

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

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ABSTRACT

Cytochrome P450 (P450, CYP) 17A1 plays a critical role in steroid metabolism, catalyzing both the 17 α -hydroxylation of pregnenolone and progesterone and the subsequent 17 α ,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 17 α -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 for DHEA from pregnenolone, indicating a distributive character of the enzyme. However, we observed processivity in pregnenolone/DHEA pulse-chase experiments. (*S*)-Orteronel was 3 times more inhibitory toward the conversion of 17 α -hydroxypregnenolone to DHEA than toward the 17 α -hydroxylation of pregnenolone. IC₅₀ values for (*S*)-orteronel were identical for blocking DHEA formation from pregnenolone and for 17 α -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 17 α -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.

The cytochrome P450 (P450 or CYP³) 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, 51A1, plus some others involved in catabolism) and vitamins (human P450s 2R1, 24A1, 26A1, 26B1, 26C1, 27A1, 27B1, 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 17 α -OH

progesterone to androstenedione (Andro) and also the oxidation of pregnenolone to 17 α -OH pregnenolone to dehydroepiandrosterone (DHEA) (Fig. 1A). 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 16 α -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 17 α -OH progesterone lyase activity of purified porcine testicular P450 17A1 3- to 4-fold (10, 20). Kominami *et al.* (21) also showed stimulation of guinea pig P450 17A1 by bovine b_5 , both the 17 α -hydroxylation of progesterone and, to a fractionally larger extent, the 17 α -OH progesterone lyase reaction. However, b_5 was inhibitory at a b_5 :P450 17A1 ratio > 0.5 (21). In 1995 Katagiri *et al.* (11) reported that human b_5 (added in a concentration equimolar to P450) stimulated several catalytic activities of human P450 17A1. Progesterone 17 α -hydroxylation was not stimulated by b_5 but pregnenolone 17 α -hydroxylation was enhanced 2.5-fold; lyase activities toward both 17 α -OH progesterone and 17 α -OH pregnenolone were enhanced ~ 10-fold by b_5 (11). No qualitative difference was observed in the binding of 17 α -OH pregnenolone 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 the Waterman group (11). The catalytic efficiency of 17 α -hydroxylation was increased 2-fold for pregnenolone but not for progesterone, as in the report of the Waterman group (11). However, no lyase activity with either 17 α -OH progesterone or 17 α -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 peroxide (FeO_2^-) or Compound I (perferryl oxygen; 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 17 α -OH steroid product remains bound to the enzyme for the succeeding lyase reaction, but in a *distributive* reaction the 17 α -OH product dissociates and then re-binds 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 17 α -OH progesterone and 17 α -OH pregnenolone (formed following 2-electron reduction of 17 α -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 selective inhibition of the androgen-producing lyase reaction, preserving the ability to generate 17 α -OH steroids as precursors for biosynthesis of glucocorticosteroids and avoiding adverse effects caused by insufficient glucocorticoid and excess

mineralocorticoid production. Some selectivity for lyase > 17 α -hydroxylation activity has been reported for abiraterone (36), orteronel (Takeda TAK-700) (40), the secosteroid (*S*)-seviteronel (VT-464) (41), and two other non-steroidal pyridine derivatives (36). X-ray crystal structures of human P450 bound to abiraterone (6) and to both enantiomers of orteronel (42) have been published by Scott and co-workers. A completely processive reaction should not have differential sensitivities to a single inhibitor. Even in a dissociative mechanism, the question arises as to how a drug can occupy a single site on the enzyme and have different K_i values for two reactions (42, 43).

For the above reasons, we examined the kinetics of binding of human P450 17A1 to its primary physiological substrates and products and also an enantiomer of orteronel. We also utilized pre-steady-state reaction kinetics and global data analysis to develop 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, 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 17 α -OH pregnenolone to DHEA confounds the results for formation of 17 α -OH pregnenolone from pregnenolone (Fig. 2A), but the lyase reaction (using 17 α -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 17 α -OH pregnenolone in the absence of b_5 (Figs. 2A, 2B) nor was very much Andro formed from progesterone or 17 α -OH

progesterone (Figs. 2C, 2D). 16 α -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_d values we estimated in preliminary assays, which were done using the "Type I" Soret spectral shifts (increase in absorbance at 390 nm, 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 seconds after additions and used these results, utilizing a quadratic equation for fitting. The apparent K_d values for pregnenolone, progesterone, 17 α -OH progesterone, and 17 α -OH pregnenolone were all sub-micromolar (Fig. 3). The K_d value for DHEA was 1.7 μ M and that for Andro was $\sim 19 \mu$ M, although the K_d 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 decreased dissociation of 17 α -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, 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*)-orteronel is 40 nM (42), which we confirmed (data not presented)). With 10 μ M (*S*)-orteronel, the apparent on-rate was 6.1 s⁻¹ (Fig. 4). Mixing of equimolar concentrations (2 μ M) of P450 17A1 and each steroid with 10 μ M (*S*)-orteronel provided a means of estimating k_{off} values, in that these were 1-2 orders of magnitude slower. Spectral data and analysis for 17 α -OH pregnenolone (as an example) are presented in Fig. 5 (k_{off} 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_{off} rates for all steroid substrates and products

(Table 2). In no case did b_5 substantially affect k_{off} values.

Time courses of individual P450 17A1 reactions

P450 17A1 reactions were initiated with equal concentrations (2 μM) 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 in order 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 Data Fig. S1).

All reactions were stimulated by the presence of b_5 , with the exception of the rate of progesterone disappearance (Figs. 6E, 6F). As expected, all secondary products showed lags in formation, including the lyase products DHEA and Andro (Figs. 6B, 6F).

Pulse-chase assays with pregnenolone oxidation to DHEA

The lag phases for formation of lyase products (DHEA from pregnenolone, Andro from progesterone) (Figs. 6B, 6F) 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 seconds, Fig. 2F) than progesterone (no Andro appearing until 5 seconds, Fig. 2B), and we examined this reaction further utilizing pulse-chase assays (Fig. 7).

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

In pre-steady-state reactions, patterned after the experiment in Fig. 6B, a chase of 80 μM unlabeled $17\alpha\text{-OH}$ pregnenolone was added to the reaction at varying times after the reaction started, and the reaction proceeded for another 5 seconds

(Fig. 7B). Even when the chase (unlabeled) $17\alpha\text{-OH}$ pregnenolone was added as early as 50 milliseconds, the production of [^3H]-DHEA was only blocked by \sim one-third. Collectively these results indicate a partially processive mechanism (Fig. 7), which is more pronounced with pregnenolone oxidation to DHEA than for progesterone conversion to Andro (Figs. 6B, 6F)).

Inhibition of P450 17A1 reactions by orteronel enantiomers

One of the goals in prostate cancer chemotherapy is attenuation of androgen production by selective inhibition of the lyase reaction without disturbing the formation of $17\alpha\text{-OH}$ products, which are necessary for the formation of other steroids (2).

The (S) enantiomer of orteronel (Supplemental Data, Fig. S2) was more inhibitory than the (R) enantiomer in all reactions (Fig. 8). In the case of progesterone, both enantiomers were slightly more inhibitory for the lyase reaction than $17\alpha\text{-hydroxylation}$ (Table 4). In the reactions with pregnenolone, the IC_{50} value for the lyase reaction was \sim one-third that for $17\alpha\text{-hydroxylation}$. When the overall conversion of pregnenolone to DHEA was analyzed (Fig. 8B), the IC_{50} values (for both enantiomers) were similar to those measured for $17\alpha\text{-hydroxylation}$ (Fig. 8A).

Development of kinetic models

For initial model development we used the measured k_{off} and K_d values to estimate apparent k_{on} rates (Table 5). The k_{on} rates were not affected by the presence of b_5 (Supplemental Data, Fig. S3)). We also measured some k_{on} rates directly but these are complex and will be considered separately (see below, Fig. 11). Only the systems containing b_5 were considered. The oxidation step rates are those measured in the single-turnover reactions presented in Figs. 6B, 6D, 6F, and 6G.

Using the simplified model and values listed in Fig. 9, it was possible to fit the data using KinTek Explorer® software (Fig. 10) for single-turnover (Fig. 10A, 10B), steady-state (Fig. 10C, 10D), and binding reactions (Fig. 10E, 10F, 10G). 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, 17 α -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_d values (Table 5). In addition, the estimated k_{on} values are all $< 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and therefore probably not diffusion-limited (50).

An alternate 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 X-ray crystallography and spectroscopy work of Scott, Laurence, and their colleagues (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, 17 α -OH pregnenolone, and DHEA to P450 17A1 (Fig. 12).

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 paper 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 17 α -OH steroid products dissociating from the enzyme. However, there is some processivity, particularly in the case of pregnenolone, i.e., part of the 17 α -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 17 α -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 17A work (34), the first of any with P450

17A1 to consider the role of b_5 in a processivity study, which is critical (Figs. 2, 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 17 α - (and 16 α -) hydroxylation and lyase reactions, plus diene formation (Fig. 6B). b_5 did not affect the affinity of P450 17A1 for 17 α -OH steroids (Fig. 3, 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, Table 1). Lee-Robichaud *et al.* (12) reported no detectable lyase activity with either 17 α -OH pregnenolone or 17 α -OH progesterone in the absence of b_5 , but both we (Figs. 2, 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 17A1 varies among species. With zebrafish P450 17A1, the stimulations by (zebrafish) b_5 were only ~ two-fold (34). Mice in which b_5 was globally deleted were still viable and reproduced, even though the formation of androgens from progesterone and 17 α -OH 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 17 α -hydroxylation of progesterone was also reduced \sim one-half. 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 17 α -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, 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, in order to simplify the system. Our work indicates that substrate binding is more complex than a 1-step/2-state system (Fig. 12), but we modeled the system accordingly to satisfy several data sets (Figs. 9, 10). In a similar way, we know that each oxidation sequence (either 17 α -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, 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 α -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 (FeO_2^-) 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 argues against this conclusion (Fig. 2, Table 1). Previous evidence that electron transfer 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 in the system, is also augmented by b_5 (16).

The stimulatory effects of b_5 (Table 1, 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. 6C, 6D, 6G, 6H). What is less clear is why the effect of b_5 on 17 α -hydroxylation in the steady-state kinetic assays (Fig. 2, 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 α -hydroxylation reactions is quite clear (Fig. 2A, 2C).

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 Mn-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 \sim 4% of that measured here (Table 1, Fig. 2) or elsewhere (16) (see also other rates (11, 12, 47)), and a control b_5 experiment with reconstituted Fe-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 $\text{Fe}^{2+}\cdot\text{O}_2$ 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).

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 2-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 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, 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 (13, 22), reductase and non-reductase-bound forms, FeO_2^- and 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, in order to avoid side effects. A number of reports of selectivity for the lyase reaction > 17 α -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*)-orterone. Some of the 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 17 α -hydroxylation of progesterone has been compared with lyase activity towards 17 α -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*)-orterone: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 alternate consideration for achieving reaction-specific inhibition of P450 17A1 is targeting residues that bind b_5 (needed for efficient

17 α -OH pregnenolone lyase activity) (Fig. 2B, 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, Arg-449) where natural mutations attenuate lyase activity but not 17 α -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, 17 α -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 17 α -OH steroids). The individual sequential reactions are inherently distributive but more processive in the case of pregnenolone than with progesterone (e.g., Fig. 6B, 6F lags). There is some processivity, especially with pregnenolone, as demonstrated by the pulse-chase experiments (Fig. 7). Our kinetic model has 17 α -OH pregnenolone undergo a 1:6 partitioning between proceeding to the lyase reaction and dissociation from P450 17A1 (Figs. 9, 11). In addition to the pulse-chase data, another point of evidence in favor of some processivity of the 17 α -hydroxylation and lyase reactions is the orteronel inhibition results with pregnenolone (Table 3). With both enantiomers of orteronel, the lyase reaction was \sim 3-fold more sensitive to orteronel, confirming the results reported by Hara *et al.* (40) and Petrunak *et al.* (42) with (*S*)-orteronel. When DHEA production was measured from pregnenolone, the IC₅₀ value was similar to the value for the 17 α -hydroxylation reaction, not the lyase reaction (measured beginning with 17 α -OH pregnenolone) (Table 3). This result is consistent with the view that a part of the P450 17A1 proceeds through the reaction without its 17 α -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 while 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-Aldrich or Steraloids (Wilton, NH). DHEA was purchased from Waterstonetech (Carmel, IN). [7-³H(N)]-pregnenolone was purchased from PerkinElmer (catalog number NET039001MC). [4-¹⁴C]-Progesterone (ARC1398) and 17 α -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 17 α -OH pregnenolone was prepared by enzyme-mediated conversion of [7-³H(N)]-pregnenolone (65), with modification. 17 α -OH-[7-³H(N)]-pregnenolone was obtained from a 30-second incubation with the enzyme system described below, devoid of b_5 . The radiolabeled steroid was purified by the chromatographic method used for ³H-17 α -OH pregnenolone assays, and fractions were collected (with the β -RAM system only used to locate the t_R 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 17 α -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) (catalog number A4326), and the compound was dissolved in CH₃OH for purification of the enantiomers. The (*S*) and (*R*) enantiomers were resolved on a Chiralcel® OJ-RH column (4.6 mm \times 150 mm) with an isocratic 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 Data, 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 CH_2Cl_2 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 (CD_3OH). (Supplemental Data, Fig. S2); UV (H_2O) ϵ_{238} $98\text{ mM}^{-1}\text{ cm}^{-1}$, ϵ_{273} $13\text{ mM}^{-1}\text{ cm}^{-1}$; NMR (400 MHz, CD_3OH) δ 2.98 (s, 3H, CH_3), 3.10 (m, 4H, pyrrolidinol methylenes), 7.65-8.00 (m, 6H, naphthyl protons), 8.10 and 8.35 (s, 2H, imidazole ring protons) (solvent impurities at δ 1.19 and 3.6, $\text{C}_2\text{H}_5\text{OH}$; δ 1.25, hexanes; δ 3.31 CH_3OH ; δ 4.87, H_2O); high resolution mass spectrum: calc. for $\text{C}_{18}\text{H}_{18}\text{N}_3\text{O}_2^+$ (MH^+) 308.1394, (*R*) m/z , (*S*): m/z , fragmentation of either yielded dominant ions at m/z 280 (loss of 18, i.e. loss of H_2O) and m/z 186 (naphthyl methylcarboxamide, loss of pyrrolidinolpyrrole entity)); optical rotation (*S*): $[\alpha]_{\text{D}}^{24^\circ\text{C}} +96 \pm 6$ ($n=3$ readings) (c 0.03, CH_3OH) (compare to $+83.8$ (61)) (sufficient (*R*)-orteronel was not obtained to accurately measure $[\alpha]$).

Enzymes

Escherichia coli recombinant human P450 17A1 was prepared as described (16). *E. coli* recombinant rat NADPH-P450 reductase and human b_5 were prepared as described by Hanna *et al.* (66) and Guengerich (67), respectively. Cholesterol oxidase (from *Streptomyces* sp.) was purchased from Sigma-Aldrich (catalog 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 b_5 (when included), and 16 μM L- α -1,2-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: 0.01 μM for incubations with pregnenolone and 17 α -OH pregnenolone, 0.01 μM and 0.5 μM for progesterone 17 α -hydroxylation and 16 α -hydroxylation (respectively), and 0.5 μM in 17 α -OH progesterone lyase assays. Substrate was added at concentrations ranging from 0.2 to 20 μM . Duplicate samples were pre-warmed 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 2 $\mu\text{g/ml}$ yeast glucose 6-phosphate dehydrogenase) (68). The mixtures were incubated for 5 min at 37°C . Reactions were quenched with 2 ml of CH_2Cl_2 , mixed with a vortex device, and placed on ice. The samples were centrifuged to separate the organic and aqueous layers, and 1.8 ml of the organic phase was transferred to a new vessel and evaporated to dryness under a nitrogen stream. The extracts from progesterone and 17 α -OH progesterone incubations were dissolved in 100 μl of a CH_3CN - H_2O (1:1, v/v) mixture, and 10 μl aliquots were analyzed by UPLC-UV. The pregnenolone and 17 α -OH pregnenolone incubation extracts were dissolved in 50 μl CH_3OH , mixed with 200 μl of cholesterol oxidase (0.5 units/reaction) in 50 mM potassium phosphate buffer (pH 7.4) (14, 34), and incubated at 30°C (with shaking at 200 rpm) for 6-12 hours. The extraction procedure was then repeated, and the samples were redissolved and analyzed as progesterone, 17 α -OH progesterone, and Andro (derived from pregnenolone, 17 α -OH pregnenolone, and DHEA, respectively). The reaction products were resolved on an Acquity BEH C18 UPLC octadecylsilane column (2.1 mm \times 100 mm, 1.7 μm) with mobile phases A (70% $\text{CH}_3\text{OH}/30\%$ H_2O , v/v) and B (CH_3CN), at a 0.2 ml/min flow rate. The mobile phase linear gradient proceeded as follows: 0-1 min, 95% A; 4 min, 70% A; 4.5 min, 60% A; 4.55-6.75 min, 5% A; 7-10 min, 95% A (all v/v). The column temperature was maintained at 40°C , and the sample chamber was held at 4°C . The reaction products were identified by co-elution with commercial standards and quantified by the A_{243} peak areas. k_{cat} and K_{m} values were estimated using non-linear regression of hyperbolic fits in Prism software (GraphPad, San Diego, CA).

Single-turnover conditions

Single-turnover incubations were performed using an RQF-3 quench-flow instrument (KinTek Corp, Snow Shoe, PA). The instrument operates through a series of rapid mixing steps through two in-line incubation chambers, where each step dilutes the samples two-fold. The instrument temperature was conditioned at 37 °C. The enzyme substrate mixture in these reactions included 4 μ M steroid, 4 μ M human P450 17A1, 8 μ M rat NADPH-P450 reductase, 4 μ M human *b*₅ (when noted), and 32 μ M DLPC (lipid vesicles) in 100 mM potassium phosphate buffer (pH 7.4). The following radiolabeled substrates were used in these studies: [³H]-pregnenolone (10³ μ Ci/ μ mol), [³H]-17 α -OH pregnenolone (700 μ Ci/ μ mol), [¹⁴C]-progesterone (60 μ Ci/ μ mol), and [³H]-17 α -OH progesterone (10³ μ Ci/ μ mol). 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 seconds, at which time the reactions were quenched with HCl (1 M) as they were pushed through the second reaction into the collection vessel. Four time point replicates were collected into the same vessel for analysis to compensate for the low specific activity of [¹⁴C]-progesterone, and the procedure was applied for the other substrates. The samples were centrifuged at 2,000 \times g for 5 min to eliminate precipitated protein, and the supernatant was directly injected for HPLC-UV-in-line liquid scintillation counting. The reaction products were resolved on a Zorbax RX-C8 octylsilane column (4.6 mm \times 250 mm, 5 μ m) with mobile phase solvent mixtures A (95% H₂O/5% CH₃OH, v/v) and B (95% CH₃OH/5% H₂O, v/v). With the column at ambient temperature, the following linear gradients (1 ml/min) were used: [³H]-pregnenolone, [¹⁴C]-progesterone, and [³H]-17 α -OH progesterone assays: 0-10 min: 70% B; 15-18 min, 100% B; 20-30 min, 70% B; [³H]-17 α -OH pregnenolone assays: 0-15 min, 65% B, 20-23 min, 100% B, 25-35 min, 65% B (all v/v). The scintillation fluid (LiquiscintTM, National Diagnostics, Atlanta, GA) flow rate was 3 ml/min. The reaction products were identified by co-elution with commercial standards (monitored at *A*₂₁₆ (pregnenolone, 17 α -

OH pregnenolone, and DHEA) and *A*₂₄₃ (progesterone, 17 α -OH progesterone, 16 α -OH progesterone, and Andro)) and quantified by radiochromatogram peak areas using the β -RAM software (LauraTM, 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 μ M human P450 17A1, and (ii) 50 μ M [³H]-pregnenolone (13 μ Ci/ μ mol). One minute after the reactions were started with the NADPH-generating system, unlabeled 17 α -OH pregnenolone was added to the mixtures, at varying concentrations (5-75 μ M (final concentration) and vehicle), and the incubations continued for ten minutes before quenching with HCl (0.67 M 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 gradient: 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, while 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 μ Ci/ μ mol, and 80 μ M unlabeled 17 α -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 seconds 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: 0.005-100 μM (*S*)- or (*R*)-orteronel, 5 μM substrate, 0.01 μM human P450 17A1 (0.1 μM in assays with 17 α -OH progesterone), 0.5 μM human *b*₅, 2 μM NADPH-P450 reductase, and 16 μM DLPC (lipid vesicles) in 50 mM potassium phosphate buffer (pH 7.4). In order to resolve an inconsistent orteronel migration profile, the mobile phase solvents were changed to 5 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ /70% CH_3OH /30% H_2O for A and 5 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ /90% CH_3CN /10% H_2O for B (all v/v). IC₅₀ values were estimated in GraphPad Prism using the Onesite-Fit logIC50 formula: $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{-(X - \text{logIC}_{50})})$.

Binding Studies

Ligand binding

Initial binding studies were performed using previously reported methods (34). In a 10-cm cell (Starna Cells, Atascadero, CA, catalog no. 34Q-100, 25 ml) and using an OLIS-Cary 14 spectrophotometer (On-Line Instrument Systems, Bogart, GA), the steroid ligands were incrementally added to 0.1 μM human P450 17A1 in 50 mM potassium phosphate buffer (pH 7.4). Experiments were conducted as a series of additions, and the enzyme samples were exposed to ambient temperature, with mixing and exposure to a strong light beam (OLIS-Cary14 instrument) for periods > 60 min. Subsequent control experiments, where ligand was not added, exhibited spectral instability in the enzyme over the same period of time. Specifically, a time-dependent decrease in the Soret band was observed, which is problematic considering that the change in the Soret band (upon ligand binding) is the outcome analyzed in the assays (no obvious precipitation occurred).

Steroid binding was reanalyzed, with fresh enzyme samples for each steroid concentration, using a stopped-flow instrument equipped with a 20-mm cell and a rapid scanning monochrometer (OLIS RSM-1000, On-Line Instrument Systems). The stopped-flow sample syringes were conditioned at 37 °C and filled with either 2 μM human P450 17A1 or 0.2-20 μM steroid in 100 mM potassium phosphate buffer (7.4) with 120 μM DLPC (lipid vesicles). After mixing, the instrument recorded the absorbance spectra from 350-500 nm at one millisecond intervals for up to 7 seconds. The differential absorbance maxima and minima were determined by subtracting the final and initial (0 seconds) spectra. The composite absorbance changes, $\Delta (A_{\text{max}} - A_{\text{min}})$, from three replicates were averaged and plotted against the ligand concentrations. The data were fit with a quadratic binding formula to estimate binding constants (K_d) 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*)-orteronel as the trapping inhibitor. The on-rate for (*S*)-orteronel was first measured by mixing 4 μM human P450 17A1 in 100 mM potassium phosphate buffer (7.4) (with 120 μM DLPC (lipid vesicles)) and 20 μM (*S*)-orteronel (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 second (one scan per millisecond). The data was 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 (k_{off}), the steroid ligands (4 μM) and human *b*₅ (4 μM , when included) were added to the enzyme solution. The time component was 0-10 seconds (averaged mode, 62 scans per second) and data were analyzed as stated.

Kinetic analysis

Steroid association rate (k_{on}) parameters were calculated from the corresponding dissociation rates (k_{off}), estimated from the inhibitor trapping assays, and the dissociation constants (K_d), derived from the steroid binding experiments, using the equation:

$$K_d = k_{\text{off}} / k_{\text{on}}$$

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 (k_{on} , k_{off} , K_d , 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).

In order 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 $k_{\text{forward}} = 100 \mu\text{M}^{-1} \text{s}^{-1}$ ($10^8 \text{M}^{-1} \text{s}^{-1}$) and $k_{\text{reverse}} = 100 \text{s}^{-1}$, with the intention of forcing the step to be rapidly reversible in order 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 Ligand binding) using KinTek Explorer® software. The data were reduced by taking the difference of the kinetic traces (~ 7 seconds trace, 1 scan per millisecond) at the wavelengths previously designated for A_{max} and A_{min} (e.g. A_{391} and A_{426} kinetic traces were subtracted for pregnenolone assays; see Ligand binding and Figs. 5, 12). Three replicate difference traces for each steroid concentration were averaged and then normalized to $t = 0$. The kinetic traces were then reduced once more by boxed averaging ($n = 5$) with standard deviation before import into the KinTek® software.

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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. This manuscript constitutes part of the Ph. D. thesis of E. G. at Vanderbilt University.

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FOOTNOTES

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 This article contains supplemental Figs. S1-S3.

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³ The abbreviations used are: Andro, androstenedione; *b*₅, cytochrome *b*₅; Compound I, formal FeO³⁺ oxidized form of a hemoprotein; DHEA, dehydroepiandrosterone; DLPC, L- α -1,2-dilauroyl-*sn*-glycero-3-phosphocholine; OH, hydroxy; P450 (or CYP), cytochrome P450; SVD, singular value decomposition.

Table 1. Steady-state parameters for human P450 17A1 reactions^a

Substrate	Reaction	$k_{\text{cat}}, \text{s}^{-1}$		$K_{\text{m}}, \mu\text{M}$		$k_{\text{cat}}/K_{\text{m}}, \mu\text{M}^{-1} \text{s}^{-1}$		+/- b_5 ratio
		$-b_5$	$+b_5$	$-b_5$	$+b_5$	$-b_5$	$+b_5$	
Pregnenolone	17 α -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	^b	0.045 ± 0.003	^b	0.49 ± 0.14	^b	0.092 ± 0.027	
17 α -OH pregnenolone	Lyase	0.0059 ± 0.0012	0.071 ± 0.005	0.57 ± 0.46	0.91 ± 0.24	0.010 ± 0.009	0.078 ± 0.021	8
Progesterone	17 α -Hydroxylation	0.086 ± 0.004	0.17 ± 0.01	9.7 ± 0.8	4.3 ± 0.4	0.0089 ± 0.0009	0.040 ± 0.004	4
	16 α -Hydroxylation	0.0064 ± 0.0009	0.025 ± 0.003	1.1 ± 0.6	5.3 ± 1.3	0.0058 ± 0.0030	0.0047 ± 0.0012	0.8
17 α -OH progesterone	Lyase	0.00031 ± 0.00001	0.019 ± 0.001	2.9 ± 0.3	7.1 ± 0.3	0.00011 ± 0.00001	0.0027 ± 0.0001	25

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

^bThe rate was too low to accurately measure the steady-state kinetic parameters (see Fig. 6A below),

Table 2. k_{off} rates estimated by singular value decomposition (SVD) analysis

	$k_{\text{off}}, \text{s}^{-1}$					
	$-b_5$			$+b_5$		
		\pm			\pm	
Pregnenolone	0.23	\pm	0.02	0.29	\pm	0.03
17 α -OH pregnenolone	0.32	\pm	0.02	0.41	\pm	0.03
DHEA	0.80	\pm	0.05	0.77	\pm	0.05
Progesterone	0.46	\pm	0.02	0.42	\pm	0.03
17 α -OH progesterone	0.81	\pm	0.08	0.92	\pm	0.04
Andro	1.8	\pm	0.2	1.4	\pm	0.1

Results are presented as means \pm S.D. for at least triplicate determinations.

Table 3. Rates of substrate disappearance in single-turnover reactions ^a

Substrate	Presence of b_5	Rate, s ⁻¹
Pregnenolone	—	0.28 ± 0.01
	+	0.43 ± 0.02
17 α -OH pregnenolone	—	0.0074 ± 0.0006
	+	0.77 ± 0.01
Progesterone	—	0.46 ± 0.02
	+	0.47 ± 0.02
17 α -OH progesterone	—	0.0066 ± 0.0005
	+	0.029 ± 0.001

^a Values are from the experiments in Fig. 6. All values are first-order and include the S. E. values calculated from curve fitting. See Supplemental Data Fig. S1 for plots and fitting.

Table 4. IC₅₀ values for inhibition of steroid oxidation by orteronel enantiomers

Substrate	Product	IC ₅₀ (95% CI), μM^a	
		(S)-Orteronel	(R)-Orteronel
Pregnenolone	17 α -OH pregnenolone	1.5 (1.1-1.9)	6.4 (4.7-8.6)
	DHEA	1.4 (1.2-1.8)	6.3 (4.0-9.7)
17 α -OH pregnenolone	DHEA	0.49 (0.22-1.1)	2.8 (1.1-7)
Progesterone	17 α -OH progesterone	0.32 (0.22-0.48)	2.1 (1.6-2.8)
	16 α -OH progesterone	0.23 (0.15-0.35)	1.3 (0.86-2.0)
17 α -OH progesterone	Andro	0.23 (0.17-0.31)	1.5 (1.0-2.3)

^aSee data in Fig. 8. Fitting was done in GraphPad Prism (Onesite-Fit logIC₅₀).

Table 5. k_{on} rates calculated from K_{d} and k_{off}

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

See Fig. 3 and Table 1 for K_{d} and k_{off} values. See also Supplemental Data Fig. S3.

Fig. 1

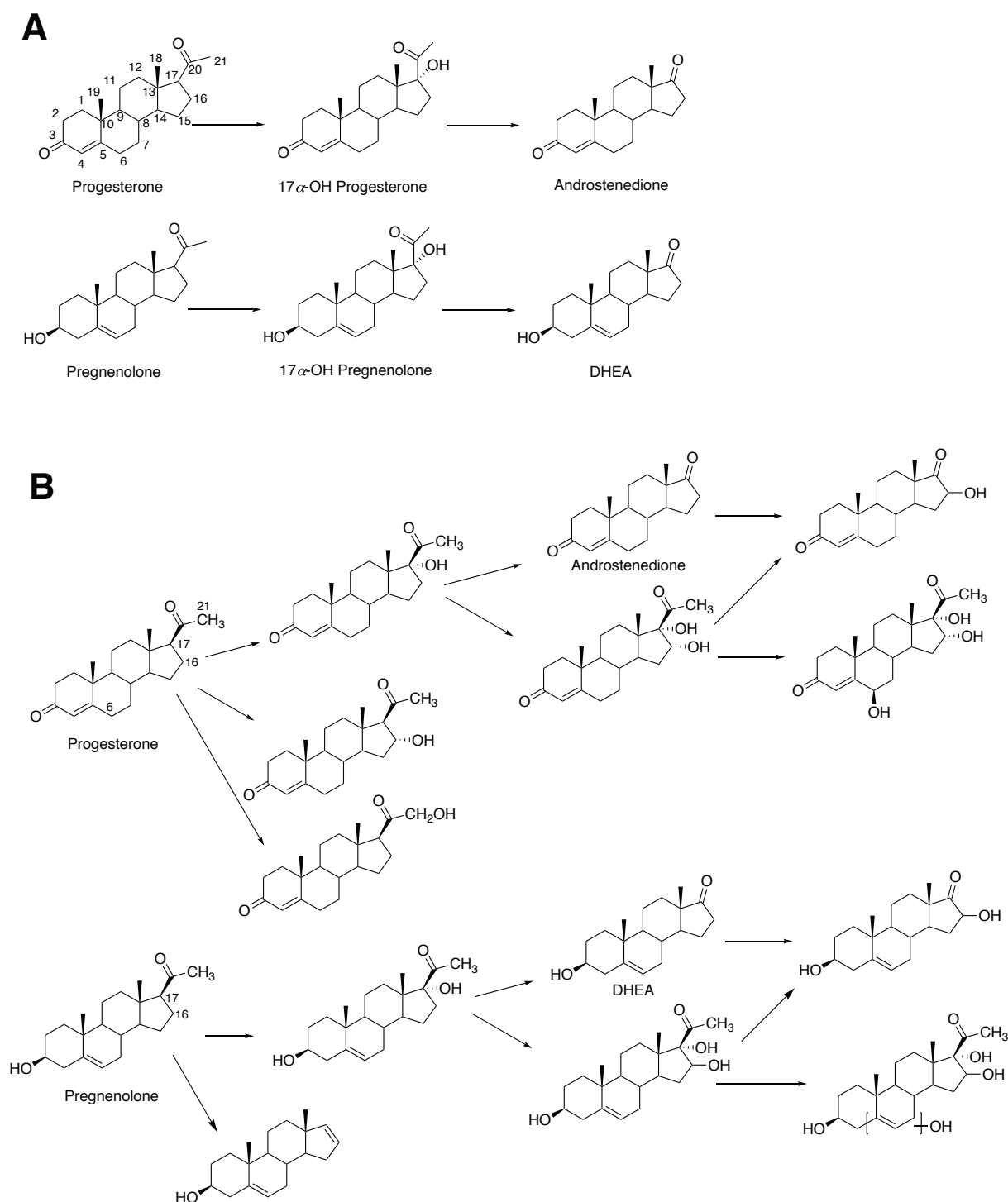


Figure 1. Oxidations catalyzed by P450 17A1. A, major classical reactions; B, expanded repertoire of reactions (14-16).

Fig. 2

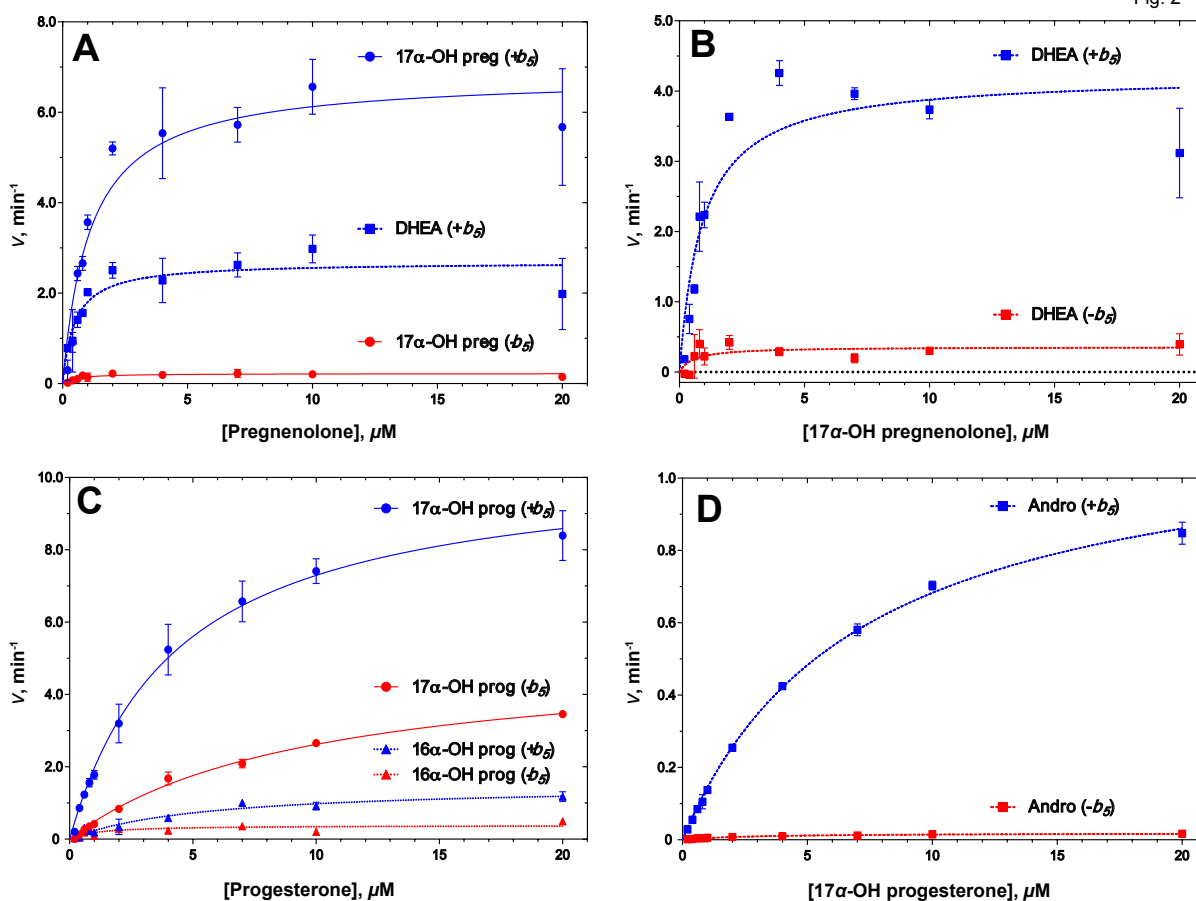


Figure 2. Steady-state kinetics of major reactions catalyzed by P450 17A1 and the effect of b_5 . The data points are means of duplicate assays (\pm range). Fitting was done by non-linear regression analysis of hyperbolic data in GraphPad Prism. See Table 1 for calculated values. A, pregnenolone; B,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

Fig. 3

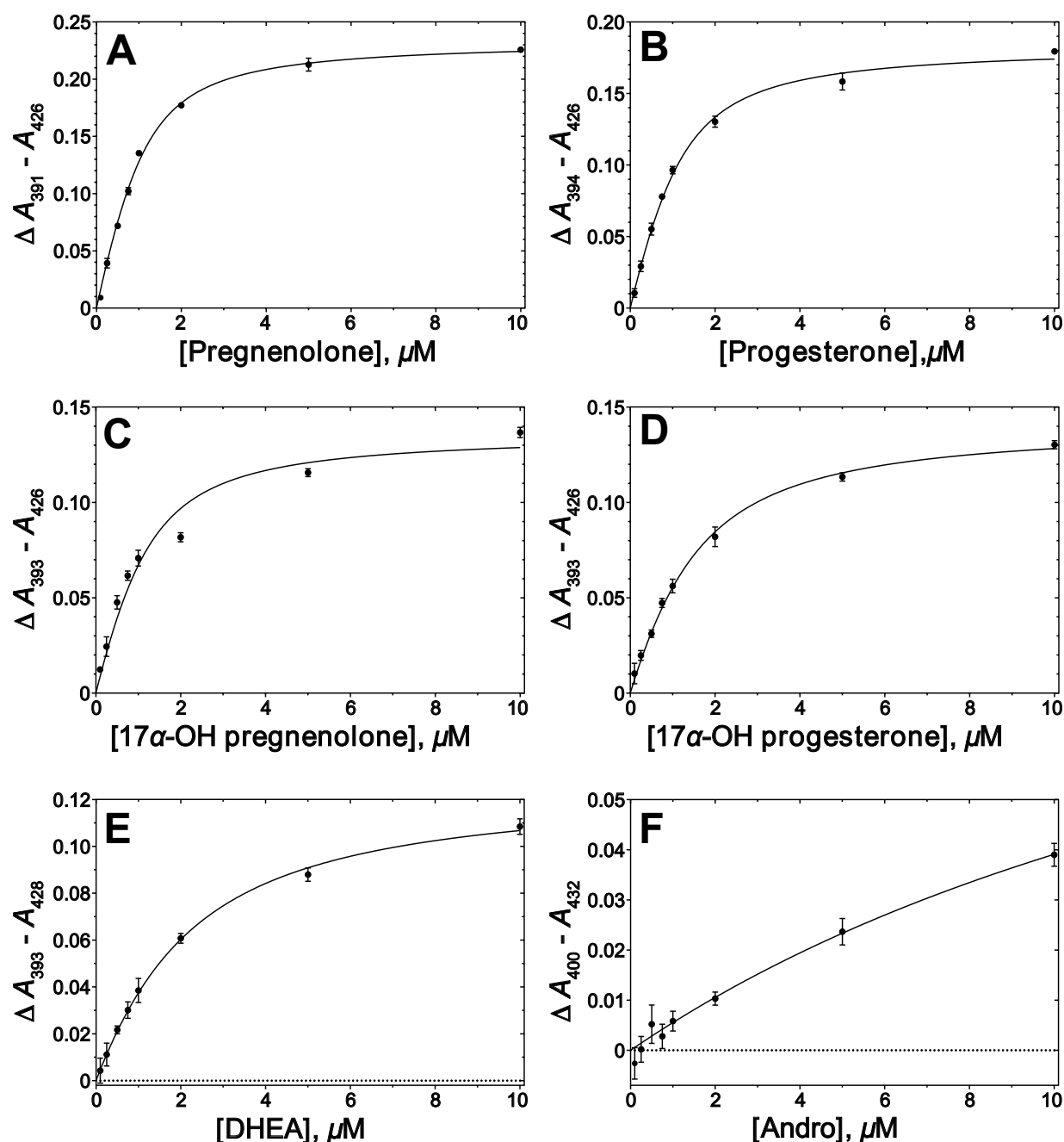


Figure 3. Binding of substrates and products to P450 17A1. Absorbance measurements were made following mixing in a stopped-flow spectrophotometer as described in detail in the Experimental Procedures section. At least three data points were collected at each ligand concentration, and the points were fit to hyperbolic curves using Prism software and non-linear regression analysis (S.E. calculated from curve fitting). A, pregnenolone (K_d $0.37 \pm 0.03 \mu\text{M}$); B, progesterone (K_d $0.47 \pm 0.04 \mu\text{M}$); C, 17 α -OH pregnenolone (K_d $0.52 \pm 0.10 \mu\text{M}$); D, 17 α -OH progesterone (K_d $0.95 \pm 0.08 \mu\text{M}$); E, DHEA (K_d $1.7 \pm 0.2 \mu\text{M}$); F, Andro (K_d $19 \pm 9 \mu\text{M}$).

Fig. 4

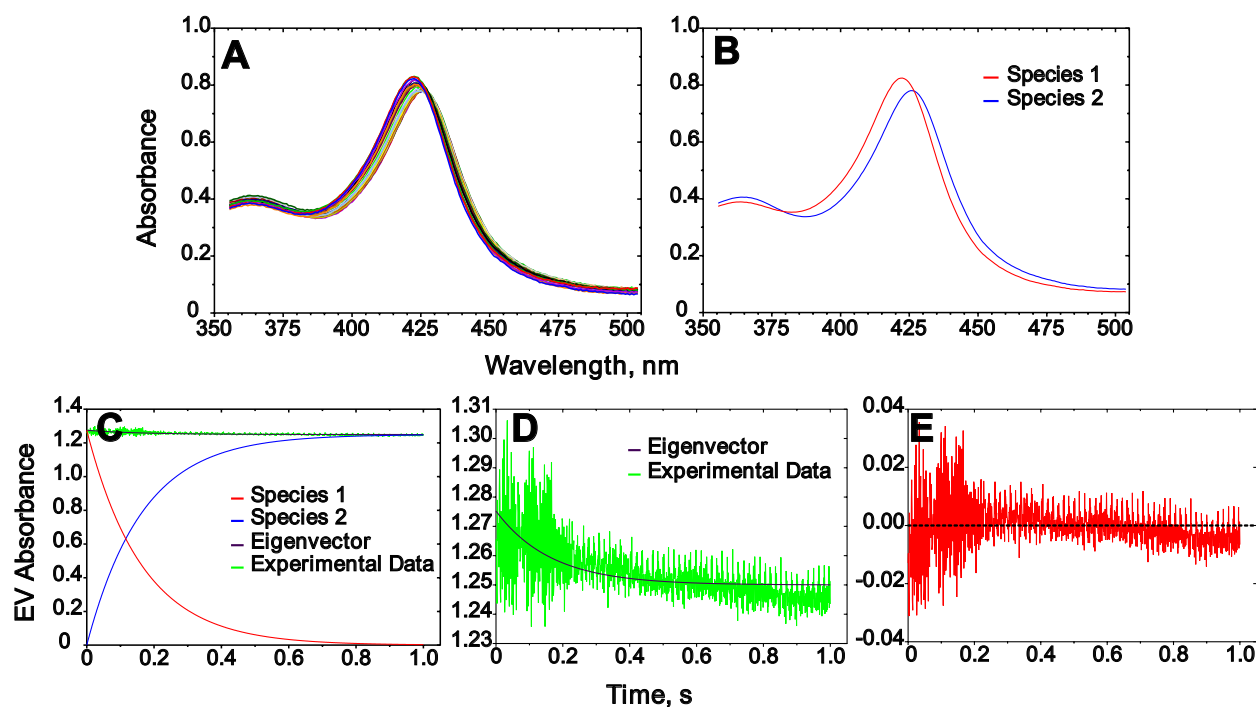


Figure 4. (S)-Orteronel binding to P450 17A1. P450 17A1 ($2 \mu\text{M}$) and (S)-orteronel ($10 \mu\text{M}$) in 100 mM potassium phosphate buffer (pH 7.4) were mixed together in the stopped-flow spectrophotometer. A, series of spectra collected at 1 millisecond intervals over a total period of 1.0 second (only a subset is shown). B, calculated beginning and ending spectra after SVD analysis using a 2-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 parts C and D).

Fig. 5

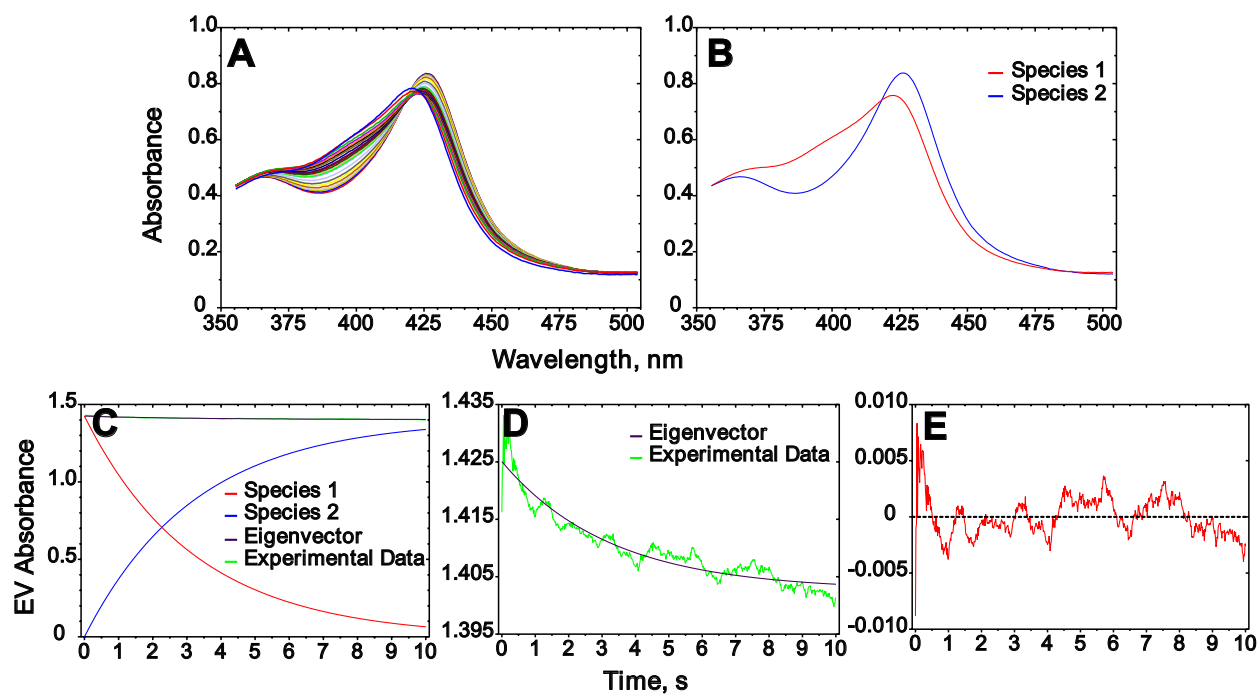


Figure 5. Estimation of k_{off} rate for 17α -OH pregnenolone from P450 17A1 using (S)-orteronel trapping. The experiment of Fig. 4C was repeated except that the P450 17A1 in the starting system was bound in a 1:1 molar complex with 17α -OH pregnenolone and the scan was extended to 10 seconds using the averaging mode (62 scans/second). Under these conditions ($4\ \mu\text{M}$ P450 17A1 (E) and $4\ \mu\text{M}$ 17α -OH pregnenolone (S) and using the K_d value of $0.52\ \mu\text{M}$ (Fig. 3), 70% of the P450 17A1 is in the substrate-bound form ($K_d = [E]_{\text{free}}[S]_{\text{free}}/[ES]$). The calculated k_{off} rate was $0.4\ \text{s}^{-1}$ (part D, with the residuals shown in part E). This is a single experiment; for the entire set of averaged experiments see Table 2.

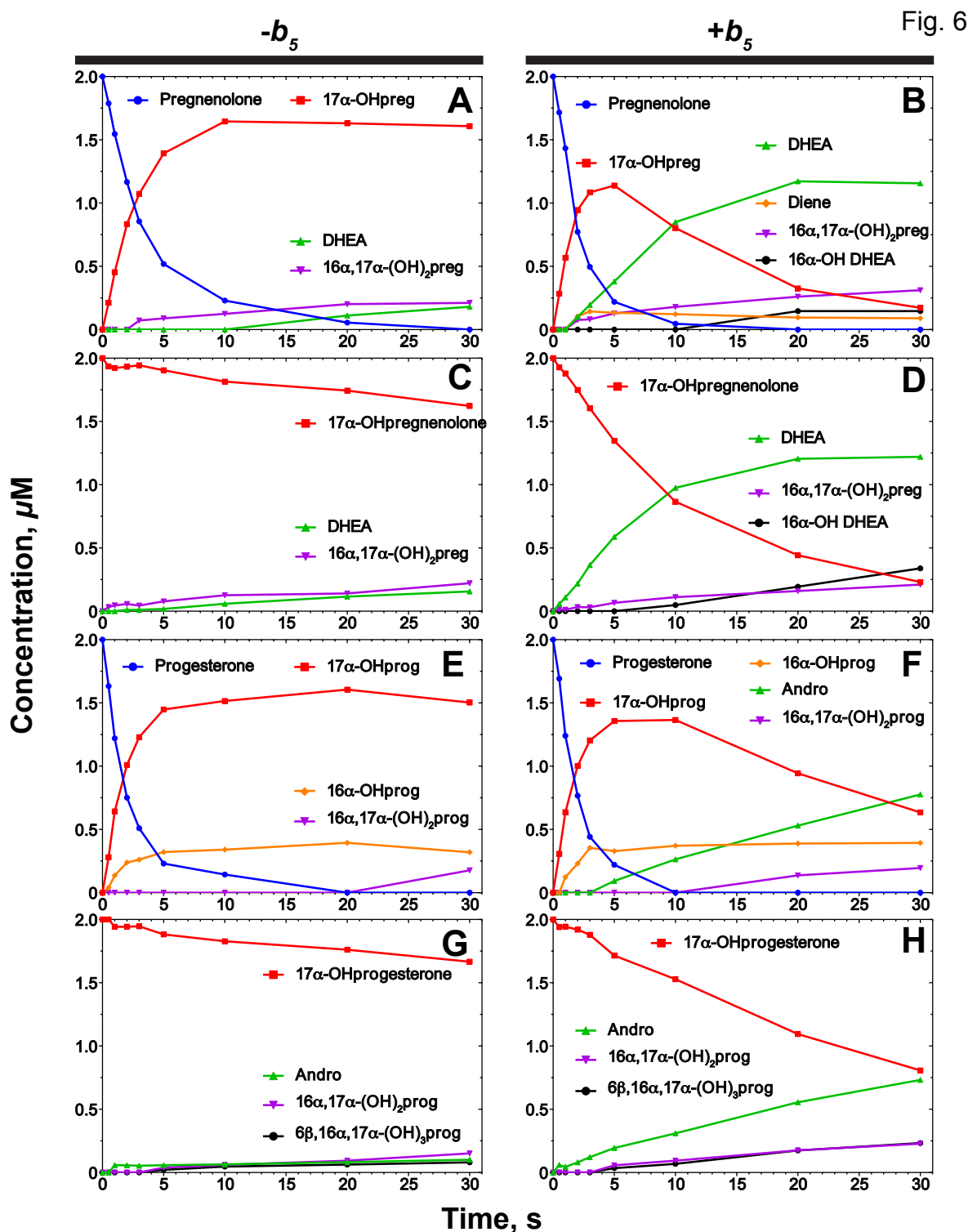


Figure 6. “Single-turnover” kinetics of 1:1 molar complexes of P450 17A1 with various substrates. In each case a mixture of 4 μ M P450 17A1, 8 μ M NADPH-P450 reductase, 32 μ M DLPC, 0.10 M potassium phosphate buffer (pH 7.4), and (when indicated) 4 μ M b_5 were mixed with an equal volume of the 0.10 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 ^{14}C -labeled substrate was used (^3H]-pregnenolone ($10^3 \mu\text{Ci}/\mu\text{mol}$), ^3H]-17 α -OH pregnenolone ($700 \mu\text{Ci}/\mu\text{mol}$), ^{14}C]-progesterone ($60 \mu\text{Ci}/\mu\text{mol}$), or ^3H]-17 α -OH progesterone ($10^3 \mu\text{Ci}/\mu\text{mol}$)). Each point is derived from a single time-point analysis. Parts A, C, E, and G did not include b_5 ; parts B, D, F, and H included b_5 . Traces are shown for reactions in which the substrate was: A and B, pregnenolone; C and D, 17 α -OH pregnenolone; E and F, progesterone; G and H, 17 α -OH progesterone. See Supplemental Data Fig. S2 and Table 3 for calculated rates of substrate disappearance.

Fig. 7

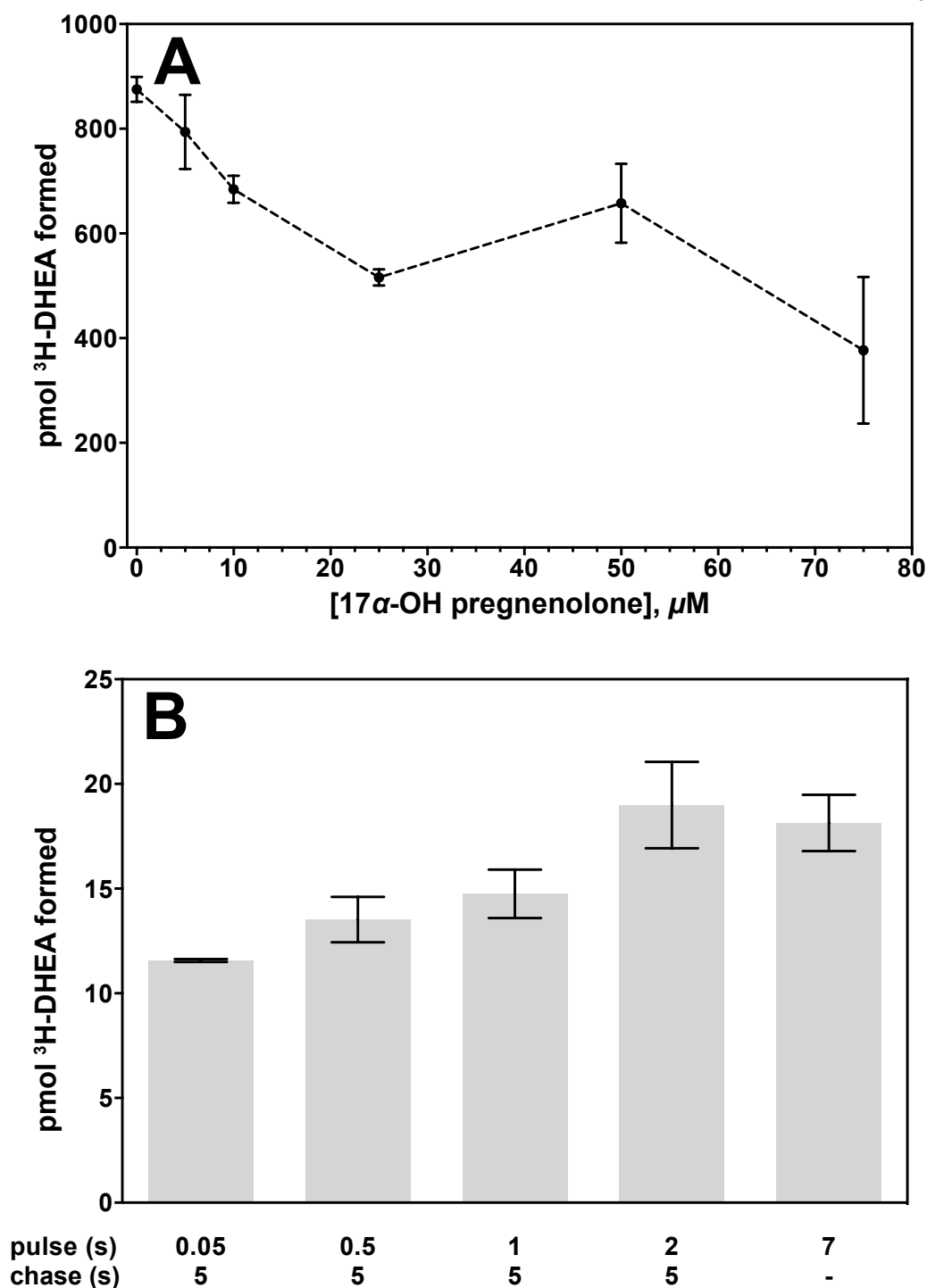


Figure 7. Pulse-chase assays of conversion of pregnenolone to DHEA. *A*, steady-state reaction. A reaction was initiated by mixing P450 17A1 (0.5 μ M), NADPH-P450 reductase (2 μ M), b_5 (0.5 μ M), DLPC (16 μ M), and [3 H]-pregnenolone (13 μ Ci/ μ mol, in 50 mM potassium phosphate buffer) with an NADPH-generating system (68). After 60 seconds, the indicated concentration of unlabeled 17 α -OH pregnenolone was added and the reaction was continued for another 10 minutes, 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 \pm SD (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 $\mu\text{Ci}/\mu\text{mol}$. The radiolabeled DHEA was measured after the addition of 80 μM unlabeled $17\alpha\text{-OH}$ pregnenolone at the indicated times following initiation of the reaction with NADPH. The results are presented as means \pm S.D. (range) of two individual experiments.

Fig. 8

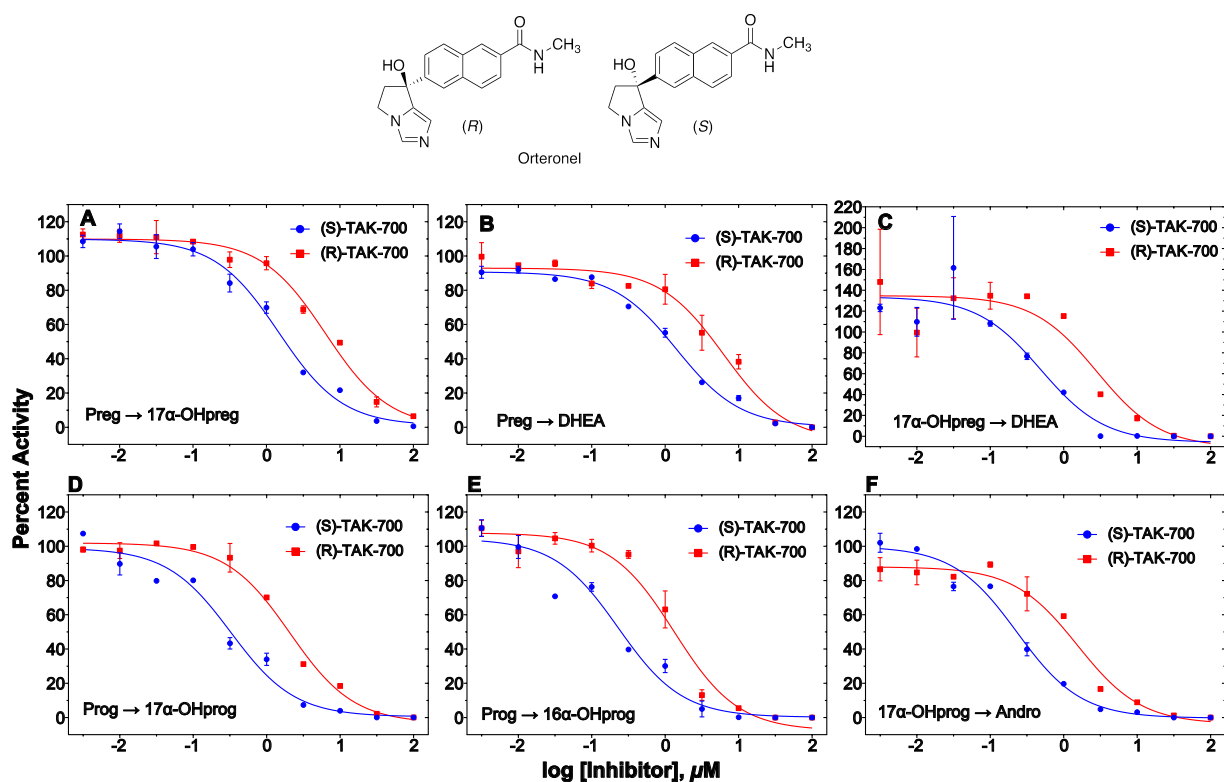


Figure 8. Inhibition of P450 17A1 reactions by (R) and (S)-orteronel. Steady-state reactions were run with 0.01 μM P450 17A1 (0.1 μM for $17\alpha\text{-OH}$ progesterone), in the presence of 0.5 μM b_5 , for 5 minutes in the presence of the indicated concentrations of the resolved enantiomers of orteronel. Results are presented as means of two determinations \pm range, with fitting as described under Experimental Procedures.

Fig. 9

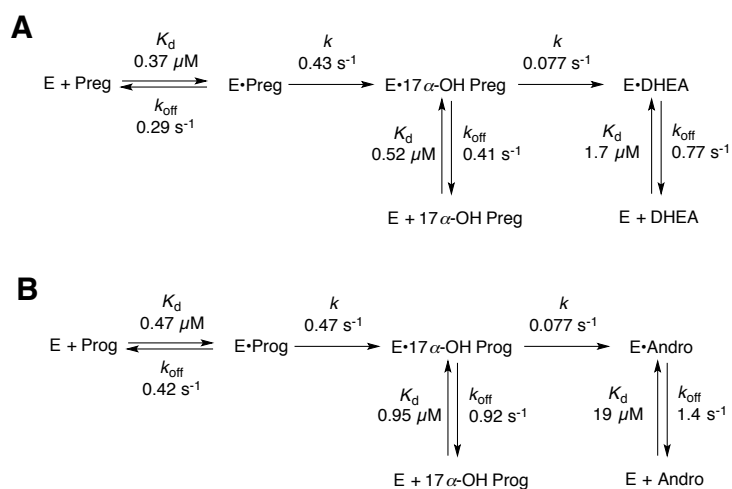


Figure 9. A kinetic scheme for oxidations of pregnenolone and progesterone using optimized rate constants and dissociation constants. See Fig. 10 for kinetic and binding data.

Fig. 10

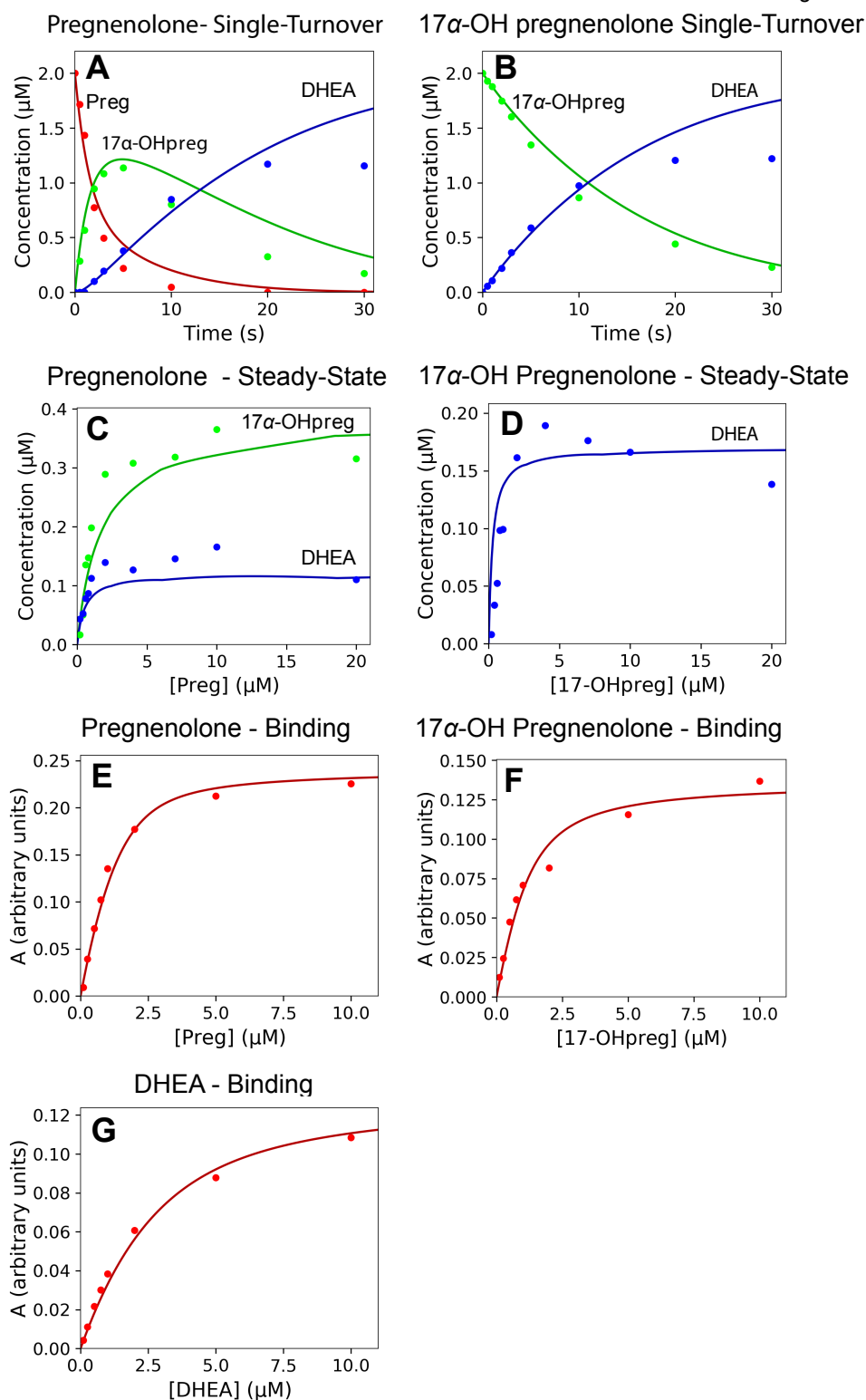


Figure 10. Fitting of pregnenolone kinetic and binding data with rate constants and K_d values (from Figure 9). Parts A and B are from Figs. 5G and 5H respectively (single turnover results). Parts C and D are steady-state kinetic results from Figs. 2A and 2B, respectively. A, single-turnover kinetics

beginning with pregnenolone. *B*, single-turnover kinetics beginning with 17α -OH pregnenolone. *C*, steady-state kinetics for conversion of pregnenolone to 17α -OH pregnenolone and DHEA. *D*, steady-state kinetics of 17α -OH progesterone oxidation to DHEA. *E*, steady-state binding of pregnenolone to (ferric) P450 17A1. *F*, steady-state binding of 17α -OH pregnenolone to (ferric) P450 17A1. *G*, steady-state binding of DHEA to (ferric) P450 17A1.

Fig. 11

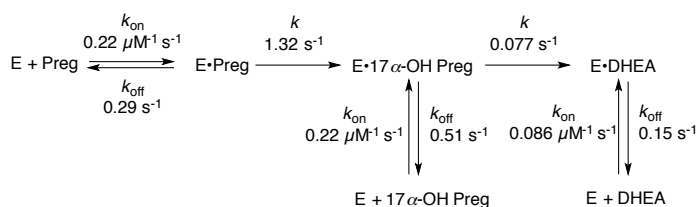


Figure 11. Results from KinTek Explorer® data analysis with experimental K_d values as constants.

Fig. 12

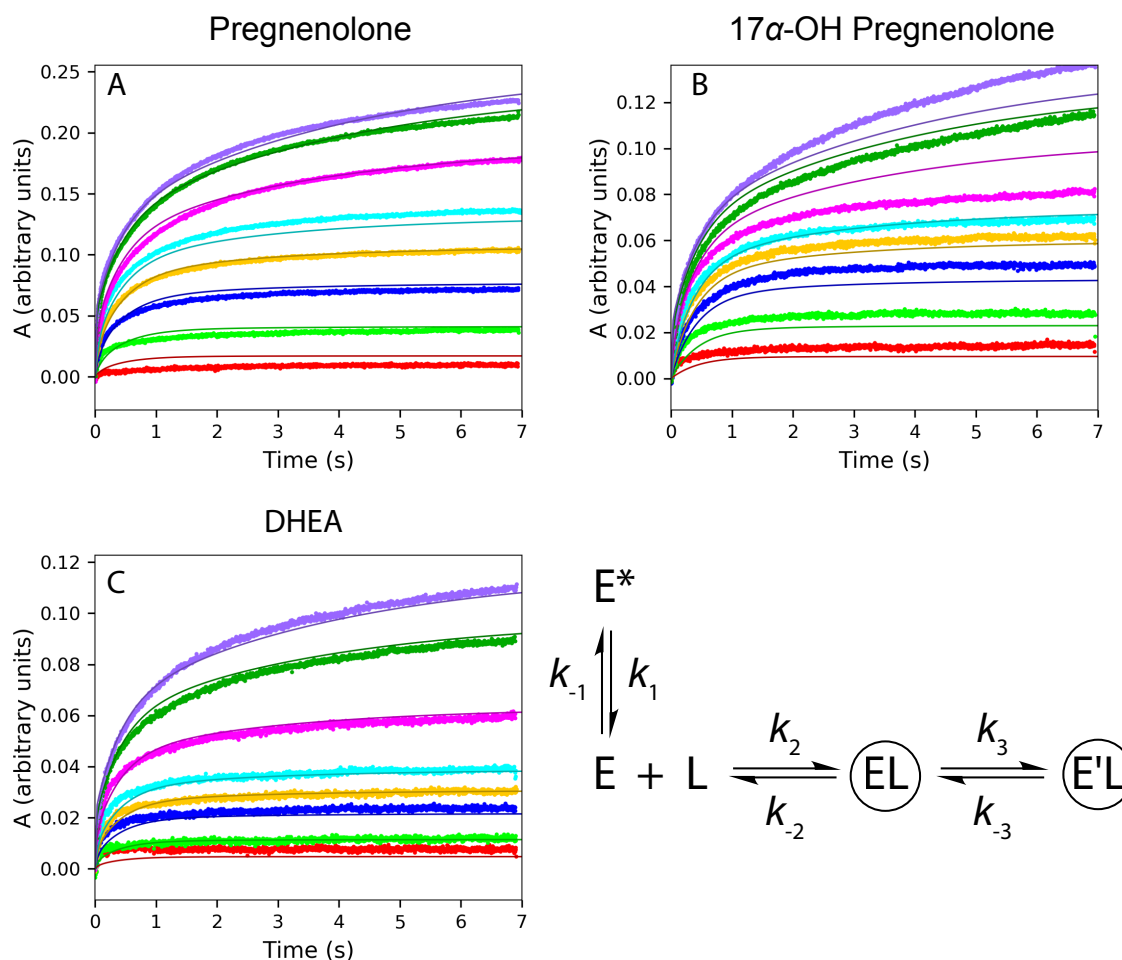


Figure 12. Analysis of P450 17A1 steroid binding kinetics. P450 17A1 (2 μ M) was mixed with increasing concentrations of pregnenolone, 17 α -OH pregnenolone, or DHEA as described in general under Experimental Procedures (see Fig. 3 for concentrations, corresponding to the data points there—note: b_5 was not present). Stopped-flow changes are shown in arbitrary units in the KinTek Explorer® global analysis, corresponding to $\Delta A_a - A_b$ changes. A, pregnenolone; B, 17 α -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'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 α -OH pregnenolone, and 393 and 428 for DHEA. The values used were $k_1 = 0.23 \text{ s}^{-1}$ and $k_{-1} = 0.21 \text{ s}^{-1}$ (for all cases) and the following for each ligand: pregnenolone, $k_2 = 1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-2} = 4.4 \text{ s}^{-1}$; $k_3 = 0.50 \text{ s}^{-1}$, and $k_{-3} = 1.9 \text{ s}^{-1}$; 17 α -OH pregnenolone, $k_2 = 8.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{-2} = 2.2 \text{ s}^{-1}$, $k_3 = 0.093 \text{ s}^{-1}$, and $k_{-3} = 2.3 \text{ s}^{-1}$; DHEA, $k_2 = 1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-2} = 12 \text{ s}^{-1}$; $k_3 = 0.10 \text{ s}^{-1}$, and $k_{-3} = 2.3 \text{ s}^{-1}$.

Fig. 13

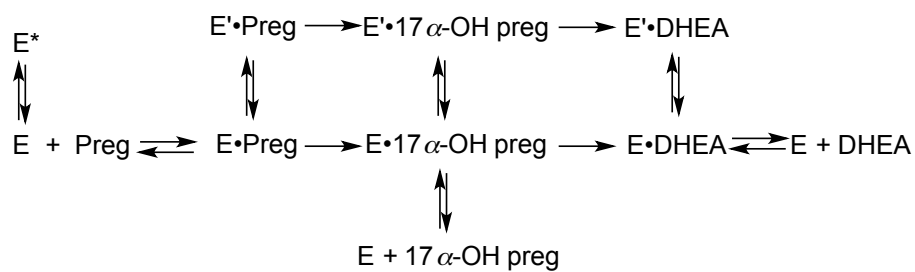


Figure 13. A hypothetical P450 17A1 reaction scheme with additional conformations of ligand-bound enzyme. Δ^4 steroids are shown but the model can also be considered for Δ^5 steroids.

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

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