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The action of cytochrome b_5 on both CYP2E1 and CYP2C19 activities requires the anionic residues D58 and D65

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Abstract

The capacity of cytochrome b_5 (b_5) to influence cytochrome P450 activities has been extensively studied and physiologically validated. Apo- b_5 enhances the activities of CYP3A4, CYP2A6, CYP2C19, and CYP17A1 but not of CYP2E1 or CYP2D6, suggesting that the b_5 interaction varies amongst P450s. We previously showed that b_5 residues E48 and E49 are required to stimulate the 17,20-lyase activity of CYP17A1, but these same residues might not mediate b_5 activation of other P450 reactions, such as CYP2E1-catalyzed oxygenations, which are insensitive to apo- b_5 . Using purified P450, b_5 , and reductase (POR) in reconstituted assays, mutation D58G+D65G, residues located in a hydrophilic α -helix of b_5 , totally abolished the ability to stimulate CYP2E1-catalyzed chlorzoxazone 6-hydroxylation. In sharp contrast, the D58G+D65G mutation retained full capability to stimulate the 17,20 lyase activity of CYP17A1. Mutation D58G+D65G competes poorly with wild-type b_5 for binding to the CYP2E1•POR complex yet accepts electrons from POR at a similar rate. Furthermore, the phospholipid composition markedly influences P450 turnover and b_5 stimulation and specificity, particularly for CYP17A1, in the order phosphatidylserine > phosphatidylethanolamine > phosphatidylcholine. Mutation D58G+D65G also failed to stimulate CYP2C19-catalyzed (*S*)-mephenytoin 4-hydroxylation, whereas mutation E48G+E49G stimulated these activities of CYP2C19 and CYP2E1 equivalent to wild-type b_5 . We conclude that b_5 residues D58 and D65 are essential for the stimulation of CYP2E1 and CYP2C19 activities and that phospholipid composition significantly influences the b_5 -P450 interaction. At least two surfaces of b_5 differentially influence P450 activities, and the critical residues for individual P450 reactions cannot be predicted from sensitivity to apo- b_5 alone.

Cytochrome b_5 (b_5) is a small acidic protein with pleiotropic functions in human physiology, including hematology, endocrinology and metabolism, and xenobiotic metabolism. Encoded by a single *CYP5A* gene, a soluble form of b_5 is found in erythrocytes, while a membrane-bound form is found in the endoplasmic reticulum in many tissues such as liver (1). The b_5 protein has a heme prosthetic group with two histidine nitrogen atoms stably coordinating the heme iron, and b_5 functions as a physiological one-electron transfer component of several reactions, including methemoglobin reduction, elongation and desaturation of fatty acids, and cholesterol biosynthesis (2). In addition, Hildebrandt and Estabrook first suggested in 1971 that b_5 participates in cytochrome P450 (P450) reactions (3), and b_5 thus stimulates androgen synthesis and regulates xenobiotic catabolism. Several lines of evidence support the physiological importance of b_5 in these various biochemical reactions. Genetic

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Supporting Information Available

Supporting information for this publication is available. Table S1 lists all of the b_5 mutations studied and the sequences of the oligonucleotide primers used for their construction and expression. Figure S1 shows UV-visible spectra of purified, recombinant wild-type b_5 and mutations D36G, E48G, E49G, D58G, D65G, E48G+E49G, and D58G+D65G, both reduced and oxidized forms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

defects in b_5 and more commonly in b_5 reductase cause congenital methemoglobinemia (4, 5). The few carefully characterized male patients with inactivating *CYP5A* mutations bear a disorder of male sex development (46,XY DSD) with defective testosterone synthesis, specifically due to loss of CYP17A1-mediated 17,20-lyase activity and thus 19-carbon steroid synthesis (6–8). Furthermore, b_5 is expressed in only one zone of the human adrenal gland, the zona reticularis, which is the major site of 19-carbon steroid synthesis in the human adrenal (9, 10). Finally, mice with liver-specific deletion of b_5 show impaired metabolism of several drugs (11), which demonstrates that the capacity of b_5 to stimulate P450-catalyzed oxidations is not an artifact of *in vitro* biochemical assays.

Whereas the essential function of b_5 in these biochemical systems is firmly established, the mechanism of its action in P450-catalyzed reactions remains unresolved and controversial. Depending on the specific P450 and substrate, b_5 can be either an obligatory component or a modulator of reactions, which would occur in the absence of b_5 (2, 12). Some P450s, including CYP2E1 and CYP2D6, are stimulated by b_5 but not by b_5 devoid of heme (apo- b_5), suggesting that b_5 functions as an electron transfer protein in these reactions. In contrast, other reactions, including those catalyzed by CYP3A4, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A5, CYP4A7, and CYP17A1, show equivalent stimulation *in vitro* upon addition of either b_5 or apo- b_5 , suggesting that b_5 might allosterically activate some P450s rather than directly participating in electron transfer (13–16). The heterogeneity amongst assay systems employed in these studies, the inconsistent results, and the potential for artifacts from heme transfer reactions (17) cloud our understanding of b_5 action on P450 reactions and preclude the genesis of a unified model to explain these effects. The spectral changes induced upon b_5 binding to various P450s have been employed to probe this interaction, but these differences are often small, slow, and difficult to reproduce (18), which limits the interpretations derived from these experiments (19).

After many years of study, four different mechanisms have been proposed for the action of b_5 in P450-mediated catalysis (2, 20). These mechanisms propose that 1) b_5 directly transfers the second electron in the rate-limiting step of the P450 reaction; 2) b_5 enhances the coupling between P450 and POR for product formation; 3) b_5 accepts and holds an electron transferred first from POR to the P450, which facilitates rapid and consecutive transfer of two electrons to the P450 in a heterotrimeric complex, the first from POR and following molecular oxygen binding to the P450, the second from b_5 ; and 4) b_5 acts as an allosteric effector, which interacts with the P450•POR complex to stimulate catalysis without direct electron transfer. These models all invoke binary or ternary complex formation, which requires specific binding sites on b_5 for the P450 and/or POR. Consequently, several studies have endeavored to identify the specific P450 residues responsible for interactions with b_5 . The CYP2B4 mutations R122A, R126A, R133A, F135A, M137A, K139A, and K433A, all located on the proximal surface of the P450 near the heme ligand, exhibit attenuated affinity for b_5 and reduced b_5 -stimulated methoxyflurane dehalogenation (21). More recently, mass spectrometry studies identified two intermolecular cross-links, between K428 and K434 of CYP2E1 with D53 and D56 of b_5 (22), respectively, which provided the first direct experimental evidence for the interacting residues of a microsomal P450 and b_5 . Mutations R347H and R358Q in CYP17A1 cause isolated 17,20-lyase deficiency (23), and these mutations impair CYP17A1 interactions with both POR and b_5 (24).

In contrast to these studies defining key P450 residues, little attention has been directed to determining the critical residues of b_5 required for P450 modulation. Our laboratory (25) established that E48 and E49 in human b_5 are essential for stimulating 17,20 lyase activity of CYP17A1. The b_5 mutation E48G+E49G retained only trace capacity to stimulate the

17,20-lyase reaction of CYP17A1 yet did not impair stimulation by wild-type b_5 or lose capacity to bind heme and to accept an electron from POR. These results suggest that an allosteric interaction driven by electrostatic forces dominates the action of b_5 on the CYP17A1•POR complex, which is consistent with the equivalence of b_5 and apo- b_5 in previous experiments (26).

Given that apo- b_5 does not stimulate CYP2E1-mediated oxidations and that mass spectrometry data suggest critical b_5 residues other than E48 and E49 for these reactions, we probed the action of b_5 on CYP2E1. We used a panel of b_5 point mutations and targeted double mutations in reconstituted assays with POR and phospholipid to identify functionally important residues on b_5 required for CYP2E1-catalyzed chlorzoxazone 6-hydroxylation. For comparison, we also studied the influence of these b_5 mutations on the 17,20-lyase reaction of CYP17A1 and CYP2C19-catalyzed (*S*)-mephenytoin 4-hydroxylation.

Experimental Procedures

Materials

Phenylmethylsulfonyl fluoride (PMSF), 5-aminolevulinic acid, Nonidet P-40, octylglucoside (*n*-octyl- β -D-glucoside), DL-dithiothreitol (DTT), ampicillin, chloramphenicol, isopropyl- β -D-thiogalactopyranoside (IPTG), 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (DAPE), 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (DAPS), 17-hydroxypregnenolone, and chlorzoxazone were obtained from Sigma (St Louis, MO). L-(+)-arabinose was purchased from Alfa Aesar (Ward Hill, MA). Complete mini-protease inhibitor was obtained from Roche Diagnostics (Indianapolis, IN). Bactotryptone and yeast extract were purchased from Difco (Detroit, MI). Molecular biology reagents, including restriction enzymes, ligases, and Phusion polymerase, were obtained from New England BioLabs (Beverly, MA), and 6-hydroxychlorzoxazone was obtained from Cayman Chemical Co. (Ann Arbor, MI). (*S*)-Mephenytoin and 4-hydroxy-(*S*)-mephenytoin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and pregnenolone was purchased from Waterstone Technologies (St. Carmel, IN). Progesterone was obtained from Calbiochem (San Diego, CA). Radiochemicals were purchased from PerkinElmer (Shelton, CT).

Plasmids

The expression plasmids were generous gifts obtained from the following investigators: human CYP2E1 in pKK233-2 from Professor James R. Halpert (University of California, San Diego, CA); human CYP17A1 in pCW and N-27-human POR in pET22 from Professor Walter L. Miller (University of California, San Francisco, CA); human b_5 in pLW01-b5H4 from Professor Lucy Waskell (University of Michigan); and human CYP2C19 in pCW from Dr. Joyce A. Goldstein (National Institutes of Health, NIEHS, Research Triangle Park, NC). The plasmid for CYP2C19 was modified to add a G₃H₆ motif at the C-terminus to facilitate purification as described for CYP17A1. The b_5 mutation plasmids (Table S1 of Supporting Information) were prepared as described previously (25). Plasmid pGro7 encoding the chaperones GroEL and GroES was purchased from ThermoFisher (Hudson, NH).

Expression and purification of recombinant proteins in *E. coli*

The plasmids encoding modified CYP2E1, POR, or b_5 protein were individually transformed in *E. coli* strain C41(DE3) cells (OverExpress, Lucigen, Middleton, WI), and the plasmids encoding modified human CYP17A1 or CYP2C19 were transformed in *E. coli* strain JM109 cells containing GroEL/GroES chaperones (pGro7 plasmid) to increase expression of active enzymes. In all cases, 1 liter of Terrific Broth supplemented with 0.5 mM 5-aminolevulinic acid and appropriate antibiotics was inoculated with 20 mL of an

overnight pre-culture. The cells were grown at 37 °C with shaking at 250 rpm until the A₆₀₀ reached 0.6–1.2 AU, at which time the culture was induced with 0.4 mM IPTG (10 mM L-arabinose was also added during pGro7 co-expression) and grown for 48 h at 25–27°C. After cell lysis with a French press, the recombinant proteins were solubilized using mild non-denaturing detergents: 0.8% octylglucoside/0.5% cholate for CYP2E1 and CYP2C19; 0.8% Nonidet P-40 for CYP17A1; 0.2% cholate/0.2% Triton X-100 for POR. After centrifugation at 100,000 × *g* for 45 min, the supernatant was mixed with Ni-Ni-NTA affinity resin and purified to homogeneity in a single step upon elution with 300 mM imidazole, followed by buffer exchange using PD-10 columns. Yields averaged approximately 100 nmol pure protein per liter of culture. The protocol for expression and reconstitution of recombinant human *b*₅ was based on the procedure of Mulrooney and Waskell (27). Yeast microsomes containing native human CYP17A1 and POR and control microsomes without human P450 enzymes were prepared from strain YiV(B) transformed with V60-c17 or native V60 plasmids, respectively, as described (28).

Preparation of [7-³H]-17-hydroxypregnenolone

The [7-³H]-17-hydroxypregnenolone was prepared by enzymatic conversion of [7-³H]-pregnenolone using recombinant CYP17A1 and POR used in these experiments. The [7-³H]-pregnenolone (0.25 mCi in ethanol) was divided into 4 equal aliquots in 2 mL polypropylene tubes and concentrated to dryness under a nitrogen stream. The residues were each carefully dissolved in 10 μL ethanol and slowly diluted with 0.4 mL of 50 mM potassium phosphate buffer (pH 7.4, 20% glycerol) with 6 mM potassium acetate, 10 mM MgCl₂, and 1 mM glutathione. Purified CYP17A1 (80 nmol/L), POR (320 nmol/L), DLPC (30 μM), and 1 mM NADPH were added, and the reaction mixtures were incubated at 37 °C for 40 min. We found that by using DLPC detergent, the 17,20-lyase activity was very poor, which enabled us to run the reaction to completion with maximal 17-hydroxypregnenolone yield yet minimal formation of the subsequent metabolite, dehydroepiandrosterone (DHEA). The steroids were extracted twice with 1 mL dichloromethane, and the combined extracts were dried under nitrogen. The residue was dissolved in ~25 μL dichloromethane and loaded onto a silica gel (Dynamic Adsorbents Inc, Norcross, GA) column in a 2 mL disposable glass pipette conditioned with hexanes. The steroids were eluted with a stepwise gradient of ethyl acetate in hexanes: 5 mL 10%, 10 mL 20%, 10 mL 30%, and 10 mL 40% ethyl acetate. Fractions (2 mL) were collected and analyzed by HPLC, and fractions containing pure 17-hydroxypregnenolone were pooled and further concentrated. Pregnenolone eluted with 10% ethyl acetate in hexanes, followed by DHEA and then 17-hydroxypregnenolone, both with 20% ethyl acetate in hexanes or higher. The yield of [7-³H]-17-hydroxypregnenolone was 120 × 10⁶ CPM or ~50%.

Reconstituted enzyme assays

In a 2 mL polypropylene tube, each purified human P450 was mixed with a 2–4-fold molar excess of POR, various amounts of *b*₅, and 30 μM DLPC (or control yeast microsomes [CYMS] as an alternative lipid source, 20 μg protein) in less than 10 μL volume and incubated for 5 min. The reaction mixture was then diluted to 0.2 mL with 40 mM HEPES buffer (pH = 7.4), 30 mM MgCl₂, 2.4 mM glutathione, and substrates (*S*)-mephenytoin for CYP2C19 (0.1 mM, 0.6% methanol), chlorzoxazone for CYP2E1 (0.2 mM, 0.9 mM KOH), or 7-[³H]-17-hydroxypregnenolone for CYP17A1 (0.6 μM with 80,000 CPM, 2% methanol). Experiments with microsomes containing native CYP17A1 and POR from transformed yeast used 50 mM potassium phosphate buffer (pH 7.4) instead of HEPES. The resulting mixture was pre-incubated at 37 °C for 3 min before adding NADPH (1 mM) and incubating at 37 °C for another 20–30 min. The reaction mixture was extracted with 1 mL methylene chloride, and the organic phase was dried under nitrogen flow.

Chromatography and data acquisition

Reaction products were analyzed using the Agilent 1260 Infinity HPLC system with UV detector and β -RAM4 in-line scintillation counter (LabLogic, Brandon, FL). Incubation extracts were dissolved in 20 μ L of methanol, and 5 μ L injections were resolved with a 50×2.1 mm, 2.6 μ m, C₈ Kinetex column (Phenomenex, Torrance, CA) equipped with a guard column at a flow rate of 0.4 mL/min. Aqueous methanol linear gradients were employed as follows: for CYP2E1 chlorzoxazone 6-hydroxylation assay, 10% methanol for 2.5 min, gradient to 75% methanol over 3 min, and isocratic 75% methanol to 9 min; for CYP2C19 (*S*)-mephenytoin 4-hydroxylation assay, 27% methanol for 5 min and gradient to 100% methanol over 8 min; for CYP17A1 17,20-lyase assay, 27% methanol from 0 to 0.5 min, jump to 39% methanol, and gradient from 39% to 75% methanol over 30 min. Products were identified by retention times of external standards chromatographed at the beginnings and ends of the experiments using absorbance at 290 nm (A_{290}) for CYP2E1 and 210 nm (A_{210}) for CYP2C19. For CYP17A1, radiochemical detection of [³H]-steroids employed Bio-SafeII scintillation cocktail (Research Products International, Mount Prospect, IL) at a flow rate of 1.2 mL/min. The data were processed with Laura4 software (LabLogic) and graphed with GraphPad (GraphPad Software, San Diego, CA).

Reduction of b_5 by NADPH and POR

POR (32 pmol) was incubated with b_5 (1 or 4 nmol) and 1.1 mM NADPH in 0.2 M potassium phosphate, pH 7.5, with 0.05% CHAPS in a final volume of 0.3 mL. The absorbance at 424 nm (A_{424}) was monitored for 2.5 min at 25 °C with data points collected every 5 s using a Shimadzu 2600 UV-visible spectrophotometer (Addison, IL). The A_{424} data as a function of time were fit to an exponential growth curve using Origin 7.5 (OriginLab, Northampton, MA):

$$y(t) = A \times (1 - e^{-kt}) + C$$

where $y(t)$ is the absorbance y at time t , C is the initial absorbance, A is a constant, and k is the first-order rate constant; therefore, the rate of reduction is:

$$y'(t) = A \times k e^{-kt} \quad \text{and} \quad y'(0) = A \times k$$

in AU·s⁻¹. Rates were converted to turnover numbers (min⁻¹) using $\epsilon = 90 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 424 nm, 1 cm path length, and [POR] = 0.11 μ M, for [b_5] = 3.3 or 13.3 μ M.

Results

Human cytochrome b_5 residues D58 and D65 are essential for stimulating CYP2E1 chlorzoxazone-6-hydroxylation

To probe the interaction of CYP2E1 with b_5 , site-directed mutagenesis was utilized to generate a series of b_5 variants with glycine substitutions in conserved surface-exposed residues around the heme-binding pocket. All mutations displayed absorption spectra comparable to that of the wild-type b_5 in the reduced and oxidized state (Figure S1 of Supporting Information), indicating that these mutations did not significantly affect the iron coordination or the electronic structure of the heme moiety.

Each mutation was tested for its capacity to increase the rate of chlorzoxazone 6-hydroxylation, a characteristic b_5 -stimulated CYP2E1 activity (Figure 1A). The presence of wild-type b_5 increased the rate of chlorzoxazone metabolism as great as a 15-fold when the

reaction is catalyzed by a reconstituted system containing purified CYP2E1, POR and DLPC (P450:POR:*b*₅ molar ratio 1:3:3). The activity of *b*₅ mutations K33G, D36G, D58G and D65G was reduced to less than 60% that of wild-type *b*₅, suggesting that acidic residues at these positions might be important for the association of *b*₅ with CYP2E1 and/or POR. In contrast, the other mutations stimulated chlorzoxazone 6-hydroxylation similar to wild-type *b*₅, indicating that these other residues are not critical for *b*₅ binding and electron transfer within the CYP2E1•POR catalytic complex.

Comparison between *b*₅ action on CYP2E1 and CYP17A1 using purified proteins reconstituted with DLPC versus yeast microsomal assay systems

We previously showed that, in contrast to CYP2E1, residues E48 and E49 of *b*₅ are essential for stimulating the 17,20-lyase activity of CYP17A1, both in yeast microsomes and in a reconstituted system with purified, modified CYP17A1 and POR. As a negative control, we again found reduced stimulation of 17,20-lyase activity for *b*₅ mutations E48G, E49G, E48G+E49G, and E52G (Table 1). Mutations D36G and D58G also showed significantly reduced stimulation of 17,20-lyase activity, but only in the reconstituted system containing DLPC. These data suggest that the contributions of *b*₅ residues D36 and D58 to the CYP17A1-catalyzed 17,20-lyase activity varies as a function of the lipids present in the assay system.

To explore this phenomenon further, we asked whether the lipids in yeast microsomes could restore to the reconstituted assay system with purified, modified CYP17A1 and POR the behavior observed in microsomes from yeast expressing native CYP17A1 and POR. For these experiments, microsomes from yeast strain YiV(B) transformed with empty plasmid V60 were used as a lipid source (control yeast microsomes, CYMS) for reconstitution. The 8 mutations of *b*₅ were again tested for their capacity to stimulate the 17,20-lyase activity of purified CYP17A1 and POR when reconstituted with control yeast microsomal lipid. The results for assays with purified, modified CYP17A1 and POR reconstituted with CYMS were indistinguishable from those obtained with yeast microsomes containing native CYP17A1 and POR (Table 1). These data provide strong evidence that E48 and E49 are the critical *b*₅ residues required for CYP17A1-catalyzed 17,20-lyase activity and androgen formation.

For CYP2E1, reconstitution with CYMS restored the stimulatory action of *b*₅ mutations K33G and D36G but not D58G and D65G (Figure 1B). Only *b*₅ mutations D58G and D65G showed markedly reduced capacity to stimulate CYP2E1-catalyzed chlorzoxazone 6-hydroxylation in both conditions. Finally, to assess the individual contributions of D58 and D65 of *b*₅ to stimulating CYP2E1-catalyzed chlorzoxazone 6-hydroxylation, the double mutation D58G+D65G was constructed, expressed and purified. The double mutation D58G+D65G was no longer capable of enhancing CYP2E1-catalyzed chlorzoxazone 6-hydroxylation (Figure 1C) yet retained full capacity to stimulate the 17,20-lyase activity of CYP17A1 reconstituted with CYMS (Table 1). These data corroborate initial results and identify *b*₅ residues D58 and D65 as indispensable components for catalytically productive interactions with CYP2E1 but not CYP17A1.

Effect of phospholipids on *b*₅ stimulation of CYP17A1 and CYP2E1 activities

The lipid composition of yeast microsomes contains 50% phosphatidylcholine (PC), 20–30% phosphatidylethanolamine (PE), 7–10% phosphatidylserine (PS), and smaller amounts of other phospholipids (29, 30). To dissect the contributions of these individual phospholipids to *b*₅ action on the P450•POR complex, experiments were carried out with the purified, reconstituted system in the presence of various phospholipids, as presented in Figure 2. For CYP17A1, wild-type *b*₅ stimulated 17,20 lyase activity when reconstituted with any phospholipid in the order PS>PE>PC; activity with anionic PS was about twice

that with cationic PC. Combinations of two phospholipids gave similar or higher activity than either phospholipid alone, and reconstitution with PE+PS gave the highest 17,20-lyase activity with wild-type b_5 , about 3-fold higher than assays using PC alone (Figure 2A).

In contrast, b_5 mutation D36G stimulated 17,20-lyase activity equivalent to or better than wild-type b_5 using PS and/or PE but minimally in assays reconstituted with PC or PC+PE. The increase in 17,20-lyase activity for incubations with CYP17A1, POR, and b_5 mutation D36G reconstituted with PS+PE was dramatic, up to 16-fold higher than assays using PC alone (Figure 2A). Finally, b_5 mutation E48G+E49G failed to stimulate 17,20-lyase activity in the assays reconstituted with any of the three phospholipids alone or in combination, consistent with results using yeast microsomes. These data support the model that b_5 residues E48 and E49 are critical for stimulating the 17,20-lyase activity of CYP17A1, whereas other residues such as D36 and D58 are required under some conditions.

Compared to results with CYP17A1, a similar influence of phospholipid was observed for the action of wild-type b_5 and b_5 mutation D36G on CYP2E1-catalyzed chlorzoxazone 6-hydroxylation. CYP2E1 assays reconstituted with PS and PE alone and in combination gave higher activity than those with PC alone, although the difference between wild-type b_5 and b_5 mutation D36G in PC was difficult to assess and minor compared to the significant difference seen with CYP17A1 (Figure 2B). Thus, optimal interaction between b_5 mutation D36G and the CYP17A1 and CYP2E1 catalytic complexes requires the presence of PS or PE. Consistent with results of assays reconstituted with yeast microsomal lipids, the b_5 double mutation D58G+D65G did not stimulate chlorzoxazone 6-hydroxylation under any conditions tested, which confirms the importance of residues D58 and D65 for interactions with CYP2E1.

Human cytochrome b_5 residues D58 and D65 are also essential for stimulating CYP2C19 progesterone 21-hydroxylation and (S)-mephenytoin 4-hydroxylation

Given the contrasting effects of apo- b_5 on CYP17A1 and CYP2E1, we asked whether the vulnerability to mutations in b_5 residues D58 and D65 was a property unique to P450 reactions for which apo- b_5 cannot substitute for holo- b_5 . Consequently, we determined the amino acids required for the stimulatory action of b_5 on CYP2C19 catalysis using two different substrates, progesterone and (S)-mephenytoin. Compared to the 15-fold increase in CYP2E1-catalyzed chlorzoxazone 6-hydroxylation, wild-type b_5 stimulates CYP2C19-catalyzed progesterone and (S)-mephenytoin hydroxylation only 2- or 3-fold, respectively. In a reconstituted system containing purified CYP2C19, POR, and DLPC, b_5 mutations D36G, D58G, and D65G afforded lower activity compared to wild-type b_5 for both progesterone 21-hydroxylation and (S)-mephenytoin 4-hydroxylation (Figure 3A and 3B). When the assay was reconstituted using CYMS, however, only b_5 mutations D58G and D65G demonstrated reduced capacity to enhance (S)-mephenytoin 4-hydroxylation (Figure 3C). Furthermore, the double mutation D58G+D65G totally abolished the ability of b_5 to enhance these CYP2C19-catalyzed reactions, demonstrating that b_5 residues D58 and D65 are required for stimulating not only CYP2E1 but also CYP2C19 catalysis. A similar preferential influence of PS and PE phospholipids, as observed for the action of b_5 and b_5 mutation D36G action on CYP17A1 and CYP2E1, was also observed in reconstituted assays with CYP2C19, POR, and b_5 (Figure 3D). We conclude that, as for CYP2E1, b_5 residues D58 and D65 are critical for stimulating these CYP2C19-catalyzed oxygenations.

Competition assays using wild-type b_5 and mutation D58G+D65G in CYP2E1 chlorzoxazone 6-hydroxylation and CYP2C19 (S)-mephenytoin 4-hydroxylation

To explain the inability of b_5 mutation D58G+D65G to stimulate CYP2E1-catalyzed chlorzoxazone 6-hydroxylation and CYP2C19-catalyzed (S)-mephenytoin 4-hydroxylation,

we considered two likely possibilities. Either b_5 mutation D58G+D65G binds to the P450s similar to wild-type b_5 but fails to elicit the biophysical changes required to augment turnover, or the double-mutation binds poorly to the P450s. To distinguish these two possibilities, we performed *competition assays* using the double mutation D58G+D65G and wild-type b_5 in a reaction with CYP2E1, POR, and CYMS as described previously (25). In the first experiment, the concentration of wild-type b_5 was held constant at the optimum 3:1 b_5 /CYP2E1 molar ratio, and increasing amounts of mutation D58G+D65G were added. As shown in Figure 4A, b_5 mutation D58G+D65G did not decrease chlorzoxazone 6-hydroxylation activity until a 5-fold molar excess was added and the total b_5 /CYP2E1 molar ratio reached 18:1. When the reaction was assayed using wild-type b_5 and CYP2E1 at a molar ratio of 15:1, the activity was also reduced, consistent with a distinct effect of excess b_5 proteins on turnover. Previously, we suggested that this inhibitory phenomenon might derive from competition between b_5 and the P450 for limiting POR, since POR transfers electrons to both hemoproteins *in vitro*. Nevertheless, these data suggest that b_5 mutation D58G+D65G does not compete with wild-type b_5 for the binding site on the CYP2E1•POR complex, which is required to stimulate chlorzoxazone-6-hydroxylation.

The competition experiments were also performed in the reverse scenario, in which chlorzoxazone-6-hydroxylation was assayed in the presence of a constant 3:1 ratio of mutation D58G+D65G to CYP2E1, and increasing amounts of wild-type b_5 were added. In these experiments, the presence of mutation D58G+D65G did not impair the capacity of wild-type b_5 to stimulate chlorzoxazone-6-hydroxylation activity at wild-type b_5 /CYP2E1 molar ratios as low as 0.3:1 (Figure 4A). The activity peaked at a wild-type b_5 /CYP2E1 molar ratio of 3:1 and declined progressively thereafter, consistent with b_5 competition with the P450 for limiting POR. These data suggest that b_5 interacts with the CYP2E1•POR complex using a binding site that includes residues D58 and D65. Similar results were obtained for CYP2C19-catalyzed (*S*)-mephenytoin 4-hydroxylation assays, in competition experiments between wild-type b_5 and b_5 mutation D58G+D65G (Figure 4B). These experiments provide additional evidence for the prominent contributions of the D58 and D65 side chains in the interaction of b_5 with the CYP2C19•POR complex as well.

Kinetics of b_5 and b_5 mutation reduction by POR and NADPH

To probe the mechanism of impaired P450 stimulation, we asked whether impaired kinetics of flavoprotein reduction could explain the altered function of these b_5 mutations, particularly for CYP2E1-catalyzed chlorzoxazone 6-hydroxylation. As we found previously in a slightly different assay system, POR reduced mutations E48G, E49G, and E48G+E49G at rates similar to that of wild-type b_5 (Table 2). In contrast, the rates of b_5 reduction for mutations D36G, D58G, and D65G were 66%, 61%, and 41% the rate for wild-type b_5 ; however, the rate of reduction for the inactive double-mutation D58G+D65G was 64% the rate for wild-type b_5 . Consequently, the rates of flavoprotein-mediated electron transfer to b_5 mutations of D36, D58, and D65 are somewhat lower than for wild-type b_5 , but the magnitude of this change is too small to fully account for the perturbed function of these mutations, and the magnitude of the decrease in these reduction rates does not correlate with their impaired stimulation of P450 activities. The discrepancies in rates of electron transfer and magnitudes of P450 stimulation for these b_5 mutations suggest that conformational or binding influences of b_5 , rather than flavoprotein reduction, mediate the stimulatory action on these P450-catalyzed reactions.

Discussion

The ability of a single b_5 protein to influence the activities of multiple P450 enzymes and to function concurrently in other electron transfer systems constitutes a paradox of biochemistry. The promiscuous nature of b_5 actions, however, offers a platform to study the

biochemistry and biophysics of the b_5 interactions with different P450 enzymes in detail. Using site-directed mutagenesis to probe these interactions, we generated multiple lines of evidence, which all implicate D58 and D65 as two b_5 residues essential for stimulating hydroxylation reactions catalyzed by CYP2E1 and CYP2C19; however, D58 and D65 appear to be dispensable for enhancing CYP17A1-catalyzed 17,20-lyase activity. Conversely, E48 and E49 of b_5 are required for stimulating high 17,20-lyase activity, but these residues are not necessary to stimulate the catalytic activities of CYP2E1 and CYP2C19. Based on our results, we propose a model in which b_5 binds to P450s via at least two distinct sets of anionic surfaces.

An amino acid sequence alignment of microsomal b_5 from mammals, yeast, and plants is shown in Figure 5. Of the 134 amino acids, 23 are acidic and highly conserved, including E49 as well as D58 and D65, which were identified as key residues in this study. A ribbon diagram of human b_5 based on its NMR solution structure is shown in Figure 6A, highlighting the bound b-type heme prosthetic group and the location of acidic amino acid residues identified in this study. The b_5 structure belongs to the α + β protein fold, with two hydrophobic core domains separated by a five-stranded β -sheet. The heme is inserted in the larger hydrophobic core domain between four α -helices, and two imidazole side chains of completely conserved histidines, H68 and H44, coordinate the heme iron and prevent direct interaction of b_5 with molecular oxygen (2). The smaller hydrophobic core domain, which might function primarily to maintain structural integrity, is formed by spatially juxtaposed N-terminal and C-terminal segments. The highly conserved residues E48 and E49, which are critical for interactions with CYP17A1, are located adjacent to the exposed heme edge at the start of the short α -helix 3, and D36 resides at the end of the β -strand 4, closer to the heme itself and less exposed to solvent than E48 and E49. D65 is located near the entrance of the heme-pocket crevice on the C-terminus of α -helix 4, close to the heme axial ligand H68, while D58 which is located at β -bridge 1, near the bottom of the heme pocket on a contiguous surface with D65, formed primarily by α -helix 4 (Figure 6B). Our data suggest that electrostatic forces generated by this surface region stabilize the binding of b_5 to CYP2E1 and CYP2C19 and/or their P450•POR complexes.

Since many laboratories have employed a variety of approaches with several different P450s to study the P450- b_5 interaction, it is difficult to generalize conclusions from the existing data to all P450s. Chemical cross-linking and tandem mass spectrometry analysis identified two intermolecular cross-links, K428 and K434 of CYP2E1 with D53 and D56 of b_5 , respectively (22). These data led to a structural model of the CYP2E1• b_5 complex, which suggested seven more ion pairs between the two proteins. No attempts have been made, however, to determine the functional significance of these residues on b_5 . Our functional data corroborate one of these ion pairs containing the conserved acidic residue D53 of rat b_5 , which corresponds to D58 of human b_5 . In contrast, we found that E56 of rat b_5 , which corresponds to E61 of human b_5 , is not essential for stimulating CYP2E1 chlorzoxazone 6-hydroxylation, whereas D65 is also required under our experimental conditions. It is possible that K434 on CYP2E1 closely approaches E61 during turnover, but this ion pair appears to contribute little to catalysis and complex stability.

Assays with apo- b_5 , which retains most of the tertiary structure of holo- b_5 but is inactive as an electron carrier, have been employed to discriminate the redox and allosteric effects of the b_5 ; however, the potential for apo- b_5 reconstitution with free heme might introduce artifacts into these experiments (17). Indeed, many b_5 actions in reconstituted P450 assays are also observed with redox-inert apo- b_5 (13–16) or Mn- b_5 (31), including the stimulation of human CYP2C19-catalyzed (*S*)-mephenytoin 4-hydroxylase activity and of CYP17A1-catalyzed 17,20-lyase activity (14, 26). In contrast, CYP2E1-catalyzed chlorzoxazone 6-hydroxylation is stimulated by b_5 but not by apo- b_5 (14), suggesting at least two distinct

mechanisms of b_5 action. We had hypothesized that the few P450s for which apo- b_5 has not been shown to substitute for holo- b_5 , such as CYP2E1, would interact with b_5 in a unique manner. Our data, however, indicate that residues D58 and D65 are essential for the b_5 interaction with both CYP2E1 and CYP2C19 but not CYP17A1, leading us to reject this hypothesis. We cannot exclude the possibility that some of the reasons why b_5 mutation D58G+D65G fails to stimulate CYP2E1 catalysis are different than for CYP2C19, but the inability of this double mutation to antagonize the stimulatory effect of wild-type b_5 on both P450s (Figure 4) suggests that all normal binding interactions are lost for the two P450s. Our results have identified at least two interaction surfaces of b_5 with P450•POR complexes (Figure 6), but we have not determined specific properties of these reactions, which predict the residues of b_5 essential for modulating each P450-catalyzed activity.

The molecular details of how b_5 influences P450•POR complexes remain elusive. For the CYP17A1-catalyzed 17,20-lyase activity, some studies suggest that b_5 does not act alone as an electron donor, but rather functions in concert with POR to aid catalysis, possibly by an allosteric mechanism (32). A computer modeling study suggests that the heme group of b_5 contacts neither POR nor CYP17A1, favoring an allosteric action (33), which is induced by changes in the energy landscape as these proteins approach (34). On the other hand, either POR or reduced b_5 alone can provide the second electron transfer to oxyferrous CYP2B4 during the catalytic cycle, which is 10–100 times faster with b_5 than with POR (35). High b_5 :POR molar ratios inhibit CYP2B4 catalysis, because the acidic, convex surface of b_5 competes with POR for the basic, concave surface of CYP2B4 (31) and the reduction of the oxyferrous P450. These studies suggest that D65 and V66 of b_5 are in contact with R122, R126, and K433 of CYP2B4 and that a catalytically competent POR• b_5 •CYP2B4 ternary complex cannot form (36). In addition, solid-state NMR has been used to identify interactions between full-length P450 and full-length b_5 , including those within the membrane (37). Despite these advances, the reasons why b_5 performs distinct functions with individual P450 enzymes, such as electron transfer with CYP2E1 and CYP2B4 or allosteric effects with CYP17A1 and CYP2C19, are not known.

The phospholipid composition has been shown to influence the activity of multiple P450 enzymes (38, 39); however, this contribution is often neglected. For CYP17A1, wild-type b_5 stimulated 17,20-lyase activity in the presence of all phospholipids tested, with 2-fold higher activity as the head group changes from strong cationic (PC) to weaker cationic (PE) to anionic (PS). Moreover, b_5 mutation D36G was 16-fold more potent with PS as compared to PC and stimulated 17,20-lyase activity equivalent to wild-type b_5 in PS (Figure 1B). A similar but less convincing pattern was observed for CYP2E1-catalyzed chlorzoxazone 6-hydroxylation with wild-type b_5 and mutation D36G (Figure 2B). In all reconstituted assays studied with any combination of phospholipids, however, b_5 mutations E48G+E49G or D58G+D65G consistently failed to stimulate the characteristic activities of CYP17A1 or CYP2E1 and CYP2C19, respectively, confirming the importance of these residues for these reactions. The poor ability of b_5 , particularly mutation D36G, to stimulate these P450 activities when reconstituted with PC might derive from the strongly cationic head group of PC or from the relatively short 12-carbon fatty acyl chains of the DLPC used in our assays. Although we did not study other PC lipids in detail, CYP2E1 chlorzoxazone 6-hydroxylation assays reconstituted with 1,2-dioctadecyl-*sn*-glycero-3-phosphocholine (DOPC, 18-carbon fatty acyl chains) gave results similar to DLPC (not shown), suggesting that the head group is the more important variable. The PE and PS lipids used were mixtures with various fatty acyl chains, limiting insight to the importance of chain length in regulating b_5 action in assays reconstituted with PS or PE.

The interaction with phospholipid membranes induces complex conformational changes in proteins (40), including a structural transition in the heme-binding domain of soluble b_5 to a

partially unfolded state with molten globule-like properties at high phospholipid/protein ratios (41), which enhances its binding to the membrane. By comparison, phospholipids increase the α -helical content and lower the β -sheet content of CYP1A2 and CYP2B1 and induce a shift from low-spin to high-spin heme iron states (38, 39). Because the membrane-spanning C-terminal helix of b_5 is essential for its capacity to influence P450 activities (42), the properties of these protein-phospholipid interactions are undoubtedly critical for the actions of b_5 on P450s. Although little is known about the specific structural changes these protein-phospholipid interactions induce, b_5 mutation D36G might be a useful tool to probe this process.

Our systematic study of b_5 mutations with CYP17A1, CYP2E1, and CYP2C19 has provided data consistent with a model in which b_5 interacts with P450 or P450•POR complexes via at least two critical surface domains, which incorporate the critical residues E48 and E49 or D58 and D65 (Figure 6). Our data do not permit us to conclude whether these critical residues directly participate in b_5 interactions with P450 or P450•POR complexes or explain how these interactions enhance catalysis. An understanding of this mechanism of b_5 action on specific P450•POR complexes, for example, might lead to a drug that disrupts the b_5 action on CYP17A1, thus selectively inhibiting 17,20-lyase activity and androgen biosynthesis without causing 17-hydroxylase deficiency. Such an agent could be used to treat castration-resistant prostate cancer, similar to the CYP17A1 inhibitor abiraterone acetate (43), yet lack the side effects derived from simultaneously inhibiting 17-hydroxylase activity (44).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

b_5	cytochrome b_5
CYMS	control yeast microsomes
CYP17A1	cytochrome P450c17
DAPS	1,2-diacyl- <i>sn</i> -glycero-3-phospho-L-serine
DHEA	dehydroepiandrosterone
DLPC	1,2-didodecanoyl- <i>sn</i> -glycero-3-phosphocholine
DAPE	1,2-diacyl- <i>sn</i> -glycero-3-phosphoethanolamine
DTT	DL-dithiothreitol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
IPTG	isopropyl- β -D-thiogalactopyranoside
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)

PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidyl serine
POR	cytochrome P450 oxidoreductase
UV	ultraviolet light

References

1. Ozols J. Structure of cytochrome *b*₅ and its topology in the microsomal membrane. *Biochim Biophys Acta*. 1989; 997:121–130. [PubMed: 2752049]
2. Schenkman JB, Jansson I. The many roles of cytochrome *b*₅. *Pharmacol Ther*. 2003; 97:139–152. [PubMed: 12559387]
3. Hildebrandt A, Estabrook RW. Evidence for the participation of cytochrome *b*₅ in hepatic microsomal mixed-function oxidation reactions. *Arch Biochem Biophys*. 1971; 143:66–79. [PubMed: 4397839]
4. Hegesh E, Hegesh J, Kaftory A. Congenital methemoglobinemia with a deficiency of cytochrome *b*₅. *N Engl J Med*. 1986; 314:757–761. [PubMed: 3951505]
5. Sakai Y, Yanase T, Takayanagi R, Nakao R, Nishi Y, Haji M, Nawata H. High expression of cytochrome *b*₅ in adrenocortical adenomas from patients with Cushing's syndrome associated with high secretion of adrenal androgens. *J Clin Endocrinol Metab*. 1993; 76:1286–1290. [PubMed: 8496319]
6. Giordano SJ, Kaftory A, Steggles AW. A splicing mutation in the cytochrome *b*₅ gene from a patient with congenital methemoglobinemia and pseudohermaphroditism. *Hum Genet*. 1994; 93:568–570. [PubMed: 8168836]
7. Kok RC, Timmerman MA, Wolffenbuttel KP, Drop SL, de Jong FH. Isolated 17,20-lyase deficiency due to the cytochrome *b*₅ mutation W27X. *J Clin Endocrinol Metab*. 2010; 95:994–999. [PubMed: 20080843]
8. Idkowiak J, Randell T, Dhir V, Patel P, Shackleton CH, Taylor NF, Krone N, Arlt W. A missense mutation in the human cytochrome *b*₅ gene causes 46,XY disorder of sex development due to true isolated 17,20 lyase deficiency. *J Clin Endocrinol Metab*. 2012; 97:E465–475. [PubMed: 22170710]
9. Suzuki T, Sasano H, Takeyama J, Kaneko C, Freije WA, Carr BR, Rainey WE. Developmental changes in steroidogenic enzymes in human postnatal adrenal cortex: immunohistochemical studies. *Clin Endocrinol (Oxf)*. 2000; 53:739–747. [PubMed: 11155097]
10. Auchus RJ, Rainey WE. Adrenarche - physiology, biochemistry and human disease. *Clin Endocrinol (Oxf)*. 2004; 60:288–296. [PubMed: 15008992]
11. Finn RD, McLaughlin LA, Ronseaux S, Rosewell I, Houston JB, Henderson CJ, Wolf CR. Defining the in Vivo Role for cytochrome *b*₅ in cytochrome P450 function through the conditional hepatic deletion of microsomal cytochrome *b*₅. *J Biol Chem*. 2008; 283:31385–31393. [PubMed: 18805792]
12. Gorsky LD, Coon MJ. Effects of conditions for reconstitution with cytochrome *b*₅ on the formation of products in cytochrome P-450-catalyzed reactions. *Drug Metab Dispos*. 1986; 14:89–96. [PubMed: 2868871]
13. Yamazaki H, Shimada T, Martin MV, Guengerich FP. Stimulation of cytochrome P450 reactions by apo-cytochrome *b*₅: evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome *b*₅ or heme oxygenase. *J Biol Chem*. 2001; 276:30885–30891. [PubMed: 11413149]
14. Yamazaki H, Gillam EM, Dong MS, Johnson WW, Guengerich FP, Shimada T. Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with cytochrome P450 3A4 and other forms: effects of cytochrome P450-P450 and cytochrome P450- *b*₅ interactions. *Arch Biochem Biophys*. 1997; 342:329–337. [PubMed: 9186495]

15. Yamazaki H, Johnson WW, Ueng YF, Shimada T, Guengerich FP. Lack of electron transfer from cytochrome *b*₅ in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome *b*₅. *J Biol Chem*. 1996; 271:27438–27444. [PubMed: 8910324]
16. Yamazaki H, Nakamura M, Komatsu T, Ohyama K, Hatanaka N, Asahi S, Shimada N, Guengerich FP, Shimada T, Nakajima M, Yokoi T. Roles of NADPH-P450 reductase and apo- and holo-cytochrome *b*₅ on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. *Protein Expr Purif*. 2002; 24:329–337. [PubMed: 11922748]
17. Guryev OL, Gilep AA, Usanov SA, Estabrook RW. Interaction of apo-cytochrome *b*₅ with cytochromes P4503A4 and P45017A: relevance of heme transfer reactions. *Biochemistry*. 2001; 40:5018–5031. [PubMed: 11305918]
18. Guengerich FP, Johnson WW. Kinetics of ferric cytochrome P450 reduction by NADPH-cytochrome P450 reductase: rapid reduction in the absence of substrate and variations among cytochrome P450 systems. *Biochemistry*. 1997; 36:14741–14750. [PubMed: 9398194]
19. Guengerich FP. Cytochrome P450: what have we learned and what are the future issues? *Drug Metab Rev*. 2004; 36:159–197. [PubMed: 15237850]
20. Miller WL. Molecular biology of steroid hormone synthesis. *Endocr Rev*. 1988; 9:295–318. [PubMed: 3061784]
21. Bridges A, Gruenke L, Chang YT, Vakser IA, Loew G, Waskell L. Identification of the binding site on cytochrome P450 2B4 for cytochrome *b*₅ and cytochrome P450 reductase. *J Biol Chem*. 1998; 273:17036–17049. [PubMed: 9642268]
22. Gao Q, Doneanu CE, Shaffer SA, Adman ET, Goodlett DR, Nelson SD. Identification of the interactions between cytochrome P450 2E1 and cytochrome *b*₅ by mass spectrometry and site-directed mutagenesis. *J Biol Chem*. 2006; 281:20404–20417. [PubMed: 16679316]
23. Geller DH, Auchus RJ, Mendonca BB, Miller WL. The genetic and functional basis of isolated 17,20-lyase deficiency. *Nat Genet*. 1997; 17:201–205. [PubMed: 9326943]
24. Geller DH, Auchus RJ, Miller WL. P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome *b*₅. *Mol Endocrinol*. 1999; 13:167–175. [PubMed: 9892022]
25. Naffin-Olivos JL, Auchus RJ. Human cytochrome *b*₅ requires residues E48 and E49 to stimulate the 17,20-lyase activity of cytochrome P450c17. *Biochemistry*. 2006; 45:755–762. [PubMed: 16411751]
26. Auchus RJ, Lee TC, Miller WL. Cytochrome *b*₅ augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *J Biol Chem*. 1998; 273:3158–3165. [PubMed: 9452426]
27. Mulrooney SB, Waskell L. High-level expression in *Escherichia coli* and purification of the membrane-bound form of cytochrome *b*₅. *Protein Expr Purif*. 2000; 19:173–178. [PubMed: 10833404]
28. Sherbet DP, Tiosano D, Kwist KM, Hochberg Z, Auchus RJ. CYP17 mutation E305G causes isolated 17,20-lyase deficiency by selectively altering substrate binding. *J Biol Chem*. 2003; 278:48563–48569. [PubMed: 14504283]
29. Gaigg B, Simbeni R, Hrastnik C, Paltauf F, Daum G. Characterization of a microsomal subfraction associated with mitochondria of the yeast, *Saccharomyces cerevisiae* Involvement in synthesis and import of phospholipids into mitochondria. *Biochim Biophys Acta*. 1995; 1234:214–220. [PubMed: 7696296]
30. Zinser E, Sperka-Gottlieb CD, Fasch EV, Kohlwein SD, Paltauf F, Daum G. Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. *J Bacteriol*. 1991; 173:2026–2034. [PubMed: 2002005]
31. Zhang H, Hamdane D, Im SC, Waskell L. Cytochrome *b*₅ inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4. *J Biol Chem*. 2008; 283:5217–5225. [PubMed: 18086668]
32. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011; 32:81–151. [PubMed: 21051590]

33. Pandey AV, Miller WL. Regulation of 17,20 lyase activity by cytochrome *b*₅ and by serine phosphorylation of P450c17. *J Biol Chem*. 2005; 280:13265–13271. [PubMed: 15687493]
34. Csermely P, Palotai R, Nussinov R. Induced fit, conformational selection and independent dynamic segments: an extended view of binding events. *Trends Biochem Sci*. 2010; 35:539–546. [PubMed: 20541943]
35. Zhang H, Im SC, Waskell L. Cytochrome *b*₅ increases the rate of product formation by cytochrome P450 2B4 and competes with cytochrome P450 reductase for a binding site on cytochrome P450 2B4. *J Biol Chem*. 2007; 282:29766–29776. [PubMed: 17693640]
36. Im SC, Waskell L. The interaction of microsomal cytochrome P450 2B4 with its redox partners, cytochrome P450 reductase and cytochrome *b*₅. *Arch Biochem Biophys*. 2011; 507:144–153. [PubMed: 21055385]
37. Durr UH, Yamamoto K, Im SC, Waskell L, Ramamoorthy A. Solid-state NMR reveals structural and dynamical properties of a membrane-anchored electron-carrier protein, cytochrome *b*₅. *J Am Chem Soc*. 2007; 129:6670–6671. [PubMed: 17488074]
38. Yun CH, Song M, Kim H. Conformational change of cytochrome P450 1A2 induced by phospholipids and detergents. *J Biol Chem*. 1997; 272:19725–19730. [PubMed: 9242629]
39. Yun CH, Ahn T, Guengerich FP. Conformational change and activation of cytochrome P450 2B1 induced by salt and phospholipid. *Arch Biochem Biophys*. 1998; 356:229–238. [PubMed: 9705213]
40. McIntosh TJ, Simon SA. Roles of bilayer material properties in function and distribution of membrane proteins. *Annu Rev Biophys Biomol Struct*. 2006; 35:177–198. [PubMed: 16689633]
41. Basova LV, Tiktopulo EI, Kutysenko VP, Mauk AG, Bychkova VE. Phospholipid membranes affect tertiary structure of the soluble cytochrome *b*₅ heme-binding domain. *Biochim Biophys Acta*. 2008; 1778:1015–1026. [PubMed: 18275841]
42. Lee-Robichaud P, Kaderbhai MA, Kaderbhai N, Wright JN, Akhtar M. Interaction of human CYP17 (P-450(17 α), 17 α -hydroxylase-17,20-lyase) with cytochrome *b*₅: importance of the orientation of the hydrophobic domain of cytochrome *b*₅. *Biochem J*. 1997; 321(Pt 3):857–863. [PubMed: 9032476]
43. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB Jr, Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW, Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN, Ellard SL, Flechon A, Saleh M, Scholz M, Efstathiou E, Zivi A, Bianchini D, Loriot Y, Chieffo N, Kheoh T, Haqq CM, Scher HI. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med*. 2011; 364:1995–2005. [PubMed: 21612468]
44. Attard G, Reid AH, Auchus RJ, Hughes BA, Cassidy AM, Thompson E, Oommen NB, Folkard E, Dowsett M, Arlt W, de Bono JS. Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer. *J Clin Endocrinol Metab*. 2012; 97:507–516. [PubMed: 22170708]

Figure 1.

Stimulation of CYP2E1-catalyzed chlorzoxazone 6-hydroxylation by b_5 and b_5 mutations. (A) Effect of b_5 and b_5 mutations on catalytic activities of CYP2E1 in the presence of DLPC. Incubations contained 150 μ M chlorzoxazone and a molar ratio of P450:POR: b_5 at 1:3:3. Results are shown as the percentage activity compared to wild-type b_5 values (=100%) from triplicate determinations. (B) Comparison of b_5 action on CYP2E1 catalysis in the presence of DLPC vs. control yeast microsomes (CYMS) as phospholipid. Purified CYP2E1 (50 pmol with DLPC or 30 pmol with CYMS) was mixed with POR and b_5 at 1:3:3 molar ratio for reconstitution. Results are shown as the percentage activity compared to wild-type b_5 values in DLPC (=100%) from triplicate determinations. (C) Comparison of wild-type b_5 and selected b_5 mutations on CYP2E1 catalysis in the presence of CYMS with molar ratio of P450:POR: b_5 at 1:3:3 (black bars) or 1:3:6 (white bars). Results are shown as

the percentage activity compared to wild-type b_5 values with 1:3:3 ratio of P450:POR: b_5 (=100%) from triplicate determinations.

Figure 2.

Effect of phospholipids on b_5 -stimulated catalysis of CYP17A1 and CYP2E1. (A) Purified CYP17A1 (10 pmol) and POR were reconstituted with wild-type b_5 or mutations E48G +E49G and D36G in the presence of various phospholipids at a P450:POR: b_5 molar ratio of 1:4:10. Incubation was carried out with 6 μ M 17-hydroxypregnenolone and [3 H]-17-hydroxypregnenolone ($\sim$ 0.4 μ Ci, 4 nM), and the formation of DHEA was determined by HPLC analysis. Results are shown as the percentage activity compared to wild-type b_5 values in PE+PS (=100%) from triplicate determinations. In the absence of b_5 , conversion to DHEA is $1.3 \pm 0.3\%$ for assays reconstituted with DLPC. (B) Purified CYP2E1 (30 pmol) and POR were reconstituted with wild-type b_5 or mutations D58G+D65G and D36G in the presence of various phospholipids at a molar ratio of 1:3:6. Incubations were conducted with 150 μ M chlorzoxazone, and the formation of 6-hydroxychlorzoxazone was determined by HPLC analysis as described under Experimental Procedures. Results are shown as the percentage activity compared to wild-type b_5 values in PE+PS (=100%) from triplicate determinations. In the absence of b_5 , conversion to 6-hydroxychlorzoxazone is $0.3 \pm 0.1\%$ for assays reconstituted with DLPC.

Figure 3.

Stimulation of CYP2C19 catalysis by *b*₅ and *b*₅ mutations. Purified CYP2C19 (30 pmol) was reconstituted with POR, various *b*₅ mutations, and DLPC at a P450:POR:*b*₅ molar ratio of 1:2:3. Catalytic activities toward (A) progesterone at 10 μ M substrate concentration and (B) (*S*)-mephenytoin at 100 μ M substrate concentration were analyzed by HPLC. Results are shown as the percentage activity compared to wild-type *b*₅ values (=100%) from triplicate determinations. (C) (*S*)-Mephenytoin 4-hydroxylation activity of CYP2C19 was examined with CYMS and selected *b*₅ mutations with molar ratio of P450:POR:*b*₅ at 1:2:3 (black bars) and 1:2:6 (white bars). Results are shown as the percentage activity compared to wild-type *b*₅ values at P450:POR:*b*₅ molar ratio of 1:2:3 (=100%) from triplicate determinations. (D) Purified CYP2C19 (30 pmol) and POR were reconstituted with wild-type *b*₅ or mutations D58G+D65G and D36G in the presence of various phospholipids at a molar ratio of 1:2:3. Incubation was conducted with 100 μ M (*S*)-mephenytoin, and the

formation of (*S*)-4-hydroxymephenytoin was determined by HPLC analysis as described under Experimental Procedures. Results are shown as the percentage activity compared to assays reconstituted with wild-type *b₅* and PE+PS (=100%) from triplicate determinations

Figure 4. Competition assays. (A) CYP2E1-catalyzed formation of 6-hydroxychlorzoxazone or (B) CYP2C19-catalyzed (*S*)-4-hydroxymephenytoin formation. Reactions were conducted with a constant P450:POR molar ratio of 1:3 for CYP2E1 or 1:2 for CYP2C19 in the presence of constant wild-type *b*₅ and varying amounts of mutation D58G+D65G, or in the presence of constant mutation D58G+D65G and varying amounts of wild-type *b*₅. Error bars indicate mean $\pm$ standard deviation of triplicate assays.

	<div style="display: flex; justify-content: space-around; font-size: small;"> *** 40 ***** 50 * *** 60 ***** 70 * </div>
<i>Homo sapiens</i>	HHKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREM
<i>Bos taurus</i>	HYKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREL
<i>Sus scrofa</i>	HHKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREL
<i>Ovis aries</i>	HYKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREL
<i>Oryctolagus cuniculus</i>	HHKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREL
<i>Rattus norvegicus</i>	HHKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREL
<i>Mus musculus</i>	HHKVYDLTKF LEEHPGGEEV LREQAGGDAT ENFEDVGHST DAREL
<i>Candida orthopsilosis</i>	NGKVYNVSSY I DEHPGGEEV I LDVAGTDAT EAFED IGHSD EAHEI
<i>Saccharomyces cerevisiae</i>	DDKVYDVVSQF KDEHPGGDEI IMDLGGQDAT ESFVD IGHSD EALRL
<i>Brassica oleracea</i> var. <i>botrytis</i>	SGKVYDVTPF MDDHPGGDEV LLS STGKDAT NDFEDVGHSD TARDM

Figure 5.

Alignment of cytochromes b_5 from different species, including human, bovine, pig, sheep, rabbit, rat, mouse, yeast, and broccoli. Asterisks above alignments indicate fully conserved residues, which including axial ligands histidine 44 and 68, and three acidic residues: E49, D58, and E65.

Figure 6.

Location of acidic residues D36, E48, E49, D58, and D65, on the b_5 surface. VMD (<http://www.ks.uiuc.edu/Research/vmd/>) package was used for molecular visualization. Panel A, ribbon diagram of entire b_5 structure with heme in red, D36 in blue, E48 and E49 in yellow, and D58 and D65 in cyan. The surface-exposed α -helix containing E48 and E49 is in green, and the α -helix containing D58 and D65 is in magenta. Panel B, expanded view of surface-exposed α -helix containing D58 and D65, found to be critical for stimulating CYP2E1 and CYP2C19 but not CYP17A1 activities, with same coloring scheme as in A.

Table 1

Stimulation of 17,20-Lyase Activity by b_5 and b_5 Mutations

no b_5	Reconstituted DLPC ^a			Reconstituted CYMS ^b		Yeast microsomes ^c	
	30:30	30:30	30:300	9±3	10:100	1:10	1:100
pmol P450:pmol b_5							
wild-type	33±3	16±2	3±1	46±3	29±6	62±3	29±6
D36G	12±1	3±1	43±2	18±1	60±3	64±5	60±3
E48G	8±1	4±1	9±2	32±4	34±1	18±1	18±2
E49G	9±3	4±1	13±3	21±3	23±1	49±6	55±1
R52G	10±3	5±0	9±1	50±4	46±4	50±2	43±4
D58G	5±1	2±1	5±1	5±1	12±1	9±1	12±1
D65G	22±4	14±3	57±6	23±4	51±7		
E48G+E49G	4±2	2±1					
D58G+D65G	5±2	2±0					

All data are expressed as percent conversion of 17-hydroxypregnenolone to DHEA product

^aThe reconstitution mixture contained purified CYP17A1 (0.15 μM), POR (0.6 μM), b_5 (0.15 or 1.5 μM) and 30 μM DLPC in 0.2 mL. The incubation was carried out with 20 μM 17-hydroxypregnenolone and [³H]-17-hydroxypregnenolone (~0.4 μCi, 4 nM). Data represent the mean ± standard deviation for three experiments.

^bThe same experiment as described in *a* except that purified CYP17A1 (0.05 μM) and POR (0.2 μM) was reconstituted with 0.5 μM b_5 and control yeast microsomal lipid (CYMS, 22 μg protein). The incubations contained 2 μM [³H]-17-hydroxypregnenolone.

^cThe same experiment as described in *b* except that yeast microsomes containing CYP17A1 and POR (22 μg protein, 1 pmol P450) replaced the purified CYP17A1 and POR with CYMS.

Table 2Reduction Kinetics of b_5 and b_5 Mutations by POR and NADPH

b_5 protein	turnover number (min^{-1})	
	1 nmol b_5	4 nmol b_5
Wild-type	74 $\pm$ 4	176 $\pm$ 6
D36G	49 $\pm$ 3	95 $\pm$ 15
E48G	86 $\pm$ 5	158 $\pm$ 12
E49G	73 $\pm$ 5	149 $\pm$ 9
E48G+E49G	73 $\pm$ 2	166 $\pm$ 7
D58G	45 $\pm$ 2	115 $\pm$ 11
D65G	31 $\pm$ 3	67 $\pm$ 8
D58G+E65G	47 $\pm$ 1	135 $\pm$ 12

Data represent the mean $\pm$ standard deviation of the mean for three experiments.