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Cytochrome b_5 Binds Tightly to Several Human Cytochrome P450 Enzymes[S]

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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) 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 used 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 b_5 (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-b_5 reductase and b_5 and augment electron flow (Hildebrandt and Estabrook, 1971; Correia and Mannering, 1973). However, purification and reconstitution experiments indicated that b_5 was not an obligatory component of P450 systems (Lu and Coon, 1968). Further research in multiple laboratories showed that the addition of b_5 to reconstituted P450 systems could either stimulate, inhibit, or have no effect (Gorsky and Coon, 1986). Evidence that b_5 was not only an effector in reconstituted systems but also important in the endoplasmic reticulum came from immunochemical experiments with antib5 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 (1986).

A number of proposals have been addressed to explain the effect of b_5. One is electron transfer (Bhatt et al., 2017). 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 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-b_5 reductase, 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 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, coexpression 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

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ABBREVIATIONS: b_5, cytochrome b_5 (CYB5A); K_d, dissociation constant; P450, cytochrome P450; POR, NADPH-cytochrome P450 reductase; M_r, molecular mass; SPR, surface plasmon resonance.

b_5 interactions with several P450s using site-directed mutagenesis and chemical crosslinking studies (Gao et al., 2006; Peng et al., 2014; Bridges et al., 1999), 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. Regarding 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.

NMR measurements (Ahuja et al., 2013; Estrada et al., 2013) are problematic in that high (>100 μM) concentrations of the proteins are needed, and estimation of what might be sub-μM 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 used 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),

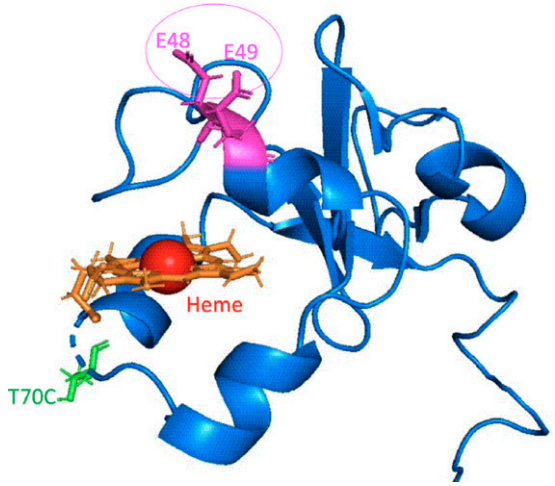

Fig. 1. Structure of human b_5. The solution structure was determined by NMR spectroscopy (Protein Data Bank 2I96). The positions of heme, the T70C mutation site, and two residues implicated in binding to P450 17A1 (E48, E49) are indicated.

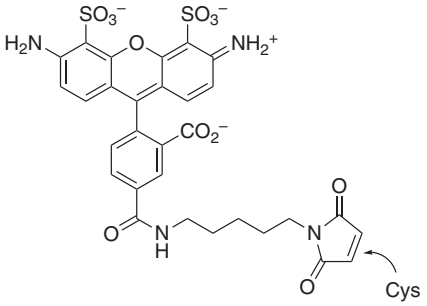

Fig. 2. Alexa 488 maleimide and site of attachment to Cys.

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 $\Delta\varepsilon_{450\text{-}490}$ = 91,000 M^{-1} cm^{-1} for the Fe^{2+}-CO versus Fe^{2+} difference spectra (Omura and Sato, 1964). The concentration of b_5 was estimated using the extinction coefficient ε_{423}= 100,000 M^{-1} cm^{-1} (Spatz and Strittmatter, 1971) or the difference extinction coefficient $\Delta\varepsilon_{424\text{-}409}$ = 180,000 M^{-1} cm^{-1} for the Fe^{2+} versus Fe^{3+} difference spectra (Velick and Strittmatter, 1956). The concentration of POR was estimated using the extinction coefficient ε_{455} = 23,600 M^{-1} 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 spectrofluorometer (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,

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

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)*(1/E)*((Kd + E + X)-sqrt((Kd + 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 S.D., 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 mass (M_r) of protein complexes were estimated using fitting to a curve developed by plotting $\log_{10} M_r$ versus corrected elution volume (corrected for void volume based on elution volume of blue dextran), using chicken ovalbumin (43 kDa), chicken conalbumin (75 kDa), 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_5 mutant (T70C) with a dye based on a previous approach used to monitor the binding of bacterial $P450_{cam}$ and rat b_5 (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_5 was still capable of stimulating the lyase activity of P450 17A1, one of the P450 reactions most sensitive to b_5 stimulation (Kim et al., 2021). The fluorescence of Alexa 488-T70C-b_5 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_5 (Yamazaki et al., 2002).

Titrations of Human P450s. Alexa 488-T70C-b_5 was titrated with increasing concentrations of P450 3A4 (Fig. 3), with the attenuation of fluorescence indicative of the binding between Alexa 488-T70C-b_5 and P450 3A4. As noted before with an acrylodan-labeled b_5 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_d value of 13 nM for the affinity of Alexa 488-T70C-b_5 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 (Supplemental Fig. 1).

The titration analysis of Alexa 488-T70C-b_5 was extended to nine other human P450 enzymes (Supplemental Fig. 2). P450s 1A2, 2B6, 2C9, 2D6, 2S1, and 4A11 showed attenuation of fluorescence, indicating tight binding between Alexa 488-T70C-b_5 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 (Supplemental Fig. 2, Table 1). However, P450 2A6 did not significantly decrease the fluorescence (Supplemental Fig. 2, Fig. 4).

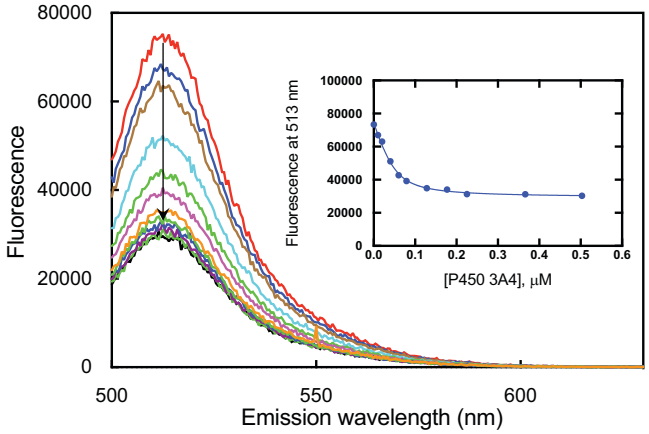

Fig. 3. Changes in fluorescence of Alexa 488-T70C-b_5 with added concentrations of P450 3A4. Inset: plot of $F_{480/513}$ data. See Table 1 for parameters.

The effects of P450 3A4 substrates and inhibitor on the binding affinity of b_5 were examined (Supplemental Fig. 3). 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 (Supplemental Fig. 3), which are still indicative of tight binding. This result suggests that there is still tight binding of Alexa 488-T70C-b_5 to P450 3A4 in the presence of substrate or inhibitor, or at least those that we used.

Rate of Association of P450 3A4 and b_5. The rate of binding of Alexa 488-T70C-b_5 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-b_5 and 0.50 μM P450 3A4 (Fig. 5), similar to but somewhat slower than the rate measured for the binding of b_5 and P450 17A1 (Kim et al., 2021).

TABLE 1

Binding affinities of human P450 enzymes to Alexa 488-T70C-b_5 and reported effects of b_5 on catalysis

P450	Alexa 488-T70C-b_5 Titration		Effect of b_5 on Activity[b]	Evidence for b_5 Electron Transfer to P450
	K_d	$\Delta Amplitude_{max}$		
	μM	%		
1A2	0.013 ± 0.003	−67	0 to −36%	–[b]
2A6	ND[a]	−11	+50 to +100%	–[b]
2B6	0.061 ± 0.015	−53	+25 to +75%	–[b]
2C8	0.15 ± 0.03	−55	+34 to +55%	±[b]
2C9	0.015 ± 0.004	−53	0 to +50%	–[b]
2D6	ND[a]	ND[a]	−11 to +2%	–[b]
2E1	0.015 ± 0.008	−32	+153 to +160%	+[b]
2S1	0.014 ± 0.003	−80	0[c]	
4A11	0.043 ± 0.011	−51	+100%[d]	+[d]
3A4	0.013 ± 0.002	−61	+25 to +80%	–[b, e]
17A1	0.0025 ± 0.0006	−70	≥ +1000% (lyase)[f]	–[g]

[a]Not determined. See Supplemental Fig. 1.
[b](Yamazaki et al., 2002).
[c](Fekry et al., 2019).
[d](Kim et al., 2014).
[e](Yamazaki et al., 1996a; Yamazaki et al., 2001; Yamazaki et al., 2002).
[f](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).
[g](Auchus et al., 1998; Lee-Robichaud et al., 1998; Guengerich et al., 2019).

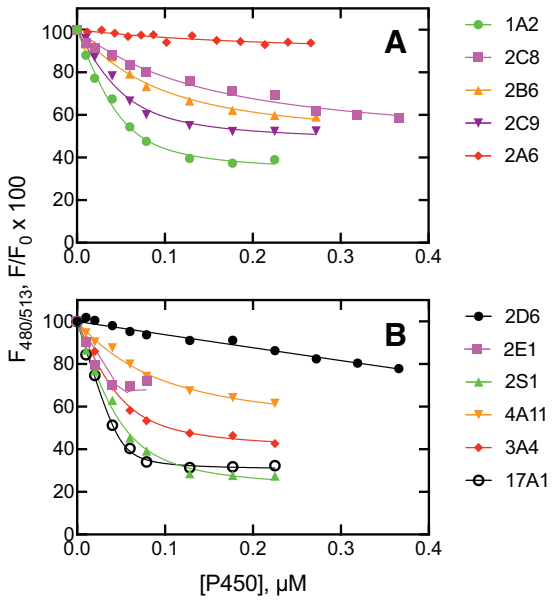

Fig. 4. Titrations of Alexa 488-T70C-b_5 with human P450 enzymes. The concentration of Alexa 488-T70C-b_5 was 50 nM, in 1 mM potassium phosphate buffer (pH 7.4). (A) P450s 1A2, 2A6, 2B6, 2C8, 2C9. (B) P450s 2D6, 2E1, 2S1, 4A11, 3A4, 17A1. See Table 1 for K_d and amplitude values. The titration with P450 17A1 was from a previously reported study (Kim et al., 2021).

Interaction of POR with a P450 3A4:b_5 Complex. Neither unlabeled b_5 nor POR (up to 2.65 μM) attenuated the fluorescence of Alexa 488-T70C-b_5 (50 nM), arguing against any inner filter effects. The attenuated fluorescence of Alexa 488-T70C-b_5 after 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-b_5, 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:b_5 complex was 0.089 μM, suggesting lower affinity than b_5 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, Supplemental Fig. 2A) did not attenuate the fluorescence despite having more absorbance.

Demonstration of a P450 3A4-b_5-POR Ternary Complex Using Gel Filtration. The fluorescence titration results (Fig. 6) suggested that P450 3A4, POR, and b_5 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. b_5 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 b_5 yielded peaks in the regions for P450 3A4 and b_5 plus a larger complex eluting earlier, as verified with gel electrophoresis (Fig. 7). A mixture of POR, P450 3A4, and b_5 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. 7, C and D, 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 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

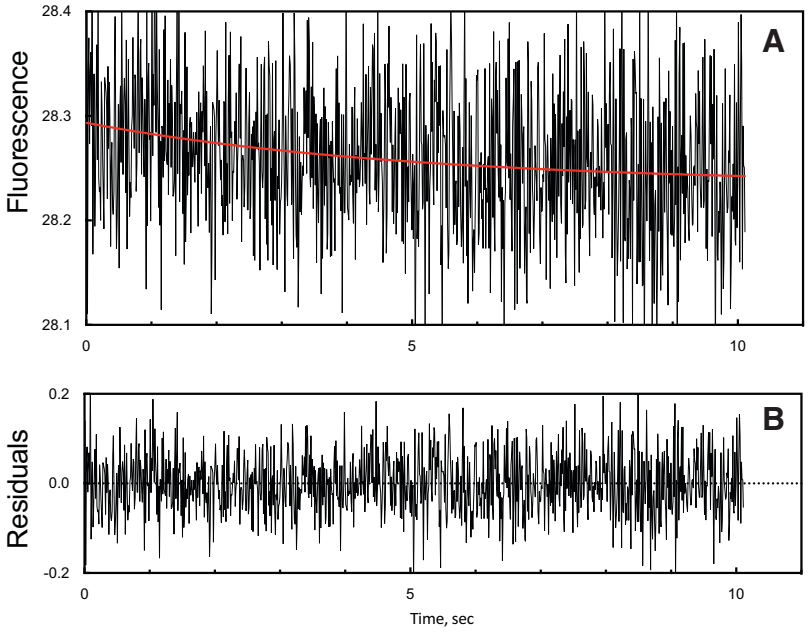

Fig. 5. Rate of binding of P450 3A4 and Alexa 488-T70C-b_5. (A) Each syringe contained a 1.0 μM solution of P450 3A4 or Alexa 488-T70C-b_5. The rate (first order) was 0.22 ± 0.03 second^{-1}. (B) Residuals trace from Part A.

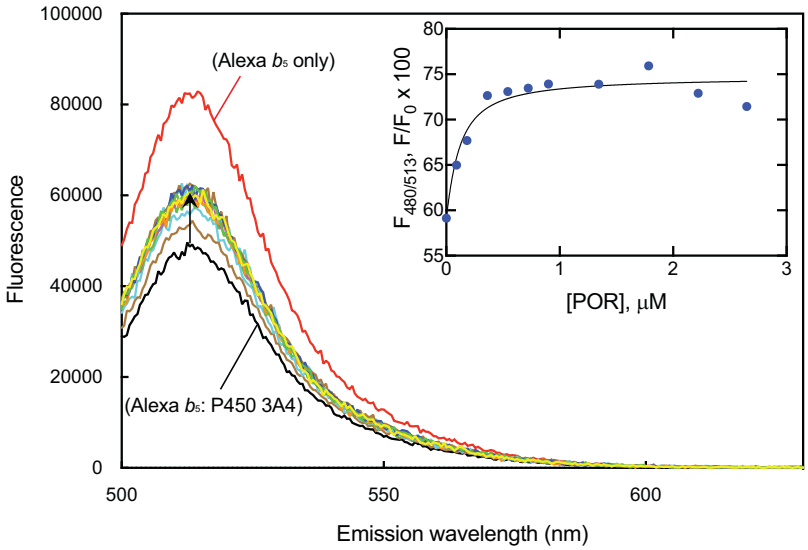

Fig. 6. Fluorescence titration spectra of a 1:1 (molar) complex of Alexa 488-T70C-b_5: P450 3A4 (50 nM) with increasing amounts of POR. The inset indicates the plot of $F_{480/513}$ data. The apparent K_d was 0.089 ± 0.036 μM.

affect b_5 affinity (Supplemental Fig. 3). 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 and 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. (1981) also reported catalytic activity toward 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, (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 b_5 were done in the absence of added phospholipids or detergents, so the forces involved in interaction of b_5 with the P450s are presumed to be ionic. P450 3A4-b_5 binding was not affected very much by the presence of a substrate (or inhibitor) (Supplemental Fig. 3). 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 b_5 affinity and stimulation of catalytic activity (Table 1). However, there are some anomalies. For instance, P450 1A2 showed strong binding of b_5 (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. (2011) reported that rabbit b_5 changed the balance of the products of ellipticine oxidation by rabbit P450 1A2. Jeřábek et al. (2016) modeled the interactions. However, in other work, b_5 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 b_5 in our own laboratory or others (Yamazaki et al., 2002), with the exception of in vivo work in mice by Henderson et al., (2015). However, 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 ω-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 (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 (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-

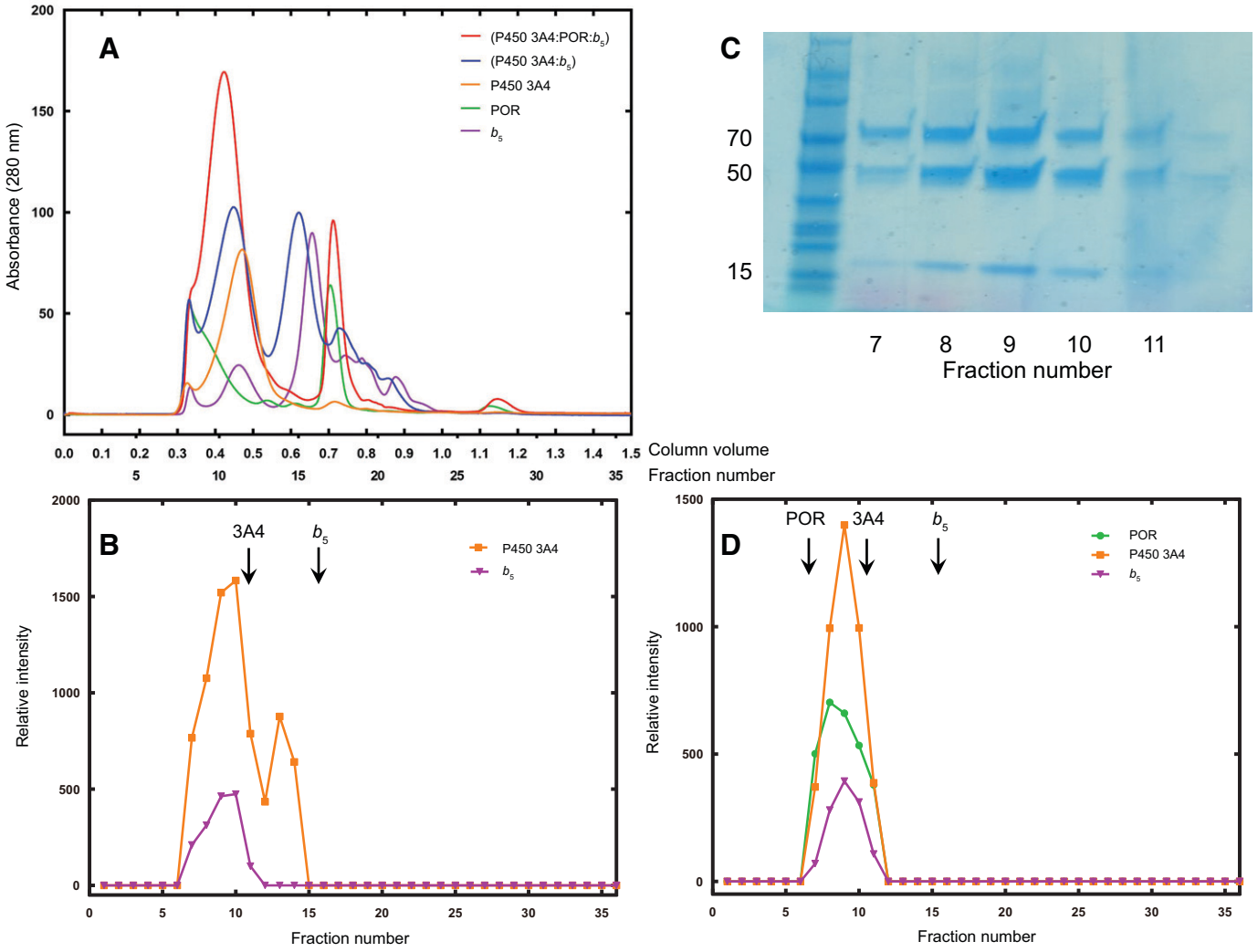

Fig. 7. Gel filtration analysis of complexes of P450 3A4, b_5, and POR. All analyses were done using a Superose 12 10/300 fast protein liquid chromatography (FPLC) column. (A) A_{280} profiles of individual proteins: POR (green), P450 3A4 (orange), b_5 (purple), a binary mixture of b_5 and P450 3A4 (blue), 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 (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 (B) and (D).

methoxycoumarin or 7-ethoxycoumarin was. 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 with 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, 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 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-b_5 could be rapidly displaced, however, by excess unmodified b_5 (Kim et al., 2021). The structure of human b_5 (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 b_5 (or its derivative) for multiple human P450s.

In summary, we used a fluorescent derivative of b_5 to show that it binds tightly to many human P450 enzymes, most of which have been shown to have b_5-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 b_5 is formed and is hypothesized to be important in catalysis, in contrast to a mechanism in which POR and b_5 shuttle at a common site. A ternary complex rationalizes interactions of reactive oxidized forms of P450 with individual proteins (POR and b_5), which would have to sequentially bind to and then vacate the P450 Fe^{2+}, $Fe^{2+}O_2$, and Fe^{3+}-O_2^- entities. At this point, we cannot extend the ternary complex evidence to P450s other than 17A1 (Kim et al., 2021) and 3A4 (Figs. 6 and 7), however.

Authorship Contributions

Participated in research design: D. Kim, Guengerich.
Conducted experiments: D. Kim, V. Kim, Tateishi.
Contributed new reagents or analytic tools: D. Kim.
Performed data analysis: D. Kim, V. Kim, Tateishi, Guengerich.
Wrote or contributed to the writing of the manuscript: D. Kim, Guengerich.

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