CYP17 Mutation E305G Causes Isolated 17,20-Lyase Deficiency by Selectively Altering Substrate Binding

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Cytochrome P450c17 (CYP17) converts the C21 steroids pregnenolone and progesterone to the C19 androgen precursors dehydroepiandrosterone (DHEA) and androstenedione, respectively, via sequential 17α-hydroxylation and 17,20-lyase reactions. Disabling mutations in CYP17 cause combined 17α-hydroxylase/17,20-lyase deficiency, but rare missense mutations cause isolated loss of 17,20-lyase activity by disrupting interactions of redox partner proteins with CYP17. We studied an adolescent male with clinical and biochemical features of isolated 17,20-lyase deficiency, including micropenis, hypospadias, and gynecomastia, who is homozygous for CYP17 mutation E305G, which lies in the active site. When expressed in HEK-293 cells or Saccharomyces cerevisiae, mutation E305G retains 17α-hydroxysteroid activities, converting pregnenolone and progesterone to 17α-hydroxysteroids. However, mutation E305G lacks 17,20-lyase activity for the conversion of 17α-hydroxyprogrenenolone to DHEA, which is the dominant 17α-hydroxysteroid pathway. In contrast, mutation E305G exhibits 11-fold greater catalytic efficiency (kcat/Km) for the cleavage of 17α-hydroxyprogesterone to androstenedione compared with wild-type CYP17. We conclude that mutation E305G selectively impairs 17,20-lyase activity for DHEA synthesis despite an increased capacity to form androstenedione. Mutation E305G provides genetic evidence that androstenedione formation via 17α-hydroxyprogesterone is dispensable for complete formation of the male phenotype in humans.

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The 6.4-kb CYP17 gene (24) was amplified by PCR using primer pairs c17geneS1a + I4AS1 and I5AS1 + c17geneA1 (see Table I) to amplify the 5'- and 3'- halves, respectively (25). Each PCR contained 1 μg of DNA, 50 pmol of each primer, 200 μM dNTPs, 1.5 μl of dimethyl sulfoxide, and 2 units of ExTaq polymerase (PanVera, Madison, WI) in a total volume of 50 μl of the manufacturer’s buffer. The following PCR conditions were employed: 94 °C for 3 min, followed by 42 cycles at 65 °C for 1 min, 70 °C for 3 min, and 95 °C for 30 s and a final annealing/extension cycle at 65 °C for 1 min and 70 °C for 5 min. The resulting PCR products were precipitated with ethanol and 0.3 M sodium acetate, purified on a 1% agarose gel, and isolated using the QIAEX-II gel extraction kit (QIAGEN Inc., Valencia, CA). The exons and flanking intronic segments of the amplicons were directly sequenced as described (25). For the other kindred members analyzed, PCR amplification of only exons 4 and 5 of the CYP17 gene was performed as described above using primers I3S1 and I5AS, except that extension parameters were 2 min at 70 °C. Exon 5 of the CYP17 amplicons was sequenced using primer I4S.

Site-directed Mutagenesis—Mutation E305G was introduced into the CYP17 cDNA by a sequential PCR using overlapping mutagenic oligonucleotides (25). Two separate PCRs utilized primer pair T7 + c17E305GS1α + c17E305GS1 + pLWAS1 in addition to plasmid pLW01-c17 as a template, 1.25 units of ExTaq polymerase, and 200 μM dNTPs in a total volume of 50 μl of the manufacturer’s buffer. The PCR cycling conditions were as follows: 94 °C for 3 min, followed by 26 cycles at 50 °C for 30 s, 72 °C for 1.5 min, and 94 °C for 1 min and a final annealing/extension cycle at 50 °C for 30 s and 72 °C for 4 min. Aliquots of these reactions were diluted 1:10, and 1 μl of each dilution was combined and used as a template in a third reaction to construct a full-length mutated cDNA using primers T7 and pLWAS1 and the same conditions used for the first two PCRs. The resulting amplicon was gel-purified, digested with BamHI and EcoRI, gel-purified again, and ligated into the BamHI/EcoRI sites of the mammalian expression vector pcDNA3 (Invitrogen). The inserts from several positive colonies were gel-purified, digested with BamHI and EcoRI, gel-purified again, and ligated into the BamHI/EcoRI sites of the mammalian expression vector pcDNA3 (Invitrogen). The inserts from several positive colonies were sequenced in their entirety to ensure that only the desired nucleotide substitution was incorporated,2 affording plasmid pcDNA3-c17E305G. A subsequent BamHI/EcoRI digestion released the E305G cDNA insert, which was gel-purified and subcloned into the BamHI/EcoRI sites of the yeast expression vector V60 (26), yielding V60-c17E305G. Wild-type vector pNYS1 (27) was previously described.

Transformation and Steroid Metabolism in HEK-293 Cells—HEK-293 cells were grown in T-75 flasks with Dulbecco’s modified Eagle’s medium containing 5.84 μg/liter glutamine, 10% fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Cellgro, Mediatech, Inc., Herndon, VA) and transfected with the FuGENE 6 reagent (Roche Applied Science) as previously described for COS-7 cells (27). Cells were seeded to 60–80% confluence in 6-well plates and transfected with 1 μg/well plasmid pcDNA3 or 2 μg of plasmid for incubations with 17α-hydroxyprogesterone. The next day, all but 0.5 ml of medium was removed from each well and replaced with 3 ml of fresh medium containing the indicated steroid (60,000 cpm/ml) plus unlabeled steroid to give a final concentration of 0.1 μM. Aliquots (1 ml) were removed at 2, 4, or 8 h; extracted; separated by thin-layer chromatography; and visualized as described (7).

Generation of Yeast Strain YIV(B)—Genomic DNA was isolated from Saccharomyces cerevisiae strain W303B by disruption with glass beads (28). DNA fragments homologous to segments in the 5’- and 3’- ends of the yeast NCPI gene (homolog of the human CPR gene) were generated by PCR using oligonucleotides Yred5’/5-Yred3’ (Not-Bam-5’), Yred5’/5-Yred3’ (Not-Bam-3’), and Yred5’/5-Hind-Bgl-3’. PCRs contained 1 μg of DNA, 100 pmol of each primer, 200 μM dNTPs, 2 mM MgCl2, 1.5 μl of dimethyl sulfoxide, and 2.5 units of Taq polymerase (Promega, Madison, WI) in a 50-μl total reaction volume. Thermocycling conditions were as follows: 94 °C for 3 min, followed by 40 cycles at 55 °C for 30 s, 72 °C for 1 min, and 94 °C for 1 min and a final annealing/extension cycle at 55 °C for 30 s and 72 °C for 3 min. The amplifications of 580 and 380 bp, respectively, were gel-purified, cloned into vector pGEM-T (Promega) using the A-overhang method, and sequenced. The Hind-Bgl-3’ fragment was excised by digestion with HindIII and BglII and ligated into the HindIII and BglII sites of vector pLW01, yielding vector pLW01–3’. The Not-Bam-5’ fragment was excised with NotI and BamHI and ligated into the HindIII and BglII sites of vector pLW01, yielding vector pLW01–3’. The Not-Bam-5’ fragment was excised with NotI and BamHI and ligated into the corresponding sites in vector pcDNA3 to acquire the convenient adjacent restriction sites HindIII (3) and Xhol (5′) from the vector (pcDNA3–5’). An extended Not-Bam-5’ fragment was excised from vector pcDNA3–5’ with Xhol and HindIII and ligated into LW01–3’ digested with Xhol and HindIII, yielding vector pLW01–5′–3’. A cassette containing the yeast phosphoglycerate kinase promoter, the human CPR gene, with modified early exons to improve expression, and the yeast phosphoglycerate kinase terminator was excised from vector Y10-OR (3) with BamHI and HindIII and ligated into vector pLW01–5′–3’ digested with BamHI and HindIII, yielding vector pLW01–5′–CRP-3′. Finally, the yeast ura3 gene, flanked by hisG repeats (which enhance homologous recombination), was excised from vector pYNK51 (29) as a BamHI/BglII fragment and ligated into the BamHI site, located between the yeast Not-Bam-5’ fragment and the CPR cassette, of vector pLW01–5′–CRP-3′, yielding the final targeting vector pLW01-YIV.

Vector pLW01-YIV (2 μg) was linearized with XhoI and used to transform10 cells of strain W303B, and clones were selected and restreaked on uracil-deficient minimal medium (1.7 g/liter Difco yeast nitrogen base [BD Biosciences], 0.3 g/liter ammonium sulfate, 20 g/liter glucose, 2% agar supplemented with 40 mg/liter l-tryptophan, 40 mg/liter adenine hemisulfate, 60 mg/liter l-uracil, and 20 mg/liter l-histidine). Several colonies were restreaked on YPD medium (10 g/liter yeast extract, 10 g/liter peptone, 20 g/liter dextrose, and 2% agar) then finally streaked onto minimal medium plates containing the above nutrients plus 50 μg/ml carbenicillin and 250 μg/ml 5-foa, yielding a small number of clones with the ura3 gene deleted. Genomic DNA was isolated from several clones to confirm that homologous recombination had occurred, as shown by a 1.9-kb PCR fragment from the phosphoglycerate kinase promoter to sequences farther 5’ of the targeting construct on yeast chromosome 8 (primers YRED5’ U4 and pPGK2-3’ (Table I). One positive clone, named YIV(B), was propagated on YPD medium and used for transformation and expression. Strain engineering is shown in Fig. 2.

Yeast Transformation, Microsome Preparation, and Enzyme Assays—S. cerevisiae strains YIV(B) and W303B were transformed with 1

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1 A silent change of one base (GTT for GTG) was inadvertently introduced by oligonucleotide c17E305GS1 at Leu105.

### Table I

| Primer | Sequence|
|--------|---------|
| cc1ggcggcgttcatcgttcgcc | ccaccccccggcgttcatcgttgctcc |
| ctcattctgcccgttctcttggtt | ccctacctgttggttggtgggtcctagt |
| cgtcagaaacccacccaaggcacaggg | ccagagtttggcctgcgggtgttag |
| c17geneS1a | c17geneA1 |
| isAS | I4S |
| I5AS | I4S |
| I4S | I5AS |
| lplWS1 | Yred5’/5’ |
| Yred5’/3’ | Yred3’/5’ |
| Yred3’/3’ | Yred5’/U4 |
| pPGKAS2 | pPGK2-3’ |

2 Restriction sites and mutagenic codons are underlined.
μg of plasmid V60 (26) containing the cDNA for either wild-type CYP17 or mutation E305G as described (25). Transformants were selected on minimal medium plates as described above.

For microsome preparation, 2-ml precultures of liquid minimal medium with supplements were inoculated with YIV(B) yeast transformed with either V60-c17 or V60-c17E305G and shaken at 30 °C overnight. The following morning, the 2-ml preculture was added to 10 ml of the same medium and grown for 10 h, at which time a flask containing 500 ml of 10 g/liter yeast extract, 10 g/liter peptone, 5 g/liter glucose, and 3% ethanol was inoculated with the 10-h culture. This culture was grown for 24 h at 30 °C, induced by addition of 60 ml of 200 g/liter galactose, and allowed to grow overnight. Cells were harvested by centrifugation at 3000 × g for 5 min; resuspended in 10 ml of 50 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.1 mM KCl; and centrifuged again. The cell pellet was resuspended in TES buffer (50 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.6 mM sorbitol) to a total volume of 20 ml and added to 20 g of glass beads (425–600 μm) in the small chamber of a BeadBeater (Biospec Products, Inc., Bartlesville, OK) with 100 μl of protease inhibitor mixture for fungalyeast cultures (Sigma). The chamber was thoroughly chilled, and cells were disrupted by pulsing three times for 1 min with several minutes of icing between pulses. The homogenate was transferred to a 50-ml centrifuge bottle. The beads were then washed with 10 ml of TES buffer, which was then added to the initial homogenate. The combined suspension was centrifuged twice at 10,000 × g, and the final supernatant was centrifuged at 100,000 × g for 45 min. Microsomes were prepared by resuspending the pellet in 1 ml of 50 mM Tris-HCl (pH 8), 1 mM EDTA, and 20% glycerol and shearing through a 27-gauge needle.

Quantitation of microsomal cytochrome P450 and protein content, incubations with radiolabeled substrates, steroid extraction, and chromatography were performed as described (3). For incubations with purified recombinant human cytochrome b5 (PanVera), microsomes were preincubated for 2 min at 37 °C with 30 molar eq of cytochrome b5 and 0.6 μM steroid prior to addition of NADPH to start the reaction. The kinetic constants \( K_{\text{m,app}} \) and \( V_{\text{max}} \) were calculated from iterative hyperbolic fits of the data to the Michaelis-Menten equation (\( v = V_{\text{max}}[S]/(K_{\text{m,app}} + [S]) \)) using Origin Version 6.0 (OriginLab Corp., Northampton, MA). Because kinetic data from experiments in the presence of competitive inhibitors encompass only the linear portions of the \( v \) versus [S] plots, hyperbolic curves cannot be fit to the data; consequently, \( K_{\text{m,app}} \) values were obtained from constants derived from least-squares fits to Lineweaver-Burk plots as described (27). Turnover experiments with hydrogen peroxide and cumene hydroperoxide (Sigma) were performed with 1 pmol of cytochrome P450, 2 pmol of \([\text{H}]\)progestosterone, and 10 μM oxidant in 200 μl as described (21). Spectral binding constants (\( K_{\text{d}} \)) were obtained from titration curves in intact W303B cells expressing wild-type CYP17 or the E305G mutation (27).

**RESULTS**

**Clinical Presentation of the Index Case and Genetic Analysis**—The subject presented at age 15 for evaluation of gynecomastia. The pregnancy was uncomplicated, and the parents were first cousins. Hypoplasia of micropenis was noted at birth, and the hypospadias was surgically repaired at age 4. Physical examination showed Tanner stage 4 breasts and pubic hair, normal (15–20 ml) testes, and a small (3 cm) phallus. Electrolytes and plasma renin activity were normal. Basal and stimulated hormone values are listed in Table II. The combination of elevated gonadotropins, low testosterone, and extremely low DHEA sulfite suggested a defect in CYP17, which is required for the conversion of C\(_{19}\) steroids to C\(_{18}\) steroids in both the adrenal glands and gonads (24). Unlike classical 17-hydroxylase deficiency, however, circulating concentrations of most 17-hydroxysteroids were elevated, suggesting selective impairment of 17,20-lyase activity (ILD). Amplification and direct sequencing of all the exons of the CYP17 gene from this subject showed a homozygous GAG-to-GGG missense mutation at codon 305 in exon 5 (data not shown). This mutation substitutes a glycine for the highly conserved glutamate at this position, which resides within the active-site pocket.

**Enzyme Activity in Transfected HEK-293 Cells**—To confirm that the CYP17 mutation alone explains the clinical and laboratory data, E305G was introduced into the CYP17 cDNA, and the mutation was expressed in HEK-293 cells. Cells expressing mutation E305G metabolized progesterone approximately the same as those expressing wild-type CYP17, except that the mutation appeared to produce more androstenedione compared to metabolism by wild-type CYP17 or the E305G mutation (27).
expression vectors for CYP17 and leaves some uncertainty that CPR plasmid amplification and thus CPR expression may vary among yeast clones and cultures, potentially complicating comparisons of 17,20-lyase activity. To increase the expression of CYP17 and to afford more consistent coexpression of human CPR, we engineered yeast strain YIV(B), in which one copy of the human CPR cDNA is stably integrated into the NCP1 locus (CPR homolog) on chromosome 8 of strain W303B. With this strain, the galactose-inducible vector (26) V60-c17 can be used to achieve high CYP17 expression, consistently 300–400 nmol/liter (27). Furthermore, genomic CPR content is uniform, enhancing the consistency of CPR expression and augmenting 17α-hydroxylase activity—5-fold over strain W303B (data not shown).

Microsomes prepared from strain YIV(B) expressing wild-type CYP17 or mutation E305G 17α-hydroxylated pregnenolone with comparable kinetics (Fig. 4A). Progesterone metabolism by mutation E305G was somewhat more rapid than that by wild-type CYP17, particularly at low concentrations (Fig. 4B), reflecting the lower $K_m$ for E305G, but the similar $V_{max}$ for the two enzymes (Table III). Microsomes containing wild-type CYP17 converted 17α-hydroxyprogrenolone to DHEA, and this conversion was stimulated by addition of cytochrome $b_5$ (Fig. 4C). In contrast, DHEA production by mutation E305G was barely detectable, even when cytochrome $b_5$ was added to the incubation (Fig. 4C). In yeast microsomes, mutation E305G also converted 17α-hydroxyprogesterone to androstenedione slightly more rapidly compared with wild-type CYP17, and for both enzymes, cytochrome $b_5$ stimulated this reaction (Fig. 4C). For mutation E305G, turnover of 17α-hydroxyprogrenolone was too slow for detailed comparative kinetic studies, but kinetic parameters were determined for the 17α-hydroxyprogesterone substrate (Fig. 4D). Consistent with the qualitative data from transfected HEK-293 cells, mutation E305G exhibited a 2-fold lower $K_m$ and a 6-fold higher $V_{max}$ compared with wild-type CYP17 for 17α-hydroxyprogesterone. However, these activities are still low compared with the robust metabolism of 17α-hydroxyprogrenolone to DHEA by wild-type CYP17, particularly in the presence of cytochrome $b_5$ (3, 4, 9).

These data suggest that mutation E305G has a higher affinity for Δ4-steroids, particularly 17α-hydroxyprogesterone, compared with wild-type CYP17. In contrast, the kinetic constants for pregnenolone are comparable for the two enzymes, yet poor turnover precludes estimates of 17α-hydroxyprogrenolone affinity for mutation E305G from these experiments alone. To measure the affinity of mutation E305G for 17α-hydroxysteroids directly, we performed competition experiments with yeast microsomes. Addition of 17α-hydroxyprogrenolone or 17α-hydroxyprogesterone to assays of [3H]pregnenolone hydroxylation increased the slopes of the lines in Lineweaver-Burk plots, but did not change the $y$ intercepts ($V_{max}$), consistent with competitive inhibition (Fig. 5). From these data, we extracted $K_{i}$ values with mutation E305G of 34 and 6.5 μM for 17α-hydroxyprogrenolone and 17α-hydroxyprogesterone, respectively; in contrast, these values are 1.2 and 13 μM for wild-type CYP17 (Table III). These data show that mutation E305G demonstrates both markedly reduced affinity for 17α-hydroxyprogrenolone and severely reduced turnover of this Δ4-steroid, which is normally the preferred substrate for the 17α,20-lyase activity of human CYP17.

Spectral Binding Constants for Δ4-Steroids with CYP17 and Mutation E305G—To characterize the binding of 17α-hydroxyprogesterone to these enzymes further, we recorded type I difference spectra in yeast strain W303B expressing wild-type CYP17 (27) or mutation E305G (Fig. 5, inset). Endogenous acyl-

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**Fig. 3.** Steroid metabolism in transfected HEK-293 cells. Medium containing [3H]labeled steroids was incubated with cells transfected with plasmid pcDNA3 containing no insert (empty vector control (Vc)); wild-type CYP17 cDNA (WT), or CYP17 cDNA containing the human CYP17 (WT), and Mutation E305G (right). The migration positions of substrates and metabolites are indicated. Prog, progesterone; AD, androstenedione; 17Prog, 17α-hydroxyprogesterone. A, transfected cells incubated with [3H]pregnenolone (left) or [3H]17α-hydroxyprogrenolone (right). The migration positions of substrates and metabolites are indicated. Prog, pregnenolone; 17Prog, 17α-hydroxyprogrenolone.
transferase activity (31) precluded binding experiments with Δ5-steroids, but saturable binding curves (data not shown) for progesterone and 17α-hydroxyprogesterone yielded $K_I$ values of 0.17 and 1.2 μM, respectively, with wild-type CYP17, values consistent with results obtained previously with this assay (27). In contrast, experiments with mutation E305G yielded $K_I$ values of 0.052 and 0.22 μM for progesterone and 17α-hydroxyprogesterone, respectively (Table III). These data confirm that mutation E305G exhibits higher affinity for Δ5-steroids compared with wild-type CYP17, in addition to enhanced turnover of 17α-hydroxyprogesterone to androstenedione.

**DISCUSSION**

The 17,20-lyase activity of CYP17 shows a greater dependence on the abundance of CPR than does its 17α-hydroxylase activity (6, 7). The 17,20-lyase reaction is also markedly stimulated by optimal molar ratios of the cofactor cytochrome b$_5$ (3, 4, 9), emphasizing the importance of redox partner interactions

**Fig. 4.** Activities of yeast microsomes containing CPR and wild-type CYP17 or mutation E305G. Microsomes from yeast strain YIV(B) transformed with expression plasmid V60 containing either wild-type CYP17 cDNA (WT; ◼) or cDNA for mutation E305G (●) were incubated in the presence of the indicated steroids. Lines were drawn by least-squares fits of the data points ($r > 0.99$ in all cases), where each data point represents the mean ± S.D. of three independent experiments (some error bars are unseen because they lie within the data points). A, Lineweaver-Burk plot of microsomes incubated with pregnenolone (Prog). B, Lineweaver-Burk plot of microsomes incubated with progesterone (Prog). C, autoradiogram of a thin-layer chromatogram of steroids (0.6 M) 0.052 0.22 M) 0.17 1.2 M, respectively, with wild-type CYP17, values consist-

**Fig. 5.** Lineweaver-Burk plot of [3H]pregnenolone 17α-hydroxylation by yeast microsomes containing CYP17 mutation E305G in the presence of 17α-hydroxy steroids. Microsomes from yeast strain YIV(B) expressing CYP17 mutation E305G were incubated with [3H]pregnenolone and 2 μl of ethanol vehicle (+ EtOH; ◼) or with either 40 μM 17α-hydroxyprogrenolone (17Prog; ●) or 20 μM 17α-hydroxyprogesterone (17Prog; ▲) both added in 2 μl of ethanol. Lines were drawn by least-squares fits of the data points ($r > 0.99$ in all cases), where each data point represents the mean ± S.D. of three independent experiments (some error bars are unseen because they lie within the data points). Lines intersect at the $y$ intercepts, allowing calculation of $K_{iapp}$ values for competitive inhibition (Table III). The inset shows the type I difference spectrum obtained when a suspension of yeast expressing mutation E305G was incubated with 10 μM 17α-hydroxyprogesterone. Error bar, 0.02 absorbance units.

**TABLE III**

| Enzyme/constant | Substrate/ligand | $K_{iapp}$ (μM) | $V_{max}$ (μM) | $K_S$ (μM) | $K_{iapp}$ (μM) |
|-----------------|-----------------|----------------|----------------|-------------|----------------|
| WT CYP17        | Pregnenolone    | 1.8 ± 0.6      | 5.5 ± 0.8      | 0.17        | 1.2            |
|                 | 17Preg          | 3.2 ± 0.1      | 5.6 ± 0.1      | 0.072 ± 0.001 | 13            |
|                 | Progesterone    | 3.4 ± 0.1      | 0.17           | 1.2         | 13             |
|                 | 17Prog          | 3.4 ± 0.1      | 0.17           | 1.2         | 13             |

* $K_{iapp}$ and $V_{max}$ values are constants ± error obtained following exhaustive iteration from Origin Version 6.0 hyperbolic curve-fitting calculations (see “Experimental Procedures”).

**Experimental Procedures**

Microsomes from yeast strain YIV(B) expressing CYP17 mutation E305G were incubated with [3H]pregnenolone and 2 μl of ethanol vehicle (+ EtOH; ◼) or with either 40 μM 17α-hydroxyprogrenolone (17Prog; ●) or 20 μM 17α-hydroxyprogesterone (17Prog; ▲) both added in 2 μl of ethanol. Lines were drawn by least-squares fits of the data points ($r > 0.99$ in all cases), where each data point represents the mean ± S.D. of three independent experiments (some error bars are unseen because they lie within the data points). Lines intersect at the $y$ intercepts, allowing calculation of $K_{iapp}$ values for competitive inhibition (Table III). The inset shows the type I difference spectrum obtained when a suspension of yeast expressing mutation E305G was incubated with 10 μM 17α-hydroxyprogesterone. Error bar, 0.02 absorbance units.

The 17,20-lyase activity of CYP17 shows a greater dependence on the abundance of CPR than does its 17α-hydroxylase activity (6, 7). The 17,20-lyase reaction is also markedly stimulated by optimal molar ratios of the cofactor cytochrome b$_5$ (3, 4, 9), emphasizing the importance of redox partner interactions.
in 17,20-lyase chemistry. Consistent with the known dependence of the 17,20-lyase reaction on the redox partners CPR and cytochrome b5, mutations in the redox partner-binding site of CYP17 can cause preferential impairment of 17,20-lyase activity and manifest clinically as ILD (18, 19). Positive charges in the redox partner-binding site of microsomal cytochromes P450 are believed to direct interaction with negative charges in the FMN domain of CPR, driving electron transfer and contributing in other subtle ways to catalysis. In the case of CYP17, neutralization of these positive charges, particularly Arg347 and Arg358 (18), Lys39 (21), and Arg448 (32), dramatically reduces 17,20-lyase activity while leaving 17α-hydroxylase activity largely intact.

In contrast, mutation E305G resides within the active-site pocket and causes ILD by a novel mechanism. Whereas both the affinity of E305G for pregnenolone and its 17α-hydroxylase activities are equivalent to those of wild-type CYP17, both the affinity and turnover of 17α-hydroxyprogrenenolone are selectively and dramatically impaired by mutation E305G. Thus, mutation E305G causes ILD not by disrupting interactions with redox partners, but by selectively altering the binding and turnover of 17α-hydroxyprogrenenolone. Surprisingly, the affinity and turnover of the Δ5-steroids progesterone and especially 17α-hydroxyprogesterone are enhanced by mutation E305G, and androstenedione production is normally stimuated by cytochrome b5. Thus, mutation E305G also reverses the relative efficiencies of the 17,20-lyase reactions in the Δ4- and Δ5-steroid pathways, rendering androstenedione production the preferred pathway (Fig. 1).

Glutamic acid and Thr395 compose a highly conserved pair of polar residues bearing a carboxylate group (glutamate or aspartate) and a hydroxyl group (almost always threonine) in the I-helix near the heme center of most cytochromes P450 (33). Mutations E305Q and T306A in modified human CYP17 expressed in Escherichia coli show impaired 17α-hydroxylase and 17,20-lyase activities, with rates 6–35% of the wild-type enzyme in reconstituted assays (34). Mutations E305Q and E305A/T306A exhibit 6–9-fold increased Ks values for 17α-hydroxyprogrenenolone, with lesser impairments of pregnenolone binding (34). However, the metabolism and binding of Δ5-steroids by these mutations were not reported, and the single mutations E305A and E305G were not characterized in this study. Glu305 and Thr395 lie in a region of the I-helix corresponding to cytochrome P450 substrate recognition site-4 (35), so each substitution of these residues may uniquely influence the binding of various substrates.

Mutation of the corresponding Thr395 to alanine in cytochrome P450cam uncouples most electron input from P450 chemistry, yielding H2O2 and unreacted substrate (36). These data suggest that the threonine, perhaps in conjunction with tightly bound water molecule(s) (37), stabilizes the oxy-P450 complex and suppresses uncoupling reactions. In contrast, mutation of Asp351 in P450cam to the isosteric asparagine reduces the turnover rate by 100-fold, and turnover for mutation D251N remains highly coupled (38). Furthermore, turnover in mutation D251N is pH-dependent, with efficiency increasing as the pH is reduced from 8 to 5 (38); and spectroscopic data suggest that the oxy-P450 species accumulates in the steady state (39). The corresponding D309A and D309N mutations in human aromatase likewise show dramatically impaired reaction rates (40). Based on these mutagenesis studies, the (Glu/Asp)-Thr residue pair has been ascribed the critical role of orchestrating the dynamic organization of active-site water molecules. This network of hydrogen-bonding residues and waters must deliver the proton(s) that drive O–O bond scission prior to dissociation of peroxide from the oxyheme species (38, 39). Hence, these two residues are implicated as critical for maintaining both efficient turnover and tight coupling.

Despite the slow turnover of other CYP17, aromatase, and cytochrome P450cam mutations that involve Glu305 and corresponding residues, theoretical considerations may explain why E305G behaves differently from alanine or glutamine substitutions. The absence of a hydrophobic side chain in glycine might allow one or more water molecules to occupy the space vacated by the carboxylate of Glu305, whereas the side chains of alanine and glutamine might preclude equivalent active-site hydration. Thus, substitution E305G appears to uniquely replace the carboxylate group with ordered water molecules (41) that functionally compensate and deliver proton(s) to the oxyheme at a rate sufficient to maintain efficient 17α-hydroxylation. In contrast, mutation D251G markedly impairs the turnover rate for P450cam (42); consequently, it is not possible to predict the properties of glycine mutations of the corresponding residues of other cytochromes P450.

Only a few known cytochromes P450 do not possess glutamate or aspartate at the position corresponding to Glu305 in human CYP17, and these isoforms belong to the CYP7, CYP8, CYP11, and CYP74 families (33). Some of these enzymes, such as the allene-oxide synthase activity of CYP74, do not require O2 and NADPH and function as peroxidases (43). In the case of human CYP17 mutation E305G, neither hydrogen peroxide nor cumene hydroperoxide reconstituted catalysis in our hands (data not shown), equivalent to results seen with the wild-type enzyme (21). Consequently, E305G appears to primarily alter substrate binding and to selectively impair 17α-hydroxyprogrenenolone metabolism, yet the mechanism of catalysis does not change. We are unaware of other examples in which replacement of the I-helix carboxylate does not drastically impair turnover rates of a cytochrome P450.

In comparing isoforms from different species, all published CYP17 cDNAs code for glutamate at the position corresponding to Glu305 in human CYP17, and these isoforms belong to the CYP7, CYP8, CYP11, and CYP74 families (33). Some of these enzymes, such as the allene-oxide synthase activity of CYP74, do not require O2 and NADPH and function as peroxidases (43). In the case of human CYP17 mutation E305G, neither hydrogen peroxide nor cumene hydroperoxide reconstituted catalysis in our hands (data not shown), equivalent to results seen with the wild-type enzyme (21). Consequently, E305G appears to primarily alter substrate binding and to selectively impair 17α-hydroxyprogrenenolone metabolism, yet the mechanism of catalysis does not change. We are unaware of other examples in which replacement of the I-helix carboxylate does not drastically impair turnover rates of a cytochrome P450.

In comparing isoforms from different species, all published CYP17 cDNAs code for glutamate at the position corresponding to Glu305 in the human enzyme (Fig. 6). Inspection of the alignment in Fig. 6 does not reveal any residues adjacent to Glu305 that track with Δ5- or Δ4-steroid preference for the 17,20-lyase reaction, and it is not known if glycine substitution of the corresponding residues of other CYP17 isoforms will also alter the relative rates of 17α-hydroxysteroid cleavage reactions. Nonetheless, the rate of the 17,20-lyase reaction with the Δ5-steroid 17α-hydroxyprogesterone for mutation E305G cannot be considered rapid, certainly not when compared with efficient enzymes in the Δ5-steroid pathway such as Xenopus CYP17 (12, 44). This analysis indicates that residues elsewhere in the molecule ordinarily confer efficient 17,20-lyase activity in the Δ5-steroid pathway to certain CYP17 isoforms.

Despite modestly increased activity in the Δ5-steroid pathway (cleavage of 17α-hydroxyprogesterone to androstenedione)
CYP17 Mutation E305G

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