Human Cytochrome b_5 Requires Residues E48 and E49 to Stimulate the 17,20-Lyase Activity of Cytochrome P450c17†

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ABSTRACT: Cytochrome P450c17 (CYP17) catalyzes both the 17α-hydroxylase and 17,20-lyase reactions in human steroid biosynthesis. Cytochrome b_5 (b5) stimulates the rate of the 17,20-lyase reaction 10-fold with little influence on 17α-hydroxylase activity. Studies with apo-b5 suggest that stimulation of 17,20-lyase activity results from an allosteric action on the hCYP17•POR complex, rather than electron transfer by b5. We hypothesized that specific residues on b5 interact with the hCYP17•POR complex and that targeted mutation of surface-exposed residues might identify b5 residues critical for stimulating 17,20-lyase activity. We constructed, expressed, and purified 14 single plus 3 double b5 mutations and assayed their ability to stimulate 17,20-lyase activity. Most mutations did not alter the capacity of b5 to stimulate 17,20-lyase activity or appeared to modestly alter the affinity of b5 for the hCYP17•POR complex. In contrast, mutation of E48, E49, or R52 reduced the maximal stimulation of 17,20-lyase activity. In particular, b5 mutation E48G + E49G lost over 95% of the capacity to stimulate 17,20-lyase activity, yet this mutation retained normal electron transfer properties. In addition, mutation E48G + E49G did not impair stimulation of 17,20-lyase activity by wild-type b5, suggesting that the mutation binds poorly to the site of the hCYP17•POR complex occupied by b5. These data suggest that a specific allosteric binding site on b5, which includes residues E48, E49, and possibly R52, mediates the stimulation of 17,20-lyase activity.

All androgen production requires the activities of cytochrome P450c17 (17α-hydroxylase/17,20-lyase; CYP17).[1] CYP17 from various species are 57 kDa microsomal P450s that contain only one active site, yet these enzymes catalyze two fundamentally distinct chemical transformations that convert 21-carbon steroids to 19-carbon androgen precursors (*1*). In the first reaction, CYP17 17α-hydroxylates both pregnenolone and progesterone to their 17-hydroxy derivatives. In the second step, the 17,20-lyase reaction, the 21-carbon,17-hydroxysteroids are cleaved to 19-carbon steroids and acetic acid. For human CYP17 (hCYP17), the cleavage of 17α-hydroxypregnenolone (17preg) to dehydroepiandrosterone (DHEA) is much more efficient than the corresponding conversion of 17α-hydroxyprogesterone to androstenedione (*2, 3*).

Whereas the 17α-hydroxylase activity of hCYP17 is robust and constant, the 17,20-lyase reaction is strongly regulated in human physiology. In the adrenal zona fasciculata, hCYP17 lacks 17,20-lyase activity, which mandates that steroidogenesis stops at 21-carbon,17-hydroxysteroids such as cortisol. In contrast, the adrenal zona reticularis and gonads produce 19-carbon steroids because hCYP17 in these tissues exhibits high 17,20-lyase activity. This dichotomy is explained by additional factors that preferentially or exclusively activate 17,20-lyase activity and enable some cells that express hCYP17 to produce 19-carbon steroids.

At least four additional factors regulate the 17,20-lyase activity of the hCYP17 catalytic system in vitro and in vivo. First, genetic (*4*−*6*) and targeted mutagenesis studies (*7, 8*) demonstrate that single amino acid substitutions in hCYP17 can selectively disrupt 17,20-lyase activity while preserving most 17α-hydroxylase activity. Second, a phosphorylation/dephosphorylation cycle (*9, 10*) contributes to regulation of the 17,20-lyase activity. Third, the 17,20-lyase reaction is particularly sensitive to interactions with the electron-donating flavoprotein cytochrome P450 oxidoreductase (POR). In reconstituted assays, increased POR abundance preferentially activates 17,20-lyase activity (*11*), and missense mutations in POR from patients with disordered steroidogenesis preferentially impair 17,20-lyase activity over 17α-hydroxylase activity (*12*−*14*). Fourth, both solubilized hCYP17 in reconstituted assays (*15, 16*) and native hCYP17 in yeast microsomes (*2*) exhibit poor 17,20-lyase in the absence of cytochrome b_5 (b5). Furthermore, b5 is abundant in cells that contain high 17,20-lyase activity and low in cells that express hCYP17 but lack significant 17,20-lyase activity (*17, 18*). Thus, b5 appears to be a major physiologic regulator of 17,20-lyase activity and 19-carbon steroid production. Consequently, elucidating the mechanism of how b5 stimulates 17,20-lyase activity is critical for developing strategies to regulate androgen production in pathologic states.

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[1] Abbreviations: 17preg, 17α-hydroxypregnenolone; b5, cytochrome b_5; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; DHEA, dehydroepiandrosterone; hCYP17, human cytochrome P450c17; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; POR, cytochrome P450-oxidoreductase; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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The catalytic cycle of hCYP17 and all microsomal P450s involves two electron transfers from reduced nicotinamide adenine dinucleotide phosphate (NADPH) via the two flavins of POR to the heme moiety of the P450. Since b5 is a hemoprotein that also undergoes redox chemistry during fatty acid biosynthesis (*19*) and methemoglobin reduction (*20*), it is possible that b5 functions as an alternative electron donor for the second electron-transfer step. However, apo-b5, which contains no heme and therefore cannot transfer electrons, also stimulates the 17,20-lyase activity of hCYP17 at least as well as holo-b5 (*2*). Consequently, b5 might rather function as an allosteric facilitator of the 17,20-lyase reaction, acting in a CYP17·POR·b5 complex. Conflicting available data do not exclude either plausible model (*21*), so the role of b5 in the 17,20-lyase reaction remains elusive.

Several residues of hCYP17, including R347 and R358 (*4*, *8*), have been implicated as part of the binding site that interacts with POR and/or b5 during the 17,20-lyase reaction. However, the amino acids of b5 that are involved in the stimulation of 17,20-lyase activity are not known, and evidence for a direct interaction of hCYP17 and b5 are sparse. The form of b5 that stimulates 17,20-lyase activity is the 16.5 kDa membrane-bound species containing two acidic core domains, one of which binds heme via histidines 44 and 68, plus a membrane-spanning C-terminal helix (*22*). The C-terminal helix of b5 is required to stimulate the 17,20-lyase activity of hCYP17 (*23*), as well as activities of other P450s (*24*). In contrast, several charged residues of b5 have been identified as essential components of the interaction of b5 with other electron transport proteins including cytochrome *c* (*25*, *26*) and b5 reductase (*27*). We hypothesized that specific surface residues of b5 interact with the hCYP17·POR complex and that the integrity of these surface residues is also essential for the stimulation of 17,20-lyase activity. Consequently, we systematically mutated surface-exposed charged residues of b5 to determine which residues are required for the stimulation of 17,20-lyase activity.

EXPERIMENTAL PROCEDURES

General Methods. Chemicals, reagents, and medium components were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO) unless indicated. Radiochemicals were purchased from PerkinElmer Life Sciences (Shelton, CT). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and used without purification. DNA modification enzymes were from New England Biolabs (Beverly, MA) unless noted.

Plasmids. Plasmid pLW01-b5H4 (pLW01 was a gift from Dr. Lucy Waskell (*28*)), which contains a T7 promoter and a high-copy replication origin from pBluescript, was used to express wild-type human b5 in *Escherichia coli*. A tetrahistidine tag was added to the N-terminus by amplifying the b5 cDNA with the primers b5H4S1Bam and b5AS1Eco (Table 1) and then inserting the PCR amplicon back into pLW01 after digesting both plasmid and insert with *Nco*I and *Eco*RI. PCR reactions were assembled with 0.2 mM deoxynucleoside triphosphates, 1−10 ng of template plasmid, 100 pmol each primer, and 1.25 U of TrueFidelity DNA polymerase (Continental Laboratory Products, San Diego, CA) in 50 μL of the manufacturer's buffer. Thermocycling conditions were 25 cycles of 94 °C, 1 min; 50 °C, 0.5 min; and 72 °C, 4 min. Plasmid pcDNA3-b5H4 was constructed for expression in HEK-293 cells by inserting the same PCR amplicon into pcDNA3 (Invitrogen, Carlsbad, CA) after digestion with *Bam*HI and *Eco*RI. The cDNAs for b5 mutations were constructed with PCR reactions using two sets of primers: T7 plus the antisense mutagenic primer and SP6 plus the sense mutagenic primer (Table 1) with pcDNA-b5H4 as template. The b5 cDNA fragments were then amplified together by a third PCR reaction using primers T7 and SP6. The final PCR product was inserted into pLW01 after digestion with *Nco*I and *Eco*RI. All cDNAs were sequenced to confirm that only the desired changes were introduced during PCR.

Recombinant Proteins. The protocol for expression and reconstitution of recombinant human b5 was based on the procedure of Mulrooney and Waskell (*29*) with some modifications. Plasmids for expressing either wild-type b5 or b5 mutations were transformed into C41(DE3) cells (OverExpress, Avidis, St. Beauzire, France) and spread onto plates of Luria−Bertani medium containing 0.1 mg/mL carbenicillin (US Biochemicals, Cleveland, OH). After an overnight incubation at 37 °C, 500 mL of Terrific Broth supplemented with 0.5 mM δ-aminolevulinic acid (Sigma) and 0.1 mg/mL carbenicillin was inoculated with a single colony. The culture was incubated at 37 °C with shaking at 140 rpm until the A_{600} reached 0.35−0.4, at which time, the cells were induced with 10 μM isopropyl-β-D-thiogalactopyranoside (Fisher) and grown for an additional 16−20 h at 37 °C. The culture was chilled on ice, and the cells were harvested by centrifugation at 11 000*g* for 10 min at 4 °C. The pellet was resuspended in 20−30 mL of cold Buffer A (10 mM potassium phosphate and 1 mM EDTA, pH 7) and centrifuged again at 11 000*g* for 10 min at 4 °C. The cell pellet was stored at −70 °C until needed. The cell pellet was thawed and resuspended in 40 mL of Buffer A with 10 μg/mL aprotinin (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The cells were lysed by high-pressure extrusion (EmulsiFlex-C5, Avestin, Ottawa, Canada) and centrifuged at 3000*g* to remove cell debris.

To determine the amount of heme required for reconstitution of the apoprotein, a 50 μL aliquot of lysed cells was added to 1.5 mL of Buffer D (20 mM Tris·HCl, 1 mM EDTA, pH 8, and 0.1% CHAPS [Pierce Biotechnology, Rockford, IL]). The apo-b5 was titrated with the hemin chloride solution (ferriprotoporphyrin IX chloride, Fluka Biochemika, Steinheim, Switzerland, prepared in 50:50 v/v ethanol/water (*29*)) by monitoring the absorbance spectrum from 350 to 600 nm before and after each addition of hemin chloride. The titration is complete when a distinct increase in absorbance at 385 nm is observed due to the accumulation of Tris-ligated (nonprotein bound) heme. Based on the amount of heme needed to reconstitute the aliquot, we calculated the quantity of heme needed for the total cell lysate, and a 10% molar excess was added. The cell lysate was centrifuged at 109 000*g* for 1 h at 4 °C to pellet the membrane fraction. The bright red gelatinous pellet was resuspended in 25 mL of buffer B (10 mM potassium phosphate, pH 8, 2 mM 2-mercaptoethanol, and 20% glycerol) using a Dounce Homogenizer. Solid CHAPS was added to the cold suspension of stirred membranes until a

Table 1: Primers Used for Construction of His-Tagged b5 cDNA and Mutations

primer	sequence
b_5H4S1Bam[a]	5′-GGATCCATGGCACACCATCACCATGCAGAGCAGTCG-3′
b_5AS1Eco[a]	5′-GAATTCTCAGTCCTCTGCCATGTATAGGC-3′
b_5K33G-S[b]	5′-CTGCACCAC**GG**GGTGTACGATTTGAC-3′
b_5K33G-AS[b]	5′-ATCGTACACC**CC**GTGGTGCAGGATC-3′
b_5D36G-S	5′-AAGGTGTACG**G**TTTGACCAAGTTTC-3′
b_5D36G-AS	5′-CTTGGTCAAA**C**CGTACACCTTGTGG-3′
b_5K39G-S	5′-GATTTGACC**GG**GTTTCTGGAAGAGC-3′
b_5K39G-AS	5′-TTCCAGAAAC**CC**GGTCAAATCGTAC-3′
b_5E42G-S	5′-AAGTTTCTGG**G**AGAGCATCCTGGTG-3′
b_5E42G-AS	5′-AGGATGCTCT**C**CCAGAAACTTGGTC-3′
b_5E48G-S	5′-CCTGGTGG**A**G**G**AGAAGTTTTAAGGG-3′
b_5E48G-AS	5′-TAAAACTTCTCC**T**CCACCAGGATGC-3′
b_5E49G-S	5′-GGTGGGGAAG**G**AGTTTTAAGGGAAC-3′
b_5E49G-AS	5′-CCTTAAAACT**C**CTTCCCCACCAGGATG-3′
b_5R52G-S	5′-GAAGTTTTA**G**GGGAACAAGCTGGAGG-3′
b_5R52G-AS	5′-AGCTTGTTCCCC**C**TAAAACTTCTTCC-3′
b_5E53G-S	5′-GTTTTAAGGG**G**ACAAGCTGGAGGTG-3′
b_5E53G-AS	5′-TCCAGCTTGT**C**CCCTTAAAACTTC-3′
b_5Q54G-S	5′-TTAAGGGAA**GG**AGCTGGAGGTGACG-3′
b_5Q54G-AS	5′-ACCTCCAGCT**CC**TTCCCTTAAAACT-3′
b_5D58G-S	5′-GCTGGAGGTG**G**CGCTACTGAGAAC-3′
b_5D58G-AS	5′-CTCAGTAGCG**C**CACCTCCAGCTTG-3′
b_5E61G-S	5′-GACGCTACTG**G**GAACTTTGAGGATG-3′
b_5E61G-AS	5′-CTCAAAGTTC**C**CAGTAGCGTCACCTC-3′
b_5D65G-S	5′-GAACTTTGAGG**G**TGTCGGGCACTC-3′
b_5D65G-AS	5′-GTGCCCGACA**C**CCTCAAAGTTCTC-3′
b_5R73G-S	5′-GATGCC**G**GGGAAATGTCCAAAAC-3′
b_5R73G-AS	5′-GGACATTTCCC**C**GGCATCTGTAGAG-3′
b_5E74G-S	5′-GCCAGGG**G**AATGTCCAAAACATTC-3′
b_5E74G-AS	5′-TTTGGACATT**C**CCCTGGCATCTGTAG-3′
b_5E48G/E49G-S	5′-CCTGGTGG**A**G**G**AG**G**AGTTTTAAGGG-3′
b_5E48G/E49G-AS	5′-TAAAACT**C**CTCC**T**CCACCAGGATGC-3′
b_5E49G/R52G-S	5′-G**G**AGTTTTA**G**GGGAACAAGCTGGAGG-3′
b_5E49G/R52G-AS	5′-AGCTTGTTCCCC**C**TAAAACT**C**CTTCC-3′
b_5E49G/E53G-AS	5′-CACCTCCAGCTTGT**C**CCCTTAAAAC-3′

[a] BamHI, *Nco*I, and *Eco*RI restriction sites are underlined. [b] The nucleotide changes that produce the indicated mutation(s) are in bold type. Nucleotide changes in both bold type and underlined were altered to introduce silent mutation that avoid primers containing five consecutive G bases. S and AS denote sense and antisense, respectively.

concentration of 1% w/v. After stirring for an additional 1 h, the solution was centrifuged at 109 000*g* for 1 h at 4 °C to pellet any insoluble membranes. The supernatant was diluted with an equal volume of buffer B containing 400 mM KCl.

Ni−NTA agarose beads (Qiagen) equilibrated with buffer B were added to the membrane fraction and incubated 1 h at 4 °C with agitation. The beads were poured into a column and washed with 20 column volumes of buffer B containing 0.1% CHAPS, 10 mM imidazole, and 400 mM KCl. The His4-tagged b5 protein was eluted from the Ni−NTA beads with buffer B containing 0.1% CHAPS and 100 mM imidazole, and the eluate was dialyzed overnight against 300 mL of buffer C (10 mM potassium phosphate, pH 7.5, 20% glycerol, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 0.05% CHAPS) with one buffer exchange.

Assay of 17,20-Lyase Activity in HEK-293 Cells. HEK-293 cells were cultured, seeded at 50% confluency in 6-well plates, transfected with the FuGENE6 reagent (Roche, Indianapolis, IN), and assayed as described (*5*, *30*). Transfections employed 0.6−1 μg of pcDNA3-c17 and varying amounts of empty pcDNA3, pcDNA3-b5H4, or pcDNA3-POR, and incubations contained 0.01−0.1 μM pregnenolone with 67 000 cpm of [^{3}H]-pregnenolone in 2 mL of medium for 1−8 h. For spectroscopic assays, cells were broken by sonication in water, and microsomes were prepared as described (*31*).

Assay of 17,20-Lyase Activity in Yeast Microsomes. Yeast microsomes containing human CYP17 and POR were prepared from strain YiV(B) and quantitated as described (*5*). Microsomes containing 1 pmol of CYP17 were mixed with b5 proteins (0.1−50 pmol) and 0.6 μM 17preg with 80 000 cpm [^{3}H]-17preg in 0.2 mL of 50 mM potassium phosphate, pH 7.4. After an incubation at 37 °C for 3 min, 1 mM NADPH (Sigma) was added, and the reaction was continued for 30 min at 37 °C. The steroids were extracted, chromatographed, visualized, and quantitated as described (*2*).

Reduction of b5 by NADPH and POR. Recombinant POR was generously provided by Dr. Julian Peterson. POR (0.2 nmol) was incubated with b5 (1.5 nmol) and 0.33 mM NADPH in 0.2 M potassium phosphate, pH 7.5, with 0.05% CHAPS in a final volume of 1.5 mL. The absorbance at 424 nm was monitored for 15 min at 25 °C with data points collected every 10 s using an Agilent 8453 UV−visible spectrophotometer system. The data were fit to an exponential growth curve using Origin 7.5 (OriginLab, Northampton, MA):

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

where A is the concentration of b5 and k is the first-order rate constant; therefore, the rate of reduction is $A \times k$ in AU·s^{-1}. Rates were converted to turnover numbers (min^{-1}) using $\epsilon = 90$ mM^{-1}·cm^{-1} at 424 nm.

Table 2: Properties of Recombinant b5 and b5 Mutations

	DS heme[a] (pmol/μL)	protein[b] (μg/μL)	pmol DS heme/ μg protein	pmol DS heme/ pmol total b5 protein
		Group 1 Mutations		
wild-type	32	2.0	16	0.27
K33G	70	5.6	13	0.21
K39G	115	5.1	22	0.37
E53G	117	7.7	15	0.25
Q54G	111	5.4	21	0.34
E61G	120	7.5	16	0.26
R73G	94	3.9	24	0.40
		Group 2 Mutations		
D36G	23	3.0	7.7	0.13
E42G	86	3.8	23	0.38
D58G	76	6.2	12	0.20
D65G	55	1.8	30	0.49
E74G	83	3.6	23	0.38
E49G + E53G	73	6.0	12	0.20
E49G + R52G	92	3.3	28	0.46
		Group 3 Mutations		
E48G	124	6.5	19	0.31
E49G	104	7.2	15	0.24
R52G	128	7.7	17	0.27
E48G + E49G	108	4.5	24	0.40

[a] Heme content by difference spectroscopy (DS), reduced vs oxidized spectra. [b] Protein concentration using colorimetric reagent.

Competition Assays. Assays were performed with the same conditions described above for assay of 17,20-lyase activity in yeast microsomes, with one alteration. Both wild-type b5 and b5 mutation E48G + E49G were added together at various ratios. First, wild-type b5 was held constant at 1 pmol, while b5 mutation E48G + E49G was varied from 1 to 30 pmol, and 30 pmol of wild-type b5 was added to the control incubation. The converse experiment was also performed using 1 pmol of b5 mutation E48G + E49G in each incubation while wild-type b5 was varied from 1 to 30 pmol.

Protein Assays. The functional concentrations of heme-containing b5 proteins were determined by the difference spectroscopy using an Agilent 8453 UV−visible spectrophotometer system. Absorbance spectra from 350 to 500 nm of b5 proteins (~0.1 mg in 1.3 mL of 50 mM potassium phosphate, pH 7.4) were recorded before and after addition of approximately 2 mg of solid sodium dithionite (Fluka). The concentration of the b5 protein was calculated from the difference in absorbance at 424 and 409 nm ($\Delta A_{424-409} = A_{424-409}$ after addition of sodium dithionite $- A_{424-409}$ before addition of sodium dithionite) using an extinction coefficient of 180 $mM^{-1}\cdot cm^{-1}$. Total protein was measured with the Coomassie Plus Protein Assay (Pierce). Cytochrome *c* reduction assays (POR activity) were performed at 20 °C as described (*2*, *32*).

RESULTS

Mutagenesis and b5 Activity in HEK-293 Cells. Among the exposed residues of b5, 14 with charged or polar side chains distributed throughout the surface were mutated to glycine, to eliminate functional groups yet allow maximum structural flexibility in the protein (Tables 1 and 2). To screen these b5 mutations for the capacity to stimulate the 17,20-lyase activity of hCYP17, we first transiently transfected

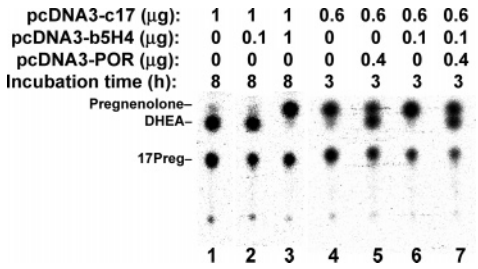

FIGURE 1: Pregnenolone metabolism in HEK-293 cells transiently transfected with expression vectors for hCYP17 (pcDNA3-c17), tetrahistidine-tagged wild-type b5 (pcDNA3-b5H4), and/or POR (pcDNA3-POR). Phosphorimage of chromatographed steroids from a representative experiment shows 0.01 μM [^{3}H]-preg conversion to [^{3}H]-17preg and [^{3}H]-DHEA in medium for transfected cells. Substantial DHEA formation occurs without overexpression of b5 (lane 1), and DHEA formation progressively decreases as the amount of pcDNA3-b5H4 plasmid is increased (lanes 2−3). Cotransfection with pcDNA3-POR (lane 5) increases 17,20-lyase activity, but cotransfection with all three plasmids does not significantly increase [^{3}H]-DHEA formation (lane 7). For assays with only pcDNA3-c17, 0.6−1 μg of empty pcDNA3 plasmid was cotransfected for consistency.

HEK-293 cells with pcDNA3-c17 and assayed conversion of [^{3}H]-pregnenolone to [^{3}H]-17preg and then [^{3}H]-DHEA in the medium. After transfection with pcDNA3-c17, endogenous POR in the HEK-293 cells reconstitutes the hCYP17 catalytic system with substantial 17,20-lyase activity, even in the absence b5 overexpression, yielding [^{3}H]-DHEA (Figure 1). Cotransfection with pcDNA3-b5H4, paradoxically, *inhibits* 17,20-lyase activity, such that [^{3}H]-DHEA formation diminishes progressively as the amount of pcDNA3-b5H4 increases. In contrast, cotransfection with pcDNA3-POR increased 17,20-lyase activity, but cotransfection with both pcDNA3-POR and pcDNA3-b5H4 further increased DHEA formation only slightly (Figure 1). These findings are consistent with a stoichiometric excess of endogenous b5 relative to hCYP17 in our HEK-293 cells, since high b5/hCYP17 molar ratios (>10−30:1) inhibit 17,20-lyase activity in yeast microsomes by competing with hCYP17 for electrons from limiting POR (*2*). On the basis of spectroscopic assays, microsomes from HEK-293 cells contained 100 pmol of b5/mg of protein, whereas P450 content was below the limits of detection (<10 $pmol\cdot mg^{-1}$), similar to P450 content in transfected COS-1 cells (*31*). The inhibition observed with b5 coexpression, which is in contrast to previous findings in HEK-293 cells (*33*), prompted us to employ another assay system with yeast microsomes and purified b5 mutations.

Stimulation of 17,20-Lyase Activity by b5 Mutations in Vitro. All 14 single plus 3 double b5 mutations were expressed in *E. coli*, reconstituted with heme, and purified. The b5 mutations varied in their migration on denaturing gel electrophoresis (SDS−PAGE) (Figure 2A) but showed comparable heme content (Table 2). Functional apo-b5 has an extremely high affinity for heme (*21*, *34*), suggesting that any b5 protein that does not bind heme is irreversibly denatured, and results with wild-type b5 indicate that these species do not interfere with hCYP17 assays. MALDI-TOF mass spectrometry confirmed that the major protein species in the preparations of wild-type b5, b5 mutation E49G (showing the most discrepant migration on SDS−PAGE), and b5 mutation E48G + E49G (showing the most altered activity, see below) exhibited molecular masses

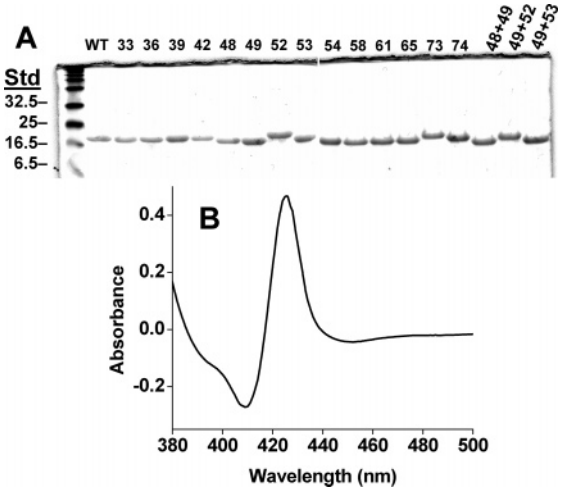

FIGURE 2: Purification and functional activity of wild-type b5 and b5 mutations expressed in *E. coli*. (A) SDS−PAGE of purified proteins (5 μg/lane) stained with Coomassie blue shows single bands with similar mobilities. Numbers at top of lanes refer to residue(s) substituted with glycine for each b5 mutation (WT = wild-type). (B) Reduced vs oxidized difference spectrum for mutation E48G + E49G shows characteristic peak at 424 nm and trough at 409 nm. Other b5 mutations gave similar difference spectra (data not shown).

within 1% of the calculated exact mass values for the full-length proteins (see Supporting Information). All reconstituted proteins demonstrated proper heme binding and redox chemistry based on spectroscopic assays (Figure 2B).

To determine which residues of b5 are essential for the stimulation of 17,20-lyase activity, purified wild-type b5 or b5 mutations were added to yeast microsomes containing hCYP17 and POR. The conversion of 17preg to DHEA was assayed at b5/hCYP17 molar ratios of 0.1:1, 1:1, and 10:1 (Table 3). Under conditions of the assay, these microsomes have low basal 17,20-lyase activity, converting 6% of the 17preg to DHEA. As anticipated, addition of wild-type b5 increased DHEA production 10-fold at a 1:1 b5/hCYP17 molar ratio with less stimulation at a 10:1 ratio. The greater stimulation of 17,20-lyase activity at equimolar b5/hCYP17 ratios than previously observed (*2*) reflects the higher ratio of hCYP17 content to POR activity in microsomes derived from the YiV(B) yeast strain (*5*) used in these experiments (220 pmol·mg^{-1} and 30 nmol cytochrome *c*·mg^{-1}·min^{-1}) than for microsomes derived from strain W303B (50 pmol·mg^{-1} and 60 nmol cytochrome *c*·mg^{-1}·min^{-1}) used previously (*2*). Control experiments using [^{3}H]-pregnenolone demonstrated that the b5 preparations did not significantly alter 17α-hydroxylase activity (see Supporting Information).

The b5 mutations segregated into three groups according to their influence on 17,20-lyase activity. Mutations Q54G, R73G, E61G, K39G, K33G, and E53G (group 1) behaved similarly to wild-type b5, with a marked increase in 17preg conversion to DHEA (50−70%) at a 1:1 b5/hCYP17 ratio and less DHEA production at a 10:1 ratio. Mutations D58G, D65G, D36G, E74G, and E42G (group 2) also stimulated DHEA production to 45−60%; however, little stimulation occurred at a 0.1:1 b5/hCYP17 ratio, and maximal DHEA synthesis occurred at the 10:1 ratio. This shift in the dose−response relationship suggests that these b5 mutations retain the capacity to stimulate 17,20-lyase activity but that their affinity for the hCYP17·POR complex might be lower than for wild-type b5. Further increases in the b5/hCYP17 ratio reduced DHEA production from the maximum for the group 2 b5 mutations as expected (data not shown).

Table 3: Stimulation of 17,20-Lyase Activity by b5 and b5 Mutations[a]

	pmol		
	0.1	1.0	10
	Group 1 Mutations		
wild-type	21 ± 3	58 ± 6	53 ± 7
K33G	25 ± 4	60 ± 11	28 ± 5
K39G	25 ± 5	61 ± 6	46 ± 6
E53G	39 ± 5	51 ± 9	17 ± 1
Q54G	32 ± 5	70 ± 4	31 ± 7
E61G	24 ± 9	58 ± 10	44 ± 8
R73G	21 ± 4	60 ± 1	46 ± 10
	Group 2 Mutations		
D36G	12 ± 2	35 ± 5	62 ± 6
E42G	15 ± 3	48 ± 5	63 ± 10
D58G	8.0 ± 1.8	24 ± 3	60 ± 4
D65G	10 ± 1	37 ± 4	59 ± 1
E74G	12 ± 1	47 ± 8	47 ± 4
E49G + E53G	8.0 ± 3.2	19 ± 4	45 ± 7
E49G + R52G	7.4 ± 0.9	24 ± 3	52 ± 5
	Group 3 Mutations		
E48G	6.1 ± 0.6	12 ± 2	20 ± 3
E49G	8.2 ± 1.3	22 ± 3	39 ± 4
R52G	5.9 ± 1.1	10 ± 1	22 ± 3
E48G + E49G	5.3 ± 0.9	5.8 ± 0.5	7.5 ± 0.8

[a] Percent of 17preg (120 pmol) converted to DHEA by yeast microsomes containing human CYP17 (1 pmol) and POR plus 0.1, 1, and 10 pmol of b5 or b5 mutation. Data are mean ± SD of triplicate determinations. Percent conversion by CYP17 microsomes only, no b5 added, is 5.6 ± 0.9.

Mutations E48G, E49G, and R52G (group 3) stimulated 17,20-lyase activity, but maximal DHEA production occurred at a 10:1 b5/hCYP17 ratio and did not exceed 40% conversion, even when the ratio was increased to 50:1. On the basis of these results, double mutations E48G + E49G, E49G + R52G, and E49G + E53G were constructed and tested. Mutations E49G + R52G and E49G + E53G exhibited the group 2 pattern of activity with >40% DHEA production at a b5/hCYP17 ratio of 10:1. In contrast, b5 mutation E48G + E49G stimulated 17,20-lyase activity by only 1.4-fold at a 10:1 b5/hCYP17 ratio (Table 3) with no additional stimulation at ratios up to 50:1 (data not shown). Consequently, b5 mutation E48G + E49G was studied in detail.

Characterization of b5 Mutation E48G + E49G. It is possible that the b5 mutations with reduced capacity to stimulate 17,20-lyase activity are folded aberrantly, but the normal reduced versus oxidized difference spectra (Figure 2B) suggest that heme binding and the overall structural integrity of the b5 mutations are grossly normal. To more carefully assess the structure and function of these proteins, we measured the kinetics of electron transfer between POR and the b5 mutations. As shown in Table 4, POR reduces all of the b5 mutations at rates comparable to the rate of reduction for wild-type b5. These data indicate that alterations in core structure and redox properties do not account for the inability of b5 mutation E48G + E49G to normally stimulate 17,20-lyase activity and suggest that these two residues comprise a critical interaction site on b5 for the hCYP17·POR complex.

Table 4: Kinetics of b5 and b5 Mutation Reduction by POR and NADPH

b5 protein	turnover number (min^{-1})
wild-type	4.0 ± 0.6
E48G	5.5 ± 0.9
E49G	5.0 ± 0.6
E48G + E49G	3.1 ± 0.6
E52G	4.7 ± 0.5
D65G	4.2 ± 0.7
R73G	3.5 ± 0.4

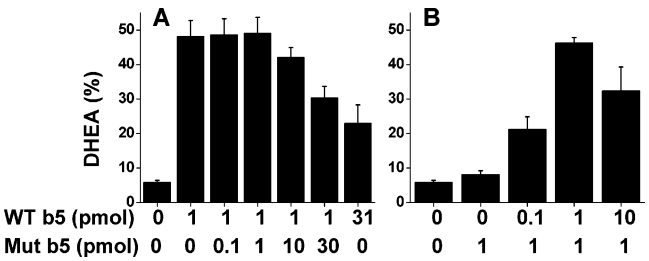

FIGURE 3: Competition assays using wild-type b5 and b5 mutation E48G + E49G. (A) DHEA formation in the presence of a 1:1 ratio of wild-type b5/hCYP17 and varying amounts of mutation E48G + E49G, showing inhibition of 17,20-lyase activity only at high total b5/hCYP17 ratios of 10:1 or greater. (B) DHEA formation in the presence of a 1:1 ratio of b5 mutation E48G + E49G/hCYP17, and varying amounts of wild-type b5, showing that the b5 mutation does not alter stimulation of 17,20-lyase activity by wild-type b5. Bars indicate mean ± SD of triplicate experiments.

Among the possible explanations for the altered activity of b5 mutation E48G + E49G, this mutation could either bind well to the hCYP17·POR complex, but not stimulate 17,20-lyase activity, or the b5 mutation could bind poorly to the hCYP17·POR complex. To distinguish between these possibilities, we assayed 17,20-lyase activity in competition experiments with both b5 mutation E48G + E49G and wild-type b5. First, we held the concentration of wild-type b5 constant at the optimum 1:1 b5/hCYP17 ratio and varied the concentration of b5 mutation E48G + E49G. If the b5 mutation competes for the same binding site on the hCYP17·POR complex as wild-type b5, 17,20-lyase activity should decrease as the concentration of b5 mutation E48G + E49G increases. Instead, addition of b5 mutation E48G + E49G does not decrease 17,20-lyase activity until a 10-fold molar excess is added and the total b5/hCYP17 ratio reaches 10:1 (Figure 3A). These data suggest that b5 mutation E48G + E49G does not compete with wild-type b5 for the binding site on the hCYP17·POR complex that stimulates 17,20-lyase activity, although the mutation does compete with hCYP17 for electrons from POR when added in excess (Table 4 and Figure 3).

We also performed the competition experiments in the reverse scenario, in which we assayed 17,20-lyase activity in the presence of a constant 1:1 ratio of b5 mutation E48G + E49G with hCYP17 and varied the amount of wild-type b5 added. In this experimental paradigm as well, the presence of b5 mutation E48G + E49G did not impair the capacity of wild-type b5 at various b5/hCYP17 molar ratios to stimulate 17,20-lyase activity (Figure 3B). These data suggest that b5 interacts with the hCYP17·POR complex using a binding site that includes residues E48 and E49. Replacement of these residues with glycine almost completely eliminates the capacity of b5 to stimulate 17,20-lyase activity but does not compromise the structural integrity or capacity to accept electrons from POR.

DISCUSSION

The cleavage of 17preg to DHEA by hCYP17 is stimulated 10-fold by b5, a biochemical process that is critical for human physiology (*35*). Comprehensive mutagenesis of surface-exposed b5 residues shows that b5 residues E48, E49, and perhaps R52 are important components of a binding site for the hCYP17·POR complex that mediates stimulation of

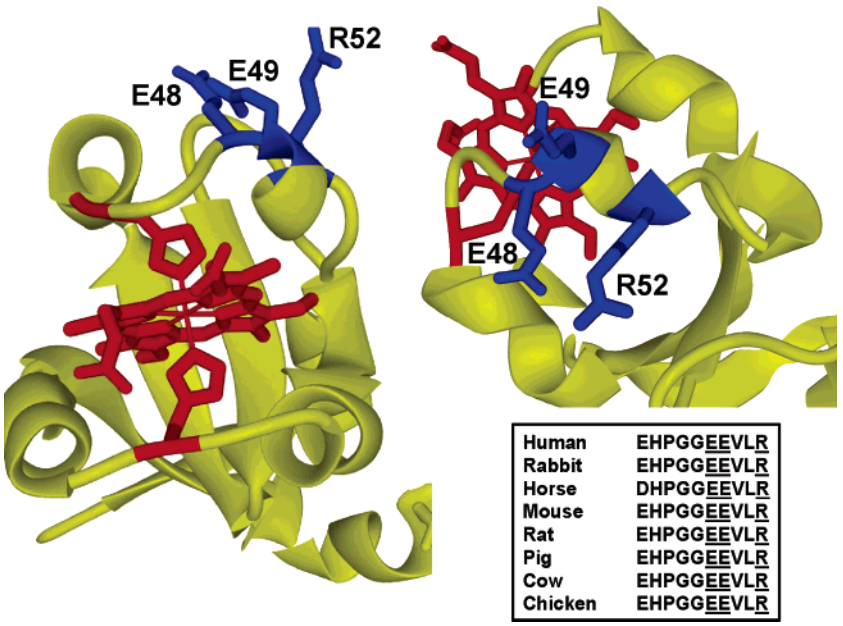

FIGURE 4: Location of residues E48, E49, and R52 on b5 surface. The crystal structure of the soluble portion of bovine b5 is used for illustration (pdb id no. 1CYO), with the heme and axial histidines in *red*, protein backbone in *yellow*, and side chains of conserved residues corresponding to E48, E49, and R52 of human b5 in *blue*. E48, E49, and R52 are located on a short α-helix adjacent to the heme in the core-1 domain of b5. Alignment of b5 (*34*) from eight mammalian species (*inset*, lower right) shows that the region containing E48, E49, and R52 (underlined, numbering for human b5) is highly conserved.

17,20-lyase activity. Mutation of b5 residues E48 and E49 both to glycine reduces the maximal stimulation of 17,20-lyase activity by over 95% yet does not impair the capacity of the protein to bind heme or to accept electrons from POR. Furthermore, the analogous b5 mutation E48A + E49A is reduced normally by b5 reductase (*27*), confirming that these residues are not essential for the reduction of b5 by flavoproteins. The presence of b5 mutation E48G + E49G does not impair the ability of wild-type b5 to stimulate 17,20-lyase activity, providing evidence that b5 mutation E48G + E49G has poor affinity for the binding site on the hCYP17·POR complex occupied by wild-type b5. Therefore, our data suggest that the electron-transfer properties of b5 and its allosteric effect on hCYP17 activity can be dissociated by targeted mutagenesis.

Few previous studies have examined the structural requirements of b5 for the stimulation of 17,20-lyase activity. Lee-Robichaud et al. showed that the carboxy-terminal membrane-spanning helix is an essential requirement for stimulating 17,20-lyase activity (*23*). Mulrooney et al. provided evidence that the interaction of CYP2B4 with b5 involves nonspecific interactions with the carboxy-terminal helix (*24*), but their studies did not include hCYP17, and other evidence from this group suggests that the stimulation of CYP2B4 methoxyflurane metabolism by b5 does involve electron transfer (*28*, *36*) and thus differs from the action of b5 on hCYP17. More recently, Yang and Hammes constructed chimeras of human b5 and *Xenopus* b5 and showed that human b5 residues 16−41 were important for stimulating the 17,20-lyase activity of hCYP17 (*37*). However, these experiments used chimeras, not single or double amino acid substitutions, and thus are not directly comparable to our results.

Among the limitations of our studies, we were unable to convincingly demonstrate a reproducible spectral change in hCYP17, purified from *E. coli* (a type I difference spectrum), upon addition of wild-type b5. Consequently, we could not use spectroscopic assays to measure the binding of the b5 mutations to hCYP17. In contrast, the action of the three groups of b5 mutations on hCYP17 in yeast microsomes was replicated with purified bovine CYP17 in reconstituted assays (see Supporting Information), strengthening the validity of our conclusions. Furthermore, our ability to quantify the affinity of our b5 mutations for the hCYP17·POR complex using activity assays is limited, because very high b5/hCYP17 molar ratios inhibit all hCYP17 activities (*2*). Because catalysis probably occurs in the hCYP17·POR complex, however, we believe that b5 affinity measurements are ideally based on studies using hCYP17·POR complexes.

Our data do not provide a complete description of either the mechanism of how b5 stimulates the 17,20-lyase activity of CYP17 or of the surfaces of b5 and the hCYP17·POR complex that interact during the 17,20-lyase reaction. The structure of hCYP17 is not known, although computational models exist (*8*), and this deficiency is a major impediment to the study of the action of b5 on hCYP17 chemistry. In addition, Pandey and Miller showed that the influence of phosphorylation and b5 are additive and not mutually exclusive (*38*). Consequently, a comprehensive understanding of the mechanism in which b5 stimulates the 17,20-lyase activity of hCYP17 requires at a minimum a high-resolution structure of hCYP17, ideally with POR bound, and knowledge of hCYP17 phosphorylation sites.

Nevertheless, our data suggest that the surface incorporating E48, E49, and possibly R52 is involved in the stimulation of 17,20-lyase activity. Since these residues are situated on a short α-helix of only one turn that is highly conserved (*34*) (Figure 4), we speculate that unwinding or disordering of this helix may be involved in the interaction of b5 with the hCYP17·POR. Future studies to characterize this interaction and to elucidate the mechanism of b5 action in detail will provide powerful information that might be used to develop novel strategies to selectively inhibit the 17,20-lyase reaction and thus block androgen and estrogen synthesis without disrupting cortisol production. Agents based on this knowledge will be useful in the treatment of cancers and hyperplasias of the prostate and breast, as well as polycystic ovary syndrome and sexual precocity.

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SUPPORTING INFORMATION AVAILABLE

MALDI-TOF mass spectra for wild-type b5 and b5 mutations E49G and E48G + E49G; pregnenolone 17-hydroxylation by hCYP17 in the absence and presence of b5; and influence of wild-type b5 and b5 mutations D65G, R73G, and E48G + E49G on 17,20-lyase activity of purified bovine CYP17 in reconstituted assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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