An Incredibly Fast Apparent Oxygen Rebound Rate Constant for Hydrocarbon Hydroxylation by Cytochrome P-450 Enzymes

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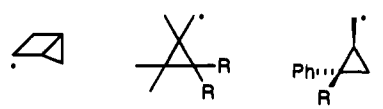
The consensus mechanism[2] for hydrocarbon hydroxylation by cytochrome P-450 enzymes involves hydrogen atom abstraction from the hydrocarbon by a high-valent iron−oxo species followed by homolytic substitution by the alkyl radical thus formed in the so-called "oxygen rebound" step[3] (Scheme 1). Alternative mechanisms that have been considered are direct "oxene" insertion into the C−H bond and formation of a cationic intermediate by hydride abstraction or hydrogen abstraction followed by electron transfer. Strong qualitative evidence for the radical pathway of Scheme 1 has come from studies with probe substrates such as deuterium-substituted cyclohexene[4] and bicyclo[2.1.0]pentane[5] that were oxidized by P-450 to give rearranged products, thus implicating some type of intermediate. A requisite cationic intermediate was excluded because substrates such as norcarane[6] and methylcyclopropane[5] were oxidized at the cyclopropylcarbinyl positions without rearrangement.

Given the importance of qualitative radical probe results in the deduction of the P-450 mechanism, the results of quantitative "radical clock"[7] studies are disturbing. Ortiz de Montellano and Stearns reported that bicyclo[2.1.0]pentane was hydroxylated by P-450 to give both unrearranged and rearranged alcohol products before the rate constant for ring opening of the bicyclo[2.1.0]pentan-2-yl radical (**1**) was determined.[5] In an attempt to measure the rate constant of the oxygen rebound step (k_{ox} in Scheme 1), Atkinson and Ingold[8] employed a series of calibrated radical clocks including **1** and calculated k_{ox} values from the ratios of unrearranged and rearranged oxidation products.[9] For



1 **2**: R = H, **3**: R = Me **4**: R = H, **5**: R = Ph

the substrates that would give radicals **1**−**5**, they[8] found apparent rate constants for oxygen rebound of 1.4×10^{10} (**1**), $(2.3-2.5) \times 10^{11}$ (**2** and **3**), and $(2-7) \times 10^{12}$ s^{-1} (**4** and **5**). These disparate results were rationalized in part by noting that radical clocks **4** and **5** might have "run slow" in the enzyme's active site due to steric effects,[8] but it is now known that both enantiomers of the hydrocarbon precursor to **4**, which almost certainly interact differently with the enzyme, give comparable apparent k_{ox} values upon P-450 hydroxylation.[10] We now report results of cytochrome P-450 hydroxylation of a new hypersensitive radical probe that are more clearly inconsistent with the radical pathway in Scheme 1.

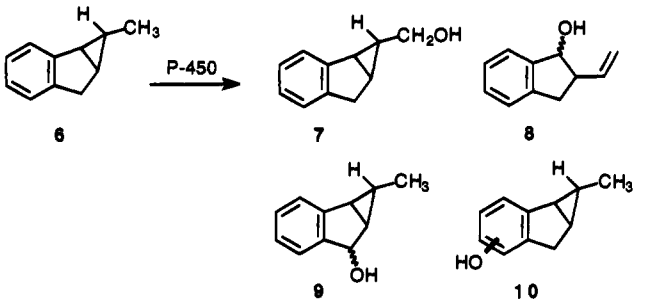
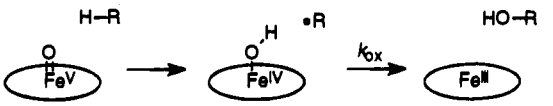


Figure 1. Products from cytochrome P-450 hydroxylation of **6**.

Scheme 1



Oxidations[11] of hydrocarbon **6** with rat liver microsomal P-450 and with the purified P-450 isozyme CYP2B1[12] from rat gave the products shown in Figure 1 in 0.5−4.8% total yield. Authentic samples of **7**, both diastereomers of **8**, and both diastereomers of **9** were prepared for mass spectral characterizations.[13] A minor oxidation product was tentatively assigned as a phenol (**10**) from its mass spectral fragmentation pattern. GC response factors for products **7** and **8**, the products arising from initial oxidation at the methyl position in **6**, were determined against a standard with mixtures of authentic samples. Response factors for **9** and **10** were estimated.

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(2) For discussions of the mechanism of cytochrome P-450-catalyzed oxidations of unactivated hydrocarbons, see the following: McMurry, T. J.; Groves, J. T. In *Cytochrome P-450 Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986; Chapter 1. Ortiz de Montellano, P. R. In *Cytochrome P-450 Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986; Chapter 7. Woggon, W.-D.; Fretz, H. In *Advances in Detailed Reaction Mechanisms*; Coxon, J. M., Ed.; JAI: Greenwich, CT, 1992; Vol. 2, pp 111−147.

(3) Groves, J. T.; McClusky, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154−160.

(4) Groves, J. T.; Subramanian, D. V. *J. Am. Chem. Soc.* **1984**, *106*, 2177−2181.

(5) Ortiz de Montellano, P. R.; Stearns, R. A. *J. Am. Chem. Soc.* **1987**, *109*, 3415−3420.

(6) White, R. E.; Groves, J. T.; McClusky, G. A. *Acta Biol. Med. Ger.* **1979**, *38*, 475−489.

(7) The term "radical clock" was applied in a seminal review of the concept by Griller and Ingold. See the following: Griller, D.; Ingold, K. U. *Acc. Chem. Res.* **1980**, *13*, 317−323.

(8) Atkinson, J. K.; Ingold, K. U. *Biochemistry* **1993**, *32*, 9209−9214.

(9) See also the following: Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1991**, *113*, 5699−5707.

(10) Atkinson, J. K.; Hollenberg, P. F.; Ingold, K. U.; Johnson, C. C.; Le Tadic, M.-H.; Newcomb, M.; Putt, D. A. *Biochemistry* **1994**, *33*, 10630−10637.

(11) The microsomes were prepared from livers of phenobarbital-treated rats as previously described.[10] The microsomal oxidation method was as before;[10] the reaction contained 5 nmol of P-450 enzymes. Purified CYP2B1[12] (0.6 nmol) was reconstituted with reductase (1.2 nmol) and dilauroylphosphatidylcholine (96 nmol) in a total volume of 2 mL of buffer, and the oxidations were conducted as previously described.[10]

(12) Nelson, D. R.; Kamataki, T.; Waxman, D. J.; Guengerich, F. P.; Estabrook, R. W.; Feyereisen, R.; Gonzalez, F. J.; Coon, M. J.; Gunsalus, I. C.; Gotoh, O.; Okuda, K.; Nebert, D. W. *DNA Cell Biol.* **1993**, *12*, 1−51.

(13) Alcohol **7**[14] was prepared by $LiAlH_4$ reduction of the corresponding ethyl ester.[15] Substrate **6**[14] was prepared from alcohol **7** by *in situ* conversion to the mesylate (methanesulfonyl chloride in THF at −5 °C) followed by reaction with $NaBHEt_3$; the mixture was treated with mcpba to epoxidize olefinic side products, and **6** was purified by silica gel chromatography. Reaction of the mesylate of **7** in water gave both diastereomers of **8**,[14] which were purified (as a mixture) by silica gel chromatography; we tentatively assign the structure of **8a** as *trans* and that of **8b** as *cis*. Compounds **9** were prepared as reported.[16]

(14) Characterized by ^1H and ^{13}C NMR spectroscopy and HRMS.

(15) Martin-Esker, A. A.; Johnson, C. C.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **1994**, *116*, 9174−9181.

(16) Friedrich, E. C.; Taggart, D. B.; Saleh, M. A. *J. Org. Chem.* **1977**, *42*, 1437−1443.

(17) A complete table of results is in the supplementary material.

(18) In one control, the analysis of an enzyme product mixture was repeated 24 h after the initial analysis; the U/R ratio decreased from 30 to 14. In another, a mixture of **7** and **8** with an initial **7**/**8** ratio of 71 was analyzed 24 h after preparation and was found to have a **7**/**8** ratio of 46. Compound **7** (but not compounds **8**) partially decomposes thermally in a GC injection port at >125 °C to give 2-vinylindene. 2-Vinylindene was not observed in product mixtures from P-450 oxidations of **6** when the GC injection port temperature was maintained below 125 °C.

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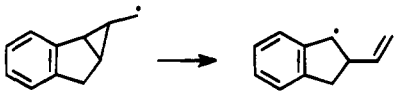
Oxidation of **6** occurred predominantly at the benzylic position, giving alcohols **9**. The products formed from oxidation of the methyl group in **6** are **7** and **8**; the ratio of unrearranged (**7**) to rearranged (**8**) products (U/R) was 48 ± 13 (1σ) for 11 CYP2B1 oxidations and 37 for a microsomal oxidation. In time course studies, enzyme activity was maintained, and the U/R ratio was consistent.[17] Control reactions showed that the values of U/R are minimum values[18] and that **6**, **7**, and **8** were recovered in high yields.[19]

Radical **11** is the putative intermediate for **7** and **8**. At 37 °C, ring opening of **11** to radical **12** occurs with $k_r = 3 \times 10^{11}$ s^{-1}.[15] The structural constraints in **11** ensure that steric crowding



11 **12**

in the enzyme's active site will have little effect on the dihedral angle between the aromatic system and the breaking bond, and, in any event, the kinetic results with **11** and related constrained systems showed that the rates of ring opening were essentially insensitive to these dihedral angles.[15] In the context of the radical formation−oxygen rebound mechanism for P-450 hydroxylation, the U/R product ratios require an incredible apparent k_{ox} value of 1.4×10^{13} s^{-1}.[20]

One might consider how the results could be in error. Selective oxygen rebound trapping of an equilibrating pair of radicals is not possible because the rate constant for cyclization of ring-opened radical **12** is too small.[21] Similarly, formation of radical **12** that was not trapped is unlikely because ring-opened products from "slow" clocks are formed[5,8,9] and the fate of untrapped **12** cannot be explained; mechanism-based inhibition clearly did not occur. Nevertheless, one might concede a possible factor of 2 error in the ring opening rate constant of **11**, which would give an apparent k_{ox} value from hydroxylation of **6** that was just at an acceptable upper rate limit, *i.e.*, equal to the rate constant for decomposition of a transition state.[20]

Even if one concludes that the apparent k_{ox} value for hydroxylation of **6** is possible, the combination of our results with those from other quantitative studies[5,8,10] indicates that there is an error either in the mechanism of P-450 hydroxylation or in the kinetic scale for fast radical reactions. According to the mechanism for P-450 hydroxylation in Scheme 1, one would expect that the amounts of rearranged alcohols from the corresponding probes will correlate with the rate constants for rearrangement of the putative radical intermediates, but Figure 2 shows that they do not. Figure 2 is a scatter plot in the formalism of a linear free energy relationship. It has a least-squares slope of 0.18 ± 0.65 at the 95% CI, and the correlation coefficient is quite poor ($r = 0.37$). Figure 2 demonstrates graphically the fact that one cannot reconcile apparent lifetimes of putative radical intermediates that differ by 3 orders of magnitude ($\Delta\Delta G^{\ddagger} = 4.3$ kcal/mol) when the oxygen rebound trapping reaction must occur with zero or nearly zero activation free energy for some intermediates (*i.e.*, **4**, **5**, and **11**).

What is the error indicated by Figure 2? It would seem that either the rate constants for the radical reactions are grossly in error or the mechanism of Scheme 1 is inaccurate or incomplete.

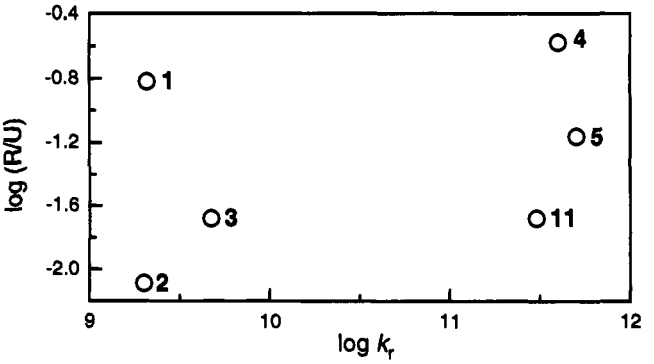


Figure 2. Scatter plot of the log of the ratio of rearranged to unrearranged alcohol products (R/U) obtained in oxidations of probes by phenobarbital-treated rat liver microsomes and purified CYP2B1 isozyme against the log of the rate constants for rearrangements of the putative radical intermediates. The numbers on the graph indicate the putative radical. Product ratios are from refs 5, 8, 10, and this work. Rate constants are compiled in ref 22 or from ref 15.

In our opinion, it is unlikely that the rate constants for rearrangements of radicals **4**, **5**, and **11** are in error by the 3 orders of magnitude necessary to give a good correlation in Figure 2, because the radical kinetic determinations are interrelated,[22] and this would require that the aryl-substituted cyclopropylcarbinyl radicals rearrange with rate constants approximately equal to those for the polymethyl-substituted radicals **2** and **3**. We believe that the mechanism of Scheme 1 for the P-450 hydroxylations is less secure. It is deduced primarily from the qualitative observation of rearranged products in probe studies and is supported by kinetic isotope effect measurements.[23] However, the intermediates required for rearrangements could be carbocations formed *after the oxidation step* from alcohol products.[24] The isotope effects are permissive for an abstraction process but not exclusive of other processes.[25] The results of P-450 oxidation studies with hypersensitive radical probes designed[26] to distinguish between radical and cationic intermediates should be interesting.[27]

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Supplementary Material Available: Table of product yields from oxidations of **6** (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(19) Unreacted **6** was recovered in 77−87% yield; the same amount was recovered when a blank mixture was subjected to the workup and the evaporative concentration procedure was employed for the enzyme studies.[11] Authentic samples of **7** and **8** in the approximate amounts obtained in P-450 oxidations of **6** were added to CYP2B1 isozyme oxidations of a test substrate. Compound **7** (98% yield) and compounds **8** (96% yield) were recovered with no detectable amount of isomerization.

(20) In the context of transition state theory, this rate constant requires a negative value for $\Delta G^{\ddagger}$ which is not possible. When $\Delta G^{\ddagger} = 0$ (*i.e.*, the activation free energy for decomposition of a transition state), the rate constant at 37 °C is 6×10^{12} s^{-1}.

(21) The ring opening of radical **11** is estimated[15] to be exothermic by 18 kcal/mol, giving an equilibrium constant for ring opening to **12** at 37 °C of 5×10^{12}. Therefore, cyclization of radical **12** has a rate constant at 37 °C of ca. 0.06 s^{-1}, which is slower than the observed rate of substrate turnover in all runs, in some cases by nearly an order of magnitude.

(22) Newcomb, M. *Tetrahedron* **1993**, *49*, 1151−1176.

(23) See ref 10 for examples of KIE measurements, a discussion, and references to other works.

(24) Unrearranged alcohols have been shown to be stable in the buffered media of the enzyme reactions (refs 5, 8, 10, and this work). Therefore, the proposed sequence requires an unprecedented ionic process occurring within the enzyme's active site after the oxidation event. A rearrangement might occur by Lewis acid catalysis or if the initial products formed were protonated alcohols produced by insertion of OH. We thank Prof. Robert D. Bach (private communication to M.N.) for suggesting the latter possibility.

(25) Large primary (k_H/k_D) KIE values[23] are compatible with almost any mechanism for C−H functionalization. Normal α-secondary KIE values[23] indicate increased *s* character for carbon in the nonreacting C−H bonds, an expected consequence of considerable stretching of the reacting C−H bond in a transition state that does not involve significant bonding to carbon by an attacking group. An abstraction process accommodates the secondary KIEs, but so also does an insertion process in which the transition state is described by extensive C−H stretching and slight C−O bonding.

(26) Newcomb, M.; Chestney, D. L. *J. Am. Chem. Soc.* **1994**, *116*, 9753−9754.

(27) It would be inappropriate to generalize our mechanistic conclusions for all cytochrome P-450 hydroxylations. However, we note that the consensus mechanism of Scheme 1 is itself a generalization based largely on studies with one enzyme (specifically CYP2B1[12] from rat) or microsomes from phenobarbital-treated rats in which CYP2B1 is the predominantly expressed isozyme. All of the data in Figure 2 is from studies with CYP2B1 or these microsomes.