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Cooperative Effects on Radical Recombination in CYP3A4-Catalyzed Oxidation of the Radical Clock β -Thujone**

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Abstract

The oxidation of hydrocarbons by cytochrome P450 enzymes is commonly thought to involve hydrogen atom abstraction by a ferryl species comparable to that of peroxidase Compound I, followed by radical recombination of the resulting carbon radical with the equivalent of an iron-bound hydroxyl radical.^{1,2} This radical rebound mechanism, first proposed in 1978, is supported by a variety of experimental results, including (a) rearrangement and inversion reactions prior to the radical recombination step, (b) the large magnitude (up to $k_H/k_D \sim 13$) of the intrinsic isotope effect for hydrogen abstraction, and (c) computational modeling of the reaction pathway. However, radical clock substrates, in which the radical undergoes a rearrangement at a known rate prior to radical recombination, have provided conflicting evidence on the radical lifetime. Although several radical clocks support a radical recombination mechanism, so-called ultrafast radical clocks yield radical lifetime estimates more consistent with a transition state than an actual intermediate.³ This discrepancy has led to postulates that hydroxylation may involve concerted insertion into the C-H bond or the involvement of multiple oxidizing species. An alternative explanation is provided by computational studies that invoke a reaction manifold with a radical intermediate that exists in two different spin states.⁴ A further possible explanation is provided by the observation that ultrafast radical clocks generally involve primary radical rearrangements, whereas slower radical clocks generally involve secondary radical rearrangements. The recombination rates of primary and secondary radicals may be differentially susceptible to modulation by interactions with the active site and the iron-oxo species. However, there is little direct evidence that the radical complex exists in two different spin states, or that the radical recombination rates can be influenced by the active site environment.

Keywords

cytochrome P450; radical reactions; allosterism; carbon hydroxylation; reaction mechanisms; ferryl

CYP3A4 is responsible for a majority of all P450-catalyzed drug oxidations in humans. One of the salient features of CYP3A4 catalysis is that it is subject to homotropic and heterotropic cooperativity (allosterism) in that one substrate molecule can alter the oxidation rate and/or regioselectivity of another.⁵ The molecular basis of P450 allosterism remains incompletely understood. Currently, most results fall into three models: multi-substrate binding, peripheral effector binding, and conformational heterogeneity.⁶ Progesterone has

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specifically been shown to function as an allosteric modulator of its own CYP3A4-catalyzed oxidation as well as that of other substrates.^{7,8} Given that allosterism tunes the CYP active site environment, its effect on the reaction of a radical clock substrate may provide valuable mechanistic information associated with the CYP mechanism. In this communication we report that the oxidation of a radical clock, β -thujone (**1**, shown as its C4 radical in Scheme 1), by CYP3A4 is subject to a concentration-dependent heterotropic cooperative effect of progesterone binding on the manifold of products that derive from C4 oxidation. The results are consistent with acceleration by progesterone of the radical recombination that traps the C4 radical with concomitant suppression of all the rearrangement pathways.

We have previously shown that α - and β -thujones function as two-zone radical clocks in that C4 oxidation triggers two concurrent timing reactions, one involving ring opening of the cyclopropyl ring and the other inversion of the stereochemistry of the C4 methyl group (Scheme 1).⁹ The α - and β -thujone C4-radical ring-opening reaction rates are $4.4 \times 10^7 \text{ s}^{-1}$ and $1.0 \times 10^8 \text{ s}^{-1}$, respectively, both of which are consistent with the lifetimes of radical intermediates. This is also consistent with the observation of a significant extent of methyl inversion in these reactions. In addition to hydroxylated products, this reaction manifold gives rise to two minor desaturated products, one with a C4–C10 double bond and another (**6**, carvacrol) in which the ring aromatizes to give a phenol product. Formation of these two products was attributed to a second oxidation of the C4 radical intermediate. The latter was previously shown to be generated from a cationic intermediate.¹⁰

In this study the CYP3A4 reaction of α - or β -thujone (100 μM) was carried out in the presence of a concentration of progesterone increasing from 0 to 150 μM . The incubation mixture contains purified, recombinant human CYP3A4, NADPH-cytochrome P450 reductase, and cytochrome b_5 in a 1:2:1 ratio, respectively, all in a reconstituted lipid environment.¹¹ The identified metabolites include 7-hydroxyl- α (or β)-thujone, 7,8-dehydro- α -thujone, 2-hydroxyl- α (or β)-thujone, 4-hydroxyl- α -thujone (**3**), 4-hydroxyl- β -thujone (**2**), carvacrol (**6**), and the ring-opened product **5**.⁹ These are all the major metabolites formed in the reaction and account for ~95% of the total conversion yield for α -thujone and ~90% for β -thujone. 4,10-Dehydrothujone was previously identified in the reactions of the thujones with P450cam and P450_{BM3} but not with mammalian P450 enzymes.⁹ This metabolite was found to conjugate with glutathione in the CYP3A4 solution and the conjugate could not be extracted with the hydrophobic metabolites (Data not shown). It was reported recently for norcarane that desaturation products and their secondary metabolites might compromise its use to measure radical lifetimes.^{12,13} This was not observed in the CYP3A4 oxidation of thujones, for which no secondary metabolites of the desaturated products were detected at a significant level (>1%, see Figure S1).

The distribution and yields of the metabolites in the CYP3A4 oxidation of β -thujone in the presence of various concentrations of progesterone are summarized in Tables 1 and S1. Not unexpectedly, a decrease from 15.4 to 11.7% in the proportion of 7-hydroxylation of β -thujone is observed as the progesterone concentration rises from 0 to 150 μM . A smaller progesterone-dependent decrease is also seen in 2-hydroxylation and desaturation of the isopropyl group. These conventional metabolic shifts reflect repositioning of the substrate prior to the oxidation step and provide no information on the reaction mechanism. However, the alterations in the product distributions that stem from oxidation of C4, the carbon that is pivotal for the radical clocks mechanisms of β -thujone (Scheme 1), are more informative. As shown in Figure 1, the proportion of 4-hydroxy- β -thujone (**2**), the unrearranged product, increases systematically from 65.6% to 78.3% as the progesterone concentration increases from 0 to 150 μM . In contrast, there is a corresponding systematic decrease in the yields of 4-hydroxy- α -thujone (**3**), in which the methyl stereochemistry is inverted (from 10.7% to 6%), metabolite **5** in which the cyclopropyl ring is opened (from 0.61% to 0.24%), and

carvacrol (**6**), the aromatic product produced by cyclopropyl ring opening and cation formation (from 3.5% to 1.5%). A direct correlation exists between the decrease in the combined rearrangement products (**3** + **5** + **6**) and the increase in the unrearranged alcohol (**2**) (Figure 2). This shows that the loss in the rearranged products is quantitatively correlated with the increase in formation of the unrearranged alcohol (**2**). While it has been well documented that allosterism alters the reaction rates and regioselectivity of CYP oxidation on the different reaction sites of a substrate, to our knowledge, this is the first report of allosteric modulation of the product distribution profile derived from a single reaction site. This differs from a conventional metabolic shift in that it can only happen after the hydrogen abstraction reaction. As all of these reactions stem from initial C4 oxidation, this result demonstrates that the changes in the product profile depend on progesterone allosteric effects *associated with or subsequent to the C4 hydrogen abstraction reaction*.

The oxidation of α -thujone, in contrast to β -thujone, shows a progesterone concentration-dependent increase in 2-hydroxylation compensated by a decrease in 7-hydroxylation, but no systematic changes are observed in the products from C-4 oxidation (Table S2). While the formation of 4-hydroxy- β -thujone (**2**) and carvacrol slightly decreases with increasing progesterone concentration, the trends in the formation of the other two C4 products are ambiguous.

The results from the oxidation of β -thujone indicate that the alteration of the CYP3A4 active site cavity caused by the simultaneous binding of progesterone decreases the *apparent* lifetime of radical **1** generated from β -thujone by the P450 activated species. The apparent radical recombination rate changes from $1.3 \times 10^{10} \text{ s}^{-1}$ in the absence of progesterone to $3.5 \times 10^{10} \text{ s}^{-1}$ in the presence of 150 μM progesterone (Table 1). These numbers represent the ratio of the C4 products **2**, **3**, plus **5** over the ring opened product **5** multiplied by the rate of the ring opening reaction.⁹ The apparent change in the radical lifetime can be rationalized if (a) the binding of progesterone restricts the motion of the β -thujone radical in a manner that favors radical recombination, effectively accelerating the trapping of the unrearranged radical, or (b) reduces the rates of the rearrangements themselves. In either case, the decrease in the formation of all the rearrangement products results in an apparent change in radical lifetime. If the observed radical lifetime reflects the ratio between high spin and low spin Cpd I species, as suggested by one theory,⁴ this result would indicate that the low spin Cpd I species with lower energy barrier increases in response to progesterone allosterism. However, the observed apparent radical rebound rate change is more to result from allosterically-induced physical changes in the enzyme active site.

The observation of products derived from cationic intermediates has been used as an indicator of the involvement of alternant oxidizing species in the P450 catalytic cycle, one of which involves a direct insertion of a hydroxyl cation into the C-H bond.³ In the present study the cationic product, carvacrol (**6**), decreases in a trend very similar to that of the radical rearrangement products **5** and **3** as a result of the progesterone allosteric effect (Figure 1). The coordinated changes we observed among the four C4 products strongly suggests that the cationic product carvacrol shares the same precursor as the other radical products and is a secondary product of the C4 radical. This is consistent with our previous observation that the C4 product distribution profile was not altered by a highly-deuterated medium.⁹

In conclusion, we have observed that progesterone exerts heterotropic cooperative effects on the C4 derived oxidation product profile in the CYP3A4-catalyzed oxidation of β -thujone. Our results demonstrate (a) that all the C-4 derived products stem from a common radical precursor, and (b) that the rate of radical recombination to give the unrearranged hydroxylated product is accelerated by the allosteric effector. This is the first demonstration

that the apparent timing of a radical clock can be specifically altered by modulation of the active site topology or properties by an allosteric effector.

Experimental Section

Chemicals

Synthesis of β -thujone (99%) and the metabolite standards have been reported previously.⁹ L- α -dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), L- α -diloleoyl-*sn*-glycero-3-phosphocholine (DOPC), and L- α -dilauroyl-*sn*-glycero-3-phosphoserine (DLPS) were from Avanti Polar Lipids Inc (Alabaster, AL). These lipids were used to reconstitute the membranes required for CYP3A4 reaction.¹¹

Enzymes

Human CYP3A4, rat cytochrome P450 reductase (CPR), and rat cytochrome b₅ were expressed and purified according to published protocols.^{14–16} Optical spectra were recorded on a CARY UV-visible scanning spectrophotometer (Varian) in 100 mM potassium phosphate buffer (pH 7.4) at 20 °C.

GC-MS Analyses of Thujones and their Metabolites

GC-MS instrument and methods used to analyze thujones and their metabolites were previously described.⁹

Enzyme Incubations

The CYP3A4 reconstitution system was prepared according to a published protocol.¹¹ The ratio of CYP3A4, cytochrome P450 reductase, and b₅ was 1:2:1. To the reconstituted enzyme solution was added α - or β -thujone (100 μ M in CH₃CN) and the corresponding amount of progesterone in methanol (5 μ L). After incubation at 37 °C for 3 min, the reaction was initiated by addition of NADPH (1 mM). The final reaction volume was 1 mL, containing CYP3A4 (1 μ M) and substrate (100 μ M). The reaction mixtures were incubated at 37 °C for 1 h and quenched by adding ice-cold ethyl acetate (2 mL). The aqueous phase was saturated with NaCl and further extracted with ethyl acetate (2 mL). Control reactions were carried out without enzymes, and separately, without NADPH. The combined organic extract was dried over anhydrous Na₂SO₄, filtered, and evaporated to 50–100 μ L under a stream of nitrogen at room temperature for GC-MS analysis of a 3.0 μ L aliquot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Percents of 4-hydroxylated β -thujone (**2**), 4-hydroxylated α -thujone (**3**), cyclopropyl ring-opened thujone alcohol (**5**), and the aromatized product carvacrol (**6**) formed in the oxidation of β -thujone by CYP3A4 as a function of the progesterone concentration.

Figure 2.

Increase in the yield of the unarranged compound **2** versus the combined yields of the rearranged products **3** + **5** + **6**, showing a direct correlation between the increase of **2** at the expense of the rearranged products. The data points from left to right correspond to the following progesterone concentrations: 0, 5, 25, 50, 80, 100, 150 μM .

Scheme 1.

Reaction manifold showing the intermediate generated by C-4 hydrogen abstraction from β -thujone and the products that derive from this initial intermediate.

Table 1

Allosteric effects of progesterone on the CYP3A4 metabolism of β -thujone at C4^{a,b}

PG (μ M)	4OH β T 2	4OH α T 3	ROP 5	Carv 6	k_1^c ($\times 10^{10}$ s ⁻¹)
0	65.6 (1.00)	10.7 (1.00)	0.61 (1.00)	3.5 (1.00)	1.3
5	68.9 (1.05)	9.9 (0.92)	0.54 (0.88)	3.3 (0.94)	1.5
25	72.8 (1.11)	8.4 (0.78)	0.4 (0.66)	2.5 (0.71)	2.0
50	74.2 (1.13)	7.5 (0.70)	0.39 (0.64)	2.2 (0.63)	2.1
80	75.8 (1.16)	7.0 (0.65)	0.33 (0.53)	1.9 (0.54)	2.5
100	77.2 (1.18)	6.6 (0.62)	0.28 (0.46)	1.7 (0.49)	3.0
150	78.3 (1.19)	6.0 (0.56)	0.24 (0.39)	1.5 (0.43)	3.5

^aThe yield of the product is given as a percent of the total products. In parenthesis is the relative amount of the product at a given progesterone concentration relative to the yield, set at 1.0 for each metabolite, in the absence of progesterone. The plots in Figure 2 are based on these relative yields. The abbreviations are: PG, progesterone; 4OH β T, 4-hydroxy- β -thujone (2); 4OH α T, 4-hydroxy- α -thujone (3); ROP, ring opened product 5; Carv, carvacrol (6).

^bThe results are the average values from a set of three incubations.

^cThe value of 1.0×10^8 s⁻¹ for the rate of cyclopropyl ring opening of β -thujone was employed in calculating the recombination rates. $k_1 = (1.0 \times 10^8) \times ([4OH\beta T] + [4OH\alpha T]) / ROP$.