Kinetic processivity of the two-step oxidations of progesterone and pregnenolone to androgens by human cytochrome P450 17A1

Received for publication, May 5, 2017, and in revised form, June 24, 2017 Published, Papers in Press, July 6, 2017, DOI 10.1074/jbc.M117.794917

Eric Gonzalez* and F. Peter Guengerich**

From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Edited by Ruma Banerjee

Cytochrome P450 (P450, CYP) 17A1 plays a critical role in steroid metabolism, catalyzing both the 17α-hydroxylation of pregnenolone and progesterone and the subsequent 17α,20-lyase reactions to form dehydroepiandrosterone (DHEA) and androstenedione (Andro), respectively, critical for generating glucocorticoids and androgens. Human P450 17A1 reaction rates examined are enhanced by the accessory protein cytochrome b$_5$ (b$_5$), but the exact role of b$_5$ in P450 17A1-catalyzed reactions is unclear as are several details of these reactions. Here, we examined in detail the processivity of the 17α-hydroxylation and lyase steps. b$_5$ did not enhance reaction rates by decreasing the k$_{off}$ rates of any of the steroids. Steroid binding to P450 17A1 was more complex than a simple two-state system. Pre-steady-state experiments indicated lag phases for Andro production from progesterone and from DHEA from pregnenolone, indicating a distributive character of the enzyme. However, we observed processivity in pregnenolone/DHEA pulse–chase experiments. (S)-Orteronel was three times more inhibitory toward the conversion of 17α-hydroxypregnenolone to DHEA than toward the 17α-hydroxylation of pregnenolone. IC$_{50}$ values for (S)-orteronel were identical for blocking DHEA formation from pregnenolone and for 17α-hydroxylation, suggestive of processivity. Global kinetic modeling helped assign sets of rate constants for individual or groups of reactions, indicating that human P450 17A1 is an inherently distributive enzyme but that some processivity is present, i.e. some of the 17α-OH pregnenolone formed from pregnenolone did not dissociate from P450 17A1 before conversion to DHEA. Our results also suggest multiple conformations of P450 17A1, as previously proposed on the basis of NMR spectroscopy and X-ray crystallography.

This work was supported by National Institutes of Health Grants R01 GM118122 (to F. P. G.), R01 GM103937 (to F. P. G.), and T32 ES007028 (to E. G.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supplemental Figs. S1–S3.

1 This paper constitutes part of the requirements to fulfill a Ph.D. thesis at Vanderbilt University. Present address: National Center for Advancing Translational Sciences (NCATS), National Institutes of Health, Bethesda, MD 20892.

2 To whom correspondence should be addressed: Dept. of Biochemistry, Vanderbilt University School of Medicine, 6388 Robinson Research Bldg., 2200 Pierce Ave., Nashville, TN 37232-0146. Tel.: 615-322-2261; Fax: 615-343-0704; E-mail: f.guengerich@vanderbilt.edu.

The cytochrome P450 (P450 or CYP)$^3$ enzymes collectively have the most diverse set of substrates, at least among oxidoreductases (1). In particular, P450s are very important catalysts in the metabolism of steroids (human P450s 1B1, 7A1, 7B1, 8B1, 11A1, 11B1, 11B2, 17A1, 19A1, 21A2, 24A1, 27A1, 39A1, 46A1, and 51A1, plus some others involved in catabolism) and vitamins (humans P450s 2R1, 24A1, 26A1, 26B1, 26C1, 27A1, 27B1, and 27C1) (2, 3). The steroidogenic P450s are particularly important, and homozygous or dual heterozygous deficiencies are often debilitating (2, 4). P450 enzymes also have major roles in the metabolism of exogenous ( xenobiotic) chemicals, including drugs and carcinogens (3), and some P450s oxidize both endogenous and xenobiotic substrates (5).

One of the steroidogenic P450s, P450 17A1, has a critical role in the production of androgens and also glucocorticoids (2). At least 50 variants with P450 17A1 insufficiency have been identified clinically (4, 6). The enzyme catalyzes the two-step oxidation of progesterone to 17α-OH progesterone to androstenedione (Andro) and also the oxidation of pregnenolone to 17α-OH pregnenolone to dehydroepiandrosterone (DHEA) (Fig. 1). The second reaction in each series (Fig. 1A) is termed a “lyase” (or “desmolase“) reaction, in that cleavage of the 17,20 C–C bond occurs. Although these were first considered to be separate enzymatic activities, they are now known to be catalyzed by a single protein, P450 17A1 (7–9). The presence of another hemoprotein, cytochrome b$_5$ (b$_5$), has been shown to greatly stimulate the lyase activity (10–13), at least in some species. The process is complicated by accompanying minor reactions (Fig. 1B). The minor products have all been identified in vitro (14–16), and 16α-OH progesterone has been measured in human serum (17).

A role for b$_5$ in enhancing P450 catalytic activity was first shown in liver microsomes in 1971 (18, 19). In 1982, b$_5$ was shown to stimulate the 17α-OH progesterone lyase activity of purified porcine testicular P450 17A1 3–4-fold (10, 20). Komnami et al. (21) also showed stimulation of guinea pig P450 17A1 by bovine b$_5$, both the 17α-hydroxylation of progesterone and, to a fractionally larger extent, the 17α-OH progesterone lyase reaction. However, b$_5$ was inhibitory at a b$_5$/P450 17A1 ratio of >0.5 (21). In 1995, Katagiri et al. (11) reported that

$^3$ The abbreviations used are: P450 (or CYP), cytochrome P450; Andro, androstenedione; b$_5$, cytochrome b$_5$; compound I, formal FeO$_3$$^+$, oxidized form of a hemoprotein; DHEA, dehydroepiandrosterone; DLPC, l-α-1,2-dilauroyl-sn-glycero-3-phosphocholine; OH, hydroxy; SVD, singular value decomposition.
human $b_5$ (added in a concentration equimolar to P450) stim-
ulated several catalytic activities of human P450 17A1. Proges-
terone 17α-hydroxylation was not stimulated by $b_5$, but preg-
genolone 17α-hydroxylation was enhanced 2.5-fold; lyase
activities toward both 17α-OH progesterone and 17α-OH
pregnenolone were enhanced ~10-fold by $b_5$ (11). No qualita-
tive difference was observed in the binding of 17α-OH preg-
nenolone to P450 17A1. In the same year (1995), Lee-
Robichaud et al. (12) also demonstrated stimulation of both
porcine and human P450 17A1 activities by porcine $b_5$,
although the results differed from those of Waterman and co-
workers (11). The catalytic efficiency of 17α-hydroxylation was
increased 2-fold for pregnenolone but not for progesterone, as
in the report of Waterman and co-workers (11). However, no

Figure 1. Oxidations catalyzed by P450 17A1. A, major classical reactions; B, expanded repertoire of reactions (14–16).
lyase activity with either 17α-OH progesterone or 17α-OH pregnenolone (as substrate) could be detected in the absence of $b_5$, but activities were seen in the presence of a 5-fold molar excess of $b_5 > P450 17A1$ (12).

There are several enigmas about P450 17A1. One is the exact role of $b_5$, which NMR studies indicate occupies the same site as and competes with the obligate electron donor NADPH-P450 reductase (13, 22). $b_5$ does not appear to transfer electrons to P450 at any point in the catalytic cycle (23, 24). Another controversy is whether a ferric peroxy ($\text{FeO}_2^-$) or compound I (perferryl oxygen; $\text{FeO}_3^+$) species is involved in the lyase reaction (15, 16, 25–27).

One of the issues regarding the two-step, net four-electron oxidation (Fig. 1A) is the processivity of the overall reaction. In a processive reaction, the 17α-OH steroid product remains bound to the enzyme for the succeeding lyase reaction, but in a distributive reaction, the 17α-OH product dissociates and then rebinds for the lyase reaction (Fig. 1A). A variety of reports on the topic of processivity have appeared, with varying conclusions. On the basis of work in human embryonic kidney (HEK-293) cells, Soucy and Luu-The (28) concluded that this is a distributive sequence of reactions. However, studies with rat, guinea pig, and bovine P450 17A1 enzymes led to conclusions that the two steps are processive (29–33). These studies involved a number of approaches and designs, but none included $b_5$, which had already been shown to stimulate the lyase reaction (10, 11). Our own work with zebrafish P450 17A1 and $b_5$ indicated that the reactions were distributive when progesterone was the substrate and more processive when pregnenolone was the substrate, although only a relatively weak effect of zebrafish or human $b_5$ is seen in that system (34).

The matter of processivity is important for several reasons. One is that 17α-OH progesterone and 17α-OH pregnenolone (formed following 2-electron reduction of 17α-OH progesterone) serve as precursors to glucocorticoids (2, 3). Another issue is that prostate cancer is androgen-stimulated (35), and P450 17A1 is a major drug target for therapy (6, 36–39). A goal is the development of a model of the human P450 17A1 reactions.

Results

Steady-state reaction kinetics

These studies began with steady-state reaction kinetics, which included an analysis of the effect of $b_5$ (Fig. 2 and Table 1). Some of the lack of ideal curve fitting may be due to the formation of additional products (Fig. 1B) (15, 16), which were not included here for the sake of simplification. The efficient conversion of 17α-OH pregnenolone to DHEA confounds the results for formation of 17α-OH pregnenolone from pregnenolone (Fig. 2A), but the lyase reaction (using 17α-OH pregnenolone as the starting substrate (Fig. 2B)) was more straightforward. The decrease in apparent rates of DHEA formation in the presence of $b_5$ (Fig. 2B) may be the result of facile 16-hydroxylation of DHEA (16). Our $k_{cat}$ and $K_m$ values are similar to those of Lee-Robichaud et al. (12), and the $k_{cat}$ values are as high or higher than reported for any human P450 17A1 preparations by others (11, 44–48).

$b_5$ stimulated all of the reactions examined (Fig. 2). Very little DHEA was formed from pregnenolone or 17α-OH pregnenolone in the absence of $b_5$ (Fig. 2, A and B) nor was very much Andro formed from progesterone or 17α-OH progesterone (Fig. 2, C and D). 16α-Hydroxylation of progesterone was also enhanced by $b_5$ (Fig. 2C). The major effects of $b_5$ were on $k_{cat}$ values (Table 1).

Binding of substrates and products to P450 17A1

Initial experiments were done with low concentrations of P450 17A1 in a 10-cm cell (34) because of the low $K_m$ values we estimated in preliminary assays, which were done using the “type I” Soret spectral shifts (increase in absorbance at 390 nm and decrease near 420 nm (49)). Because of issues we found regarding the instability of P450 17A1 during the protracted measurements, we collected data (following stopped-flow mixing) 7 s after additions and used these results, utilizing a quadratic equation for fitting. The apparent $K_m$ values for pregnenolone, progesterone, 17α-OH progesterone, and 17α-OH pregnenolone were all sub-micromolar (Fig. 3). The $K_m$ value for DHEA was 1.7 μM and that for Andro was ∼19 μM, although the $K_m$ fitting for the latter product was not very accurate.

Measurement of $k_{off}$ rates of substrates and products of P450 17A1

One of the possible roles of $b_5$ in the enhancement of the lyase reactions could be the decreased dissociation of 17α-OH steroids (Fig. 1A). If this were the case, the $k_{off}$ rates would be expected to be lower in the presence of $b_5$.

Because of the different Soret spectra observed with various ligands (Figs. 4 and 5) and the interaction of the imidazole group of orteronel with the heme iron (34), we were able to use this compound as a trap (the estimated $K_d$ for (S)-oteronel is 40 nM (42), which we confirmed (data not presented)). With 10 μM (S)-oteronel, the apparent on-rate was 6.1 s$^{-1}$ (Fig. 4). Mixing of equimolar concentrations (2 μM) of P450 17A1 and each steroid with 10 μM (S)-oteronel provided a means of estimating $k_{off}$ values, in that these were 1–2 orders of magnitude slower. Spectral data and analysis for 17α-OH pregnenolone (as an example) are presented in Fig. 5 ($k_{off}$...
The trapping method was used to measure $k_{\text{off}}$ rates for all steroid substrates and products (Table 2). In no case did $b_5$ substantially affect $k_{\text{off}}$ values.

**Time courses of individual P450 17A1 reactions**

P450 17A1 reactions were initiated with equal concentrations (2 $\mu$M) of P450 17A1 and each substrate (Fig. 6). (These experiments are often termed “single-turnover” assays, although the term is not strictly correct in that substrate binding and release are occurring on similar time scales as substrate turnover.) In these assays, we also measured minor products to better account for reaction stoichiometry. Several apparent first-order rates of substrate disappearance were calculated and are presented in Table 3 (see also supplemental Fig. S1).

**Figure 2. Steady-state kinetics of major reactions catalyzed by P450 17A1 and the effect of $b_5$.** The data points are means of duplicate assays (± range). Fitting was done by non-linear regression analysis of hyperbolic data in GraphPad Prism. See Table 1 for calculated values. A, pregnenolone (preg); B, 17α-OH pregnenolone; C, progesterone (prog); D, 17α-OH progesterone.

**Table 1**

**Steady-state parameters for human P450 17A1 reactions**

See Fig. 2 for plots. The S.E. values for $k_{\text{cat}}$ and $K_m$ are calculated from within each experiment, as estimated using Prism non-linear regression analysis. In terms of comparing $k_{\text{cat}}$ values to most literature rates for this enzyme, be aware that most have been expressed in units of min$^{-1}$. Those are expressed here in units of s$^{-1}$ in order to link these to other reaction rates considered in this work.

| Substrate | Reaction       | $k_{\text{cat}}$ $(s^{-1})$ | $K_m$ $(\mu$M$)$ | $k_{\text{cat}}/K_m$ $(s^{-1} \mu$M$^{-1}$) | $b_5$ ratio |
|-----------|----------------|-----------------------------|-------------------|--------------------------------------------|-------------|
| Pregnenolone | 17α-Hydroxylation | 0.0037±0.0005 | 0.11±0.01 | 0.5±0.3 | 1.1±0.2 | 0.0074±0.0044 | 0.10±0.02 | 14 |
| Lyase | | 0.0059±0.0012 | 0.074±0.005 | 0.045±0.003 | 0.49±0.14 | 0.0010±0.0009 | 0.078±0.021 | 8 |
| 17α-OH pregnenolone | Lyase | 0.0068±0.0004 | 0.17±0.01 | 0.57±0.46 | 0.91±0.24 | 0.0089±0.0009 | 0.040±0.004 | 4 |
| Progesterone | 17α-Hydroxylation | 0.0064±0.0009 | 0.25±0.003 | 0.11±0.06 | 0.53±1.3 | 0.0058±0.0030 | 0.0047±0.0012 | 0.8 |
| Lyase | | 0.000031±0.00001 | 0.019±0.001 | 2.9±0.3 | 7.1±0.3 | 0.00011±0.00001 | 0.0027±0.0001 | 25 |

* The rate was too low to accurately measure the steady-state kinetic parameters (see Fig. 6A).

0.4 s$^{-1}$ in that example, 0.32 ± 0.02 s$^{-1}$ for an average of more experiments in Table 2).

The trapping method was used to measure $k_{\text{off}}$ rates for all steroid substrates and products (Table 2). In no case did $b_5$ substantially affect $k_{\text{off}}$ values.
**Figure 3. Binding of substrates and products to P450 17A1.** Absorbance measurements were made following mixing in a stopped-flow spectrophotometer as described in detail under “Experimental procedures.” At least three data points were collected at each ligand concentration, and the points were fit to hyperbolic curves using Prism software and non-linear regression analysis (S.E. calculated from curve fitting). A, pregnenolone (K_\text{d} 0.37 ± 0.03 μM); B, progesterone (K_\text{d} 0.47 ± 0.04 μM); C, 17α-OH pregnenolone (K_\text{d} 0.52 ± 0.10 μM); D, 17α-OH progesterone (K_\text{d} 0.95 ± 0.08 μM); E, DHEA (K_\text{d} 1.7 ± 0.2 μM); F, Andro (K_\text{d} 19 ± 9 μM).

**Figure 4. (S)-Orteronel binding to P450 17A1.** P450 17A1 (2 μM) and (S)-orteronel (10 μM) in 100 mM potassium phosphate buffer (pH 7.4) were mixed together in the stopped-flow spectrophotometer. A, series of spectra collected at 1-ms intervals over a total period of 1.0 s (only a subset is shown). B, calculated beginning and ending spectra after SVD analysis using a two-species model in the OLIS GlobalWorks® software. C, SVD analysis of data for disappearance of starting species and appearance of final species, along with OLIS EV (Eigenvector) absorbance and OLIS normalized experimental data. D, eigenvector (solid line) and normalized experimental data. E, residuals analysis of fit to a rate of 6.1 s^{-1} (from C and D).
All reactions were stimulated by the presence of b$_2$, with the exception of the rate of progesterone disappearance (Fig. 6, E and F). As expected, all secondary products showed lags in formation, including the lyase products DHEA and Andro (Fig. 6, B and F).

**Pulse–chase assays with pregnenolone oxidation to DHEA**

The lag phases for formation of lyase products (DHEA from pregnenolone and Andro from progesterone) (Fig. 6, B and F) are indicative of distributive mechanisms, in that a completely processive mechanism should proceed without a lag. The lag phase was shorter in the case of pregnenolone (no DHEA appearing until 2 s, Fig. 2F) than progesterone (no Andro appearing until 5 s, Fig. 2B), and we examined this reaction further utilizing pulse–chase assays (Fig. 7).

Reactions were initiated with $[^{3}$H]pregnenolone. In steady-state assays (Fig. 7A), unlabeled 17α-OH pregnenolone was added 60 s after the reaction was initiated, and the reaction proceeded for another 10 min. Even with adding a concentration of unlabeled 17α-OH pregnenolone as high as 75 μM, only about one-half of the $[^{3}$H]DHEA formed from $[^{3}$H]pregnenolone could be blocked.

In pre-steady-state reactions, patterned after the experiment in Fig. 6B, a chase of 80 μM unlabeled 17α-OH pregnenolone was added to the reaction at varying times after the reaction started, and the reaction proceeded for another 5 s (Fig. 7B). Even when the chase (unlabeled) 17α-OH pregnenolone was added as early as 50 ms, the production of $[^{3}$H]DHEA was only $\frac{1}{3}$ that for 17α-OH hydroxylation (Fig. 8).

For initial model development, we used the measured $k_{\text{off}}$ and $K_{d}$ values to estimate apparent $k_{\text{on}}$ rates (Table 5). The $k_{\text{on}}$ rates were not affected by the presence of b$_2$ (supplemental Fig. S3). We also measured some $k_{\text{on}}$ rates directly, but these are complex and will be considered separately (see below, Fig. 11). Only the systems containing b$_2$ were considered. The oxidation step rates are those measured in the single-turnover reactions presented in Fig. 6, B, D, F, and G.

Using the simplified model and values listed in Fig. 9, it was possible to fit the data using KinTek Explorer® software (Fig. 9).
Human P450 17A1 kinetics

Figure 6. “Single-turnover” kinetics of 1:1 molar complexes of P450 17A1 with various substrates. In each case a mixture of 4 μM P450 17A1, 8 μM NADPH-P450 reductase, 32 μM DLPC, 0.10 M potassium phosphate buffer (pH 7.4), and (when indicated) 4 μM b5, were mixed with an equal volume of the 0.10 M potassium phosphate buffer (pH 7.4) containing 1 mM NADPH to initiate reaction in a KinTek RP-3 rapid quench apparatus. At each indicated time point, the reaction was terminated, and the products and residual substrate were separated and quantitated by radio-HPLC as described under “Experimental procedures.” In each case, [3H]- or [14C]-labeled substrate was used ([3H]pregnenolone (103 Ci/mmol), [3H]17α-OH pregnenolone (700 μCi/mmol), [3H]progesterone (60 μCi/mmol), or [3H]17α-OH progesterone (103 μCi/mmol)). Each point is derived from a single time point analysis. A, C, E, and G did not include b5; B, D, F, and H included b5. Traces are shown for reactions in which the substrate was as follows: A and B, pregnenolone; C and D, 17α-OH pregnenolone; E and F, progesterone; G and H, 17α-OH progesterone. See supplemental Fig. S2 and Table 3 for calculated rates of substrate disappearance. prog, pregnenolone; prog, progesterone.

10) for single-turnover (Fig. 10, A and B), steady-state (Fig. 10, C and D), and binding reactions (Fig. 10, E–G). As mentioned in the presentation of Fig. 2 (see above), some of the discrepancy may be due to not including all secondary products (14–16).

We also considered the binding of pregnenolone, 17α-OH pregnenolone, and DHEA in detail, using global analysis of the actual kinetic binding curves (Fig. 11). The $k_{off}$ values used were approximately those measured in the trapping experiments (Table 2). However, the $k_{on}$ rates are slower than estimated from consideration of $k_{off}$ and $K$ values (Table 5). In addition, the estimated $k_{on}$ values are all $<10^6$ M$^{-1}$ s$^{-1}$ and therefore probably not diffusion-limited (50).

An alternative binding scheme is proposed, one in which there exist multiple interconverting conformations of P450 17A1 in the absence of ligands. Independent evidence for the existence of multiple conformations comes from the NMR...
NADPH-generating system (68). After 60 s, the indicated concentration of labeled [3H]pregnenolone having a specific radioactivity of 830 Ci/mmol, in 50 mM potassium phosphate buffer (pH 7.4, final), and the radiolabeled DHEA was measured by radio-HPLC with [3H]progesterone and [3H]-OH progesterone to P450 17A1 (Fig. 12).

| Substrate | Presence of \( b_5 \) | Rate \( \times 10^{-3} \) s⁻¹ |
|-----------|----------------------|-----------------------------|
| Pregnenolone | − | 0.28 ± 0.01 |
| + | 0.43 ± 0.02 |
| 17α-OH pregnenolone | − | 0.0074 ± 0.0006 |
| + | 0.77 ± 0.01 |
| Progesterone | − | 0.46 ± 0.02 |
| + | 0.47 ± 0.02 |
| 17α-OH progesterone | − | 0.0066 ± 0.0005 |
| + | 0.029 ± 0.001 |

Discussion

P450 17A1 plays a critical role as the immediate enzyme involved in steroid metabolism following transformation of cholesterol to pregnenolone by another P450, P450 11A1 (2). P450 17A1 deficiency is a major endocrinological issue (3, 4), but the same enzyme is also a current target in the treatment of prostate cancer (39). In this study, we investigated several kinetic aspects of how this enzyme works in the catalysis of two successive reactions (Fig. 1A). We demonstrate that this is an inherently distributive process, in regard to the two successive reactions, with some of the 17α-OH steroid products dissociating from the enzyme. However, there is some processivity, particularly in the case of pregnenolone, i.e. part of the 17α-OH steroid pool does not dissociate but remains bound to the enzyme for the second oxidation step. Accordingly, some of the product DHEA is derived from 17α-OH pregnenolone that has not dissociated from the enzyme (Fig. 7). This is, to our knowledge, the first detailed study to consider the processivity of isolated human P450 17A1 and, with the exception of our zebrafish P450 17A1 model (34), the first of any with P450 17A1 to consider the role of \( b_5 \) in a processivity study, which is critical (Figs. 2 and 6). The fish enzyme is only modestly stimulated by fish or human \( b_5 \) (34). We conclude that \( b_5 \) stimulates both the human P450 17A1 17α- (and 16α-) hydroxylation and lyase reactions, plus diene formation (Fig. 6B). \( b_5 \) did not affect the affinity of P450 17A1 for 17α-OH steroids (Fig. 3 and Table 2).

We believe that multiple conformations of P450 17A1 (Fig. 13) are necessary to explain several of the kinetic results, in support of NMR spectroscopic (22) and X-ray crystallographic evidence (42, 44) for the existence of multiple conformations of the protein.

One previous study concluded that human P450 was a processive enzyme, but this work was only done at a cellular level (HEK-293 cells) (28). Others concluded that the rat (31) and guinea pig (29) enzymes are processive with the substrate progesterone, based on microsomal studies. Another rat microsomal study (30) with progesterone was unclear regarding conclusions about processivity but can be interpreted in the context of a partially processive mechanism. Tagashira et al. (32) concluded that the guinea pig enzyme was processive with the substrate progesterone, and Yamazaki et al. (33) concluded that the bovine enzyme was “20% processive”; all of these studies (except those using microsomes) were done in the absence of \( b_5 \).

It is clear that any results obtained in the absence of \( b_5 \) are problematic (Fig. 2 and Table 1). Lee-Robichaud et al. (12) reported no detectable lyase activity with either 17α-OH pregnenolone or 17α-OH progesterone in the absence of \( b_5 \), but both we (Figs. 2 and 6) and others (11, 47) did observe low activity with human P450 17A1. \( b_5 \) has been proposed to play a critical role in the balance of steroid metabolism, particularly in the production of androgens (11, 12). One issue is that the role of \( b_5 \) in lyase reactions catalyzed by P450 171 varies among species. With zebrafish P450 17A1, the stimulations by \( b_5 \) are 2-fold (34). Mice in which \( b_5 \) were only −2-fold (34). Mice in which \( b_5 \) was globally deleted were still viable and reproduced, even though the formation of androgens from progesterone and 17α-OH pregnenolone or 17α-OH progesterone to P450 17A1 (Fig. 12).

Figure 7. Pulse–chase assays of conversion of pregnenolone to DHEA. A, steady-state reaction. A reaction was initiated by mixing P450 17A1 (0.5 μM), NADPH-P450 reductase (2 μM), b5 (0.5 μM), DLPC (16 μM), and [3H]pregnenolone (13 μCi/μmol, in 50 mM potassium phosphate buffer) with an NADPH-generating system (68). After 60 s, the indicated concentration of unlabeled 17α-OH pregnenolone was added, and the reaction was continued for another 10 min, at which time the reaction was quenched by the addition of HCl (0.67 M, final), and the radiolabeled DHEA was measured by radio-HPLC as described under “Experimental procedures.” Results are presented as means ± S.D. (range) of duplicate determinations. B, rapid quench pulse-chase experiment. A similar approach was used, utilizing the rapid-quench apparatus with [3H]pregnenolone having a specific radioactivity of 830 μCi/μmol. The radiolabeled DHEA was measured after the addition of 80 μM unlabeled 17α-OH pregnenolone at the indicated times following initiation of the reaction with NADPH. The results are presented as means ± S.D. (range) of two individual experiments.

X-ray crystallography and spectroscopy work of Scott and coworkers (22, 42, 44). Using a model in which both the ligand-free and ligand-bound forms of the enzyme have multiple, inter-convertible conformations, we were able to fit the raw data files for binding of pregnenolone, 17α-OH pregnenolone, and DHEA to P450 17A1 (Fig. 12).
progesterone in testis microsomes was severely attenuated, and the (in vivo) intratesticular levels of testosterone were reduced by one-half (51). In vitro (murine) microsomal 17α-hydroxylation of progesterone was also reduced \( \frac{1}{2} \). Again, there is species variability, and the application to humans is not direct.

In humans, some clinical endocrine disorders involving low levels of androgens have been linked to defective variants of \( b_5 \) (52, 53). In the two cases cited (52, 53), the (in vivo) levels of 17α-OH steroids were not compromised. P450 17A1 is primarily an adrenal enzyme, and one question is what the relative levels of \( b_5 \) and P450s are there. Some information is available, but the situation is complex in that there are four zones in the adrenal gland: glomerulosa, fasciculate, reticularis, and medulla. To our knowledge, the levels of the relevant enzymes have not been measured in human adrenal tissue. Hamamoto et al. (54) quantitated several enzymes in bovine adrenals using immunochemical methods. P450 17A1 was not measured but another microsomal P450 (21A2), and two mitochondrial P450s (11A1 and 11B1) were. The levels of \( b_5 \) were lower than any of the three P450s in all four zones (54). This situation contrasts with

![Figure 8. Inhibition of P450 17A1 reactions by (R)- and (S)-orteronel. Steady-state reactions were run with 0.01 \( \mu \)M P450 17A1 (0.1 \( \mu \)M for 17α-OH progesterone), in the presence of 0.5 \( \mu \)M \( b_5 \), for 5 min in the presence of the indicated concentrations of the resolved enantiomers of orteronel. Results are presented as means of two determinations ± range, with fitting as described under “Experimental procedures.”](image)

**Table 4**

| Substrate                | Product              | IC\(_{50}\) (95% CI)\(^a\) |
|--------------------------|----------------------|-----------------------------|
| Pregnenolone             | 17α-OH pregnenolone  | 1.5 (1.1–1.9) \( \mu \)M    |
| 17α-OH pregnenolone      | DHEA                 | 1.4 (1.2–1.8) \( \mu \)M    |
| Progesterone             | 17α-OH progesterone  | 0.49 (0.22–1.1) \( \mu \)M  |
| 16α-OH progesterone      | DHEA                 | 0.32 (0.22–0.48) \( \mu \)M  |
| 17α-OH progesterone      | Andro                | 0.23 (0.15–0.35) \( \mu \)M  |

\(^a\) See data in Fig. 8. Fitting was done in GraphPad Prism (Onesite-Fit logIC\(_{50}\)).

**Table 5**

| Substrate      | \( k_{\text{on}} \) \( \text{M}^{-1} s^{-1} \) \( +b_5 \) | \( k_{\text{off}} \) \( \text{s}^{-1} \) \( -b_5 \) |
|----------------|---------------------------------|-------------|
| Pregnenolone   | 0.62                            | 0.78        |
| 17α-OH pregnenolone | 0.62                          | 0.79        |
| DHEA           | 0.47                            | 0.45        |
| Progesterone   | 0.98                            | 0.89        |
| 17α-OH progesterone | 0.85                          | 0.97        |
| Andro          | 0.095                           | 0.074       |

In humans, some clinical endocrine disorders involving low levels of androgens have been linked to defective variants of \( b_5 \) (52, 53). In the two cases cited (52, 53), the (in vivo) levels of 17α-OH steroids were not compromised.

P450 17A1 is primarily an adrenal enzyme, and one question is what the relative levels of \( b_5 \) and P450s are there. Some information is available, but the situation is complex in that there are four zones in the adrenal gland: glomerulosa, fasciculate, reticularis, and medulla. To our knowledge, the levels of the relevant enzymes have not been measured in human adrenal tissue. Hamamoto et al. (54) quantitated several enzymes in bovine adrenals using immunochemical methods. P450 17A1 was not measured but another microsomal P450 (21A2), and two mitochondrial P450s (11A1 and 11B1) were. The levels of \( b_5 \) were lower than any of the three P450s in all four zones (54). This situation contrasts with
liver, where the microsomal concentrations of (total) P450 and \( b_5 \) are similar (55).

In modeling our results, we focused on grouping several individual steps together, to simplify the system. Our work indicates that substrate binding is more complex than a one-step/two-state system (Fig. 12), but we modeled the system accordingly to satisfy several data sets (Figs. 9 and 10). In a similar way, we know that each oxidation sequence (either \( 17\alpha \)-hydroxylation or the lyase step) done by P450 17A1 probably involves at least nine distinct steps (56, 57). We have modeled each of these sets with a single rate or rate constant (Figs. 9 and 11), without trying to discern rate-limiting steps (there is evidence that C–H bond breaking is at least partially rate-limiting in the \( 17\alpha \)-hydroxylation step (14)).

\( b_5 \) showed stimulation of all of the P450 17A1 reactions (Fig. 2). In some previous work, the conclusion was reached that \( b_5 \) selectively enhanced the lyase reaction, possibly by stabilizing the ferric peroxide (Fe\( _{3+} \)) form of the enzyme proposed to be selectively involved in the lyase reaction (12, 58). Our findings that multiple reactions are stimulated by \( b_5 \) argue against this conclusion (Fig. 2 and Table 1). Previous evidence that electron transfer from \( b_5 \) to P450 or its oxygenated complex by \( b_5 \) is not involved in the stimulation of P450 17A1 catalytic activity (23, 24) is complemented by our more recent findings that P450 17A1 activity supported by the oxygen surrogate iodosylbenzene, in the absence of NADPH-P450 reductase, is also augmented by \( b_5 \) (16).

The stimulatory effects of \( b_5 \) (Table 1 and Fig. 2) are clearly not related to differential effects on binding of intermediate products (Table 2). The effects of \( b_5 \) on the lyase reactions with both pregnenolone and progesterone were seen in single-turnover experiments (Fig. 6, C, D, G, and H). What is less clear is why the effect of \( b_5 \) on \( 17\alpha \)-hydroxylation in the steady-state kinetic assays (Fig. 2 and Table 1) is much greater than seen in the single-turnover studies (Fig. 6). Recently, Peng et al. (47) provided evidence for improvement of electron coupling of P450 17A1 by \( b_5 \), but presumably the same factors would influence abortive oxygen formed in the steady-state and under pre-steady-state conditions. We do not have a clear definition of exactly what the \( k_{cat} \) or \( K_m \) parameters mean in this complex system, although the stimulation of both \( 17\alpha \)-hydroxylation reactions is quite clear (Fig. 2, A and C).

\( b_5 \) is still an enigma in terms of its role in the P450 17A1 system. As already pointed out, there is considerable evidence against a role for electron transfer, i.e. catalytic activity in assays with the heme deleted from \( b_5 \) (23) or replaced by manganese-substituted heme (24) and with the oxygen surrogate iodosylbenzene (16). A recent publication by Duggal et al. (48) argues for electron transfer from \( b_5 \), based on negative results of experiments with Mn\( ^{2+} \)-substituted \( b_5 \), in contrast to others (24). However, the rate of lyase activity with the control \( b_5 \) was only \(~4\%\) of that measured here (Table 1 and Fig. 2) or elsewhere (16 and also see also other rates in Refs. 11, 12, 47), and a control \( b_5 \) experiment with reconstituted iron-substituted heme was not included. The weight of evidence is that \( b_5 \) induces conformational changes in P450 17A1 (and probably several other P450s in which it plays a non-redox role (59) in facilitating product formation (13)), possibly through more productive coupling (47). Exactly how this occurs is unclear. There is NMR evidence that \( b_5 \) occupies the same site of P450 17A1 that NADPH-P450 reductase does (22). An enigma is that this evidence suggests that the P450 17A1–iron oxygen complex, whatever it may be, receives all of its electrons and is in an activated state before the reductase leaves and is replaced by \( b_5 \) which induces a more favorable conformation for catalysis. This process must happen rapidly, occurring in every reaction cycle. Assuming the above conclusion that \( b_5 \) and NADPH-P450 reductase compete for the same P450 17A1 site and cannot be bound simultaneously (22), it is possible that \( b_5 \) could bind earlier, but it would have to be released for NADPH-P450 reductase to rebind (to the Fe\( ^{2+} \)–O\( _2 \) complex) for electron delivery, and then rebind. We previously reported that \( b_5 \) could stimulate the lyase activity of P450 17A1 supported by the oxygen surrogate iodosylbenzene (16).

---

**Figure 9.** Kinetic scheme for oxidations of pregnenolone (Preg) and progesterone (Prog) using optimized rate constants and dissociation constants. See Fig. 10 for kinetic and binding data.
A role for multiple P450 17A1 conformations, indicated by NMR spectroscopy (13) and X-ray crystallography (42, 44), is supported by some of our own results. As indicated, we were unable to fit the substrate-binding data with only a simple two-state system (Fig. 12). We propose that not only the ground state form of P450 17A1 but also the substrate-bound form (13, 42, 44) and probably several of the electronic intermediates in the reaction pathway have multiple conformers (Fig. 13). Such a diagram is consistent with current general thoughts about enzyme mechanisms (60) and is probably operative here (Fig. 13). Attempts to expand our kinetic analysis to include extra conformations with KinTek Explorer® were unsuccessful due

Figure 10. Fitting of pregnenolone kinetic and binding data with rate constants and $K_d$ values (from Fig. 9). A and B are from Fig. 5, G and H, respectively (single-turnover results). C and D are steady-state kinetic results from Fig. 2, A and B, respectively. A, single-turnover kinetics beginning with pregnenolone (preg). B, single-turnover kinetics beginning with $17\alpha$-OH pregnenolone. C, steady-state kinetics for conversion of pregnenolone to $17\alpha$-OH pregnenolone and DHEA. D, steady-state kinetics of $17\alpha$-OH progesterone oxidation to DHEA. E, steady-state binding of pregnenolone to (ferric) P450 17A1. F, steady-state binding of $17\alpha$-OH pregnenolone to (ferric) P450 17A1. G, steady-state binding of DHEA to (ferric) P450 17A1.
to the complexity of the system, and even if more rate constants could be attached to the system (in Fig. 13), we do not have enough data constraints to judge the validity of any modeling that might fit. Therefore, our kinetic conclusions about processivity rely on models in which reaction steps are grouped together (Figs. 9 and 11). The nature of multiple conformations proposed in the scheme in Fig. 13 is unknown. These could be only structural conformers. However, these could also be $b_{5}$-bound and non-$b_{5}$-bound forms, reductase- and non-reductase-bound forms, $\text{FeO}_{2}$ and $\text{FeO}^{3+}$ forms poised to do different reactions (a possibility we have not ruled out (16)), or forms that preferentially bind orteronel instead of the substrate and product. With an enzyme in which there appears to be only one ligand-binding site, multiple conformations (Fig. 13) are required to explain preferential inhibition of individual reactions by any drug. Alternatively, a second enzyme site can exist and might be involved (42).

A potential issue related to the processivity of P450 17A1 is application to discovery and development of drugs (for prostate cancer) that would only inhibit the lyase reaction, to avoid side effects. A number of reports of selectivity for the lyase reaction $>17\alpha$-hydroxylation have appeared (40–42, 61, 62). However, repeated assays with purified P450 17A1/reductase/b_{5} systems have yielded less selectivity, and some of the reports of high selectivity have not been confirmed (36, 42). Our own results with orteronel show IC_{50} values (Table 4) similar to those reported very recently by Petrunak et al. (42), although we did not observe an 11-fold selectivity for $(R)$-orteronel. Some of the

---

**Figure 11.** Results from KinTek Explorer® data analysis with experimental $k_{a}$ values as constants.

**Figure 12.** Analysis of P450 17A1 steroid binding kinetics. P450 17A1 (2 μM) was mixed with increasing concentrations of pregnenolone, $17\alpha$-OH pregnenolone, or DHEA as described in general under “Experimental procedures” (see Fig. 3 for concentrations, corresponding to the data points there; note, b_{5} was not present). Stopped-flow changes are shown in arbitrary units in the KinTek Explorer© global analysis, corresponding to $\Delta A_\alpha - A_\alpha$ changes. A, pregnenolone; B, $17\alpha$-OH pregnenolone; C, DHEA. In the model, there are two forms of the enzyme, E and E*, and only one binds the ligand (to form EL). EL is in equilibrium with a second ligand-bound form, $E'\cdot L$. In the fitting, both EL and E*L show the observed spectral perturbation (and are therefore circled). Raw data are presented in the rough traces, and the overlaid lines are the fits from the KinTek Explorer© software. The wavelengths used for a and b changes were 391 and 426 for pregnenolone, 393 and 426 for $17\alpha$-OH pregnenolone, and 393 and 428 for DHEA. The values used were $k_{a_1} = 0.23 \text{s}^{-1}$ and $k_{a_1} = 0.21 \text{s}^{-1}$ (for all cases) and the following for each ligand: pregnenolone, $k_{2} = 1.2 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$; $k_{2} = 2.2 \text{s}^{-1}$; $k_{3} = 0.50 \text{s}^{-1}$; and $k_{3} = 1.9 \text{s}^{-1}$; $17\alpha$-OH pregnenolone, $k_{2} = 8.4 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$; $k_{2} = 1.2 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$; $k_{2} = 1.2 \text{s}^{-1}$; $k_{3} = 0.10 \text{s}^{-1}$; and $k_{3} = 2.3 \text{s}^{-1}$.

---

**Figure 13.** Hypothetical P450 17A1 reaction scheme with additional conformations of ligand-bound enzyme. Δ⁴ steroids are shown, but the model can also be considered for Δ⁵ steroids.
human P450 17A1 kinetics

discrepancies among IC_{50} values are attributed to the use of different substrate concentrations, which necessarily affect IC_{50} estimates. Some reported selectivity comparisons are not directly related to processivity, in that 17α-hydroxylation of progesterone has been compared with lyase activity toward 17α-OH pregnenolone (36). In some cases, more selectivity has been reported in cell cultures, based only on measurement of endogenous steroids (36, 40, 62), although the reasons are not clear.

Two findings of the recent Petrunak et al. report (42) are particularly important: (i) the two different protein conformations for binding the (R) and (S) enantiomers of orteronel, and (ii) extra binding space near the F/G helix region only in the case of the (S)-orteronel–P450 17A1 complex. The finding of multiple (or at least two) conformations of ligand-bound P450 17A1 is consistent with the complexity of some of our kinetic data (Fig. 12). Another point is that if there is a second peripheral ligand-binding site in P450 17A1, then it may be possible to have an induced fit component (63) instead of, or in addition to, a conformational selection model (Fig. 13) for P450 17A1. In principle, the second site of ligand binding could possibly be involved in generating reaction-specific inhibition with drugs targeting P450 17A1.

An alternative consideration for achieving reaction-specific inhibition of P450 17A1 is targeting residues that bind b_5 (needed for efficient 17α-OH pregnenolone lyase activity) (Fig. 2B and Table 1). An issue here is that one would need to identify (b_5 binding) sites that are not needed to bind NADPH-P450 reductase (22). One approach would be developing drugs to bind residues (e.g. Glu-305, Arg-347, Arg-358, and Arg-449) where natural mutations attenuate lyase activity but not 17α-hydroxylation activity (6, 64). Arg-347, Arg-358, and Arg-449 were identified as being involved in the interaction of P450 17A1 with b_5 using NMR spectroscopy (22), and Glu-305 is located in the canonical active site (6).

In conclusion, we have analyzed several kinetic aspects of catalysis by human P450 17A1, particularly the coupling of the two major steps, 17α-hydroxylation and the lyase step, with both the substrates progesterone and pregnenolone. b_5 has an ancillary role in several reactions, and some possible roles were ruled out (e.g. enhancing binding of 17α-OH steroids). The individual sequential reactions are inherently distributive but more processive in the case of pregnenolone than with progesterone (e.g. Fig. 6, B and F, lags). There is some processivity, especially with pregnenolone, as demonstrated by the pulse-chase experiments (Fig. 7). Our kinetic model has 17α-OH pregnenolone undergo a 1:6 partitioning between proceeding where natural mutations attenuate lyase activity but not 17α-OH pregnenolone being available for exchange (to allow orteronel to enter the active site). In considering the hypothetical scheme presented in Fig. 13, it is conceivable that one or more conformational form(s) of the enzyme might be in a “processive” mode, whereas others are in a “distributive” mode, and the composite would be the results we observe here. The results have relevance for the development of prostate cancer treatment drugs that selectively inhibit the individual reactions.

Experimental procedures

Reagents

Most of the steroids were obtained from either Sigma or Steraloids (Wilton, NH). DHEA was purchased from Waterstonetech (Carmel, IN). [7-3H]Pregnenolone was purchased from PerkinElmer Life Sciences (catalogue number NET039001MC). [4-14C]Progesterone (ARC1398) and 17α-OH-[1,2,6,7-3H]progesterone (ART 0638) were purchased from American Radiolabeled Chemicals (St. Louis, MO). The (S)-orteronel enantiomer was a generous gift of Millennium Pharmaceuticals (Cambridge, MA).

Radiolabeled 17α-OH pregnenolone was prepared by enzyme-mediated conversion of [7-3H]pregnenolone (65), with modification. 17α-OH-[7-3H]pregnenolone was obtained from a 30-s incubation with the enzyme system described below, devoid of b_5. The radiolabeled steroid was purified by the chromatographic method used for 3H-17α-OH pregnenolone assays, and fractions were collected (with the β-RAM system only used to locate the t_0 of the product, disconnected for the preparative mode). The steroid was extracted from the mobile phase with CH2Cl2, the organic (lower) layer was transferred to a new vessel, and the solvent was evaporated under a nitrogen stream. The dried extract was dissolved in C2H5OH, and co-elution with commercial 17α-OH pregnenolone was used to further validate the identity of the radiolabeled compound.

Purification of (R)-orteronel

A racemic orteronel mixture was purchased from ApexBio (Houston, TX) (catalogue number A4326), and the compound was dissolved in CH2OH for purification of the enantiomers. The (S) and (R) enantiomers were resolved on a Chiralel® OJ-RH column (4.6 × 150 mm) with an isotropic 63.5% CH3OH, 36.5% H2O mobile phase (v/v), with baseline resolution. The two enantiomeric fractions with absorbance peaks at 238 nm were collected (supplemental Fig. S2). The (S) and (R) fractions were identified using a standard (S)-orteronel sample, which eluted first. (R)-Orteronel was extracted from the aqueous solution with CH2Cl2 and the solvent was evaporated in vacuo. The dried solid was stored at −20 °C until further use. The two enantiomers had identical UV, NMR, and mass spectra, except for a slight difference in the chemical shifts attributed to the solvent (CD2OH) (supplemental Fig. S2). UV (H2O) ε_{238} 98 mm^{-1} cm^{-1}; ε_{273} 13 mm^{-1} cm^{-1}; NMR (400 MHz, CD2OH) δ 2.98 (s, 3H, CH3), 3.10 (m, 4H, pyrroolidinol methylenes), 7.65–8.35 (m, 8H, naphthyl and imidazole ring protons) (solvent impurities at δ 1.19 and 3.6, C8H12OH; δ 1.25, hexanes; δ 3.31 CH3OH; δ 4.87, H2O); high resolution mass spectrum,
calculated for C_{18}H_{18}N_{3}O_{2}^{+} (MH^+) 308.1394, (R): m/z 308.1399 (Δ 1.6 ppm), (S): m/z 308.1398 (Δ 1.3 ppm), fragmentation of either yielded dominant ions at m/z 280 (loss of 18, i.e. loss of H2O) and m/z 186 (naphthyl methylcarboxamide, loss of pyrrolidinolpyrrole entity); optical rotation (S): [α]_{24}° +96 ± 6 (n = 3 readings) (c 0.03, CH3OH) (compare with +83.8 (61)) (sufficient (R)-orteronol was not obtained to accurately measure [α]).

**Enzymes**

_Escherichia coli_ recombinant human P450 17A1 was prepared as described (16). _E. coli_ recombinant rat NADPH-P450 reductase and human b5 were prepared as described by Hanna et al. (66) and Guengerich (67), respectively. Cholesterol oxidase (from _Streptomyces_ sp.) was purchased from Sigma (catalogue number C8649).

**Catalytic assays**

_Steady-state incubations_—The steady-state catalytic assays were generally conducted using a reconstituted enzyme system in a final reaction volume of 0.5 ml. The reaction mixtures typically contained 0.01—0.5 μM human P450 17A1, 2 μM rat NADPH-P450 reductase, 0.5 μM human b5 (when included), and 16 μM 1α,2α-dilauroyl-sn-glycero-3-phosphocholine (DLPC) (added as lipid vesicles after sonication of a 1 mg/ml aqueous stock) in 50 mM potassium phosphate buffer (pH 7.4). The P450 17A1 enzyme concentrations used were as follows: 0.01 μM for incubations with pregnenolone and 17α-OH pregnenolone; 0.01 and 0.5 μM for progesterone 17α-hydroxylation and 16α-hydroxylation (respectively); and 0.5 μM in 17α-OH progesterone lyase assays. Substrate was added at concentrations ranging from 0.2 to 20 μM. Duplicate samples were prewarmed at 37 °C for 5 min (water bath with shaking), and reactions were initiated with the addition of an NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mM NADP, and DHEA, respectively). The reaction products were resolved in a final reaction volume of 0.5 ml. The reaction mixtures typically contained 0.01—0.5 μM human P450 17A1, 2 μM rat NADPH-P450 reductase, 4 μM human b5 (when noted), and 32 μM DLPC (lipid vesicles) in 100 mM potassium phosphate buffer (pH 7.4). The following radiolabeled substrates were used in these studies: [3H]pregnenolone (103 μCi/μmol); [3H]17α-OH pregnenolone (700 μCi/μmol); [14C]progesterone (60 μCi/μmol); and [3H]17α-OH progesterone (103 μCi/μmol). The reactions were initiated by rapid mixing with 1 mM NADPH in 50 mM potassium phosphate buffer (7.4) in the first reaction chamber. The samples were incubated from 0.5 to 30 s, at which time the reactions were quenched with HCl (1 M) and the sample chamber was held at 4 °C. The reaction products were identified by co-elution with commercial standards and quantified by the radiochromatogram peak areas using the β-RAM software (Laura™, LabLogic Systems, Brandon, FL).

_Pulse–chase assays_—Pulse–chase experiments were performed to follow the two-step conversion of [3H]pregnenolone to [3H]DHEA under steady-state and single-turnover conditions. The reconstituted enzyme/substrate mixture used in the steady-state pulse–chase assays was the same as for the protocol described for steady-state incubations, with the following alterations: (i) 0.5 μM human P450 17A1, and (ii) 50 μM [3H]pregnenolone (13 μCi/μmol). One minute after the reactions were started with the NADPH-generating system, unlabeled 17α-OH pregnenolone was added to the mixtures, at varying concentrations (5–75 μM (final concentration) and

---

_Human P450 17A1 kinetics_

_J. Biol. Chem. (2017) 292(32) 13168 –13185_ 13181
Vehicle), and the incubations continued for 10 min before quenching with HCl (0.67 mM final concentration). The mixtures were centrifuged to clear precipitated protein, and the steroid products were resolved and quantified by the HPLC methods employed in the single-turnover experiments (see above), with the following linear gradients: 0–9 min, 75% B; 13.4 min, 86% B; 13.5–15 min, 90% B; 16–25 min, 75% B (all v/v).

The pulse–chase assays done with single-turnover conditions followed a protocol similar to that outlined above, with some differences. The standard single-turnover procedure included one reaction phase, whereas a pulse–chase experiment requires two incubation periods. This was achieved in the rapid quench instrument by utilizing the second reaction chamber for incubating with a chase compound, with the quenching agent placed in the collection vial. The reagent concentrations were increased so that, after two dilutions, the working concentrations in the chase step (second phase) matched those from the single-turnover incubations. The specific activity of [3H]pregnenolone was adjusted to 830 μCi/μmol, and 80 μM unlabeled 17α-OH pregnenolone was used as the chase compound. The pulse length was varied between 0.05 and 2 s, and the chase continued for 5 s before the reaction was quenched in the collection vessel with HCl (0.6 M final concentration). The mixtures were centrifuged to clear precipitated protein, and the steroid products were resolved and quantitated by the method described for the steady-state pulse–chase assays.

Orteronel inhibition—Competitive inhibition studies with the orteronel enantiomers were performed and analyzed by the methods described for steady-state assays. The inhibitors were added to the enzyme substrate mixture with the final composition as follows: 0.005–100 μM (S)- or (R)-oteronel, 5 μM substrate, 0.01 μM human P450 17A1 (0.1 μM in assays with 17α-OH progesterone), 0.5 μM human b5, 2 μM NADPH-P450 reductase, and 16 μM DLPC (lipid vesicles) in 50 mM potassium phosphate buffer (pH 7.4). To resolve an inconsistent migration profile, the mobile phase solvents were changed to 5 mM NH4CH3CO2, 70% CH3OH, 30% H2O or A and 5 mM NH4CH3CO2, 90% CH3CN, 10% H2O for B (all v/v). IC50 values were estimated in GraphPad Prism using the Onesite-Fit binding formula to estimate binding constants (Kd) using GraphPad Prism, with the equation $Y = B + (A/2)(1/E)((K_d + E + X) - ((K_d + E + X)^2 - (4EX))^{1/2}$.

Inhibitor trapping—Spectral enzyme–inhibitor trapping assays were used to measure dissociation rates for the steroid ligands. The analysis is dependent upon a faster inhibitor binding rate versus the dissociation rates for the steroids. The experiments were performed at ambient temperature using the stopped-flow apparatus described above and (S)-oteronel as the trapping inhibitor. The on-rate for (S)-oteronel was first measured by mixing 4 μM human P450 17A1 in 100 mM potassium phosphate buffer (pH 7.4) (with 120 mM DLPC (lipid vesicles)) and 20 μM (S)-oteronel (in the same buffer). The rate was estimated by singular value decomposition (SVD) analysis of the experimental data matrix with the following parameters: absorbance, measured value; wavelength, 350–500 nm (200 points); and time, 0–1 s (one scan/ms). The data were fit with a two-species sequential model (simple first-order), and the calculated rates from at least three replicates were averaged. To estimate the dissociation rates (koff), the steroid ligands (4 μM) and human b5 (4 μM, when included) were added to the enzyme solution. The time component was 0–10 s (averaged mode, 62 scans/s), and data were analyzed as stated.

Kinetic analysis—Steroid association rate (kass) parameters were calculated from the corresponding dissociation rates (koff), estimated from the inhibitor trapping assays, and the dissociation constants (Kd) were derived from the steroid binding experiments, using Equation 1,

$$K_d = k_{off}/k_{on} \quad (Eq. 1)$$

The experimental data from the catalytic assays, under single-turnover and steady-state conditions, and steroid titrations were concurrently fit to a minimal kinetic model using KinTek Explorer® software (KinTek, Snow Shoe, PA). The predetermined rate parameters (kass, koff, and Kd) and single-turnover substrate conversion rates were used as starting values in the model, and in some cases, the values were fixed. The limits for the molar extinction coefficients that were applied to the spectral binding experiments were derived from the prior fitting with a quadratic formula in GraphPad Prism (see above). The imported data sets included the averaged data points with standard deviation (when applicable).
To accurately fit the ligand titration and catalytic experiments in a single model, a “kinetically silent” second-order activation step was added to prevent the software from regarding the binding assay as a catalytic experiment. The activation step corresponds to the combined P450 17A1 reaction steps that lead to the chemically competent iron–oxygen species (i.e., reduction by NADPH-P450 reductase, binding of molecular oxygen, protonation, and loss of water), which were not evaluated. The concentration of the “activating reagent” was fixed at 1 μM, and a $K_{eq}$ of 1 μM was set with $k_{\text{forward}} = 100 \mu M^{-1} s^{-1}$ ($10^8 M^{-1} s^{-1}$) and $k_{\text{reverse}} = 100 s^{-1}$, with the intention of forcing the step to be rapidly reversible to prevent any kinetic influence on the other parameters. Additionally, the reversibility accounted for uncoupling (loss of iron–oxygen species to reactive oxygen species or $H_2O$, resulting in $Fe^{3+}$) that can occur during compound I (or ferric peroxide) formation. Any rate limitations derived from uncoupling were considered to be factored into the rate of the chemical reaction step. Simulations without the activation step produced similar results with the same constraints.

Steroid binding kinetics was evaluated by applying different enzyme–ligand binding models to the kinetic data obtained in stopped-flow binding assays (see under “Ligand binding”) using KinTek Explorer® software. The data were reduced by taking the difference of the kinetic traces (~7 s trace, 1 scan/ms) at the wavelengths previously designated for $A_{\text{max}}$ and $A_{\text{min}}$ (e.g., $A_{391}$ and $A_{426}$). Kinetic traces were subtracted for pregnenolone and $H_2O$, resulting in $Fe^{3+}$, that can occur during compound I (or ferric peroxide) formation. Any rate limitations derived from uncoupling were considered to be factored into the rate of the chemical reaction step. Simulations without the activation step produced similar results with the same constraints.

**Author contributions**—E. G. purified the enzymes, did the kinetic analyses, analyzed the data, and did the model fitting. F. P. G. conceived the experimental plan, participated in some of the stopped-flow kinetic studies, analyzed the data, and reviewed all results. E. G. and F. P. G. both wrote the manuscript.

**Acknowledgments**—We thank M. V. Martin, L. M. Folkmann, L. D. Nagy, and T. T. N. Phan for preparing NADPH-P450 reductase and $b_5$ and K. Trisler for assistance in preparation of the manuscript. We also thank T. T. N. Phan and K. M. Johnson for acquiring spectra of the orteronel enantiomers and P. L. Polavarapu for the use of the polarimeter.

**References**

1. Rendic, S., and Guengerich, F. P. (2015) Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem. Res. Toxicol.* 28, 38–42

2. Auchus, R. J., and Miller, W. L. (2015) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed) 4th Ed., pp. 851–879, Springer, New York

3. Guengerich, F. P. (2015) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed) 4th Ed., pp. 523–785, Springer, New York

4. Auchus, R. J. (2017) Steroid 17-hydroxylase and 17,20-lyase deficiencies, genetic and pharmacologic. *J. Steroid Biochem. Mol. Biol.* 165, 71–78

5. Guengerich, F. P. (2017) Intersection of roles of cytochrome P450 enzymes with xenobiotic and endogenous substrates. Relevance to toxicity and drug interactions. *Chem. Res. Toxicol.* 30, 2–12

6. DeVore, N. M., and Scott, E. E. (2012) Structures of cytochrome P450 17A1 with prostate cancer drugs abiraterone and TOK-001. *Nature* 482, 116–119

7. Nakajin, S., Shively, J. E., Yuan, P. M., and Hall, P. F. (1981) Microsomal cytochrome P-450 from neonatal pig testis: two enzymatic activities (17α-hydroxylase and C17,20-lyase) associated with one protein. *Biochemistry* 20, 4037–4042

8. Zuber, M. X., Simpson, E. R., and Waterman, M. R. (1986) Expression of bovine 17α-hydroxylase cytochrome P-450 cDNA in nonsteroidogenic (COS-1) cells. *Science* 234, 1258–1261

9. Chung, B. C., Picado-Leonard, J., Hanu, M., Bienkowski, M., Hall, P. F., Shively, J. E., and Miller, W. L. (1987) Cytochrome P450c17 (steroid 17α-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proc. Natl. Acad. Sci. U.S.A.* 84, 407–411

10. Katagiri, M., Suhara, K., Shiroo, M., and Fujimura, Y. (1982) Role of cytochrome $b_5$ in the cytochrome P-450-mediated C21-stereoid 17,20-lyase reaction. *Biochem. Biophys. Res. Commun.* 108, 379–384

11. Katagiri, M., Kagawa, N., and Waterman, M. R. (1995) The role of cytochrome $b_5$ in the biosynthesis of androgens by human P450c17. *Arch. Biochem. Biophys.* 317, 343–347

12. Lee-Robichaud, P., Wright, J. N., Akhtar, M. E., and Akhtar, M. (1995) Modulation of the activity of human 17α-hydroxylase-17,20-lyase (CYP17) by cytochrome $b_5$: endocrinological and mechanistic implications. *Biochem. J.* 308, 901–908

13. Estrada, D. F., Skinner, A. L., Laurence, J. S., and Scott, E. E. (2014) Human cytochrome P450 17A1 conformational selection: modulation by ligand and cytochrome $b_5$. *J. Biol. Chem.* 289, 14310–14320

14. Yoshimoto, F. K., Zhou, Y., Peng, H. M., Stidd, D., Yoshimoto, J. A., Sharma, K. K., Matthew, S., and Auchus, R. J. (2012) Minor activities and transition state properties of the human steroid hydroxylases cytochromes P450c17 and P450c21, from reactions observed with deuterium-labeled substrates. *Biochemistry* 51, 7064–7077

15. Yoshimoto, F. K., and Auchus, R. J. (2015) The diverse chemistry of cytochrome P450 17A1 (P450c17, CYP17A1). *J. Steroid Biochem. Mol. Biol.* 151, 52–65

16. Yoshimoto, F. K., Gonzalez, E., Auchus, R. J., and Guengerich, F. P. (2016) Mechanism of 17α,20-lyase and new hydroxylation reactions of human cytochrome P450 17A1. $^{18}$O labeling and oxygen surrogate evidence for a role of a peroxynitrite oxygen. *J. Biol. Chem.* 291, 17143–17164

17. Turcu, A. F., Rege, I., Chomic, R., Liu, J., Nishimoto, H. K., Else, T., Moraits, A. G., Palapattu, G. S., Rainey, W. E., and Auchus, R. J. (2015) Profiles of 21-carbon steroids in 21-hydroxylation deficiency. *J. Clin. Endocrinol. Metab.* 100, 2283–2290

18. Estabrook, R. W., Hildebrandt, A. G., Baron, J., Netter, K. J., and Leibman, K. (1971) A new spectral intermediate associated with cytochrome P-450 in liver microsomes. *Biochem. Biophys. Res. Commun.* 42, 132–139

19. Hildebrandt, A., and Estabrook, R. W. (1971) Evidence for the participation of cytochrome $b_5$ in hepatic microsomal mixed-function oxidation reactions. *Arch. Biochem. Biophys.* 143, 66–79

20. Onoda, M., and Hall, P. F. (1982) Cytochrome $b_5$ stimulates purified testicular microsomal cytochrome P-450 (C21 side-chain cleavage). *Biochem. Biophys. Res. Commun.* 108, 454–460

21. Kominami, S., Ogawa, N., Morimune, R., De-Ying, H., and Takemori, S. (1992) The role of cytochrome $b_5$ in adrenal microsomal steroidogenesis. *J. Steroid Biochem. Mol. Biol.* 42, 57–64

22. Estrada, D. F., Laurence, J. S., and Scott, E. E. (2013) Substrate-modulated cytochrome P450 17A1 and cytochrome $b_5$ interactions revealed by NMR. *J. Biol. Chem.* 288, 17008–17018

23. Auchus, R. J., Lee, T. C., and Miller, W. L. (1998) Cytochrome $b_5$ augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *J. Biol. Chem.* 273, 3158–3165
Human P450 17A1 kinetics

24. Lee-Robichaud, P., Akhtar, M. E., and Akhtar, M. (1998) Control of androgen biosynthesis in the human through the interaction of Arg347 and Arg358 of CYP17 with cytochrome b5. Biochem. J. 332, 293–296

25. Lee-Robichaud, P., Shyadehi, A. Z., Wright, J. N., Akhtar, M. E., and Akhtar, M. (1995) Mechanistic kinship between hydroxylation and desaturation reactions: acyl-carbon bond cleavage promoted by pig and human CYP17 (P-450c17, 17α-hydroxylase-17,20-lyase). Biochemistry 34, 14104–14113

26. Akhtar, M., Wright, J. N., and Lee-Robichaud, P. (2011) A review of mechanistic studies on aromatase (CYP19) and 17α-hydroxylase-17,20-lyase (CYP17). J. Steroid Biochem. Mol. Biol. 125, 2–12

27. Mak, P. J., Gregory, M. C., Denisov, I. G., Sligar, S. G., and Kincaid, J. R. (2011) Evidence that the cytochrome P450 17A1 (CYP17) active site proton delivery and the lyase activity of human cytochrome P450 17A1 by increasing the coupling of NADPH consumption to androgen production. Biochemistry 55, 4356–4365

28. Duggal, R., Liu, Y., Gregory, M. C., Denisov, I. G., Kincaid, J. R., and Sligar, S. G. (2016) Evidence that cytochrome b5 acts as a redox donor in CYP17A1 mediated androgen synthesis. Biochem. Biophys. Res. Commun. 477, 202–208

29. Schenken, J. B., Remmer, H., and Estabrook, R. W. (1967) Spectral studies of drug interaction with hepatic microsomal cytochrome P-450. Mol. Pharmacol. 3, 113–123

30. Fesht, A. (1999) Structure and Mechanism in Protein Science. pp. 158–161, W. H. Freeman & Co., New York

31. McLaughlin, I. A., Ronseaux, S., Finn, R. D., Henderson, C. J., and Roland Wolf, C. (2010) Deletion of microsomal cytochrome b5 profoundly affects hepatic and extrapечен drug metabolism. Mol. Pharmacol. 78, 269–278

32. Kok, R. C., Timmerman, M. A., Wolfetten, L. K. P., Drop, S. L., and de Jong, F. H. (2010) Isolated 17,20-lyase deficiency due to the cytochrome b5 mutation W27X. J. Clin. Endocrinol. Metab. 95, 994–999

33. Idkowiak, J., Randell, T., Dhir, V., Patel, P., Shackleton, C. H., Taylor, N. F., Denisov, I. G., and Sligar, S. G. (2016) Structural and kinetic basis of steroid 17α,20-lyase activity in teledost fish cytochrome P450 17A1 and its absence in cytochrome P450 17A2. J. Biol. Chem. 290, 3248–3268

34. Bonomo, S., Hansen, C. H., Petrunak, E. M., Scott, E. E., Styrishave, B., Jørgensen, F. S., and Olsen, L. (2016) Promising tools in prostate cancer research: selective non-steroidal cytochrome P450 17A1 inhibitors. Sci. Rep. 6, 29468

35. Tin, L., and Hu, Q. (2014) CYP17 inhibitors—arboriterone, C17,20-lyase inhibitors and multi-targeting agents. Nat. Rev. Urol. 11, 32–42

36. Toren, P. J., Kim, S., Pham, S., Mangali, A., Adomat, H., Guns, E. S., Zoubedi, A., Moore, W. E., and Gleave, M. E. (2015) Anticancer activity of a novel selective CYP17A1 inhibitor in preclinical models of castrate-resistant prostate cancer. Mol. Cancer Ther. 14, 59–69

37. Nuñez, V. C., and Brodie, A. M. (2015) Discovery and development of galeterone (TOK-001 or VN/124–1) for the treatment of all stages of prostate cancer. J. Med. Chem. 58, 2077–2087

38. Hara, T., Kouno, J., Kaku, T., Takeuchi, T., Kusaka, M., Tasaka, A., and Yamaoka, M. (2013) Effect of a novel 17,20-lyase inhibitor, orteronel (TAK-700), on androgen synthesis in male rats. J. Steroid Biochem. Mol. Biol. 134, 80–81

39. Rafferty, S. W., Eisner, J. R., Moore, W. R., Schotzinger, R. J., and Hoekstra, W. J. (2014) Highly-selective 4-(1,2,3-triazole)-based P450c17a 17,20-lyase inhibitors. Bioorg. Med. Chem. Lett. 24, 2444–2447

40. Petrunak, E. M., Rogers, S. A., Aube, J., and Scott, E. E. (2017) Structural and functional evaluation of clinically-relevant inhibitors of steroidogenic cytochrome P450 17A1 (CYP17A1). Drug Metab. Dispos. 45, 635–645

41. Yadav, R., Petrunak, E. M., Estrada, D. F., and Scott, E. E. (2017) Structural insights into the function of steroidogenic cytochrome P450 17A1. Mol. Cell Endocrinol. 441, 68–75

42. Petrunak, E. M., DeVore, N. M., Porubsky, P. R., and Scott, E. E. (2014) Structures of human steroidogenic cytochrome P450 17A1 with substrates. J. Biol. Chem. 289, 32952–32964

43. Gregory, M. C., Denisov, I. G., Grinkova, Y. V., Khatri, Y., and Sligar, S. G. (2013) Kinetic solvent isotope effect in human P450 CYP17A1-mediated androgen formation: evidence for a reactive peroxoamine intermediate. J. Am. Chem. Soc. 135, 16245–16247

44. Khatri, Y., Gregory, M. C., Grinkova, Y. V., Denisov, I. G., and Sligar, S. G. (2014) Active site proton delivery and the lyase activity of human cytochrome P450 17A1 by increasing the coupling of NADPH consumption to androgen production. Biochemistry 55, 4356–4365

45. Higuchi, A., Kominami, S., and Takemori, S. (1991) Kinetic control of androgen biosynthesis in the human through the interaction of Arg347 and Arg358 of CYP17 with cytochrome b5. Biochem. J. 332, 293–296

46. Mak, P. J., Gregory, M. C., Denisov, I. G., Sligar, S. G., and Kincaid, J. R. (2015) Unveiling the crucial intermediates in androgen production. Proc. Natl. Acad. Sci. U.S.A. 112, 15856–15861

47. Soucy, P., and Luu-The, V. (2000) Conversion of pregnenolone to DHEA by human 17α-hydroxylase/17,20-lyase (P450c17). Evidence that DHEA is produced from the released intermediate, 17α-hydroxyprogrenenolone. Eur. J. Biochem. 267, 3243–3247

48. Pallan, P. S., Nagy, L. D., Lei, L., Gonzalez, E., Kramlinger, V. M., Azumaya, C. M., Wawrzak, Z., Waterman, M. R., Guengerich, F. P., and Egli, M. (2015) Structural and kinetic basis of steroid 17α,20-lyase activity in teleost fish cytochrome P450 17A1 and its absence in cytochrome P450 17A2. J. Biol. Chem. 290, 3248–3268

49. Tagashira, H., Kominami, S., and Takemori, S. (1995) Kinetic studies of cytochrome P450 c17α,20β dependent androstenedione formation from progesterone. Biochemistry 34, 10939–10945

50. Yamazaki, T., Ohno, T., Sakaki, T., Akiyoshi-Shibata, M., Yabusaki, Y., Imai, T., and Kominami, S. (1998) Kinetic analysis of successive reactions catalyzed by bovine cytochrome P450 c17α,20β. Biochemistry 37, 2800–2806
utility in the treatment of prostate cancer. *Bioorg. Med. Chem.* **19**, 6383–6399

62. Yamaoka, M., Hara, T., Hitaka, T., Kaku, T., Takeuchi, T., Takahashi, J., Asahi, S., Miki, H., Tasaka, A., and Kusaka, M. (2012) Orteronel (TAK-700), a novel non-steroidal 17,20-lyase inhibitor: effects on steroid synthesis in human and monkey adrenal cells and serum steroid levels in cynomolgus monkeys. *J. Steroid Biochem. Mol. Biol.* **129**, 115–128

63. Koshland, D. E., Jr., Némethy, G., and Filmer, D. (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* **5**, 365–385

64. Geller, D. H., Auchus, R. J., Mendonça, B. B., and Miller, W. L. (1997) The genetic and functional basis of isolated 17,20-lyase deficiency. *Nat. Genet.* **17**, 201–205

65. Peng, H.-M., and Auchus, R. J. (2013) The action of cytochrome b$_5$ on CYP2E1 and CYP2C19 activities requires anionic residues D58 and D65. *Biochemistry* **52**, 210–220

66. Hanna, I. H., Teiber, J. F., Kokones, K. L., and Hollenberg, P. F. (1998) Role of the alanine at position 363 of cytochrome P450 2B2 in influencing the NADPH- and hydroperoxide-supported activities. *Arch. Biochem. Biophys.* **350**, 324–332

67. Guengerich, F. P. (2005) Reduction of cytochrome b$_5$ by NADPH-cytochrome P450 reductase. *Arch. Biochem. Biophys.* **440**, 204–211

68. Guengerich, F. P. (2014) in *Hayes’ Principles and Methods of Toxicology* (Hayes, A. W., and Kruger, C. L., eds) 6th Ed., pp. 1905–1964, CRC Press-Taylor & Francis, Boca Raton, FL