Structural and functional effects of cytochrome $b_5$ interactions with human cytochrome P450 enzymes

The small heme-containing protein cytochrome $b_5$ can facilitate, inhibit, or have no effect on cytochrome P450 catalysis, often in a P450-dependent and substrate-dependent manner that is not well understood. Herein, solution NMR was used to identify $b_5$ residues interacting with different human drug-metabolizing P450 enzymes. NMR results revealed that P450 enzymes bound to either $b_5$-ae-5 (CYP2A6 and CYP2E1) or this region and a2-3 (CYP2D6 and CYP3A4) and suggested variation in the affinity for $b_5$. Mutations of key $b_5$ residues suggest not only that different $b_5$ surfaces are responsible for binding different P450 enzymes, but that these different complexes are relevant to the observed effects on P450 catalysis.

Cytochrome P450 (P450) monooxygenases have vital roles both in the metabolism of xenobiotics, including drugs, and in the biosynthesis of endogenous compounds, such as steroids, vitamins, fatty acids, and eicosanoids. Although the ability of P450 enzymes to oxidize such substrates requires interaction with a redox partner protein, catalysis can additionally be modulated by interactions with the membrane-bound heme protein cytochrome $b_5$ ($b_5$). Cytochrome $b_5$ has a complex influence, such that P450 catalysis can be stimulated, not affected, or even inhibited, prompting numerous investigations into the mechanism(s) by which these varied responses are elicited (1, 2). Proposals have largely focused on $b_5$ functioning 1) in a purely redox role of electron delivery or 2) as an allosteric modulator of P450 conformation. Metal-substituted, redox-silent forms of $b_5$ (Fig. 1A) transiently interacting with the concave, largely positively charged, proximal face of P450 enzymes (Fig. 1B). This proximal P450 face is the same surface to which the required redox partners bind (NADPH-cytochrome P450 oxidoreductase (CPR) for microsomal P450 enzymes), underscoring the necessity of transient interactions between P450 and its protein partners (3, 8).

Regardless of the mechanism, the capability of $b_5$ to modulate P450 catalysis relies on direct binding between $b_5$ and a P450. Insights into individual P450/$b_5$ complexes gained from mutagenesis (3, 4), chemical modification (5), and cross-linking studies (6, 7) are consistent with the convex, negatively charged, heme-exposed face of $b_5$ (Fig. 1A) transiently interacting with the concave, largely positively charged, proximal face of P450 enzymes (Fig. 1B). This proximal P450 face is the same surface to which the required redox partners bind (NADPH-cytochrome P450 oxidoreductase (CPR) for microsomal P450 enzymes), underscoring the necessity of transient interactions between P450 and its protein partners (3, 8).

Fundamental questions persist with regard to P450/$b_5$ interactions. How variable is the binding interface between $b_5$ and different P450 enzymes? Do the affinities vary? Could differences in the $b_5$/P450 interaction underlie the different effects $b_5$ has on P450 catalysis? To complicate matters, the effects that $b_5$ has on catalysis can depend not only on the P450 enzyme, but also the P450 substrate being metabolized (9–11) and experimental parameters, including the ratio of P450 to $b_5$ (10). Differences in $b_5$ effects with different substrates have fueled interest in whether conformational linking exists between the P450 active site cavity and the P450 proximal face interacting with $b_5$ and CPR (8, 12, 13).

Because there are no X-ray structures available of $b_5$ interacting with any P450, solution NMR is an attractive high-resolution approach to decoding these transient binding interactions. Previous studies of $^{15}$N-labeled $b_5$ binding to increasing concentrations of unlabeled human steroidogenic CYP17A1 enzyme or rabbit CYP2B4 revealed only small chemical shift perturbations, but more pronounced decreases in the intensity of $b_5$ resonances (8, 14). These line-broadening effects may result from a number of potential sources. Binding of the smaller ~16-kDa $b_5$ to a larger P450 enzyme (~50–55 kDa) would enhance the transverse relaxation rate, but could also result in paramagnetic effects from the P450 heme and/or alter the protein-protein interaction to fall within the intermediate-exchange time scale (8, 14). Thus, whereas progressive line broadening occurs upon formation of increasing amounts of the reversible P450/$b_5$ complex, there is not necessarily a 1:1 correspondence between signal loss and amount of complex formed, which prevents the determination of meaningful $K_d$ values. Regardless, the degree of broadening has varied between these two different P450 enzymes, the P450 ligands present, and experimental conditions, such as the presence of lipid

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2 The abbreviations used are: P450, cytochrome P450; CPR, NADPH-cytochrome P450 oxidoreductase; CYP, cytochrome P450; $b_5$, cytochrome $b_5$; CZN, chlorozoxazone; pNP, para-nitrophenol; DXM, dextromethorphan; NFP, nifedipine; Ni-NTA, nickel-nitrilotriacetic acid; HSQC, heteronuclear single quantum coherence; PDB, Protein Data Bank.
provides a physical basis to begin understanding the ability of the complex of the two proteins when P450 substrate is present (5). In this study, classical substrates with a single or at least one major metabolite were preferred, as this might be expected to simplify interaction modes by promoting a more homogeneous P450 conformation as well as permitting quantification of a single product in catalytic assays. Thus, CYP2E1 was saturated with the muscle relaxant chlorzoxazone (denoted as CYP2E1 (CZN)), CYP3A4 with the hypotensive drug nifedipine (denoted as CYP3A4(NFP)), CYP2D6 with the cough suppressant dextromethorphan (denoted as CYP2D6(DXM)), and CYP2A6 with coumarin and p-nitrophenol (denoted as CYP2A6(pNP)). Of these substrates, the addition of coumarin, chlorzoxazone, and dextromethorphan to CYP2A6, CYP2E1, and CYP2D6, respectively, yielded shifts in the difference spectra indicating a transition from low spin to high spin, facilitating the ability to monitor protein saturation. The intrinsic absorbance of nifedipine and p-nitrophenol impaired such observations for CYP3A4 and CYP2A6, respectively. Under these conditions, after collection of NMR spectra none of the samples resulted in visible precipitation, and the reduced-carbon monoxide difference spectra were unchanged from the freshly purified P450 protein.

Whereas NMR-observed titrations necessarily involved various concentrations of P450, other experiments were designed to reflect ratios of P450:CPR:b5 often used in P450 functional assays in the literature. This is important, as thoroughly demonstrated in studies on the rabbit CYP2B4 by Waskell and coworkers (10). Herein, all catalytic assays used 1:2:2 ratios of P450 to CPR to b5, rather than optimizing these ratios for maximal turnover with individual P450/substrate combinations as is frequently done. In NMR experiments, this same ratio of P450 to b5 was used to evaluate the b5 mutants (0.5:1 P450:b5, which is the same as 1:2 in the assays). To ensure that these ratios are accurate, special precautions were taken to avoid frequent problems with the quality and quantification of reductase and b5. Cytochrome b5 was reconstituted with heme during purification to avoid large amounts of apo-b5 that can otherwise occur when polypeptide production exceeds heme production and that could also possibly bind P450. Reductase was purified to remove as much as possible of the proteolyzed form that can reduce cytochrome c and might be able to bind P450 but is not able to reduce P450 and promote catalysis. Thus, reductase was quantitated by flavin content instead of cytochrome c reduction. Reductase was also quantitated by total protein from the bicinchoninic acid assay using bovine serum albumin as a standard and was no more than 30% higher than quantitation by flavin absorbance. Both b5 and reductase were the human forms to match the human P450 enzymes, rather than the rat versions often employed. Finally, because electrostatics are probably involved in both steering and binding between b5 and P450 enzymes, to provide additional consistency, the same buffer was used in NMR experiments and all catalytic assays, with the exception of the CYP3A4 nifedipine...
metabolism assay, which required slightly different conditions (see below).

Finally, although the effects of \( b_5 \) on P450 catalysis are often simply evaluated by monitoring differences in metabolite formation in the presence and absence of \( b_5 \), \( b_5 \) may also have other effects not readily detected by this approach. It has been reported that \( b_5 \) can change the amount of NADPH consumed per amount of product formation (percent coupling) (1), which would not necessarily alter the amount of product formed. For this reason, NADPH consumption and its coupling to product formation were also evaluated in the absence and presence of wild-type and mutant \( b_5 \) proteins.

**Interaction and catalytic effects of \( b_5 \) on CYP2A6**

When the 2D HSQC spectrum of \(^{15}\text{N}\)-labeled \( b_5 \) alone is collected (e.g. see Fig. 1C (black)), the intensity of each resonance is by definition 100%. As reported previously for CYP17A1 and CYP2B4, titrations of \(^{15}\text{N}\)labeled \( b_5 \) with increasing concentrations of 2A6(coumarin) result in few chemical shifts, but line broadening occurs and the intensity of most resonances decreases (Fig. 1C), with more marked reductions for specific residues (Fig. 1D). Because the dominant features are changes in intensity and because such overlaid spectra are difficult to comprehend visually, each resonance in each series of spectra was analyzed, and the percentage of intensity remaining was plotted graphically (e.g. Fig. 2A).

For CYP2A6(coumarin), reductions in the average \( b_5 \) signal intensity occur even at very low P450: \( b_5 \) ratios. At a CYP2A6(coumarin): \( b_5 \) ratio of 0.1:1, an average of ~68% of the original signal remained. As expected with increasing formation of the complex, the average intensities continued to decrease as more and more CYP2A6(coumarin) was added. At 0.2:1, on average, ~41% of the original signal intensity remained (Fig. 2A, black dashed line). At 0.3:1, 0.4:1, and 0.5:1, ~35%, 26%, and 22% of the original signal remained.

At titration points with ~40–60% reduction in intensity, there is enough P450: \( b_5 \) complex formed to clearly identify specific \( b_5 \) amino acids whose resonances are differentially broadened compared with the average. For CYP2A6(coumarin), this corresponds to the 0.2:1 ratio, which reveals that a number of \( b_5 \) resonances were broadened more than one S.D. value (Fig. 2A, red dashed line) from the average (Fig. 2A, black dashed line), consistent with the involvement of these \( b_5 \) residues in binding CYP2A6(coumarin). These \( b_5 \) resonances correspond to residues Thr-60, Asp-65, His-68, Ser-69, Thr-70, and Arg-73, which cluster on \( b_5 \) helices 4 and 5 and the loop connecting them (Fig. 2B, red).

To further examine the role of specific \( b_5 \) residues interacting with CYP2A6(coumarin), a series of mutations were examined. Residues Asp-65 and Glu-49 were selected based on 1) their location on the \( b_5 \) surface, 2) previous evidence that electrostatic pairing is important in \( b_5 \)/P450 complex formation (4, 8), 3) locations on distinct \( b_5 \) faces (Fig. 1A), and 4) their location in or near binding interfaces identified herein (see below). Asp-65 is located in \( b_5 \) α4, whereas Glu-49 is in \( b_5 \) α3. The effects of the charge-neutralizing E49Q and D65N mutations on P450 interactions with \( b_5 \) were evaluated at a P450: \( b_5 \) ratio of 0.5:1 for all P450 enzymes. As indicated above, under these conditions with wild-type \( b_5 \), the intensity for the average \( b_5 \) resonance decreases to ~22% of the original signal (Fig. 2C, left). However, when the \( b_5 \) mutant D65N was mixed with CYP2A6(coumarin) at the same ratio, the overall line broadening was much less, to only 76% of the original (Fig. 2C, middle), consistent with decreased complex formation between \( b_5 \) D65N and CYP2A6 (coumarin) compared with wild-type \( b_5 \). Conversely, mixing the \( b_5 \) mutant E49Q with CYP2A6(coumarin) yielded average line broadening (to ~25%; Fig. 2C, right) similar to wild-type \( b_5 \) (22%; Fig. 2C, left), suggesting that this mutation did not adversely affect formation of the \( b_5 \)/CYP2A6(coumarin) complex. These results suggest that whereas the anionic charge on Asp-65 in \( b_5 \) helix 4 has a significant role in \( b_5 \) binding to CYP2A6(coumarin), Glu-49 in \( b_5 \) helix 3 has little to no effect on complex formation.

To relate these structural observations to enzymatic function, coumarin metabolism assays were subsequently employed. Under our conditions, there was no significant effect of \( b_5 \) on CYP2A6 7-hydroxycoumarin formation. Neither \( k_{\text{cat}} \) nor \( K_{\text{m}} \) was substantially altered in the presence of \( b_5 \) (Fig. 2D). Thus, it was not surprising that neither the D65N nor E49Q \( b_5 \) mutants altered coumarin metabolism (Fig. 2D).

Although the presence of wild-type \( b_5 \) did not alter formation of the 7-hydroxycoumarin product, coupling studies revealed that NADPH consumption was reduced by ~60% (Fig. 2E, red). Thus, wild-type \( b_5 \) increased productive coupling of the reaction by ~1.9-fold (Fig. 2F, red). The \( b_5 \) D65N mutant, which appeared to decrease \( b_5 \) binding to CYP2A6(coumarin) in the NMR experiments, yielded a much smaller decrease in NADPH consumption (Fig. 2E, green) and had coupling similar to that when \( b_5 \) was not present (Fig. 2F, green versus black). The E49Q \( b_5 \), which did not appear to alter \( b_5 \) binding to CYP2A6 (coumarin) in the NMR experiments, functioned more like wild-type \( b_5 \), with a similar NADPH consumption (Fig. 2E, blue versus red) and productive coupling (Fig. 2F, blue versus red). Overall, wild-type and E49Q \( b_5 \) acted similarly, with 1.9- and 1.6-fold increases in coupling, respectively, whereas D65N has little effect on coupling (a 1.2-fold increase in coupling over reactions without \( b_5 \)).

 Whereas the effects of \( b_5 \) on NADPH consumption observed herein are consistent with the ~50% reduction previously reported for CYP2A6 coumarin 7-hydroxylation (22, 24), there have also been reports that \( b_5 \) can increase product formation in the range of 1.5–2.5-fold (19, 22–24). However, no such increase in 7-hydroxycoumarin was observed herein. This apparent discrepancy could be due to other differences in experimental conditions. Of particular importance are the protein ratios used for P450:CPR: \( b_5 \) in catalytic assays. Experimental evidence to date strongly supports mutually exclusive, overlapping binding sites for CPR and \( b_5 \) on the proximal face of P450 enzymes. As a result, the ratio and relative affinities of reductase and \( b_5 \) for a particular P450 would dictate the observed effects (1). At high relative ratios and/or affinities, \( b_5 \) could inhibit required reduction of ferric P450 by reductase, whereas at lower relative ratios and/or affinities, \( b_5 \) may exert stimulatory effects on other steps of the P450 catalytic cycle to increase coupling (13). That \( b_5 \) significantly inhibits NADPH consumption without changes in product formation suggests
that for this enzyme(substrate) combination, the 1:2:2 ratio may balance the inhibitory and stimulatory effects of \(b_5\). However, other significant differences, for example in the species and quantitation of \(b_5\) and reductase, the presence of lipid, etc., may also contribute to different observations across the literature with respect to \(b_5\) effects on product formation.

Regardless, NMR and functional assays herein consistently suggest that D65N in \(b_5\) helix 4 is important in both binding to CYP2A6(coumarin) and in improving coupling of NADPH consumption to product formation. More broadly, the NMR data suggest that \(b_5\) surface residues in both helix 4 and helix 5 are involved in binding to CYP2A6(coumarin).
Figure 2. Interaction of CYP2A6(coumarin) with [15N]b5 by NMR and b5 modulation of CYP2A6 catalysis. A, a single NMR spectrum of 0.2:1 CYP2A6(coumarin):[15N]b5 is shown graphically. The intensity of each b5 resonance (circle) corresponding to an individual b5 residue (x axis) is plotted as a percentage of that resonance’s intensity in the absence of P450 (y axis). Fine lines between circles indicate continuous assignments for sequential b5 amino acids. Gaps in the line between circles indicate that one or more intervening b5 amino acids have not been assigned to an individual resonance in the spectrum (also shown in white in B). The average (µ) and average minus one S.D. (µ−1) are indicated on the plot by black and red dashed lines, respectively, and constitute the selection criteria of b5 residues involved in P450 binding (red circles). B, on the surface of the human soluble domain b5 structure (PDB entry 2I96), residues displaying differential broadening effects are colored red, whereas residues that have assigned NMR resonances that are not differentially affected by P450 addition are colored gray, and unassigned residues are colored white. C, resonance intensity plots as in A, but comparing the effects of line broadening between WT b5 and b5 mutants D65N and E49Q at a fixed 0.5:1 CYP2A6(coumarin):[15N]b5 ratio. D, effects of wild-type and mutant b5 proteins on Michaelis–Menten kinetic parameters of CYP2A6-mediated coumarin 7-hydroxylation. Each sample was generated in duplicate with S.D. illustrated by error bars. Steady-state kinetic constants below are shown with S.D. E, measurement of NADPH consumed (nmol of NADPH/min/nmol of CYP2A6) for the CYP2A6 coumarin reaction at a fixed coumarin concentration of 128 µM. Samples were generated in duplicate with the S.D. illustrated by error bars. F, percent coupling of CYP2A6-mediated coumarin 7-hydroxylation of coumarin. Samples were generated in duplicate with the S.D. illustrated by error bars. ns, p > 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001.
Interaction and catalytic effects of b5 on CYP2E1

CYP2E1-mediated oxidation of many different substrates is reportedly stimulated by b5. These include chlorzoxazone, acetaminophen, aniline, and N-nitrosodimethylamine (17, 18). Studies suggest that b5 may play a redox role with CYP2E1, as apo-b5 does not increase product formation (19, 20).

NMR experiments performed in this study revealed that titration of b5 with CYP2E1(chlorzoxazone), like CYP2A6 (coumarin), also caused fairly substantial overall broadening at moderate P450:b5 ratios. At 0.25:1, for example, an average of ~55% of signal remained (Fig. 3A), and by 0.5:1, only ~28% of the original signal was present (Fig. 3C). The resonances for b5 residues Val-34, His-68, Ser-69, Thr-70, and Ser-76 had signal losses more than one S.D. below this average (Fig. 3B). Each of these residues except for Val-34 reside on the original signal was present (Fig. 3C). The resonances for b5 residues Val-34, His-68, Ser-69, Thr-70, and Ser-76 had signal losses more than one S.D. below this average (Fig. 3A). Each of these residues except for Val-34 reside on α5 or the preceding loop (Fig. 3B). This result is consistent with a monofacial interaction between b5 and CYP2E1(CZN) involving α5 and nearby residues.

Consistent with the above idea, when evaluated at a consistent P450:b5 ratio of 0.5:1, the b5 D65N mutant had less loss of average signal intensity (to 66%; Fig. 3C, middle) than wild-type b5 (Fig. 3C, left). This suggests that the D65N b5 mutant reduces binding to CYP2E1(CZN). Notably, the E49Q mutant on the opposite b5 surface had an intermediate effect on average signal intensity to ~41% of the original signal.

Comparisons of the mutational effects are clearer when evaluating chlorzoxazone 6-hydroxylation. Consistent with previous literature reports (28, 29), the effect of wild-type b5 mutant performed very similarly to wild-type b5 in these reactions yielded substrate metabolism was not present at all (Fig. 3B). Using solution NMR to visualize the interaction between CYP3A4(NFP) and b5, the effect of wild-type b5 on CYP2E1-mediated oxidation of many different substrates is typically very poor for CYP2E1 in general. Under the current conditions in the absence of b5, CYP2E1 coupling was only ~3%. The addition of wild-type b5 significantly decreased the NADPH consumption by 48% (Fig. 3E) while increasing product formation, resulting in increased coupling of the reaction by ~2.5-fold (Fig. 3F). The b5 mutant E49Q behaved similarly to wild-type b5, in that NADPH consumption was decreased to a similar extent (Fig. 3E), and combined with increased product formation, the coupling increased ~3.1-fold (Fig. 3F), slightly more than in the presence of wild-type b5. The b5 mutant D65N also reduced NADPH consumption (~38%; Fig. 3E) but had little effect on product formation, thus increasing coupling by ~1.7-fold when compared with reactions in the absence of b5 (Fig. 3F).

Interactions between b5 and CYP2E1 have previously been studied using cross-linking of the complex coupled with mass spectrometry. Those studies also identified residues on the same surface of b5: Asp-58 and Glu-61 in one study (6) and Asp-58 and Asp-65 in another study (30). In the latter study, these interactions were further tested for their functional role, and both residues appeared to be important in b5 stimulation of the 6-hydroxylation of CZN (30). Unfortunately, the resonance for Asp-58 in b5 has not been assigned, but it would be predicted to undergo differential line broadening. Overall, the current experiments and previous studies support the concept that the region surrounding Asp-65 is involved in both physical interaction between b5 and CYP2E1 and increases observed in product formation.

Interaction and catalytic effects of b5 on CYP3A4

Influence of b5 on CYP3A4 has been reported broadly. A number of CYP3A4-mediated reactions are stimulated by both holo- and apo-b5 (21), which may support a more allosteric role for b5. The current studies evaluated interactions and the effects of b5 on the hypotensive drug NFP.

Using solution NMR to visualize the interaction between CYP3A4(NFP) and b5, it was clear that considerable broadening occurred for the average b5 resonance with low concentrations of CYP3A4(NFP), suggesting substantial formation of the P450:b5 complex. With a CYP3A4(NFP):b5 ratio of 0.1:1, 85% of the average b5 signal remained. Additional CYP3A4(NFP) resulted in further decreases in the average resonance signal (0.2:1, ~59%; 0.3:1, ~59%; 0.4:1, ~41%; 0.5:1, ~29%).

Although the trends were similar throughout the titration range, differential line broadening was most distinct at a 0.2:1 ratio of CYP3A4(NFP):b5 (Fig. 4A). Residues of b5 differentially broadened more than one S.D. below the average are Leu-41, Glu-42, His-44, Gly-47, Glu-48, Leu-51, Arg-52, His-68, Ser-69, Ala-72, and Arg-73 (Fig. 4A). These residues cluster on helices 2, 3, and 5, as well as the loop between helices 2 and 3, and the loop preceding helix 5 (Fig. 4B). This suggests that these two faces of b5 are likely to interact with CYP3A4(NFP).

To evaluate the significance of these two surfaces, the b5 mutants D65N and E49Q were used to further evaluate the physical interaction between b5 and CYP3A4(NFP). As with the other enzymes, this was conducted at the P450:b5 ratio of 0.5:1. Whereas CYP3A4(NFP) resulted in a signal reduction to an average of 29% for wild-type b5 (Fig. 4C, left), this effect was dampened for both b5 mutants. At this same ratio, ~45% of the average signal remained for D65N (Fig. 4C, middle) and ~60% for E49Q (Fig. 4C, right). Thus, both mutations appeared to reduce CYP3A4(NFP):b5 complex formation. Notably, although the overall average intensities were higher for each b5 mutant compared with wild type, distinctive differential broadening still occurred (Fig. 4C, middle and right), suggesting that specific interactions between b5 and 3A4(nifedipine) were not completely disrupted by either single point mutation.

CYP3A4 converts nifedipine to dehydronifedipine. The addition of wild-type b5 to such reactions resulted in inhibition of this reaction (Fig. 4D). The addition of b5 both decreased the kcat and increased the Km, such that the kcat/Km was almost half that when b5 was not present. Literature has reported varied stimulation of nifedipine by b5, typically ~1.3–3-fold increased product formation (19, 31). The differences in these observations may lie in the protein ratios used to perform the experi-
ments. Peng and Auchus (31) used a 1:2:4 ratio in their assays, whereas Yamazaki et al. (19) used a 1:4:1 ratio. The overlapping binding sites and the variability in these results under different conditions may suggest that at higher concentrations of \(b_5\) relative to reductase, \(b_5\) may begin to outcompete reductase binding, resulting in an overall reduction in product formation, whereas lower relative concentrations of \(b_5\) could be stimulatory. However, there were also other potentially signifi-

Figure 3. Interaction of CYP2E1(chlorzoxazone) with \[^{15}N\]b5, by NMR and b5 modulation of CYP2E1 catalysis. A, a single NMR spectrum of 0.25:1 CYP2E1(CZN):[^{15}N]b5 is shown graphically as described in Fig. 2A. B, mapping of differentially affected residues (red) on human b5. The color code is as described in the legend to Fig. 2B. C, resonance intensity plots comparing line broadening for wild-type b5 and mutated b5 on Michaelis-Menten kinetic parameters of CYP2E1-mediated chlorzoxazone 6-hydroxylation. Each sample was generated in triplicate with S.D. illustrated by error bars. Steady-state kinetic constants below are shown with S.D. E, NADPH consumed (nmol of NADPH/min/nmol of CYP2E1) for CYP2E1-mediated chlorzoxazone metabolism at a fixed chlorzoxazone concentration of 600 \(\mu\)M. Samples were generated in duplicate with the S.D. illustrated by error bars. F, percent coupling of CYP2E1-mediated chlorzoxazone 6-hydroxylation. Samples were generated in duplicate with the S.D. illustrated by error bars. ns, \(p > 0.05\); **, \(p \leq 0.01\); ***, \(p \leq 0.001\).
significant differences between how the assays were accomplished in different reports, including the presence of lipid or the constructs or buffer used. CYP3A4-mediated metabolism seems to be more sensitive to these environmental parameters than many other P450 enzymes. Notably, the single $b_5$ mutants D65N and E49Q had effects on nifedipine oxidation most similar to wild-type $b_5$. Thus, the interactions between CYP3A4(NFP) and $b_5$ are not disrupted enough by either single-point mutation to ameliorate the effect of $b_5$ on product formation. Unfortunately, NADPH consumption and coupling of the 3A4(nifedipine) reaction were not successful due to low turnover rates of the reaction under the conditions employed for all of the assays herein.
Cytochrome P450/b₅ interactions

Overall, the differential line broadening of NMR signal suggests that CYP3A4(NFP) interacts with b₅ over the widest surface area for any of the P450/b₅ complexes in this study. Interactions between CYP3A4 and b₅ have been reported previously in cross-linking and mutational studies. Zhao et al. (7) used mass spectrometry to identify cross-links between the two proteins; residues on human b₅ identified were Glu-42, Glu-48, and Glu-61, which are located on α₂, α₃, and the start of α₄. Another study reported similar residues, where mutations in human b₅ residues Glu-48, Glu-49, Asp-58, and Asp-65 had significant decreases in the ability of b₅ to enhance CYP3A4-mediated testosterone and nifedipine oxidation (31). Moreover, the b₅ double mutant D58G/D65G significantly impaired the ability to form cross-links with CYP3A4, whereas the double mutant E48G/E49G had reduced stimulation but was highly dependent on the type of phospholipid in the assay (31). Overall, the current NMR data of the CYP3A4/b₅ complex are consistent with reported interactions in these previous studies but highlight additional residues.

Interaction and catalytic effects of b₅ on CYP2D6

CYP2D6 metabolizes numerous pharmaceuticals, including many antidepressants and antipsychotics. It exhibits substantial polymorphism in humans (25). To assess whether 2D6 forms a complex with b₅, NMR-monitored titrations of [¹⁵N]b₅ with CYP2D6 were carried out using a saturating amount of its common antitussive substrate DXM. The titrations resulted in line broadening, consistent with complex formation between the two proteins, but required much higher concentrations of CYP2D6 than any of the other drug-metabolizing enzymes in this study. For example, it takes a 1:1 CYP2D6(DXM):b₅ ratio before the average signal intensity drops to about half (48%; Fig. 5A). In comparison, CYP2A6(coumarin) had more signal reduction by a 0.2:1 ratio.

Within these data, the resonances for four residues are broadened more than one S.D. from the average. Gly-47, Glu-49, His-68, and Ser-69 are all differentially broadened (Fig. 5A). On the b₅ surface, these residues fall into two disconnected regions of b₅: the start of α₃ (Gly-47 and Glu-49) and the loop between α₄ and α₅ (His-68 and Ser-69) (Fig. 5B). These data suggest that when a complex is formed at higher concentrations of CYP2D6(DXM), both faces of b₅ are involved.

To probe the importance of these distinct faces, the E49Q and D65N b₅ mutants were also evaluated for their ability to bind CYP2D6(DXM). At the uniform ratio of 0.5:1, wild-type b₅ retained an average signal intensity of 69% (Fig. 5C, left), but both mutants retained even more signal intensity. E49Q and D65N retained ~80 and ~84% of the signal intensity, respectively (Fig. 5C, right and middle). This is consistent with both charges contributing to the CYP2D6(DXM):b₅ complex when it does occur.

To link these structural observations with the effects of b₅ on CYP2D6 dextromethorphan metabolism, both wild-type and mutant b₅ proteins were employed in steady-state turnover assays. Similar to other in vitro reports on CYP2D6 reactions (19, 32), the presence of wild-type b₅ had no significant effects on kinetics of metabolite formation from dextromethorphan (Fig. 5D). Thus, not surprisingly, neither b₅ mutant altered product formation.

When NADPH consumption was measured, however, the presence of b₅ did result in a two-thirds reduction (Fig. 5E) and therefore an increase in coupling (from ~23 to ~72%; Fig. 5F). Each of the single E49Q and D65N b₅ mutants also reduced NADPH consumption (to ~59 and ~65%, respectively; Fig. 5E), but not as effectively as wild-type b₅. Thus, the ~2.0-fold increases in coupling with E49Q and D65N b₅ were less than the ~3-fold increase in coupling observed for wild-type b₅ (Fig. 5F).

CYP2D6(DXM) interaction with b₅ appeared to be similar to CYP3A4(NFP) in that opposite surfaces of b₅ were implicated, but extensive broadening of b₅ resonances did not occur until an equal molar (1:1) ratio of 2D6(DXM):b₅, a feature that would be consistent with a weaker interaction with CYP2D6(DXM) in vitro bufuralol 1′-hydroxylation, tamoxifen 4-hydroxylation, or acetaminophen conversion to its toxic metabolite N-acetyl-p-benzoquinone imine (19, 20, 26, 27). There is one conflicting report indicating that b₅ could modulate CYP2D6 metabolite formation in vivo, as mice humanized for CYP2D6 have decreased bufuralol and desbroquino turnover upon hepatic deletion of the b₅ gene. This could be ameliorated by the addition of membranes containing b₅ (33).

Interaction and catalytic effects of b₅ on CYP2A6 saturated with p-nitrophenol

To begin to probe the effects that the identity of a given P450 substrate might have on interactions with b₅, the interactions of CYP2A6 saturated with pNP were also investigated using NMR and functional assays and compared with earlier results for CYP2A6(coumarin).

When [¹⁵N]b₅ was titrated with CYP2A6(pNP), differential line broadening was observed for very similar residues observed with 2A6(coumarin). Specifically, b₅ residues significantly affected were Glu-64, His-68, Ser-69, Thr-70, and Arg-73 (Fig. 6A), which comprise b₅ α₄, α₅, and the intervening loop (Fig. 6B). However, the average reduction in signal was not as pronounced when CYP2A6 was saturated with pNP as it was when CYP2A6 was saturated with coumarin. For example, at a P450:b₅ ratio of 1:0.5, ~49% of the average b₅ signal remained for the CYP2A6(pNP)/b₅ mixture (Fig. 6C, left), compared with ~22% remaining signal for the CYP2A6(coumarin)/b₅ mixture (Fig. 2C, left).

The importance of Asp-65 in forming the complex between b₅ and CYP2A6(pNP) was similar to that observed with the substrate coumarin. At a uniform 0.5:1 P450:b₅ ratio, the b₅ D65N mutation retained more signal average intensity (~86%; Fig. 6C, middle) than wild-type b₅ (49% signal remaining; Fig. 6C, left), consistent with reduced complex formation for the mutant. By comparison, under the same conditions, the b₅ E49Q mutant had an average intensity (54%; Fig. 6C, right) much more similar to that of wild-type b₅, suggesting that this b₅ residue does not play a significant role in binding CYP2A6(pNP).

The effects of these b₅ mutants on formation of a CYP2A6(pNP)/b₅ complex correlated with observations of the kinetics (Fig. 5D). Thus, not surprisingly, neither b₅ mutant altered product formation.
of CYP2A6 para-nitrophenol metabolism to 4-nitrocatechol. Whereas wild-type $b_5$ modestly stimulated the reaction with both an increase in $k_{cat}$ and decrease in $K_m$, resulting in an overall ~1.5-fold increase in catalytic efficiency (Fig. 6D), the $b_5$ mutant E49Q performed very similarly (Fig. 6D), suggesting that this residue is not critical for $b_5$ simulation of CYP2A6-

Figure 5. Interaction of CYP2D6(dextromethorphan) with $[^{15}N]b_5$ as determined by NMR and catalytic modulation of $b_5$ on CYP2D6-mediated metabolism of dextromethorphan. A, $b_5$ resonance intensity plot at a 1:1 (CYP2D6(DXM):$[^{15}N]b_5$) ratio normalized to the free $b_5$ resonance intensity. The color code is as described in the legend to Fig. 2B. B, human soluble domain $b_5$ structure (PDB entry 2I96) with residues displaying differential broadening effects colored red, residues that are assigned in the NMR spectrum colored gray, and unassigned residues colored white. C, $b_5$ resonance intensity plots comparing the effects of line broadening between WT $b_5$ and $b_5$ mutants D65N and E49Q at a fixed 0.5:1 (CYP2D6(DXM):$[^{15}N]b_5$) ratio. D, effect of WT $b_5$ and mutants on Michaelis–Menten kinetic parameters of CYP2D6-mediated O-demethylation of dextromethorphan. Each sample was generated in triplicate with S.D. illustrated by error bars. Steady-state kinetic constants below are shown with S.D. E, measurement of NADPH consumed (nmol of NADPH/min/nmol of CYP2D6) for CYP2D6 dextromethorphan reaction at a fixed dextromethorphan concentration of 1 mM. Samples were generated in duplicate with the S.D. illustrated by error bars. F, percent coupling of CYP2D6-mediated O-demethylation of dextromethorphan. Samples were generated in duplicate with the S.D. illustrated by error bars. *, $p\leq 0.05$; **, $p\leq 0.01$; ***, $p\leq 0.001$. 

| Condition | $k_{cat}$ (min$^{-1}$) | $K_m$ (µM) | $k_{cat}/K_m$ (µM$^{-1}$ min$^{-1}$) |
|-----------|-----------------------|------------|-----------------------------------|
| - $b_5$   | 0.76 ± 0.02           | 96 ± 12    | 0.0080 ± 0.0010                   |
| + WT $b_5$| 0.79 ± 0.03           | 102 ± 14   | 0.0080 ± 0.0010                   |
| + D65N $b_5$| 0.77 ± 0.03 | 122 ± 15  | 0.0063 ± 0.0008                   |
| + E49Q $b_5$| 0.69 ± 0.03 | 73 ± 11   | 0.0090 ± 0.0010                   |

of CYP2A6 para-nitrophenol metabolism to 4-nitrocatechol. Whereas wild-type $b_5$ modestly stimulated the reaction with both an increase in $k_{cat}$ and decrease in $K_m$, resulting in an overall ~1.5-fold increase in catalytic efficiency (Fig. 6D), the $b_5$ mutant E49Q performed very similarly (Fig. 6D), suggesting that this residue is not critical for $b_5$ simulation of CYP2A6-

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mediated pNP metabolism. In contrast, reactions substituting the $b_5$ mutant D65N resulted in steady-state kinetic parameters more similar to reactions containing no $b_5$ at all (Fig. 6D), suggesting that Asp-65 is critical for the $b_5$ stimulation of CYP2A6-mediated pNP oxidation. It has previously been reported that CYP2A6-mediated pNP oxidation is not significantly altered by the presence of $b_5$ (34). However, these results were obtained at a single pNP concentration that was relatively low ($5 \mu M$) and at
a CYP2A6:CPR ratio of 1:5.8 (34). The CYP2A6 pNP assays performed in the current study used a range of pNP concentrations (25–1600 μM) and a CYP2A6:CPR ratio of 1:2. Thus, differences in experimental parameters could contribute to observed differences in \( b_5 \) stimulation and again suggest that the ratio of \( b_5 \) to CPR is likely to determine the outcome because these two proteins both bind, and probably compete for, the P450 proximal surface.

Evaluation of NADPH coupling herein revealed that wild-type \( b_5 \) decreased NADPH consumption by \( \sim 30\% \) (Fig. 6E) and increased coupling of NADPH consumption to formation of the 4-nitro catechol product formation 1.8-fold (Fig. 6F). By comparison, neither the E49Q or D65N mutation significantly altered NADPH consumption (Fig. 6E). However, the increased product formation observed for the \( b_5 \) E49Q results in a \( \sim 1.4\)-fold increase in coupling, whereas the absence of effect of D65N \( b_5 \) on product formation and NADPH consumption meant coupling was the same as in the absence of \( b_5 \) (Fig. 6F). Thus, these studies also support a significant role for Asp-65, but less so for Glu-49.

Overall, the NMR and mutation data herein suggest that the \( b_5 \) surface involved in binding to CYP2A6(pNP) (Fig. 6B) is very similar to the \( b_5 \) surface binding CYP2A6(coumarin) (Fig. 6B). Mutation studies confirmed that Asp-65 on one face of \( b_5 \) plays a significant role in both CYP2A6/\( b_5 \) interactions, whereas Glu-49 on the opposing \( b_5 \) face had relatively little contribution. The greater degree of line broadening occurring with the substrate coumarin compared with pNP is potentially consistent with a stronger \( b_5 \) interaction with CYP2A6(coumarin) compared with CYP2A6(pNP). However, we cannot exclude effects due to possible differences in CYP2A6 paramagnetism when bound to different ligands. CYP2A6(coumarin) results in almost complete conversion to high spin, whereas the CYP2A6 (pNP) spin state cannot be readily assessed because of significant pNP absorbance in the region of interest. Using immobilized CYP2A6 and biotinylated \( b_5 \), Guengerich and co-workers (27) found no difference in binding for unliganded CYP2A6 versus CYP2A6 bound to coumarin. Like the structural data, the effects of \( b_5 \) mutants in turnover assays support an important role for D65N in coupling and metabolite formation. In contrast, E49Q performed more similarly to wild-type enzyme, suggesting that this residue is not critical for changes in CYP2A6-mediated pNP metabolism. In both CYP2A6-mediated coumarin and pNP metabolism, \( b_5 \) increased coupling \( \sim 2\)-fold. However, this mostly stemmed from a decrease in NADPH utilization for coumarin hydroxylation, whereas for pNP oxidation, both decreased NADPH consumption and increased metabolite formation contributed. Thus, although the \( b_5 \) residues binding CYP2A6 are conserved across these two substrates, the effects on CYP2A6 catalysis vary.

**Comparison of P450/\( b_5 \) interactions across P450 enzymes and substrates**

Of the four human P450 enzymes surveyed, all of them were found to interact with cytochrome \( b_5 \) and to do so predominately on at least one of the surfaces surrounding the heme-exposed face of \( b_5 \). This is in general agreement with studies assessing \( b_5 \) interactions with CYP17A1 (4, 8), CYP2B4 (14), CYP3A4 (7, 31), and CYP2E1 (6, 30). However, the use of NMR allows one to simultaneously probe all possible \( b_5 \) residues involved in the interaction without modifying either interacting partner aside from isotopic labeling. As a result, this study identified two \( b_5 \) surfaces differentially interacting with different xenobiotic P450 enzymes. All four P450 enzymes in this study interacted with His-68 and Ser-69 on the loop between \( \alpha_4 \) and \( \alpha_5 \). Three of them (CYP2A6, CYP2E1, and CYP3A4) additionally interacted with adjacent residues in \( b_5 \) \( \alpha_5 \). In addition to this patch on one side of the heme, CYP2D6 and CYP3A4 can also bind to the opposite surface of \( b_5 \) \( \alpha_3 \), with CYP3A4 having the broadest interaction surface for both regions. Notably, CYP2A6 saturated with either coumarin or pNP interacted with the same face of \( b_5 \), but it remains to be seen whether this is true for other P450/substrate pairs. It is clear that across these P450/substrate pairs and across the different binding interfaces observed, \( b_5 \) consistently increases coupling but may or may not alter product formation, as was also seen for rabbit CYP2B4 (10).

Although the current NMR studies provide detailed structural information about the P450/\( b_5 \) interaction, they are not well suited to the determination of dissociation constants. However, it is notable that CYP2D6(DXM) was a very distinct outlier in terms of the amount of line broadening observed for \( b_5 \) resonances compared with CYP3A4(NFP), CYP2A6(coumarin or pNP), CYP2E1(CZN), and even CYP17A1 in a previous parallel study (8). Although other explanations are possible, the simplest explanation is that this observation is consistent with less complex formation. A study evaluating the physical interaction between P450 enzymes immobilized on a plastic plate and biotinylated \( b_5 \) ranked the affinities as unliganded CYP3A4 > CYP2A6 ~ CYP2D6 > CYP2E1 but noted that much less CYP2D6/\( b_5 \) complex was formed in these experimental conditions as well (27).

In conclusion, it appears that cytochrome \( b_5 \) interacts with different drug-metabolizing P450 enzymes with both shared and distinct surfaces. Disruption of these surfaces correlates with functional effects on metabolite production and/or NADPH consumption. Thus, in the absence of X-ray structures, solution NMR is a high-resolution technique to examine these transient P450 interactions with other proteins. Further work remains to map the P450 residues involved in binding \( b_5 \), to compare \( b_5 \) and reductase competition for binding different P450 enzymes, and to determine the mechanism(s) by which \( b_5 \) modulates P450 catalysis.

**Experimental procedures**

*Generation of the soluble domain of human cytochrome \( b_5 \) with \( ^{15}N \)-labeling for NMR experiments*

A synthetic, codon-optimized gene encoding the soluble domain (residues 1–108) of human microsomal cytochrome \( b_5 \) with a C-terminal His₆ tag (Genewiz) was cloned into the NcoI and BamHI restriction sites of pET15b and transformed into *E. coli* C41 (DE3) cells already containing the pGro7 plasmid (Takara Bio) for expression of GroEL/GroES chaperones. Transformed cells were selected by growing cells for \( \sim 18 \) h at 37 °C on a non-inducing minimal medium plate (MDAG-11)
Cytochrome P450/b₅ interactions

(35) supplemented with carbenicillin (100 µg/ml) and chloramphenicol (20 µg/µl) to select for the b₅ and chaperone plasmids, respectively. All subsequent cultures contained these antibiotics. A single colony was picked and grown for ~16 h at 37 °C with shaking (250 rpm) in a 50-ml liquid culture of non-inducing minimal medium MDAG-135 (35). Expression cultures consisted of 1 liter of a defined minimal medium (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM ¹⁵N NH₄Cl, 53 mM glucose, 4 mM MgSO₄, and trace metals) inoculated with 5 ml of the liquid starter culture. Cells were grown with shaking (250 rpm) at 37 °C, to an optical density at 600 nm (A₆₀₀) of 0.3, at which point the heme precursor δ-aminolevulinic acid (to 1 mM) and the chaperone inducer L-arabinose (to 13 mM) were added. After A₆₀₀ reached 0.7–0.8, b₅ expression was induced by adding 0.4 mM isopropyl 1-thio-D-galactopyranoside, and the temperature and shaking were reduced to 30 °C and 225 rpm, respectively. Cultures were subsequently grown for 20 h before harvesting and freezing the cell pellet at ~80 °C. Purification was initiated by resuspending cells in lysis buffer (500 mM potassium phosphate, 100 mM NaCl, 15% glycerol, 1 mM EDTA, pH 7.4) with the addition of 1 mM PMSF. Resuspended cells were lysed by a French press, and heme reconstitution was performed as described (36). Lysed cells were clarified by ultracentrifugation at 140,000 × g and loaded onto a pre-equilibrated 25-ml Ni-NTA column (Qiagen) with loading buffer (100 mM potassium phosphate, 20% glycerol, 200 mM NaCl, 20 mM imidazole, pH 7.4), washed with an additional 8 column volumes of loading buffer, and eluted using a 6 column volumes of elution buffer (loading buffer with 200 mM imidazole). Eluted fractions with A₄₃/A₂₈₀ > 2.0 were pooled, concentrated, and loaded on a Sephacryl S-200 HR column (GE Healthcare) equilibrated and run with gel filtration buffer (50 mM potassium phosphate, 20% glycerol, 100 mM NaCl, 0.05% (w/v) CHAPS, pH 7.4) and exchanged into storage buffer as described above. Protein purity was assessed on SDS-PAGE, and UV-visible spectroscopy was used to confirm degree of heme incorporation (A₄₃/A₂₈₀ = 3.0) and quantify dithionite-reduced b₅ (36).

Generation of full-length human NADPH-cytochrome P450 reductase for catalytic assays

A synthetic, codon-optimized gene for full-length human NADPH-cytochrome P450 reductase (Integrated DNA Technologies) preceded by the ompA signal peptide and followed by a His₆ tag was cloned into pET-29a(+) using NdeI and HindIII. Expression of reductase was performed as described previously for a truncated CPR construct (38) with modifications. Modifications to the expression included 1) transformation and generation of the starter culture as described for soluble b₅, 2) supplementation of expression cultures with 2 mg/liter riboflavin, and 3) shaking at 200 rpm after induction. Full-length CPR was also purified in a manner similar to a previously described method (38), with modifications: 1) membrane fractions after cell lysis were isolated by ultracentrifugation at 140,000 × g for 25 min before detergent extraction, 2) omission of the ammonium sulfate precipitation step, and 3) the addition of a second nickel affinity column after elution from Octyl-Sepharose 4 Fast Flow (GE Healthcare) resin to deplete detergent. Final reductase samples were evaluated on SDS-PAGE and by UV-visible spectroscopy. CPR was quantitated by flavin absorbance of the fully oxidized protein at 454 nm with an extinction coefficient of 21.4 mM⁻¹ cm⁻¹ (39).

Generation of P450 enzymes for NMR and catalytic assays

All cytochrome P450 enzymes were the forms used previously to generate crystallographic structures and resulted in catalytically active protein omitting the N-terminal transmembrane helix and adding a C-terminal His tag. Constructs and expression and purification of human CYP2A6 (40) and CYP2E1 (41) were reported previously. Synthetic, codon-optimized genes encoding CYP2D6 and CYP3A4 were generated (GenScript) to match the reported constructs (42, 43), cloned into the pCWori vector, and expressed and purified as described in these same publications with some modifications. Briefly, E. coli DH5α containing the pGro7 plasmid (Takara Bio) was used to express the P450 enzymes. Transformation and starter cultures were performed as described for soluble b₅. Proteins were purified by isolating spheroplasts (44), lysis by

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**20830 J. Biol. Chem. (2017) 292(51) 20818 - 20833**
Magnetic Resonance Data Bank (accession number 6921) (16).ferred from the deposited chemical shifts in the Biological
and constant between samples (500
the P450. Concentration of the P450 substrate was saturating
as a percentage of the resonance peak height in the absence of
P450 enzyme, the peak height for each resonance was expressed
P450 enzyme was added to the sample. For samples including
was prepared, and a defined molar amount of an unlabeled

M NFP for 3A4). NMR experiments were performed with both
M coumarin for 2A6, 500
muM coumarin for 2A6, 500
muM CZN for 2E1, 2 mm DXM for 2D6, 5 mm pNP for 2A6, or 200
muM NFP for 3A4). NMR experiments were performed with both
b5 and P450 in their oxidized states. At the conclusion of each
NMR experiment, samples were collected from the NMR
tube, centrifuged to determine whether any precipitation had
occurred, and evaluated in terms of the reduced-carbon mono-
oxide difference spectrum.

**Cytochrome P450 catalytic assays**

All catalytic assays were performed by incubating a P450 with
full-length human P450 reductase and full-length human b5
(when applicable) at a 1:2:0 ratio for minus b5 reactions or a
1:2:2 ratio for b5 containing reactions for 20 min at room tem-
perature. The amounts of P450 enzyme were 10 pmol (couma-
rin assay) or 50 pmol (pNP assay) of CYP2A6, 50 pmol of
CYP2D6 (DXM assay), 50 pmol of CYP2E1 (CZN assay), and 50
pmol of CYP3A4 (NFP assay). This protein mixture was added
to the same buffer used for NMR experiments, except for the
nifedipine assay, which used an assay buffer consisting of 40 mm
HEPES, 30 mm MgCl2, pH 7.4. The reactions also contained the
respective P450 substrate (0–128 mM coumarin, 0–600 mM
CZN, 0–1600 mM pNP, 0–1000 mM DXM, 0–250 mM nifedip-
ine). For the nifedipine assay, all reactions were performed in
amber vials. The reactions were preincubated at 37 °C for 3 min
and then initiated with 1 mM NADPH and allowed to proceed at
37 °C for 10 min (coumarin, CZN, and pNP assays), 15 min
(DXM assay), or 20 min (nifedipine assay). Reactions were term-
inated by the addition of diluted perchloric acid (acetonitrile
for the nifedipine assay), placed on ice, and centrifuged at
5000 × g for 5 min before injection onto a Luna C18 (5 μm,
150 × 4.60 mm; Phenomenex) column at a flow rate of 1
ml/min (0.75 ml/min for the nifedipine assay). Separation on
HPLC was obtained using the following mobile phase solutions
for each of the respective assays. For separation of coumarin
and its 7-OH metabolite, the mobile phase consisted of 50%/50%/
20 mM potassium phosphate, pH 2.8/methanol. For CZN
and its 6-OH metabolite, the mobile phase consisted of 75%/25%/20
mM potassium phosphate, pH 2.8/acetonitrile to elute product
and then a sharp gradient to 40%/60% to elute sub-
trate, followed by reequilibration to 75%/25%. For pNP and its
4-nitrocatechol metabolite, the mobile phase consisted of 73%/27%
10 mM potassium phosphate, pH 3.5/acetonitrile. For
DXM and its O-demethylated metabolite dextrophan, the
mobile phase consisted of 50%/50% 10 mM potassium phos-
phate, pH 3.5/50% acetonitrile and 100% methanol (250:200,
v/v). For nifedipine and its metabolite dehydronifedipine,
the mobile phase consisted of 45%/55% water/methanol. Meta-
obolite detection occurred by fluorescence for coumarin (355-nm
excitation, 460-nm emission) and DXM (280-nm excitation,
310-nm emission) assays and by UV absorbance for CZN (287
nm), pNP (345 nm), and NFP (270 nm) assays. The amounts
of the different metabolites produced were calculated using
authentic standards prepared by the same method as the sam-
ple. Each reaction at each substrate concentration was per-
formed at least in duplicate. Data were fit to the Michaelis–
Menten equation using GraphPad Prism.

**Measurement of NADPH consumption**

NADPH consumption during the various P450 reactions was
measured by creating reaction samples similar to the catalytic
assays except for the following: 1) 100 pmol P450 was used to
increase signal/noise, 2) reactions were scaled up to 1 ml in the
respective assay buffer containing the maximum substrate con-
centration used in turnover reactions, 3) NADPH concentra-
tion was reduced to 0.5 mM to reduce background, and 4) reac-
tions were allowed to proceed for 10 min. CYP2A6(pNP)
samples required the use of 2-mm path length cuvettes due to
high substrate absorbance, so these reactions used 50 pmol of
P450 and a final volume of 500 μl. Rates of NADPH consump-
tion were measured by monitoring linear decreases in absorb-
bance at 340 nm. The amount of NADPH consumed was cal-
culated using an extinction coefficient of 6.22 mm−1 cm−1.
Control reactions omitting P450 provided the background NADPH consumption, which was subtracted. The amount of product at the end of each reaction was measured using the same methods described for each catalytic assay. Coupling efficiencies were calculated by dividing metabolite product formation in these experiments by their respective NADPH consumption. All measurements were performed in duplicate.

**Author contributions**—A. G. B. and E. E. S. conceptualization; A. G. B. data curation; A. G. B. and E. E. S. formal analysis; A. G. B. and E. E. S. investigation; A. G. B. visualization; A. G. B. and E. E. S. methodology; A. G. B. and E. E. S. writing—original draft; A. G. B. and E. E. S. writing—review and editing; E. E. S. supervision; E. E. S. funding acquisition; E. E. S. project administration.

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Cytochrome P450/b₅ interactions
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