Deuterium Isotope Effects on A-Ring and D-Ring Metabolism of Testosterone by CYP2C11: Evidence for Dissociation of Activated Enzyme–Substrate Complexes

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ABSTRACT: Cytochrome P450 systems are unusual in that many of them can convert a substrate to a number of different metabolites by several possible kinetic mechanisms. Steady-state equations describing the deuterium isotope effects for mechanisms in which different orientations of the substrate relative to the perferryl oxygen in the active site of the enzyme are achieved before a hydrogen (or possibly an electron) is abstracted have been derived and solved (Gillette et al., 1994). These equations have been used to elucidate the kinetic mechanisms by which CYP2C11 converts testosterone to 2α-hydroxytestosterone on the one hand and 16α-hydroxytestosterone and androstenedione on the other. We have synthesized testosterone-*2,2,4,6,6-*$^2H_5$ and compared its metabolism by CYP2C11 with that of nondeuterated testosterone. In this system, deuterated 2α-hydroxytestosterone would be formed by a deuterium abstraction pathway via the active oxygen intermediate (EOS_w) and the D-ring metabolites would be formed by non-deuterium abstraction pathways from active oxygen intermediates represented by (EOS_x). The results revealed that testosterone in the activated enzyme–substrate complexes, (EOS_w) and (EOS_x), does not change orientations while it is in the active site of CYP2C11. Instead, two of the noncompetitive experiments indicated that testosterone is able to dissociate from the (EOS) complexes and reassociate in either the same or different orientations. A third noncompetitive experiment suggested that testosterone in the (EOS) complexes does not change orientations while it is in the active site of CYP2C11, nor does it dissociate from the (EOS) complexes; instead, the pattern of metabolite formation is governed almost solely by the orientation of testosterone in the (ES_w) and (ES_x) complexes.

CYP2C11 is known to convert testosterone to 2α-OHT,[1] 16α-OHT, Andro, and 6β-OHT (Guengerich et al., 1982; Waxman et al., 1983; Cheng & Schenkman, 1983) (Figure 1). Since CYP2C11 is able to form both 2α-OHT and 16α-OHT, we wondered how the same enzyme was able to hydroxylate opposite ends of the molecule.

Several kinetic mechanisms may be envisioned by which a single enzyme may catalyze the formation of several metabolites from a substrate. In each of these mechanisms the substrate may combine with the enzyme in several different orientations to form a set of (ES) complexes. These complexes may then become activated by way of a series of intermediate steps to form a set of (EOS) complexes; the orientation of the substrate in each of these determines which hydrogen is abstracted and thereby which metabolite is formed. The flexibility and stability of these complexes govern the kinetic mechanism. In the parallel pathway mechanism, the orientations of the substrate in the active site of the enzyme are fixed and the pattern of metabolism is dependent solely on the orientations of the the substrate in the (ES) complexes. In the nondissociative mechanism, the substrate in the active site of the (EOS) complexes may change orientations before the hydrogen is abstracted from the substrate; in this mechanism, the rate constants for the interconversion to different orientations approach or exceed the rate constant for the abstraction of the hydrogens to form the metabolites. In the dissociative mechanism the substrate dissociates from the (EOS) complexes to form (EO) and then reassociates in either the same or different orientations; in this mechanism the rate constants for the dissociation and reassociation must approach or exceed the rate constants for the hydrogen abstraction reactions that lead directly to the formation of the metabolites.

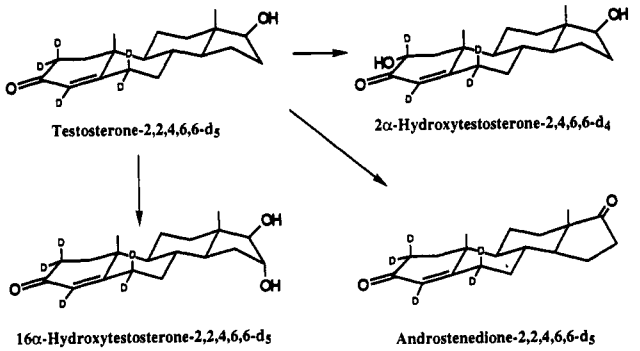

FIGURE 1: Pathways of testosterone metabolism by CYP2C11.

Gillette et al. (1994) have derived equations which indicate that these kinetic mechanisms may be differentiated by studies

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; 2α-OHT, 2α-hydroxytestosterone; 6β-OHT, 6β-hydroxytestosterone; 16α-OHT, 16α-hydroxytestosterone; 16α-OHA, 16α-hydroxyandrostenedione; Andro, androstenedione; (EOS_w), active oxygen substrate complex leading to the formation of 2α-OHT; (EP_w), enzyme–2α-OHT complex; (EOS_x), active oxygen substrate complex leading to the formation of a D-ring metabolite; (EP_x), enzyme–D-ring metabolite complex; d_0-T, unlabeled testosterone; d_5-T, testosterone-*2,2,4,6,6-*$^2H_5$; MOX, methoxamine hydrochloride; Tri-Sil BSA, *N,O*-bis(trimethylsilyl)acetamide/trimethylchlorosilane (1:1 v/v); MO-TMS, *O*-methoximine trimethylsilyl ether.

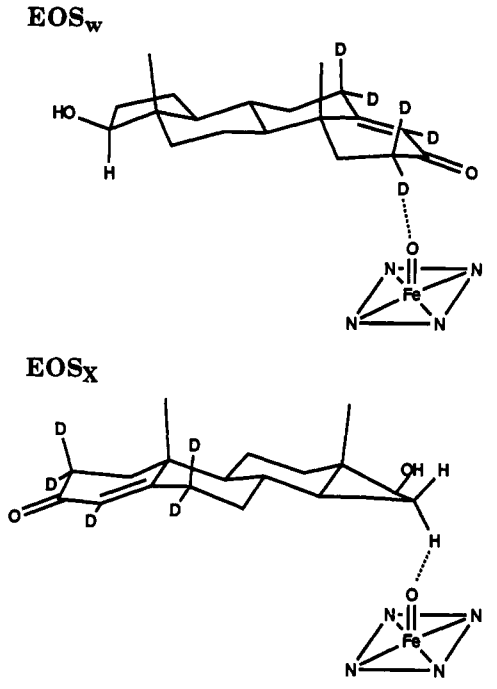

FIGURE 2: Orientations of testosterone in the (EOS_w) and (EOS_x) complexes.

of the deuterium isotope effects on the metabolism of substrates that are converted to at least two metabolites: one, P_w, by a pathway that requires the abstraction of a deuterium (the deuterium abstraction pathway) from a deuterated substrate in an (EOS_w) complex, and the other, P_x, by a pathway that does not require the abstraction of a deuterium (the non-deuterium abstraction pathway) from the deuterated substrate in an (EOS_x) complex. In this approach it is first necessary to establish that the isotope effect on the V_{max}/K_m for P_w is normal i.e., that V_{max}/K_m for P_w is greater for the nondeuterated substrate than for the deuterated substrate, $(V_{max,pw}/K_m)_H > (V_{max,pw}/K_m)_D$. The mechanisms then may be differentiated by studies of the deuterium isotope effect on P_x. The approach requires that the isotope effects be determined both in competitive experiments (in which the incubation mixtures contain both deuterated and undeuterated substrates and the data are normalized to equal concentrations of the substrates) and in noncompetitive experiments (in which the V_{max}/K_m values for both substrates are determined in separate experiments).

In the present study, we have used this approach to elucidate the mechanisms by which CYP2C11 metabolizes testosterone. For this purpose we have synthesized testosterone-*2,2,4,6,6-*$^2H_5$, (d_5-T) and used it as the deuterated substrate. The formation of the 2α-OHT thus occurs by the deuterium abstraction pathway, i.e., P_w via (EOS_w), whereas the formation of 16α-OHT and Andro occur by non-deuterium abstraction pathways, i.e., P_x via (EOS_x) (Figure 2). As shown in Table 1, the kinetic mechanism will be the parallel pathway mechanism when the ratio $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for 16α-OHT or Andro in noncompetitive experiments and the ratio $(v)_H/(v)_D$ for these metabolites in competitive experiments equal 1.0; that is, there is no isotope effect. The mechanism will be nondissociative when both $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ and $(v)_H/(v)_D$ for these metabolites are less than 1.0; that is, there is an inverse isotope effect in both kinds of experiments. In the dissociative mechnaism the $(v)_H/(v)_D$ for these metabolites equals 1.0, that is, there is no isotope effect on the ratio of metabolites in the competitive experiment; but $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ in the noncompetitive experiment may range from <1.0 to >1.0, although it usually will be <1.0 (Table 1, footnote *b*).

Table 1: Expected Deuterium Isotope Effects Produced by Different Kinetic Mechanisms

type of expt	non-dissociative mechanism	parallel pathway mechanism	dissociative mechanism
competitive:[a] $(v_{px})_H/(v_{px})_D$	<1.0	1.0	1.0
noncompetitive:[a] $(V_{max,px}/K_m)_H/(V_{max,px}/K_m)_D$	<1.0	1.0	<1 to >1[b]

[a] "px" refers to the formation of a metabolite by a non-deuterium abstraction pathway; in this study it refers to any of the D-ring metabolites. [b] The value will usually be <1.0. Nevertheless, it is theoretically possible that the value can be >1.0; this would happen in the unlikely event that (EOS_w) and (EOS_x) dissociate to (EO) and substrate, but only (EOS_w) is formed from (EO) (Gillette et al., 1994).

MATERIALS AND METHODS

Materials. Sulfuric acid-d_2 (D, 99.5%) and methanol-d_1 (D, 99.5%) were purchased from Cambridge Isotope Laboratories (Woburn, MA); MOX and Tri-Sil BSA were from Pierce Chemical Co. (Rockford, IL); testosterone, Andro, EDTA, NADPH, and Sephadex LH-20 were from Sigma Chemical Co. (St. Louis, MO); and 1,2-dilauroyl-*sn*-glycerophosphocholine was from Calbiochem (La Jolla, CA). 11α-Hydroxyprogesterone, 16α-OHA, and 6β-, 2α-, and 16α-OHT were purchased from Steraloids (Wilton, NH).

Cytochrome P450 reductase was prepared as described by Dutton et al. (1987) and Van Der Hoeven and Coon (1974), and CYP2C11 was prepared with slight modifications by the method described by Nagata et al. (1985).

Synthesis of Substrate. d_5-T was prepared by treating testosterone with 5% $^2H_2SO_4$ in methanol-d_1 at 50 °C for 4 days as previously described (Korzekwa et al., 1990). It was then purified by successive preparatory HPLC on a silica column (8 × 150 mm) with a mobile phase gradient of ethyl acetate/hexane ranging from 30:70 to 60:40 over 30 min. Mass spectrometry revealed that 89.9% was d_5-T, 9.4% was d_4-T, and 0.7% was d_0-T. Studies with ^1H and ^{13}C NMR (300 mHz) revealed that protons were not present in the 2- and 6-positions; thus the d_4-T contaminant must be testosterone-*2,2,6,6-*$^2H_4$. Since the isotope effects for the metabolism of d_4-T would be virtually identical to those for d_5-T, its presence would not affect the results.

Incubation Assay. Mixtures containing 50 pmol of CYP2C11, 100 pmol of P450 reductase, 10 μg of dilauroylphosphatidylcholine, 1 mM EDTA, 1 mM NADPH, and testosterone (0–250 μM) in 1 mL of potassium phosphate buffer (50 mM, pH 7.4) were incubated at 37 °C for 2 min. The reactions were stopped by the addition of 1 mL of ethyl acetate, 0.75 nmol of 11α-hydroxyprogesterone was added (as an internal standard), and the samples were extracted (3 × 1 volume) with ethyl acetate. The extracts were then combined and evaporated to dryness under nitrogen.

HPLC Analysis. The testosterone metabolites were assayed by a modification of the procedure of Bornheim et al. (1987). A Waters 600 solvent delivery system was equipped with a Waters C18 4-μm Radial Pak reverse-phase column and operated at 1.2 mL/min at room temperature. The column was equilibrated with 50:49:1 water/methanol/acetonitrile and a linear solvent gradient developed between 44:55:1, 36:62:2, and 23:75:2 over 15, 8, and 1.5 min, respectively, before returning to the equilibrium conditions over 2.5 min. Metabolites were detected with a Waters multiwavelength UV

detector operating at 242 nm. Peaks were integrated with a Waters Maxima 820 data collecting system. Peak areas of the metabolites were compared to those of the internal standard, and the amounts were calculated by relating these area ratios to standard curves generated with authentic standards. Retention times of the standards, expressed in minutes, were as follows: 9.80, 6β-OHT; 13.00, 16α-OHA; 13.63, 16α-OHT; 19.43, 2α-OHT; 25.43, 11α-hydroxyprogesterone; 29.32, Andro; and 34.20, testosterone.

Derivatization and Analysis by GC/MS. Samples were analyzed as their MO-TMS derivatives formed by a modification of the procedure used by Smith et al. (1992). Where indicated, the HPLC peaks corresponding to the metabolites were collected, and the solvent removed under nitrogen. The residue was then dissolved in MOX reagent (2% w/v MOX in pyridine, 50 μL), and the reaction was allowed to proceed for 30 min at 80 °C. To this was added Tri-Sil BSA (50 μL), and the mixture was heated to 80 °C for 2 h. Upon completion of the reaction, the excess silylating reagent was removed under nitrogen at 40 °C, and the residue was dissolved in *n*-hexane/trichloromethane (1:1 v/v). The solution was then added to a short column of Sephadex LH-20 slurry, packed in a Pasteur pipet, and eluted with *n*-hexane/trichloromethane (1:1 v/v). The first 2 mL of eluate, which contained the steroid derivatives, was saved, and the solvent was removed under nitrogen.

The residue of the eluate was then dissolved in *n*-hexane/trichloromethane (1:1 v/v) and analyzed by GC/MS, performed on a Hewlett-Packard 5890 gas chromatograph, equipped with a poly(dimethylsiloxane)-fused silica column (Heliflex, AT-1, 30 m × 0.25 mm i.d., 0.1-mm film: Altech), and interfaced to a Hewlett-Packard 5971 mass-selective detector. Helium was used as the carrier gas (head pressure 5 psi). The GC program used an initial temperature of 50 °C for 1 min, which was increased to 300 °C at 20 min and maintained for 5 min. The retention times of the MO-TMS derivatives, expressed in minutes, were as follows: 12.2, testosterone; 12.5, 2α-OHT; 13.1, 16α-OHT; and 12.1, Andro. Mass spectrometry was conducted in the electron impact mode, and selective molecular ions of the MO-TMS derivatives were monitored: d_0-T and d_5-T at m/z 389 and 394 (M^+); d_0-2α-OHT and d_4-2α-OHT at m/z 446 and 450 ($M^+ - OCH_3$); d_0-16α-OHT and d_5-16α-OHT at m/z 477 and 482 (M^+); and d_0-Andro and d_5-Andro at m/z 344 and 349 (M^+), respectively. The data were corrected for the presence of metabolites formed from the d_4-T present in the substrate preparation.

Statistical analyses were performed with the use of Symphony (Lotus Development Corp., Cambridge, MA) and SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

Competitive Experiments. CYP2C11 was incubated with mixtures of d_0-T and d_5-T, and the amounts of the metabolites were determined by HPLC analysis as described in Materials and Methods. As expected, the enzyme catalyzed the formation of 2α-OHT, 16α-OHT, and small amounts (<1%) of 6β-OHT (Guengerich et al., 1982; Waxman et al. 1983; Cheng & Schenkman, 1983). In addition, it also formed 16α-OHA.

In the first of these experiments, the concentration of d_5-T was held constant and the concentration of d_0-T was varied. The amounts of the various metabolites were measured, and the data were calculated as stated in Table 2. In this experiment the ratio of $(2\alpha\text{-OHT})_H/(2\alpha\text{-OHT})_D$ was greater than 1.0, but the ratio of $(16\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_D$ was not significantly different from 1.0. The data appeared to be consistent, because the isotope effect on the ratio of the metabolites, i.e., $[(2\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_H]/[(2\alpha\text{-OHT})_D/(16\alpha\text{-OHT})_D]$, was virtually identical to the isotope effect on the formation of 2α-OHT.

Table 2: Competitive Experiment (1): Isotope Effects on Ratios of Metabolites[b]

	ratio (or ratio of ratios)	mean ± SD
	ratios:	
1	$\dfrac{(16\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_D}{d_0\text{-T}/d_5\text{-T}}$	1.03 ± 0.13[a]
2	$\dfrac{(2\alpha\text{-OHT})_H/(2\alpha\text{-OHT})_D}{d_0\text{-T}/d_5\text{-T}}$	4.32 ± 0.82
3	$(2\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_H$	0.67 ± 0.15
4	$(2\alpha\text{-OHT})_D/(16\alpha\text{-OHT})_D$	0.17 ± 0.09
5	$\{[(2\alpha\text{-OHT})_H + (16\alpha\text{-OHT})_H]/[(2\alpha\text{-OHT})_D + (16\alpha\text{-OHT})_D]\}/(d_0\text{-T}/d_5\text{-T})$	1.46 ± 0.15
	ratio of ratios:	
6	$\dfrac{(2\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_H}{(2\alpha\text{-OHT})_D/(16\alpha\text{-OHT})_D}$	4.33 ± 1.12

[a] 1.0 lies between the values for the 35 and 65 percentiles. [b] CYP2C11 was incubated with 3.23 μM testosterone-d_5 and various concentrations of testosterone-d_0 as described in Materials and Methods, and the total amounts of each metabolite formed from both substrates were measured by HPLC. The ratio of the metabolites formed in each incubation mixture was determined by GC/MS and divided by the ratio of the substrate concentrations present in the incubation mixture. The ratios are reported as the mean ± SD.

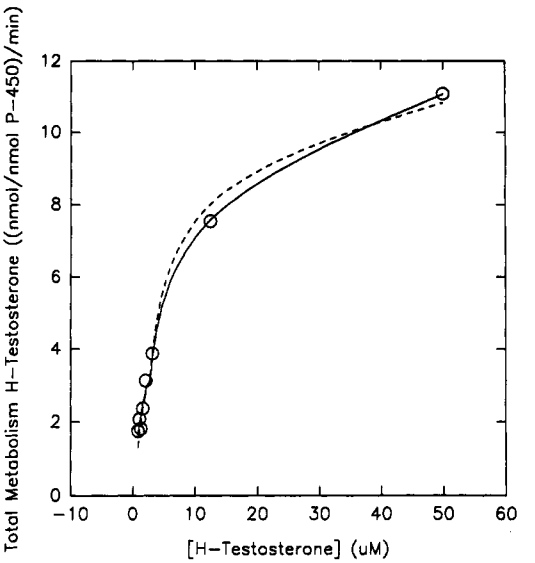

FIGURE 3: Metabolism of d_0-T in the presence of d_5-T. CYP2C11 was incubated in various concentrations of d_0-T and 3.23 μM d_5-T as described in Materials and Methods. The amounts of the metabolites formed from the d_0-T were determined by HPLC followed by GC/MS and are represented by the symbol (O). The curve fitting procedures of SigmaStat were based on the equations $v = (a + bS)S/(c + dS + eS^2)$ (—) and $v = aS/(c + dS)$ (- - -).

Since the equations for the rates of formation of the metabolites by the dissociative and the nondissociative mechanisms differ (Gillette et al., 1994), it seemed possible that the mechanisms might be distinguished by fitting the data to both equations (Figure 3). Although the equation for the dissociative mechanism simulated the data slightly better than did the Michaelis–Menten equation, we did not believe that the results provided conclusive evidence for the dissociative mechanism.

Table 3: Competitive Experiment (2): Isotope Effects on Ratios of Metabolites[b]

ratios	mean ± SD
$(16\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_D$	1.12 ± 0.06
$(2\alpha\text{-OHT})_H/(2\alpha\text{-OHT})_D$	5.10 ± 0.09
$(\text{Andro})_H/(\text{Andro})_D$	1.23 ± 0.14[a]
$(16\alpha\text{-OHA})_H/(16\alpha\text{-OHA})_D$	1.21 ± 0.09

[a] 1.0 lies between the values for the 1 and 99 percentiles. The values for the other ratios were significantly greater than 1.0 ($P < 0.01$). [b] CYP2C11 was incubated with 124 μM d_0-T and 125 μM d_5-T as described in Materials and Methods. The ratio of the amounts of each metabolite formed from the two substrates was determined by GC/MS. The results were corrected for the difference in concentration of the two substrates and are reported as the mean ± SD of 6 replicates.

In the second competitive experiment, CYP2C11 was incubated with virtually a 50:50 mixture of d_0-T and d_5-T and the ratio of each metabolite formed from the two substrates was determined by GC/MS (Table 3). Again, the ratio $(2\alpha\text{-OHT})_H/(2\alpha\text{-OHT})_D$ was greater than 1.0. Moreover, the ratios $(16\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_D$, $(\text{Andro})_H/(\text{Andro})_D$, and $(16\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_D$ also tended to be >1.0. Because the isotope effects on the formation of the D-ring metabolites were never less than 1.0 in either of the competitive experiments, we concluded that a nondissociative mechanism need not be further considered (Table 1).

Noncompetitive Experiments. Noncompetitive experiments were needed to distinguish between the parallel pathway and dissociative mechanisms. In these experiments, CYP2C11 was incubated with various concentrations of either d_0-T or d_5-T, and the amounts of the various metabolites were determined by HPLC, as described in Materials and Methods (Tables 4–6).

In the first and third noncompetitive experiments (Tables 4 and 6), the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ values were greater than 1.0 for the formation of 2α-OHT, but less than 1.0 for the formation of 16α-OHT, Andro, and 16α-OHA and total D-ring oxidation. These experiments provided evidence that the metabolites formed by the enzyme occur by the dissociative mechanism.

By contrast, in the second noncompetitive experiment (Table 5) there were virtually no isotope effects for the formation of 16α-OHT, Andro, or 16α-OHA. The reason for the difference between this experiment and the other two is unknown, but perhaps even slightly different ways of reconstituting the system may lead to different kinetic mechanisms.

The $(V_{max})_H/(V_{max})_D$ values were greater than 1.0 for 2α-OHT in all three noncompetitive experiments (Tables 4–6). But they were virtually 1.0 for the formation of 16α-OHT, Andro, and 16α-OHA in experiments 1 and 3 and slightly less than 1.0 in experiment 2.

In all three noncompetitive experiments, the ratios of the ratios, $(2\alpha\text{-OHT}/16\alpha\text{-OHT})_H/(2\alpha\text{-OHT}/16\alpha\text{-OHT})_D$ and $(2\alpha\text{-OHT}/\text{D-ring metabolites})_H/(2\alpha\text{-OHT}/\text{D-ring metabolites})_D$, were greater than 1.0, but were variable, ranging from about 3.9 to about 5.3.

Table 4: Noncompetitive Experiment (1): Isotope Effects on V_{max}/K_m, V_{max}, and Ratios of the Metabolites of Testosterone Formed by CYP2C11[d]

	(nmol of metabolite/min)/{(nmol of P450)[testosterone]}		
(V_{max}/K_m)/P450	d_0-T	d_5-T	d_0-T/d_5-T
total metabolism	3.54 ± 0.29	4.42 ± 0.31[c]	0.81 ± 0.09[a]
2α-OHT	1.27 ● 0.13	0.55 ± 0.09[c]	2.37 ● 0.38
16α-OHT	1.64 ± 0.14	2.73 ± 0.19[c]	0.60 ± 0.06
Andro	0.45 ± 0.10	0.74 ● 0.23[c]	0.62 ± 0.10
16α-OHA	0.19 ● 0.06	0.40 ± 0.15[c]	0.50 ± 0.06
D-ring metabolites	2.27 ± 0.17	3.87 ± 0.25[c]	0.59 ± 0.06
	(nmol of metabolite/min)/nmol of P450		
V_{max}/P450	d_0-T	d_5-T	d_0-T/d_5-T
total metabolism	15.9 ± 1.3	12.37 ± 0.87[c]	1.29 ± 0.14
2α-OHT	5.71 ± 0.56	1.53 ± 0.24[c]	3.80 ± 0.61
16α-OHT	7.35 ± 0.61	7.64 ± 0.53	0.97 ± 0.10[a]
Andro	2.00 ● 0.43	2.08 ± 0.63	0.99 ± 0.16[a]
16α-OHA	0.85 ± 0.27	1.11 ± 0.41[b]	0.80 ± 0.16[a]
D-ring metabolites	10.20 ± 0.80	10.83 ● 0.94[b]	0.95 ± 0.09[a]
ratios:			
2α-OHT/16α-OHT	0.78 ± 0.04	0.20 ± 0.02[c]	3.93 ± 0.50
2α-OHT/(D-ring metabolites)	0.56 ± 0.03	0.14 ± 0.02[c]	4.01 ± 0.48

[a] 1.0 lies between the values for the 1 and 99 percentiles. [b] $P < 0.05$. [c] $P < 0.001$, that the difference from the undeuterated substrate is due to chance. [d] CYP2C11 was incubated with various concentrations of either d_0-T or d_5-T as described in Materials and Methods. The K_m values were estimated from regression of a v/S versus v plot for the total metabolism of the substrates. The estimates for K_m were 4.49 μM with d_0-T and 2.80 with d_5-T. With these estimates, the V_{max}/K_m values for each of the metabolites were calculated from the equation $V_{max}/K_m = v[(1/S) + (1/K_m)]$, where v is the amount of metabolite formed in each mixture; the means ± SD were then calculated from the set of such calculations. The estimates of the values for V_{max} were calculated from the equation $V_{max} = v[1 + (K_m/S)]$ for each metabolite in each incubation mixture. The estimates of the isotope effects (d_0/d_5) were calculated from the values obtained at a given concentration of d_0-T divided by the values obtained with the corresponding concentration of d_5-T, and the means ± SD were calculated from the resulting values.

DISCUSSION

As shown in Tables 2–6, we have confirmed that CYP2C11 oxidizes testosterone to 2α-, 16α-, and 6β-OHT and Andro, but the formation of 6β-OHT was barely detectable. In addition, however, we found evidence that the enzyme also catalyzed the formation of 16α-OHA, which theoretically could be formed from released 16α-OHT or released Andro or directly from either primary metabolite while they remained in the active site of CYP2C11. Because the source of 16α-OHA can affect measurements of the apparent isotope effects, it became important to discover the principal pathway for the formation of the metabolite. The finding that as the concentration of testosterone was increased, the 16α-OHA fraction decreased while that of Andro increased and the fractions of 2α-OHT and 16α-OHT remained relatively constant (data not shown) indicated that 16α-OHA was formed to a greater extent from released Andro than from released 16α-OHT. Subsequent experiments revealed that 16α-OHT was a very poor substrate for CYP2C11 (unpublished results). Moreover, Waxman et al. (1983) reported that CYP2C11 converts Andro to 16α-OHA. We therefore focused attention on the measurements of 2α-OHT, 16α-OHT, and total D-ring metabolites as estimates of the rates of formation of the primary metabolites.

To elucidate the mechanism by which this enzyme catalyzed the oxidation of testosterone in both the A-ring and the D-ring, we studied the metabolism of d_0-T and d_5-T in both competitive and noncompetitive experiments. With this combination of substrates, the formation of 2α-OHT and 6β-OHT would occur by deuterium abstraction pathways, while the formation of the other metabolites would occur by non-deuterium abstraction pathways.

The finding that in competitive experiments the ratio $(16\alpha\text{-OHT})_H/(16\alpha\text{-OHT})_D$ and the ratio $(\text{D-ring metabolites})_H/(\text{D-ring metabolites})_D$ were virtually 1.0 (Tables 2 and 3)

Table 5: Noncompetitive Experiment (2): Isotope Effects on V_{max}/K_m, V_{max}, and Ratios of the Metabolites of Testosterone Formed by CYP2C11[d]

	(nmol of metabolite/min)/{(nmol of P450)[testosterone]}		
(V_{max}/K_m)/P450	d_0-T	d_5-T	d_0-T/d_5-T
total metabolism	11.29 ± 1.01	7.92 ± 0.37[c]	1.45 ± 0.15
2α-OHT	4.12 ± 0.41	0.79 ± 0.09[c]	5.36 ± 0.89
16α-OHT	5.25 ± 0.52	5.14 ± 0.31	1.04 ± 0.11[a]
Andro	1.35 ± 0.26	1.44 ± 0.24	0.95 ± 0.08[a]
16α-OHA	0.57 ± 0.19	0.55 ± 0.14	1.07 ± 0.23[a]
D-ring metabolites	7.18 ± 0.63	7.13 ± 0.37	1.02 ± 0.11[a]
	(nmol of metabolite/min)/nmol of P450		
V_{max}/P450	d_0-T	d_5-T	d_0-T/d_5-T
total metabolism	35.0 ± 3.1	29.8 ± 1.5[c]	1.23 ± 0.12
2α-OHT	12.8 ± 1.3	2.89 ± 0.34[c]	4.53 ± 0.76
16α-OHT	16.3 ± 1.6	18.8 ± 1.1[c]	0.88 ± 0.09[a]
Andro	4.18 ± 0.82	5.30 ± 0.88[b]	0.81 ± 0.07
16α-OHA	1.78 ± 0.58	2.01 ± 0.53	0.90 ± 0.19[a]
D-ring metabolites	22.3 ± 2.0	26.1 ± 1.4[c]	0.87 ± 0.09[a]
ratios:			
2α-OHT/16α-OHT	0.78 ± 0.03	0.15 ± 0.02[c]	5.19 ± 0.85
2α-OHT/(D-ring metabolites)	0.57 ± 0.03	0.11 ± 0.01[c]	5.26 ± 0.82

[a] 1.0 lies between the values for the 1 and 99 percentiles. [b] $P < 0.05$. [c] $P < 0.001$, that the difference from undeuterated substrate is due to chance. [d] CYP2C11 was incubated with various concentrations of either d_0-T or d_5-T as described in Materials and Methods. K_m, V_{max}, V_{max}/K_m, and (d_0/d_5) values were estimated as described in the footnotes to Table 4. The K_m values for this experiment were 3.10 μM with d_0-T and 3.67 μM with d_5-T.

Table 6: Noncompetitive Experiment (3): Isotope Effects on V_{max}/K_m, V_{max}, and Ratios of the Metabolites of Testosterone Formed by CYP2C11[d]

	(nmol of metabolite/min)/{(nmol of P450)[testosterone]}		
(V_{max}/K_m)/P450	d_0-T	d_5-T	d_0-T/d_5-T
total metabolism	9.20 ± 0.30	7.95 ± 0.36[c]	1.16 ± 0.07
2α-OHT	3.48 ± 0.12	1.03 ± 0.09[c]	3.42 ± 0.28
16α-OHT	4.24 ± 0.16	4.97 ± 0.26[c]	0.86 ± 0.05
Andro	1.02 ± 0.22	1.38 ± 0.28[c]	0.74 ± 0.07
16α-OHA	0.46 ± 0.13	0.57 ± 0.14[c]	0.80 ± 0.13[a]
D-ring metabolites	5.72 ± 0.23	6.92 ± 0.29[c]	0.83 ± 0.05
	(nmol of metabolite/min)/nmol of P450		
V_{max}/P450	d_0-T	d_5-T	d_0-T/d_5-T
total metabolism	41.4 ± 1.4	28.5 ± 1.3[c]	1.46 ± 0.09
2α-OHT	15.64 ± 0.55	3.67 ± 0.33[c]	4.29 ± 0.36
16α-OHT	19.05 ± 0.72	17.80 ± 0.92[b]	1.07 ± 0.07[a]
Andro	4.60 ± 0.97	4.94 ± 1.00	0.93 ± 0.09[a]
16α-OHA	2.06 ± 0.57	2.04 ± 0.49	1.01 ± 0.16[a]
D-ring metabolites	25.7 ± 1.0	24.8 ± 1.0	1.04 ± 0.07[a]
ratios:			
2α-OHT/16α-OHT	0.82 ± 0.02	0.21 ± 0.01[c]	4.00 ● 0.23
2α-OHT/(D-ring metabolites)	0.61 ± 0.03	0.15 ± 0.01[c]	4.13 ± 0.21

[a] 1.0 lies between the values for the 1 and 99 percentiles. [b] $P < 0.05$. [c] $P < 0.001$, that the difference from the undeuterated substrate is due to chance. [d] CYP2C11 was incubated with various concentrations of either d_0-T or d_5-T as described in Materials and Methods. K_m, V_{max}, V_{max}/K_m, and (d_0/d_5) values were estimated as described in the footnotes to Table 5. The K_m values for this experiment were 4.49 μM with d_0-T and 1.26 μM with d_5-T.

indicated that the A-ring and D-ring metabolites were not formed by a nondissociative mechanism. As pointed out by Gillette et al. (1994), the equations for the nondissociative mechanisms predict that these values should be less than 1.0. But the ratios cannot discriminate between the parallel pathway and the dissociative mechanisms, because in competitive experiments both mechanisms predict that the values should be 1.0.

Theoretically, competitive experiments in which the concentration of one of the substrates is held constant and that of the other varied can differentiate between parallel pathway and dissociative mechanisms. The rate equations for the parallel pathway mechanism may be arranged to a Michaelis equation for a competitive inhibitor, whereas the rate equation for the dissociative mechanism will contain quadratic equations in both the numerator and the denominator and cannot be rearranged to a Michaelis equation (Gillette et al., 1994). As shown in Figure 3, the simulation based on the equation for the dissociative mechanism tended to be better than that for the parallel pathway, but the differences between them were not statistically significant. In this instance, therefore, the results were equivocal, and we were unable to distinguish between the dissociative and the parallel pathway mechanisms solely from the competitive experiments.

Isotope effects on parameters in noncompetitive experiments provide the most sensitive tests for differentiating the parallel pathway and dissociative mechanisms. For the dissociative mechanisms, the equations predict that the ratio $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for 16α-OHT and the total D-ring metabolites in noncompetitive experiments usually will be <1.0, whereas for the parallel pathway mechanism the equations predict these ratios should be 1.0 (Gillette et al., 1994). The finding that the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the formation of 16α-OHT and for the total D-ring metabolites was <1.0 in the first and third noncompetitive experiments (Tables 4 and 6) thus provided evidence for the dissociative mechanism.

For some unknown reason, the second noncompetitive experiment (Table 5) indicated that the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the formation of 16α-OHT and total D-ring metabolites was not significantly different from 1.0, which raises the possibility that, under some experimental conditions, the system may approach a parallel pathway mechanism. If the metabolites were formed by a parallel pathway mechanism, the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the formation of 2α-OHT would be >1.0 only if (EOS_w) were reduced to (ES_w) and water, and the rate constant for reduction (k_{w42}) approached or exceeded the rate constant for the abstraction of the 2α-hydrogen from testosterone (Gillette et al., 1994).

Alternatively, the finding that the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the formation of 2α-OHT was greater in the second experiment (Table 5) than in the other two noncompetitive experiments (Tables 4 and 6) lends support for the idea that both k_{w42} and k_{x42} were unusually large in the second experiment. This would tend to increase the $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ ratios for all of the metabolites, including the D-ring metabolites, and thereby diminish the expected inverse isotope effects.

Although the isotope effects on the V_{max} values for the formation of the various metabolites are not useful in differentiating between dissociative and nondissociative mechanisms of multimetabolite formation, they are useful in elucidating other aspects of the mechanism. As shown in Tables 4–6, the ratios $(V_{max})_H/(V_{max})_D$ for the formation of 2α-OHT were >1.0 while those for 16α-OHT and the total D-ring metabolites were not statistically different from 1.0. But such results would be expected if, at equally high concentrations of the substrates, the concentrations of $(EOS_w)_H$ and $(EOS_x)_H$ in the mixtures containing d_0-T were only slightly higher than the concentrations of $(EOS_w)_D$ and $(EOS_x)_D$ in

the mixtures containing d_5-T. Several situations may be envisioned in which high concentrations of d_0-T and d_5-T would result in rather minor differences in the concentrations of these intermediate forms. Suppose, for example, that at very high concentrations of d_0-T virtually all of the enzyme existed as $(EOS_w)_H$; substitution of d_5-T would not be expected to change significantly the relative concentrations of the intermediates, and the ratio $(V_{max})_H/(V_{max})_D$ for the 2α-OHT would approach the intrinsic isotope effect (k_{w46H}/k_{w46D}), while the isotope effects on the D-ring metabolites would be virtually 1.0. On the other hand, suppose that virtually all of the enzyme existed as either (EOS_x) or (EP_x); in these cases the $(V_{max})_H/(V_{max})_D$ for the 2α-OHT would be less than the intrinsic isotope effect (k_{w46H}/k_{w46D}), while the isotope effects on the D-ring metabolites would still be virtually 1.0. The parallel pathway mechanism offers another scenario: Suppose that at very high concentrations of d_0-T virtually all of the enzyme existed as (ES_w) or (ES_x). Substitution of d_5-T might decrease the V_{max} rate of formation of 2α-OHT by increasing the rate of reduction to water without altering the V_{max} for the formation of the D-ring metabolites.

While the evidence presented here suggests that substrate dissociates from the active site of the enzyme in order to account for the isotope effects on the A-ring and D-ring metabolites, it does not preclude the possibility that the (EOS_x) complexes leading to the D-ring metabolites are sufficiently flexible to interconvert while the substrate is in the active site of the enzyme. It is entirely possible that both mechanisms may occur in the same system, i.e., the dissociative mechanism, to account for the isotope effects on the formation of A-ring and D-ring metabolites, but that superimposed upon the dissociative mechanism is a nondissociative mechanism for the oxidations within the D-ring. Studies with preparations of testosterone deuterated in the 16α-position would be needed to clarify these issues.

As discussed by Gillette et al. (1994), estimates of the intrinsic isotope effect should be obtained by the ratio, (2α-OHT/D-ring metabolites)$_H$/(2α-OHT/D-ring metabolites)$_D$, when the rate constants for the dissociation and reassociation of the (EOS) complexes are very large. Calculations by the semiempirical quantum chemical method, AM1, of the relative stabilities of the C-2 and C-7 radicals of testosterone, however, suggest that the intrinsic isotope effect for the formation of 2α-OHT and 7α-OHT should be about the same (unpublished calculations), namely, about 9 (Korzekwa et al., 1990). The finding that the values for (2α-OHT/D-ring metabolites)$_H$/(2α-OHT/D-ring metabolites)$_D$ were always <5.3, therefore, suggests that the rate constants for dissociation and reassociation are not sufficiently large to allow full expression of the intrinsic isotope effect; instead, they are probably of the same order of magnitude as the rate constants for the abstraction of the hydrogen.

The discovery that a cytochrome P450 can metabolize substrates by dissociative mechanisms has implications of broad significance. For example, as long as it was thought that the formation of several metabolites occurred solely by nondissociative mechanisms, the meaning of values for $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ greater than 1.0 for the total metabolism of a substrate was puzzling. As pointed out by Northrop (1975), when the immediate precursor of the isotope-sensitive step is formed by an irreversible reaction, the value of $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ for the total metabolism of the substrate should be 1.0 as long as there is no other pathway emanating from the (EOS_w) complex. Thus, it has been assumed that when the isotope effects on V_{max}/K_m have been greater than 1.0 for the total metabolism of substrates by cytochrome P450 enzymes, the (EOS_w) complex must be reduced to water. For this reason, in the parallel pathway mechanism values for $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ greater than 1.0 can occur only when the (EOS_w) complex, which leads to the 2α-hydroxylation of testosterone, can be reduced to water. No such assumption is needed for dissociative mechanisms. In these mechanisms, values for $(V_{max}/K_m)_H/(V_{max}/K_m)_D$ greater than 1.0 can be explained by isotope effects on the concentration of (EO). At low concentrations of the undeuterated and deuterated substrates the concentrations of (EO) will be greater with the deuterated substrate than with the undeuterated substrate; consequently, the concentration of (E) will be lower with the deuterated substrate and the rate of formation of a metabolite produced by the deuterium abstraction pathway will be decreased while the rate of formation of a metabolite produced by a non-deuterium abstraction pathway will be increased. In dissociative mechanisms, however, the perferryl oxygen in (EO) must be stable; it must be sufficiently long-lived that the dissociation and reassociation constants become dominant. Indeed, in the dissociative mechanisms considered by Gillette et al. (1994), it was assumed that (EO) did not undergo any reactions other than the formation of (EOS_w) and (EOS_x). If (EO) were reduced to water, the rate equations for the formation of the various metabolites in noncompetitive experiments would consist of quadratic equations in both the numerator and the denominator (unpublished derivation); it would not be possible to rearrange them to Michaelis forms, and the Lineweaver–Burk and other plots would be curves rather than straight lines. Nevertheless, it should be intuitively obvious that in these cases the reduction of (EO) to (E) and water would tend to increase the relative rates of formation of the metabolites produced by either the deuterium abstraction pathway or the non-deuterium abstraction pathway. At any given low concentration of the substrates in noncompetitive experiments, the $(v)_H/(v)_D$ values for both kinds of metabolites would tend to be larger than they would be if (EO) were not reduced. In competitive experiments, the $(P_x)_H/(P_x)_D$ would still be 1.0.

The addition of an inhibitor or another substrate to a system acting by a dissociative mechanism may cause other unusual effects. For example, even at low concentrations of both the substrate and the inhibitor, the inhibitor may either increase or decrease the metabolism of the substrate (Gillette et al., 1994). Moreover, the addition of the inhibitor theoretically may change the relative concentrations of (E) and (EO) and thereby change the relative proportions of the metabolites of the substrate. However, we were unable to show that the addition of progesterone altered the pattern of testosterone metabolism by CYP2C11 (unpublished results).

In conclusion, the equations derived for the isotope effects of the general models for multimetabolite formation (Gillette et al., 1994) have led to experiments that in combination have greatly clarified the mechanism by which CYP2C11 metabolizes testosterone. They have led to the first definitive evidence that this enzyme–substrate combination may act through a dissociative mechanism. The equations should be useful in elucidating whether other cytochrome P450 and substrate combinations act through dissociative, nondissociative, or parallel pathway mechanisms.

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