_2 values obtained from the curve-fitting procedure, indicating that the equilibrium concentration of the high-spin species is controlled entirely by K_1 and K_2. Simultaneous presence of the substrate and product decreased K_1 of the substrate and K_2 of the product, indicating that there is interaction between the substrate-preferred and the product-preferred sites. A possible functional significance of the differences in the site preferences of the substrate and product is discussed.

In principle all cytochrome P450s share a common reaction cycle with regard to structure and redox states of the heme. The cytochrome has two ferric states which differ in optical spectra, spin state, coordination number, and ability to undergo enzymatic reduction (White & Coon, 1980; Narasimhulu, 1993b). In the absence of exogenous ligands, most P450s are low-spin and hexacoordinated with their Soret absorption band around 417−419 nm. It has been known for a long time that exogenous ligands can elicit two major types of spectral changes in cytochrome P450s (Remmer et al., 1966). These have been referred to as Type I and Type II. The Type I complexes are high-spin with their Soret absorption peaks around 388−394 nm, and Type II complexes are low-spin with their Soret absorption around 417−426 nm (Mitani & Horie, 1969a,b; Whysner et al., 1969, 1970; White & Coon, 1982). The addition of substrates (Type I ligands) shifts the Soret absorption peaks of P450s from 417−419nm to 388−394nm. The Type I spectral shift is indicative of transformation of the heme from a hexacoordinated low-spin to a pentacoordinated high-spin state. This transition is probably brought about by substrate interacting with mainly the protein moiety (Raag & Poulos, 1989), and it reflects bimolecular substrate binding reaction (Griffin & Peterson, 1972; Marden & BonHoa, 1987; Narasimhulu, 1991, 1993a). Various types of spectroscopic investigations have shown that addition of Type II ligands to substrate-bound or substrate-free high-spin P450s or their addition to substrate-free low-spin p450s generates new low-spin species with the added ligand at L_6 position (Holm et al., 1976; Collman & Sorrell, 1977; White & Coon, 1982; Yoshida et al., 1982; Dawson et al., 1982; Dawson & Sono, 1987). It is thought that Type II transition is a result of a Type II ligand replacing the native L_6 ligand. This requires that the exogenous ligand has higher affinity for the L_6 position than the native ligand. Many substrate-free low-spin P450s are stable, suggesting that the native L_6 ligand may not be easily replaceable by all exogenous Type II ligands. A second possibility is that a Type II ligand can also elicit Type I transition, converting the low-spin heme to the pentacoordinated high-spin state, and that this transition precedes binding of the exogenous ligand to the L_6 position. That a designated type of ligand can cause both types of transitions is indicated by studies reported on camphor binding to $P450_{cam}$ (Marden & BonHoa, 1987), as well as binding of substrate and product to $P450_{2B4}$ (Narasimhulu, 1994). At low concentrations camphor converts low-spin $P450_{cam}$ to high-spin state, and at high concentrations it converts high-spin back to a low-spin state (Lang et al., 1977; Marden & BonHoa, 1987). Marden & BonHoa (1987) proposed a three-state model in which at low concentrations camphor binds, converting the heme to high-spin state, and then at high concentrations a second molecule binds, forming a ternary complex converting the high-spin back to a low-spin state. These authors concluded that $P450_{cam}$ contains a low-affinity second substrate binding site. They did not consider product binding. No further reports regarding the two sites have appeared.

Many Type II ligands are oxygen donors (e.g., hydroxy compounds) or nitrogen donors (e.g., amines) (White & Coon, 1982; Dawson & Sono, 1987). This is of interest especially because products of P450-catalyzed reactions are formed by interacting with the active oxygen at the L_6

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position. The products are hydroxylated and some final products are amines formed by oxidative dealkylation. This raises a question as to whether or not the product of a P450-catalyzed reaction has significant affinity for the L_6 position and whether or not it can play a role in the regulation of P450 activity via Type II interactions.

In the present study, spin-state transitions in $P450_{2B4}$ caused by the substrate benzphetamine and the product desmethylbenzphetamine have been investigated. In this paper the Type I ligand binding site will be designated as site 1, and the Type II ligand binding site as site 2. Results indicating the following are presented: (a) site 1 is preferred by substrate and site 2 is preferred by product, and (b) the relative association constants of a given ligand to the two sites (K_1/K_2) determine the equilibrium concentration of the high-spin state. Little attention has been paid to product−P450 interactions. Therefore a hypothesis regarding possible functional significance of product−P450 interactions is also discussed.

MATERIALS AND METHODS

Glycerol, benzphetamine hydrochloride, and most other chemicals were purchased from Sigma Chemical Co. Highly purified phenobarbital-induced rabbit liver $P450_{2B4}$ was a gift from Drs. Alfin D. Vaz and M. J. Coon. Desmethylbenzphetamine was a gift from Drs. Steve Pernecky and M. J. Coon. Dilaurylphosphatidylcholine (DLPC) was from Avanti Co.

Purity of Benzphetamine and Desmethylbenzphetamine. The purities of benzphetamine and desmethylbenzphetamine were ascertained by mass spectrometric analysis. Both were 100% pure as reflected by the abscence of any other peaks on the gas chromatographic tracing.

Spectrophotometry. All spectra were obtained with a split-beam Perkin-Elmer spectrophotometer, Model 277, equipped with electronic baseline corrector and a head-on photomultiplier, or a Model U-3000 Hitachi spectrophotometer.

Determination of Ligand Binding Parameters. Spectral titrations were performed as follows: Microliter volumes of aqueous solutions of either benzphetamine hydrochloride or desmethylbenzphetamine hydrochloride were added to the experimental cuvette and equal volumes of water were added to the reference cuvette. The stock solutions were either 0.05 or 0.025 M. Enzyme dilution (no more than 10%) was taken into account for calculations. No visible turbidity was observed. In addition, blank titrations were also performed with the substrate as well as the product in order to detect any ligand-dependent scatter changes. After each addition, the difference spectrum was scanned from 360 to 500 nm. The ΔA at the desired wavelengths were obtained from the digital readout as well as from the difference spectrum. The $\Delta\Delta A$(388−470 nm) or $\Delta\Delta A$(470−420 nm) values were used for analysis. In addition, titrations were performed in a low-noise and high-sensitivity dual-wavelength filter photometer by a semimicro technique (Narasimhulu, 1976). In this technique the sample is constantly stirred with a magnetic stirring attachment during titration, and the $\Delta\Delta A$ is recorded with a strip chart recorder. By this technique $\Delta\Delta A$ between selected pairs of wavelengths could be obtained more precisely, whereas the former technique allowed examination of the entire difference spectrum.

Data Analysis. The curve-fit programs used were the same as those in previous studies (Narasimhulu, 1993a). The standard error of estimate, randomness of distribution of residuals, reduced χ^2, and the standard deviations of the individual parameters of the model were considered in evaluating the goodness of fit.

Temperature-Jump Relaxation Measurements. The T-jump relaxation measurements were made using the apparatus manufactured by Dialog (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. This apparently did not cause any damage to the enzyme as indicated by the absence of significant amounts of P420 as determined from the CO difference spectrum of the dithionite-reduced enzyme.

RESULTS

Absolute Spectra. The UV−visible spectrum of substrate-free $P450_{2B4}$ was that of low-spin with the Soret peak at 417 nm and α and β bands at 533 and 571 nm (Figure 1A, curve 1). The Soret band decreased considerably when desmethylbenzphetamine was added (curves 2 and 3). There was little or no shift in the Soret peak (416−417 nm). There were small shifts and intensity changes in α and β bands. Similar Soret peak positions and decreases in peak intensities have been reported for low-spin complexes of P450s with certain nitrogen donor ligands. These ligands are probably coordinated to L_6 positions of P450s (Dawson et al. 1982; White & Coon, 1982; Yoshida et al., 1982). A small increase in absorption in the high-spin region (390 nm) could be observed at the concentrations of the product shown in the figure (curves 2 and 3). The high-spin absorption could be observed more clearly in the difference spectra (Figure 1C).

Difference Spectra. The shape of ligand-induced difference spectra of $P450_{2B4}$ depended on the ligand and its concentration as well as on the experimental conditions. In the presence of the phospholipid DLPC, benzphetamine (substrate) produced normal Type I spectra at the concentrations shown (Figure 1B). The peak [$\Delta\Delta A$(388−406 nm)] to trough [$\Delta\Delta A$(406−420 nm)] ratios remained essentially the same, indicating that only the two spectrally well-separated species (substrate-free low-spin and substrate-bound high-spin) are probably involved in producing these spectra. Desmethylbenzphetamine (product) elicited a much smaller distorted Type I spectrum with loss of the isosbestic point (Figure 1C). Similar substrate-induced normal Type I spectra and product-induced distorted spectra have also been observed with another P450 (Kominami et al., 1980).

Ligand Binding Parameters. Depending upon experimental conditions the $\Delta\Delta A$ versus substrate/product concentration data could be fit to the two-sites model (eqs 1 and 2) satisfactorily by the criteria indicated under Materials and Methods.

$$E^{LS} + L \overset{K_1}{\leftrightarrow} EL_1{}^{HS} \qquad EL_1{}^{HS} + L \overset{K_2}{\leftrightarrow} EL_1L_2{}^{LS} \qquad (1)$$

Subscripts 1 and 2 refer to sites 1 and 2; L = S (substrate) or P (product); K_1 and K_2 are apparent association constants.

Binding of Benzphetamine to $P450_{2B4}$. Figure 2A shows a $\Delta\Delta A$(388−470 nm) versus benzphetamine concentration

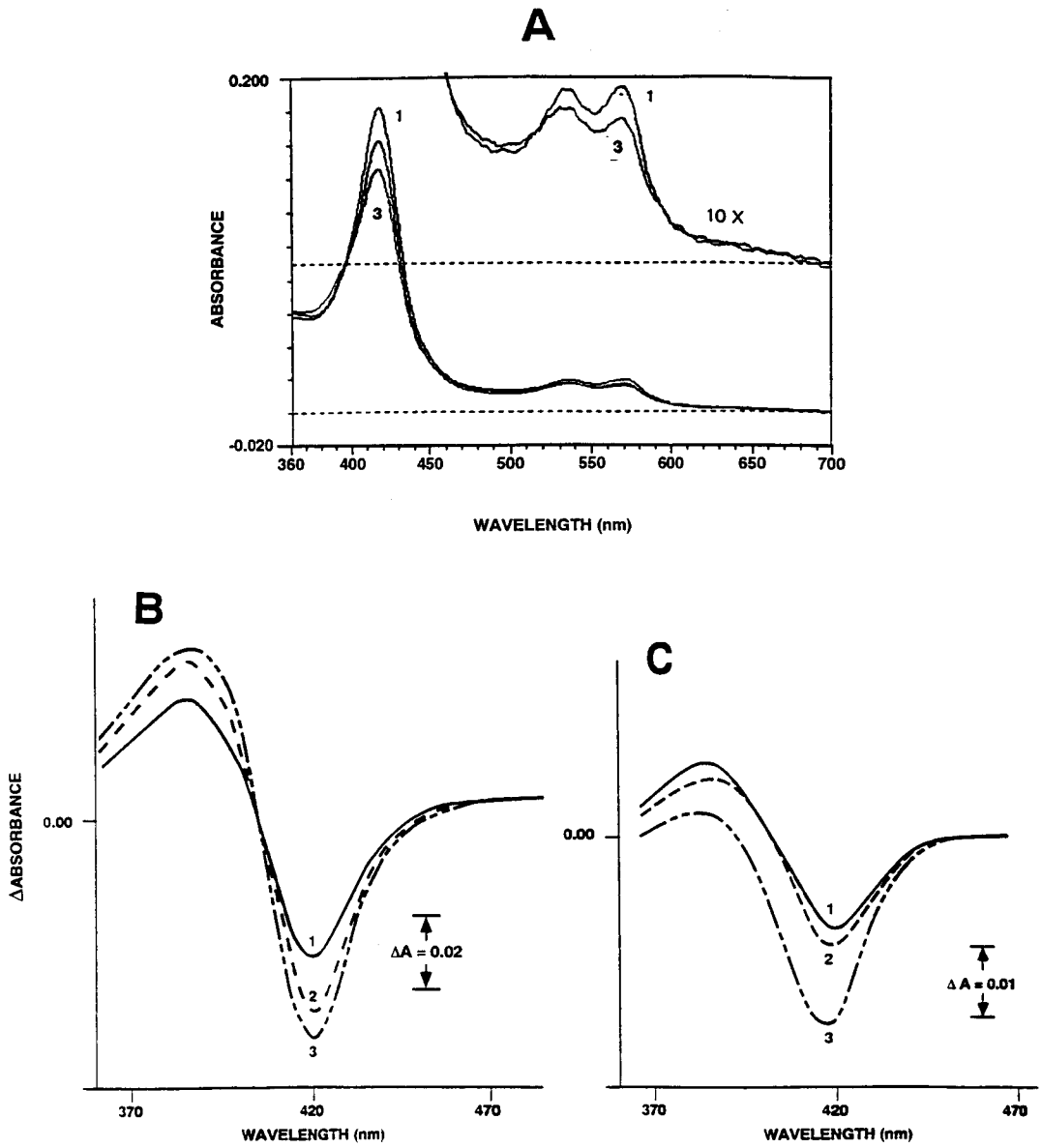

FIGURE 1: Effects of Substrate and Product on Spectrum of $P450_{2B4}$. The reaction medium contained 100 mM potassium phosphate buffer, pH 7.3, 50 μg/mL DLPC, 20% glycerol, and 1.5 μM P450. (A) Curve 1, absolute spectrum; curves 2 and 3, in the presence of 110 and 440 μM desmethylbenzphetamine. Panels B and C show difference spectra. (B) Curves 1−3 in the presence of 44, 133, and 220 μM benzphetamine. (C) Curves 1−3 in the presence of 500 μM, 600 μM, and 1.2 mM desmethylbenzphetamine. Temperature was approximately 25 °C.

curve in the absence of DLPC. The solid line is the theoretical curve for eq 2:

$$(\Delta A_1 - \Delta A_2) = lE_T K_1[(\epsilon_{EL1} - \epsilon_{EL2} + \epsilon_{E2} - \epsilon_{E1})L + (\epsilon_{ELL1} - \epsilon_{ELL2} + \epsilon_{E2} - \epsilon_{E1})K_2L^2]/(1 + K_1L + K_1K_2L^2) \quad (2)$$

ϵ represents difference extinction coefficients of the various species. The different species are indicated by subscripts. The subscripts are followed by the numeral 1 or 2 which represents λ_1 or λ_2. The association constant K_1 (1803 ± 95 M^{-1}) was greater than K_2 (810 ± 41 M^{-1}), indicating that the substrate has much higher affinity for site 1 than for site 2 (Table 1). The ϵ's of the free enzyme would be zero. The $\Delta\epsilon$s at 388−470 nm for ES^{HS} and for ESS^{LS} were 65.4 ± 2 mM^{-1} cm^{-1} and negligibly small, respectively. Figure 2B shows simulated curves for E^{LS}, $ES_1{}^{HS}$, and $ES_1S_2{}^{LS}$, using the K_1 and K_2 values obtained by the curve-fitting procedure.

Table 1 shows benzphetamine−P450 binding parameters obtained under different experimental conditions. A decrease in temperature resulted in a decrease in K_1 as well as in K_2. Since the substrate has very low affinity for site 2, there was little transformation of the high-spin back to low-spin state. Therefore there was little change in the maximum high-spin species ($ES^{HS}{}_{max}$). However, omission of the lipid considerably decreased K_1 and increased K_2. This probably resulted in a decrease in the maximum high-spin. The observed $ES^{HS}{}_{max}$ values were reasonably close to those calculated according to eq 3, using the values for K_1 and K_2 obtained by the curve-fitting procedure:

$$EL^{HS}{}_{max} = \frac{E_T K_1 (1/K_1K_2)^{1/2}}{2 + K_1(1/K_1K_2)^{1/2}} \quad (3)$$

Binding of Desmethylbenzphetamine to $P450_{2B4}$. The $\Delta\Delta A$ versus product concentration curves obtained using the two

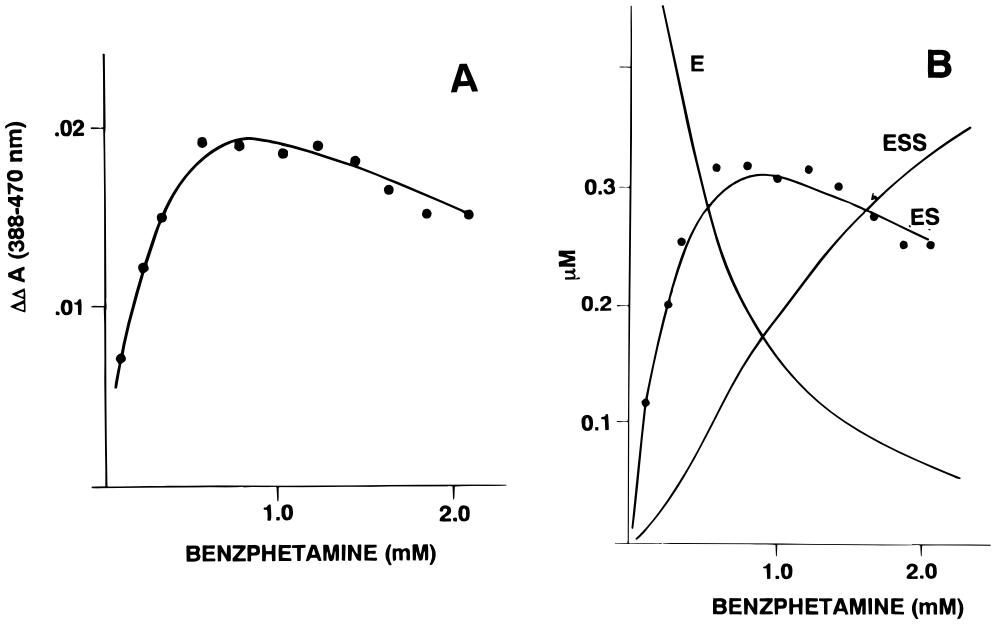

FIGURE 2: Titration of $P450_{2B4}$ with Benzphetamine in the Absence of Lipid. The reaction medium was 100 mM potassium phosphate buffer, pH 7.3, + 20% glycerol. E_T (total enzyme) = ~0.7 μM $P450_{2B4}$. (A) The solid line is the theoretical curve of eq 2. (B) Solid curves are simulated curves using the K_1 and K_2 values obtained by the curve-fitting procedure (Table 1). The dots are concentrations of the ES_1^{HS} complex calculated using $\Delta\Delta\epsilon$E of 62 mM^{-1} cm^{-1} (Table 1). $E^{LS} = E_T$/denom; $ES^{HS} = E_T K_1 S$/denom; $ESS^{LS} = E_T K_1 K_2 S^2$/denom. Denom = $(1 + K_1 S + K_1 K_2 S^2)$. Temperature was about 25 °C.

Table 1: Parameters of benzphetamine−$P450_{2B4}$ Binding under Different Experimental Conditions[a]

additions	temp (°C)	association constant (M^{-1}) K_1	K_2	ES^{HS}_{max} (μM) obs	calc	S.E.E. (% Median *Y*)
+DLPC	26	26341 ± 3089	250 ± 78	0.59	0.58	2.3
+DLPC	21	17738 ± 2000	153 ± 73	0.53	0.60	3.8
+DLPC	14	6766 ± 675	137 ± 19	0.58	0.50	3.2
0	26	1803 ± 95	810 ± 41	0.32	0.29	3.1

[a] In all instances $\Delta\Delta A$(388−470 nm) obtained from difference spectra versus benzphetamine concentration was used for analysis as described under Materials and Methods. The Δ difference extinction coefficients of ES^{HS} varied from 65 to 70 mM^{-1} cm^{-1} and those of ESS^{LS} were negligibly small.

pairs of wavelengths, 388−470 nm and 407−420 nm, are shown in Figure 3. The parameter values (K_1 and K_2) obtained at both pairs of wavelengths (Table 2) were similar. K_2 was much greater than K_1, indicating that the product has much higher affinity for site 2 than for site 1.

The difference extinction coefficient at 388−470 nm of EP_1^{HS} (85 mM^{-1} cm^{-1}, Table 2) was considerably greater than that of ES_1^{HS} (65 mM^{-1} cm^{-1}), indicating that ES_1^{HS} is probably not spectrally identical to EP_1^{HS}. As with the substrate, the calculated EP^{HS}_{max} was similar to the observed value. Since $K_2 > K_1$ (Table 2) for the product and $K_1 > K_2$ (Table 1) for the substrate, the concentration of EP^{HS}_{max} was only 12−16% that of ES^{HS}_{max}.

Binding of Product to Substrate−$P450_{2B4}$ Complex. The addition of the product to substrate−P450 complex decreased the 388−390-nm absorption and increased the 420-nm absorption, and the normal Type I spectrum (Figure 4A, curve 1) was replaced by a distorted spectrum (Figure 4A, curve 2), similar to that produced by product alone (Figure 1C). As indicated earlier, the Soret bands of substrate-free low-spin ferric P450s are around 417−419 nm and those of substrate-bound high-spin P450s are around 388−394 nm. Then the decrease in the 388-nm absorption and increase in the 420-nm absorption upon addition of product to the substrate−P450 complex is indicative of transformation of the high-spin P450 back to a low-spin state. The decrease in the relative $\Delta A/\Delta A_{max}$ magnitude of the substrate-induced Type I spectral change with increase in product concentration remained essentially the same at all substrate concentrations tested (Figure 4B). A two-sites model (eq 4) in which the

$$E^{LS} = S \overset{K_1^*}{\leftrightarrow} ES_1^{HS} \qquad ES_1^{HS} + P \overset{K_2^*}{\leftrightarrow} ES_1P_2^{LS} \qquad (4)$$

substrate (S) binds to site 1 and the product (P) binds to site 2 was found to give an acceptable fit (S.E.E. = 7.7% of median *Y*) to the data (Figure 4B). Since $K_1 \gg K_2$ when L in eq 1 was S, and $K_2 \gg K_1$ when L was P, it was assumed that binding of substrate to site 2 in the presence of the product and binding of the product to site 1 in the presence of the substrate were negligible at the concentrations of S and P studied. Therefore, when both S and P were present simultaneously (eq 4), K_1* and K_2* were considered as the apparent association constants for binding of substrate to site 1 and product to site 2, respectively. Then in eq 2, *L* was replaced by [S] and L^2 by [S][P]. The difference extinction coefficient at 388−470 nm for ES_1^{HS} was 62 mM^{-1} cm^{-1}, and that for $ES_1P_2^{LS}$ was negligibly small. The values obtained for K_1* (2834 ± 435 M^{-1}) and K_2* (1026 ± 194 M^{-1}) at 330 μM substrate were considerably less than those when P450 was titrated with substrate or product alone (Tables 1 and 2). Apparently, presence of the product decreased the free energy ($-\Delta G$) of binding of the substrate to site 1 from −6 to −4.7 kcal M^{-1}, and the presence of the substrate decreased the $-\Delta G$ of binding of the product to site 2 from −5.6 to −4.1 kcal M^{-1}, suggesting interaction between the two sites.

Since relatively high (millimolar) concentrations of the product (P) were used in experiments shown in Figure 4, it was important to find out whether or not the substrate-

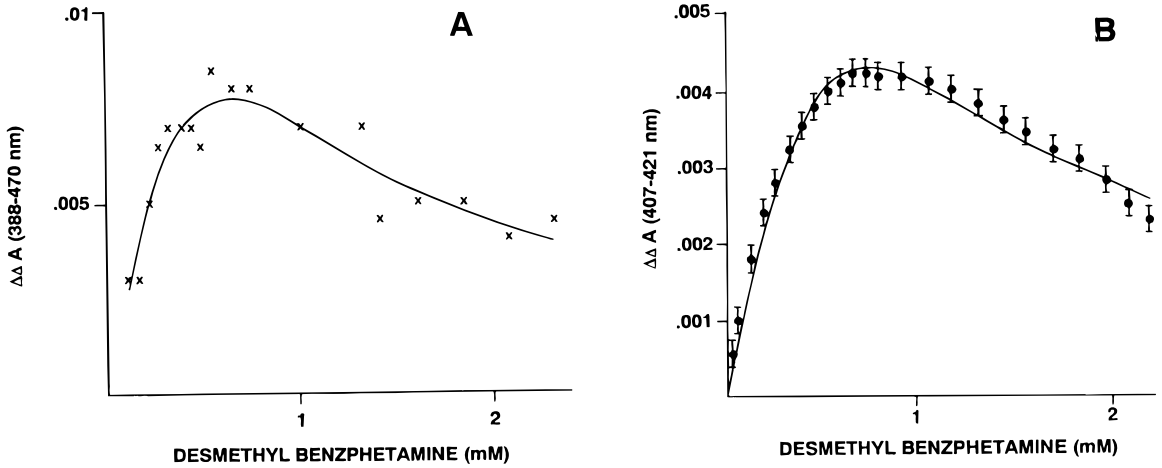

FIGURE 3: Titration of $P450_{2B4}$ with Desmethylbenzphetamine. The reaction medium was 100 mM potassium phosphate buffer, pH 7.3, + 20% glycerol + 50 μg/mL DLPC. $E_T = 1.5$ μM. (A) ΔA(388−470 nm) versus [P], ≈26 °C; S.E.E. = 9% of median *Y*. (B) $\Delta\Delta A$(407−421 nm) versus [P] data obtained by the more precise technique (Materials and Methods) at 24.8 °C. The error bars indicate the constant error input for each data point in the curve-fitting procedure; S.E.E = 3.7%. The solid lines are theoretical curves of eq 2, assuming that the difference extinction coefficient of the free enzyme ($\Delta\epsilon E^{LS}$) is zero. The parameter values are shown in Table 2.

Table 2: Parameters of Desmethylbenzphetamine−$P450_{2B4}$ Binding Reaction: Results of Analysis of $\Delta\Delta A$ versus Ligand Concentration[a]

				$[EP_1^{HS}]_{max}$ (μM)	
$\lambda_1-\lambda_2$ (nm)	parameter	(M^{-1})	ϵ (mM^{-1} cm^{-1})	obs	calc
(388−470)[b]	K_1	211 ± 17			
	K_2	12730 ± 1130			
	$\Delta\epsilon EP_1 - \Delta\epsilon EP_2$		85 ± 4	0.1	0.09
(407−421)[a]	K_1	124 ± 1			
	K_2	14828 ± 143			
	$\Delta\epsilon EP_1 - \Delta\epsilon EP_2$		66 ± 0.4	0.07	0.06

[a] The goodness of fit of the data to the two-sites model (eq 2) is indicated by S.E.E. (% median *Y*) and χ^2. They were 9 and 1.04 for (*b*) and 3.7 and 1.06 for (*c*). The difference extinction coefficients for the free enzyme ($\Delta\epsilon E$) would be zero. The ($\Delta\epsilon EPP^1 - \Delta\epsilon EPP_2$) at the selected wavelengths were negligible. The curves are shown in Figure 3.

dependent absorption at 388−390 nm can be restored when the product is removed. The enzyme−buffer mixture containing the P450 (1.4 μm), the substrate (0.6 mM), and the product (2.5 mM) was diluted stepwise with the buffer containing only the substrate. Spectral evaluation after each addition and dilution showed that the substrate-induced 390-nm absorption was completely restored, indicating that the product-induced decrease in the 390-nm absorption is probably not due to any irreversible changes. In addition, a number of data points sufficient for analysis were obtained by the more precise technique at much lower product concentrations (0.04−1 mM). The same two-sites model (eq 4) gave an excellent fit (S.E.E. = 0.45% of median *Y*) to the data, confirming the results obtained at the higher concentrations. Considering the difference between the precision of the two techniques, the values for K_1* and K_2* were not significantly different from those indicated above.

The possibility of formation of several complexes has to be considered in the simultaneous presence of the substrate and the product. These are ES_1^{HS}, EP_1^{HS}, $ES_1S_2^{LS}$, $ES_1P_2^{LS}$, $ES_2P_1^{LS}$, and $EP_1P_2^{LS}$. Therefore, it is possible that more species than those in eq 4 must be taken into account when concentrations of the substrate and product are outside the concentration ranges used in the present experiments. For example, contributions made by high concentrations of product (relative to that of substrate) competing with substrate for site 1 and high concentrations of substrate (relative to that of product) competing with product for site 2 will have to be taken into account. In addition, as indicated earlier there may be interaction between the two sites, and the two sites may be influenced differently by different experimental conditions. Therefore the values for the various parameters reported are applicable only to the ligand and enzyme concentrations used and the present experimental conditions. Factors influencing the interactions of the substrate and the product with $P450_{2B4}$ are beyond the scope of this paper.

It should be pointed out that models in which binding of the product to the P450−substrate complex releases the substrate failed to fit the data.

Effects of Benzphetamine and Desmethylbenzphetamine on the Binding of 1-Benzylimidazole. This was investigated by the more sensitive technique. The benzylimidazole is a strong Type II ligand to $P450_{2B4}$ with a K_d of about 1 μM [White and Coon (1982) and the present study]. Binding of Type II ligands to high-spin P450s decrease the high-spin absorption (388−390 nm). The Lineweaver−Burk plots of decrease in $\Delta\Delta A$(390−470 nm) versus the imidazole concentrations obtained in the presence of the product as well as in the presence of the substrate were linear with correlations between 0.997 and 0.9999. In the presence of the product (0.9 and 1.9 mM) the *y*-intercept remained the same whereas the *x*-intercept decreased and the slope increased with increase in the product concentration. Such intercept and slope effects are consistent with competitive inhibition (Dixon and Webb, 1979). In the presence of the substrate (0.99 and 1.47 mM) the *x*-intercept remained essentially the same whereas the *Y*-intercept as well as the slope increased

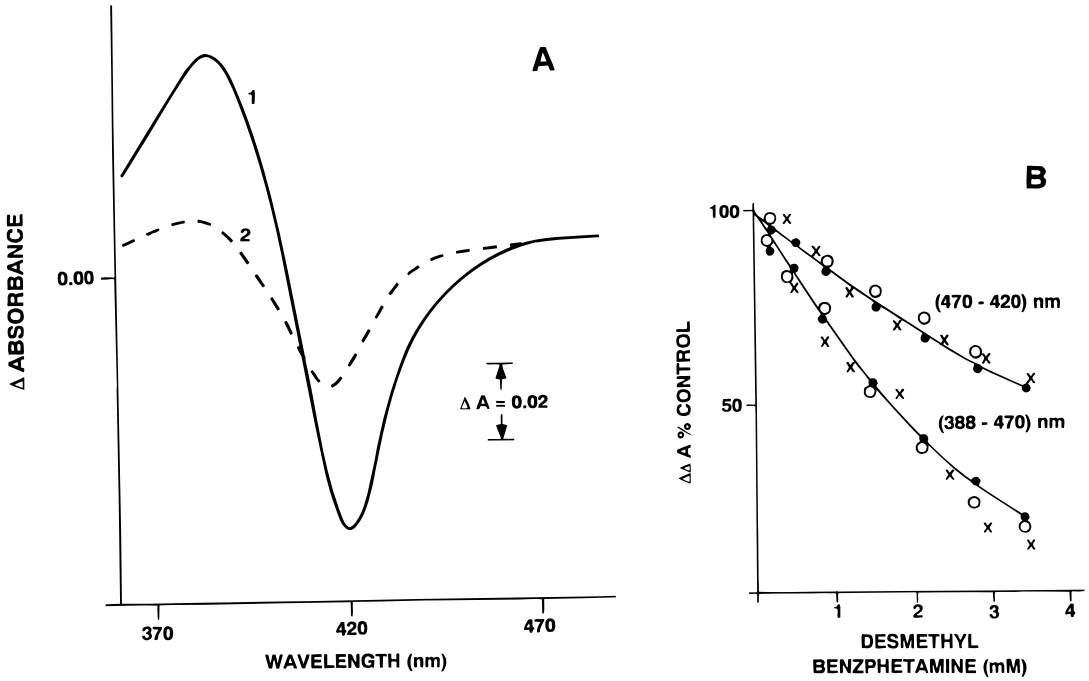

FIGURE 4: Effect of Desmethylbenzphetamine on the Benzphetamine-Induced Difference Spectrum in $P450_{2B4}$. The reaction medium was 100 mM potassium phosphate, pH 7.3, + 20% glycerol + 50 μg/mL DLPC. $E_T = 1.4$ μM. (A) Curve 1: Difference spectrum induced by approximately 331 μM substrate. Curve 2: Curve 1 + 2 mM product. Temperature was about 26 °C. (B) Plots showing that 388-nm peak and 420-nm trough of the substrate-induced difference spectrum decrease disproportionately when titrated with the product, and that the curves obtained in the presence of 110 μM (○), 330 μM (●), and 880 μM (×) substrate were essentially the same.

with increase in the substrate concentration. Such effects are consistent with a noncompetitive type of interaction (Dixon and Webb, 1979). However, it should be emphasized that due to interaction between the Type I and the Type II sites, the present graphic picture is expected to be applicable strictly to the concentrations of S, P, the imidazole, and E_T (0.6 μM) used in this study. Precise effects of S, P and the imidazole interactions on the parameter values are a subject for another paper. However, since the plots were linear within a wide range (0.4−8 μM; 15−20 data points) of the imidazole concentrations, the present graphic picture is considered as supportive evidence for the two-sites model and the site preferences of the substrate and the product.

Temperature-Jump Relaxation Experiments. The biphasicity of the interactions of the substrate as well as the product with $P450_{2B4}$ were also observed in the time scale of the T-jump relaxation technique. This is shown for the substrate benzphetamine in Figure 5. The measuring wavelength was 417 nm. The T-jump (23−25 °C) decreased the 417-nm absorption (Figure 5, top), which is indicative of increased substrate binding. This is consistent with the positive enthalpy of substrate binding to $P450_{2B4}$ observed in static titration experiments (Taniguchi et al., 1984; Narasimhulu, 1993a). The K_{obs} ($1/\tau$) versus [enzyme + substrate] concentration curve exhibited a clear maximum (Figure 5, bottom). Although the k_{obs} versus the [enzyme + product] curve was also biphasic, the addition of the product (0.1−1.2 mM) in the presence of the substrate (1.12 mM) decreased the k_{obs} exhibiting only the second phase. This is also similar to the results of static spectral titration experiments (Figure 4B), in that only the second phase was observed when the substrate−P450 complex was titrated with the product. Considering the overall resemblance of these results of relaxation experiments and the static titration experiments (Figures 2−4), it is reasonable to assume that the decrease in k_{obs} (Figure 5) is due to binding of ligand to site 2 (L_6). It is possible that the hexacoordinated low-spin P450 may not exhibit any relaxation similar to the native low-spin $P450_{2B4}$ (Narasimhulu, 1993a). It is also possible that relaxation due to binding of exogenous ligand to site 2 changes the absorption in the opposite direction (high-spin to low-spin), annihilating the relaxation due to site 1 binding. Further studies are needed to find out the mechanism of the decrease in k_{obs}.

The transient amplitudes observed with substrate and product at comparable relaxation rates in the initial phases of their k_{obs} versus concentration curves are shown in Table 3. The measuring wavelength was 390 nm. This absorption increased with increase in temperature (23−25 °C), which is indicative of increased Type I binding. The maximum transient high-spin amplitude observed with the product was about 50% of that observed for the substrate at comparable relaxation rates. Depending upon the product concentration the amplitudes varied from 30% to 60% of the maximum observed with the substrate (around 100 μM). These values are considerably greater than the 12−16% observed for $EP_1^{HS}{}_{max}/ES_1^{HS}{}_{max}$ in static titration experiments (Tables 1 and 2). This suggests that product binding to site 1 may be sufficiently faster than binding to site 2 to reveal more of the product-produced high-spin form than in the static titration experiments. At present this can only be inferred from the amplitude observed in the presence of the product relative to that observed in the presence of the substrate at the same P450 concentration under the same experimental conditions and the same relaxation rates. More direct evidence for the precedence of site 1 binding must come from a more detailed kinetic analysis which will be the subject for another paper. The purpose in presenting these data is to indicate that (a) biphasic effects of substrate and product on P450 spin state were observed in static titration

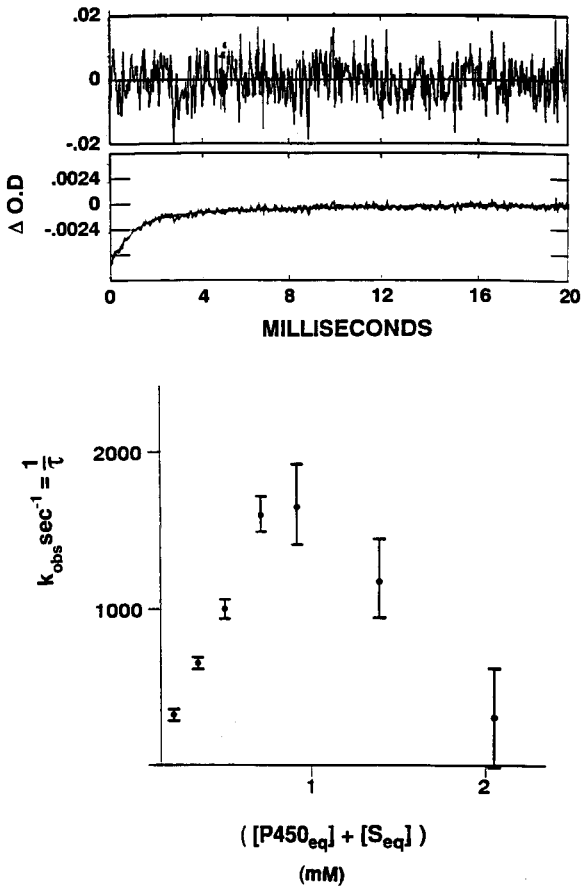

FIGURE 5: T-Jump Relaxation Data Obtained at 417 nm. The reaction medium contained 180 mM potassium phosphate buffer, pH 7.4, + 50 μg/mL DLPC + 5% glycerol + 2 μM $P450_{2B4}$. The temperature jump was from 23 to 25 °C. (Top) Represenative relaxation trace obtained in the presence of 430 μM benzphetamine. The measuring wavelength was 417 nm. Upward deflection of the curve indicates decrease in absorbance. (Bottom) k_{obs} versus [enzyme + substrate] plot. The error bar represents ±1 standard deviation.

Table 3: Comparison of Benzphetamine and Desmethylbenzphetamine with Respect to Temperature-Jump (23−25 °C) Relaxation Transient Amplitudes Observed at 390 nm[a]

	k_{obs} (s^{-1})	amplitude $EL^{HS}_{t=\infty} - EL^{HS}{}_{t=0}$ (μM)
benzphetamine (170 μM)	700 ± 60	0.04 ± 0.004
desmethylbenzphetamine (176 μM)	730 ± 40	0.02 ± 0.0003

[a] The reaction medium was 0.18 M KPO_4 buffer, pH 7.4, + 5% glycerol + 2 μM $P450_{2B4}$. The concentrations of the high-spin complexes were calculated using absolute extinction coefficients ϵ (mM^{-1}/cm^{-1}) of 116 for $ES_1{}^{HS}$ and 135 for $EP_1{}^{HS}$. Corrections were made for the spectral overlap of the substrate-free low-spin enzyme using ϵ (mM^{-1} cm^{-1}) of 55 at the measuring wavelength (390 nm).

experiments as well as in the time scale of the T-jump technique, and (b) although relative $EP_1{}^{HS}/ES_1{}^{HS}$ values are probably not precise, the relaxation amplitudes may be revealing more of the $EP_1{}^{HS}$ than the static spectra.

DISCUSSION

The spectral titration data revealed two different ligand binding sites in $P450_{2B4}$. The site 1 binding elicited the Type I spectral change and site 2 binding elicited the Type II spectral change, indicating that the two sites are none other than the well-known Type I and Type II sites. However, the preferential binding of the substrate to site 1 ($K_1 > K_2$), the preferential binding of the product to site 2 ($K_2 > K_1$), and formation of the ternary complex S−P450−P observed in the present study are novel findings. The present results indicate that the two sites may behave as two separate sites, rather than parts of a single site as currently viewed. The results also show that the maximum equilibrium concentration of the high-spin complex ($EL^{HS}{}_{max}$) that can be observed in the static spectrum entirely depends upon the relative affinities of the ligand to the two sites (K_1/K_2). Due to their site preferences, the substrate forms predominantly high-spin complex, and the product forms predominantly low-spin complex. As a result the product-produced high-spin P450 ($EP_1{}^{HS}{}_{max}$) was only 12−16% of that produced by the substrate ($ES_1{}^{HS}{}_{max}$), as observed in the static spectra. However, the T-jump relaxation amplitudes apparently revealed more of $EP_1{}^{HS}$ (50% of $ES_1{}^{HS}$) which is consistent with the two-sites model (eqs 1 and 2) in that site 1 binding must precede site 2 binding regardless of the ligand's site preference. The competition between the product and the Type II ligand (1-benzylimidazole) and noncompetitive interaction between the substrate and the same Type II ligand indicate that the product binds to the same site as the Type II ligand, and the substrate binds to a different site. This then strongly supports the two-sites model and the site preferences of the substrate and the product.

The P450 spin state in the simultaneous presence of the substrate and product are relevant to function because both are present under the turnover conditions. From the foregoing discussion, it is reasonably clear that the substrate is predominantly a Type I ligand whereas the product is predominantly a Type II ligand, and the ternary complex S−P450−P is formed when S and P are both present. However, the presence of the substrate apparently decreased the free energy of binding of the product to site 2, and the presence of the product decreased the free energy of binding of substrate to site 1, suggesting interaction between the two sites. It is possible that the decreased free energies are more relevant to catalysis. This would be in line with the currently favored hypothesis that destabilization of ES complex is absolutely essential for enzymatic catalysis (Jencks, 1987). Assuming that the observed decreases in $-\Delta G$s for $ES_1{}^{HS}$ and $EP_2{}^{LS}$ complexes are due to increases in their Gibbs energies, it is conceivable that these increases may contribute toward destabilization of ES_1 and EP_2 complexes.

The existence of substrate-preferred and product-preferred sites may have functional significance. A reasonable hypothesis is as follows: Substrate may lose its affinity to site 1 upon hydroxylation and the product formed may gain affinity to site 2. While substrate binding to site 1 controls conversion of nonreducible (low-spin) P450 to the active reducible (high-spin) intermediate of the catalytic cycle (Narasimhulu, 1971a,b, 1993b), dissociation/release of product from site 2 controls the hydroxylation rate, keeping the system fully coupled. This is explained as follows: When substrate accepts the active oxygen at the L_6 position, forming the hydroxy product, the hydroxyl group of the product may be coordinated to the iron as L_6 before the product dissociates. Product−site 2 complex, then, would be similar to the Type II complexes of P450 enzymes. The present results indicate that desmethylbenzphetamine, which is the N-demethylation product of benzphetamine, binds preferentially to site 2. Obviously product formed in the first cycle must

be released from site 2 and high-spin pentacoordinated structure must be restored for initiation of the next cycle. Therefore slow product dissociation/release can slow down the overall catalytic cycle. Slow product dissociation/release can keep the system fully coupled, provided that the kinetics of various steps involved are optimal for efficient conversion of $Fe^{2+}O_2$ to $Fe^{3+}O$. If the product is released faster than rate of acceptance of the active oxygen by the substrate, the system could become uncoupled. Thus the substrate and product dynamics may play major roles in determining the rate of hydroxylation as well as extent of coupling. This is further discussed in the following paragraph.

As also indicated earlier, P450s are very similar with regard to effects of ligands on optical spectra, spin state, and heme structure. Ligand-induced Type I and Type II transitions have been observed in most P450s. Therefore it would be of interest to find out whether or not the present hypothesis has any support from the existing literature. As also indicated earlier, the same two-sites model had been proposed for binding of the substrate camphor to P450cam (Marden & BonHoa, 1987). The product hydroxycamphor is predimnantly a Type II ligand (Li et al., 1995), similar to the product of $P450_{2B4}$ in the present study. Since most products of P450s are formed by accepting the active oxygen at the L_6 position, it is possible that products of other P450s are also predominantly Type II ligands. The recent kinetic evidence that two different molecules can be simultaneously bound to the same P450 (CYP3A4) active site (Shou et al., 1994) is in favor of the present hypothesis in that the $P450_{2B4}$ can bind to substrate and product, forming a ternary complex. The effects of mutations of the highly conserved Thr (Thr-252 in P450cam, implicated in O_2 binding reaction) on the rate of hydroxylation by $P450_d$ depended on the structure of the substrate (Furya et al., 1989). The bacterial enzyme P450cam could be converted from fully coupled system to partially or fully uncoupled system by merely altering the structure of the substrate (Poulos & Raag, 1992; Dawson et al., 1995; Kadkhodayan et al., 1995). $P450_{C21}$ was fully coupled in the presence of 17-OH progesterone and completely uncoupled in the presence of androstenedione which cannot accept the active oxygen (Narasimhulu, 1971b). These are consistent with the present proposal that substrate and/or product dynamics may play major roles in determining the rate of hydroxylation as well as extent of coupling in P450s. It is possible that the state of the iron and its environment have a major influence on the kinetics of various steps involved in efficient conversion of $Fe^{2+}O_2$ to $Fe^{3+}O$. We know that the Type I and the Type II ligands have a profound influence on P450 conformation, spin state of the iron, and structure of the heme. It is possible that the structure of the substrate plays a major role in determining the precise conformation of the active site, required for efficient coupling.

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