Cytochrome $b_5$ Increases the Rate of Product Formation by Cytochrome P450 2B4 and Competes with Cytochrome P450 Reductase for a Binding Site on Cytochrome P450 2B4

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The kinetics of product formation by cytochrome P450 2B4 were compared in the presence of cytochrome $b_5$ (cyt $b_5$) and NADPH-cyt P450 reductase (CPR) under conditions in which cytochrome P450 (cyt P450) underwent a single catalytic cycle with two substrates, benzphetamine and cyclohexane. At a cyt P450:cyt $b_5$ molar ratio of 1:1 under single turnover conditions, cyt P450 $b_5$ catalyzes the oxidation of the substrates, benzphetamine and cyclohexane, with rate constants of $18 \pm 2$ and $29 \pm 4.5 \text{s}^{-1}$, respectively. Approximately 500 pmol of norbenzphetamine and 21 pmol of cyclohexanol were formed per nmol of cyt P450. In marked contrast, at a cyt P450:CPR molar ratio of 1:1, cyt P450 $b_5$ catalyzes the oxidation of benzphetamine $100$-fold ($k = 0.15 \pm 0.05 \text{s}^{-1}$) and cyclohexane $10$-fold ($k = 2.5 \pm 0.35 \text{s}^{-1}$) more slowly. Four hundred picomoles of norbenzphetamine and 21 pmol of cyclohexanol were formed per nmol of cyt P450. In the presence of equimolar concentrations of cyt P450, cyt $b_5$, and CPR, product formation is biphasic and occurs with fast and slow rate constants characteristic of catalysis by cyt $b_5$ and CPR. Increasing the concentration of cyt $b_5$ enhanced the amount of product formed by cyt $b_5$ while decreasing the amount of product generated by CPR. Under steady-state conditions at all cyt $b_5$:cyt P450 molar ratios examined, cyt $b_5$ inhibits the rate of NADPH consumption. Nevertheless, at low cyt $b_5$:cyt P450 molar ratios $\leq 1:1$, the rate of metabolism of cyclohexane and benzphetamine is enhanced, whereas at higher cyt $b_5$:cyt P450 molar ratios, cyt $b_5$ progressively inhibits both NADPH consumption and the rate of metabolism. It is proposed that the ability of cyt $b_5$ to enhance substrate metabolism by cyt P450 is related to its ability to increase the rate of catalysis and that the inhibitory properties of cyt $b_5$ are because of its ability to occupy the reductase-binding site on cyt P450 $b_5$, thereby preventing reduction of ferric cyt P450 and initiation of the catalytic cycle. It is proposed that cyt $b_5$ and CPR compete for a binding site on cyt P450 $b_5$. The cytochromes (cyt)$^2$ P450 are a superfamily of heme-containing enzymes that catalyze the biotransformation of a large number of endogenous and xenobiotic compounds by utilizing reducing equivalents from NADPH. The mammalian hepatic microsomal cytochromes P450 receive electrons from their redox partner, NADPH-cyt P450 reductase (CPR). To be able to catalyze the oxidation of substrates, cyt P450 requires two electrons and two protons. Its catalytic cycle is complex with multiple steps (1). The first step is the binding of substrate, which may increase the redox potential of the heme. CPR, not cyt $b_5$, then delivers the first electron to reduce the ferric heme to the ferrous state. The ferrous iron binds oxygen to form the oxyferryl complex ($\text{Fe}^{2+}\text{O}^2\text{O}^{-}$), whose potential is high enough to accept a second electron from either CPR or cyt $b_5$ to yield the peroxo intermediate ($\text{Fe}^{2+}\text{O}^2\text{O}^2\text{O}^2\text{O}^{-}$). The peroxo intermediate is rapidly protonated (first proton) to form the hydroperoxo intermediate ($\text{Fe}^{2+}\text{O}^2\text{O}^2\text{O}^{-}$). A second proton is delivered to the distal oxygen, resulting in cleavage of the oxygen molecule to form water and the oxyferryl intermediate [Fe$^{2+}$O = O], generally considered to be the active hydroxylating species. An atom of oxygen is inserted into the substrate, and product release follows.

It has been recognized for 3 decades that under steady-state conditions, cyt $b_5$ alters the rate of catalysis by selected cytochromes P450 (for recent reviews see Refs. 2–5). Cyt $b_5$ has been reported to affect the catalytic activity of more than 20 cyt P450 isozymes, including the majority of the human drug-metabolizing cyt P450 isozymes like cyt P450 3A4, 2B6, 2C9, 2C19, and 2E1 (6–9). Cyt $b_5$ may enhance, inhibit, or not alter catalysis by microsomal cyt P450 depending on the specific cyt P450 isozyme, the substrate, and the experimental conditions (10, 11). It has been suggested that cyt $b_5$ modulates cyt P450 catalysis by donating the second electron to cyt P450 and/or by acting as an allosteric modifier of the oxygenase (3). In the case of cyt P450 $b_5$, it has been shown that the electron-donating properties of cyt $b_5$ are required for its stimulatory activity (10–12). Its inhibitory properties, which are also manifest by manganese-protoporphyrin IX cyt $b_5$ are likely related to its ability to displace CPR from its binding site on cyt P450 $b_5$ (11–13). Recent experiments performed under single turnover conditions suggest that both the electron-donating ability and effec-

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Tables S1 and S2.

2 The abbreviations used are: cyt, cytochrome; CPR, NADPH-cytochrome P450 reductase; DLPC, dilauroylphosphatidylcholine; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry.
tor function of cyt b5 may be operating to enhance catalysis by cyt P450 2B4 (14).

It has also been reported that apo-cyt b5 devoid of heme can stimulate catalysis (9, 15–18). However, some investigators have disputed that apo-cyt b5 can stimulate cyt P450 catalysis (19). These conflicting reports indicate that the mechanism by which cyt b5 affects the catalysis of cyt P450 remains poorly understood. Hildebrandt and Estabrook (20) proposed that cyt b5 functions by transferring one of the two reducing equivalents to cyt P450 based on the observation in microsomes that cyt b5 partially reoxidized upon the addition of NADH to a microsomal drug-metabolizing reaction under steady-state conditions. This proposal is supported by in vitro experiments demonstrating that ferrous cyt b5 is an efficient electron donor to oxyferrous cyt P450 (Fe3+/OO−) (21, 22). Furthermore, stimulation of benzphetamine and methoxyflurane metabolism by cyt P450 2B4 is only observed in the presence of holo-cyt b5 but not in the presence of apo-cyt b5 or cyt b5 containing manganese protoporphyrin IX (11, 12). Chlormezoxime metabolism by cyt P450 2E1 is also stimulated by cyt b5, but not by apo-cyt b5, in a purified reconstituted system as well as in membranes in which cyt P450 2E1 and CPR have been coexpressed (8, 9). These results are supportive of an electron transfer role for cyt b5 in cyt P450 catalysis. Stimulation of cyt P450 2E1 activity was originally attributed to more rapid electron transfer to oxyferrous cyt P450 by cyt b5 than CPR. However, data from our laboratory have recently demonstrated that cyt b5 and CPR reduce cyt P450 at a similar rate but that catalysis nevertheless occurs more slowly in the presence of reductase (14). Presumably, oxyferrous cyt P450 exists in different conformations in the presence of cyt b5 and CPR.

Attempts to characterize the interaction of cyt P450 at the individual steps in its reaction cycle with its amphipathic redox partners have been extremely challenging because membrane-binding proteins form heterogeneous aggregates between and among themselves in aqueous solution. It is also likely that cyt P450 changes conformation during its reaction cycle and that its various conformations may react differently with cyt b5 and CPR (23). Cyt P450 2B4 forms a 1:1 complex with CPR and with cyt b5 in reconstituted systems. The binding of the redox partner is enhanced in the presence of substrates, and substrates enhance the affinity for the redox partner (21, 23–25). A site-directed mutagenesis study of the interactions of cyt P450 2B4 with CPR and cyt b5 has identified residues, primarily in the C-helix on the proximal side of cyt P450 2B4, that participate in binding both CPR and cyt b5 (13). These data demonstrate that CPR and cyt b5 have nonidentical but nevertheless overlapping binding sites on the proximal surface of cyt P450 2B4 and predict that cyt b5 and CPR will compete for this binding site on cyt P450. Model complexes of cyt P450 2B4 with cyt b5 and CPR also support the hypothesis of an overlapping binding site (4). On the contrary, Schenken and co-workers (26) have proposed a ternary complex of cyt P450, cyt b5, and CPR based on the finding that a carbodiimide cross-linked cyt b5–cyt P450 2B4 complex was able to accept electrons from CPR for the metabolism of para-nitroanisole. The experiment with the cross-linked complex led to the conclusion that cyt b5 and CPR had distinct binding sites on cyt P450 2B4 and that it was possible for cyt P450, cyt b5, and CPR to form an active ternary complex. The experiments described in this work address this issue using a recently developed procedure that directly measures product formation from a single catalytic cycle of cyt P450 2B4. They also enable us to compare the rate of product formation with the rate of reduction of cyt P450 2B4 determined spectrophotometrically (14).

In view of the complexity of the multistep reaction cycle of cyt P450, the fact that the first electron to the ferric cyt P450 can only be efficiently delivered by CPR, and the heterogeneously aggregated state of the three membrane proteins in the reconstituted system, we elected to bypass the early steps in the catalytic cycle and to examine product formation by oxyferrous cyt P450 2B4 under single turnover conditions. The ferric cyt P450 2B4 was stoichiometrically reduced by dithionite, eliminating the requirement for CPR for the delivery of the first electron. Catalysis was initiated by mixing the reduced cyt P450 complex with an oxygen-containing solution. By directly measuring the rate at which product formation occurred during a single catalytic cycle, it was possible to determine whether cyt b5 or CPR was responsible for catalysis. These experimental conditions circumvent many of the problems associated with investigating enzymatic activity under steady-state conditions and enabled us to show that increasing the concentration of cyt b5 leads to more catalysis by cyt b5 at the expense of catalysis by CPR. The data were interpreted to indicate that cyt b5 and CPR are competing for a mutually exclusive binding site on cyt P450 2B4.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals used are of ACS grade unless otherwise specified. Cyclohexane, cyclohexanol-d4, NADPH, benzphetamine, and sodium dithionite were purchased from Sigma. Dilauroylphosphatidyl-choline (DLPC) was purchased from Doosan Serdary Research Laboratory (Toronto, Canada).

**Protein Expression and Purification**—The membrane-bound forms of cyt P450 2B4, rabbit cyt b5, and rat CPR were expressed and purified from *E. coli* as described previously (14). The concentration of cyt P450 was determined using an extinction coefficient of Δε450–490 nm of 91 μM−1 cm−1 for the cyt P450-CO complex, as described by Omura and Sat0 (27). The concentration of CPR was determined using an extinction coefficient of 21 μM−1 cm−1 at 456 nm for oxidized enzyme (28). The concentration of cyt b5 was determined using an extinction coefficient of 185 μM−1 cm−1 for the absorbance change at 426 minus 409 nm between ferrous and ferric cyt b5, respectively (29).

**Determination of the Rate of Product Formation under Single Turnover Conditions in a Rapid Chemical Quench Apparatus**—Measurements of the kinetics of product formation were performed on a QFM-400 chemical quench flow apparatus (Molecular Kinetics, Inc., Indianapolis, IN). The QFM-400 apparatus was housed in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI), where the oxygen level was maintained at ~5 ppm. The temperature of the reaction chamber of the QFM-400 apparatus was maintained with a circulating water bath.
The complexes of cyt P450 with its redox partners were pre-formed by incubating cyt P450 with either CPR or cyt b5 or both overnight at −4 °C in an anaerobic chamber in Buffer A (0.1 M potassium phosphate, pH 7.4, 15% glycerol). The overnight incubation prevents precipitation and ensures the sample is anaerobic. DLPC was included in the protein mixture at a molar ratio of DLPC:cyt P450 of 60:1. Methyl viologen was present at a concentration of 0.1 μM. The substrate of cyt P450, either cyclohexane or benzphetamine, was added to a final concentration of 1 mM. Cyclohexane was added from a stock of methanolic solution, and the methanol concentration in the reaction mixture was ≤0.25% (v/v). The cyt P450-cyt b5 complex was reduced stoichiometrically in a tonometer under anaerobic conditions, using a standardized solution of dithionite. The reduction was monitored by following absorbance changes at 422 and 438 nm for cyt b5 and cyt P450, respectively. Likewise, the cyt P450-CPR complex was reduced with dithionite, and the reduction was monitored at 585 and 385 nm for CPR and cyt P450, respectively. The cyt P450-cyt b5 complex was reduced to a ferrous state (cyt P4502+ and cyt b52+), whereas the cyt P450-CPR complex was reduced by 3-electron equivalents (ferrous cyt P450 and 2e-reduced CPR). When all three proteins were present, the preformed complexes were reduced with 4-electron equivalents to ferrous cyt P450, ferrous cyt b5, and 2e-reduced CPR.

The reduced protein complex was loaded into Syringe A of the chemical quench flow apparatus inside the anaerobic chamber. With cyclohexane as the substrate, the protein concentration in Syringe A was 10–15 μM for the cyt P450-cyt b5 complex and 15 μM for the cyt P450-CPR complex. The concentration of the cyt P450-cyt b5 and cyt P450-CPR complex was 30 μM when benzphetamine was used as substrate. Syringe B contained air-saturated Buffer A and 1 mM substrate. The reaction was initiated by mixing the contents of Syringe A (containing the reduced proteins) with an equal volume from Syringe B (containing substrate and air-saturated Buffer A ([O2] = ~250 μM)). At designated times the reaction was rapidly quenched with an equal volume of 1 M NaOH. The reaction temperature was 30 °C with cyclohexane and 15 °C with benzphetamine. The last sample was collected ~10 s after mixing in the presence of cyclohexane and at ~60 s in the presence of benzphetamine. The sample at time = 0 was prepared by first mixing the reduced protein complex with 1 M NaOH to inactivate the enzymes and then adding air-saturated buffer. Typically 15–30 samples were collected during the time course of a reaction. The amount of cyclohexanol in each reaction mixture was measured using gas chromatography-mass spectrometry (GC-MS), whereas the amount of norbenzphetamine was quantified by liquid chromatography-mass spectrometry (LC-MS). The kinetics of product formation were determined from a plot of the amount of product formed versus time.

Quantification of Cyclohexanol with Gas Chromatography-Mass Spectrometry (GC-MS)—The amount of cyclohexanol produced by cyt P450 was quantified with a GC-MS method developed in this laboratory with a detection limit of 6 pmol at a signal-to-noise ratio of ≥3. At higher concentrations of cyclohexanol, the error was ±10–15%. Immediately after the reaction was quenched, it was spiked with 267 pmol of the internal standard, cyclohexanol-d14. The samples containing cyclohexanol and perdeuterated cyclohexanol were then extracted twice with 3 ml of dichloromethane. The extracts were concentrated with a stream of nitrogen to ~1–5 μl and redissolved in 50 μl of methanol. Standard solutions containing known amounts of both the internal standard and cyclohexanol (6–500 pmol) were prepared and processed under the same conditions as the quenched unknown samples each time an experiment was performed. The only difference was that the proteins in the samples used for the standard curve had not been reduced by dithionite.

Quantitative analysis was performed on an HP6890/MSD5893 GC-MS instrument (Agilent Technologies). A 2-μl aliquot of the methanolic solution containing cyclohexanol and cyclohexanol-d14 was injected at 250 °C in the pulse-splitless mode. Cyclohexanol and its perdeuterated analogue were chromatographed on a capillary DB-210 column (0.20 mm × 0.18 μm; J & W Scientific) using helium as carrier gas. A deactivated fused silica guard column (0.2 mm × 10 m; Agilent Technologies) was placed in front of the capillary DB-210 column. Following injection of the samples, the oven temperature was maintained at 50 °C for 3 min and then increased to 100 °C at a rate of 10 °C/min. Both cyclohexanol and cyclohexanol-d14 eluted at ~7.4 min. The mass spectrometer detector was operated in the selective ion monitoring mode with electronic ionization at 70 eV. In the selective ion monitoring mode, the intensities of only four ions were recorded, i.e. m/z 57 and 82 from fragmentation of cyclohexanol and m/z 61 and 92 from fragmentation of cyclohexanol-d14. Product was quantified by generating a standard curve with 6–500 pmol of cyclohexanol, using cyclohexanol-d14 as the internal standard. It gave rise to a straight line with R2 ≥ 0.98 in the range of calibration. The amount of cyclohexanol in the unknown samples was calculated by using the ratio of the area under the curve from the chromatogram of the selected ions at m/z 82 and m/z 92 based on the calibration curve performed on the same day.

Quantification of Norbenzphetamine with LC-MS/MS—The amount of norbenzphetamine produced under single turnover conditions was quantified with an LC-MS/MS method developed in this laboratory. After the reaction was quenched with NaOH, 0.3 nmol of the internal standard, norbenzphetamine-d5, was spiked into each sample. Norbenzphetamine and norbenzphetamine-d5 were extracted twice with 3 ml of methyl tert-butyl ether. The extracts were dried with a stream of nitrogen gas and redissolved in 0.4 ml of a solution of 50% acetonitrile, 50% H2O for LC-MS/MS analyses. An aliquot of 10 μl of the solution was injected into the LC-MS for quantification. Standard solutions containing a known amount of norbenzphetamine (0.02–5 nmol) and 0.3 nmol of norbenzphetamine-d5 were prepared under the same conditions as the quenched samples except that the protein complex was not reduced.

LC-MS/MS analysis was performed on a Thermo Finnigan TSQ Quantum mass spectrometer (Thermo Scientific, MA). Norbenzphetamine was separated from benzphetamine on a Zorbax SB-C18 column (2.1 × 150 mm, 3.5 μm; Agilent Technologies). The column temperature was maintained at 35 °C. The mobile phase consisted of 100% acetonitrile (A) and 20 mM...
ammonium formate (B). The column was equilibrated with 50% A, 50% B for 6 min at a flow rate of 0.4 ml/min. Norbenzphetamine and norbenzphetamine-$d_5$ were eluted with a linear gradient from 50% A, 50% B to 90% A, 10% B in 5 min, and the mobile phase was held at 90% A, 10% B for another 2 min. Norbenzphetamine and norbenzphetamine-$d_5$ eluted at $\sim$3.2 min, whereas benzphetamine eluted at $\sim$4.3 min. It is of note that commercially available benzphetamine is contaminated with $\approx$0.01% norbenzphetamine that gives a background signal during LC-MS/MS analysis of the samples from the single turnover experiments. This background norbenzphetamine was quantified in the time = 0 sample and subtracted from the experimental samples. With a typical norbenzphetamine background $\approx$10 pmol of norbenzphetamine can be detected with a signal/noise ratio of 3.

The mass spectrometer was operated in the selected reaction monitoring mode. Norbenzphetamine and norbenzphetamine-$d_5$ were ionized by electrospray ionization at $\sim$3500 V. Protonated parental ions ([M + H]$^+$) of norbenzphetamine (m/z 226.1) and norbenzphetamine-$d_5$ (m/z 231.1) were admitted into the first quadrupole and fragmented by collision with nitrogen gas in the second quadrupole. The fragment ions of m/z 91.1 from parental ion of m/z 226.1 and m/z 96.1 from parental ion of m/z 231.1 were detected in the third quadrupole, and the integrated area of the two fragment ions, m/z 91.1 versus m/z 96.1, were used for quantification.

Determination of the Rate of Product Formation under Pre-steady-state Conditions in a Rapid Chemical Quench Apparatus—The kinetics of product formation were measured under pre-steady-state conditions using the chemical quench flow apparatus to determine whether product release was the rate-limiting step in catalysis. The experiments were conducted at 30 °C in a manner identical to that described for the single turnover conditions except that Syringe A contained the pre-formed (at 4 °C overnight) oxidized complexes in aerobic Buffer at 30 °C in a manner identical to that described for the single rate-limiting step in catalysis. The experiments were conducted in a spectrophotometric quartz cuvette. Cyt P450 (0.3 nmol), varying amounts of cyt $b_5$, and product, respectively. The calculated product constant values for the cyt P450-CPR complex were derived from the modeling were consistent with the experimental data. The $K_d$ values for the cyt P450-CPR and cyt P450-cyt $b_5$ complexes were calculated using the calculated ratio of $k_2/k_1$ and $k_4/k_3$.

RESULTS

Kinetics of Cyclohexanol Formation in the Presence of Cyt $b_5$ and CPR under Single Turnover Conditions—In an attempt to gain further insight into the interactions of cyt P450 with its redox partners, CPR and cyt $b_5$, we have investigated the ability of cyt P450 2B4 to oxidize cyclohexane under conditions in which cyt P450 2B4 can undergo only a single catalytic cycle. Hence the terminology “single turnover conditions” is used. In these experiments, a 1:1 complex was preformed by incubating equimolar amounts of cyt P450 with either cyt $b_5$ or CPR over-night at 4 °C. The preformed cyt P450-cyt $b_5$ complex was stoichiometrically reduced to ferrous cyt P450 and ferrous cyt $b_5$, whereas the cyt P450-CPR complex was reduced to the ferrous cyt P450 and the 2-electron-reduced state of CPR. The 2e-re-

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P + C \xrightarrow{k_1} PC \quad \xrightarrow{k_2} P + C
\]

\[
P + B \xrightarrow{k_3} PB \quad \xrightarrow{k_4} P + B
\]

Scheme 1. Competitive model. P, cyt P450; B, cyt $b_5$; C, reductase; Pr, product.
The concentration of cyt P450 and cyt b₅ is 10 μM. The dashed line is the spectrum of the ferric cyt P450-cyt b₅ complex at the beginning of the titration, and the solid line is the spectrum of the ferrous cyt P450-cyt b₅ complex at the end of the titration. The inset shows changes in absorbance at 422 nm (●) and 438 nm (□) used to monitor cyt b₅ and cyt P450, respectively.

Reduced state of CPR exists as a mixture primarily of FMN hydroquinone (FMNH₂, FAD) and disemiquinone (FMNH+, FADH⁻) (31). Because of the rapid interflavin electron transfer (70 s⁻¹) from FAD to FMN (32), the existence of more than one form of CPR will not affect the kinetics of product formation that occurs at much slower rates (0.15–2.5 s⁻¹; see below). Multiple turnovers do not occur because only the FMNH₂ form of CPR is able to reduce cyt P450 (28). The 1e-reduced CPR and ferrous cyt b₅ cannot donate electrons to ferric cyt P450 2B₄ at a significant rate because of their high unfavorable redox potential. The mid-point redox potentials of the 1e-reduced CPR (FMN/FMNH₂) and cyt b₅ are -110 and +25 mV versus normal hydrogen electrode, respectively, which are much higher than that of substrate-bound cyt P450 2B₄ (-245 mV) (14, 31, 33). Once the proteins were stoichiometrically reduced to the appropriate oxidation state, catalysis was initiated by the rapid addition of oxygen to form oxyferrous cyt P450, which immediately accepts an electron from either ferrous cyt b₅ or the FMN hydroquinone of the 2e-reduced CPR to undergo a single catalytic cycle. Under our experimental conditions, CPR and cyt P450 are present at equal concentrations, thus ensuring that only a single catalytic cycle occurs. In the experiments in which cyt b₅ was present at a higher concentration than cyt P450, multiple turnovers of cyt P450 did not occur because cyt b₅ does not reduce ferric cyt P450.

Fig. 1 shows an example of the stoichiometric reduction of the preformed cyt P450-cyt b₅ complex with aliquots of dithionite in the presence of 1 mM cyclohexane. The absorbance changes at 438 and 422 nm were monitored to follow the reduction of cyt P450 and cyt b₅ respectively, as cyt b₅ has an isobestic point at 438 nm, and >95% of the absorbance changes at 422 nm arise from reduction of ferric cyt b₅ to ferrous cyt b₅. As shown in Fig. 1 (inset), reduction of cyt P450 and cyt b₅ proceeded in the order of their redox potentials. The first electron equivalent of dithionite reduced cyt b₅ as the absorbance change at 422 nm increased, whereas the absorbance at 438 nm remained virtually unchanged. Ferric cyt P450 was reduced to ferrous cyt P450 with addition of the second electron equivalent. The spectrum recorded at the end of the titration (Fig. 1, solid line) shows a reduced complex as evidenced by the intense Soret band at 424 nm characteristic of ferrous cyt b₅ and the disappearance of the charge transfer band at 648 nm indicative of high spin ferric cyt P450 (34). The stoichiometric reduction of the cyt P450-CPR complex was performed in an analogous manner. Three electron equivalents, one for cyt P450 and two for CPR, are required to reduce the cyt P450-CPR complex for the purpose of the single turnover experiments.

The kinetics of cyclohexanol formation by the cyt P450-cyt b₅ and cyt P450-CPR complexes under single turnover conditions are shown in Fig. 2. The amount of cyclohexanol produced increases exponentially after mixing the reduced cyt P450 complexes with oxygen. In the presence of cyt b₅, cyclohexanol formation reaches a plateau in ~0.25 s (Fig. 2, ●). In contrast, it takes nearly 10 times longer, 2 s, before product formation levels off in the presence of CPR (Fig. 2, □). Both kinetic traces were well fit with a single exponential, giving average rate constants of 29 ± 4.5 and 2.5 ± 0.35 s⁻¹ for the cyt P450-cyt b₅ and cyt P450-CPR complexes, respectively. Furthermore, the average amount of cyclohexanol produced per pmol of cyt P450 is higher in the presence of cyt b₅ (58 pmol) than in the presence of CPR (21 pmol) as shown in Table 1. These results demonstrate that catalysis occurs ~10-fold faster in the presence of cyt b₅ than in the presence of CPR. Possible explanations for the faster catalysis in the presence of cyt b₅ will be considered under “Discussion.”
Cyt b₅ and P450 Reductase Compete for a Binding Site on P450

TABLE 1
Kinetic parameters for cyclohexanol formation by cyt P450 2B4 in the presence of cyt b₅ and CPR under single turnover conditions at 30 °C

| Molar ratio P450:CPR:b₅ | Fast phase | Slow phase | pmol product/ nmol cyt P450 |
|--------------------------|------------|------------|----------------------------|
|                          | A₁%        | kₚ        | A₂%          | kᵢₚ     |                      |
| 1:0:1                    | 100         | 29 ± 4.5  | 100          | 2.5 ± 0.35 | 58                    |
| 1:1:0                    | 27 ± 4.4    | 34 ± 5.4  | 73 ± 6       | 1.6 ± 0.25 | 26                    |
| 1:1:1                    | 34 ± 6      | 26 ± 7    | 66 ± 5       | 2.6 ± 0.7  | 31                    |
| 1:1:2                    | 59 ± 7      | 38 ± 3.5  | 41 ± 7       | 5.3 ± 1.1  | 45                    |
| 1:1:3                    | 0.35 ± 0.02 | 0.28 ± 0.02 | 0.25 ± 0.02 | 0.7 ± 0.05 | 73 ± 6%               |

*a A% is the percentage of total product formed with the indicated rate constant.

FIGURE 3. Kinetics of cyclohexanol formation by the cyt P450-CPR complex in the presence of varying amounts of cyt b₅ at 30 °C. Cyt P450 concentration in the presence of both cyt b₅ and CPR. Under these conditions, cyt b₅ and CPR will provide the "second electron" and reduce oxyferrous cyt P450 to generate the active oxidizing species of cyt P450 and ultimately product. If cyt b₅ and CPR compete for a binding site on cyt P450, higher molar ratios of cyt b₅ to CPR should increase the amount of catalysis mediated by cyt b₅. The unique rate constants for catalysis was used to readily distinguish whether cyt P450 and CPR was responsible for the catalysis. Fig. 3 shows the kinetics of cyclohexanol formation at four different cyt P450:Cyt P450 molar ratios. Multiple catalytic cycles were prevented by maintaining a 1:1 ratio of cyt P450:CPR, whereas the cyt b₅ concentration was increased. In the presence of the three proteins, i.e. cyt P450, cyt b₅, and CPR, the kinetics of cyclohexanol formation are no longer monophasic as observed in the presence of CPR or cyt b₅ alone, but are biphasic, indicating that both cyt P450 and CPR are contributing to the generation of product (Fig. 3 and Table 1). Fitting the kinetic trace of product formation obtained at a cyt P450:Cyt b₅:CPR molar ratio of 1:1:1 required two exponentials. The two rate constants, 34 ± 5.4 and 1.6 ± 0.25 s⁻¹, correspond within experimental error to the rate of product formation by cyt b₅ and CPR, respectively, whereas the amplitude of the individual phases corresponds to the fraction of total product generated by cyt b₅ and CPR. At equimolar concentrations of the three proteins, CPR is responsible for 73 ± 6% of catalysis, whereas cyt b₅ catalyzes 27%, suggesting that CPR binds tighter to oxyferrous cyt P450 2B4 than cyt b₅ under our experimental conditions.

The addition of increasing amounts of cyt b₅ to a constant amount of cyt P450 and CPR resulted in more product being formed by cyt b₅ and less by CPR. At a cyt P450:Cyt P450 molar ratio of 1:1:3, 59% of the product was formed with a rate constant characteristic of cyt b₅ versus 27% at an equimolar ratio of the three proteins. This result is consistent with our hypothesis that cyt b₅ and CPR compete for a site on the surface of cyt P450 2B4.

Determination of the Rate of Cyclohexanol Formation under Pre-steady-state Conditions in the Presence of Excess NADPH—
The effect of cyt b₅ on cyt P450 catalysis was examined under pre-steady-state conditions to gain insight into where in the catalytic cycle cyt b₅ was exerting its effect. The experiments described in this section were designed to test whether product dissociation was rate-limiting and whether cyt b₅ modifies product dissociation. If product dissociation is the rate-limiting step in the catalytic cycle of an enzyme, a "burst" of product formation usually occurs before the reaction achieves the steady state (35). In this experiment, the oxidized protein complex (ferric cyt P450 and fully oxidized CPR), not the reduced one as in the single turnover experiments, was rapidly mixed with O₂ and NADPH in the rapid chemical quench apparatus, and the time course of product formation was quantified. The results are presented in Fig. 4. When only cyt P450 and reductase were present at an equimolar ratio, the kinetic trace was linear, indicating that the reaction did not exhibit burst kinetics (Fig. 4, □) and that product release was not rate-limiting. A steady-state activity of 9.0 ± 1.1 nmol of cyclohexanol/min/nmol of cyt P450 at 30 °C was calculated from the slope of the trace. A 3-fold molar excess of cyt b₅ compared with cyt P450 and CPR did not modify product release, which was linear. However, the activity in the presence of cyt b₅ was decreased by 65% to 2.9 ± 0.28 nmol of cyclohexanol/min/nmol of cyt P450.
Cyt b₅ and P450 Reductase Compete for a Binding Site on P450

TABLE 2

| Molar ratio | NADPH | Cyclohexanol | Coupling* |
|-------------|--------|--------------|-----------|
| P450:CPR:b₅ | nmol/min/nmol of cyt P450 | nmol/min/nmol of cyt P450 | % |
| 1:1:0 | 47.0 ± 1.1 | 6.2 ± 0.80 | 13.3 |
| 1:1:0.5 | 45.8 ± 4.3 | 10.8 ± 2.1 | 24.1 |
| 1:1:1 | 32.2 ± 3.3 | 8.2 ± 1.6 | 25.6 |
| 1:1:3 | 7.5 ± 1.5 | 1.4 ± 0.22 | 18.8 |
| 1:1:5 | 3.9 ± 0.87 | 0.30 ± 0.063 | 7.6 |

* Coupling refers to the percentage of NADPH that was used to form product.

NADPH Consumption and Metabolism of Cyclohexane under Steady-state Conditions—If cyt b₅ and CPR compete for a binding site on cyt P450 2B4, cyt b₅ should be capable of competing with CPR and decreasing NADPH consumption and product formation by cyt P450 2B4. To test this hypothesis, the rates of cyclohexane metabolism and NADPH consumption by cyt P450 2B4 were examined under steady-state conditions at various concentrations of cyt b₅. To obtain statistical significance for our results, the reaction was conducted in triplicate in five separate experiments for a total of 15 measurements. The results are summarized in Table 2. At equimolar concentrations of cyt P450 and CPR, the rate of NADPH consumption is 47 nmol/min/nmol of cyt P450 in the absence of cyt b₅. With increasing levels of cyt b₅, NADPH consumption progressively declines. Although at a 0.5 M equivalent of cyt b₅, the average rate is minimally, but not statistically significantly diminished. In a later section a similar observation was made with benzphetamine as the substrate. In the presence of a 5-fold molar excess of cyt b₅, NADPH consumption is inhibited by 92% and product formation by 95%. Paradoxically, at equimolar ratios of the three proteins, the metabolism of cyclohexane is stimulated by ≈33%, whereas NADPH consumption is inhibited by ≈32%, signifying that the coupling of the reaction is increased by 12%. Previous investigators have also observed that cyt b₅ stimulates the metabolism of benzphetamine and 7-ethoxycoumarin at low (≤1) molar ratios of cyt b₅:cyt P450, while inhibiting activity at higher molar ratios (5, 10, 11). For decades, investigators have been intrigued and perplexed by the slow phase (see supplemental Fig. S1 for a plot of data from Fig. 5 on a log scale). As summarized in Table 3, the rate constants for the fast and slow phases corresponding to the rate constants observed in the presence of cyt b₅ and CPR individually (Fig. 5 and Table 3). The biphasic feature is more prominent in the presence of benzphetamine compared with cyclohexane because of the ≈10-fold versus ≈10-fold difference, respectively, in rate constants between the fast and slow phases (see supplemental Fig. 1 for a plot of data from Fig. 5 on a log scale). As summarized in Table 3, the rate constants for the fast and slow phases are 17 and 0.18 s⁻¹, respectively, at a molar ratio of P450:CPR:cyt b₅ of 1:1:1. The relative amplitude of the fast phase is 32% and represents product formation by cyt b₅, whereas the relative amplitude of the slow phase is 68%, representing product formation by CPR. Because equimolar amounts of the three proteins were preincubated overnight at 4 °C, the fact that 68% of product is formed with a rate constant characteristic of CPR was interpreted to mean that under our experimental conditions CPR binds tighter to cyt P450 than cyt
b5. Increasing the cyt b5 molar ratio in the protein mixture did not alter the magnitude of the rate constant but significantly altered the amplitudes of the phases. The amplitude of the fast phase increases with increasing concentrations of cyt b5 at the expense of the slow phase. For example, upon increasing the molar ratio of cyt b5 from 1 to 3, the amplitude of the fast phase went from 32 to 74% of the total, whereas the slow phase decreases proportionally. Comparison of the data obtained with the two substrates, cyclohexane and benzphetamine (Tables 1 and 3), demonstrates that the relative amount of formed product by cyt b5 and CPR at the different molar ratios is similar. Both with substrates at a 1:1 molar ratio, CPR catalyzes 70% and cyt b5 30% of product formation.

**Modeling of the Kinetics of Benzphetamine Oxidation at Different Molar Ratios of Cyt b5**—In an attempt to determine whether the kinetics of norbenzphetamine formation at different molar ratios of cyt b5 can be fit to Scheme I, in which cyt b5 and CPR are competing for cyt P450, the data were analyzed using the DYNAFIT program (30). The DYNAFIT program was developed for fitting the time course of enzyme reactions to a molecular mechanism represented by a set of chemical equations. The data from the four kinetic traces in Fig. 5 with CPR, obtained with different concentrations of cyt b5, were simultaneously and globally fit to Scheme I. As shown, all four experimental traces fit well with a single set of kinetic parameters to Scheme I, which depicts a model in which cyt b5 and CPR compete for a binding site on cyt P450 2B4 (see supplemental Fig. S2). The fit yields rate constants for the fast (k4) and slow (k3) phases of 35 and 0.23 s⁻¹, respectively, and are in reasonable agreement with the experimental values of 18 and 0.15 s⁻¹. The calculated Kd values for the cyt P450-cyt b5 and the cyt P450-CPR complexes are 2.8 and 0.021 μM, respectively. The Kd value for the cyt P450-CPR complex has been reported to range from 0.02 to 0.05 μM (13, 23–24). A much wider range of Kd values (0.02–7.5 μM) has been reported for the cyt P450-cyt b5 complex (13, 21, 25–26). The agreement between the experimental and calculated values supports the validity of the competitive model. A similar analysis was also performed for the kinetics of cyclohexanol formation obtained at 30°C using DYNAFIT software. The kinetic trace of cyclohexanol formation is fit reasonably well to Scheme I, but the goodness of fit is poorer compared with that of norbenzphetamine formation because of lower product formation. The data are included in the Supplemental Material. The kinetic analysis yielded estimated Kd values of 0.0043 μM for the cyt P450-CPR complex and 0.31 μM for the cyt P450-cyt b5 complex. The estimated k2 and k3 values are 2.5 and 30 s⁻¹, respectively.

**NADPH Consumption and Metabolism of Benzphetamine under Steady-state Conditions**—The effects of cyt b5 on the rate of NADPH consumption, benzphetamine metabolism, and coupling of NADPH consumption to product formation were also studied under steady-state conditions and compared with our observations with cyclohexane (Tables 2 and 4). Basically our results with benzphetamine and cyclohexane metabolism were similar, although there is some variation in the details. Overall, the catalytic reaction with benzphetamine is greater than 50% coupled compared with cyclohexane, where catalysis was maximally coupled at 25%. The effect of cyt b5 on product formation under steady-state conditions is the net effect of the stimulatory and inhibitory actions of cyt b5 on cyt P450 2B4 catalysis. The inhibitory effect of cyt b5 on catalysis is a consequence of its ability to decrease NADPH consumption. This effect is significant at cyt b5:CPR molar ratios ≥1. Cyt b5 also increases the efficiency of NADPH utilization 23–30%. We and others have previously observed that at cyt b5:CPR molar ratios of =1:1, cyt b5 enhanced catalytic efficiency by =20% (5, 10). In general, stimulation of benzphetamine metabolism is observed at low cyt b5:cyt P450 molar ratios, whereas inhibition of benzphetamine metabolism occurs at higher, >1, molar ratios of cyt b5:cyt P450. Briefly, our explanation for the stimulatory effect of cyt b5 is its faster rate of catalysis, whereas it is proposed that its inhibitory effect is because of its ability to bind on the proximal surface of cyt P450 2B4, thereby preventing CPR from reducing ferric cyt P450 2B4 and initiating catalysis.

**DISCUSSION**

Our experiments under single turnover conditions with preformed reduced complexes of cyt P450 2B4 demonstrated that cyt b5 and CPR catalyze product formation monophagously with readily distinguishable rate constants for two substrates, cyclohexane and benzphetamine (Tables 1 and 3). The exact molecular mechanism responsible for the much faster rate of catalysis in the presence of cyt b5 is not understood at this time.

One plausible explanation for the more rapid catalysis in the presence of cyt b5 is that cyt b5 either induces and/or binds a cyt P450 conformation in which the second proton required for cyt P450 oxidations is delivered more rapidly to the hydroperoxo P450 conformation in which the second proton required for cyt P450 oxidations is delivered more rapidly to the hydroperoxo P450 intermediate. The hypothesis that the proton may be delivered to the hydroperoxo intermediate more quickly in the presence of cyt b5 includes the following. 1) Cyt b5 and CPR reduce oxyferric cyt P450 at similar rates. 2) Oxyferric cyt P450 turns over to ferric cyt P450 more slowly in the presence of CPR via a putative intermediate that could neither be detected spectrally nor distinguished from oxyferric cyt P450. The rate constant for the disappearance of oxyferric cyt P450 and the simultaneous appearance of the ferric protein in the presence of CPR (~0.09

| Molar Ratio | NADPH Coupling* | Formaldehyde | Coupling |
|-------------|------------------|--------------|----------|
| P450:CPR:b5 | nmol/min/nmol   | nmol/min/nmol | %        |
| 1:1:0       | 83 ± 5.5         | 47 ± 3.3     | 56       |
| 1:1:0.5     | 81 ± 2.1         | 56 ± 0.8     | 69       |
| 1:1:1       | 66 ± 3.2         | 48 ± 1.0     | 73       |
| 1:1:3       | 36 ± 6.3         | 30 ± 3.8     | 82       |
| 1:1:5       | 25 ± 1.3         | 21 ± 1.8     | 85       |

* Coupling refers to the percentage of NADPH that was used to form product.
s\(^{-1}\)) is consistent with the rate constant for norbenzphetamine formation (0.15 s\(^{-1}\)) determined in this work under similar conditions (14, 3).

Why such a large number of the cyt P450 complexes with cyt \(b_5\) and CPR are nonproductive and catalysis is so uncoupled continues to be an important unanswered question. Nevertheless, it is a well known characteristic of microsomal cyt P450, with benzphetamine being one of the most highly coupled substrates and cyclohexane being a moderately coupled substrate. The uncoupling is particularly puzzling in the case of CPR because ferric cyt P450 2B4 is rapidly and completely reduced in a preformed ferric cyt P450-CPR complex. Furthermore, the thermodynamic driving force for the reduction of oxyferrous cyt P450 is \(\approx 200\) mV greater than that for the ferric protein. Thus one is led to conclude that complex formation and reduction of the cyt P450 in the complex are necessary but not sufficient for catalysis (14, 22, 36). Because thermodynamics are not gating the process, it would appear that a conformational change is required for efficient substrate oxidation. In general, ferric cyt P450 can be reduced by a number of reductants. However, the oxyferrous cyt P450 is more particular about the electron donor for product formation. For example, putidaredoxin efficiently reduces cyt P450\(_{cat\,c}\), and gives rise to product, whereas adrenodoxin, flavodoxin, and other reductants are very sluggish and do not yield product (1).

Substrate is another important determinant of the coupling of the reaction. Tighter binding substrates like benzphetamine, in general, tumble less freely in the active site and spend more time near the active oxidizing oxyferrous species and, as a result, undergo more efficient catalysis and less side product formation (3, 10, 11). Tighter binding substrates also promote tighter binding of cyt P450 with its redox partners (23). In other words, substrate binding and cyt P450 complex formation are mutually beneficial. Our single turnover results are clearly in line with this notion as catalysis of benzphetamine is much better coupled than catalysis of cyclohexane.

Because cyt \(b_5\) and CPR catalyze product formation with readily distinguishable rate constants, it was feasible to directly determine whether cyt \(b_5\) or CPR mediated catalysis when both CPR and cyt \(b_5\) were present in the reaction mixture. The experiment enabled the hypothesis that cyt \(b_5\) and CPR compete for the binding site on cyt P450 2B4 to be tested. When equimolar concentrations of cyt P450, cyt \(b_5\), and CPR were present in the reaction mixture, \(\sim 30\%\) of product formed with a rate constant characteristic of cyt \(b_5\) and \(\sim 70\%\) with a rate constant consistent with that of CPR with the two substrates, cyclohexane and benzphetamine. With increasing concentrations of cyt \(b_5\), relatively more product was formed with a rate constant similar to that of cyt \(b_5\), although there was a decrease in the amount of product formed with a rate constant characteristic of CPR (Figs. 2 and 4; Tables 1 and 3). These data are consistent with the hypothesis that cyt \(b_5\) and CPR compete for a binding site on cyt P450 2B4. These results are not consistent with the alternative hypothesis that cyt \(b_5\) and CPR bind at separate, noninteracting sites on cyt P450 2B4 and form a ternary complex (26). At the high concentrations used in the single turnover experiments cyt \(b_5\) and CPR would both be expected to saturate their respective binding sites. In the two-site model where cyt \(b_5\) and CPR occupy two separate sites on cyt P450 2B4, cyt \(b_5\), with its 10–100-fold faster catalytic rate constant, is predicted to give rise to the great majority of product, and this amount of product should not increase with increasing cyt \(b_5\) concentration. The experiments, using rapid chemical quench under single turnover conditions, do not support the model that cyt \(b_5\) and CPR bind to distinct, separate sites on cyt P450 2B4, both of which are competent to deliver the second electron necessary for catalysis (26). The result from the kinetic modeling reinforces our interpretation that CPR and cyt \(b_5\) compete for a binding site on cyt P450 2B4.

The single turnover studies demonstrated that product is formed faster in the presence of cyt \(b_5\) and that cyt \(b_5\) competes with CPR for a binding site on cyt P450 2B4. These two effects of cyt \(b_5\) on product formation under single turnover conditions have helped clarify the mechanism of action of cyt \(b_5\) on the activity of cyt P450 under steady-state conditions. The data in Tables 2 and 4 show that at low \((\leq 1)\) molar ratios of cyt \(b_5\):CPR, cyt \(b_5\) increases the amount of product formed under steady-state conditions, although at high \((\geq 1)\) cyt \(b_5\):CPR molar ratios, cyt \(b_5\) inhibits the activity of cyt P450. It is proposed that the ability of cyt \(b_5\) to stimulate the activity of cyt P450 2B4 is related to the fact that catalysis by cyt P450 2B4 occurs faster in the presence of cyt \(b_5\) than with CPR. This enhanced rate of catalysis results in increased coupling between NADPH consumption and product formation. Our laboratory, as well as other investigators, has observed that on average cyt \(b_5\) increases the coupling of the reaction by \(\approx 15–25\%\) (5, 10). Depending on the substrate, this modest increase in coupling at the optimal cyt \(b_5\) concentration may result in either an inconsequential or significant increase in the absolute amount of product formed. In general, poorer, more uncoupled substrates experience a greater apparent enhancement in product formation.

For example, the approximately constant amount of NADPH consumption during steady-state conditions and a similar magnitude of increased coupling by cyt \(b_5\) for different substrates with a particular cyt P450 suggest an explanation of how cyt \(b_5\) can apparently exhibit strikingly different effects on the cyt P450 activity depending on the substrate. If NADPH consumption is 100 nmol/min/nmol of cyt P450 and cyt \(b_5\) at a cyt \(b_5\):reductase molar ratio of 1:1 increases the coupling of NADPH consumption to product formation 15%, then a poor substrate such as methoxyflurane, whose metabolism is 2% coupled in the absence of cyt \(b_5\), will have its activity increased from 2 to 17 nmol/min/nmol cyt P450. This is an 8-fold increase in the measured activity (10). With a moderately good substrate like cyclohexane (13% coupled without cyt \(b_5\)), product formation will increase with cyt \(b_5\) from 13 to 28 nmol/min/nmol cyt P450, a 2-fold increase in the absolute amount of product. An excellent substrate like benzphetamine (50% coupled without cyt \(b_5\)) will increase its activity from 50 to 65 nmol/min/nmol cyt P450, a 1.3-fold increase in activity. In practice, experimental variation, even among individuals and enzyme preparations from the same laboratory, is such that with benzphetamine this small change in a large number may not be detected. In this case, the investigator might erroneously conclude cyt \(b_5\) inhibited or had no effect on catalysis. Unless NADPH consumption is meas-
ured, it is difficult to ascertain the true effect of cyt b<sub>5</sub> on cyt P450 activity.

 Examination of our experimental results with the three substrates in the above example shows our explanation fits reasonably well to the data. With cyclohexane (±13% coupled), a 75% stimulation of product formation results, whereas with benzphetamine, a good substrate (±50% coupled), only a minimal ±17% enhancement in the absolute amount of product occurred (Tables 2 and 4). Trudell and co-workers (5) reported that cyt b<sub>5</sub> is most effective at stimulating cyt P450 catalysis under conditions where CPR is present at molar ratios low enough to be rate-limiting, as in microsomes where the molar ratio of CPR:cyt P450 is 1:10–20.

 It is suggested that the inhibitory effects of cyt b<sub>5</sub> at cyt b<sub>5</sub>-CPR molar ratios ≈1 can be attributed to its ability to prevent the binding of CPR to cyt P450 2B4 in agreement with the results of site-directed mutagenesis studies of cyt P450 2B4 (13). If CPR is unable to bind and reduce ferric cyt P450 2B4, catalysis and NADPH consumption will be inhibited as reported in Tables 2 and 4. Data obtained with cyt b<sub>5</sub> reconstituted with a manganese-containing protoporphyrin IX support our contention that the inhibitory effect of cyt b<sub>5</sub> is because of its competition with CPR for a binding site on cyt P450 2B4 (11). Although Mn-cyt b<sub>5</sub> has the same structure as holo-heme-containing cyt b<sub>5</sub> it remains oxidized under the aerobic conditions in which the steady-state activity of cyt P450 is measured. These properties of Mn-cyt b<sub>5</sub> predict that it should possess only inhibitory properties, because it can bind to cyt P450 2B4 like the native cyt b<sub>5</sub>, but it is unable to donate electrons to cyt P450 and stimulate its activity. As predicted the experiments demonstrated that Mn-cyt b<sub>5</sub> only inhibited the activity of cyt P450 2B4 at a constant concentration and molar ratio of cyt P450:CPR. Under no conditions did Mn-cyt b<sub>5</sub> stimulate the activity of cyt P450 2B4.

 There is another feasible mechanism for the inhibitory effects of cyt b<sub>5</sub> on catalysis, although it is considered unlikely by the authors. At high concentrations, cyt b<sub>5</sub> might form a stable complex with CPR, decreasing its effective concentration and ability to reduce cyt P450. Although CPR can reduce cyt b<sub>5</sub>, there is no experimental support for tight complex formation between the two proteins (37). Two laboratories, one using an enzyme-linked immunosorbent assay affinity approach and another using surface plasmon resonance in an optical biosensor cell, failed to detect stable complexes between cyt b<sub>5</sub> and CPR (38–40). Therefore, it is unlikely that reduction of cyt b<sub>5</sub> by CPR constitutes the main pathway for inhibition of NADPH consumption under steady state. In addition, cyt b<sub>5</sub> and CPR are both acidic proteins, and their electrostatic interactions would not be conducive to tight complex formation (3, 28, 41).

 In addition to demonstrating that catalysis occurs more rapidly in the presence of cyt b<sub>5</sub> than in the presence of CPR, the single turnover and steady-state experiments described in this work have enabled us to distinguish between two models that have been put forward to explain the complex effects of cyt b<sub>5</sub> on catalysis by cyt P450 2B4. The single-site competitive model proposes that CPR and cyt b<sub>5</sub> have unique but overlapping binding sites on cyt P450 2B4 and compete in a mutually exclusive manner for binding to cyt P450 2B4 (13). This model is supported by site-directed mutagenesis studies on cyt P450 2B4, which demonstrate that cyt b<sub>5</sub> and CPR have unique but overlapping binding sites on the proximal surface of cyt P450 2B4. In view of the mutagenesis studies, it is difficult to understand how it would be possible for the two proteins not to compete for binding to cyt P450 2B4. The two-site model hypothesizes the formation of a ternary complex of cyt P450, CPR, and cyt b<sub>5</sub> with distinct noninteracting binding sites for cyt b<sub>5</sub> and CPR. Both cyt b<sub>5</sub> and CPR are able to reduce the oxyferrous form of cyt P450, but only CPR reduces ferric cyt P450 (26). The two independent sites model predicts that cyt b<sub>5</sub> and CPR would form a ternary complex with cyt P450 under single turnover conditions. Because the rate constant for catalysis by cyt b<sub>5</sub> is 10–100-fold greater than that for CPR, cyt b<sub>5</sub> would, in a ternary complex, be virtually solely responsible for catalysis under single turnover conditions. Tables 1 and 3 demonstrate that cyt b<sub>5</sub> was not solely responsible for catalysis that was mediated by both CPR and cyt b<sub>5</sub>. In fact, CPR accounts for approximately two-thirds of product formed at an equimolar ratio of cyt P450, CPR, and cyt b<sub>5</sub>, and cyt b<sub>5</sub> contributes the remaining one-third of product. This product ratio most likely reflects the relative affinity of CPR and cyt b<sub>5</sub> to cyt P450, and it can be reasonably interpreted with a one-site competitive model but not with the two-independent sites model. Furthermore, the one-site competitive model predicts that cyt b<sub>5</sub> will prevent CPR from binding to cyt P450 2B4 and reducing ferric cyt P450 2B4, an essential early step in cyt P450 catalysis. Experimentally, NADPH consumption is progressively inhibited by cyt b<sub>5</sub> at all concentrations examined, consistent with the one-site competitive model. The two-site model does not explain this inhibition.

 Additional data significantly strengthening our hypothesis that cyt b<sub>5</sub> and CPR compete for a single site on cyt P450 2B4 are the overlapping binding sites for cyt b<sub>5</sub> and CPR in the published models of the cyt P450-cyt b<sub>5</sub> and cyt P450-CPR complexes (4). A complex of cyt P450 2B4 with CPR was generated by superimposing the heme of the closed conformation of cyt P450 2B4 and the FMN domain of CPR on the crystal structure of the complex between cyt P450 BM<sub>4</sub> and the FMN domain of the cyt P450 BM<sub>4</sub> reductase (41–43). The residues in contact in the complex obtained by overlaying cyt P450 2B4 and CPR on the bacterial cyt P450 BM<sub>4</sub>, are in excellent agreement with the residues (Arg-122, Phe-135, Met-137, Lys-139, Arg-126, Arg-133, Arg-422, and Arg-443) found by site-directed mutagenesis of cyt P450 2B4 to be involved in binding CPR (4, 13).

 In conclusion, under single turnover conditions the following could be demonstrated: 1) cyt b<sub>5</sub> catalyzed product formation 10–100-fold more rapidly than CPR, and 2) cyt b<sub>5</sub> was able to compete with CPR to form product. Faster catalysis with cyt b<sub>5</sub> predicts cyt b<sub>5</sub> should stimulate the activity of cyt P450 2B4 because there is less time for side product formation whereas the ability of cyt b<sub>5</sub> to diminish the binding of CPR to cyt P450 2B4 predicts cyt b<sub>5</sub> should decrease the activity of cyt P450 2B4 under steady-state conditions. It is suggested that these two completely opposite effects of cyt b<sub>5</sub> explain its varied effects on the activity of cyt P450 2B4 under steady-state conditions. The observed effect of cyt b<sub>5</sub> on the steady-state activity of cyt P450 2B4 will be the net result of its stimulatory and inhibitory
effects. It is hypothesized that under steady-state conditions, the stimulatory effect of cyt $b_5$ at low molar cyt $b_5$/CPR ratios is related to its ability to increase the rate of product formation by cyt P450 2B4 and consequently the coupling of NADPH consumption. At low molar ratios, cyt $b_5$ causes minimal disruption of the binding of CPR to cyt P450 2B4 and NADPH consumption. It is proposed that the inhibition of NADPH consumption and the steady-state activity of cyt P450 2B4 at high molar ($\geq 1$) ratios of cyt $b_5$ is because of inhibition of the reduction of ferric cyt P450 2B4 as a result of formation of a cyt P450-cyt $b_5$ complex, which does not permit CPR to deliver the first electron required for catalysis. At high molar ratios of cyt $b_5$, its inhibitory effects dominate its stimulatory effects. When the stimulatory and inhibitory effects are equal, activity will not change. The experiments also support the hypothesis that cyt $b_5$ and CPR compete for a binding site on P450 2B4 at two distinct, noninteracting sites on cyt P450 2B4. It remains to be determined to what extent our findings with cyt P450 2B4 apply to other microsomal cytochromes P450.

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