Cytochrome $b_5$ Inhibits Electron Transfer from NADPH-Cytochrome P450 Reductase to Ferric Cytochrome P450 2B4

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Experiments demonstrating that cytochrome (cyt) $b_5$ inhibits the activity of cytochrome P450 2B4 (cyt P450 2B4) at higher concentrations suggested that cyt $b_5$ was occupying the cyt P450 reductase-binding site on cyt P450 2B4 and preventing the reduction of ferric cyt P450 2B4 (Zhang, H., Im, S.-C., and Waskell, L. (2007) J. Biol. Chem. 282, 29766–29776). In this work experiments were undertaken with manganese-containing cyt $b_5$ (Mn-cyt $b_5$) to test this hypothesis. Because Mn-cyt $b_5$ does not undergo oxidation state changes under our experimental conditions, interpretation of the experimental results was unambiguous. The rate of electron transfer from cyt P450 reductase to ferric cyt P450 2B4 was decreased by Mn-cyt $b_5$ in a concentration-dependent manner. Moreover, reduction of cyt P450 2B4 by cyt P450 reductase was incomplete in the presence of Mn-cyt $b_5$. At a Mn-cyt $b_5$:cyt P450 2B4:cyt P450 reductase molar ratio of 5:1:1, the rate of reduction of ferric cyt P450 2B4 was decreased by 10-fold, and only 30% of the cyt P450 2B4 was reduced, whereas 70% remained oxidized. It could be demonstrated that Mn-cyt $b_5$ had its effect by acting on cyt P450, not the reductase, because the reduction of cyt c by cyt P450 reductase in the presence of Mn-cyt $b_5$ was not effected. Furthermore, under steady-state conditions in the cyt P450 reconstituted system, Mn-cyt $b_5$, which lacks the ability to reduce oxyferrous cyt P450 2B4, was unable to stimulate the activity of cyt P450. Mn-cyt $b_5$ only inhibited the cyt P450 2B4 activity. In conjunction with site-directed mutagenesis studies and experiments that strongly suggested that cyt $b_5$ competed with cyt P450 reductase for binding to cyt P450, the current investigation demonstrates unequivocally that cyt $b_5$ inhibits the activity of cyt P450 2B4 by preventing cyt P450 reductase from binding to cyt P450, a prerequisite for electron transfer from cyt P450 reductase to cyt P450 and catalysis.

Microsomal cytochromes (cyt) P450, functioning as monooxygenases, catalyze the oxidative biotransformation of numerous pharmaceuticals, carcinogens, pro-carcinogens, and endogenous compounds like fatty acids and steroids. Cyt P450 require two electrons and two protons to carry out catalysis that leads to insertion of a single oxygen atom into the substrate. In the mammalian microsomal cyt P450 system, the two electrons are delivered to cyt P450 by NADPH-dependent cytochrome P450 reductase (CPR). Like cyt P450, CPR is membrane-bound and located in the membrane of the endoplasmic reticulum. CPR contains two flavin molecules, FMN and FAD. The diflavin moiety of CPR is essential for sequential electron transfer to cyt P450 as it permits CPR to accept two electrons from NADPH and transfer one electron at a time to cyt P450. The first electron from CPR reduces ferric cyt P450 to ferrous cyt P450, which rapidly binds oxygen to form oxyferrous cyt P450. The second electron is then delivered to oxyferrous cyt P450. This is followed by protonation of the reduced oxyferrous intermediate leading to heterolytic cleavage of the oxygen bond to form water and an oxyferryl intermediate, the putative, active, oxidizing species of cyt P450. An oxygen atom is inserted into the substrate, and the more hydrophilic product dissociates from the enzyme. Readers are referred to a recent review for further details about the cyt P450 reaction cycle (1).

An alternative electron donor to cyt P450 is cyt $b_5$, another microsomal hemoprotein also located in the endoplasmic reticulum membrane. Because of its relatively high mid-point redox potential (+25 mV versus NHE), cyt $b_5$ can deliver only the second electron to oxyferrous cyt P450 but not the first electron to ferric cyt P450. It has been recognized for 3 decades that cyt $b_5$ may either increase, decrease, or not alter the activity of selected cyts P450 (2, 3). Cyt $b_5$ has been reported to affect the catalytic activity of more than 20 cyt P450 isoforms, including the majority of the human drug-metabolizing cyt P450 isoforms like cyt P450 3A4, 2B6, 2C9, 2C19, and 2E1 (1, 4–8). The effect of cyt $b_5$ has also been shown to depend on the cyt P450 isozyme and substrate (2, 9). In the case of cyt P450 2B4 and 2E1, the electron donating properties of cyt $b_5$ are required for its stimulatory activity (6, 7, 9–11), although some studies suggest that apo-cyt $b_5$ can stimulate the activity of cyt P450 3A4 via an allosteric effect (12). At present, the ability of apo-cyt $b_5$ to stimulate cyt P450 3A4 is controversial (13). Experiments performed in the reconstituted system with purified proteins have demonstrated that ferrous cyt $b_5$ can rapidly reduce oxyferrous cyt P450 2B4 (14, 15).

It is known that cyt P450 2B4 forms a 1:1 complex with CPR and with cyt $b_5$ in a purified, reconstituted system (16, 17). A site-directed mutagenesis study of the interactions of cyt P450 2B4 with CPR and cyt $b_5$ has identified residues, primarily in the...
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C-helix on the proximal side of cyt P450 2B4, that participate in binding both CPR and cyt b₅ (18). 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 the basis of a cross-linking study with a carbodiimide, Schenckman and co-workers (19) have proposed a two-site model, hypothesizing that cyt b₅ and CPR bind at two distinct, functional sites on cyt P450 and form a ternary complex.

Recently we investigated the interaction of cyt P450 2B4 with CPR and cyt b₅ by examining product formation under both single turnover and steady-state conditions in an effort to understand the complex effects of cyt b₅ on cyt P450 2B4 catalysis (20). It was possible to demonstrate under single turnover conditions that catalysis by cyt P450 2B4 occurred faster in the presence of cyt b₅ than with CPR and that at high concentrations cyt b₅ appeared to displace CPR from cyt P450 2B4. These observations suggested an explanation for the results under steady-state conditions where cyt b₅ stimulated product formation at low concentrations but inhibited activity at higher concentrations. The stimulatory activity at low concentrations but inhibited activity at higher concentrations cyt b₅ and CPR supported the hypothesis that cyt b₅ and CPR had overlapping binding sites on the proximal surface of cyt P450 2B4 (20).

The stimulatory activity of cyt b₅ at low concentrations may result from an increased rate of catalysis by cyt P450 2B4 and explains why the absorbance increase at 450 nm as a result of formation of the ferrous cyt P450-CO adduct following reduction of ferric cyt P450 2B4 by CPR. The stopped-flow experiments were performed with a Hi-Tech SF61DX2 stopped-flow spectrophotometer (Hi-Tech, Wiltshire, UK) housed in an anaerobic chamber (Belle Technology, Dorset, UK) as reported previously (24). The transient spectra were recorded with a Hi-Tech SF61DX2 stopped-flow spectrophotometer (Toronto, Canada). Carbon monoxide gas (purity > 99.9%) was purchased from Matheson Tri-Gas (Parispany, NJ). Mn(III) protoporphyrin IX chloride was purchased from Frontier Scientific Inc. (Logan, UT). Re-distilled glycerol was purchased from Roche Diagnostics.

Protein Expression and Purification—Cyt P450 2B4, cyt b₅, and CPR were expressed and purified from Escherichia coli as described previously (20). The concentration of cyt P450 2B4 was determined using an extinction coefficient of Δε₄₅₀→₄₉₀ nm of 91 M⁻¹ cm⁻¹ as described by Omura and Sato (21). The concentration of CPR was determined using an extinction coefficient of 21 M⁻¹ cm⁻¹ at 456 nm for the oxidized enzyme (22). The concentration of cyt b₅ was determined using an extinction coefficient of 185 M⁻¹ cm⁻¹ at 456 nm for the ferrous and ferric cyt b₅ (23).

Kinetics of the Reduction of Ferric Cyt P450 2B4 by CPR in the Presence of Various Concentrations of Holo-cyt b₅—The rate of electron transfer from CPR to ferrous cyt P450 2B4, or the first electron transfer, was measured to probe the interaction between cyt P450 2B4, CPR, and cyt b₅. The kinetics were determined with stopped-flow spectrophotometry by monitoring the absorbance increase at 450 nm as a result of formation of the ferrous cyt P450-CO adduct following reduction of ferric cyt P450 2B4 by CPR. The stopped-flow experiments were performed with a Hi-Tech SF61DX2 stopped-flow spectrophotometer (Hi-Tech, Wiltshire, UK) housed in an anaerobic chamber (Belle Technology, Dorset, UK) as reported previously (24).

The temperature of the stopped-flow spectrophotometer reaction chamber and observation cell was maintained at 30 °C using a circulating water bath. Cyt P450 2B4, CPR, and cyt b₅ (when present) were pre-mixed by incubating cyt P450 (3 M), CPR (3 μM), and various concentrations of cyt b₅ (0–15 μM) in 0.1 M potassium phosphate, pH 7.4, buffer containing 15% glycerol, 0.18 M DLPC, and 1 mM benzphetamine at 4 °C overnight. The anaerobic protein mixture was rapidly mixed with 0.1 M potassium phosphate buffer, pH 7.4, containing 15% glycerol, 1 mM benzphetamine, and 3 μM NADPH. Both solutions had been saturated with CO by blowing CO gas over the sample solutions. The absorbance change at 450 nm was recorded as a function of time.

Reduction of Ferric Cyt b₅ by Ferrous Cyt P450-CO—The kinetics of the reduction of ferric cyt b₅ by ferrous cyt P450-CO were determined with a stopped-flow spectrophotometer, basically as described for reduction of ferric cyt P450 2B4 by CPR. The experiment was performed under anaerobic conditions to avoid possible side reactions involving oxygen. Cyt P450 2B4 was reduced with a stoichiometric amount of dithionite in a tonometer. The ferrous cyt P450 solution was saturated with CO gas to form the cyt P450-CO adduct. Cyt P450-CO and ferric cyt b₅ were loaded into separate syringes in the stopped-flow instrument. The transient spectra were recorded with a photodiode array detector following rapid mixing of ferric cyt b₅ with cyt P450-CO. The absorbance was also measured in the single wavelength mode at 450 nm. The final concentration of cyt b₅ and cyt P450 after mixing was 5 μM in 0.1 M potassium phosphate buffer, pH 7.4, 15% glycerol.
Preparation of Full-length and Soluble Mn-Cyt b5—The full-length Mn-cyt b5 was prepared by reconstituting full-length apo-cyt b5 with Mn(III) protoporphyrin IX as described by Morgan and Coon (11). The heme of cyt b5 was removed from holo-cyt b5 by acidifying the cyt b5-containing solution to pH 2.0, and the dissociated heme was extracted from the aqueous solution with 2-butanol. Apo-cyt b5 was then reconstituted with Mn(III) protoporphyrin IX at molar ratio of 2:1 to apo-cyt b5. Mn-cyt b5 was recovered from a Sephadex G-25 size-exclusion column where free MnIII protoporphyrin was bound. Soluble Mn-cyt b5 was prepared by reconstitution of soluble bovine apocytochrome b5 with Mn(III) protoporphyrin IX as described previously (25). The concentration of Mn-cyt b5 was determined using an extinction coefficient of 57 mM−1 cm−1 at 469 nm (25).

Kinetics of Reduction of Ferric Cyt P450 2B4 by CPR in the Presence of Full-length Mn-Cyt b5—The kinetics of reduction of ferric cyt P450 by CPR in the presence of Mn-cyt b5 was measured in the same way as in the presence of holo-cyt b5 as described above. Because it is redox-silent under our experimental conditions, Mn-cyt b5 does not participate in the electron transfer processes and does not undergo spectral changes under the experimental conditions. It is therefore possible to deconvolute the end point spectra recorded in the stopped-flow spectrophotometer as only 1 M eq of NADPH was used in the reaction. Deconvolution of the spectra by linear regression gives the concentration of each species at the end of the reaction.

The concentration of each species at the end of the reaction was obtained by iterative regression of the observed spectrum as a linear combination of the standard spectrum of each species. When all three proteins were present, theoretically there are six possible species at the end of the reaction, including cyt P450-CO, ferric cyt P450 in the presence of 1 mM benzphetamine, oxidized Mn-cyt b5, reduced Mn-cyt b5, 1-electron-reduced CPR, and 2-electron-reduced CPR. The observed spectrum, $A_{obs}$, is expressed as in Equation 1,

$$A_{obs} = \sum_{i=1}^{6} \epsilon_i \times C_i \times l$$

where $\epsilon_i$ and $C_i$ represent the extinction coefficient and concentration of each of the six species, and $l$ is the 1-cm path length. Linear regression was performed with SigmaPlot software (Systat Inc., San Jose, CA). Because of the low extinction coefficient of the 1- and 2-electron-reduced forms of CPR, it was not possible to obtain a reliable estimate of the amount present.

Kinetics of Reduction of Cyt c by CPR in the Presence of Full-length Mn-Cyt b5—The effect of Mn-cyt b5 on reduction of cyt c by CPR was studied to examine whether Mn-cyt b5 forms a tight complex with CPR, capable of diminishing the ability of CPR to transfer electrons to cyt P450. A solution containing 8 mM of CPR and full-length Mn-cyt b5 (4, 8, 16, 24, and 40 mM) was preincubated at 4 °C overnight at the specified concentration in a glove box in 0.1 M potassium phosphate buffer, pH 7.4, that contained 15% glycerol and a 60-fold molar excess of DLPC with respect to CPR. The concentration of CPR in syringe 1 of the stopped-flow spectrophotometer was 8 μM before mixing, whereas the concentration of Mn-cyt b5 varied from 0 to 40 μM. The pre-mixed CPR and Mn-cyt b5 were rapidly mixed with an equal volume of the 0.1 M potassium phosphate buffer, pH 7.4, 15% glycerol, that contained cyt c (8 μM) and NADPH (8 μM). The kinetics of cyt c reduction were monitored at 550 nm, using an $\Delta A$ of 21.1 mm−1 cm−1 at 550 nm between ferric and ferrous cyt c (26).

Measurement of the Activity of Cyt P450 2B4 Under Steady-state Conditions in the Presence of Full-length Mn-Cyt b5—The rates of NADPH consumption and benzphetamine metabolism in the presence of Mn-cyt b5 were determined under steady-state conditions in the purified reconstituted cyt P450 2B4 system at 30 °C as described earlier (20). The reaction was initiated by adding excess NADPH to a final concentration of 0.3 mM and terminated after 5 min by adding 70% trifluoroacetic acid to a final concentration of 5%. The amount of formaldehyde produced from metabolism of benzphetamine was analyzed with Nash’s reagent as described (27). NADPH consumption was determined by measuring the decrease in absorbance at 340 nm using an extinction coefficient of 6.2 mm−1 cm−1.

Data Analysis—Apparent rate constants and amplitudes for the rate of electron transfer from CPR to ferric cyt P450 and cyt c were obtained by fitting the absorbance changes at the selected wavelength as a single or double exponential function using SigmaPlot software (Systat Inc.).

RESULTS

Electron Transfer from CPR to Ferric Cyt P450 in the Presence of Holo-cyt b5—In an attempt to test our hypothesis that cyt b5 inhibited the activity of cyt P450 2B4 by preventing cyt P450 reductase from binding to and reducing cyt P450 2B4, the rate of electron transfer from CPR to ferric cyt P450 was measured in the presence of cyt b5. The reduction of ferric cyt P450 2B4 was measured in the presence of carbon monoxide and 1 mM eq of NADPH in the stopped-flow spectrophotometer. Reduction of CPR by NADPH ($k \equiv 28$ s−1) and the binding of CO to ferric cyt P450 ($k \equiv 100$ s−1) occur significantly faster than reduction of cyt P450 2B4. Therefore, the rate of electron transfer to cyt P450 2B4 can be determined by observing the absorbance changes at 450 nm after mixing the pre-formed cyt P450-CPR complex with NADPH in the presence of CO (28, 29). Fig. 1A shows the absorbance changes at 450 nm during the course of the first electron transfer to cyt P450 in the presence of various concentrations of holo-cyt b5. Reduction of ferric cyt b5, which also occurs under these conditions, was monitored at 422 nm as shown in Fig. 1B. As expected, ferric cyt P450 was reduced by CPR biphosphinically in the absence of cyt b5. The biphasic rate constants are 4.1 and 0.51 s−1, and the amplitude of the fast phase, $k_1$, is 81% (see Table 1). This result is similar to that reported by other investigators (29, 30). In the presence of holo-cyt b5, the rate of reduction of ferric cyt P450 2B4 decreases as observed for cyt P450 1A2 and 2E1 (31). A 5-fold molar excess of cyt b5 completely eliminated formation of the cyt P450-CO complex as evidenced by the absence of an absorbance increase at 450 nm. In fact, a small decrease in absorbance was observed at this concentration, which reflects partial reduction of the excess cyt b5 (32). With higher concentrations of cyt b5, the
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![Graph showing kinetics of reduction of ferric cyt P450 2B4 by CPR in the presence of varying amounts of cyt b₅.](image)

**FIGURE 1.** Kinetics of reduction of ferric cyt P450 2B4 by CPR in the presence of varying amounts of cyt b₅. Cyt P450, CPR, and cyt b₅ were preincubated in the presence of D LPC and 1 mM benzphetamine under anaerobic conditions and rapidly mixed with 1 mM eq of NADPH. The final concentration of cyt P450, CPR, and NADPH after mixing was 1.5 μM, whereas the final concentration of cyt b₅ varied. For easy comparison, all kinetic traces are offset to the same baseline. A and B show absorbance changes at 450 and 422 nm, respectively. ---, no cyt b₅; –––, 1.5 μM cyt b₅; ----, 4.5 μM cyt b₅; ——, 7.5 μM cyt b₅.

**TABLE 1**

The rate constants and amplitudes observed during the electron transfer process from CPR to ferric cyt P450 in the presence of varying amounts of the membrane form cyt b₅ at 30 °C

| Concentration | Phase I | Phase II |
|---------------|---------|----------|
| P450 | CPR | Cyt b₅ | k₁ | A₁ | k₂ | A₂ |
| μM | μM | μM | % | % | % | % |
| 1.5 | 1.5 | 0 | 450 | 4.1 ± 0.32 | 81 ± 7 | 0.51 ± 0.057 | 19 ± 3.5 |
| 1.5 | 1.5 | 0.75 | 450 | 4.0 ± 0.52 | 60 ± 5 | 0.73 ± 0.091 | 40 ± 5 |
| 1.5 | 1.5 | 1.5 | 450 | 1.7 ± 0.13 | 49 ± 6 | 0.54 ± 0.065 | 51 ± 8 |
| 422 | 0.39 ± 0.04 | 100 ± 11 | 422 | 0.60 ± 0.04 | 100 ± 12 | 422 | 0.38 ± 0.05 | 100 ± 8 |
| 422 | 0.69 ± 0.09 | 100 ± 13 | 422 | 0.25 ± 0.042 | 100 ± 11 | 422 | 0.87 ± 0.056 | 100 ± 14 |
| 1.5 | 1.5 | 3.0 | 450 | 1.2 ± 0.23 | 100 ± 12 | 422 | 0.38 ± 0.05 | 100 ± 8 |
| 1.5 | 1.5 | 4.5 | 422 | 1.2 ± 0.23 | 100 ± 12 | 422 | 0.38 ± 0.05 | 100 ± 8 |
| 1.5 | 1.5 | 7.5 | 422 | 1.2 ± 0.23 | 100 ± 12 | 422 | 0.38 ± 0.05 | 100 ± 8 |

The absorbance at 422 nm increased rapidly. The absorbance change at 422 nm in the presence of the membrane form cyt b₅ is a net result of reduction of cyt b₅ and formation of cyt P450-CO. The former process results in an absorbance increase at 422 nm (Δε = 110 mM⁻¹ cm⁻¹), whereas the latter results in an absorbance decrease (Δε = −20 mM⁻¹ cm⁻¹) as shown by the absorbance decrease in the absence of cyt b₅ (Fig. 1B, solid line). The increase in absorbance at 422 nm demonstrates that cyt b₅ is reduced. In the presence of a 5-fold excess of cyt b₅, cyt b₅ is reduced at an apparent rate constant of 1.2 s⁻¹.

The decrease in the rate of reduction of ferric cyt P450 in the presence of cyt b₅ is the result of the following processes: 1) cyt b₅ binding to the proximal surface of cyt P450 2B4, which inhibits the binding of CPR; 2) oxidation of cyt P450-CO by ferric cyt b₅; and 3) reduction of cyt b₅ by CPR. The three simultaneous reactions occurring under the experimental conditions are illustrated in Scheme 1. It is well documented that CPR reduces cyt b₅ (33, 34). Peterson et al. (35) have reported that cyt b₅ is capable of oxidizing ferrous carbon monoxide cyt P450, but the rate was not determined. A rate of 45 s⁻¹ was calculated for oxidation of the CO adduct of cyt P450 1A2 by ferric cyt b₅ based on kinetic simulation (31). To better understand the relative importance of the different electron transfer processes, we have directly measured the rate of reduction of ferric cyt b₅ by cyt P450 2B4-CO.

Reduction of Ferric Cyt b₅ by Ferrous Cyt P450-CO—Experiments were performed anaerobically in the stopped-flow spectrophotometer to directly measure the rate of reduction of ferric cyt b₅ by ferrous cyt P450-CO by mixing equimolar amounts of cyt P450-CO with ferric cyt b₅. The results are presented in Fig. 2. As shown, the absorbance at 450 nm bleaches whereas the absorbance at 422 and 555 nm increases over time (Fig. 2A). The bleaching at 450 nm indicates that CO dissociates from cyt P450, presumably because of oxidation of ferrous cyt P450 to ferric cyt P450, whereas the increase in intensity at 422 and 555 nm is indicative of reduction of cyt b₅. The absorbance change at 450 nm is fit to give biphasic rate constants of 0.6 and 0.1 s⁻¹ (fast phase, 45%). Fitting the kinetic trace at 422 nm gave rate constants of 0.8 and 0.2 s⁻¹ (fast phase, 50%). The simultaneous change in absorbance at 450 and 422 nm indicates that electron transfer from ferrous cyt P450-CO to ferric cyt b₅ occurs without an observable intermediate. Approximately 40% of the cyt P450-CO is oxidized under these conditions. The rate of electron transfer from ferrous cyt P450 2B4 to ferric cyt b₅ in the absence of CO was reported to be ~2.7 and 0.44 s⁻¹ at 5 °C (15). The slower oxidation of cyt P450-CO by ferric cyt b₅ may result from the increased redox potential of cyt P450-CO or the slow dissociation of CO from cyt P450-CO and subsequent reduction of cyt b₅ by ferrous cyt P450. It has been estimated that the binding of CO to ferrous cyt P450 raises the mid-point potential by ~180 mV versus NHE (36) relative to the ferri-ferrous couple in the presence of 1 mM benzphetamine (~245 mV versus NHE) (37). Increasing the potential would reduce the driving force for electron transfer compared with the ferrous protein. It has also been reported that CO dissociates from cyt P450-CO with a biphasic rate constant (k₉) of 0.4 and 0.049 s⁻² at 20 °C.
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A UV-visible spectra were recorded in the stopped-flow spectrophotometer at 0, 0.1, 0.5, 1, 3, 5, and 10 s following mixing. Arrows indicate the time-dependent changes at selected wavelengths. B, absorbance changes at 450 and 422 nm. Cyt P450 (5 μM) was reduced stoichiometrically with dithionite in the presence of DLPC and 1 mM benzphetamine. A stream of CO gas was introduced to ferrous cyt P450 solution to form cyt P450-CO. The cyt P450-CO complex was then rapidly mixed with a CO-saturated solution containing ferric cyt P450 (5 μM) to reduce it stoichiometrically with dithionite in the presence of DLPC overnight at 4 °C. Benzphetamine was added to the samples to a final concentration of 1 mM. The protein sample was mixed with CO-saturated buffer containing 1 mM benzphetamine and 1 eq of NADPH.

The visible spectra (380–700 nm) recorded, ~60 s after mixing in the stopped-flow spectrophotometer, when the reaction was essentially complete, are shown in Fig. 4A (open circles). In the absence of Mn-cyt b₅, ferric cyt P450 was completely reduced by 1 M eq of NADPH. In the presence of increasing

interpretation. The kinetics of the reduction of ferric cyt P450 2B4 by CPR in the presence of Mn-cyt b₅ are shown in Fig. 3. As observed for holo-cyt b₅, full-length Mn-cyt b₅, decreases the rate of electron transfer from CPR to ferric cyt P450. Both the rate and extent of cyt P450-CO formation progressively decrease with increasing concentrations of Mn-cyt b₅. The apparent rate constants and relative amplitudes are summarized in Table 2. At an equimolar ratio of cyt P450, CPR, and Mn-cyt b₅, the apparent rate constant decreases ~60% to 1.7 and 0.28 s⁻¹. Similar rate constants were reported by Reed and Hollenberg (38). In the experiments conducted with a 5-fold molar excess of Mn-cyt b₅, the rate constant of the fast phase decreases by ~16-fold. In contrast to holo-cyt b₅, where no cyt P450 was reduced in the presence of a 5-fold molar cyt b₅ excess, ~30% of cyt P450 was reduced in the presence of Mn-cyt b₅.

The visible spectra (380–700 nm) recorded, ~60 s after mixing in the stopped-flow spectrophotometer, when the reaction was essentially complete, are shown in Fig. 4A (open circles). In the absence of Mn-cyt b₅, ferric cyt P450 was completely reduced by 1 M eq of NADPH. In the presence of increasing


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FIGURE 4. A, UV-visible spectra during the reduction of ferric cyt P450 by CPR in the presence of a 0, 1–3, and 5-fold excess of Mn-cyt b₅. The spectra were recorded 60 s after mixing. The experimental conditions are the same as described in Fig. 3. B, fit concentration of cyt P450-CO (○) and ferric cyt P450 (●) obtained from the iterative fitting of the end point spectra described above.

amounts of Mn-cyt b₅, the absorbance at 450 nm decreases, whereas the absorbance at 470 nm increases. The peak at 470 nm is characteristic of oxidized Mn-cyt b₅ (25). Potentially there are six possible species at the end of the reaction as follows: ferric cyt P450 in the presence of 1 mM benzphetamine, cyt P450-CO, 1e-reduced CPR, 2e-reduced CPR, oxidized Mn-cyt b₅, and reduced Mn-cyt b₅. Because the standard spectra of the six species are known and only 1 eq of NADPH was used, the end point spectra can be deconvoluted, and the concentration of each species can be estimated through iterative curve-fitting.

As expected, the iterative fitting reveals that Mn-cyt b₅ remains oxidized (see the supplemental material). At the 5-fold molar excess of Mn-cyt b₅, the final concentrations of cyt P450-CO (0.2 μM) and ferric cyt P450 (0.9 μM) estimated from the spectra acquired using a photodiode array detector are displayed in Fig. 4B. The presence of Mn-cyt b₅ during the first electron transfer process results in a decrease in the final concentration of cyt P450-CO and an increase in the final concentration of ferric cyt P450, indicating that Mn-cyt b₅ inhibits reduction of cyt P40 2B4 by CPR. At a Mn-cyt b₅:cyt P450:CPR ratio of 5:1:1, the concentration of cyt P450-CO at the end of the reaction, estimated by deconvoluting the data in Fig. 4A, is only 20% of the total cyt P450, in good agreement with the ~30% determined from measurements at a single wavelength (450 nm) in Fig. 3. As illustrated in Fig. 4, A and B, the higher the Mn-cyt b₅ concentration, the greater the inhibition of cyt P450 reduction by CPR and the less cyt P450-CO is formed. The spectra of the 1- and 2-electron-reduced CPR are similar and have a low extinction coefficient so the fitted concentrations were deemed unreliable and, in any case, were not relevant to the cytochrome concentrations.

Even though the membrane-bound form of Mn-cyt b₅ substantially inhibits the reduction of ferric cyt P450 by CPR, the soluble-form Mn-cyt b₅ is without effect (data not shown). The indifference of the first electron transfer to the presence of the soluble-form of Mn-cyt b₅ underscores the importance of the hydrophobic membrane-binding domain of cyt b₅ for its interaction with cyt P450. It is evident that the degree of inhibition of ferric cyt P450 is proportional to the concentration of Mn-cyt b₅. Fig. 5 shows the concentration dependence of the inhibition of CPR-mediated reduction of cyt P450 2B4 by cyt b₅ and Mn-cyt b₅ is consistent with our hypothesis that cyt b₅ inhibits reduction of cyt P450 2B4 by competing with reductase for a binding site on the proximal surface of cyt P450 2B4. An unlikely alternative explanation for this observation is that cyt b₅ is binding to the reductase and decreasing its ability to reduce cyt P450 (39, 40). To test this possibility, we examined electron transfer from CPR to cyt c in the presence of various concentrations of Mn-cyt b₅.

Mn-cyt b₅ Does Not Inhibit the Reduction of Cyt c by CPR—Evidence indicates that cyt P450 2B4 and cyt c bind to the same or an overlapping site on cyt P450 reductase (41, 42). Therefore, we investigated the ability of Mn-cyt b₅ to form a tight complex with CPR that would prevent it from reducing cyt P450 2B4 by
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The experiment was performed as described under "Materials and Methods."

| Molar ratio P450:CPR:Mn-cyt b₅ | NADPH | Formaldehyde |
|-------------------------------|-------|--------------|
|                               | nmol/min/nmol of cyt P450 | nmol/min/nmol of cyt P450 |
| 1:1:0                         | 78 ± 5.7 | 51 ± 1.2   |
| 1:1:0.5                      | 70 ± 7.7 | 48 ± 1.5  |
| 1:1:1                       | 44 ± 4.3 | 32 ± 0.62 |
| 1:1:3                      | 15 ± 0.76 | 12 ± 0.28 |
| 1:1:5                      | 7.8 ± 0.38 | 6.0 ± 0.20 |

TABLE 3
Rate of reduction of cyt c by CPR in the presence of Mn-cyt b₅ at 30 °C

CPR and varying amounts of Mn-cyt b₅ were in one syringe and cyt c and NADPH in the other syringe. The final concentration of NADPH, CPR, and cyt c was 6 μM.

| Mn-cyt b₅/CPR ratio | k  | Cyt c reduced % |
|---------------------|----|----------------|
| 0                   | 3.3 ± 0.67 | 91 ± 5.5 |
| 0.5                 | 3.6 ± 0.92 | 96 ± 7.0 |
| 1                   | 3.9 ± 1.1  | 95 ± 3.8 |
| 2                   | 3.8 ± 0.78 | 95 ± 1.8 |
| 3                   | 4.1 ± 1.0  | 95 ± 3.8 |
| 5                   | 4.7 ± 1.0  | 91 ± 2.8 |

TABLE 4
Effect of cyt b₅ on the rate of NADPH consumption and benzphetamine metabolism by cyt P450 2B4 under steady-state conditions at 30 °C

The data in Table 4 are from Ref. 20.

| Molar ratio P450:CPR:b₅ | NADPH | Formaldehyde |
|-------------------------|-------|--------------|
|                         | nmol/min/nmol of cyt P450 | nmol/min/nmol of cyt P450 |
| 1:1:0                   | 83 ± 5.5 | 47 ± 3.3   |
| 1:1:0.5                 | 81 ± 2.1 | 56 ± 0.8  |
| 1:1:1                   | 66 ± 3.2 | 48 ± 1.0  |
| 1:1:3                   | 36 ± 6.3 | 30 ± 3.8  |
| 1:1:5                   | 25 ± 1.3 | 21 ± 1.8  |

Measurements of the ability of CPR to reduce cyt c in the presence of varying concentrations of Mn-cyt b₅. The results, summarized in Table 3, demonstrate that Mn-cyt b₅ did not inhibit the reduction of cyt c by CPR even at a molar ratio of Mn-cyt b₅:CPR of 5:1. Regardless of the Mn-cyt b₅ concentration, cyt c was reduced completely with an apparent rate constant of ~3.9 s⁻¹. In view of the data indicating that cyt c and cyt P450 2B4 compete for a binding site on CPR, lack of inhibition of cyt c reduction by Mn-cyt b₅ indicates that Mn-cyt b₅ does not form a tight complex with CPR, in agreement with results from other laboratories (39, 40). This experiment and data favor our argument that inhibition of reduction of ferric cyt P450 in the presence of Mn-cyt b₅ is because of formation of a tight Mn-cyt b₅-cyt P450 2B4 complex.

Comparison of the Effect of Holo-cyt b₅ and Mn-cyt b₅ on the Steady-state Activity of Cyt P450 2B4—Cyt b₅ can either stimulate, inhibit, or not affect the rate of catalysis by cyt P450 2B4 in a purified, reconstituted system, depending on the cyt b₅:cyt P450 2B4 molar ratio (2, 11, 20). The stimulatory activity of cyt b₅ is attributed to the ability of oxyferrous cyt P450 2B4 to form product faster in the presence of low concentrations of cyt b₅ than in the presence of CPR (20). High concentrations of cyt b₅ inhibit the activity of cyt P450 2B4 because it can effectively compete with CPR for a binding site on cyt P450 2B4. If the latter two statements are true, the prediction is that Mn-cyt b₅ should not stimulate, only inhibit the activity of cyt P450 2B4 in a reconstituted system.

Tables 4 and 5 summarize and compare the activity of cyt P450 2B4 with the substrate benzphetamine and the amount of NADPH consumed during turnover under steady-state conditions in the presence of cyt b₅ and Mn-cyt b₅. At low concentrations, cyt b₅ stimulates product formation and increases the efficiency of the reaction (efficiency refers to the fraction of NADPH that is utilized to form product) by decreasing the amount of superoxide formed (10). High concentrations of cyt b₅ decrease NADPH consumption and product formation by binding to cyt P450 2B4 and preventing CPR from binding to and reducing cyt P450 2B4, processes essential for catalysis. As Mn-cyt b₅ is incapable of undergoing either oxidation or reduction under our experimental conditions, it should not be able to stimulate the activity or increase the efficiency of catalysis. As predicted, product formation is not stimulated by Mn-cyt b₅ at low concentrations as it is with cyt b₅. In fact, Mn-cyt b₅ decreases both the rate of NADPH consumption and product formation. At a Mn-cyt b₅:cyt P450 molar ratio of 5:1, the rate of the NADPH consumption and product formation decrease by ~90%. At similar molar ratios, Mn-cyt b₅ is more inhibitory than cyt b₅, consistent with its inability to reduce oxyferrous cyt P450 and stimulate catalysis. For example, NADPH consumption and product formation are inhibited by 70 and 55%, respectively, in the presence of 5-fold molar excess of holo-cyt b₅. In contrast, both activities decrease by 90% in the presence of a 5-fold molar excess of Mn-cyt b₅, which is consistent with the proposal that there is not a separate, functional binding site for Mn-cyt b₅.

DISCUSSION

We have demonstrated that cyt b₅ and Mn-cyt b₅ substantially hinder the reduction of ferric cyt P450 2B4 by CPR, a critical step in the oxidative transformation of substrates. Both holo- and Mn-cyt b₅ decrease the rate of reduction and the amount of ferric cyt P450 reduced when only 1 μM of NADPH is available. Our experiments indicate that full-length Mn-cyt b₅ inhibits the reduction of cyt P450 2B4 by binding on its proximal surface, preventing CPR from binding and reducing ferric cyt P450.

Mn-cyt b₅ is redox-silent under our experimental conditions and thus can only exert its effect through its binding to cyt P450 and CPR. Cyt b₅, presumably Mn-cyt b₅ as well, can form a complex with both cyt P450 and CPR. It is well established through kinetic, biochemical, mutagenic, cross-linking, and spectrophotometric experiments that cyt b₅ forms a cyt P450-cyt b₅ complex with Kₛ values in the range of 0.02–7.5 μM depending on experimental conditions (14, 18–20). Association of cyt b₅ with cyt P450 2B4 is fast as ferrous cyt b₅ is capable of transferring an electron to oxyferrous cyt P450 at ~2 s⁻¹ when the two proteins were mixed from separate syringes in the stopped-flow spectrophotometer (15). In contrast, under simi-
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lar conditions, the association of cyt P450 with CPR is a slow \((t_{1/2} = 2\) min), rate-limiting process \((44)\). Therefore, the rate of reduction of cyt P450 by CPR is typically measured by rapidly mixing a preformed cyt P450-CPR complex with NADPH. Cyt P450 forms a 1:1 complex with CPR with an apparent \(K_d\) of \(-0.03\) \(\mu M\) \((17, 18, 45, 46)\).

Our results with Mn-cyt b₅ indicate that Mn-cyt b₅ also functions as a competitive inhibitor of the reduction of ferric cyt P450 2B4 with a \(K_i\) of 1.7 \(\mu M\) (Fig. 5), which is similar to the \(K_d\) value for the cyt P450-cyt b₅ complex. The observation that Mn-cyt b₅ decreases the rate of reduction of ferric cyt P450 by CPR and that only some of the ferric cyt P450 is able to accept an electron from CPR demonstrate that a certain fraction of ferric cyt P450 forms a cyt P450-Mn-cyt b₅ complex that is incapable of accepting an electron from CPR. At higher concentrations of Mn-cyt b₅, more cyt P450-Mn-cyt b₅ complex is formed at the expense of the cyt P450-CPR complex. This accounts for the dependence of the reduction of ferric cyt P450 by CPR on the concentration of Mn-cyt b₅. Our results do not support formation of a ternary complex of cyt P450-CPR-cyt b₅ because formation of a ternary complex with separate functional binding sites for cyt b₅ and CPR predicts that Mn-cyt b₅ should NOT substantially inhibit the reduction of ferric cyt P450 \((19)\). Because Mn-cyt b₅ has no effect on reduction of CPR by NADPH (data not shown) and does not inhibit the activity of CPR, ferric cyt P450 should be fully reduced by 1 eq of NADPH according to a ternary complex model. In fact, only 30\% of ferric cyt P450 is reduced at a Mn-cyt b₅:P450 molar ratio of 5 (Figs. 3 and 4).

Another feasible, though considered unlikely, mechanism for the inhibitory effect of Mn-cyt b₅ on reduction of ferric cyt P450 by CPR is formation of a stable Mn-cyt b₅-CPR complex. This would require Mn-cyt b₅ to have a high affinity for CPR and be capable of competing with cyt P450 for binding to CPR. The interprotein interaction between cyt P450 and CPR or cyt b₅ has an electrostatic component and involves complementary charge pairing at the protein interface \((2, 18, 47)\). Because both CPR and cyt b₅ are negatively charged proteins, it is unlikely that CPR would form a tight complex with cyt b₅. In fact, two laboratories have failed to detect a stable complex between CPR and cyt b₅ using an enzyme-linked affinity approach and surface plasmon resonance in a biosensor cell \((39, 40)\). The observation that Mn-cyt b₅ has no effect on cyt c reduction by CPR also strongly argues against a significant role for a Mn-cyt b₅-CPR complex in inhibiting the reduction of cyt P450 2B4.

Holo-cyt b₅ inhibits reduction of ferric cyt P450 to a greater extent than Mn-cyt b₅. At a cyt b₅/cyt P450 ratio of 5:1, ferric cyt P450 2B4 was not reduced (Fig. 1). At the same molar ratio of Mn-cyt b₅, 30\% of ferric cyt P450 was reduced. This additional inhibition of cyt P450-CO formation by holo-cyt b₅ in the presence of limiting NADPH arises from oxidation of cyt P450-CO and CPR by ferric cyt b₅. We have experimentally confirmed that cyt P450-CO is oxidized by ferric cyt b₅ at a rate similar to CO dissociation from ferrous cyt P450. This reaction is thermodynamically and kinetically feasible because the mid-point potential of cyt b₅ is estimated to be ~175 mV higher than that of cyt P450-CO, and oxidation of ferrous cyt P450 by ferric cyt b₅ proceeds at a rate of ~2.5 s⁻¹ \((15, 36)\). The reduction of cyt b₅ by CPR is also well documented \((33, 34)\).

Our previous work, which measured product formation under single turnover conditions, showed that ferrous cyt b₅ and reduced CPR compete for a binding site on ferrous cyt P450 2B4 to deliver the second electron to generate product \((20)\). Global analysis of the kinetics of product formation yielded a \(K_d\) of 2.8 \(\mu M\) for the ferrous cyt P450-ferrous cyt b₅ complex. In this study, we have demonstrated that this competitive binding also applies to the oxidized enzymes during the first electron transfer. Interestingly, although totally different approaches were used, both our studies yield a similar binding affinity between cyt P450 and cyt b₅ \((2.8 \text{ versus } 1.7 \mu M)\) irrespective of protein oxidation state. Whether cyt P450 utilizes exactly the same site for the first and second electron transfer remains to be tested. These two studies, nonetheless, provide a better understanding of the complex role of cyt b₅ in cyt P450 catalysis.

In the case of cyt P450 2B4, the stimulatory effect of cyt b₅ arises from its ability to more efficiently use NADPH for generating product than CPR. N-Demethylation of benzphetamine by oxyferrous cyt P450 2B4 is 100-fold faster with ferrous cyt b₅ than with CPR \((20)\). This ability to rapidly form product increases the catalytic efficiency of cyt P450 under steady-state conditions \((10–20\%\). Association of cyt b₅ with ferric cyt P450, however, also has a negative effect on cyt P450 catalysis as it inhibits reduction of ferric cyt P450 by CPR and thus blocks an early step in the cyt P450 catalytic cycle. This is why stimulation of cyt P450 activity is only observed at the low cyt b₅:cyt P450 molar ratio and inhibition predominates at higher cyt b₅ concentrations \((11, 20, 48)\). A similar phenomenon has been observed for cyt P450 1A2 \((49)\) and 2C9 \((8)\) even though the optimal cyt b₅:cyt P450 ratio may vary among different cyt P450 isoforms. It remains to be seen to what extent our hypothesis is applicable to other cyt P450 isozymes.

In conclusion, the role of cyt b₅ in cyt P450 2B4 catalysis has been examined under both pre-steady-state and steady-state conditions, and the mechanism by which cyt b₅ inhibits the activity has been elucidated. Both holo- and Mn-cyt b₅ substantially reduce the rate of reduction of ferric cyt P450 2B4 by CPR, and the amount of ferric cyt P450 reduced by CPR. This has been shown to be due to the ability of cyt b₅ to bind to cyt P450, thereby preventing CPR from binding to and reducing cyt P450 2B4. To account for the complex role of cyt b₅ under steady-state conditions, we propose the following: 1) the inhibitory effect of cyt b₅ on cyt P450 catalysis is because of the fact that cyt b₅ and CPR compete for a binding site on the proximal surface of cyt P450 2B4, and formation of a cyt b₅-P450 complex prevents ferric cyt P450 from accepting an electron from CPR and initiating the catalytic cycle; and 2) the stimulatory effect of cyt b₅ is because of more rapid and efficient catalysis in the presence of cyt b₅ compared with CPR. When the two opposite effects are equal, cyt b₅ has no effect on cyt P450 catalysis.

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