P450c17 Mutations R347H and R358Q Selectively Disrupt 17,20-Lyase Activity by Disrupting Interactions with P450 Oxidoreductase and Cytochrome b_5

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Cytochrome P450c17 catalyzes steroid 17α-hydroxylase and 17,20-lyase activities and hence is a key enzyme in the production of human glucocorticoids and sex steroids. These two activities are catalyzed in a single substrate-binding site but are regulated independently in human physiology. We have recently shown that cytochrome b_5 facilitates 17,20-lyase activity by allosterically promoting the interaction of P450c17 with P450 oxidoreductase (OR) and that the human P450c17 mutations, R347H and R358Q, selectively destroy 17,20-lyase activity while sparing 17α-hydroxylase activity. We transfected COS-1 cells with vectors for these P450c17 mutants and found that an excess of OR and b_5 restored a small amount of 17,20-lyase activity, suggesting the mutations interfere with electron donation. To determine whether these mutations selectively interfere with the interaction of P450c17 and its electron-donating system, we expressed each P450c17 mutant in yeast with or without OR, b_5, or both, and measured enzyme kinetics in yeast microsomes using pregnenolone and 17α-hydroxypregnenolone as substrates. The apparent Michaelis-Menten (K_m) values for the R347H mutant with and without coexpressed OR were 0.2 and 0.6 μM, respectively, and for the R358Q mutant with and without OR they were 0.3 and 0.4 μM, respectively; these values did not differ significantly from the wild-type values of 0.4 and 0.8 μM with and without OR, respectively. Furthermore, coincubation with 17α-hydroxypregnenolone showed a competitive mechanism for interference of catalysis. The similar kinetics and the competitive inhibition prove that the mutations did not affect the active site. Coexpression of the mutants with OR yielded insignificant 17,20-lyase activity, but addition of a 30:1 molar excess cytochrome b_5 to these microsomes restored partial 17,20-lyase activity, with the R358Q mutant achieving twice the activity of the R347H mutant. These data indicate that both mutations selectively interfere with 17,20-lyase activity by altering the interaction of P450c17 with OR, thus proving that the lyase activity was disrupted by interfering with electron transfer. Furthermore, the data offer the first evidence that R347 is a crucial component of the site at which b_5 interacts with the P450c17·OR complex to promote electron transfer. (Molecular Endocrinology 13: 167–175, 1999)

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INTRODUCTION

Steroid hormone biosynthesis requires a relatively small number of P450 enzymes to catalyze numerous steroidal interconversions (1). Human P450c17 serves as the qualitative regulator for steroid production, catalyzing sequential 17α-hydroxylation and 17,20-bond scission (17,20-lyase) reactions (2–4). P450c17 also has a modest degree of 16α-hydroxylase activity (5–7). There is only a single P450c17 protein moiety in both the adrenal and the gonad, encoded by a single gene (8) that expresses a single species of mRNA (9), which catalyzes these three steroidal transformations on a single active site. Sex steroid production requires both 17α-hydroxylase and 17,20-lyase activities, and deficiencies in one or both actions of P450c17 leads to ambiguity in male genital formation and lack of progression into puberty in females (4).

More than 125 patients have been described with clinically apparent defects in P450c17, most causing complete loss of all P450c17 activity (10). Of the 23 mutant alleles studied at the molecular level before 1997, all caused defects that affected both the 17α-hydroxylase and 17,20-lyase activities of

P450c17 in equal proportion when expressed in transfected cells (11). Seventeen patients with clinical and hormonal profiles consistent with an isolated deficiency of 17,20-lyase activity have been described (11) since the initial description of apparent isolated 17,20-lyase deficiency (12). However, subsequent molecular genetic and clinical studies showed that one of the initial patients had both 17α-hydroxylase and 17,20-lyase deficiency (13, 14); thus the existence of patients with isolated 17,20-lyase deficiency was in doubt until recently. We then reported two new cases of 17,20-lyase deficiency in whom we identified the P450c17 mutations R347H and R358Q (15). These two mutations selectively ablated more than 95% of 17,20-lyase activity while retaining about 65% of 17α-hydroxylase activity (15) and correspond to two site-directed mutants in rat P450c17 that also preferentially impair 17,20-lyase activity (16). These mutations lie in a region that appears to be critical for binding P450 oxidoreductase (OR) (4, 15, 17, 18), which donates electrons to all microsomal cytochrome P450 enzymes. Thus, these mutations suggested a novel mechanism for steroidogenic P450 enzyme defects, in which substrate binding remains intact, while electron coupling and/or transfer within the P450-electron donor complex is disrupted (15). Initial kinetic studies in transfected COS-1 cells suggested that 17α-hydroxypregnenolone could inhibit the 17α-hydroxylation of pregnenolone by the R347H and R358Q mutants, but this experiment could not determine whether this inhibition was competitive. A quantitative demonstration that the mode of inhibition is competitive is needed to prove that the mutations do not affect the active site. Furthermore, the endogenous COS-1 cell expression of cytochrome b_5 also precluded studies of the potential mechanism by which these mutations might alter the interaction of P450c17 with b_5.

To investigate rigorously the mechanism by which the R347H and R358Q mutations selectively disrupt 17,20-lyase activity, it was first necessary to describe the mechanisms and regulation of 17,20-lyase activity in the wild-type enzyme. Because the R347H and R358Q mutants are located in the proposed redox partner-binding domain, the contributions of the electron donors OR and cytochrome b_5 to 17,20-lyase activity are of great significance. By examining the catalysis of wild-type P450c17 in genetically engineered yeast, we have recently shown that 1) lyase activity requires OR; 2) human cytochrome b_5 cannot support catalysis by itself; 3) cytochrome b_5 can augment lyase activity, but only in the presence of OR; 4) b_5 promotes lyase activity by an allosteric mechanism and does not participate detectably in direct electron transfer; and 5) *in vitro*, cytochrome b_5 modulates lyase activity in a biphasic manner, with inhibition at very high molar ratios of b_5 to P450c17 (19). To delineate the specific interactions with electron transfer proteins that are disrupted by the R347H and R358Q mutants, and to quantitate the affinity of the mutant enzymes for 17α-hydroxypregnenolone, we evaluated the kinetics of these mutants in yeast microsomes.

RESULTS

Expression of R347H and R358Q in COS-1 Cells

Expression of the P450c17 mutants R347H and R358Q in transfected COS-1 cells showed the mutants retained about 65% of wild-type 17α-hydroxylase activity but less than 5% of wild-type 17,20-lyase

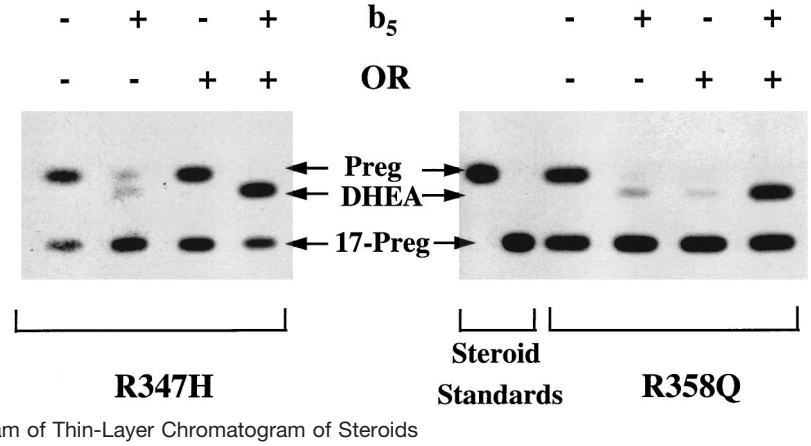

Fig. 1. Autoradiogram of Thin-Layer Chromatogram of Steroids

COS-1 cells cotransfected with vectors expressing P450c17 mutants and the redox partners P450 OR and cytochrome b_5 were incubated with [^{3}H]pregnenolone (21.1 Ci/mmol, NEN Life Sciences, Boston MA), and the steroids present in the culture media were analyzed. *Left*, Cells expressing R347H; *right*, cells expressing R358Q. Each mutant exhibits 17α-hydroxylase activity, converting some pregnenolone (Preg) to 17α-hydroxypregnenolone (17 Preg), but insignificant amounts of DHEA are produced by the two mutants, either alone or when singly cotransfected with vectors expressing OR or b_5. However, each mutant acquires some 17,20-lyase activity when coexpressed with both OR and b_5, as evidenced by the appearance of DHEA.

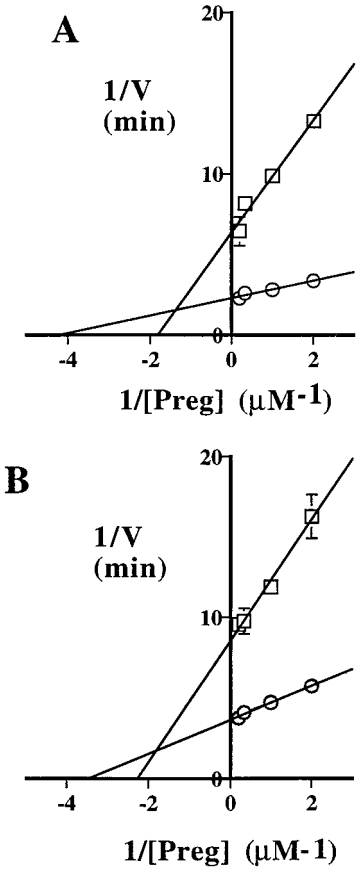

Fig. 2. Lineweaver-Burk Plots of 17α-Hydroxylase Activity for the P450c17 Mutants R347H and R358Q

Lines were derived from least-squares fit to the data points. The apparent K_m and V_{max} values obtained from these data are shown in Table 1. Microsomes were prepared from W303B yeast cotransfected with either V10-c17 and the empty cDE2 vector (*squares*) or with cDE2-OR (*circles*). All incubations were performed with [^{14}C]pregnenolone. Data are the mean of three independent experiments; where no error bars (± SD) are seen, the error bars lie within the symbol for the data point. A, Assays with the R347H mutant; B, assays with the R358Q mutant.

activity, and our modeling studies indicated that these mutants lie in the redox-partner binding site of P450c17 (15). Therefore, we hypothesized that the presence of a substantial excess of the redox partner, OR, might partially overcome this selective 17,20-lyase deficiency. Transfection of COS-1 cells confirmed that each mutant retained substantial 17α-hydroxylase activity but lacked detectable 17,20-lyase activity (Fig. 1). Coexpression of each mutant with OR appeared to augment the 17α-hydroxylase activity, similarly to our previous observations with wild-type P450c17 (7, 15), but unlike those previous experiments with wild-type enzyme, the presence of excess OR did not foster significant 17,20-lyase activity (Fig. 1). We recently demonstrated that cytochrome b_5 selectively increases the 17,20-lyase activity of human P450c17 by allosterically facilitating its interaction with OR (19). However, cotransfection of cells with P450c17 and b_5, in which the only available OR is that present endogenously in the COS-1 cells, also failed to confer detectable 17,20-lyase activity, consistent with our recent demonstration that b_5 cannot act as an electron donor to human P450c17 (19). However, when either the R347H or R358Q mutants are expressed together with vectors overexpressing both OR and b_5, significant 17,20-lyase activity is restored to each mutant. Thus the R347H and R358Q mutants appear to lie in the redox partner binding site, so that partial 17,20-lyase activity can be restored by a substantial molar excess of both OR and b_5. To elucidate the molecular mechanisms by which the combination of OR and b_5 achieves the qualitative results seen in

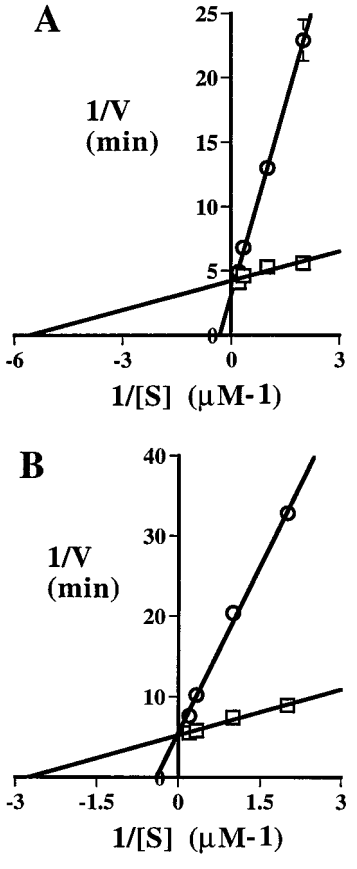

Fig. 3. Lineweaver-Burk Plots of 17α-Hydroxylase Activity for the P450c17 Mutants R347H and R358Q in the Presence of 17α-Hydroxypregnenolone

Lines were derived from least-squares fit to the data points. The apparent K_m and K_i values obtained from these data are shown in Table 1. Microsomes were prepared from W303B yeast cotransfected with vectors V10-c17 and cDE2-OR. Incubations were performed in the absence (*circles*) or presence (*squares*) of 5 μM unlabeled 17α-hydroxypregnenolone and [^{14}C]pregnenolone as the starting substrate. Data are the means of three independent experiments; where no error bars (±SD) are seen, the error bars lie within the symbol for the data point. A, Assays with the R347H mutant; B, assays with the R358Q mutant.

Table 1. Kinetic Constants

	WT	WT/OR	R347H	R347H/OR	R358Q	R358Q/OR
K_m^{app} (μM)[a]	0.8[b]	0.4[b]	0.6	0.2	0.4	0.3
V_{max} (min^{-1})	0.7[c]	3[c]	0.2	0.4	0.1	0.3
K_i^{app} (μM)[d]	N.D.[e]	0.7	N.D.	0.3	N.D.	0.8

[a] Apparent K_m for pregnenolone as substrate.
[b] Lineweaver-Burk plots not shown.
[c] Data from Ref. 19 (Table 3).
[d] Apparent K_i for 17α-hydroxypregnenolone as inhibitor.
[e] Not done.

COS-1 cells, we used our recently described (19) application of a yeast microsome system (20).

Mutant 17α-Hydroxylase Kinetics

Endogenous yeast OR can support both the 17α-hydroxylase and 17,20-lyase activities of P450c17, but the catalytic efficiency (V_{max}/K_m) of each reaction is 10-fold higher in the presence of human OR whether or not the yeast OR is also present (19). Therefore we used the parental yeast strain W303B doubly transfected with vector V10 expressing either of the two mutant forms of P450c17, and with vector cDE2 expressing the cDNA for either human OR or, in the desired absence of human redox partner, with an equal mass of empty vector. Microsomal preparations of these transfectants were then used for kinetic analysis of the K_m and V_{max} by Lineweaver-Burk plots (Fig. 2). Yeast transfected with either of the P450c17 mutants but without human OR are capable of catalyzing 17α-hydroxylase activity, indicating that despite the apparent perturbation in redox partner binding caused by the amino acid substitutions, some 17α-hydroxylase activity can occur utilizing the endogenous yeast OR. As with wild-type P450c17 (19), the V_{max} for the 17α-hydroxylase activity of each mutant is augmented by the presence of cotransfected human OR, while the apparent K_m is reduced (Table 1). In yeast microsomes, the apparent K_m for pregnenolone is 0.6 μM for R347H and 0.4 μM for R358Q; coexpression with OR decreases these values to 0.2 μM and 0.3 μM, respectively, similar to the 2-fold effect seen with the wild-type enzyme (Table 1); this is consistent with the mutations altering the interaction of P450c17 with OR even though the mutants retain considerable 17α-hydroxylase activity. The V_{max} increases 2- to 3-fold in the presence of the OR, slightly less than the 4- to 5-fold effect seen when the wild-type P450c17 is cotransfected with human OR (Table 1). Although these mutants cause a relatively selective loss of lyase activity, there is also an effect on 17α-hydroxylation, as each mutant retains about 65% of wild-type 17α-hydroxylase activity but less than 5% of the native 17,20-lyase activity in transfected COS-1 cells (15). Similarly, the V_{max} obtained for the hydroxylase activity of each P450c17 mutant in yeast, with or without coexpressed OR, is only 10–30% of that observed for the wild-type enzyme.

Effect of 17-OH-Pregnenolone Intermediate on Mutant Hydroxylase Activity

Our previous work in COS-1 cells indicated that 17-OH-pregnenolone inhibited the 17α-hydroxylation of pregnenolone by the wild-type and both mutant P450c17 proteins (15). Although these data suggested that neither of the mutations resulted in a major conformational change in the steroid hormone binding (active) site, the inherent limitations of kinetic measurements obtained in whole transfected cells precluded our determining the nature of this inhibition. Therefore, we used the yeast microsomal system to perform hydroxylase assays in the presence and absence of unlabeled 17α-hydroxypregnenolone at a final concentration of 5 μM (5- to 10-fold K_m). The Lineweaver-Burk plots demonstrate the competitive nature of this inhibition for each mutant, as there is no change in V_{max} (Fig. 3). The apparent K_m values of 0.2–0.4 μM pregnenolone and K_i values of 0.3–0.8 μM 17α-hydroxypregnenolone in the presence of OR are remarkably similar for all three forms of P450c17 (Table 1) and compare favorably to the half-maximal inhibition values of 0.3 to 1.0 μM derived from the qualitative COS-1 cell experiments (15). This demonstration that 17α-hydroxypregnenolone is a competitive inhibitor of the 17α-hydroxylase reaction in both mutants confirms the hypothesis that substrate binding at the active site is not significantly impaired by the R347H and R358Q mutations.

Effect of Human Cytochrome b_5 on Mutant Enzymatic Activity

Addition of OR increases both 17α-hydroxylase and 17,20-lyase activity in environments where OR is limiting (7, 19). By contrast, cytochrome b_5, either coexpressed in yeast with P450c17 and OR or exogenously added to yeast microsomes containing P450c17 and OR, will increase 17,20-lyase activity but has no appreciable effect on the already maximal 17α-hydroxylase activity (19). Coexpression of human OR with the R347H and R358Q mutants of P450c17 increased their 17α-hydroxylase activities (Fig. 4A) similarly to the effect seen with the wild-type enzyme (19), but the coexpression of OR had no detectable effect on the 17,20-lyase activity of the mutants despite the presence of endogenous yeast OR (Fig. 4B). However,

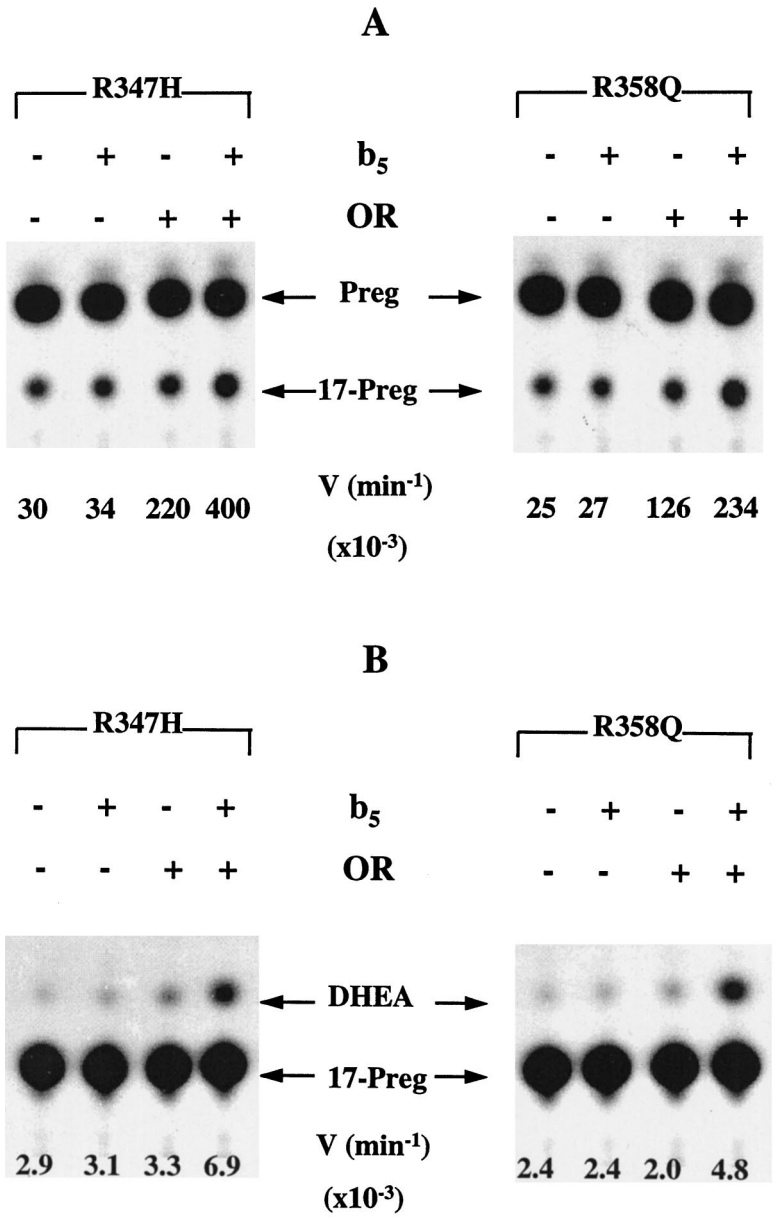

Fig. 4. Effect of Exogenously Added Human b_5 on Mutant P450c17 Activities

Microsomes from W303B yeast expressing the R347H or R358Q mutants were cotransfected with cDE2 expressing no protein (−) or expressing OR (+). Where indicated (+), purified human b_5 was added at a 30:1 molar ratio to P450c17. A, Microsomes were incubated with 1 μM [^{14}C]pregnenolone, and 17α-hydroxylase was measured as conversion to 17α-hydroxypregnenolone by TLC and autoradiography as shown, after which the spots for the two steroids were cut out and counted to generate the enzymatic velocity data shown below the chromatogram. B, Microsomes were incubated with 50 nM of [^{3}H]17α-hydroxypregnenolone, and 17,20-lyase activity was measured as conversion to DHEA and analyzed as in panel A.

unlike the wild-type, exogenous addition of purified human cytochrome b_5 increased both the hydroxylase and lyase activities of the mutants (Fig. 4). This is consistent with the view that b_5 allosterically fosters the interaction of P450c17 and OR, and that the R347H and R358Q mutations interfere with this interaction. To test this, we examined the effects of exogenously added b_5 on the kinetics of the 17,20-lyase activity of each mutant.

Initial rate kinetic analysis of the 17,20-lyase activity of the R347H and R358Q mutants, either in the presence or absence of cotransfected OR, was not possible, as there was too little activity for meaningful quantitation. Because b_5 increases the 17,20-lyase activity of wild-type (19, 21–23) and mutant (Fig. 4) P450c17, we examined the effect of varying the ratio of b_5 to P450c17 for the wild-type and each mutant in the presence of OR. When examined over a range of b_5:

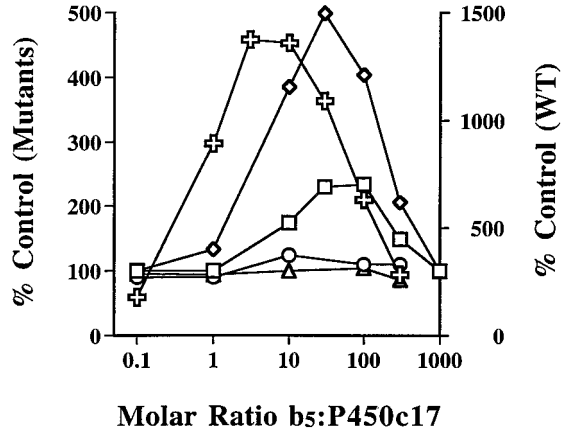

Fig. 5. Biphasic Effect of Cytochrome b_5 on 17,20-Lyase Activity

In the absence of OR, increasing molar ratios of b_5 to P450c17 conferred no 17,20-lyase activity on the P450c17 mutants R347H (*triangles*) or R358Q (*circles*). In the presence of human OR, 17,20-lyase activity was maximal at b_5 to P450c17 ratios of 3:1 to 10:1 for wild type P450c17 (*plus signs*) but higher ratios were needed for maximal activity of R347H (30 to 100:1) (*squares*) and R358Q (30:1) (*diamonds*). Production of DHEA from 50 nM [^{3}H]17α-hydroxypregnenolone was measured in yeast microsomes containing the amounts of b_5 shown and is plotted as the percent of conversion by the microsomes without added b_5. The data for the mutants are graphed with respect to the scale on the *left*, and the wild type data are graphed with respect to the scale on the *right*.

P450c17 molar ratios of 0.1:1 up to 1000:1, the peak response of the wild-type enzyme is 2.5- to 6-fold greater than that seen with either mutant (Fig. 5). This peak response is shifted to at least 10-fold higher ratios for the mutants (30 to 100:1) than for wild-type enzyme (1 to 3:1). The two mutants also respond differently to the presence of excess cytochrome b_5: there is a sharper and more dramatic effect on the 17,20-lyase activity of R358Q than the broader response seen for R347H. Finally, in the absence of human OR, there is no detectable 17,20-lyase activity for either mutant, even at b_5:P450c17 ratios up to 300:1. This is consistent with our previous observation that OR must be present for P450c17 to catalyze any 17,20-lyase activity (19). The differences in the b_5 curves (Fig. 5) indicate that even though the R347H and R358Q mutations alter the charge distribution in the redox-partner binding site (15), the two mutants can be distinguished biochemically by the differential effect of b_5 stimulation. These data suggest that R347 is the more critical residue for the interaction of b_5 with the P450c17·OR complex (19). These differences in the b_5 titration curves of the R347H and R358Q mutants provide a more detailed understanding of the interactions of redox partners with P450c17 during catalysis.

DISCUSSION

Because human P450c17 catalyzes very little conversion of 17α-hydroxyprogesterone to Δ^4-androstenedione (19), almost all circulating human sex steroids derive from dehydroepiandrosterone (DHEA) synthesized by the sequential 17α-hydroxylase and 17,20-lyase activities of P450c17. Consequently, all mutations in P450c17 studied before 1997 reduced both activities comparably (11, 24, 25). The discovery of patients whose P450c17 mutations abolished almost all lyase activity while preserving most of the 17α-hydroxylase activity provides a unique opportunity to study the structural requirements for the proper assembly of the catalytic complex that performs the 17,20-lyase reaction (18). Somewhat surprisingly, the computational predictions (15) and the direct evidence presented in this paper demonstrate unequivocally that these two mutations do not change the enzyme's affinity for the 17α-hydroxypregnenolone intermediate. Instead, mutations R347H and R358Q impair the ability of these mutants to form productive interactions with OR and, to a variable extent, with b_5.

The study of the R347H and R358Q mutants shows that these amino acid replacements do not alter the active site and that 17,20-lyase activity is more vulnerable to disruptions in redox partner interactions than is the 17α-hydroxylase reaction. While it is theoretically possible that some hypothetical mutation in the active site of P450c17 could preferentially reduce affinity for 17α-hydroxypregnenolone to cause isolated 17,20-lyase deficiency, no such mutations have been found, either by examining mutations found in patients or by site-directed mutagenesis (17). It is of interest that a third mutation of P450c17, F417C, was recently reported that also caused selective impairment of 17,20-lyase activity, although with a greater impairment of 17α-hydroxylase activity than is found in the R347H or R358Q mutants (26). While F417 is rather distant in its location in the linear amino acid sequence, our molecular modeling indicates that F417 lies near R358 and R347 in three-dimensional space, but unlike the solvent-exposed arginines, F417 is buried and forms a bulging ridge at the presumed edge of the redox-partner binding site (18). Thus, interference with either the electrostatic surface charges or the shape of the redox-partner binding site can cause selective loss of 17,20-lyase activity.

Although R347 and R358 appear to be close in three-dimensional space, the biochemistry of these two mutants can be distinguished by their activities in the presence of cytochrome b_5. Whereas b_5 stimulates the wild-type enzyme's 17,20-lyase activity more than 13-fold (19), b_5 stimulates the lyase activity of the R358Q and R347H mutants only 5- and 2.5-fold, respectively. These results suggest that R347 is a critical component of the P450c17-b_5 interaction site on P450c17. Our previous studies suggest that the 17,20-lyase reaction occurs in the P450c17·OR complex,

with b_5 acting on this complex as an allosteric facilitator (19). This model is consistent with the observation that the mutations of the redox-partner binding site eliminate detectable 17,20-lyase activity in the presence of the low levels of OR endogenously provided by the yeast or COS-1 cells. A trace of lyase activity was observed in the presence of human (but not yeast) OR, and we can demonstrate significant lyase activity only in the presence of both human OR and human b_5. Thus our genetic, biochemical, and computational studies of wild-type and mutant P450c17 proteins are beginning to delineate the structural details of how this complex is assembled and why it performs this unique oxidative carbon-carbon bond cleavage reaction only with 17-OH-pregnenolone. Furthermore, the differences between the biochemistry of the R347H and R358Q mutants demonstrate that binding of b_5 is necessary but not sufficient for maximal lyase activity and that residues that interact preferentially with OR and b_5 can be functionally distinguished by specific mutations in P450c17.

The ratio of 17,20-lyase activity to 17α-hydroxylase activity is regulated in normal human adrenal physiology. DHEA concentrations (reflecting 17, 20-lyase activity) rise dramatically at adrenarche and then wane during aging (27), while cortisol concentrations (reflecting 17α-hydroxylase activity) remain constant. The regulation of this ratio of activities may be altered in the polycystic ovary syndrome, which affects 5% of women of reproductive age (28). These individuals have both ovarian and adrenal hyperandrogenism with normal cortisol, suggesting a disorder at the level of 17,20-lyase activity (4, 29). Our studies of mutations causing isolated 17,20-lyase deficiency show that the surface charges in the redox partner binding site are crucial for optimizing lyase activity. Obviously, the physiological regulation of lyase activity will not involve changes in the amino acid sequence of P450c17, but regulated posttranslational modification might alter surface charges in the redox partner binding site, providing a mechanism for the physiological regulation of the hydroxylase-lyase ratio. Serine/threonine phosphorylation of human P450c17 by an unidentified cAMP-dependent protein kinase increases 17,20-lyase activity, and dephosphorylation of P450c17 diminishes 17,20-lyase activity without altering its 17α-hydroxylase activity (29). Consistent with this view, a preliminary report indicates that the P450c17 mutant F419C, found in a patient with isolated 17,20-lyase deficiency, cannot be phosphorylated normally (30). The similarity of the dephosphorylation data (29) and our present results with mutations in the redox partner binding site suggests that specific phosphorylated serine residues serve to provide surface charges that optimize the interaction of P450c17 with OR and/or b_5 to optimize electron transfer for the lyase reaction. Thus, understanding the precise mechanisms contributing to the ratio of 17,20-lyase to 17α-hydroxylase activity is of substantial interest and importance.

Finally, our understanding of how mutations in P450c17 can selectively destroy lyase activity suggests novel approaches to the design of inhibitors of this enzyme that occupies a central role in determining flux through the steroidogenic pathways. We hypothesize that compounds that bind to the redox partner binding site of P450c17 might be highly potent inhibitors of sex steroid biosynthesis, which would be therapeutically useful in polycystic ovary syndrome and in cancers of the breast and prostate. Furthermore, such compounds would not bind to the active site and therefore need not resemble steroids; hence they should not produce side effects often associated with steroids. Therefore, principles deduced from the study of two patients with a rare defect in androgen biosynthesis might yield widespread benefits in the understanding of physiology and treatment of disease.

MATERIALS AND METHODS

Yeast Strains and Expression Vectors

Wild-type yeast strain W303B (JC104) (*trp1*-1; *ura3*-1; *ade2*-1; *can1*-100, mat α) and the yeast expression vector cDE2 (pYcDE-2) were generous gifts of Drs. Gregory Petsko and Ira Herskowitz. The yeast expression vector V10 (pYeDP10) was a generous gift from Dr. Denis Pompon (CNRS, Gif-sur-Yvette, France). Wild-type human P450c17 cDNA (9) was PCR amplified using primers previously described (19) and digested with *Bam*HI and *Eco*RI, facilitating directional cloning into the complementary ends of *Bgl*II-*Eco*RI-digested V10 vector. This destroys the *Bgl*II site and places the P450c17 cDNA under the control of the constitutive *pgk* promoter, producing the vector V10-c17. Human OR cDNA was PCR amplified from pECE-OR (7) using primers previously described (19). The human OR cDNA was then cloned into the *Eco*RI site of the cDE2 vector under the control of the constitutive *adc1* promoter, with a *trp1* selectable marker. P450c17 cDNAs containing the mutations R347H or R358Q were PCR amplified from previously constructed pMT2 expression vectors as templates (15) using *pfu* polymerase (Stratagene, La Jolla, CA) and the primers c17S-1 and c17AS-1 (19), and then sequenced in their entirety to ensure the presence of only the desired mutation. These mutant cDNAs were then digested with *Bam*HI and *Eco*RI and ligated into the V10 vector as with the wild-type c17 cDNA described above.

Yeast Transformation and Growth

Yeast were transformed using 700 μl of 40% polyethylene glycol 3350, 0.1 M lithium acetate, 10 mM Tris-HCl (pH 8), 1 mM EDTA. About 10^6 yeast cells were transformed in 100 μl of 0.1 M lithium acetate, 10 mM Tris-HCl (pH 8), 1 mM EDTA with 1 μg of plasmid DNA, and 50 μg of denatured herring sperm DNA as carrier (31). Cells were washed in 100 μl of 1 M sorbitol before final resuspension in 100 μl of 10 mM Tris-HCl (pH 8), 1 mM EDTA, for plating onto selective media. Expression of mutant or wild-type P450c17 was always under the control of the constitutive *pgk* promoter. For microsome preparations, transformed yeast were cultured in minimal SD media containing 20 g/liter D-glucose, 1.7 g/liter yeast nitrogen base without amino acids or ammonium sulfate (Difco, Detroit, MI), 5 g/liter ammonium sulfate, and 45 mg/liter adenine.

Microsome Preparation and Characterization

Yeast cells harvested at a density of 4.5–6 $\times$ 10^7 cells/ml were disrupted by manual breakage with 450- to 600-μm glass beads for 5 min (20), stopping at 1-min intervals, and icing the cells for 30 sec between disruptions. Three microliters of 1 M ethanolic phenylmethylsulfonyl fluoride were added after the first minute of breakage. For each 300 ml of culture, the crude extracts together with the glass beads were washed twice with 5–7 ml of 50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.4 M sorbitol, and the cellular debris was collected by centrifugation at 4 C twice for 10 min each at 14,000 $\times$ *g*. The microsomes were pelleted by centrifugation at 4 C for 60 min at 100,000 $\times$ *g* and were resuspended in 50 mM Tris-HCl (pH 8), 1 mM EDTA, 20% glycerol at a final concentration of 10–20 μg/μl total protein. Each preparation was homogenized by shearing the microsomes with passage through a 27-gauge needle 10 times; aliquots were kept frozen at −70 C. Microsomal protein content was determined colorimetrically. Microsomal P450 content was measured spectrophotometrically (32) using either a Cary 3E or a Shimazdu UV 160U spectrophotometer.

P450c17 Enzyme Assay

Microsomes were assayed for hydroxylase activity under initial rate kinetics by preincubation in 50 mM potassium phosphate buffer (pH 7.4) with 0.5–5.0 μM steroid (added in 4 μl of ethanol) in a total volume of 200 μl, at 37 C for 3 min before the addition of 1 mM NADPH to initiate the reaction. For 17α-hydroxylase assays, each reaction contained 10,000 cpm of [^{14}C]pregnenolone (55.4 Ci/mol, NEN Life Science Products, Inc., Boston, MA). For the 17,20-lyase assays, including the b_5 titration experiments, each reaction contained 100,000 cpm of [^{3}H]17α-hydroxypregnenolone (21.2 Ci/mmol, NEN Life Science Products) at a final 17α-hydroxypregnenolone concentration of 50 nM. Steroids were extracted with 400 μl of ethyl acetate-isooctane (1:1, vol/vol), concentrated under nitrogen, separated by TLC (Whatman PE SIL G/UV silica gel plates, Maidstone, Kent, UK) using a 3:1 chloroform-ethyl acetate solvent system, and quantitated as described (15). Purified recombinant human cytochrome b_5 was obtained from PanVera (Madison, WI). Kinetic behavior was approximated as a Michaelis-Menten system for data analysis, and all error bars shown represent SDs. K_i values for 17-OH-pregnenolone were calculated from the equation $K'm = (K_m/K_i)[I] + K_m$ where $K'm$ is the K_m in the presence of inhibitor I at the concentration [I] (33).

COS-1 Cell Transfections

COS-1 cells were grown to 50% confluence in 10% FCS and at 5% CO_2 and transfected with 2–5 μg of cDNA in the appropriate expression vectors, as previously described (34). Cells received vectors expressing either mutant P450c17 cDNA alone, mutant P450c17 plus OR cDNA [in the pECE expression vector, (7)], mutant P450c17 cDNA plus b_5 cDNA (19, 35, 36), or mutant P450c17 plus the vectors for OR and b_5. The total amount of transfected cDNA was standardized by cotransfection with empty pMT2 vector DNA. Transfection efficiency was monitored by cotransfection with a Rous sarcoma virus-luciferase construct. Transfected cells were washed with PBS and incubated in fresh medium for 36 h before addition of either [^{3}H]pregnenolone or [^{3}H]17α-hydroxypregnenolone in 4 ml of medium, at a final concentration of 0.6 nM. Steroids were extracted and analyzed as described above.

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