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Evaluation of Norcarane as a Probe for Radicals in Cytochome P450- and Soluble Methane Monooxygenase-Catalyzed Hydroxylation Reactions

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Abstract: Norcarane was employed as a mechanistic probe in oxidations catalyzed by hepatic cytochome P450 enzymes and by the soluble methane monooxygenase (sMMO) enzyme from *Methylococcus capsulatus* (Bath). In all cases, the major oxidation products (>75%) were *endo*- and *exo*-2-norcaranol. Small amounts of 3-norcaranols, 2-norcaranone, and 3-norcaranone also formed. In addition, the rearrangement products (2-cyclohexenyl)methanol and 3-cycloheptenol were detected in the reactions, the former possibly arising from a radical intermediate and the latter ascribed to a cationic intermediate. The formation of the cation-derived rearrangement product is consistent with one or more reaction pathways and is in accord with the results of previous probe studies with the same enzymes. The appearance of the putative radical-derived rearrangement product is in conflict with other mechanistic probe results with the same enzymes. The unique implication of a discrete radical intermediate in hydroxylations of norcarane may be the consequence of a minor reaction pathway for the enzymes that is not manifest in reactions with other probes. Alternatively, it might reflect a previously unappreciated reactivity of norcaranyl cationic intermediates, which can convert to (2-cyclohexenyl)methanol. We conclude that generalizations regarding the intermediacy of radicals in P450 and sMMO enzyme-catalyzed hydroxylations based on the norcarane results should be considered hypothetical until the origin of the unanticipated results can be determined.

The mechanistic details of enzyme-catalyzed hydroxylation reactions have intrigued chemists and biochemists for decades. Alkane hydroxylations are readily achieved at ambient temperature by the cytochome P450 enzymes (P450),[1] and methane monooxygenase (MMO) enzymes can accomplish the remarkably difficult hydroxylation of methane with ease.[2] Simple chemical analogies of these reactions are not available, although some complex model systems behave in a biomimetic manner. Increasingly sophisticated instrumental techniques[3−5] and high-level computational studies[6−9] undoubtedly provide important details about the mechanisms of the hydroxylation reactions, but much information currently available comes from probe studies that seek evidence for transients formed in the reactions.

Mechanistic probe substrates for oxidations are straightforward in concept, although interpreting the implications of their application can sometimes be difficult. One identifies a characteristic process of a transient of interest, such as a cation or radical, and then employs a substrate that might be used to detect the formation of the transient. For example, oxidation of a chiral compound at the asymmetric center might occur with racemization if a long-lived radical or cation formed in the reaction but with retention or possibly inversion if a concerted process occurred. Alternatively, a strained cyclopropyl substrate could ring open if a radical or cation were produced at the cyclopropylcarbinyl position but not in a concerted process. Both chiral substrates and substituted cyclopropanes have been employed as probes of enzyme-catalyzed hydroxylation reactions.

One mechanistic probe that has been employed in studies of enzyme-catalyzed hydroxylations is bicyclo[4.1.0]heptane (nor-

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carane). In principle, norcarane is a highly advanced probe because the radical and cation produced at C2, the cyclopropylcarbinyl position, react differently. The norcaran-2-yl radical ring opens predominantly (ca. 50:1) to the (2-cyclohexenyl)methyl radical,[10] and the rate constant for this ring opening reaction, 2×10^8 s^{-1}, can be estimated from the results of tin hydride trapping studies[10] and the rate constant for reaction of a radical with tin hydride.[11] C2 cationic species from norcarane react mainly by cleavage of the bridge bond to give ring-expanded products derived from 3-cycloheptenol, but cleavage of an exocyclic cyclopropyl bond to give products from (2-cyclohexenyl)methanol also occurs.[12] The formation of the latter products from cationic reactions of 2-norcaranol appears to have been overlooked in previous studies that employed norcarane as a mechanistic probe. In addition, the cationic manifold is biased against ring cleavage in solvolytic reactions; for example, solvolyses of 2-norcaranol 3,5-dinitrobenzoate esters afforded mainly mixtures of *endo*- and *exo*-2-norcaranol.[13]

Norcarane was employed in mechanistic studies with cytochome P450 by White et al.,[14] but no rearranged alcohol products were detected. More recently, Austin et al. used norcarane in a study of hydroxylation by the AlkB hydroxylase enzyme from *Pseudomonas oleovorans*.[15] Consistent with earlier probe results that implicated a relatively long-lived radical intermediate in hydroxylations by this enzyme,[16] they observed partial rearrangement in the alcohol products that led to an estimated radical lifetime in the oxidation reaction of about 1 ns.[15] Quite recently, while the present work was in its final stages, Brazeau et al. reported a study of norcarane oxidation by the soluble methane monooxygenase (sMMO) from *Methylosinus trichosporium* (*Ms. trichosporium*) OB3b wherein small amounts of rearrangement products were found; the authors concluded that both a cationic species and a discrete radical intermediate were produced.[17] Although evidence for cationic intermediates in sMMO hydroxylations had been reported previously,[18,19] other probe studies of hydroxylation catalyzed by the sMMO of this species and by the related sMMO from *Methylococcus capsulatus* (*Mc. capsulatus*) (Bath) were consistent with reactions proceeding *without* formation of discrete radical intermediates.

The latter mechanistic inconsistency with norcarane is mirrored in oxidations that we have studied with both P450 and sMMO enzymes. We report here the results of hydroxylations of norcarane catalyzed by hepatic P450 enzymes and by the sMMO enzyme from *Mc. capsulatus* (Bath). In all cases, small amounts of rearrangement products derived from putative cationic intermediates and radical intermediates were found, much like the recent results with *Ms. trichosporium* OB3b.

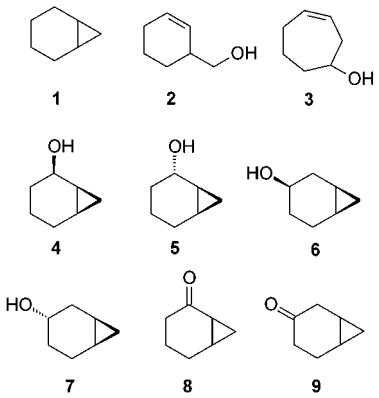

Figure 1. Norcarane (**1**) and products observed from its enzyme-catalyzed hydroxylation reactions.

These results are in stark contrast to those obtained for the same enzymes with other probes. We conclude that mechanistic generalizations based on results with norcarane are unwarranted. The small amounts of rearrangement products likely result from multiple pathways in the enzyme-catalyzed hydroxylation reactions.

Results

Enzyme-Catalyzed Oxidations. Norcarane (**1**) was oxidized with P450 enzymes that were overexpressed in *Escherichia coli* (*E. coli*) and purified. P450 2B4 is the phenobarbital-induced hepatic P450 from rabbit, and P450 Δ2B4 is an expressed version of the same enzyme with a short deletion at the N-terminus. P450 Δ2E1 is the truncated version of the ethanol-inducible hepatic P450 from rabbit, and P450 Δ2E1 T303A is a mutant of this enzyme in which threonine in the active site was replaced with alanine. The preparations and purifications of these enzymes were reported previously.[20−22] The reconstituted P450 enzyme preparations contained the purified P450 enzyme, 2 equiv of P450 reductase, and NADPH in DLPC liposomes in buffer solutions. The oxidation reactions were allowed to proceed for 30 min at 37 °C.

Norcarane also was oxidized with the sMMO system from *Mc. capsulatus* (Bath). In these reactions, the enzyme system was a 1:2:0.5 mixture of the hydroxylase, coupling protein, and reductase components. Reactions were conducted at 45 °C, the optimal growth temperature of the organism.

Following an extractive workup, products were identified by GC-mass spectral comparisons to authentic samples and quantitated by GC analysis. The products are shown in Figure 1. Oxidations at the 2- and 3-positions gave both endo and exo alcohols (**4**−**7**). Overoxidation of these alcohols gave the corresponding ketones **8** and **9** that were detected in low yields. In addition, small amounts of both possible rearranged products from oxidation of the C2 position, 3-(hydroxymethyl)cyclohexene (**2**) and 3-cycloheptenol (**3**), were detected in all cases. Table 1 lists the regioselectivity of the oxidations, the stereoselectivity in the alcohol products from oxidation at C2 and C3 of the substrate, and the percentages of rearrangement products

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Table 1. Results of Enzyme-Catalyzed Oxidations of Norcarane

enzyme[a]	regio[b]	stereo (C2)[c]	stereo (C3)[d]	% **2**[e]	% **3**[e]
2B4	91:9	76:24	49:51	0.31 ± 0.06	0.4 ± 0.1
Δ2B4	92:8	78:22	53:47	0.4	0.5
Δ2E1	94:6	27:73	62:38	0.5 ± 0.1	0.16 ± 0.01
Δ2E1 T303A	82:18	52:48	80:20	3.1 ± 0.1	0.6 ± 0.4
Mc. capsulatus (Bath)	90:10	65:35	33:67	3.3 ± 0.1	2.6 ± 0.6
Ms. trichosporium OB3b[f]	91:9	63:37		1.5	1.2

[a] The first four enzymes listed are cytochromes P450. The last two enzymes are sMMOs. [b] Regioselectivity: ratio of products from oxidation of norcarane at C2 and C3; standard deviations from multiple run experiments are <1. [c] Stereoselectivity at C2: ratio of endo/exo alcohols (**4**:**5**). [d] Stereoselectivity at C2: ratio of endo/exo alcohols (**6**:**7**). [e] Percentage of products **2** and **3** relative to all products from oxidation at C2; standard deviations are given for multiple runs. [f] Results from ref 17.

Table 2. Results of Stability Control Studies[a]

product	nmol added[b]	nmol found[c]
2	0	3.1
	10	9.7
3	0	0.7
	15	16.7
4	0	190
	200	380

[a] Reactions conducted with 1 nmol of P450 2B4. [b] Amount of product added before oxidation reaction. [c] Yield of product in nmol.

2 and **3** relative to the total amount of products from oxidation at C2.

The rearrangement products **2** and **3** are important with respect to mechanistic interpretations, and they were found in low yields. Various studies confirmed that these two products were formed in the oxidation reactions. In multiple run experiments, the deviations in yields of **2** and **3** were small. A product mixture from a P450 2B4 oxidation and one from an sMMO oxidation were analyzed in another laboratory using a different GC-MS protocol, and the results confirmed the presence of both **2** and **3**.[23] In the case of P450 2B4, we determined the stability of **2** and **3**, as well as alcohol **4**, under enzyme-catalyzed hydroxylation conditions. In matched experiments, P450 2B4 oxidations were conducted with **1** and with mixtures of norcarane that contained products **2**−**4**. This type of control reaction provides realistic information about the stability of the products as they are being formed in the enzyme reactions because substrate **1** is always present in large excess during the reaction and might serve as a competitive inhibitor for oxidation of the small amounts of products. The results are listed in Table 2. Product **2** was slightly degraded in the control reaction, and product **3** was stable.

The major products were from oxidation of the C2 position of norcarane, consistent with the reduced C−H bond energy at a cyclopropylcarbinyl position of about 3 kcal/mol.[24] The relatively large differences in regioselectivity, stereoselectivity, and the amounts of rearrangement products formed between P450 Δ2E1 and its T303A mutant are noteworthy because the mutation involves replacement of a highly conserved threonine that is thought to be involved in the protonation reactions in the evolution of the active oxidants. Similar differences in product distributions have been reported for this pair previously,[22,25] and the T303A mutant gave larger amounts of cationic rearrangement products than the wild-type (wt) enzyme.[26] The near identity in the results for P450 2B4 and its expressed version containing an N-terminal deletion, P450 Δ2B4, indicate that there is little difference in the reactions of the natural and truncated enzymes. The product distribution found in oxidation by the sMMO of *Mc. capsulatus* (Bath) is similar to that reported from oxidation by the sMMO of *Ms. trichosporium* OB3b.[17]

Solvolysis Studies. Previously reported solvolysis studies of the 3,5-dinitrobenzoate esters of 2-norcaranol conducted at 80 °C gave predominantly bicyclic alcohols.[13] We briefly investigated reactions of 2-norcaranyl mesylates at lower temperatures. The mesylate from *endo*-2-norcaranol was prepared from reaction of the alcohol with methanesulfonyl chloride and Et_3N in tetrahydrofuran (THF) at −20 °C and allowed to stand at −20 °C for 1 h. Water was then added at −20 °C, and the mixture was stirred at room temperature for 12 h. Following an extractive workup procedure, GC analysis of the products showed a mixture of *endo*-2-norcaranol (**4**), *exo*-2-norcaranol (**5**), and 3-cycloheptenol (**3**) in a 67:28:5 ratio in approximately 50% yield. To the limit of detection (ca. 0.5%), no (2-cyclohexenyl)methanol (**2**) was formed. When the reaction sequence was repeated using a mixture of *endo*- and *exo*-2-norcaranol (30:70) for preparation of the mesylate, a similar mixture of products was obtained. The same product ratios were obtained when the reactions were repeated with the exception that the mesylates were prepared at room temperature.

The results of the mesylate solvolysis reactions are similar to those found in the solvolysis of the 3,5-dinitrobenzoate esters in aqueous acetone, where a 67:23:10 ratio of **4**, **5**, and **3** were found from both the endo and exo esters.[13] The consistent ratio of products found from mesylates prepared from the endo alcohol and from the mixture of endo and exo alcohols shows that the mesylates were formed and reacted in solvolyses reactions in high yields.

Discussion

Mechanistic probe studies of enzyme-catalyzed oxidations have proven to be quite difficult to understand. Part of the confusion comes from probe studies that sought to test for a radical intermediate but employed probes that provided no method for differentiation between cationic and radical intermediates, and much of the mechanistic work reported for P450 and sMMO enzymes falls into this category. When no rearrangement or racemization of such a probe is observed, one can deduce that neither transient is produced with an appreciable lifetime, but a calculation of a radical lifetime from an observed product distribution is essentially meaningless if the probe gives the same products from a radical and a cationic intermediate. In addition, experimental results have been misinterpreted and products misidentified. Another layer of complexity arises from the possibility that more than one hydroxylation reaction pathway might exist for these enzymes.

Figure 2 displays the iron−oxygen species that form in the evolution of the oxidants in P450 and sMMO. In P450, the

(23) GC-mass spectral analyses were conducted with a low-polarity column that gave a different order of product elution. We are grateful to Prof. J. T. Groves for providing us with these results.

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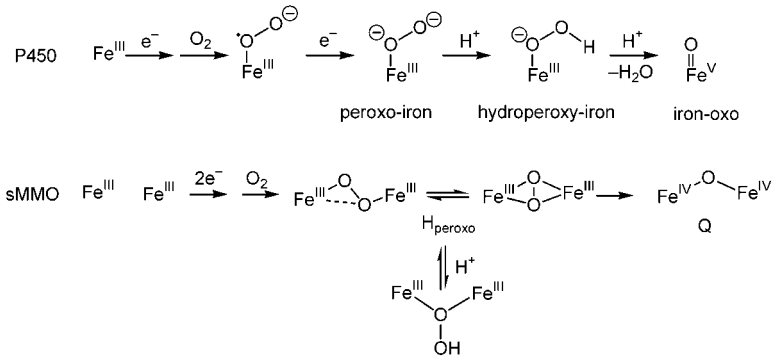


Figure 2. Iron−oxygen intermediates produced in P450 and sMMO oxidations. See refs 1 and 5.

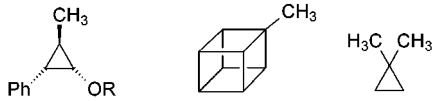
resting enzyme is reduced to an iron(II) species that can bind dioxygen. Further reduction gives a peroxo−iron species that converts to a hydroperoxy−iron unit upon protonation. A second protonation and loss of water give the ultimate oxidant, an iron−oxo species. Recent "cryoreduction" studies resulted in the EPR detection of the peroxo−iron and hydroperoxy−iron intermediates,[3,27] but the iron−oxo species apparently is too short-lived to detect,[3] despite its tentative identification in an earlier report.[4] In sMMO, two iron atoms are reduced to the Fe(II) level, and dioxygen binding affords a species termed H_{peroxo}. H_{peroxo} converts to the ultimate oxidant, an oxo-bridged diiron(IV) species termed Q. For the *Ms. trichosporium* OB3b enzyme the H_{peroxo} to Q conversion apparently depends on protonation of H_{peroxo},[28] but this conversion is independent of pH for sMMO from *Mc. capsulatus* (Bath).[29] Unlike with P450, the H_{peroxo} and Q intermediates accumulate and are detectable during the reaction cycle of sMMO,[30] although the structures depicted in Figure 2 for these species are speculative.

Evidence has been reported that two electrophilic oxidants are formed in both P450 and sMMO oxidations. For P450, the evidence involves changes in regioselectivity in oxidations catalyzed by wild-type and mutant P450s,[22,25] similar to the differences found here for P450 Δ2E1 and its T303A mutant, and the finding that rearranged and unrearranged alcohols from oxidation of the methyl group in a methylcyclopropane mechanistic probe are formed with different Michaelis parameters, different kinetic isotope effects, and different solvent isotope effects.[31] The two oxidants are presumed to be the iron−oxo species and either the hydroperoxy−iron intermediate or iron-complexed hydrogen peroxide. For the sMMO from *Mc. capsulatus* (Bath), single-turnover studies revealed that H_{peroxo} or its immediate successor in the reaction cycle is capable of epoxidizing propene[29] and other substrates,[32] adding a second oxidizing species to Q, the active methane oxidant.

Computational studies of the iron−oxo species in P450 indicate that two reactive spin states are accessible, a low-spin ensemble that reacts by insertion and a high-spin ensemble that reacts by H-atom abstraction from substrate to afford a radical,[6] and multiple spin state reactions of iron−oxo in P450 are supported by recent experimental results.[31] Theoretical analysis of sMMO hydroxylation of ethane[33] indicates that the transition state can evolve into alcohol through both concerted and bound radical pathways. These pathways do not represent "fundamentally different reaction mechanisms," as sometimes stated,[17] but rather reflect the character of a late transition state.

The experimental and computational evidence for multiple reactive species and multiple reaction channels in hydroxylations catalyzed by P450 and sMMO enzymes indicates the difficulty one has in interpreting the results from any mechanistic probe in isolation. With that caution, we evaluate the results with norcarane in the context of other experimental and theoretical results. The mechanistic issues involve whether one can implicate discrete cationic and radical intermediates.

Cationic Rearrangement Product. The implication of a cationic intermediate in norcarane oxidation is consistent with previous mechanistic studies employing probes that give unique rearrangements upon formation of a cation. In the case of P450, the methylcyclopropane substrates **10** and methylcubane (**11**) afforded some cation-derived rearrangement products with several hepatic P450s, including the P450 enzymes studied in this work.[26,34] For sMMO, evidence for production of cationic transients comes from oxidation studies with 1,1-dimethylcyclopropane[18] (**12**), probe **10a**,[19] and methylcubane (**11**).[19,35] In all of these studies, cation-derived products were obtained, but one cannot estimate the extent of cation formation because the rates, partitioning, and even the identities of the cationic intermediates are unknown. The norcarane results provide this same type of evidence, namely, that a cationic species is implicated, but the extent of its occurrence cannot be determined.



10a: R = Me **10b:** R = *t*-Bu **11** **12**

It is important to note that the detection of cationic rearrangement products does not necessarily demand that a norcaranyl C2 carbocation was produced. There exist two reasonable routes to cationic species in the enzyme-catalyzed

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oxidations. One is formation of a radical that is subsequently oxidized to a cation. This route is unlikely in the case of the hypersensitive probes **10** because the cyclopropylcarbinyl radicals produced from such species ring open with rate constants of $(5-8) \times 10^{11}$ s^{-1} to give benzylic radical products,[36] but benzyl-substituted products were not formed in substantial amounts in the enzyme-catalyzed oxidations of probes **10**.[19,26,34]

A second route to cationic products involves insertion of the elements of "OH^+", the expected reaction effected by the iron−hydroperoxy species in P450 or by a protonated form of H_{peroxo} in sMMO. Protonated alcohols thus formed could be deprotonated to give unrearranged product or react by loss of water with concomitant cationic rearrangement. We have previously argued the logic of this route to cationic products.[19,26,34] In their recent study of norcarane oxidation by the sMMO from *Ms. trichosporium* OB3b, Brazeau et al. attempted to detect reactions of H_{peroxo} (or its immediate successor) by stopped-flow kinetics methods but found no kinetic effect to suggest that norcarane was oxidized by these species.[17] The authors assumed that the amount of cationic intermediate formed in the sMMO reaction with norcarane was significantly greater than the amount of cationic rearrangement product **3** they detected in the products.[17] If, however, the ca. 1.2% of **3** they report[17] represents most of the norcarane that reacted by the cationic pathway in steady-state turnover, then perhaps no deviation in the observed single-turnover stopped-flow kinetics would be detectable.

We therefore conclude that the detection of cationic rearrangement products from norcarane provides supporting evidence for cationic intermediates similar to that found in previous studies with probes that distinguish between cationic and radical intermediates.[18,19,26] Given the high reactivity of cations and the small amounts of cationic products from all probes, it is likely that "cationic" reaction pathways are minor in both P450 and sMMO.

Radical Rearrangement Product. Although there is now general agreement for both P450 and sMMO hydroxylation reactions that the transition state for C−H bond activation has radical character, *the key question is whether there is evidence for discrete radical intermediates. That is, does a radical species "live" significantly longer than the transition-state lifetime of the reaction, which is about 0.2 ps at ambient temperatures?* Despite long histories of this claim for both types of enzymes, most of the evidence for discrete radicals was circumstantial. With little exception,[18] early mechanistic work involved the use of probes that gave the same rearrangement product from radicals and cations, and the formation of rearranged products was assumed to involve radicals. Now that one has strong evidence that some type(s) of cationic species is (are) formed in P450 and sMMO-catalyzed hydroxylations, that assumption is seen to be without foundation.

The detection of radical-derived rearrangement product **2** from the P450 enzymes, the sMMO enzyme from *Mc. capsulatus* (Bath) studied in this work, and the sMMO from *Ms. trichosporium* OB3b,[17] is, therefore, apparently spectacular. It is seemingly unequivocal evidence for production of discrete radical intermediates by these enzymes, and it can lead to a mechanistic conclusion regarding discrete radical intermediates that is diametrically opposed to that deduced from studies with other probes that permit "radical lifetime" estimates.

Mechanistic probes that do not permit differentiation between a radical and a cationic intermediate provide equivocal information about the lifetime of a radical when rearrangement products are observed. The absence of any rearrangement products from such a probe is not equivocal because it eliminates the possibility of any intermediate with a lifetime adequate for formation of detectable amounts of rearranged product. For P450 enzymes, hydroxylations of several probes gave only unrearranged alcohol products to the limits of detection, which were typically less than 1% relative yield. These include methylcyclopropane,[37,38] dimethylcyclopropanes,[38] and isopropylcyclopropane,[38] each of which is a potential precursor to a cyclopropylcarbinyl radical that ring opens with a rate constant similar to that of the norcaran-2-yl radical, on the order of 1×10^8 s^{-1}. Interestingly, even norcarane was reported to be hydroxylated by P450 without formation of any rearrangement products,[14] but the result probably reflects low analytical sensitivity in that study because the enzyme employed was P450 2B4, the same enzyme used here in a reconstituted system. The observation of radical-derived product **2** from P450-catalyzed hydroxylation of norcarane in the present work is in marked contrast to other results.

In the case of sMMO-catalyzed hydroxylations, the dichotomy is more apparent. No rearranged products were detected from the *Mc. capsulatus* (Bath) sMMO oxidations[39] of *trans*-1,2-dimethylcyclopropane and bicyclo[2.1.0]pentane, radicals from which ring opening rate constants are 2×10^8 and 2×10^9 s^{-1}, respectively.[40,41] Nor was any rearrangement found in the *Mc. capsulatus* (Bath) hydroxylations of "hypersensitive" radical probes, the radicals from which rate constants for radical ring openings exceed 1×10^{11} s^{-1}.[39,42] The detection of product **2** in norcarane hydroxylation catalyzed by this MMO is clearly out of character.

Although equivocal concerning the origins of the rearranged products, the product ratios when rearrangement is found from probes that do not differentiate between cations and radicals can be used to establish *upper limits* on the lifetimes of a putative radical. The general trend in P450 mechanistic studies has been that smaller upper limits for radical lifetimes occur as the rate constants for the radical rearrangements increase. That is, the relative amounts of rearranged products change little, and the upper limits on the radical lifetimes are calculated to be smaller when the rate constants for the radical rearrangements are greater. For example, the limit for a radical lifetime from hydroxylation of bicyclo[2.1.0]pentane[37,38] (k for the radical of 2×10^9 s^{-1})[40,41] is 100 ps, the limit from hydroxylation of hexamethylcyclopropane[38] (k for the radical of 5×10^9 s^{-1} at 37 °C)[40] is 4 ps, and the limit from hydroxylation of several aryl-substituted methylcyclopropanes[38,43−45] (k for the radicals

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in the range of $(1-5) \times 10^{11}$ s^{-1}) is about 0.3 ps. These values are limits to a radical lifetime. Direct calculations of radical lifetimes in P450 hydroxylations, however, are available from results with probes **10** that differentiate between radicals and cations; those results give radical lifetimes in the range of 0.08−0.2 ps,[26,34] or about the lifetime of a transition state.

Radical lifetimes for the sMMO hydroxylations also are on the order of the lifetime of a transition state. Very short upper limits are calculated from the partial racemizations observed in hydroxylation of chiral (by virtue of isotopic substitution) ethane by the sMMO from *Ms. trichosporium* OB3b[46] and of chiral ethane and chiral butane by the sMMO from *Mc. capsulatus* (Bath).[47] Assuming that fast rotation of the alkyl radical ($k = 5 \times 10^{12}$ s^{-1})[48] is the limiting process for racemization, one calculates an upper limit on the radical lifetime of less than 0.2 ps from the chiral alkane oxidations. As discussed elsewhere,[33] the rotation of a bound radical must be accompanied by recoil from the hydroxylating OH group, but even with inclusion of this vibrational component the bound radical lifetime is only 0.3 ps. The radical lifetime upper limit calculated from the oxidation of an aryl-substituted methylcyclpropane by the sMMO from *Ms. trichosporium* OB3b was less than 0.2 ps.[35,39] In the hydroxylation of probe **10b** by the sMMO from *Mc. capsulatus* (Bath), the calculated radical lifetime was 0.25 ps.[19]

Thus, radical lifetimes for P450 and sMMO oxidation reactions obtained from studies with probes that can differentiate between radicals and cations are 0.25 ps or less. In many cases, results from studies with probes that cannot differentiate between radicals and cations give upper limits on the radical lifetimes of 0.3 ps or less. Much larger upper limits for the radical lifetimes can be calculated from studies with some probes that do not differentiate between radicals and cations, but to do so is pointless because they are, after all, only limits. The preponderance of probe-derived evidence, then, is that the radical has a lifetime on the order of that of a transition state or about 0.2 ps.

In comparison to the above, the present results with norcarane suggest that radical intermediates are formed with relatively long lifetimes *if* one pathway gives alcohols **2**, **4**, and **5**. A rate constant of 2×10^8 s^{-1} for the norcaran-2-yl radical ring opening was previously estimated[15] from product distribution results in tin hydride trapping reactions.[10] Using that value, one calculates apparent radical lifetimes in the P450-catalyzed oxidations of norcarane of 15−160 ps. For the oxidation of norcarane by the sMMO from *Mc. capsulatus* (Bath), one calculates an apparent radical lifetime of 150 ps, and, using the results for norcarane oxidation by the sMMO of *Ms. trichosporium* OB3b,[17] one calculates an apparent radical lifetime in the range 20−150 ps. Although these are short lifetimes, they are 2−3 orders of magnitude greater than those determined previously.

The calculation of radical lifetimes from the norcarane results *requires* the assumption of a single pathway giving rearranged alcohol **2** and unrearranged alcohols **4** and **5**. If that condition is assumed, as it was by Brazeau et al.,[17] then the conclusion that hydroxylation proceeds through a radical intermediate is predetermined.

We reject the assumption of a single reaction pathway on the basis of the preponderance of experimental and computational evidence that multiple oxidants and multiple pathways exist for both P450 and sMMO. It seems likely that the amounts of rearranged alcohol **2** from norcarane oxidations are not reporting on the lifetimes of radical intermediates at all but instead reflect the amounts of substrate that react by different channels, one involving an insertion process and the other involving a bound radical. For both P450 and sMMO, computational work indicates that such reaction channels are available.

In the case of P450, a two-state model for reaction of the iron−oxo species was presented and has been further refined by Shaik and co-workers.[6,49] Both low-spin and high-spin reaction ensembles are found computationally for iron−oxo plus substrate. The low-spin reaction pathway resembles a hydrogen-abstraction reaction, but collapse of the nascent radical with oxygen is barrier-free, resulting in an insertion reaction. The high-spin reaction pathway gives a radical from substrate because a barrier to collapse exists.[49] Recent computational work by Yoshizawa et al. suggests that a barrier should exist on both the low-spin and high-spin surfaces, but limited dynamic simulations suggest a short (ca. 200 fs) radical lifetime.[8]

The barriers for iron−oxo oxidation of methane on the two spin surfaces are computed to be similar,[8,49] but the transition states for the reactions have different degrees of polarization with the low-spin TS being more highly polarized.[49] An increase in the donor properties of the substrate will favor reaction on the low-spin surface. This property seems to be an especially attractive explanation for why evidence for a discrete radical can be found for norcarane but not with probes **10**. Alkyl substitution and the cyclopropane ring in norcarane will increase the donor character of this substrate relative to methane, but the aryl and alkoxy groups in substrates **10** undoubtedly increase the donor character even more and would further favor reaction on the low-spin surface. Recent experimental results provided evidence that supports the two-state model for reactions of iron−oxo in P450, and the probe in that study was a methylcyclopropane that should have donor character similar to that of norcarane.[31]

Computations of hydroxylation by sMMO[9,33] by density functional and dynamics calculations find two approximately isoenergetic channels evolving from a common transition state in the reaction of Q with methane or ethane. One channel can be considered a bound radical recoil/rebound pathway; the other, a nonsynchronous concerted reaction.[9] Varying distribution into these channels by different probe substrates may be responsible for the lack of correlation between k_r and the amount of rearranged product, although a more detailed description will have to await the completion of computations for these larger hydrocarbons. Molecular dynamics calculations of the activation of ethane by Q, however, does indicate that the partition ratio between bound radical recoil/rebound and concerted mechanisms

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is influenced by the mass of the substrate.[33] As the mass of the substrate is increased, the insertion reaction becomes more highly favored.

For both enzyme types, the computations offer one possible rationale for the small amount of norcarane that appears to be processed by a radical channel. For P450, perhaps the spin state is important. For sMMO, a radical must somehow escape the bound radical state, perhaps because its secondary carbon atom cannot approach the bridging oxygen atom in Q as closely as the primary carbon atoms in the more massive substrates **10**. It appears reasonable that the radical channels might be insignificant for the latter substrates.

Although one can rationalize the results with norcarane in the context of minor hydroxylation pathways proceeding through a radical, they are so novel that one should use caution in drawing such a conclusion. Moreover, there exists a particularly worrisome aspect of the cation chemistry of norcarane that might be important in evaluating its reliability as a mechanistic probe. The premise that alcohol product **2** was formed by a radical reaction pathway is based on the assumption that the norcaran-2-yl cation does not react to give products derived from the (2-cyclohexenyl)methyl cation, but that is not correct. Acid-catalyzed acetolysis of 2-norcaranol was found to give predominantly the acetate from alcohol **3**, but the acetate from alcohol **2** also was formed as a minor product.[12,50] The solvolysis results previously reported for the 3,5-dinitrobenzoate esters of 2-norcaranol[13] and found here for the corresponding mesylates show that alcohol **2** is not formed in measurable amounts in these reactions. The solvolysis products are skewed heavily toward the cyclic system, 2-norcaranol, and the results are similar to those found in many reactions that involve putative cyclopropylcarbinyl cations that are trapped to give predominantly cyclopropylmethanol derivatives.[51] Thus, it would seem logical to anticipate that alcohol products **2** and **3** would be minor products from a cationic reaction in the enzyme-catalyzed oxidations, but, then, they were minor products in the reactions we studied. A better understanding of the norcaranyl cation chemistry would therefore appear to be important for interpreting the results of norcarane probe studies of P450 and sMMO.

In conclusion, small amounts of cation- and putative radical-derived rearrangement products are found in P450 and sMMO enzyme-catalyzed hydroxylations of norcarane. Formation of the cationic rearrangement product is consistent with the growing consensus that some type of cation-forming reaction is possible with these enzymes, perhaps involving reactions of predecessors of the ultimate oxidants that insert OH^+ into a C−H bond of substrate. Formation of small amounts of the radical rearrangement product might indicate the extent of radical formation by minor pathways of the ultimate oxidants in the enzymes and is not necessarily a measure of the radical lifetimes in a single reaction pathway. It is also possible that the production of the radical-derived product is artifactual and reflects an unappreciated aspect of norcaranyl cation chemistry. One is well-advised not to formulate generalizations about P450- and sMMO-catalyzed hydroxylation pathways from the unexpected findings with norcarane.

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Experimental Section

Bicyclo[4.1.0]heptane[52] (norcarane, **1**), 3-(hydroxymethyl)cyclohexene[53] (**2**), 3-cycloheptenol[10] (**3**), *endo*-bicyclo[4.1.0]heptan-2-ol[54] (*endo*-2-norcaranol, **4**), *endo*-bicyclo[4.1.0]hetptan-3-ol[55] (*endo*-3-nocaranol, **6**), bicyclo[4.1.0]heptan-2-one[56] (2-norcaranone, **8**), and bicyclo[4.1.0]heptan-3-one[55] (3-norcaranone, **9**) were prepared by literature methods. *exo*-Bicyclo[4.1.0]heptan-2-ol (*exo*-2-norcaranol, **5**) was obtained as a mixture with **4** (70:30, **5**:**4**) from LAH reduction of **8**,[56] and *exo*-bicyclo[4.1.0]heptan-3-ol (*exo*-3-norcaranol, **7**) was obtained as a mixture with **6** (13:87, **7**:**6**) from LAH reduction of **9**.[55]

Solvolysis Reactions. In a representative reaction, alcohol **4** (125 mg, 1.1 mmol) was added to a solution of freshly distilled MsCl (96 μL, 1.21 mmol) and Et_3N (0.23 mL) in 10 mL of dry THF at −20 °C. After 1 h at −20 °C, the mixture was treated with 0.07 mL of water. The mixture was stirred at −20 °C for 1 h and at room temperature for 12 h. The reaction was quenched by addition of excess water, and products were isolated by an extractive workup (ether, acid). The ethereal solution was dried over $MgSO_4$ and analyzed by GC.

P450-Catalyzed Oxidations. The P450 enzymes were prepared as previously reported.[20−22] In a typical reaction, a mixture of 1 nmol of P450 Δ2B4 and 2 nmol of P450 reductase was allowed to stand in an ice bath for 5 min. A mixture of 0.96 nmol of DLPC suspended in 20 mM potassium phosphate buffer (pH = 7.4), sonicated before use, was added to the enzyme mixture. The mixture was diluted to a volume of 2 mL with buffer. A solution of 10 μL of **1** in MeOH (15 mg/mL) was added. The mixture was incubated at 37 °C for 5 min. The oxidation was initiated by the addition of NADPH buffer solution; the final concentration of NADPH was 1.2 mM. The mixture was gently shaken at 37 °C for 30 min and then extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phase was dried ($MgSO_4$) and filtered. A solution of tridecane was added as an internal standard, and the solution was concentrated to ca. 0.05 mL by fractional distillation with a 60−70 °C oil bath.

The resulting mixture was analyzed by GC and GC-MS on 0.52 and 0.25 mm, respectively, Carbowax columns. Products **2** and **6** were not resolved and appeared as a merged peak. Whereas the MS of both **2** and **6** contained ions at $m/z = 79$, the MS of **2** had an ion at $m/z = 81$ that was not present in the MS of **6**. Using authentic samples of **2** and **6**, the relative intensities of the ions at $m/z = 79$ were determined as well as the relative intensities of the ions from **2** at $m/z = 79$ and $m/z = 81$. The ratio of **2**:**6** was then determined in the product samples from the GC-MS results and the measured ion ratios.

sMMO-Catalyzed Oxidations. The sMMO hydroxylase was purified from batch cultures of *Mc. capsulatus* (Bath) according to published procedures.[57] The reductase and coupling protein components of sMMO were purified from recombinant expression systems.[57,58]

Norcarane hydroxylations with sMMO were carried out as follows. A 10 nmol portion of MMOH, 20 nmol of MMOB, and 5 nmol of MMOR were combined in a total volume of 400 μL of 25 mM MOPS, pH 7.0, in a septum-capped vial. The enzyme was incubated for 1 min at 45 °C before addition of 8 μL of norcarane (neat) was added by means of a gastight syringe. The reaction was initiated by addition of 1.6 μmol of NADH and was incubated at 45 °C for 20 min with stirring. Addition of 2 mL of methylene chloride quenched the reaction by causing the proteins to precipitate. The organic layer was removed and the aqueous layer extracted twice more with 2 mL portions of methylene chloride each time. The organic layers were combined, dried with

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$MgSO_4$, and filtered. The product mixture was concentrated by distillation, reducing the volume of the organic fraction from ~6 to ~0.5 mL. Concentrated extracts were analyzed by GC-MS as described above, and the yields are reported on the basis of the relative ionizations from each product.

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Note Added in Proof: Another work describing P450-catalyzed oxidations of norcarane was recently reported; Auclair, K.; Hu, Z.; Little, D. M.; Ortiz de Montellano, P. R.; Groves, J. T. *J. Am. Chem. Soc.* **2002**, *124*, 6020−6027. The work contains significant differences in data interpretation to which we draw the reader's attention.

Supporting Information Available: Product yield table and a representative GC-mass spectral run (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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