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Published on Web 05/02/2002

Revisiting the Mechanism of P450 Enzymes with the Radical Clocks Norcarane and Spiro[2,5]octane

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Received January 15, 2002

Abstract: Norcarane (**1**) and spiro[2.5]octane (**2**) yield different product distributions depending on whether they are oxidized via concerted, radical, or cationic mechanisms. For this reason, these two probes were used to investigate the mechanisms of hydrocarbon hydroxylation by two mammalian and two bacterial cytochrome P450 enzymes. Products indicative of a radical intermediate with a lifetime ranging from 16 to 52 ps were detected during the oxidation of norcarane by $P450_{cam}$ (CYP101), $P450_{BM3}$ (CYP102), CYP2B1, and CYP2E1. Trace amounts of the cation rearrangement product were observed with norcarane for all but CYP2E1, while no cation or radical rearrangement products were observed for spiro[2.5]octane. The results for the oxidation of norcarane with a radical rearrangement rate of 2×10^8 s^{-1} are consistent with the involvement of a two-state radical rebound mechanism, while for the slower (5×10^7 s^{-1}) spiro[2,5]-oct-4-yl radical rearrangement products were beyond detection. Taken together with earlier data for the hydroxylation of bicyclo[2.1.0]pentane, which also suggested a 50 ps radical lifetime, these three structurally similar and functionally simple substrates show a consistent pattern of rearrangement that supports a radical rebound mechanism for this set of cytochrome P450 enzymes.

Introduction

The cytochrome P450 (P450) enzyme family encompasses heme proteins that catalyze a large diversity of transformations, the most common of which are hydroxylation, epoxidation, and heteroatom oxidation.[1] The most generally accepted mechanism for substrate hydroxylation by P450 enzymes consists of 7 steps (Figure 1): (a) the 6-coordinate low-spin ferric enzyme binds a substrate with a concomitant shift to a 5-coordinate high-spin state; (b) transfer of one electron from a redox partner reduces the ferric enzyme to the ferrous state; (c) the ferrous enzyme binds molecular oxygen to produce the Fe^{II}-O_2 complex; (d) a second electron followed by a proton are transferred to the ferrous-dioxy species to afford an iron-hydroperoxo (Fe^{III}-OOH) intermediate; (e) the O−O bond is cleaved to release a molecule of water and an iron-oxo ferryl (formally Fe^{V}=O) oxidizing species; (f) a hydrogen atom is abstracted from the substrate by the iron-oxo intermediate to yield a one-electron reduced ferryl species (Fe^{IV}-OH) and a substrate radical (R•), followed by radical recombination to give the enzyme−product complex; and (g) release of the product (ROH) to regenerate the initial low-spin ferric enzyme. Despite decades of intensive research, many details of this mechanism remain unclear. Crystal structures have recently been determined in the case of $P450_{cam}$ (CYP101) for several of the intermediates along this reaction pathway.[2,3] However, to date, the iron-oxo porphyrin π-cation radical of P450 enzymes has not been unambiguously observed, although its presence has been inferred by low-temperature EPR, spectroscopic, and possibly crystallographic studies.[3−5] The

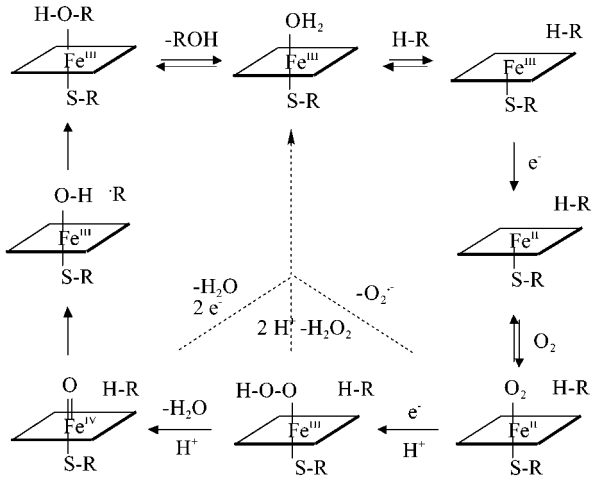

Figure 1. Hydroxylation mechanism for P450 enzymes. The square represents heme bound to the enzyme (R).

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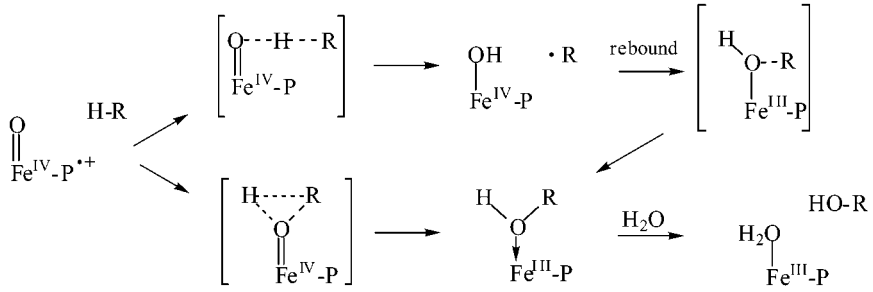

Figure 2. Concerted versus rebound mechanisms by an oxoiron(IV)porphyrin cation radical species. The porphyrin is designated as P and the protein is omitted.

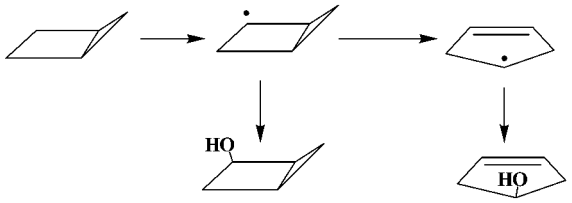

Figure 3. Structure of bicyclo[2.1.0]pentane and its radical and cationic rearrangement products.

quest for the exact mechanism of oxidation by the iron-oxo species has given rise to contradictory conclusions over the past two decades. The initial view of a concerted mechanism (Figure 2) was dismissed in the late 1970s based on the observation of large intramolecular kinetic isotope effects ($k_H/k_D > 10$),[6] loss of stereochemistry,[6−8] and substrate rearrangements.[6,9,10a] A nonconcerted hydroxylation pathway, referred to as the oxygen rebound mechanism, was first suggested by Groves et al.[10b] to account for these rearrangements. This process involves a hydrogen atom abstraction from the substrate by the iron-oxo species to yield a carbon-centered radical intermediate. Subsequent transfer of the iron-bound hydroxyl group to the substrate radical would give the product alcohols in this scenario. However, the high level of stereospecificity (>90% retention) observed in most experiments suggested either that any radical intermediate would have to be short-lived or that substrate motion was restricted within the enzyme active site.[11] The first use of radical clock substrates in the P450 area by Ortiz de Montellano and Stearns,[12] followed by timing of the clock,[13,14] suggested a lifetime of 50 ps for the radical intermediate formed during the P450 oxidation of bicyclo[2.1.0]pentane (Figure 3). Subsequent extensive work by Newcomb and co-workers with ultrafast radical clock substrates able to differentiate between radical and cationic pathways (Figure 4) indicated radical life-

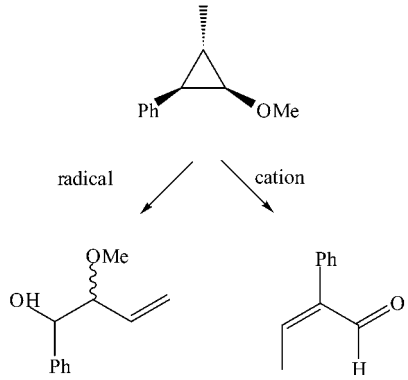

Figure 4. One example of an ultrafast radical clock capable of differentiating radical from cationic intermediates. See ref 16.

times of 80−200 fs, which are in the order of the lifetimes of transition states rather than intermediates.[15,16] These experiments, which also yielded products indicative of cationic intermediates, led them to propose the involvement of two oxidizing species, the iron-oxo complex that inserts an oxygen atom, and an iron-hydroperoxo intermediate that inserts an OH^+ species.

In an effort to reconcile the divergent results obtained with radical clocks, Shaik and co-workers have proposed a "two-state reactivity" process.[17−20] Using density functional theory, they showed that the iron-oxo species adopts two reactive states that explain the formation of products via both the rebound (radical) and insertion (concerted) mechanisms. The low spin doublet state leads to a substrate radical transition state with no energy barrier to rebound collapse, whereas the high spin quartet generates a radical with a significant barrier. This clearly suggests a finite lifetime for the radical intermediate, especially for the high-spin reaction pathway. Although the proposed two-state rebound mechanism explains the formation of radical transition states or intermediates, the model is not complete and provides no explanation for some of the available observations (e.g. variations in isotope effects, or cationic products). The

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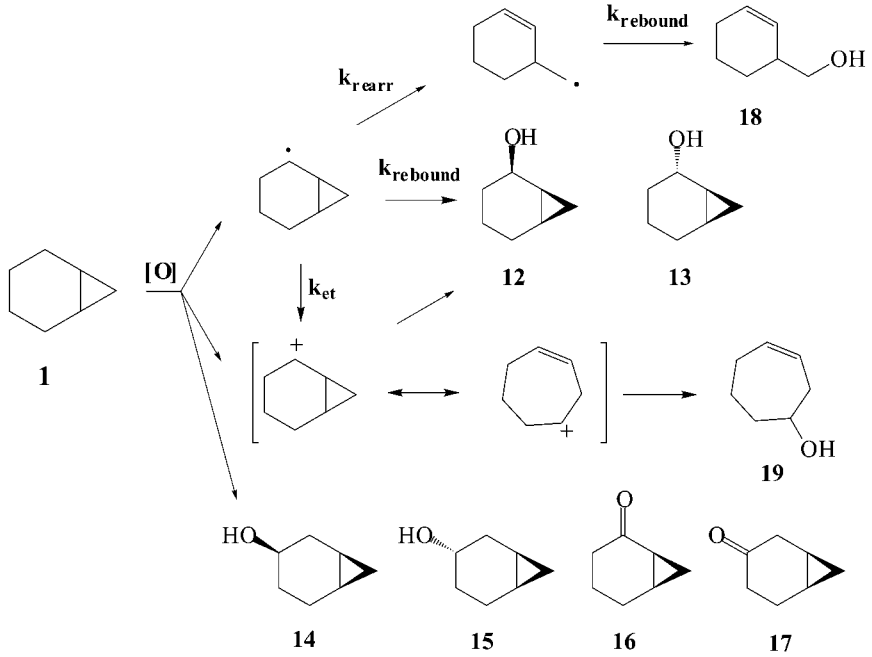

Figure 5. Rearrangement pathways and products resulting from formation of a radical or a cation intermediate upon oxidation of norcarane (**1**) by P450 enzymes.

alternative reaction intermediates to the iron-oxo species have recently been reviewed.[21]

More recently, Hata and co-workers examined the mechanism of methane oxidation by the iron-oxo species using density functional theory.[22] The template model used was based on the structure of $P450_{cam}$ and consisted of a porphine ligated to CH_3-S^-. Their calculations suggested a four-step process: (a) formation of Fe^{III}-OH and a substrate radical; (b) rotation of the OH group of Fe^{III}-OH; (c) interaction of the substrate radical with Fe^{III}-OH; and (d) elimination of the hydroxylated product. Consistent with the isotope effect reported earlier and the proposed radical intermediate, they calculated the rate-limiting step to be the hydrogen abstraction (step 1, 15 kcal/mol).

It is interesting to note that bicyclo[2.1.0]pentane, the only diagnostic substrate that allowed the measurement of a lifetime for the radical intermediate, is also the only clock that involves oxidation at a methylene group in a small and otherwise featureless molecule.[12] This led us to consider whether steric hindrance at the hydroxylation site, the strength of the scissile C−H bond of the substrate, and unencumbered molecular rotation may play decisive roles in the rebound mechanism. We therefore examined the P450-catalyzed oxidation of hydrocarbon radical clocks of a similar nature containing only secondary and tertiary carbons and no other complicating functionality. Norcarane (**1**) has been a valuable substrate in mechanistic studies of other oxidative enzymes and model systems because it can unambiguously differentiate between cation and radical intermediates (Figure 5).[23a−c] For example, norcarane has been used to establish the formation of a radical intermediate with a lifetime of ∼1 ns during catalysis by the non-heme diiron monooxygenases AlkB.[24a] The oxidation of norcarane by methane monooxygenase has been used to demonstrate that the Fe(IV)-Fe(IV) intermediate Q performs a first oxidation leading to a substrate radical intermediate that is sometimes followed by a second oxidation to give a cationic intermediate.[24b] The second radical clock used in our experiments, spiro[2.5]octane (**2**), was selected for three reasons: (a) it differentiates radical and cationic intermediates (Figure 6); (b) it is oxidized at a secondary carbon instead of a methyl group; and (c) its radical has been shown to rearrange ca. 4 times *slower* ($k = 5 \times 10^7$ s^{-1}) than that of norcarane ($k = 2 \times 10^8$ s^{-1}). We have investigated the oxidation of norcarane and spiro[2.5]octane by four different enzymes: the two bacterial enzymes $P450_{cam}$ and $P450_{BM3}$ and the two mammalian enzymes CYP2B1 and CYP2E1. Our results clearly show the involvement of a radical intermediate in the oxidation of norcarane by the four P450 enzymes. The calculated radical lifetime in this case was found to range from 16 ps for CYP2B1 to 52 ps for $P450_{cam}$. The latter corresponds to a rebound rate of 2×10^{10} s^{-1}. Trace amounts of the cationic rearrangement product were also detected in several of the enzymatic reactions. No rearrangement products derived from either a radical or a cation intermediate were observed for the hydroxylations of spiro[2.5]octane.

Results

To validate the use of norcarane and spiro[2.5]octane as diagnostic substrates, we first spectroscopically measured their

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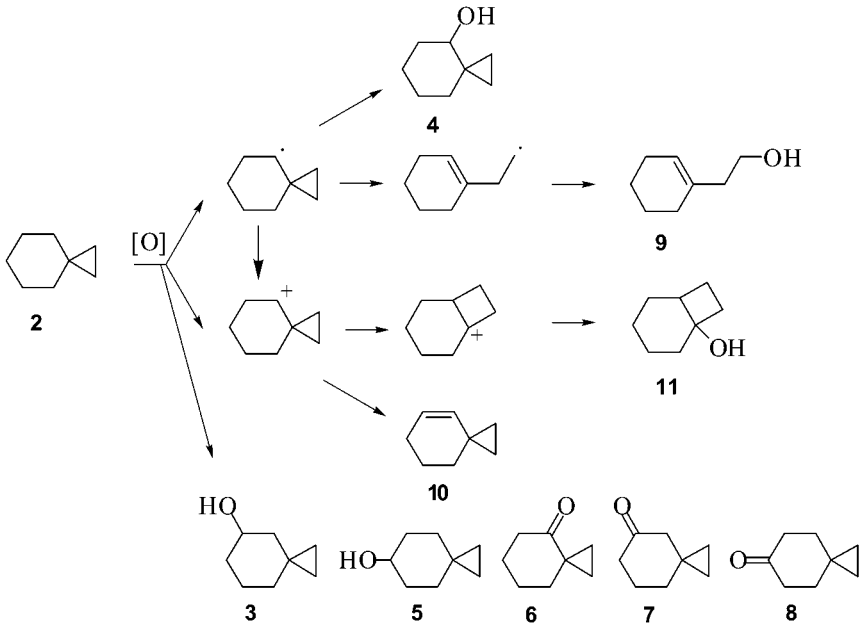

Figure 6. Rearrangement pathways and products resulting from formation of a radical or a cation intermediate upon oxidation of spiro[2.5]octane (**2**) by P450 enzymes.

Table 1. Ligand Dissociation Constants (K_d Values)

enzyme	[norcarane], μM	[spiro[2.5]octane], μM
$P450_{cam}$	14	200
$P450_{BM3}$	70	1
CYP2B1	11	4
CYP2E1	3	150

binding to $P450_{cam}$, $P450_{BM3}$, rat CYP2B1, and human CYP2E1. Both substrates produce a Type I shift (i.e., a shift to a high-spin species absorbing at 390 nm) in the Soret absorption of the four enzymes. The calculated K_d values are listed in Table 1.

To discard the possibility of oxidation by hydrogen peroxide or superoxide (uncoupling products of P450 enzymes), catalase and superoxide dismutase were added to the reaction mixtures. Control reactions did not show any oxidation products in the absence of either the P450 enzyme or NADH/NADPH. Spiro-[2.5]octane was oxidized by $P450_{cam}$, CYP2B1, and CYP2E1, whereas norcarane was a good substrate for all four enzymes.

The reaction of $P450_{cam}$ with spiro[2.5]octane (Figure 7A) yielded 63% of 5-hydroxyspiro[2.5]octane (**3**), the major product, 19% of 4-hydroxyspiro[2.5]-octane (**4**), and 18% of 6-hydroxyspiro[2.5]octane (**5**). The reaction of CYP2B1 (Figure 7B) afforded the same products but in different proportions: 74% of **5**, 12% of **4**, 11% of **3,** and 3% of the 6-ketone (**6**). The conversion was 40% based on the starting spiro[2.5]octane detected in the reaction mixture. The products of the reaction of CYP2E1 with spiro[2.5]octane did not match any of the standards available and could not be identified. None of the enzymes tested with this substrate yielded detectable amounts of the products expected from a radical intermediate, 1-(2-hydroxyethyl)cyclohexene (**9**), or a cationic intermediate, spiro-[2.5]oct-4-ene (**10**) and 1-hydroxybicyclo[4.2.0]octane (**11**).

The solvolysis of 1-(2-tosyloxyethyl)cyclohexene (**9**-**Ts**) has been shown to produce 4-hydroxyspiro[2.5]octane (**4**) and 1-hydroxybicyclo[4.2.0]octane (**11**) as the major products and

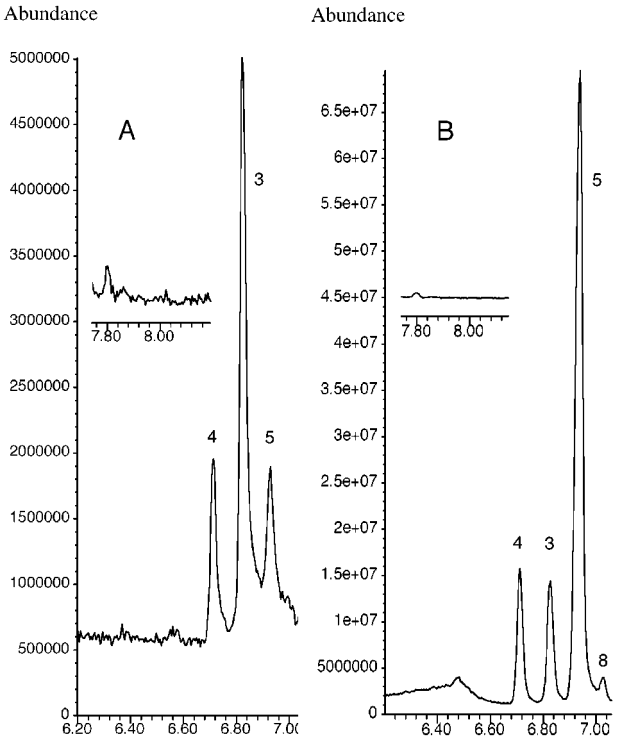

Figure 7. Total ion current chromatograms for the oxidation of spiro[2.5]-octane (**2**): Trace A shows the data resulting from the reaction of $P450_{cam}$ and trace B is the data obtained from the reaction of CY2B1. The rearranged products **9** and **11** would have appeared at 8.03 and 6.81 min, respectively. Ion chromatograms showed negligible intensity at *m*/*e* 79 (base peak of **9**) at 8.03 min in both samples and intensity at *m*/*e* 98 (base peak of **11**) corresponding to <0.5% **11** in trace A and <0.2% **11** in trace B. Compound **3** has a negligible peak at *m*/*e* 98.

2% of the primary alcohol 1-(2-hydroxyethyl)cyclohexene (**9**).[25] These results were confirmed to obtain authentic samples of these materials for GC-MS comparison. The free radical

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Table 2: Product Distributions for the Reactions of Spiro[2.5]octane

products	$P450_{cam}$	CYP2B1
3[a]	63	11
4	19	12
5	18	74
8	nd[b]	3

[a] Product yields were determined by digital integration of the total ion current signal of the GC-MS using the resident HP ChemStation software. Product identity was confirmed by retention time and fragmentation pattern as compared to authentic standards. The rearrangement products 3-(2-hydroxyethyl)cyclohexene (**9**) and 1-hydroxybicyclo[4.2.0]octane (**11**) were shown to be absent (<0.5%) by comparing the TIC trace to authentic standards. [b] Not detected.

Table 3. Product Distributions for the Reactions of Norcarane

products	$P450_{cam}$	$P450_{BM3}$	CYP2B1	CYP2E1
12[a]	100	100	100	100
13	35	49	59	188
14	16	13	11	18
16	20	nd	0.5	nd
17	3.2	9.8	5.0	7.0
18	1.6	1.3	0.5	2.0
19	0.6	0.6	0.5	nd
radical lifetime (ps)	52	44	16	35

[a] Product yields are reported relative to *endo*-2-norcaranol and were determined by digital integration of the total ion current signal of the GC-MS using the resident HP ChemStation software (see Figure 8). Product identity was confirmed by retention time and fragmentation pattern. Products reported in less than 1% relative yield were determined by comparing ion chromatograms of the four most intense ions to those of dilute standard mixtures. *endo*-3-Norcaranol (**15**) did not resolve from the *exo*-2-norcaranol peak and is reported as the sum. The mass spectrum of this peak indicated that it is mostly the latter compound. Products designated as "nd" were below detectable limits with a good baseline.

chlorinations of norcarane and spiro[2.5]octane were carried out in accord with a previous study.[26] A competitive chlorination of these two substrates afforded 4-chloromethylcyclohexene and 1-(2-chloroethyl)cyclohexene in relative ratios of 4:1 with respect to the unrearranged chlorides, indicating a 4-fold slower rearrangement rate of the intermediate radical of spiro[2.5]-octane.[27,28]

The oxidation of norcarane by $P450_{cam}$, $P450_{BM3}$, and CYP2E1 yielded *endo*-2-norcaranol (**12**) as the major product, smaller amounts of *exo*-2-norcaranol (**13**), *exo*-3-norcaranol (**14**), *endo*-3-norcaranol (**15**), 2-norcaranone (**16**), and 3-norcaranone (**17**), and 1.3−2.0% of the radical rearrangement product 4-hydroxymethylcyclohexene (**18**) (Table 3). The total ion current chromatogram in the product region for the oxidation of norcarane by $P450_{BM3}$ is presented in Figure 8. The oxidation of norcarane by CYP2B1 afforded the same products as the other enzymes but yielded only 0.5% 4-(hydroxymethyl)-cyclohexene (**18**). Small amounts (ca. 0.5%) of the product expected from a cationic intermediate, 3-cyclohepten-1-ol (**19**), were detected in the hydroxylation mixtures from $P450_{cam}$, $P450_{BM3}$, and CYP2B1, while none of this product was detected for CYP2E1, which showed the most radical rearrangement product **18**. Based on these data and the rearrangement rate of 2×10^8 sec^{-1} for norcarane, the rate of rebound is ca. 10^{10} sec^{-1}, and the lifetime of the radical formed falls in the range of 16 ps for CYP2B1 and 52 ps for $P450_{cam}$.

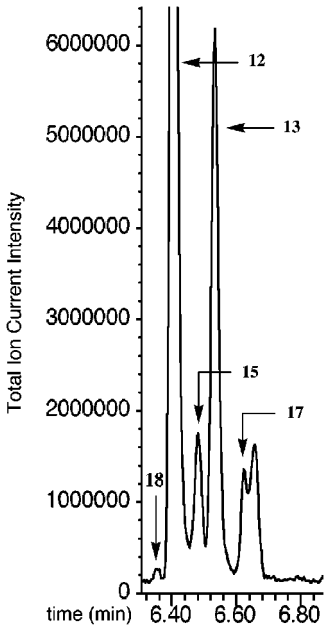

Figure 8. GC-MS total ion current (TIC) chromatogram in the region of the product alcohols from the hydroxylation of norcarane by $P450_{BM3}$.

Discussion

Our data clearly demonstrate the formation of products resulting from a radical intermediate during the hydroxylation of norcarane by the four different P450 enzymes: $P450_{cam}$, $P450_{BM3}$, CYP2B1, and CYP2E1. For CYP2B1 the amount of radical rearrangement product was significantly smaller than the others. In agreement with the reported radical rearrangement observed for bicyclo[2.1.0]pentane,[12] the maximum lifetime calculated for the 2-norcarane radical is 16−52 ps, which is in the order of magnitude that corresponds to an intermediate rather than a transition state.

No substrate radical intermediate was detected during the oxidation of spiro[2.5]octane by $P450_{cam}$, $P450_{BM3}$, CYP2B1, or CYP2E1. This is consistent with the 4-fold slower radical rearrangement rate for the spiro[2.5]oct-4-yl radical measured using the chlorination of spiro[2.5]octane by *tert*-butyl hypochlorite. Thus, each of these three closely related hydrocarbon substrates, bicyclo[2.1.0]pentane, norcarane, and spiro[2.5]-octane, gives an amount of radical rearrangement consistent with a 16−50 ps radical lifetime in a typical oxygen rebound scenario. While the rearranged cyclopentenol product derived from bicyclo[2.1.0]pentane could have derived from either a radical or cation pathway, the quantitative correlation of substrate rearrangement with the radical rearrangement rate described here supports a radical process for this substrate.

Our results are in good agreement with Shaik's two-state rebound mechanism in which the iron-oxo species may oxidize the substrate via a low-spin concerted pathway or a high-spin nonconcerted mechanism leading to a radical intermediate. Assuming that other factors change less, this model also allows the prediction that, as the radical becomes a better electron donor, the high-spin rebound barrier will decrease, and the high-spin pathway will become more concerted. This corroborates the trend observed in the oxidation of not only arylmethylpro-

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(28) Given the direct comparison reported here for the rearrangement rates of the 2-norcaranyl and spiro[2, 5]oct-4-yl radicals, an earlier estimate (ca. 4×10^8 s^{-1}) determined at 120 K by EPR spectroscopy for the rearrangement rate of the latter must be ca. 10 times too fast. Cf.: Roberts, C.; Walton, J. C. *J. Chem. Soc., Perkin. Trans. 2* **1985**, 841−846.

pane derivatives but also the three radical clocks, norcarane, bicyclo[2.1.0]pentane, and spiro[2.5]octane, in which oxidation occurs at a secondary carbon. Thus, hydrogen abstraction from norcarane or bicyclo[2.1.0]pentane leads to radicals that are relatively poor electron donors because of the strain, and are more likely to rearrange (longer lifetime observed). Conversely, because the spiro[2.5]oct-4-yl radical is a better electron donor with a 4-fold slower rearrangement rate, it is expected to yield less rearrangement products.

The detection of trace amounts of the cation product (**19**) observed for the norcarane hydroxylation for three of the P450s studied here leaves open the possibility that a cation rearrangement pathway is accessible to some small extent for this substrate. However, the absence of the cyclobutanol product (**11**) during the hydroxylation of spiro[2.5]octane argues against cationic intermediates during the hydroxylation of this substrate. How then does one explain the appearance of cationic rearrangements with some substrates, as has been reported by Newcomb et al.,[16] while for other substrates no such rearrangements are observed? While a hydroperoxyiron(III) heme intermediate has been suggested as an alternate oxidant in the P450 reaction cycle, based on changes in product ratios upon modification of the enzyme active site, there is no evidence that such a hydroperoxide would be sufficiently electrophilic at the distal oxygen to react with a C−H bond. In fact, high-level DFT calculations[17,18] indicate that such a species would be nucleophilic but not electrophilic. Furthermore, Hofmann and Sligar et al. have shown by cryo-ENDOR spectroscopy that in a single turnover hydroxylation with $P450_{cam}$, the proton of the incipient alcohol product derived from the substrate C−H bond with the alcohol oxygen coordinated to the heme iron.[4] This is as expected for an iron-oxo precursor but is not possible for an iron-hydroperoxide.

We suggest that the cationic rearranged products that sometimes appear during P450 turnover derive from an electron transfer oxidation of the incipient carbon radical that competes in some cases with oxygen rebound (Figure 5). This is an expression of the inner-sphere vs outer-sphere paradigm for carbon radical oxidation that has been extensively studied by Kochi.[29] In this very informative work, the rate of electron transfer from an alkyl radical to an iron(III)phenanthroline oxidant to form a carbocation and iron(II) was timed by competition with a homolytic bromine atom transfer from bromotrichloromethane. Significantly, carbocation formation via electron-transfer oxidation of even primary alkyl radicals was found to compete with inner-sphere atom transfer processes. Moreover, the extent of carbocation formation was a very sensitive function of the oxidation potentials of both the iron(III) oxidant and the alkyl radical while the inner-sphere processes were most sensitive to steric effects. It is very reasonable to assume that similar competitions would be at work during the stepwise hydroxylation process mediated by cytochrome P450. Here, oxygen rebound to form the product alcohol is the inner-sphere process while competing electron-transfer processes would occur to the extent that the rebound rate, taken to be about 10^{10} s^{-1} based on earlier data of Ingold[13,14] and data presented here, competes with the electron-transfer rate. Significantly, the rate of electron-transfer oxidation for secondary carbon radicals by Fe(III)phen was timed at ca. 10^8 M^{-1} s^{-1}. Thus, the electron-transfer oxidation of an incipient carbon radical at the active site in the P450 rebound process could well be oxidized by an intermediate oxoiron(IV) species to explain the products that appear to arise from cationic rearrangement processes.

Another anomaly in the body of data on P450 hydroxylations is the observation that some "fast" radical clocks have shown *less* rearrangement than slower clocks.[30] For a two-state rebound process, the transition state for hydrogen abstraction will position the active oxygen a few tenths of an ångstrom *farther* from the hydroxylated carbon atom than the transition state for the subsequent C−O bond formation. Thus, the extent of rearrangement detected by a particular probe may simply reflect a facile molecular trajectory from the hydrogen abstraction transition state to the hydroxylation transition state. Indeed, in cases for which the radical is produced within the attractive potential well of the new C−O bond, the timing of radical rearrangements, and thus the timing of the radical clocks, is likely to be affected. Such an effect would be most pronounced for hydroxylation at the C−H bond of methyl groups, with a very strong C−H bond and a small steric size. Both effects would push the reaction coordinate toward a tighter radical cage and shorten the radical lifetime.[31b] Furthermore, the effective C−H bond strength for cyclopropylcarbinyl hydrogens will depend on the stereoelectronics of the interaction of the C−H bond with the neighboring cyclopropyl group. We note that these interactions are rigidly enforced in the bicycloalkanes under investigation here. Indeed, it is the more sterically hindered 2-*endo*-hydrogen of biyclo-[2.1.0]pentane that is the more reactive.[12]

A clear case of differential reactivity of nominally similar secondary radical intermediates is observed in the hydroxylation of a chirally deuterated ethylbenzene by a chiral iron porphyrin.[31b] Thus, for this P450 model system, the hydrogen−deuterium inventory of the products showed that while the benzylic radical produced by abstraction of the *pro-R* deuterium rebounded to produce the alcohol with 95% *retention* of configuration at carbon, significant *racemization* was observed following abstraction of the *pro-S* hydrogen. Clearly, the same carbon radical is produced in both cases, but the effective *lifetime* of the *pro-R* radical, as revealed by the very small amount of rearrangement, must be shorter than that of the *pro-S* radical due to the asymmetry of the steric environment. This result is consistent with the observed epimerization at carbon observed during the hydroxylation of norbornane by cytochrome P450 in the first experiments using a mechanistically diagnostic substrate probe that was indicative of a radical rebound process.[6] Thus, radical reactions at C2 of norbornane are known to occur with epimerization while cationic processes are prevented from doing so by neighboring σ-bond participation.

Summary and Conclusions

We have reexamined the mechanism of aliphatic hydroxylation by P450 enzymes using two new diagnostic substrates: spiro[2.5]octane and norcarane. The formation of a radical

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intermediate is consistent with the oxidation products of norcarane obtained with $P450_{cam}$, $P450_{BM3}$, CYP2B1, and CYP2E1 with intermediate radical lifetimes of 52, 44, 16, and 35 ps, respectively. Although we could not detect such an intermediate during the transformation of spiro[2.5]octane, the 4-fold slower radical rearrangement rate for the spiro[2.5]oct-4-yl radical as compared to the 2-norcaranyl radical would be expected to lower the yield of radical products and be below our detection limit. The results presented here for norcarane and spiro[2.5]octane along with earlier results with bicyclo-[2.1.0]pentane are in good agreement with the two-state oxygen rebound mechanism with a consistent timing for the lifetime of the radical intermediate.

The mechanisms of aliphatic hydroxylation by metal-oxo species remain among the most interesting and enigmatic in catalytic science. The challenge for the field has been to fold the disparate and sometimes contradictory data into a comprehensible picture. We have shown here that the hydroxylation event mediated by cytochrome P450 at the methylene group of a closely related set of substrates gives a consistent set of data supporting a radical rebound process. The traces of cation products observed have been attributed to a competing electron-transfer process that has strong precedent in chemical systems. The lack of rearrangement observed for some fast rearranging probes can be understood in terms of an interplay of sensitive steric and stereoelectronic effects, especially for the hydroxylation of the C−H bond of a methyl group.

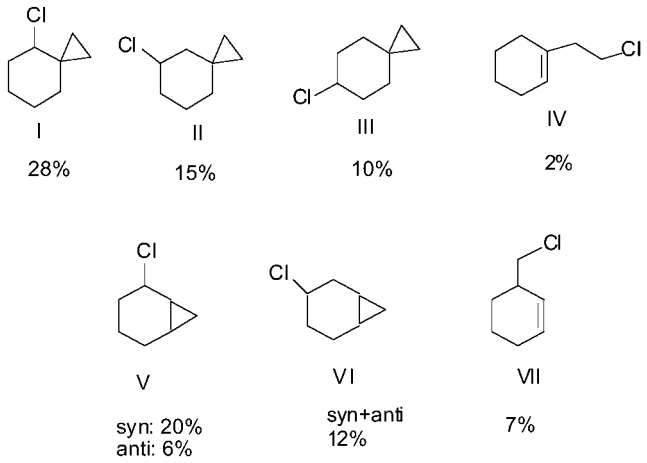
Experimental Procedures

Materials. Unless otherwise mentioned, all the reagents were from Sigma-Aldrich. The dichloromethane and toluene were Omnisolve grade and purchased from EM Science (Darmstadt, Germany). The QuickChange site-directed mutagenesis kit and the nitrilotriacetic acid resin (NTA-Agarose) were obtained from Stratagene (La Jolla, CA). Yeast extract, tryptone, 2YT, sodium chloride, EDTA, and organic solvents were obtained from Fisher Scientific (Fair Lawn, NJ). Isopropyl-β-D-thiogalactopyranoside was from FisherBiotech (Fair Lawn, NJ), and ampicillin from Promega (Madison, WI). High-purity argon (99.998%), CO (99.9%), and the oxygen adsorbent, Oxisorb-W, used to further purify the argon were purchased from Puritan-Bennett Medical Gases (Overland Park, KS). All the chemicals were used without further purification. The UV−visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer or on a Cary 1E Varian UV−visible spectrophotometer. GC-MS analyses were performed on a HP GC-MS (5890/ 5989B) with a HP-5MS cross-linked 5% PH ME Siloxane capillary column.

Synthesis. Norcarane and spiro[2.5]octane were prepared by using the Simmons−Smith reaction as previously described.[32] Authentic samples of the product norcaranols, spiro[2.5]octanols, the corresponding ketones, and the rearrangement products 4-cyclohepten-1-ol and 4-hydroxymethylcyclohexene were prepared according to published procedures.[24] The purity of all products was assessed by ^{1}H NMR and GC-MS.

Chlorination of Norcarane and Spiro[2.5]bicyclooctane with *tert*-Butyl Hypochlorite. Norcarane (196 mg, 2 mmol), spiro[2.5]octane (220 mg, 2 mmol), and *tert*-butyl hypochlorite (215 mg, 2 mmol) in 5 mL of benzene were charged into a 25 mL flask under Ar at 20 °C (water bath). The flask was irradiated with a 75 W tungsten lamp for 20 min. Analysis of the reaction mixtures by GC-MS revealed the presence of various monochlorides **I**−**VII**.

The absolute rate constant for abstraction of chlorine from *tert*-butyl hypochlorite had been determined to be 2.6×10^9 M^{-1} s^{-1} by using homoallyl free radical clock, cyclopropylcarbinyl (free radical ring-opening rate at 20 °C: $k_o = 7.9 \times 10^7$ s^{-1}).[27] Thus, the ratio of **V**:**VII** allows a confirmation of the rearrangement rate for the 2-norcaranyl radical to be 2×10^8 s^{-1}. The competition reaction between norcarane and spiro[2.5]octane with *tert*-butyl hypochlorite shows that the rate for ring opening of the 2-norcaranyl radical is about 4 times as fast as the rate for spiro[2.5]oct-4-yl radical (5×10^7 s^{-1}).



Preparation of the Enzymes and Binding Assays. $P450_{cam}$ from *Pseudomonas putida*, Pd, and PdR were expressed heterologously in *Escherichia coli* and were purified as reported previously.[33] CYP2B1[34] and rat NADPH-cytochrome P450 reductase[35] were expressed and purified as described elsewhere. Human CYP2E1 was kindly provided by F. P. Guengerich, Vanderbilt University. Cytochrome b_5 was generously provided by Almira Correia, University of California, San Francisco. K_d values for the association of norcarane or spiro[2.5]octane with the different P450 enzymes were determined by UV spectroscopy. The camphor present in the $P450_{cam}$ sample was removed using a PD10 column eluted with 10 mM potassium phosphate at pH 7.5. The binding assays were performed on the same day ($P450_{cam}$ is unstable to freezing/thawing in the absence of camphor). The decrease in absorbance of the low-spin state at 416 nm and the increase in absorbance of the high-spin species at 390 nm were monitored upon addition of norcarane or spiro[2.5]octane. K_d values for CYP2E1 were measured by displacement of the pyrazole ligand and the corrected K_d was calculated using the following equation: $K_d = K_s^{\text{apparent}}/[1 + \text{ligand concentration}/K_s^{\text{ligand}}]$. When pyrazole was used the decrease in absorbance occurred at 423 nm and the increase was again at 390 nm.

Cytochrome $P450_{BM3}$ Expression. $P450_{BM3}$ DNA in the pUC19 vector was kindly provided by Professor Armand J. Fulco (University of California, Los Angeles). Quickchange was used to add a His_6-tag on the N-terminal end of the protein with the 5′-sense oligonucleotide 5′-GA AAG AGG GAT AAC ATG CAC CAC CAC CAC CAC CAC ACA ATT AAA GAA ATG CCT CAG CC. Mutations were confirmed by DNA sequencing. The DNA was then cleaved out of pUC119 and ligated into the pCWori vector. *Escherichia coli* strain DH5α [*F′ ara* D*(lac-proAB)rpsLf*80d *lac*ZDM15 *hsd*R17] were transformed with the plasmid and spread onto Luria-Bernati medium containing agar. One-liter of 2YT liquid medium supplemented with 100 mg/L of ampicillin

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was used for the expression. $P450_{BM3}$ expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside at $OD_{600} \sim 0.8$ (after about 4 h at 37 °C and 250 rpm), then further grown at 30 °C for 11 h after another 75 mg/L of ampicillin was added. The harvested cells (75 g from 4 L cultures) were lysed for 1 h at 4 °C in 200 mL of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 0.3 g of lysosyme. The cells were further lysed by sonication. Following centrifugation, the supernatant was directly loaded onto a nickel column (15 mL) equilibrated with buffer A (20 mM Tris buffer at pH 8.0, 0.5 M NaCl, 10% glycerol, and 1 mM PMSF). The column was washed with buffer A (100 mL) and the protein was eluted with buffer A containing 100 mM imidazole. The colored fractions with Rz $\geq$ 0.4 were pooled, diluted in one volume of 100 mM potassium phosphate at pH 7.4 containing 0.5 mM of DTT, and loaded onto a 2′,5′-ADP Sepharose column (20 mL) equilibrated with the same buffer. The column was washed with 5 column volumes each of the equilibration buffer and 500 mM potassium phosphate containing 0.5 mM DTT. The protein was eluted with the latter buffer containing 10 mM 3′-AMP. The red fractions were concentrated in an Amicon cell using a YM30 membrane and dialyzed against 10 mM potassium phosphate, pH 7.4.

Reactions of the Different P450 Enzymes with Norcarane or Spiro[2.5]octane. Unless otherwise indicated, the buffer was 100 mM potassium phosphate at pH 7.4. $P450_{cam}$ reactions [$P450_{cam}$ (2 μM), Pd (4 μM), PdR (2 μM), substrate (500 μM of **1** or **2**), superoxide dismutase (2 μM), and catalase (100 μg/mL)] were dissolved in buffer. The reaction was started by the addition of NADH (200 μM). *$P450_{BM3}$ reactions*: To a solution of $P450_{BM3}$ (2 μM) in buffer were added superoxide dismutase (2 μM), substrate (500 μM), and NADPH (100 μM). *CYP2B1 reactions*: The following components were mixed in strict order, DLPC (L-α-phosphatidylcholine dilauroyl, 200 μg/mL), CYP2B1 (0.5 μM), P450 reductase (0.5 μM), cytochrome b_5 (1 μM), superoxide dismutase (0.5 μM), and catalase (100 μg/mL). The mixture was incubated at room temperature for 10 min followed by the addition, in order, of the buffer and the substrate (500 μM). After 5 min of incubation at 37 °C, the reaction was started by the addition of NADPH (1 mM). The enzymatic reactions with CYP2E1 were prepared by mixing in order and incubating at room temperature for 10 min, DLPC (30 μg/mL), P450 reductase (1.5 μM), CYP2E1 (0.5 μM), superoxide dismutase (0.5 μM), and catalase (100 μg/mL). After addition, in order, of the buffer and the substrate (500 μM) followed by incubation at 37 °C for 5 min, the reaction was initiated by mixing NADPH (1 mM). Finally, control reactions were carried out without enzyme and, separately, without NADPH/NADH. All the reaction mixtures (1 mL) were incubated at 37 °C for 1 h before being extracted with dichloromethane (3 $\times$ 300 μL). The combined organic layers were carefully washed with brine (2 $\times$ 400 μL), before injection in the GC/MS system. The rebound rates were calculated from the following equation: $k_{rebound} = k_{rearr.} \times$ [nonradical products]/[radical product]. The radical lifetime is the reciprocal of $k_{rebound}$.

Acknowledgment. We are grateful for support of this work by the National Institutes of Health (GM25515, P.R.O.M.; GM36928, J.T.G.) and by the National Science Foundation (NSF 9810248, J.T.G.) for support of the Center for Environmental Bioinorganic Chemistry.

Supporting Information Available: Ion chromatograms showing the product region for norcarane oxidation by $P450_{BM3}$ and CYP2E1 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA025608H