Mechanism of 17α,20-Lyase and New Hydroxylation Reactions of Human Cytochrome P450 17A1

18O LABELING AND OXYGEN SURROGATE EVIDENCE FOR A ROLE OF A PERFERRYL OXYGEN

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Cytochrome P450 (P450) reactions can involve C–C bond cleavage, and several of these are critical in steroid and sterol biosynthesis. The mechanisms of P450s 11A1, 17A1, 19A1, and 51A1 have been controversial, in the context of the role of ferric peroxide (FeO3-) versus perferryl (FeO3+, compound I) chemistry. We reinvestigated the 17α-hydroxyprogesterone and 17α,20-lyase reactions of human P450 17A1 and found incorporation of one 18O atom (from 18O2) into acetic acid, consonant with proposals for a ferric peroxide mechanism (Akhtar, M., Lee-Robichaud, P., Akhtar, M. E., and Wright, J. N. (1997) J. Steroid Biochem. Mol. Biol. 61, 127–132; Akhtar, M., Wright, J. N., and Lee-Robichaud, P. (2011) J. Steroid Biochem. Mol. Biol. 125, 2–12). However, the reactions were supported by iodosylbenzene (a precursor of the FeO3+ species) but not by H2O2. We propose three mechanisms that can involve the FeO3+ entity and that explain the 18O label in the acetic acid, two involving the intermediacy of an acetyl radical and one a steroid 17,20-dioxetane. P450 17A1 was found to perform 16-hydroxylation reactions on its 17α-hydroxylated products to yield 16,17α-dihydroxyprogrenenolone and progesterone, suggesting the presence of an active perferrylloxo active species of P450 17A1 when its lyase substrate is bound. The 6F-hydroxylation of 16α,17α-dihydroxyprogesterone and the oxidation of both 16α,17α-dihydroxyprogesterone and 16α,17α-dihydroxyprogrenenolone to 16-hydroxy lyase products were also observed. We provide evidence for the contribution of a compound I mechanism, although contribution of a ferric peroxide pathway in the 17α,20-lyase reaction cannot be excluded.

Cytochrome P450 (P450) enzymes catalyze oxidations of more chemicals than any other group of proteins (1). The list of reactions includes aliphatic and aromatic hydroxylations, heteroatom oxidations, epoxidations, and reactions involving both ring formation and cleavage (2–4). Many P450 reactions are important in the biosynthesis and degradation of steroids and sterols (4, 5), including several critical C–C bond cleavage reactions, i.e. those catalyzed by P450s 11A1, 17A1 (Fig. 1), 19A1, and 51A1 (6, 7).

The mechanisms of the C–C cleavage reactions have been the subject of considerable interest and debate. One of the questions with P450s 17A1, 19A1, and 51A1 has been whether the active oxidant is a ferric peroxide (FeO3-), which is an early intermediate following oxygen addition to the iron (Fig. 2, step 4) or the FeO3+ species (Fig. 2, step 6), often referred to as compound I (4, 10, 11). With P450s 17A1 and 19A1, a variety of approaches has been applied, including theoretical calculations, biomimetic models, spectroscopy, substrate atom labeling, and kinetics (12–32).

These C–C bond cleavage reactions are complex, and many of the results are ambiguous; also, a “mixed” mechanism would not be discerned in many of these experiments. One powerful approach originally used by Akhtar and co-workers (27–31) analyzes the actual reaction and can provide discrimination between the nucleophilic FeO3- and electrophilic FeO3+ reactions (Fig. 2), based on the incorporation of 18O label from O2 into the carboxylic acid products (Fig. 3) (7). However, these experiments are complicated due to the ubiquitous presence of formic acid (P450 19A1 and 51A1 reactions) and acetic acid (P450 17A1) in laboratory settings. Thus, the data from such experiments are interpreted with the most confidence when the steroid substrates are labeled with 2H or 13C isotopes to facilitate analysis (15, 33). Even then, the mass spectrometry results can be problematic, particularly if a shift of only one atomic mass unit is introduced and isotopologues derived from 18O incorporation are not discriminated from molecules containing natural abundance 13C atoms (33).

The incorporation of one atom of 18O label from O2 into formic acid (Fig. 3A) had been considered one of the most critical pieces of evidence in support of an FeO3- mechanism for P450 19A1 (14, 15, 34). Because of the importance of this evidence in the existing dogma, we re-examined this experiment using several technical improvements including the following: (i) purified recombinant P450 19A1; (ii) a new diazo reagent conditions; HRMS, high resolution mass spectrometry; LC-MS, (combined) liquid chromatography-mass spectrometry.
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**Results**

**Experimental Design for 18O Experiments**—The P450 17A1 17α,20-lyase reaction produces DHEA from 17α-hydroxyprogrenolone (Fig. 1). The product acetic acid is of particular interest in determining the mechanism of P450 17A1 catalysis (Fig. 3). To unambiguously distinguish the acetic acid formed as a product of the P450 17A1 reaction, the 17α-hydroxy substrate was d6-labeled at position C21 because of concerns about the level of endogenous acetic acid interfering with that formed in the enzyme reaction, based on our experience with 1- and 2-C carboxylic acids (36–39). Based on possible mechanisms shown in Fig. 3, the acetic acid products of 18O2 incubations with the 17α-hydroxy steroids are as follows: CD3CO18OH (mechanisms A, B, C, and F), a 1:2 molar ratio of CD2HCOOH and CD3CO18OH (mechanism D), only CD2HCOOH (mechanism E), or only CD3COOH (mechanism Fa). Because of the small amounts of acetic acid produced (1:1 stoichiometry with steroid, ~25 μmol) during incubations, the acetic acid was converted into an ester using diazoethylpyridine to facilitate characterization. In addition to the increase in mass, the ester is designed for efficient ionization attributable to the nitrogen in the pyridine ring, i.e., 2-(pyridin-2-yl)ethyl acetate (33) (m/z 166.1, "MH+") with one 18O incorporated (d6-labeled, "MH+ + 2") or one 16O incorporated (d6-labeled, "MH+ + 3"), and one 18O incorporated (d6-labeled, "MH+ + 5"). A similar approach was used for 17α-hydroxy-[2,2,4,6,6,21,21,21-2H8]progesterone.

**17α-Hydroxyprogrenolone and 17α-Hydroxyprogesterone 18O Experiments**—One 18O atom was incorporated into acetic acid without deuterium loss (Fig. 4, B–E) when 17α-hydroxy-[21,21,21,2H3]pregnenolone was used as the substrate, ruling out the mechanism in Fig. 3E.

In the case of 17α-hydroxy-[2,2,4,6,6,21,21,21-2H8]progesterone as the substrate (Fig. 5), the signal-to-noise ratio of 18O-incorporated acetate was three times greater than when...
the 17α-hydroxy-[21,21,21-2H3]pregnenolone substrate was used. This improvement in sensitivity is attributed to the extra centrifugation step to remove the emulsion when extracting the acetic acid product (cf. “Experimental Procedures”). Additionally, we observed a trideutero-pyridine acetate product with no 18O incorporation at 6 ppm mass tolerance (Fig. 5B, 5b); however, the intensity was small compared with the 18O-incorporated acetate product (≈0.1% of 18O-incorporated product), and this isotopologue is likely derived from the residual 16O2 in the 18O2 cylinder (99% 18O abundance).

Reactions with Oxygen Surrogates, Background and Previous Studies—If the ferric hydroperoxide mechanism is operative, then one might expect the reaction to be supported by the direct addition of H2O2 to ferric P450 (Fig. 2). However, Auchus and Miller (40) reported that no 17α,20-lyase activity was observed with recombinant human P450 17A1 plus H2O2 in yeast microsomes. Iodosylbenzene is a single oxygen donor and cannot support a reaction that requires two oxygens, i.e., a ferric peroxide complex (41). Iodosylbenzene also did not support the 17α,20-lyase reaction in a P450 17A1 yeast microsomal system (40).
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**P450 17A1 Reactions with Iodosylbenzene**—Preliminary experiments indicated that the most effective concentration to use was 300 μM (results not presented).

Two products were formed from 17α-hydroxyprogesterone in both the iodosylbenzene and NADPH-based systems (Fig. 6). The expected product androstenedione (Fig. 1) was characterized by co-elution with a standard and by both LC-UV and LC-MS comparisons with a standard (data not shown). The other product, which eluted just before androstenedione, was identified as 16,17α-dihydroxyprogesterone by co-elution with a standard and by both LC-UV and LC-MS comparisons with a reference standard (Fig. 7). Although the dihydroxy product co-eluted with the 16α,17α-diastereomer, we cannot exclude the presence of the 16β-stereoisomer.

The products formed from 17α-hydroxyprogrenenolone were converted to Δ4 steroids by the action of cholesterol oxidase. These were identified as 16,17α-dihydroxyprogrenenolone and androstenedione, thus indicating that the products formed from 17α-hydroxyprogrenenolone were 16,17α-dihydroxyprogrenenolone and DHEA.

The rates of formation of 16,17α-dihydroxyprogesterone and androstenedione from 17α-hydroxyprogesterone in the NADPH- and iodosylbenzene-based systems were comparable in the absence of cytochrome b5 (Fig. 8A). The iodosylbenzene-dependent reaction was stimulated 2-fold by cytochrome b5, but the stimulation of the reaction that used NADPH-P450 reductase was much greater (10-fold), so that the iodosylbenzene versus NADPH-P450 reductase comparison (with cytochrome b5 present) is more disparate (Fig. 8A).

With 17α-hydroxyprogrenenolone as substrate, a similar conclusion was reached regarding comparisons of the rates of the NADPH/reductase- and iodosylbenzene-supported reactions (Fig. 8B). When the 16-hydroxylation of the 17α-hydroxy steroids was considered, the iodosylbenzene-supported reactions were faster (Fig. 9). It is also notable that these reactions were stimulated by cytochrome b5.

**Reactions with H2O2**—H2O2 was added to purified P450 17A1 (with cytochrome b5 present), and no detectable 17α,20-lyase activity was found toward 17α-hydroxyprogrenenolone or 17α-hydroxyprogesterone, using varying concentrations of H2O2 (up to 10 mM) (Fig. 9). Under these conditions, the usual reconstituted P450 17A1/NAPD-P450 reductase/cytochrome b5 system yielded the expected products (Fig. 10, B and E).

**Additional Oxidation Products**—With both 17α-hydroxyprogesterone and 17α-hydroxyprogrenenolone, the rates of formation of the lyase products (androstenedione and DHEA) were no longer linear after 5 min (300 s) (Fig. 8).

*FIGURE 4. P450 17A1 incubation with [21,21,21-2H3]17α-hydroxyprogrenenolone (1) in the presence of 18O2, followed by derivatization and analysis by HRMS. A, scheme showing the incubation of deuterated lyase substrate (1) with P450 17A1 and cytochrome b5 in the presence of 18O2. The acetic acid product (3) was derivatized with the diazooethylpyridine reagent (4) and analyzed by liquid chromatography-HRMS. B, ion chromatograms monitoring the various isotopically labeled acetate products that were derivatized to the pyridylethyl esters (Sa-h), with 4 ppm mass tolerance parameter. a, m/z 171 window (d4 18O); b, m/z 169 window (d4 18O); c, m/z 170 window (d3 18O); d, m/z 168 window (d2 18O); e, m/z 169 window (d1 18O); f, m/z 167 window (d0 18O); g, m/z 168 window (d0 18O); h, m/z 166 window (d0 18O). C, mass spectrum of the m/z 166.5–171.3 range by selecting the ts 3.01–3.12-min time interval in the ion chromatogram corresponding to the pyridine ester retention time. Shown at m/z 167.0901 is the peak corresponding to the acetate from background acetic acid from the natural abundance of 13C isotope (Sf), expected mass, m/z 167.0896, Δ 3.0 ppm). The peak at m/z 171.1099 corresponds to the acetate derived from the enzymatic product (Sa, expected mass, m/z 171.1093, Δ 3.5 ppm). D, expansion of the mass spectrum (m/z 168.95–169.22) from C showing the absence of d4-labeled acetate with no 18O incorporation (Sb, expected mass, m/z 169.1051). E, expansion of the mass spectrum (m/z 170.95–171.22) from C showing the presence of d4-labeled acetate with 18O incorporation (Sa, expected mass, m/z 171.1093). p, profile (peaks are shown in profile mode and not “centroid”). ESI, electrospray ionization; RT, retention time; NL, normalized level. More information about the meaning of the settings can be obtained from the Xcalibur Qual Browser User Guide (Thermo Scientific).
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more obvious in the latter case, with the amount of accumulated product decreasing (Fig. 8B). The phenomenon was found to be the result of further 16-hydroxylation of the lyase products, in that we were able to identify these products (tR, UV spectra, and mass spectra) in the longer term reactions with both substrates (with 16-hydroxy-DHEA being converted to 16-hydroxyandrostenedione by cholesterol oxidase in the assays with 17α-hydroxypregnenolone) (Fig. 11). The time course of formation of these products from androstenedione and DHEA is shown in Fig. 12.

We also noted a decrease in the level of 16,17α-dihydroxy steroids with extended time (in the NADPH-supported reactions, Fig. 9). We analyzed the products formed from (commercial) 16α,17α-dihydroxyprogesterone. One product was 16α-hydroxyandrostenedione, identified above (Fig. 13). The major product formed from 16α,17α-dihydroxyprogesterone was 16α-hydroxy-DHEA, identified by its mass and NMR spectra (Fig. 14).

Identification of Steroid B Ring Hydroxylation Product—The other major product in the incubation of 16α,17α-dihydroxyprogesterone with P450 17A1 was a triol, as judged by HRMS (m/z 363.2160, calculated MH+ m/z 363.2166, Δ1.7 ppm) (Fig. 15A-15D) (42). The UV spectrum was similar to those of other Δ4 3-keto steroids except blue-shifted ~5 nm (Fig. 15B), consistent with an intact Δ4 3-keto steroid having some modification near the chromophore. The site of hydroxylation was identified as C-6 by 1H NMR (Fig. 15C and Table 1), i.e. 6β,16α,17α-trihydroxyprogesterone (43, 44). The 18, 19, and 21 methyl groups were intact (δ0.79, 1.40, and 2.28 ppm), but there was a new multiplet at δ 4.38 ppm. The NOESY spectrum (supplemental Fig. S1) showed a spatial correlation between the new hydroxymethine proton (δ 4.38 ppm) and the Δ4-proton (δ 5.70 ppm). Moreover, the HMBC spectrum (heteronuclear multiple-bond correlation (NMR) spectroscopy) (supplemental Fig. S2) indicated a 3-bond coupling interaction between the C4-carbon (δ 125 ppm) and the hydroxymethine proton (δ 4.38 ppm) suggesting either the C2-position or the C6-position for the newly identified hydroxyhexenone proton (δ 4.38 ppm). The C6-position for the hydroxymethine proton (δ 4.38 ppm) was established because the C2-methylene protons (δ 2.53 and 2.42 ppm), which had a 2-bond correlation to the C3-keto carbon atom (δ 205.1 ppm) in the HMBC spectrum, were present. Moreover, the COSY spectrum (supplemental Fig. S3) indicated a 3-bond coupling between the C6-proton (δ 4.38 ppm) and the C7-protons (δ 1.98 and 1.34 ppm) (see also HSQC spectrum, supplemental Fig. S4).

We have assigned the stereochemistry of the C6-hydroxyl group as β, based on the chemical shifts of the 7-protons of the

(Si, expected mass, m/z 167.0896, Δ 3.6 ppm). The peak at m/z 171.1099 corresponds to the acetate derived from the enzymatic product (Sa, expected mass, m/z 171.1093, Δ 4.1 ppm). D, expansion of the mass spectrum (m/z 168.95–169.22) (from C) showing the detection of d1-labeled acetate with no 13C incorporation (Sb, expected mass, m/z 169.1051, Δ 5.3 ppm). E, expansion of the mass spectrum (m/z 170.95–171.22) from C showing the presence of d1-labeled acetate with 13C incorporation (Sa, expected mass, m/z 171.1093), p, profile (peaks are shown in profile mode and not “centroid”). ESI, electrospray ionization. RT, retention time. NL, normalized level. More information about the meaning of the settings can be obtained from the Xcalibur Qual Browser User Guide (Thermo Scientific).
steroid. In considering the chemical shifts of literature compounds (see Refs. 61 and 62 and see Table 2C in Ref. 42 and references therein), corresponding to \(6\alpha\) - and \(6\beta\)-hydroxyprogesterone, the chemical shifts of the \(7\alpha\) - and \(7\beta\)-protons are very informative. In the isolated P450 17A1 product, the chemical shifts of the \(7\alpha\) - and \(7\beta\)-protons were \((\delta)\) 1.34 and 1.98 ppm, respectively, and in Ref. 42, the \(7\alpha\) - and \(7\beta\)-protons were at \((\delta)\) 1.28 and 2.02 for \(6\beta\)-hydroxyprogesterone, whereas the protons had chemical shifts of \((\delta)\) 1.11 and 2.19 for \(6\alpha\)-hydroxyprogesterone. Thus, \(6\alpha\)-hydroxy is the most likely stereochemistry of the new product. The NOESY spectrum showed spatial correlation between the C6-proton \((\delta 4.38 \text{ ppm})\) and both of the

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**FIGURE 6.** Formation of \(16,17\alpha\)-dihydroxyprogesterone and androstenedione from \(17\alpha\)-hydroxyprogesterone by P450 supported by the oxygen surrogate iodosylbenzene. Retention times \((t_R)\) and integration units are indicated on the chromatograms. A, standard compounds. B, reaction (0.5 mM P450 17A1) supported by NADPH-P450 reductase (2.0 mM), cytochrome \(b_5\) (0.5 mM), and NADPH (30-s incubation). C, reaction (0.5 mM P450 17A1 and cytochrome \(b_5\) (0.5 mM)) with 0.30 mM iodosylbenzene (30-s incubation). In control experiments with only cytochrome \(b_5\) and iodosylbenzene (2 mM) mixed with the \(17\alpha\)-hydroxysteroids, the amounts of androstenedione detected were <15% of the amounts observed in this and similar studies with both \(17\alpha\)-hydroxysteroids.

**FIGURE 7.** Identification of \(16,17\alpha\)-dihydroxyprogesterone as a product of \(17\alpha\)-hydroxyprogesterone. HRMS spectrum of \(16,17\)-dihydroxyprogesterone formed in a reaction with NADPH-P450 reductase, cytochrome \(b_5\), and NADPH. Exact mass 346.2217 (protonated species); observed for MH\(^+\), \(m/z\) 347.2184 (\(\Delta\) 9.5 ppm).
7α- and 7β-protons (δ 1.34 and 1.98 ppm). Considering a Newman projection down the C6–C7 bond axis, this NOESY interaction is supported by the 6β-hydroxy configuration (supplementary Fig. S1).

We also analyzed the products formed from 16α,17β-dihydroxypregnenolone. One product was 16α-hydroxy-DHEA. The other product was either a tetraol or an epoxytriol (5,6-epoxy-3β,16α,17α-trihydroxypregnan-20-one), as judged by HRMS (m/z 365.2305, calculated mass, m/z 365.2323, Δ 4.9 ppm). The site of oxygen incorporation was not identified. The major product isolated from this reaction was 16α-hydroxy-DHEA, as can be seen from the 1H NMR spectrum of the purified product (Fig. 14). There is a loss of the C21-methyl protons (δ 2.25 ppm) and an upfield shift of the 16β-proton (5.1 ppm to 4.4 ppm) (Fig. 14B). The proton NMR spectrum of the isolated P450 17A1 product also matched a previously reported NMR spectrum of synthetic 16α-hydroxy-DHEA (45).

**Solvent Kinetic Isotope Effects on 17α,20-Lyase Reactions—**

No C–H bond-breaking steps are involved in any steps proposed in Fig. 3 except for Fig. 3D, which is not supported by the 18O work. Thus, no C–H kinetic deuterium isotope effect studies can be applied, but solvent kinetic isotope effect experiments can be informative.

No solvent kinetic isotope effect was found for the 17α,20-lyase reaction with 17α-hydroxyprogesterone (measured rates of 1.47 ± 0.03 min⁻¹ in H₂O and 1.40 ± 0.03 min⁻¹ in 95% D₂O)

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**FIGURE 8.** Time course and effect of cytochrome b₅ on 19-carbon steroid formation in the presence of iodosylbenzene (PhIO) or the typical NADPH-supported reaction. A, oxidation of 17α-hydroxyprogesterone. B, oxidation of 17α-hydroxypregnenolone. The insets show the NADPH-supported reactions in the presence of cytochrome b₅. The points are means of duplicate assays, shown as means ± range.
Our results indicate that acetic acid recovered in the human P450 17A1 reactions with either 17α-hydroxyprogesterone or 17α-hydroxypregnenolone contained 18O atom from molecular oxygen. These results are consonant with the original analysis of Akhtar and co-workers on P450 17A1 (27, 28) and, on their own, are consistent with the FeO$_2^-$ mechanism presented in Fig. 3A (40, 46). Alternative mechanisms that are still consistent with the 18O labeling results, but involving FeO$_3^-$, are presented in Fig. 3, B, C, and F, arrow b (7, 40, 47). The mechanisms in Fig. 3, B and C involve formation of an acetyl radical, which has adequate chemical precedent (32, 48–50). The dioxetane mechanism is similar to one that has been proposed for tryptophan and indole dioxygenases (51). One of these two FeO$_3^-$ mechanisms is proposed to contribute to the lyase reaction in that (i) iodosylbenzene can support the lyase reaction, and (ii) we report that P450 17A1-17α-hydroxysteroid complexes are poised for multiple hydroxylation reactions in addition to lyase reactions.
Incubations with $^{18}$O$_2$—Previous studies of $^{18}$O$_2$ incubations with P450 17A1 concluded that $^{18}$O incorporation into the acetic acid product was the major isotopologue detected, leading to the conclusion that the ferric peroxide was the iron-active species for C–C bond cleavage (26, 52). However, these studies (i) used low resolution mass spectrometry and (ii) used microsomes from porcine testes as the source of enzyme (i.e., non-purified enzyme); and (iii) the report that used the direct lyase substrate $^{17}$H$_{9251}$-hydroxy-$[21,21,21-2$H$_3]$pregnenolone did not present raw data (26), whereas a subsequent report used [16a,17a,21,21,21-2H$_4$]pregnenolone as the substrate and not the direct substrate that results in the formation of DHEA, i.e. 17α-hydroxypregnenolone (52). Furthermore, the low resolution mass spectrometry used in these studies yielded an ambiguous determination of $^{18}$O-, 2H-, and $^{13}$C-labeled content of the acetate products (52), which resulted in the interpretation of multiple possible mechanisms for the lyase step of P450 17A1 (Fig. 3). We performed the incubation with purified P450 17A1.

**FIGURE 10. Reaction products formed from 17α-hydroxyprogesterone and 17α-hydroxypregnenolone in P450 17A1 reactions supported by various factors.** Retention times ($t_R$) and integration units are indicated on the chromatograms. A–C, 17α-hydroxyprogesterone; D–F, 17α-hydroxypregnenolone. A and D, NADPH-P450 reductase, cytochrome b$_5$, and NADPH; B and E, H$_2$O$_2$ (10 mM) (with cytochrome b$_5$); C and F, iodosylbenzene (PhI=O, 300 μM) (with cytochrome b$_5$). In these studies the $^{18}$O products (formed from 17α-hydroxypregnenolone) were oxidized to $^{18}$O products to facilitate LC-UV analysis.
and labeled lyase substrates (17α-hydroxy-[21,21,21-2H₃]pregnenolone and 17α-hydroxy-[2,2,4,6,21,21,21-2H₈]progesterone) in the presence of 18O₂ and analyzed the acetate product by HRMS after derivatization with the new diazo reagent. Our results unambiguously established that the acetate produced from the enzymatic incubation incorporated one oxygen atom from molecular oxygen in both cases, with the C-21 deuteriums retained (Figs. 4 and 5). Moreover, we did not detect significant amounts of any other acetate isotopeologues from the enzyme incubation (i.e. loss of one deuterium or lack of 18O incorporation). These experiments agree with the observation of oxygen incorporation from the reports of Akhtar and co-workers (26, 52). However, although these data can support a ferric peroxide mechanism for C–C bond cleavage (Fig. 3A), they do not rule out a compound I mechanism (Fig. 3, B, C, and F, arrow b).

**Oxygen Surrogate Studies, Iodosylbenzene—**The use of iodosylbenzene and NADPH-P450 reductase to form compound I with P450 17A1 resulted in two different activities when the 17α-hydroxyprogesterone was used as the substrate. In both conditions, 16α-hydroxylation and C–C bond cleavage activities toward 17α-hydroxyprogesterone yielded 16,17α-dihydroxyprogesterone and androstenedione, respectively (Figs. 6, 8A, and 9A). However, the product distributions were different; iodosylbenzene yielded more 16-hydroxylation relative to C–C bond cleavage (9:1, Fig. 6C) compared when NADPH-P450 reductase was used (0.1:1, Fig. 6B). The switch in reactivities depending on the oxidation system used (iodosylbenzene versus NADPH-P450 reductase) suggests a conformational change in the enzyme-substrate complex when the reductase binds to the P450 enzyme. Moreover, when 17α-hydroxypregnenolone was used as the substrate, the 16-hydroxylation activity was diminished (Fig. 10, D and F) relative to when 17α-hydroxyprogesterone was used as the substrate. Similarly, this substrate-dependent switch in reactivity is reminiscent of the different 16- versus 17-hydroxylation regioselectivities observed when two different substrates, progesterone and pregnenolone, are used for P450 17A1 (53), which is explained by the 3-keto-Δ⁴ versus 3β-hydroxy-Δ⁵ moieties in the AB-ring systems of these steroid substrates.

Moreover, the fact that P450 17A1 is catalyzing a C-H hydroxylation with its lyase substrate, 17α-hydroxypregnenolone,
supports the presence of a compound I species, which either hydroxylates the C16-position or cleaves the C17,C20-bond as shown in Fig. 3. This observation may contradict the conclusions about the active iron hydroperoxo species observed by resonance Raman spectroscopy (9, 25). However, it is possible that the iron peroxohemiketal species reported in the resonance Raman study (9), i.e. Fig. 3A, tetrahedral intermediate, was a structural misassignment and that the actual observed species was indeed an iron peroxy intermediate attached through the C17-position of the steroid (Fig. 3F). This iron peroxy intermediate can be formed from a nucleophilic attack of the C17-hydroxy group of the lyase substrate (i.e. 17α-hydroxyprogrenenolone or 17α-hydroxyprogesterone, Fig. 3F) onto compound I.

It should be pointed out that the iodosylbenzene mechanism may be more complex than just a direct oxygen transfer, as pointed out by Ortiz de Montellano (46). A possible intermediate is shown in Fig. 18, which may even have oxidant capacity of its own.

**Oxygen Surrogate Studies, Hydrogen Peroxide**—Many studies in the literature involve the use of alkyl hydroperoxides as oxygen surrogates for P450 reactions, beginning with Kadlubar et al. (54). However, although peracids can be used as reagents to generate P450 compound I (55), studies with alkyl hydroperoxides are problematic due to the production of radicals and their ensuing chemistry (46, 56). Some bacterial family 152 P450s appear to use H2O2 as a physiological cofactor (37, 57–59), and bacterial P450 101A1 (P450cam) was mutated to a species that could efficiently utilize H2O2 in reactions (60). H2O2 can support some mammalian P450 reactions after direct addition (61–66) (although generally not as well as alkyl hydroperoxides (54)), but the role of a ferric peroxide in each oxygenation reaction can only be postulated, in that the ferric peroxide can subsequently convert to compound I.

In principle, the FeO2/H2O2 complex could proceed to compound I (FeO3/H2O) through appropriate acid-base catalysis, but there are also side reactions that may diminish the progress of a putative Fe3+/H2O2 complex on to FeO3+ (Fig. 2). We made attempts to observe compound I or other intermediates by mixing P450 17A1 with H2O2 (10 mM) or iodosylbenzene (300 μM) in a stopped-flow spectrophotometer (dead time ~2 ms, rapid scanning) but were unsuccessful in seeing any distinct complexes (data not presented). However, given the difficulties encountered by others in observing these transient species even with bacterial P450s (55), negative results are inconclusive.

**Cytochrome b5 Effects**—Another issue that is still not resolved is the stimulatory effect of cytochrome b5, which is known to
promote the 17α,20-lyase reaction of P450 17A1. Results with apo-cytochrome b₅, devoid of heme, have shown that cytochrome b₅ does not donate the second electron in the catalytic cycle of this P450 (67). The stimulation of 17α,20-lyase activity by cytochrome b₅ in the iodosylbenzene-supported reaction (Fig. 8) is consistent with this view. We also note that cytochrome b₅ stimulated the 16-hydroxylation reactions with both 17α-hydroxyprogesterone and 17α-hydroxypregnenolone (Fig. 8). In other unpublished data,⁴ we have also noted the stimulation of the 17α-hydroxylation of both progesterone and pregnenolone by cytochrome b₅. Our conclusion about the stimulatory role of cytochrome b₅ in the iodosylbenzene reactions is that it is acting in an allosteric manner to facilitate these reactions (e.g. due to more ideal juxtaposition in reaction intermediates), which is the reason proposed for the normal physiological reaction (6, 7, 67).

Hydroxylations Catalyzed by P450 17A1—The 16α-hydroxylation of DHEA has previously been reported to be catalyzed by P450 3A4 (68, 69). Upon monitoring the production of DHEA from 17α-hydroxyprogrenolone by P450 17A1 over time (Fig. 8B, inset), there was a decrease in DHEA formation in the time points greater than 5 min. This observation suggested that DHEA was further being oxidized to another product. We hypothesized that this new product would correspond to 16-hydroxy-DHEA based on the other activities of P450 17A1 (16-hydroxylation of progesterone (53) and 16-hydroxylation

⁴ E. Gonzalez and F. P. Guengerich, unpublished data.

FIGURE 14. Characterization of 16α-hydroxy-DHEA. The product was formed in an incubation of an NADPH-reconstituted P450 17A1 system with 16α,17α-dihydroxypregnenolone and isolated by preparative HPLC. A, HRMS spectrum of [DHEA + 16]⁺ peak, 16α-hydroxy-DHEA (theoretical m/z 305.2111, found m/z 305.2094). B, NMR spectra of 16α,17α-dihydroxypregnenolone (a) and product (b) in CDCl₃ (600 MHz). See text for discussion.
of its 17α-hydroxylated products). The new product, which was converted to its 3-keto-Δ^4 counterpart by cholesterol oxidase, co-chromatographed with standard 16α-hydroxyandrostenedione (Fig. 10). The 16-hydroxylation product of P450 17A1 was also observed when androstenedione was used as the substrate (Fig. 11). The ability of P450 17A1 to form 16-hydroxylated androgens is physiologically relevant in that estriol, an abundant and characteristic estrogen dur-
TABLE 1
NMR shift assignments for 6β,16α,17α-trihydroxyprogesterone

| Carbon atom | δ _H_ | δ _D_ |
|-------------|-------|-------|
| 1           | 36    | 2.06, 1.74 (COSY δ 2.53) |
| 2           | 34.3  | 2.53, 2.42 |
| 3           | 205.1 (HMBC-2CH₃) |       |
| 4           | 127.7 | 5.84 (broad s) |
| 5           | 167.5 |       |
| 6           | 73.0  | 4.38 (broad s) (NOESY to δ 584) |
| 7           | 38.5  | 1.98, 1.34 (COSY δ 4.38) |
| 8           | 29.5 (43, 44) | 1.99 |
| 9           | 52.9 (HMBC 19-CH₃, 1-H) | 1.02 |
| 10          | 42.7  |       |
| 11          | 20 (43) | 1.66 (COSY δ 1.08, 1.94), δ 1.48 |
| 12          | 30.8  | 1.97, 1.53 |
| 13          | 35.4 (HMBC 18-CH₃) |       |
| 14          | 48.9 (43, 44) | 2.03 (W-coupling to δ 5.09, four bonds away) |
| 15          | 33.6  | 1.59, 1.92 (COSY δ 5.09) |
| 16          | 72.3  | 5.09 |
| 17          | 88.6  |       |
| 18          | 15.1  | 0.79 |
| 19          | 19.5  | 1.40 |
| 20          | 211.4 |       |
| 21          | 27.4  | 2.28 |

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ing human pregnancies, arises from the aromatization of 16α-hydroxy androgens (70, 71).

The current working hypotheses we favor are shown in some detail in Fig. 19. Two involve an acetyl radical and one a steroid dioxetane intermediate, which are both considered viable entities.

B Ring Hydroxylation of 16α,17α-Dihydroxyprogesterone by P450 17A1—Surprisingly the 6β-position was hydroxylated when 16α,17α-dihydroxyprogesterone was used as a substrate for P450 17A1 (Fig. 15). This shift in regioselectivity from the D-ring to the B-ring of the steroid by P450 17A1 was due to the presence of two hydroxyl groups on the 16α- and 17α-positions. Interestingly, regioselectivity was switched from the B-ring to the C–D-ring of the steroid in another P450 system when the Δ4-double bond was reduced. P450 3A4 hydroxylates the 6β-position of testosterone (B-ring of the steroid); however, with 5α-dihydrotestosterone as the substrate, P450 3A4 oxygenated the 18β-methyl group (between the C–D-ring of the steroid) (72). The causes for the switch in regioselectivities of the different P450 systems are probably not the same. Moreover, P450 17A1, which normally hydroxylates the α-face of its steroid substrates (pregnenolone and progesterone), introduced a hydroxyl group on the β-face of 16α,17α-dihydroxyprogesterone. The hydroxylation of the opposite face can be rationalized from overlaying 17α-hydroxyprogesterone and 16α,17α-dihydroxyprogesterone (Fig. 20). When the C10, C14, and O16 atoms from 16α,17α-dihydroxyprogesterone were aligned with the C14, C10, and O3 atoms of 17α-hydroxyprogesterone, the O17 atom of 17α-hydroxyprogesterone was positioned 1.4 Å away from the C6 atom of 16α,17α-dihydroxyprogesterone, the site where the new oxygen atom is introduced. Additionally, the 17-oxygen of 17α-hydroxyprogesterone has been shown to hydrogen bond to Asn-202 of P450 17A1 in the crystal structure (73). Based on our observations with 6β-hydroxylation of 16α,17α-dihydroxyprogesterone by P450 17A1 and the overlay of the two different substrates, we reason that the 16β-hydroxy group of 16α,17α-dihydroxyprogesterone hydrogen bonds to Asn-202 of P450 17A1 in the crystal structure (73). Based on our observations with 6β-hydroxylation of 16α,17α-dihydroxyprogesterone by P450 17A1 and the overlay of the two different substrates, we reason that the 16β-hydroxy group of 16α,17α-dihydroxyprogesterone hydrogen bonds to Asn-202 of P450 17A1, which in turn directs the 6β-hydrogen to the active iron center of the enzyme. Interestingly, the 17α,20-lyase product for the 16α,17α-dihydroxyprogesterone substrate (i.e. 16α-hydroxyandrostenedione) was detected by LC-MS analysis and co-elution with the standard, but this lyase product seems to be a minor product in comparison with the 6β-hydroxylation product.

17α,20-Lyase Reaction of 16α,17α-Dihydroxypregnenolone by P450 17A1—Conversely, when 16α,17α-dihydroxypregnenolone was used as the substrate for P450 17A1, the major product isolated from the incubation was 16α-hydroxy-DHEA, which is formed from the 17,20-carbon–carbon bond cleavage

Oxygen Incorporation into 16α,17α-Dihydroxypregnenolone by P450 17A1—An additional oxygenation product (M + 16 of substrate) was detected by LC-HRMS when 16α,17α-dihydroxypregnenolone was used as the substrate. However, there was not enough purified material recovered to determine the
location of the oxygen on the steroid ring by 1H NMR. A possible site of oxidation may be the C21-position. Alternatively, from the knowledge of 6β/H9252-hydroxylation reactivity of P450 17A1 with 16β/H9251,17β/H9251-dihydroxyprogesterone as the substrate, the oxygen may be incorporated in two other possible sites as follows: (i) the 5,6-double bond of the substrate to form the 5,6-epoxide or (ii) the C7-position may be hydroxylated. Epoxidation activity of P450 17A1 has been previously reported with a 16,17-steroid substrate (74). Nevertheless, the shift in favoring C–C bond cleavage reactivity over hydroxylation when using the 3β/H9252-hydroxy substrate (16β,17β-dihydroxypregnenolone) instead of the 3-keto-Δ4 substrate (16α,17α-dihydroxypregnenolone) is similar to what occurs with pregnenolone and progesterone (i.e. 17α,20-carbon, carbon bond cleavage versus 16α-hydroxylation). This observation may be related to the hydrogen bonding that occurs between the 3β/H9252-hydroxy group of the 3β/H9252-hydroxy-5 substrate and Asn-202 of the enzyme.

Kinetic Solvent Isotope Effects Do Not Support a Ferric Peroxide Mechanism—One argument against the proposed acetyl radical mechanism (Fig. 3B) is a reported inverse kinetic solvent deuterium isotope effect (0.39) reported by Sligar and co-workers (23). If the mechanism in Fig. 3, B, C, or F, arrow b, were valid, the abstraction of a hydrogen atom from the 17-hydroxyl group (Fig. 3B) or the heterolytic cleavage of an O–H bond (Fig. 3D) might be expected to be a (partially) rate-limiting step, and an inhibitory effect of hydroxyl deuteration might be expected. In contrast, a similar study by Swinney and Mak (35) reported that (30%) D2O attenuated androgen formation from 17α-hydroxyprogesterone using microsomes from pig testes as the enzyme source (kH/kD ~1.25 at pH 7), suggesting that the 17α,20-lyase reaction is dependent on compound I formation either through the pro-

FIGURE 17. Solvent kinetic deuterium isotope effects on 17α-hydroxyprogesterone and 17α-hydroxypregnenolone reactions catalyzed by P450 17A1 (in the presence of NADPH-P450 reductase and cytochrome b5). Retention times (tR) and integration units are indicated on the chromatograms. The substrate concentration was 30 μM in all cases, and the reactions were done in either H2O or 95% D2O (v/v) at pH or pD 7.4. A and B, 17α-hydroxyprogesterone; C and D, 17α-hydroxypregnenolone. A and C, H2O; B and D, 95% D2O (v/v).

FIGURE 18. Possible oxidizing alternative to compound I in the iodosylbenzene (Phl=O)-supported reactions (46).
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![Mechanisms of P450 17A1-catalyzed 17α,20-lyase reaction consistent with 18O labeling (7), oxygen surrogate results, and solvent kinetic isotope results. The course of an 18O label (from 18O2) is indicated with an asterisk (7, 40). A, compound I mechanism with hydrogen atom abstraction from the 17α alcohol followed by C17-C20 bond scission to yield an acetyl radical; B, addition of the 17α hydroxyl group to compound I to yield an iron peroxide-C17 complex, followed by decomposition via a C17-C20 dioxetane; C, compound I mechanism with hydrogen atom abstraction from the C16 carbon. See text for discussion and also Fig. 3.](image_url)

![Sites of hydroxylation of progesterone by P450 17A1. A, chair configuration of progesterone, with the four sites of attack indicated by arrows. B, wire diagram of 17α-hydroxyprogesterone (17OHP, red) and 16α,17α-dihydroxyprogesterone (16,17OHP, blue), overlaid, with the latter in an alternative configuration to show the proximity of the C-6 atom of 16α,17α-hydroxyprogesterone with the 17α-hydroxy group of 17α-hydroxyprogesterone. The model was made using Chem3D, with a minimum root mean square error of 0.1 and minimum root mean square gradient of 0.01. The C14, C10, and O3 atoms of 17α-hydroxyprogesterone were aligned respectively, by displaying the distance measurements of each pair of atoms and then running an overlay minimization calculation. The green lines indicate the pair of atoms that were aligned (after overlay minimization, the distances between C14 of 17α-OHP and C10 of 16,17-OHP; C10 of 17α-OHP and C14 of 16,17-OHP; and O3 of 17α-OHP and O17 of 16,17-OHP were 1.2, 1.3, and 1.4 Å, respectively, and are shown as green lines). The distance between the O17 atom of 17α-hydroxyprogesterone and C6 atom of 16α,17α-dihydroxyprogesterone was 1.4 Å.](image_url)

Because of the discrepancy, we reinvestigated the results in our own system (Fig. 16). Running the normal P450 17A1 reaction (with NADPH-P450 reductase and cytochrome b5) in 95% D2O showed no significant change in the rate of conversion of 17α-hydroxyprogesterone to androstenedione and a small but statistically significant change in the rate of oxidation of 17α-hydroxypregnenolone to DHEA, with an apparent isotope effect of 0.83 (Fig. 17), which is much less than the effect (0.39) reported by Gregory et al. (23). Interpretation of solvent kinetic deuterium isotope effects is complex (75), in that protonation and deprotonation can occur throughout the amino acid side chains of an enzyme, not only on an iron-oxygen complex. The reason for the small inverse isotope effect with one lyase substrate but not another (Figs. 16 and 17) is unclear. The opposite pattern between the solvent isotope effects for the 17α-hydroxypregnenolone lyase and the 16-hydroxylation reactions is qualitatively consistent with the report of Gregory et al. (23). One possibility is that the Δ5 substrate (17α-hydroxypregnenolone) 3-hydroxy group exchanges with deuterium and that this has an effect on the juxtaposition of the substrate in the active site. The hydroxyl moiety has been shown by Scott and coworkers (73, 76) to be in hydrogen bonding distance to Asn-202 of human P450 17A1. A substitution of the 3-hydroxyl group by deuterium (+OD) could shift the substrate to favor the lyase reaction versus 16-hydroxylation. However, the lack of solvent isotope effects does not allow any definite conclusions about the rate-limiting nature of the abstraction of a proton or hydrogen atom from the 17-OH group, due to the multiple complex influences from solvent deuterium on enzyme function.

Although resonance Raman spectra of what is reported to be the human P450 17A1 FeO2 complex have recently been published (9, 25), two caveats are as follows: (i) no cytochrome b5 (for which the 17α,20-lyase reaction is very dependent, e.g. Fig. 8B) was present, and (ii) the observed complex was not tested for its catalytic competence, i.e. to form product(s). Even if the FeO2 complex did form the normal products (androstenedione and DHEA, plus the 16-hydrox-

- tonation of the distal oxygen of ferric peroxide (cf. P450 catalytic cycle) or deuterium atom abstraction from the 17-hydroxy group of the substrate (Fig. 3B).
ylation products, which is unlikely) in these experiments, the simultaneous or subsequent intermediacy of an FeO$_3$ species as well could not be ruled out.

**Conclusions**—The ability of iodosylbenzene, but not H$_2$O$_2$, to support the lyase reaction provides what may be the strongest evidence in favor of a compound I mechanism, in that iodosylbenzene cannot possibly form a peroxy intermediate. The apparent rate of the lyase reaction was similar to that of the NADPH-supported reaction (without cytochrome b$_5$) in the case of the 17α-hydroxyprogesterone reaction and was somewhat less than that of the NADPH-supported reaction (without cytochrome b$_5$) in the case of the 17α-hydroxyprogrenenolone lyase reaction (Figs. 8 and 9). Ideally, the compound I form of P450 17A1 could be prepared using the approaches that Green and co-workers (8, 55, 64, 77) have used with two bacterial P450s (64), and the reaction could be investigated directly. Nevertheless, in considering all of the literature in this field and that presented here in this article, the iodosylbenzene and H$_2$O$_2$ results (Fig. 10) are difficult to dismiss, even if they are not physiological, and are interpreted as evidence for a compound I reaction (Fig. 19).

The multiple hydroxylations are probably catalyzed by FeO$_3$ intermediates, formed with P450 17A1-17α-hydroxy steroid complexes. It is possible that individual reactions (i.e. hydroxylation, lyase) proceed from different FeO complexes, although it is simpler to explain all as emanating from a single iron-oxygen intermediate. The myriad of reactions is depicted in Fig. 21 and reveals a surprising flexibility in the P450 17A1 enzymes. As indicated, P450 17A1 has been shown to catalyze 21-hydroxylation of progesterone (53). Our observed rates are not overly dominant. The biological activities of most of the products are, to our knowledge, still unknown.

In summary, we have provided evidence that a compound I-type mechanism (Fig. 19) can be involved in the 17α,20-lyase reactions. Our results do not rule out a ferric peroxy mechanism, nor do they define the fraction of the normal reaction that is catalyzed by each of the two mechanisms, if both are operative. If further research implicates compound I in this reaction, then few strong cases for P450 ferric peroxy chemistry will remain, at least in the field of steroid metabolism (33, 64, 78).

**Experimental Procedures**

**General**—Bruker instruments (400 and 600 MHz) were used to acquire NMR spectra in the Vanderbilt facility. CD$_3$CN and
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CDCl₃ residual proton peaks were referenced to δ 1.94 and 7.26 ppm, respectively, and the CDCl₃ triplet in the carbon spectrum was referenced to δ 77.16 ppm and CD₃CN was referenced to 118.26 ppm (79). Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich. A modified version of the nitrosourea reagent was synthesized according to Ref. 33 using 2-(2-pyridyl)ethylamine (instead of 3-(3-pyridyl)propylamine) as the starting material, as described in detail here.

Reagents—17α-Hydroxy-[21,21,21-2H₃]pregesterone (96.5% atomic excess as judged by ¹H NMR, 97.9% atomic excess as judged by LC-MS) was synthesized and characterized as described previously (53). 17α-Hydroxy-[21,21,21-2H₃]pregnenolone (nominal 98.4% atomic excess) and 17α-hydroxy-[2,2,4,6,6,21,21,21-2H₃]pregesterone (nominal 98.7% atomic excess) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). 16α,17α-Dihydroxyprogesterone (algestone) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). 16α-Hydroxyandrostenedione was obtained from Steraloids (Newport, RI).

Enzymes—Recombinant human P450 3A4 with a C-terminal His₄ tag was expressed in Escherichia coli and purified as described previously (80, 81). E. coli recombinant rat NADPH-P450 reductase and human liver cytochrome b₅ were prepared as described by Hanna et al. (82) and Guengerich (83), respectively.

Recombinant human P450 17A1 (with a C-terminal His₄ tag) was expressed in E. coli and purified by metal-affinity chromatography using a protocol adapted from those previously reported (76, 84, 85). Briefly, an E. coli codon-optimized cDNA, corresponding to the amino acid sequence reported by DeVore and Scott (76), was purchased (Genewiz, South Plainfield, NJ) and inserted into the pCW-Ori(+) expression vector. The construct was used to transform competent E. coli JM109 cells (Agilent), and an isolated colony was used to inoculate 100 ml of Luria-Bertani medium (containing 100 μg/ml ampicillin), which was then incubated at 37 °C with shaking at 250 rpm overnight (12–14 h). Expression ensued by inoculating 1 l of Terrific Broth medium, containing 100 mg/liter ampicillin and 250 μl/liter of trace elements (86), with 10 ml of the overnight culture and incubating at 37 °C (250 rpm) for ~4 h (OD₆₀₀ ~0.32). The expression culture was then supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside and 1 mM δ-amino-7-oxonovelic acid, and the incubation conditions were changed to 30 °C and 200 rpm. After ~40 h, the culture was centrifuged at 5000 × g for 10 min, and the bacterial pellet was resuspended in 300 ml of 100 mM Tris-HCl buffer (pH 7.6) containing 500 mM sucrose and 0.5 mM EDTA and placed on ice. The suspension was then treated with 60 μl of a 50 mg/ml lysozyme solution/g of bacterial pellet and incubated on ice for 30 min, with gentle mixing every 10 min. All subsequent steps were conducted on ice or at 4 °C. Next, a spheroplast pellet was obtained by centrifugation at 5000 × g for 10 min and resuspended in 25 ml of 300 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 6 mM Mg(CH₂CO₂)₂, 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Complete™, EDTA-free, Roche Applied Science). The spheroplasts were lysed by sonication, and debris and unbroken cells were removed by centrifugation at 9000 × g for 20 min. The cytosol was cleared of the membrane fraction by centrifugation at 100,000 × g for 60 min and was supplemented with 300 mM NaCl and 20 mM imidazole prior to loading onto a nickel-nitriotriacetic acid resin (Qiagen) bed that had been equilibrated with 300 mM potassium phosphate buffer (pH 7.4) containing 300 mM NaCl, 20% glycerol (v/v), 20 mM imidazole, and 0.1 mM DTT. The bound protein was washed with 10 bed volumes of the same buffer and eluted with the same buffer containing 250 mM imidazole. The purified enzyme was then dialyzed four times against 100-fold volumes of 200 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.1 mM EDTA, and 0.1 mM DTT and stored at ~70 °C until use.

Chemical Synthesis

(2-(Pyridin-2-yl)ethyl)urea (33)—A solution of 2-(2-pyridyl)ethylamine (0.753 g, 6.17 mmol) and benzo-terazole-1-carboxamide (1.00 g, 6.17 mmol) in tetrahydrofuran was heated under reflux for 12 h. The reaction mixture was concentrated in vacuo and purified by flash column chromatography (gradient of hexanes to 50% hexanes in ethyl acetate (v/v) to 10% CH₂OH in CH₂Cl₂ (v/v)) to afford (2-(pyridin-2-yl)ethyl)urea as a white solid (1.00 g, 6.06 mmol, 98%). R₀.28 (silica gel GF, CH₂Cl₂/CH₂OH, 9:1, v/v); ¹H NMR (600 MHz, CD₃CN) δ 8.50 (d, J = 4.8 Hz, 1H), 7.66 (dd, J = 7.7, 7.6, 2.0 Hz, 1H), 7.21 (d, J = 7.9 Hz, 1H), 7.17 (dd, J = 7.9, 4.8 Hz, 1H), 5.25 (br s, 1H), 4.54 (br s, 2H), 3.44 (d, J = 6.7 Hz, 1H), 3.42 (d, J = 6.7 Hz, 1H), 2.89 (apparent t, J = 6.8 Hz, 2H); ¹³C NMR (150 MHz, CD₃CN): δ 160.8, 159.4, 150.1, 137.3, 124.2, 122.3, 40.3, 38.9.

1-Nitroso-1-(2-(pyridin-2-yl)ethyl)urea (33)—NaNO₂ (0.40 g, 6.11 mmol) was added to a solution of (2-(pyridin-2-yl)ethyl)urea (1.0 g, 6.11 mmol) in 50 ml of 1.2 M aqueous HCl at 0 °C. After 2 h, the reaction was diluted with CH₂Cl₂ (100 ml), and the resulting mixture was washed with NaHCO₃ (saturated aqueous solution, 2 × 50 ml). The organic layer was dried with MgSO₄ and concentrated under a stream of nitrogen to afford the nitrosourea (0.45 g, 2.3 mmol, 38%) as a yellow oil, which was used without further purification as the precursor to generate the diazo reagent for derivatization of the acetic acid product of P450 17A1. R₀.42 (silica gel GF, CH₂Cl₂/CH₂OH, 9:1, v/v); ¹H NMR (600 MHz, CD₃CN) δ 8.53 (d, J = 4.8 Hz, 1H), 7.70 (dd, J = 7.7, 7.6, 1.8 Hz, 1H), 7.29 (d, J = 7.7 Hz, 1H), 7.22 (dd, J = 7.7, 4.7 Hz, 1H), 3.71 (apparent t, J = 6.6 Hz, 2H), 3.05 (apparent t, J = 6.6 Hz, 1H).

2-(Pyridin-2-yl)ethyl Acetate—2-(Pyridin-2-yl)ethanol (1.0 ml, 8.9 mmol) and acetic anhydride (840 μl, 8.9 mmol) were stirred overnight in a 4-ml screw cap vial. The reaction was dissolved in CH₂Cl₂ and treated with aqueous NaHCO₃ to remove the by-product CH₃CO₂H and the product was purified by preparative TLC (silica gel GF, 20 × 20 cm, 500 μm) using hexane/ethyl acetate (1:1, v/v) as the mobile phase. The product was scraped from the TLC plate, extracted with CH₂Cl₂, and filtered. The solvent was evaporated under a stream of nitrogen to afford 2-(pyridin-2-yl)ethyl acetate as a pale yellow liquid (980 mg, yield 67%). R₀.61 (silica gel GF, CH₂Cl₂/CH₂OH, 9:1, v/v); ¹H NMR (400 MHz, CDCl₃): δ 8.55 (d, J = 4.6 Hz, 1H), 7.61 (ddd, J = 7.7, 7.6, 1.8 Hz, 1H), 7.18 (d, J = 7.8 Hz, 1H), 7.14 (dd, J = 7.6, 5.0 Hz, 1H), 4.46 (t, J = 6.7 Hz, 2H), 3.12 (t, J = 6.7 Hz, 2H), 2.02 (s, 3H); ¹³C NMR (100 MHz, CDCl₃): 171.1, 158.1, 149.5, 136.5, 123.5, 121.7, 63.7, 37.4, 21.0;
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HRMS (ESI\(^+\)): calculated for C\(_9\)H\(_{11}\)NO\(_2\) [M + H]\(^+\) m/z 166.08626, found 166.08678 (Δ 0.3 ppm).

16\(α\), 17\(α\)-Dihydroxyprogrenolone—16\(α\), 17\(α\)-Dihydroxyprogrenolone was synthesized in two steps from commercially available 16,17-dehydroprogrenolone-3-acetate. 16,17-Dehydroprogrenolone-3-acetate was converted to 16,17-dehydroprogrenolone according to a previously reported procedure (74). 16,17-Dehydroprogrenolone was subjected to dihydroxylation conditions loosely based on a procedure that was previously reported (87); KMnO\(_4\) (105 mg, 0.67 \(μ\)mol) in acetone/H\(_2\)O (6:1, v/v) was added over 10 min with an addition funnel to 16,17-dehydroprogrenolone (209 mg, 0.67 \(μ\)mol) in acetone (50 ml) and HCO\(_2\)H (50 \(μ\)l) at 0 °C. After the addition was complete, the reaction mixture was stirred for 2 min and then washed with H\(_2\)O (100 ml) and extracted with ethyl acetate (200 ml). The organic layer was concentrated in vacuo and purified by flash column chromatography (100% hexanes to 50% ethyl acetate/hexanes, v/v), and the most pure fraction (as judged by TLC analysis) was concentrated (10 mg, white solid).

To determine the purity of the isolated material, a HPLC UV system (10 mM glucose 6-phosphate, 0.5 mM NADP\(^+\)) was added to the reaction mixture. The CH\(_3\)OH from the collection of the acetic acid derivative at a flow rate of 0.3 ml/min with the following gradient: 0–1.0 min, 100% A (v/v); 1.0–5.0 min, 98% A; 5.0–8.0 min, 96% A; 8.0–10.0 min, 90% A; 10.0–12.0 min, 88% A; 12.0–15.0 min, hold at 88% A. The solvent was evaporated under a stream of nitrogen, and residues were dissolved in CH\(_3\)CN (70 μl).

**Assays**

18\(O\)\(_2\) Incubations—The standard reconstituted P450 17A1 system contained P450 17A1 (8.4 \(μ\)mol), NADPH-P450 reductase (13 \(μ\)mol), cytochrome \(b_5\) (14 \(μ\)mol), 130 \(μ\)mol 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)pregnenolone or 100 \(μ\)mol 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)]pregnesterone, and \(17\alpha\)-2-dilauroyl-sn-glycerol-3-phosphocholine (80 \(μ\)mol) in 2.2-ml incubation mixtures containing 50 mm potassium phosphate buffer (pH 7.4). Reaction mixtures were placed in Thunberg tubes, and air was removed on a gas train equipped with a manifold (89, 90) (three exchanges of argon/vacuum; 5 min each cycle). After introduction of 18\(O\)\(_2\) (Sigma-Aldrich, 99% atomic excess, pressurized cylinder) into a Thunberg tube under vacuum, each reaction was initiated by adding an NADPH-generating system (10 mm glucose 6-phosphate, 0.5 mm NADP\(^+\), and 2 \(μ\)g/ml yeast glucose-6-phosphate dehydrogenase (91); 8% of total reaction volume) tipped from the stopper reservoir, with mixing. Incubations were conducted in a water bath at 37 °C for 30 min for 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)]pregnolone substrate or 60 min for 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)]pregnesterone substrate with shaking at 100 rpm.

The reactions were quenched with CH\(_3\)Cl\(_2\) (5 ml), and 500 \(μ\)l of 3 M HCl (chilled to 0 °C) for the 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)]pregnenolone substrate or 1 ml of 3 M HCl (chilled to 0 °C) for the 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)]pregnesterone substrate was added to decrease the pH to ~1 and facilitate extraction of acetic acid into the organic layer. For cases with 17\(α\)-hydroxy-[2,4,6,7,12\(H_5\)]pregnesterone as the substrate, the mixture (after addition of HCl) was mixed with a vortex device and centrifuged at 1500 \(×\) g for 1 min to remove the emulsion. Before the reaction of the product acetic acid with the diazo reagent, the organic extracts were collected, and residual water was removed using anhydrous MgSO\(_4\) (~50 mg for each extraction).

**Derivatization of Acetic Acid—Diazotolpyridyline was prepared by mixing 1- (pyridin-2-yl)ethyl urea (5 mg) in diethyl ether (2 ml), after treatment with KOH (1 ml of a 30% (w/v) solution in H\(_2\)O) (33). The organic layer (containing the diazo reagent) was dried with anhydrous MgSO\(_4\) (~50 mg), filtered with a cotton-plugged Pasteur pipette, and reacted with an organic extract of each P450 17A1-steroid-18\(O\)\(_2\) incubation. The solvent was evaporated under a stream of nitrogen, and residues were dissolved in CH\(_3\)CN (70 μl).

**LC-MS Analysis**—LC-MS analysis of deuterium-labeled (2-pyridin-2-yl)ethyl acetate from 18\(O\)\(_2\) incubations was performed using an Acquity UPLC system connected to a Thermo LTQ XL Orbitrap mass spectrometer operating in the electrospray ionization (ESI) positive ion mode. A Phenomenex Kinetex® 2.6-μm C8 100 A, LC column (100 × 2.1 mm) was used for separation of the acetic acid derivative at a flow rate of 0.3 ml/min with the following gradient: 0–1.0 min, 100% A (v/v); 1.0–5.2 min, 100% B (v/v); 5.2–8.0 min, 100% A (v/v); mobile phase A was 10 mm NH\(_4\)HCO\(_3\) in H\(_2\)O (v/v); and mobile phase B was 10 mm NH\(_4\)HCO\(_3\) in 95:5 CH\(_3\)CN/H\(_2\)O (v/v).

For the 18\(O\)\(_2\) incubation assays, the LTQ mass spectrometer was tuned in the electrospray ionization positive mode using synthetic 2-(pyridin-2-yl)ethyl acetate (see above). The tune settings were as follows: sheath gas flow rate, 15 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); spray voltage, 4 kV; capillary temperature, 300 °C; capillary voltage, 16 V; tube lens, 30 V.

The LTQ Orbitrap XL high resolution mass spectrometer was calibrated with the ESI-positive ion calibration solution by direct infusion (10 μl/min with a 500-μl Hamilton syringe) as done previously (33). The mass spectrometer was first tuned to the standard solution with m/z 524.3 (methionine/arginine/phenylalanine alanine acetate), and the tube lens voltage was set to 145 V to fragment caffeine (m/z 195 to 138).

**17\(α\)-Hydroxysteroid Reactions with Oxygen Surrogates**—The standard reconstituted system used for comparison included P450 17A1 (0.5 \(μ\)mol for iodosylbenzene, 0.1 \(μ\)mol for H\(_2\)O\(_2\)), NADPH-P450 reductase (2.0 \(μ\)mol), cytochrome \(b_5\) (0.5 \(μ\)mol), and \(17\alpha\)-1,2-dilauroyl-sn-glycerol-3-phosphocholine (10 \(μ\)mol) in 0.5-ml incubation mixtures containing 50 mm potassium phosphate buffer (pH 7.4) and the NADPH-generating system. Assays were done as in the case of the 18\(O\) labeling work (see above) except that the incubations were aerobic, as described previously (37), using 10 \(μ\)mol 17\(α\)-hydroxyprogesterone or 17\(α\)-
Mechanism of P450 17A1 Reactions

hydroxypregnenolone with P450 17A1 (0.5 μM) and no reductase, in the absence or presence of cytochrome b5 (0.5 μM). H2O2 (to 10 μM) or isodosylbenzene (to 2 mM) was added at varying concentrations (from aqueous stocks). The incubations were done for 5 min with H2O2 and for 30 s with isodosylbenzene (41, 92), with extraction into CH2Cl2 and analysis of the conversion of 17α-hydroxypregnenolone to androstenedione and of 17α-hydroxypregnenolone to DHEA by UPLC. The Δ5 steroids were converted to Δ4 steroids by treatment with cholesterol oxidase prior to LC-UV analysis (37). Isodosylbenzene reactions were conducted for a short time period because the reagent is very destructive to P450 heme (41).

Product analysis was done on a Waters Acquity UPLC system with a Waters Acquity UPLC Ethylene Bridged Hybrid (BEH) octadecylsilane (C18) column (2.1 × 100 mm, 1.7 μm). LC conditions were as follows: solvent A consisted of 70% CH3OH and 30% H2O (v/v), and solvent B was 95% CH3OH and 5% CH3OH (v/v), and solvent B was 100% CH3CN. The products were resolved by a 0.2 ml min−1 gradient with the following steps: 0–1 min, hold at 5% B (v/v); 1–4 min, linear gradient from 5 to 30% B (v/v); 4–4.5 min, linear gradient from 30 to 40% B (v/v); 4.5–5.5 min, 40 to 95% B (v/v); 5.5–6.75 min, hold at 95% B (v/v); 6.75–7 min, 95 to 5% B (v/v); and 7–10 min, hold at 95% B (v/v). The column temperature was maintained at 40 °C, and the Δ4 steroids were quantified by their absorbance at 243 nm.

LC-UV-MS Analysis of New Steroid Products—An LTQ Orbitrap XL mass spectrometer was tuned in the atmospheric pressure chemical ionization-positive mode with commercially available steroid solutions in a 1:1 mixture (v/v) of H2O and CH2CN (16α-hydroxyandrostenedione and 16α,17α-dihydroxyprogesterone). The tune settings were as follows: vaporizer temperature, 350 °C; sheath gas flow rate, 50 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); discharge current, 10 μA; capillary temperature, 275 °C; capillary voltage, 10 V; tube lens, 25 V. The same LC method used for UV analysis (see above) was employed for the Δ4 steroid products. The LC conditions for the Δ5 steroids were as follows: solvent A consisted of 95% H2O and 5% CH3OH (v/v), and solvent B was 95% CH3OH and 5% H2O (v/v). The gradient steps were as follows: 0–1.5 min, hold at 60% B (v/v); 1.5–7.5 min, linear gradient from 60 to 85% B (v/v); 7.5–7.75 min, hold at 85% B (v/v); 7.75–8.25 min, 85 to 60% B (v/v); and 8.25–10 min, hold at 60% B (v/v). The column was kept at ambient temperature.

Solvent Kinetic Isotope Effect Assays—These assays were also done as in the case of the oxygen surrogate experiments (see above), with the incubations done aerobically as described previously (37), using 30 μM 17α-hydroxyprogesterone or 17α-hydroxypregnenolone with P450 17A1 (0.5 μM)/cytochrome b5 (0.5 μM); NADPH-P450 reductase (2 μM); and L-α-1,2-dilauroyl-sn-glycerol-3-phosphocholine (10 μM) in 0.5 ml incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), with 1 mM NADPH. In the D2O experiments, the content of D2O was 95% (v/v), with the pD adjusted (pH = pD + 0.4) (75, 93). Incubations were for 60 s at 37 °C, and the products were analyzed as for the oxygen surrogate experiments (see above).

Isolation of 6β,16α,17α-Trihydroxyprogesterone as a Product—A 20-ml reaction mixture consisting of the same components described in the oxygen surrogate experiments (see above), using 280 units/ml of catalase and 40 μM 16α,17α-dihydroxyprogesterone, was run overnight (~20 h). The product was extracted from the aqueous mixture with 200 ml of CH2Cl2, and the solvent was evaporated. The dried product was purified using the same system outlined for the 16,17-dihydropregnenolone purification (see above) using an isocratic HPLC method (63.5% CH2OH) and peak detection at 243 nm.

Isolation of 16α-Hydroxy-DHEA as a Product—The same procedure described for the isolation of the 6β,16α,17α-trihydroxyprogesterone product (see above) was used, with the following exceptions. The enzyme concentrations were increased to 1 μM P450 17A1, 4 μM NADPH-P450 reductase, 1 μM cytochrome b5, and 3700 units/ml catalase, with 50 μM 16α,17α-dihydroxyprogrenolone and the NADPH-generating system. The incubation was run for 4 h. Purification was conducted using the same procedure detailed in the LC-UV purification of 16α,17α-dihydroxyprogesterone (see above), except that the wavelength used to detect the Δ5 product was 215 nm.

Author Contributions—F. K. Y. synthesized most of the chemicals, did all of the NMR analysis, and did the 18O analyses. E. G. purified the P450 17A1 and did most of the incubations and HPLC analyses, including some of the mass spectra. R. J. A. supervised part of the work and helped write the paper. F. P. G. oversaw the project, synthesized isodosylbenzene, and assembled the paper. All authors contributed to the writing of the paper and the conclusions.

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Note Added in Proof—In the version of this article that was published as a Paper in Press on June 23, 2016, the spectra in Fig. 14B were inadvertently duplicated from Fig. 15C. This error has now been corrected and does not affect the results or conclusions of this work.

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