Cytochrome $b_5$ Binds Tightly to Several Human Cytochrome P450 Enzymes

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ABBREVIATIONS: P450 (or CYP), cytochrome P450; $b_5$, cytochrome $b_5$ (CYB5A); POR, NADPH-cytochrome P450 reductase; $K_d$, dissociation constant.
ABSTRACT

Numerous studies have been reported in the past 50-plus years regarding the stimulatory role of cytochrome b$_5$ (b$_5$) in some but not all microsomal cytochrome P450 (P450, CYP) reactions with drugs and steroids. A missing element in most of these studies has been a sensitive and accurate measure of binding affinities of b$_5$ with P450s. In the course of work with P450 17A1, we developed a fluorescent derivative of a human b$_5$ site-directed mutant, Alexa 488-T70C-b$_5$, that could be utilized in binding assays at sub-µM concentrations. Alexa 488-T70C-b$_5$ bound to human P450s 1A2, 2B6, 2C8, 2C9, 2E1, 2S1, 4A11, 3A4, and 17A1 with estimated K$_d$ values ranging from 2.5 to 61 nM. Only weak binding was detected with P450 2D6, and no fluorescence attenuation was observed with P450 2A6. All of the P450s that bound b$_5$ have some reported activity stimulation except for P450 2S1. The affinity of P450 3A4 for b$_5$ was decreased somewhat by the presence of a substrate or inhibitor. The fluorescence of a P450 3A4•Alexa 488-T70C-b$_5$ complex was partially restored by titration with NADPH-P450 reductase (POR) (K$_{d,apparent}$ 89 nM), suggesting the existence of a ternary P450 3A4-b$_5$-POR complex, as observed previously with P450 17A1. Gel filtration evidence was also obtained for this ternary complex with P450 3A4. Overall, the results indicated that the affinity of b$_5$ for many P450s is very high and that ternary P450-b$_5$-POR complexes are relevant in P450 3A4 reactions, as opposed to a shuttle mechanism.
SIGNIFICANCE STATEMENT

High affinity binding of cytochrome $b_5$ ($b_5$) ($K_d < 100$ nM) was observed with many drug-metabolizing cytochrome P450 (P450) enzymes. There is some correlation of binding with reported stimulation, with several exceptions. Evidence is provided for a ternary P450 3A4-$b_5$-NADPH-P450 reductase complex.
Introduction

The story of the involvement of cytochrome \textit{b}_5 (\textit{b}_5) in cytochrome P450 (P450) drug oxidations began with observations on the enhancement of some NADPH-dependent microsomal catalytic activities by NADH (Hildebrandt and Estabrook, 1971). The initial explanation was that NADH could deliver electrons to P450 via the flavoprotein NADH-\textit{b}_5 reductase and \textit{b}_5 and augment electron flow (Hildebrandt and Estabrook, 1971; Correia and Mannering, 1973). However, purification and reconstitution experiments indicated that \textit{b}_5 was not an obligatory component of P450 systems (Lu and Coon, 1968). Further research in multiple laboratories showed that the addition of \textit{b}_5 to reconstituted P450 systems could either stimulate, inhibit, or have no effect (Gorsky and Coon, 1986). Evidence that \textit{b}_5 was not only an effector in reconstituted systems but also important in the endoplasmic reticulum came from immunochemical experiments with anti-\textit{b}_5 and reactions in liver microsomes (Noshiro et al., 1979; Noshiro et al., 1980; Yamazaki et al., 1996b). For a summary of some of the early literature in this area see Peterson and Prough (Peterson and Prough, 1986).

A number of proposals have been addressed to explain the effect of \textit{b}_5. One is electron transfer (Bhatt et al., 2017). \textit{b}_5 can provide the first electron in the P450 catalytic cycle (reducing ferric iron to ferrous), although the difference in redox potentials ($E_{m,7}$) is unfavorable. The more widespread proposal has been that \textit{b}_5 is providing the second electron (i.e., to the Fe$^{2+}$O$_2$ complex) (Noshiro et al., 1981), although reconstituted systems have been reported with only NADH, NADH-\textit{b}_5 reductase, \textit{b}_5, and P450 (West et al., 1974). This NADH-dependent electron transport system may be how drug metabolism occurs in liver-specific NADPH-P450 reductase (POR) knockout (Por$^{-/-}$) mice (Gu et al., 2003; Henderson et al., 2003). Another proposal is that \textit{b}_5 is acting in an allosteric manner, changing the conformation of a P450 to make it more active.
in some reactions. This hypothesis has its basis in studies showing that some (but not all) P450 activities are enhanced by apo-\(b_5\) or mangano-porphyrin-\(b_5\), which are incapable of electron transfer (Yamazaki et al., 1996a; Auchus et al., 1998; Lee-Robichaud et al., 1998; Yamazaki et al., 2001; Yamazaki et al., 2002). Further, co-expression of P450 17A1 with apo-\(b_5\) (lacking the globular head domain) stimulates the steroid lyase activity in mammalian COS-1 cells (Storbeck et al., 2012). Another proposal, related to both of the above, is that \(b_5\) somehow improves the efficiency of the NADPH-coupling system, reducing the leakage of electrons to form reactive oxygen species (Peterson and Prough, 1986; Zhang et al., 2008; Peng et al., 2016).

Discerning the mechanism of stimulation by \(b_5\) has been difficult, for a number of reasons. Electrons can flow through POR to \(b_5\) (Guengerich, 2005), as well as NADH-\(b_5\) reductase, the pathway used in fatty acid desaturation. Although a number of studies have identified sites of \(b_5\) interactions with several P450s using site-directed mutagenesis and chemical cross-linking studies (Gao et al., 2006; Peng et al., 2014 Bridges, 1998, 32285), no structures of binary complexes are yet available. There is also a conundrum regarding evidence that POR and \(b_5\) occupy the same site on some P450s (Estrada et al., 2013) and how rapid interchange of the accessory proteins can support catalysis. One proposal is that P450 dimers could bind POR on one end and \(b_5\) on the other (Holien et al., 2017).

An important issue has been the measurement of binding parameters of \(b_5\) and P450s. To our knowledge, no spectral titrations have been useful (Naffin-Olivos and Auchus, 2006), presumably because of the strong Soret absorbance of both heme proteins.\(^3\) NMR measurements (Estrada et al., 2013; Ahuja, 2013) are problematic in that high (>100 \(\mu\)M) concentrations of the proteins are needed, and estimation of what might be sub-\(\mu\)M \(K_d\) values is impossible. Surface plasmon resonance (SPR) measurements suffer from the need to bind one component, and the
rates observed are notoriously slow, not reflective of diffusion-controlled events (Johnson, 2019).

Recently we expressed a $b_5$ mutant, T70C (Fig. 1) (Stayton et al., 1988), conjugated it with a fluorescent dye (Alexa 488 maleimide) (Fig. 2), and utilized this probe in studies on the interaction of $b_5$ with P450 17A1 (Kim et al., 2021). The results were interpreted in a model of very tight binding of $b_5$ and P450 17A1, with POR binding to form a ternary complex during catalysis (Kim et al., 2021). We have now extended this approach to other human P450s, particularly those with precedents for stimulation by $b_5$. 
Materials and Methods

**Enzymes.** Recombinant human $b_5$ (Guengerich, 2005) and rat POR (Hanna et al., 1998) were expressed in *Escherichia coli* and purified as described, without the use of affinity tags. Expression (in *E. coli*) and purification of C-terminal (His)$_6$-tagged P450s was as in the indicated references: 1A2 (Sandhu et al., 1994), 2A6 (Kim et al., 2005), 2B6 (Hanna et al., 2000), 2C8 (Tang et al., 2009), 2C9 (Sandhu et al., 1993), 2D6 (Hanna et al., 2001), 2E1 (Gillam et al., 1994), 2S1 (Wu et al., 2006), 3A4 (Gillam et al., 1993; Hosea et al., 2000), 4A11 (Kim et al., 2014), and 17A1 (Gonzalez and Guengerich, 2017). These P450s all have N-terminal amino acids modified, plus deletions, for optimal heterologous expression but still show a requirement of phospholipid vesicles for maximum catalytic activity. All have been found to be catalytically active under appropriate reconstitution conditions and, in several cases, to show $b_5$ stimulation (Yamazaki et al. 2002). Alexa 488-T70C-$b_5$ was prepared as described elsewhere in a study of P450 17A1 (Kim et al., 2021). All proteins were of high purity as judged by SDS-gel electrophoresis and did not contain any obvious cleavage products. The concentrations of P450 were estimated using the extinction coefficient $\Delta\varepsilon_{450-490} = 91,000 \text{ M}^{-1} \text{ cm}^{-1}$ for the Fe$^{2+}$-CO vs. Fe$^{2+}$ difference spectra (Omura and Sato, 1964). The concentration of $b_5$ was estimated using the extinction coefficient $\varepsilon_{423} = 100,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Spatz and Strittmatter, 1971) or the difference extinction coefficient $\Delta\varepsilon_{424-409} = 180,000 \text{ M}^{-1} \text{ cm}^{-1}$ for the Fe$^{2+}$ vs. Fe$^{3+}$ difference spectra (Velick and Strittmater, 1956). The concentration of POR was estimated using the extinction coefficient $\varepsilon_{455} = 23,600 \text{ M}^{-1} \text{ cm}^{-1}$ (Yasukochi and Masters, 1976).

**Fluorescence Titrations.** A solution of 50 nM Alexa 488-T70C-$b_5$ in 1 mM potassium phosphate buffer (pH 7.4) was placed in a 1.0-ml cuvette in an OLIS DM45 spectrofluorimeter.
(On-Line Instrument Systems, Athens, GA). Excitation was at 480 nm, and the emission spectrum was scanned from 500 to 650 nm, with a peak at 513 nm. $F_{513}$ values were collected, plotted, and fit to a hyperbolic curve with a standard quadratic equation

$$Y = B + \frac{A}{2E} \left( K_d + E + X \right) - \sqrt{\left( K_d + E + X \right)^2 - 4EX}$$

in GraphPad Prism software (GraphPad, San Diego, CA), where $Y$ is the observed fluorescence, $E$ is the Alexa 488-T70C-b$_5$ concentration, $X$ is the concentration of ligand added (P450), $K_d$ is the dissociation constant, and $B$ is the intercept (this is set in Prism as: $Y=B+(A/2)*((K_d+E+X)-(K_d+E+X)^2-(4*E*X))$, with $E$ set at the value used and $B$ being the fluorescence at the starting point, $F_0$). The extrapolated endpoint was used in each case, in that there is no independent evidence that the interaction of each P450 with Alexa 488-T70C-b$_5$ will generate the same fluorescence decrease in every case. Most of the results are expressed as $F/F_0 \times 100$, where $F$ is the fluorescence at 513 nm (excitation at 480 nm) and $F_0$ is the fluorescence in the absence of any added ligand.

**Association Kinetics of Alexa 488-T70C-b$_5$ and P450 3A4.** The rate of association of Alexa 488-T70C-b$_5$ with P450 3A4 was estimated by mixing 1 µM concentrations of each (in 100 mM potassium phosphate buffer, pH 7.4), in an OLIS RSM1000 instrument (23 °C, 4 mm × 4 mm cell, 1.24 mm slits, 480 nm excitation, and detecting emission > 530 nm with an Oriel long-pass filter attached to the photomultiplier tube). The decrease in fluorescence was fit to a single-exponential, with the SD, using the OLIS GlobalWorks program. The fit was transformed using residuals analysis with the software.
**Gel Filtration Studies.** Size-exclusion chromatography was done with a Superose 12 10/300 GL column (11 µm, 10 mm × 300 mm, GE Healthcare) with an NGC Quest 100 Plus Chromatography system (BioRad). The buffer was 50 mM potassium phosphate (pH 7.4) containing 0.15 M NaCl, and the flow rate was 1.0 ml min⁻¹. The column was equilibrated for each run with 1 column volume (23.6 ml); the injection volume was 3% of the column volume (10 nmol of each protein was injected, i.e. 100 µl of 100 µM solutions). Elution was with 1.5 column volumes, and absorbance was monitored at 280 nm. Fractions were collected (1.0 ml) and analyzed by SDS-polyacrylamide gel electrophoresis (4-15% gradient gel), with staining with Coomassie Blue and densitometry using GelAnalyzer 19.1 software (www.gelanalyzer.com, Istvan Lazar and Istvan Lazar, Jr.).

The molecular weights ($M_r$) of protein complexes were estimated using fitting to a curve developed by plotting log$_{10} M_r$ vs. corrected elution volume (corrected for void volume based on elution volume of blue dextran), using chicken ovalbumin (43 kDa), chicken conalbumin (75kDa), rabbit muscle aldolase (158 kDa), ferritin (440 kDa), and blue dextran (≥2,000 kDa) (Cytiva Life Sciences).
Results

**Rationale.** In recent work with P450 17A1 (Kim et al., 2021) we labeled a human b<sub>5</sub> mutant (T70C) with a dye, based on a previous approach used to monitor the binding of bacterial P450<sub>cam</sub> and rat b<sub>5</sub> (Stayton et al., 1988; Stayton et al., 1989). The site of attachment is removed from the putative sites of binding (Glu-48, Glu-49), at least to P450 17A1 (Naffin-Olivos and Auchus, 2006), on the opposite side of the heme prosthetic group (Fig. 1). The dye Alexa 488 was attached using a maleimide linker (Fig. 2). Alexa 488-T70C-b<sub>5</sub> was still capable of stimulating the lyase activity of P450 17A1, one of the P450 reactions most sensitive to b<sub>5</sub> stimulation (Kim et al., 2021). The fluorescence of Alexa 488-T70C-b<sub>5</sub> was attenuated upon binding P450 17A1, in a concentration-dependent manner (Kim et al., 2021). We extended the approach to other human P450s, several of which are known to be stimulated by the presence of b<sub>5</sub> (Yamazaki et al., 2002).

**Titrations of Human P450s.** Alexa 488-T70C-b<sub>5</sub> was titrated with increasing concentrations of P450 3A4 (Fig. 3), with the attenuation of fluorescence indicative of the binding between Alexa 488-T70C-b<sub>5</sub> and P450 3A4. As noted before with an acrylodan-labeled b<sub>5</sub> mutant and other hemoproteins (Stayton et al., 1988; Stayton et al., 1989), the fluorescence changes were more marked at lower ionic strength, indicative of charge-charge interactions. The decrease in the fluorescence was fit to a quadratic equation and yielded an apparent K<sub>d</sub> value of 13 nM for the affinity of Alexa 488-T70C-b<sub>5</sub> and P450 3A4 (Fig. 3 inset, Table 1). These are charge-charge interactions and the presence of phospholipid (L-α-dilauroyl-sn-glycero-3-phosphocholine) did not appreciably affect the titration results (Supporting Fig. S1).
The titration analysis of Alexa 488-T70C-b5 was extended to nine other human P450 enzymes (Supporting Fig. S2). P450s 1A2, 2B6, 2C9, 2D6, 2S1, and 4A11 showed attenuation of fluorescence, indicating tight binding between Alexa 488-T70C-b5 and P450 (Fig. 4). P450s 1A2, 2C9, 2E1, and 2S1 displayed very tight binding affinities with $K_d$ values of ~ 15 nM (Table 1). In particular, P450 2S1 showed a decrease in the $F_{480/513}$ amplitude as strong as that of P450 17A1 (Supporting Fig. S2, Table 1). However, P450 2A6 did not significantly decrease the fluorescence (Supporting Fig. S2, Fig. 4).

The effects of P450 3A4 substrates and inhibitor on the binding affinity of b5 were examined (Supporting Fig. S3). The titration spectra indicated similar fluorescence changes as in the absence of ligands, with somewhat increased $K_d$ values of 73, 84, and 68 nM in the presence of the substrates testosterone and midazolam and the inhibitor/substrate ketoconazole, respectively (Supporting Fig. S3), which are still indicative of tight binding. This result suggests that there is still tight binding of Alexa 488-T70C-b5 to P450 3A4 in the presence of substrate or inhibitor, or at least those that we used.

**Rate of Association of P450 3A4 and b5.** The rate of binding of Alexa 488-T70C-b5 to P450 3A4 was measured by observing the decrease of fluorescence upon mixing the two proteins using a stopped-flow fluorimeter. Fluorescence attenuation was observed with a first order $k_{obs}$ value of 0.22 (± 0.03) s$^{-1}$ with concentrations of 0.50 µM Alexa 488-T70C-b5 and 0.50 µM P450 3A4 (Fig. 5), similar to but somewhat slower than the rate measured for the binding of b5 and P450 17A1 (Kim et al., 2021).
Interaction of POR with a P450 3A4:b₅ Complex. Neither unlabeled b₅ nor POR (up to 2.65 µM) attenuated the fluorescence of Alexa 488-T70C-b₅ (50 nM), arguing against any inner filter effects. The attenuated fluorescence of Alexa 488-T70C-b₅ following binding of P450 3A4 (1:1 molar ratio) was partially restored by titration with POR (Fig. 6), as in the case of P450 17A1 (Kim et al., 2021). The P450 interaction with POR appeared to be competitive with Alexa 488-T70C-b₅, but the original fluorescence values were never reached, which is inconsistent with complete displacement. The calculated $K_d$ value of POR for the P450 3A4:b₅ complex was 0.089 µM, suggesting lower affinity than b₅ for P450 3A4 (0.013 µM) (Fig. 3, Table 1). The lack of a complete increase to the starting amplitude is not due to an inner filter effect, in that some P450s (e.g., 2A6, Supporting Fig. S2A) did not attenuate the fluorescence despite having more absorbance.

Demonstration of a P450 3A4-b₅-POR Ternary Complex using Gel Filtration. The fluorescence titration results (Fig. 6) suggested that P450 3A4, POR, and b₅ form a ternary complex. Accordingly, we tested this hypothesis further using a different approach, i.e. gel filtration (Fig. 7). Most of the P450 3A4 eluted as a single oligomeric peak on a Superose 12 column. b₅ eluted later, as might be expected, and POR eluted as a multimer near the void volume of the column (Fig. 7). (The identity of the second A$_{280}$ peak in the POR sample is unknown and is presumed to be a small molecule, in that no proteins were visualized upon SDS-gel electrophoresis and Coomassie Blue staining, Fig. 7).

A complex of P450 3A4 and b₅ yielded peaks in the regions for P450 3A4 and b₅ plus a larger complex eluting earlier, as verified with gel electrophoresis (Fig. 7). A mixture of POR, P450 3A4, and b₅ had most of the 280 nm-absorbing material (protein) in a large peak eluting
later than free POR but earlier than the P450 3A4-\(b_5\) complex, as validated by gel electrophoresis (Fig. 7C, 7D, fraction 9). The presence of all three proteins in the ternary complex peak fractions (Fig. 7D) is documented in the gel shown in Fig. 7C. Based on calibration with \(M_r\) standard proteins, the approximate \(M_r\) of the P450 3A4-\(b_5\) complex is 480 kDa and the approximate \(M_r\) of the P450 3A4-\(b_5\)-POR ternary complex is 690 kDa, indicating the presence of multimeric complexes (although the stoichiometry is unknown).
Discussion

A fluorescent derivative of \( b_5 \), Alexa 488-T70C-\( b_5 \), was used to characterize the binding of \( b_5 \) to a number of human liver microsomal P450s involved in drug metabolism. We found that several of these have high affinity for \( b_5 \), although none was apparently as strong as what we reported for (adrenal) P450 17A1 \((K_d \text{ 2.5 nM})\) (Kim et al., 2021). Some studies with P450 3A4, historically known to be enhanced by \( b_5 \) (at least for some activities), showed that ligands did not strongly affect \( b_5 \) affinity (Fig. S3). Also, the binding of P450 3A4 to \( b_5 \) and the dissociation of the complex occurred on a time scale of ~ 1 second (Fig. 5), similar to P450 17A1 (Kim et al., 2021). POR can interact with the complex and, as with P450 17A1 and \( b_5 \) (Kim et al., 2021), our evidence supports the existence of a ternary complex of P450 3A4, \( b_5 \), and POR (Figs. 6, 7).

The complexity of \( b_5 \) interactions with P450s can be traced back to the 1970s and 1980s (Peterson and Prough, 1986). As an example, the Yamano laboratory used a \( b_5 \)-affinity column to isolate a rabbit liver P450 termed B\( 1 \) (Miki et al., 1980), now recognized as CYP3A6 (Koop et al., 1981; Schwab and Johnson, 1987; Nelson et al., 1993). The purified protein showed an absolute requirement for \( b_5 \) in the \( O \)-demethylation of \( p \)-nitroanisole (Sugiyama et al., 1980) but not in reactions with the substrates benzphetamine, aminopyrine, and aniline (Miki et al., 1980). Koop et al. (Koop et al., 1981) also reported catalytic activity towards benzphetamine, aminopyrine, \( p \)-nitroanisole, \( p \)-nitrophenetole, testosterone, and androstenedione in the absence of \( b_5 \). It is of interest to note that this is another P450 Subfamily 3A member that was shown to bind tightly with \( b_5 \) (Miki et al., 1980), as was human P450 3A4 in our own work (Fig. 4, Table 1). The variability of \( b_5 \) dependence for rabbit P450 2B4 was studied by Gorsky and Coon (Gorsky and Coon, 1986) and shown to be highly sensitive to reconstitution conditions.
As in the case of our fluorescence studies done with P450 17A1 (Kim et al., 2021), all experiments with b5 were done in the absence of added phospholipids or detergents, so the forces involved in interaction of b5 with the P450s are presumed to be ionic. P450 3A4-b5 binding was not affected very much by the presence of a substrate (or inhibitor) (Fig. S3). We did not evaluate the effects of substrates with other P450s.

Our results can be compared with earlier efforts to study complexes, mainly with SPR measurements (Shimada et al., 2005; Yablokov et al., 2017). The $K_d$ values for the complexes were generally much higher for the SPR analyses, and no binding was detected for P450 1A2 (Shimada et al., 2005) or 2C9 (Yablokov et al., 2017). Because one component of an SPR system must be immobilized, SPR measurements are hampered by mass transfer artifacts (Johnson, 2019). Moreover, the sites of attachment of labels to the proteins are generally unknown.

In general, there is some correlation between b5 affinity and stimulation of catalytic activity (Table 1). However, there are some anomalies. For instance, P450 1A2 showed strong binding of b5 ($K_d$ 13 nM) but had not been shown to stimulate either phenacetin O-deethylation or 7-ethoxyresorufin O-deethylation activity by human P450 1A2 in reconstituted systems (Shimada et al., 2005). Kotrbová et al. (Kotrbová et al., 2011) reported that rabbit b5 changed the balance of the products of ellipticine oxidation by rabbit P450 1A2. Jeřábek et al. (Jeřábek et al., 2016) modeled the interactions. However, in other work b5 had no effect on rabbit P450 1A2-catalyzed aminopyrine N-demethylation and inhibited aniline 4-hydroxylation (Gorsky and Coon, 1986).

P450 2D6 showed weak binding here and we are unaware of any reports of stimulation of P450 2D6 activity by b5 in our own laboratory or others (Yamazaki et al., 2002), with the exception of in vivo work in mice by Henderson et al. (Henderson et al, 2015). However, Bart
and Scott (Bart and Scott, 2017) did find interaction of \(b_5\) and P450 2D6 using NMR spectroscopy, but this might be the result of the high concentrations of proteins used (> 100 µM).

P450 2S1 is an interesting case, in that \(b_5\) bound tightly but we did not observe an effect of \(b_5\) on catalytic activity in previous assays (Fekry et al., 2019). However, in those oxidative reactions (fatty acid \(\omega-1\) hydroxylations) the rates were very low (with or without \(b_5\)). In our experience, P450 2S1 has shown better catalytic activities in reductive reactions (Xiao et al., 2011; Wang and Guengerich, 2012; Wang and Guengerich, 2013), but we have not examined the effect of \(b_5\) on any of those.

P450 2A6 is also an anomaly. No interaction with \(b_5\) was detected in our titrations (Fig. 2A). Bart and Scott (Bart and Scott, 2017) detected interactions with NMR spectroscopy and identified the \(b_5\) residues Thr-60, Asp-65, His-58, Ser-69, Thr-70, and Arg-73 as being involved. It is conceivable that the presence of the fluorophore (Alexa 488) on residue 70 (originally Thr-70, changed to Cys-70) blocked \(b_5\) binding and that the fluorescence was not attenuated (but see Fig. 1). However, in that NMR study (Bart and Scott, 2017) the same \(b_5\) residues were implicated in the binding of P450 2E1 to \(b_5\), and we did observe quenching of the fluorescence with P450 2E1 (Fig. 4B). The role of \(b_5\) in catalytic activity of P450 2A6 also seems spurious. Both we (Yamazaki et al., 2002; Yun et al., 2005) and others (Soucek, 1999) have observed ~ 2-fold stimulation of coumarin 7-hydroxylation by \(b_5\) but Bart and Scott (Bart and Scott, 2017) did not. In our own work (Yun et al., 2005), coumarin 7-hydroxylation was stimulated by \(b_5\) but neither the 3- or 7-hydroxylation of 7-methoxycoumarin or 7-ethoxycoumarin was. Bart and Scott (Bart and Scott, 2017) did observe \(b_5\) stimulation of chlorzoxazone 6-hydroxylation (3.5-fold) and 4-nitrophenol 2-hydroxylation (1.5-fold) (using specificity constants \((k_{cat}/K_m)\) for comparisons). Another anomaly is that some electron transfer from reduced \(b_5\) to the P450 2A6 Fe\(^{2+}\)O\(_2\) complex
could be shown, but this electron transfer was not very efficient in coumarin 7-hydroxylation (Yun et al., 2005) and apo-\(b_5\) was almost as effective as (holo) \(b_5\) in supporting steady-state coumarin 7-hydroxylation (Yamazaki et al., 2002). At this time we can conclude that the interaction of P450 2A6 with Alexa 488-T70C-\(b_5\) is weak (Fig. 4A), which may be a particular feature of our system, and that the general evidence is that the \(b_5\) stimulation of P450 2A6 catalytic activities is not a strong one, at least compared to P450s 17A1, 3A4, and 2E1 (Soucek, 1999; Yamazaki et al., 2002; Yun et al., 2005; Bart and Scott, 2017).

Fluorescent derivatives of \(b_5\) have been made previously, in order to examine the interactions of P450 17A1 and \(b_5\) in cells (Storbeck et al., 2012; Simonov et al., 2015) and of \(b_5\) with bacterial P450\(_{cam}\) and other hemoproteins (myoglobin, cytochrome \(c\)) (Stayton et al., 1988; Stayton et al., 1989). We have not characterized the biophysical nature of the interactions of our derivative, Alexa 488-T70C-\(b_5\), with P450s. Inner filter artifacts can be ruled out and Förster resource energy transfer (FRET) interactions with the P450 heme are probably not relevant, in that a 12-mer peptide derived from P450 17A1 (putative binding region) could also attenuate the fluorescence (Kim et al., 2021), as could several small molecules. It is very possible that other \(b_5\) mutants and fluorophores may prove to be more useful probes, and we are evaluating some. However, the results with Alexa 488-T70C-\(b_5\) to date are useful in estimating the affinity of \(b_5\) for individual P450s (Fig. 4) and P450 variants (Kim et al., 2021).

Some caveats need to be considered. The modification of any residue of a protein, whether by mutagenesis or chemical modification, can alter the properties in ways that are unexpected and may not be indicative of the role(s) of that residue in normal function (Means and Feeney, 1971). In principle, the changes at Thr-70 of \(b_5\) (mutagenesis or conjugation) might alter its affinity for one or more P450s, and the order of affinities (Table 1) may not be
completely accurate. Our previous work with P450 17A1 showed that Alexa 488-T70C-b5 could be rapidly displaced, however, by excess unmodified b5 (Kim et al., 2021). The structure of human b5 (Fig.1) indicates that the dye should not be in a position to directly interact with the P450 (Glu-48, Glu-49), at least P450 17A1 and probably others. We conclude that, at the least, this work demonstrates the high affinity of b5 (or its derivative) for multiple human P450s.

In summary, we utilized a fluorescent derivative of b5 to show that it binds tightly to many human P450 enzymes, most of which have been shown to have b5-stimulated catalytic activity, with some exceptions. An important conclusion with P450 3A4 is that, as in the case of P450 17A1 (Kim et al., 2021), a ternary complex of P450, POR, and b5 is formed and is hypothesized to be important in catalysis, in contrast to a mechanism in which POR and b5 shuttle at a common site. A ternary complex rationalizes interactions of reactive oxidized forms of P450 with individual proteins (POR and b5), which would have to sequentially bind to and then vacate the P450 Fe2+, Fe2+O2, and Fe3+-O2− entities. At this point we cannot extend the ternary complex evidence to P450s other than 17A1 (Kim et al., 2021) and 3A4 (Figs. 6, 7), however.
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Authorship Contributions

Participating in research design: Guengerich, D. Kim

Conducted experiments: D. Kim, V. Kim, Tateishi

Contributed new reagents or analytical tools: D. Kim

Performed data analysis: D. Kim, V. Kim, Tateishi, Guengerich

Wrote or contributed to the writing of the manuscript: Guengerich, D. Kim
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Footnotes

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3Regarding previous attempts to estimate $K_d$ values for binding of $b_5$ and other P450s by optical spectroscopy (Bridges et al., 1998; Ahuja et al., 2013), no data were presented and the tabulated results show high error and inconsistency of an order of magnitude, demonstrating the difficulty of the approach.

The authors declare no financial conflicts of interest.
Legends for figures

**Fig. 1.** Structure of human b5. The solution structure was determined by NMR spectroscopy (Protein Data Bank 2I96). The positions of heme, the T70C mutation site, and two residues implicated in binding to P450 17A1 (E48, E 49) are indicated.

**Fig. 2.** Alexa 488 maleimide and site of attachment to Cys.

**Fig. 3.** Changes in fluorescence of Alexa 488-T70C-b5 with added concentrations of P450 3A4. Inset: plot of \( F_{480/513} \) data. See Table 1 for parameters.

**Fig. 4.** Titrations of Alexa 488-T70C-b5 with human P450 enzymes. The concentration of Alexa 488-T70C-b5 was 50 nM, in 1 mM potassium phosphate buffer (pH 7.4). (A) P450s 1A2, 2A6, 2B6, 2C8, 2C9. (B) P450s 2D6, 2E1, 2S1, 4A11, 3A4, 17A1. See Table 1 for \( K_d \) and amplitude values. The titration with P450 17A1 was from a previously reported study (Kim et al., 2021).

**Fig. 5.** Rate of binding of P450 3A4 and Alexa 488-T70C-b5. (A) Each syringe contained a 1.0 µM solution of P450 3A4 or Alexa 488-T70C-b5. The rate (first-order) was 0.22 ± 0.03 s\(^{-1}\). (B) Residuals trace from Part A.

**Fig. 6.** Fluorescence titration spectra of a 1:1 (molar) complex of Alexa 488-T70C-b5: P450 3A4 (50 nM) with increasing amounts of POR. The inset indicates the plot of \( F_{480/513} \) data. The apparent \( K_d \) was 0.089 ± 0.036 µM.
Fig. 7. Gel filtration analysis of complexes of P450 3A4, $b_5$, and POR. All analyses were done using a Superose 12 10/300 FPLC column. (A) $A_{280}$ profiles of individual proteins: POR (green), P450 3A4 (orange), $b_5$ (purple), a binary mixture of $b_5$ and P450 3A4 (blue), and a ternary mixture of all three proteins (red) are shown. Individual fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis, and densitometry was done of the Coomassie Blue-stained bands corresponding to the individual proteins. The POR preparation contained uncharacterized 280 nm-absorbing material eluting near the position of free $b_5$ but not showing any protein after electrophoresis and staining. (B) Densitometry traces of P450 3A4 (orange) and $b_5$ (purple) eluted in a binary equimolar mixture of the two proteins. The migration positions of the individual proteins ((P450) 3A4 and $b_5$) are indicated. (C) Coomassie Blue staining of the proteins in a ternary complex, as eluted from the column in Part A. The numbers on the left indicate $M_r$ values of markers relevant to the three proteins of interest, which have approximate $M_r$ values of 79 kDa (POR), 57 kDa (P450 3A4), and 17 kDa ($b_5$). (D) Densitometry traces of P450 3A4 (orange), POR (green), and $b_5$ (purple) eluted in a ternary equimolar mixture of the three proteins. The migration positions of proteins (POR, (P450) 3A4, and $b_5$) are indicated in Parts B and D.
Table 1. Binding affinities of human P450 enzymes to Alexa 488-T70C-\(b_5\) and reported effects of \(b_5\) on catalysis.

<table>
<thead>
<tr>
<th>P450</th>
<th>(K_d), (\mu)M</th>
<th>(\Delta\text{Amplitude}_{\text{max}}) (%)</th>
<th>Effect of (b_5) on activity(^b)</th>
<th>Evidence for (b_5) electron transfer to P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>0.013 ± 0.003</td>
<td>-67</td>
<td>0 to -36%</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>2A6</td>
<td>ND(^a)</td>
<td>-11</td>
<td>+50 to +100%</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>2B6</td>
<td>0.061 ± 0.015</td>
<td>-53</td>
<td>+25 to +75%</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>2C8</td>
<td>0.15 ± 0.03</td>
<td>-55</td>
<td>+34 to +55%</td>
<td>(\pm)(^b)</td>
</tr>
<tr>
<td>2C9</td>
<td>0.015 ± 0.004</td>
<td>-53</td>
<td>0 to +50%</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>2D6</td>
<td>ND(^a)</td>
<td>ND(^a)</td>
<td>-11 to +2%</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>2E1</td>
<td>0.015 ± 0.008</td>
<td>-32</td>
<td>+153 to +160%</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>2S1</td>
<td>0.014 ± 0.003</td>
<td>-80</td>
<td>0(^c)</td>
<td>(_)(^b)</td>
</tr>
<tr>
<td>4A11</td>
<td>0.043 ± 0.011</td>
<td>-51</td>
<td>+100%(^d)</td>
<td>(_)(^d)</td>
</tr>
<tr>
<td>3A4</td>
<td>0.013 ± 0.002</td>
<td>-61</td>
<td>+25 to +80%</td>
<td>(_)(^b),(^e)</td>
</tr>
<tr>
<td>17A1</td>
<td>0.0025 ± 0.0006</td>
<td>-70</td>
<td>(\geq) +1,000% (lyase)(^f)</td>
<td>(_)(^g)</td>
</tr>
</tbody>
</table>

\(^a\)Not determined. See Fig. S1A.

\(^b\)(Yamazaki et al., 2002)

\(^c\)(Fekry et al., 2019)
(Kim et al., 2014)

(Yamazaki et al., 1996a; Yamazaki et al., 2001; Yamazaki et al., 2002)

(Gonzalez and Guengerich, 2017). Reported for lyase reaction with 17α-OH progesterone or 17α-OH pregnenolone. The 17-hydroxylation reactions show only slight stimulation (Kim et al., 2021).

(Auchus et al., 1998; Lee-Robichaud et al., 1998; Guengerich et al., 2019)
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(A) Fluorescence

(B) Residuals

Time, sec
Figure 6

Emission wavelength (nm)

Fluorescence

(Alexa b₅ only)

(Alexa b₅: P450 3A4)
Figure 7
SUPPLEMENTAL FILE

Cytochrome \( b_5 \) Binds Tightly to Several Human Cytochrome P450 Enzymes

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DRUG METABOLISM AND DISPOSITION

DMD-AR-2021-000475

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Figure S3. Titrations of Alexa 488-T70C-\( b_5 \) with P450 3A4 in the presence of substrate and inhibitor.

Figure S4. Calibration for estimation of molecular weights of complexes.
Figure S1. Titrations of Alexa 488-T70C-b5 with P450 17A1 and P450 3A4 in the absence and presence of phospholipid. Titrations were done with 50 nM Alexa 488-T70C-b5 as in Figs. 1 and 2 in the absence (●, filled blue circles) and presence (■, filled red squares) of 30 µM L-α-dilauroyl-sn-glycero-3-phosphocholine (DLPC). (A) P450 17A1 titration without lipid, (B) P450 17A1 titration with DLPC, (C) Plots of F513 from Parts A (●) and B (■); (D) P450 3A4 titration without lipid, (E) P450 17A1 titration with DLPC, (F) Plots of F513 from Parts D (●) and E (■).
Figure S2. Fluorescence titration spectra of Alexa 488-T70C-b<sub>5</sub> with human P450 enzymes. 
(A) P450s 1A2, 2A6, 2B6, 2C8, 2C9. (B) P450s 2D6, 2E1, 2S1, 4A11, 3A4, 17A1. The concentration of Alexa488-T70C-b<sub>5</sub> was 50 nM.
Figure S3. Titrations of Alexa 488-T70C-b5 with P450 3A4 in the presence of substrates and an inhibitor. Binding titrations were carried out using purified P450 3A4 enzyme including (A) 50 μM testosterone, (B) 100 μM midazolam, or (C) 10 μM ketoconazole. Emission spectra (scanning 500-630 nm, with an excitation wavelength at 480 nm) were recorded after subsequent additions of P450 3A4. The inset is a plot of the F₄₈₀/F₅₁₃ data. The calculated Kₐ values were 73 ±
18, 84 ± 24, and 79 ± 18 nM in presence of testosterone, midazolam, and ketoconazole, respectively.

Fig. S4

Figure S4. Calibration for estimation of molecular weights of complexes. (A) Chromatograms of Cytiva Mr standards: ovalbumin, 43 kDa; convalbumin, 75 kDa; aldolase, 158 kDa; ferritin, 440 kDa; blue dextran, ≥ 2,000 kDa. (B) Calibration plot. The estimated Mr values for the P450 3A4:b5 and P450 3A4:POR:b5 complexes were 480 and 690 kDa, respectively.