Androgen Formation by Cytochrome P450 CYP17. Solvent Isotope Effect and pL Studies Suggest a Role for Protons in the Regulation of Oxene versus Peroxide Chemistry

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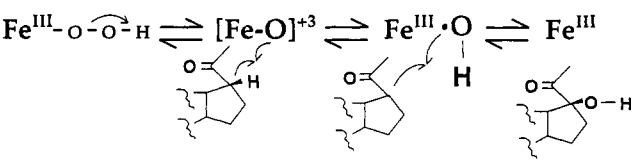
ABSTRACT: CYP17 catalyzes the cleavage of the C-17 side chain of progesterone to form androstenedione. The two-step reaction involves an initial 17α-hydroxylation catalyzed by oxene chemistry followed by cleavage of the C-17 side chain. We have recently shown that C-17 side-chain cleavage may involve the rearrangement of a peroxy intermediate via a Baeyer–Villiger rearrangement [Mak, A. Y., & Swinney, D. C. (1992) *J. Am. Chem. Soc. 114,* 8309]. Accordingly, CYP17 is proposed to catalyze oxidations via both oxene and peroxide chemistry. This study was initiated to investigate the possibility that protons may play a determining role in differentiating between the oxene and peroxide chemistries associated with product formation. The pL dependence of the deuterium solvent isotope effects associated with progesterone oxidation to 17α-hydroxyprogesterone and 17-*O*-acetyltestosterone and 17α-hydroxyprogesterone oxidation to androstenedione was determined in microsomes from pig testes. The formation of 17α-hydroxyprogesterone is assumed to occur via oxene chemistry and the formation of 17-*O*-acetyltestosterone and androstenedione by peroxide chemistry. The initial rate of progesterone oxidation to 17α-hydroxyprogesterone was associated with a pL-independent inverse solvent isotope effect ($^{H}k/^{D}k$ = 0.75–0.95, in 30% DOD), whereas the rate of oxidation to 17-*O*-acetyltestosterone was associated with a pL-independent positive solvent isotope effect in the presence of 30% DOD ($^{H}k/^{D}k$ approximately 2). In contrast, DOD inhibited the formation of androstenedione from 17α-hydroxyprogesterone in a noncompetitive, pL-dependent manner. These data strongly suggest that protons do influence the chemistry of CYP17 catalysis. We interpret the pL-independent isotope effects to represent the effect of the fraction of deuterium upon protonation of the ferric peroxy intermediate (Fe^{III}–O-O^-). DOD shifts the acid–base equilibrium to the protonated intermediate (Fe^{III}–O-O-H), increasing the rate of products formed via oxene chemistry at the expense of those formed from peroxide chemistry. This results in the inverse isotope effect observed for 17α-hydroxyprogesterone formation and the positive isotope effect for 17-*O*-acetyltestosterone formation. The pL-dependent, noncompetitive inhibition of androstenedione formation by DOD is interpreted to reflect the effect of shifting the pK of a rate-controlling ionization upon the concentration of a catalytically competent enzyme. We hypothesize that 17α-hydroxyprogesterone binds to an unprotonated form of the enzyme in a manner which interrupts a *proton shuttle* to the ferric peroxy intermediate, thus facilitating the C-17 side-chain cleavage via peroxide chemistry.

Androgens are synthesized from progesterone and pregnenolone in a two-step reaction. These reactions are the sole route for biosynthesis of the sex hormones. The first step is 17α-hydroxylation; the second step is the cleavage of the C-17 side chain. Both steps require NADPH and molecular oxygen (Lynn & Brown, 1958) and are catalyzed by a single enzyme, CYP17.[1] Both activities copurify from microsomes (Nakajin & Hall, 1981) and are expressed from a single mRNA (Zuber et al., 1986). The intermediate, 17α-hydroxyprogesterone (17-OHP),[2] is not only the precursor for testicular androgens but also the precursor for the adrenal corticoid, cortisol. The factors which regulate between the hydroxylation reaction and the side-chain cleavage reaction and their role in differentiating between the formation of cortisol and androgens are unknown.

The mechanism of cytochrome P450 hydroxylations has been extensively investigated, and the consensus is that the activated oxygen, the "oxene" (formed from heterolytic cleavage of the iron-bound peroxy intermediate, equivalent to an oxoferrylporphyrin radical cation), first abstracts a hydrogen atom from the substrate to form the equivalent of an iron-bound hydroxy radical and a substrate-based radical (Scheme 1). The two radical species then recombine to form the hydroxylated product (White & Coon, 1980; Ortiz de Montellano, 1986; Guengerich & Macdonald, 1990). There is no evidence to indicate that the 17α-hydroxylation of progesterone by CYP17 occurs any differently (Scheme 1).

Scheme 1: Hydroxylation Mechanism



The mechanism of carbon–carbon cleavage catalyzed by cytochromes P450 is not as well understood. At least four cytochromes P450 catalyze carbon–carbon bond cleavage: CYP17, lanosterol 14α-demethylase, aromatase, and cholesterol side-chain cleavage enzyme (Aktar et al., 1982; Sheets et al., 1982; Beusen et al., 1987; Cole et al., 1990; Larroque et al., 1990; Fisher et al., 1991; Korzekwa et al., 1991; Akhtar

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[1] CYP17 has been referred to as 17α/20 lyase, $P450_{17\alpha}$, and $P450_{SCCII}$.

[2] Abreviations: 17α-hydroxyprogesterone, 17-OHP; 17-*O*-acetyltestosterone, AT; pL, L is H or D; deuterium oxide, DOD; water, HOH; maximum velocity, *V*; Michaelis constant, K_m or *K*.

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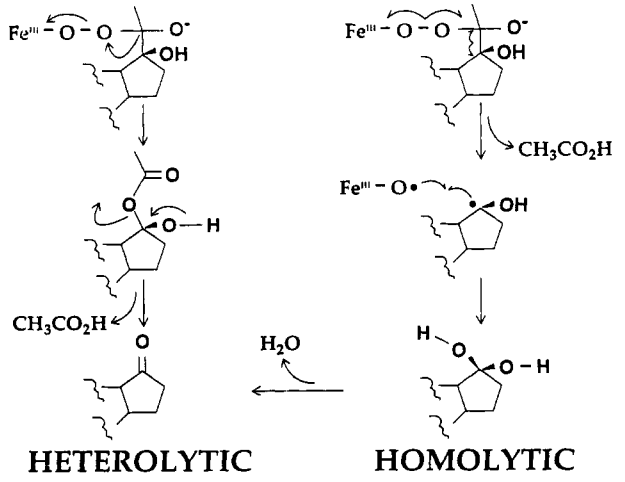
Scheme 2: Side-Chain Cleavage Mechanism



et al., 1993). There is a growing body of evidence implicating the involvement of a substrate-bound *ferric peroxy intermediate* in these reactions. Studies by Akhtar's group (Stevenson et al., 1988; Corina et al., 1991; Miller et al., 1991) as well as by our own (Mak & Swinney, 1992) support involvement of a ferric peroxy intermediate in CYP17 oxidations. We identified 17-*O*-acetoxytestosterone (AT) as a product of CYP17 oxidation of progesterone and proposed that product formation occurred via Baeyer–Villiger rearrangement of the ferric peroxy intermediate. Akhtar and co-workers observed 17α-hydroxyandrost-5-en-3β-ol to be formed from pregnenolone in microsomes from pig testes solely at the expense of cleavage of the C-17–C-20 bond of pregnenolone. Utilizing stable isotope studies, this data was rationalized by invoking the participation of a Fe^{III}–OOH intermediate. These studies indicate that CYP17 can catalzye product formation via peroxide chemistry and are consistent with the hypothesis that androstenedione is formed from 17-OHP via a ferric-peroxy-dependent pathway. The ferric peroxy intermediate could rearrange either heterolytically (via the Baeyer–Villiger rearrangement) to the hemiacetal or homolytically, as suggested by Akhtar for the formation of 17α-hydroxyandrost-5-en-3β-ol, to the gem diol. Hydrolysis of the intermediates results in the formation of androstenedione (Scheme 2).[3]

As stated above, CYP17 is not the only P450 in which evidence for the involvement of peroxide chemistry in substrate oxidations has accumulated. Cleavage of the lanosterol 32-methyl group by lanosterol 14α-demethylase is proposed by Fisher and co-workers (1991) to result from elimination of a 14α-formyloxy intermediate formed by Baeyer–Villiger rearrangement of a putative ferric-bound peroxy intermediate. They reported the isolation of the 14α-formyloxy intermediate and observed its conversion to the final demethylated product. Many mechanisms have been proposed for the final oxidation in the aromatization of the steroid A ring by aromatase, including the involvement of a ferric peroxy intermediate (Aktar et al., 1982; Beusen et al., 1987; Cole et al., 1990; Korzekwa et al., 1991; Akhtar et al., 1993). The experimental evidence to date is still consistent with the involvement of peroxide chemistry, whereas most other proposed mechanisms have been experimentally eliminated. And recently, strong evidence for the involvement of a peroxy-like intermediate in cytochrome-P450-mediated deformylation reactions has been reported by Vaz and co-workers (Roberts et al., 1991; Vaz et al., 1991). Thus, there is a growing body of evidence which strongly supports the assertion that cytochromes P450 can catalyze oxidations via peroxide chemistry as well as oxene chemistry.

Given that cytochromes P450 can catalyze reactions via both oxene and peroxide chemistry, the obvious question is *What factors differentiate between a cytochrome-P450-catalyzed oxidation via oxene chemistry versus one via peroxide chemistry*? This study was initiated to investigate the possibility that protons may play a determining role in differentiating between the oxene and peroxide chemistries associated with product formation.

EXPERIMENTAL METHODS

Materials. [1,2-^{3}H]-17-OHP and [1,2,6,7-^{3}H]progesterone were purchased from New England Nuclear Research Products (Boston, MA). [1,2,6,7-^{3}H]Progesterone was purified by HPLC before use. Nonradioactive steroids were purchased from Sigma Chemical Co. (St. Louis, MO), and deuterium oxide (99.8 atom % D) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents and chemicals were of the highest quality available.

Incubations. Incubations with neonatal pig testes microsomes, prepared by standard procedures (Mak & Swinney, 1992), contained protein (0.025–1.5 mg), NADPH (1 μmol), magnesium chloride (3 μmol), potassium phosphate buffer (100 μmol) at various pHs, and [1,2-^{3}H]-17-OHP or [1,2,6,7-^{3}H]progesterone (25 nmol in 20 μL of MeOH) in a total volume of 1 mL. The incubations were preincubated at room temperature for 10 min prior to initiation with 1 μmol of NADPH. Following agitation at 37 °C for 10 min, all reactions were terminated by the addition of 6 mL of methylene chloride. After mixing and centrifugation of the samples, the aqueous phase was removed and the organic phase was transfered to new tubes. The samples were evaporated under a stream of nitrogen and then were reconstituted with 200 μL of methanol. After sonicating for 1 min, 50 μL of each sample was injected onto the HPLC for analysis. Initial velocities were determined from the microCuries of product formed normalized for time and protein.

For the experiments with deuterium oxide, the potassium phosphate buffer was made in 30% deuterium oxide. The deuterated potassium phosphate buffer was freshly prepared prior to each experiment by mixing deuterium oxide with the phosphate buffer at the appropriate pH. The deuterium oxide did not change the apparent pH reading (Beckman Model 3500 digital pH meter). The pD of the buffers was determined using the equation $pD = pH_{obsd} + 0.3314n + 0.0766n^2$, where n is the fraction of deuterium (Salomaa et al., 1964). The use of 30% DOD results in a calculated change in the observed pH of 0.106 unit, and the buffers containing DOD were adjusted accordingly. The $^Hk/^Dk$ values were determined where the rate of the calculated ^Dk was always measured at 0.1 pL unit greater than that for the calculated ^Hk. We do not believe this adjustment affects the conclusions of these studies.

The pL-dependent isotope effects associated with progesterone metabolism were determined in duplicate experiments and those associated with 17-OHP metabolism in triplicate experiments. Single, representative experiments are shown in Figures 1, 2, and 3. Individual data points were determined in duplicate. The pK values were determined from triplicate experiments and expressed as the mean ± standard deviation.

HPLC. A Jones chromatography (Lakewood, CO) 5-μm, 25-cm ODS column was used for separation of androstenedione

[3] Studies have shown the 17-hydroxyl oxygen to be retained in C-17 side-chain cleavage (Corina et al., 1991). This suggests that either hydrolysis of the gem diol is stereoselective or heterolytic cleavage of the peroxy intermediate is preferred.

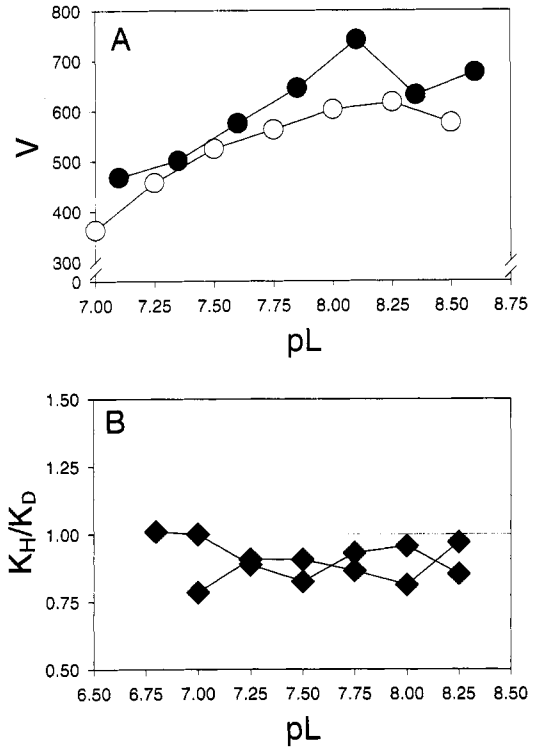


FIGURE 1: Effect of DOD upon the conversion of progesterone to 17α-hydroxyprogesterone in microsomes from pig testes. (A) Effect of pL upon rate. Data are from a single experiment and are representative of other determinations; hollow circles indicate HOH and filled circles 30% DOD. (B) Dependence of the solvent isotope effect upon pL. Data represent two separate experiments.

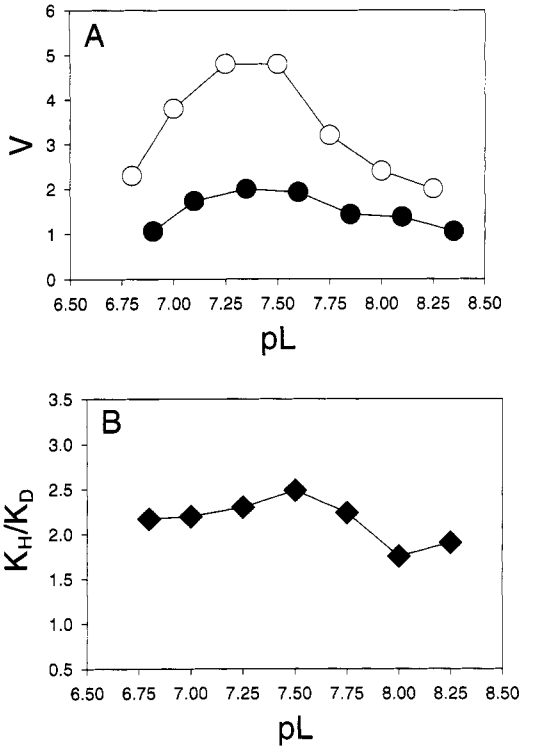


FIGURE 2: Effect of DOD upon the conversion of progesterone to 17-*O*-acetoxytestosterone in microsomes from pig testes. (A) Effect of pL upon rate; hollow circles indicate HOH and filled circles 30% DOD. (B) Dependence of the solvent isotope effect upon pL.

(t_R = 28 min), 17-OHP (t_R = 37 min), and progesterone (t_R = 44 min). The column was eluted with methanol/acetonitrile/tetrahydrofuran/water at 2 mL/min under the following conditions: 3 min isocratic at 5/6/4/85, 25-min gradient to 13/11/8/68 (curve −5), 10-min linear gradient to 17/14.5/11/57.5, 10-min linear gradient to 17/57/11/15, 4 min to 3/90/4/3. AT was eluted from the same column with methanol/acetonitrile/water at 1 mL/min under the following conditions: 95 min isocratic at 30/25/45, 2-min linear gradient to 45/45/10, 10 min isocratic at the same condition, 2-min linear gradient to 30/25/45 (t_R = 73 min). Radioequivalents were detected with a Packard A500 radioflow detector with a flow rate of 5 mL/min.

RESULTS

pL–Rate Profiles and Solvent Isotope Effects—Progesterone Oxidation. The pL profiles and solvent isotope effects associated with progesterone conversion to 17-OHP and AT in microsomes from pig testes are shown in Figures 1 and 2, respectively. The solvent isotope effects associated with the conversion of progesterone to 17-OHP and AT appear to be independent of pL. The formation of 17-OHP was associated with an inverse isotope effect ($^Hk/^Dk$ = 0.75–0.95, Figure 1B), and the formation of AT was associated with a positive isotope effect ($^Hk/^Dk$ approximately 2, Figure 2B). The rates of product formation in these experiments were determined under conditions which were as near to initial velocity as possible for accurate quantitation of AT. However, under these conditions, up to 15% of the 17-OHP formed from progesterone is further oxidized to androstendione. This small amount of conversion to androstenedione was not included in the isotope effect determinations because it did not affect the opposite nature of the isotope effects for 17-OHP and AT formation and the conclusions of the study.

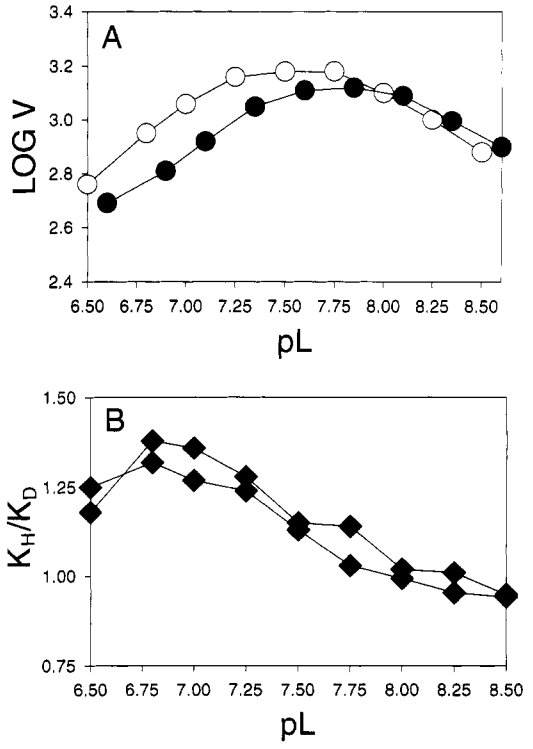


FIGURE 3: Effect of DOD upon the conversion of 17α-hydroxyprogesterone to androstenedione in microsomes from pig testes. (A) Effect of pL upon rate. Data are from a single experiment and are representative of other determinations; hollow circles indicate HOH and filled circles 30% DOD. (B) Dependence of the solvent isotope effect upon pL. Data represent two separate experiments.

17α-Hydroxyprogesterone Oxidation. The pL-dependent oxidation of 17-OHP to androstenedione is shown in Figure 3. A pL-dependent isotope effect was observed. No discernable isotope effect was detected above a pL of 8. These data suggest that, for 17-OHP oxidation, there exists a pL-

Table 1: Kinetic Constants Associated with Oxidation of Progesterone and 17α-Hydroxyprogesterone by CYP17[a]

			HOH			30% DOD		
substrate	product	pL	*V*	*K*	*V*/*K*	*V*	*K*	*V*/*K*
P	17-OHP	7.25[b]	310 (42)	13.5 (4.0)	23.0			
	AT	7.25[b]	2.9, 4.6	6.5, 3.1	1.0			
17-OHP	A	7.25[b]	2125 (392)	4.2 (0.4)	506			
	A	7.0[c]	1873	7.8	240	1267	8.3	153
	A	7.25[c]	1920	8.0	240	1469	8.0	184
	A	7.5[c]	2117	4.2	504	1641	4.1	400
	A	7.75[c]	1960	6.2	316	1936	6.7	289
	A	8.0[c]	1785	6.3	283	1537	6.8	226
	A	8.25[c]	1182	4.3	275	1122	4.8	234

[a] Kinetic constants were determined by the best fit to the Michaelis–Menton equation with the nonlinear regression program Enzfitter (Elsvier Science Publisher BV, The Netherlands). V_{max} (V) is expressed as pmol of product/min/mg of protein, and the apparent K_m values (K) are in μM. Experiments were conducted at pD values which are 0.1 unit greater than pH values. [b] Determinations from multiple experiments, $n = 4$ for P and $n = 13$ for 17-OHP; parentheses indicate standard errors. [c] Kinetic constants with and without DOD were determined in the same experiment.

sensitive group which contributes to the rate of reaction. Extrapolation of the changes in slope of the log V versus pL plots shows the apparent pK of an ionizable group to be shifted from 7.20 ± 0.04 in HOH to 7.51 ± 0.03 in 30% DOD.

DOD also decreased the maximum velocity for androstendione formation with no apparent effect upon K_m (Table 1). Lineweaver–Burk plots at pL of 7.0 and 7.5 show that deuterium acts as a noncompetitive inhibitor of the C-17 side-chain cleavage reaction (plots not shown). Deuterium is apparently acting as a noncompetitive inhibitor by increasing the pK of an ionizable group on the free enzyme and shifting the equilibrium toward the protonated form. Apparently, 17-OHP can not bind to the protonated enzyme in a catalytically competent manner. From this, it is concluded that a free base with a pK of approximately 7.2 is necessary for 17-OHP to bind to CYP17 in a catalytically competent manner.

Kinetic Constants. Similar apparent K_m values of approximately 5–10 μM were observed for both progesterone and 17-OHP oxidation (Table 1). The maximal rate of 17-OHP formation from progesterone was approximately 100 times greater than the rate of AT formation. The capacity (V/K) of the enzyme to catalyze side-chain cleavage of 17-OHP was over 500 times greater than its capacity to form AT from progesterone.

DISCUSSION

This study was initiated in order to investigate the role of protons in differentiating between the oxene and peroxide chemistry catalyzed by CYP17. From the analysis of the data, assuming that 17-OHP is formed by oxene chemistry and AT and androstenedione are formed by peroxide chemistry, we observed that the balance between the two is influenced by both the fraction of deuterium and the pL.

The push–pull mechanism for regulation of cleavage of ferric peroxy intermediates suggests that O–O cleavage is facilitated by protonation of the ferric peroxy intermediate (pull effect) (Dawson et al., 1976; Poulos, 1987; Yamaguchi et al., 1993). The protonation and cleavage can be kinetically differentiated into two steps, an initial acid–base equilibrium step (1) followed by the O–O cleavage step which does not involve another proton transfer (2). Reversible substrate protonation, if followed by a slow step not involving another proton transfer, would give specific hydrogen-ion catalysis rather than general acid catalysis (Kresge et al., 1987; Kresge, 1987). Rapid and reversible proton transfer from catalyst to substrate has been associated with inverse deuterium solvent isotope effects (Keeffe & Kresge, 1986). This is a result of DOD, a weaker base than HOH, shifting the acid–base equilibrium in the same direction as a decrease in pH.[4]

$$Fe^{3+}\text{–O-O}^- + HA \rightleftharpoons Fe^{3+}\text{–O-O-H} + A^- \quad (1)$$

$$Fe^{III}\text{–O-O-H} \rightleftharpoons [Fe\text{–O}]^{3+} + {}^-OH \quad (2)$$

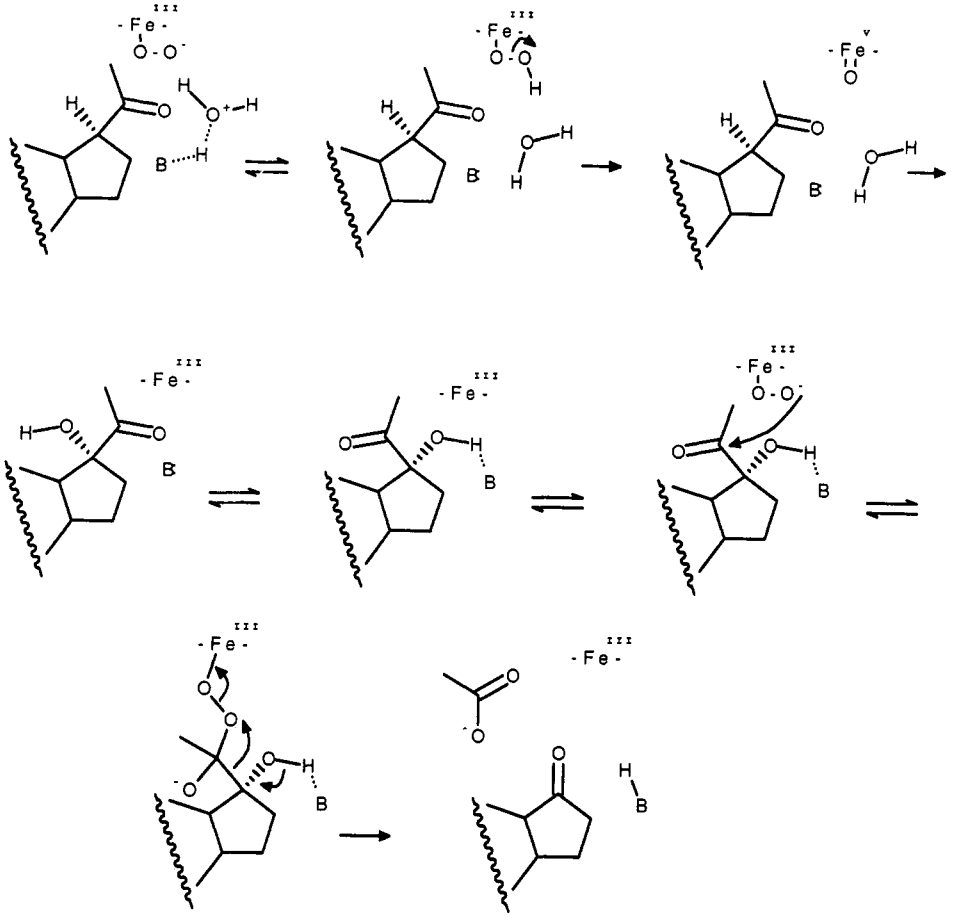
We propose the opposite relationship for the isotope effects associated with progesterone oxidation to 17-OHP and AT to be consistent with the effect of the fraction of deuterium upon acid–base equilibrium of the ferric peroxide intermediate (Figures 1 and 2). The proton is transferred to the ferric peroxy anion intermediate in a preequilibrium step followed by rate-limiting cleavage of the peroxy -O–O- bond in a step not involving proton transfer. The inverse solvent isotope effect reflects a *specific acid–base-catalyzed* mechanism and is the result of the effect of DOD upon the concentrations of [Fe^{III}–O-O^-] and [Fe^{III}–O-O-H]. This change in the equilibrium is proposed to be influenced by the fraction of protic sites which are exchanged with deuterium, not the pL. Thus, the isotope effects are made possible by the rate-limiting cleavage of the ferric peroxy -O–O- bond. These results are not consistent with a strong contribution from the "push effect" as deuterium should decrease the ability of the thiolate 5th ligand to donate electrons through the heme. This would stabilize the O–O bond, facilitate peroxy reactions, and result in a positive isotope effect for AT formation.

The observation of a dependence of the isotope effect upon pL for androstenedione formation from 17-OHP suggests that the solvent isotope effect upon side-chain cleavage was due to the shifting of the pK of a rate-controlling ionization in DOD rather than to an effect on the rate of catalysis. Upon determining the apparent pK, a shift from approximately 7.20 for HOH to 7.51 for 30% DOD was observed. This indicates that some functionality with a pK of approximately 7.2 is contributing to the rate of C-17 side-chain cleavage of progesterone. From this data and the observation that DOD acts as a noncompetitive inhibitor, we have postulated that this is consistent with a pL-dependent equilibrium between two enzyme states, of which only the unprotonated state can bind with 17-OHP to lead to the products of C-17 side-chain cleavage. We hypothesize that the unprotonated form of the enzyme interacts with 17-OHP through hydrogen bonding with the C-17 hydroxy group and, in doing so, interrupts a *proton shuttle* to the ferric peroxy complex, thereby facilitating reactions via peroxide chemistry by limiting the "pull effect".[5] The lack of a pL-dependent effect for progesterone oxidation to 17-OHP and AT is consistent with its inability to hydrogen

[4] Most acid-catalyzed reactions proceed more readily in DOD than in HOH. Deuterium oxide is believed to be less basic than water. The lower basicity of deuterium oxide is caused at least in part by the decrease in the O–H stretching force constant in going from water to oxoniom ion which is observed in the infrared spectra (Bethell & Sheppard, 1953). Thus, the zero-point energies of H–O and D–O bonds are closer in the ion than in water, making the equilibrium toward the ion less favorable in DOD than in HOH. Thus, DOD is a weaker base and a stronger acid than HOH.

[5] The C-17 hydroxy group of 17-OHP may also contribute to increasing the rate of the peroxy reactions by internal hydrogen bonding to the C-20 carbonyl. This should lower the activation energy for formation of the substrate-bound peroxy intermediate by increasing the electrophilic character of the C-20 carbon. In addition, it should stabilize the newly formed intermediate and increase the probability for reaction in the forward direction.

Scheme 3: Proposed Mechanism of CYP17 Oxidation[a]



[a] Water coordinated to the active site base transfers a proton to the ferric peroxy anion intermediate (step 1). The protonated intermediate heterolytically cleaves to form the activated "oxene" (step 2), which catalyzes the 17α-hydroxylation of progesterone (step 3). The 17α-hydroxy group coordinates to the active site base and prevents access of another molecule of water (step 4) and protonation of the newly formed ferric peroxy anion intermediate (step 5). The anion intermediate nucleophilically attacks the C-20 carbonyl group of the substrate (step 6) to form an enzyme-bound substrate intermediate which decomposes to the products acetate and androstenedione with the assistance of the active site base (step 7).

bond through a C-17 substituent and distinguish between different states of enzyme protonation. Accordingly, a base involved in the proton shuttle must accept protons from a proton donor (possibly HOH or DOD) before progesterone binds in a catalytically competent manner and then shuttle those protons to the ferric peroxy intermediate at the appropriate time (Scheme 3).

A specific interaction of 17-OHP with the enzyme may also help explain the difference in relative rates observed between the first reaction (C-17 hydroxylation) and the second (C-17 side-chain cleavage) in androstenedione formation from progesterone. The first enzyme reaction catalyzes progesterone oxidation to products primarily via oxene chemistry, whereas peroxide chemistry is thought to dominate the second reaction. The V/K associated with the products of oxene chemistry was 23 times greater (17-OHP) than that associated with peroxide chemistry (AT) for the first reaction; however, the capacity (V/K) for the enzyme to catalyze the products of peroxide chemistry increased over 500-fold, and the overall enzyme capacity increased over 20-fold for the second reaction (Table 1). To explain these observations in terms of the hypothesized mechanism, the acid–base equilibrium in eq 1 must lie to the right for C-17 hydroxylation and to the left for C-17 side-chain cleavage. Obviously, the introduction of the 17-hydroxy group dramatically increases the rate of product formation via peroxide chemistry and must prevent a shift to the right in eq 1. These observations are consistent with the proposal that 17-OHP interacts with an active site base to interupt a proton shuttle to the ferric peroxide anion and facilitate product formation via peroxide chemistry.

Kitamura et al. (1991) recently observed that the conversion of Arg 346 to alanine abolished lyase activity associated with rat testicular CYP17 and resulted in an accumulation of 17-OHP. The lyase activity was partially recovered by conversion of this mutant to Lys 346. They identified this residue as part of an arginine-rich region in the putative active site and suggested that it interacts with the 17α-hydroxyl group of 17-OHP. If the microscopic pK_a value for Arg 346 is significantly decreased in the active site of CYP17, Arg 346 could be the active site base we have hypothesized to play a role in catalysis.[6] Our data is consistent with their observation that Arg 346 is involved in C-17 side-chain cleavage and not involved in 17-OHP formation.

We interpret our results to be consistent with the following mechanism for androgen formation by CYP17. A molecule of water accompanies progesterone into the active site of CYP17 to assist acid–base catalysis via the active site base

[6] The pK_a of free arginine is approximately 12 and inconsistent with Arg 346 participating as a base catalyst since it should be protonated under physiological conditions. However, there is precedence for a significant decrease in the actual microscopic pK_a values associated with the functional groups of an enzyme active site. The pK_a for lysine in the active site of acetoacetate decarboxylase was shown to be decreased by 4 pK units (Schmidt & Westheimer, 1971), and Neidhart et al. (1991) have postulated a similar magnitude decrease for the pK_a of a lysine residue in mandelate racemase. It is possible that the proximities of Arg 357, Lys 360, and Arg 361 could destabilize the positive charge of Arg 363 and increase its acidity.

(possibly Arg 346); however, when 17-OHP is formed, it binds to the base and prevents the access of another molecule of water (Scheme 3). This interaction could contribute to a concerted oxidation mechanism by holding 17-OHP in the active site and interupting the proton shuttle. In addition, an active site base could also assist in the direct decomposition of the ferric peroxy substrate intermediate to acetate and androstenedione (structure 7, Scheme 3).

The implications of these findings with regards to other multifunctional cytochromes P450, i.e., aromatase and lanosterol 14α-demethylase, suggest that these enzymes must somehow regulate protonation of the peroxy intermediate either via the ability to exclude water from the active site or via the assistance of an active site base. Protonation may also contribute to nitric oxide synthase oxidation of arginine to citrulline and nitric oxide. Marletta (1993) has proposed the involvement of an oxene-dependent oxidation in the formation of the *N*-hydroxyarginine intermediate followed by a peroxy-dependent elimination of NO. It is tempting to postulate that the *N*-hydroxyarginine intermediate may also bind to an active site base to initiate the second oxidation and interupt a proton shuttle to the heme.

Since all cytochromes P450 are thought to have a similar catalytic cycle, they must all have some potential to catalyze oxidations via peroxide chemistry. The rate of product formation via peroxide chemistry would depend upon the suitability of the substrate to undergo these types of oxidations and the position of equilibrium between protonated and unprotonated forms of the ferric peroxy intermediate. As long as the reaction remains specific acid catalyzed, the equilibrium should allow for the formation of a finite concentration of the ferric peroxy anion. Vaz et al. (1991) have observed cytochrome-P450-dependent oxidative deformylation of aldehydes in the presence of hydrogen peroxide. These data were interpreted to support the involvement of peroxy intermediates in deformylation reactions. In light of the proposal presented in this report, we suggest that the ability of these reactions to use hydrogen peroxide as an alternative oxidant is a function of the preequilibrium step in the formation of the active oxygen species. The ferric peroxy anion formed via the preequilibrium would initiate the peroxide chemistry.

These studies were initiated to determine if the state of protonation of CYP17 in any way influences the oxene or peroxide chemistry which is thought to differentiate between product formation. We observed that the ratio of the products of the two chemistries is influenced by both the fraction of deuterium and the pL. These observations strongly suggests that protons do influence the chemistry of CYP17 catalysis. We have proposed a mechanism whereby the chemistry and the product identity is essentially determined by the state of protonation of the ferric peroxy intermediate. Further studies are needed to determine what other factors may also influence the catalytic chemistry.

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