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Xylene monooxygenase, a membrane-spanning non-heme diiron enzyme that hydroxylates hydrocarbons via a substrate radical intermediate

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Abstract The non-heme diiron enzyme xylene monooxygenase (XylM) has been shown to hydroxylate hydrocarbons via a hydrogen abstraction–carbon radical recombination mechanism (oxygen rebound). Using the radical clock bicyclo[4.1.0]heptane (norcarane) in a whole-cell assay, and observing the ratio of rearranged 3-(hydroxymethyl)cyclohexene and unrearranged 2-norcaranol products, the lifetime of the substrate radical was determined to be approximately 0.2 ns. The wild-type organism *Pseudomonas putida* mt-2 and two separate *Escherichia coli* clones expressing *xylMA* genes gave similar results. One clone produced the *Pseudomonas putida* mt-2 XylMA hydroxylase and the other produced *Sphingomonas yanoikuyae* B1 XylMA hydroxylase. Clones were constructed by inserting genes for xylene monooxygenase and xylene monooxygenase reductase downstream from an IPTG-inducible T7 promoter. Mechanistic investigations using whole-cell assays will facilitate more rapid screening of structure–function relationships and the identification of novel oxygenases. This approach should enable the construction of a picture of the key metalloenzymes and the mechanisms they

use in selected parts of the global carbon cycle without requiring the isolation of every protein involved.

Keywords Cytochrome P450 · Hydroxylase · Methane monooxygenase · Non-heme diiron enzymes · Radical clocks

Abbreviations AlkB: alkane monooxygenase · CYP: cytochrome P450 · IPTG: isopropyl- β -D-thiogalactopyranoside · sMMO: soluble methane monooxygenase · XylM: xylene monooxygenase

Introduction

Xylene monooxygenase (XylM) is a membrane-spanning, non-heme diiron enzyme with a histidine-rich active site that catalyzes the insertion of an oxygen atom into a C–H bond [1]. XylM has 25% sequence homology with alkane monooxygenase (AlkB) [2], with significant homology in both an eight-histidine motif and in the presence of three long hydrophobic domains thought to be membrane-spanning regions [3]. A detailed comparison of the amino acid sequence of a variety of hydroxylases suggests that XylM and AlkB, together with a number of membrane-spanning desaturases, form a third, distinct class of diiron enzymes, the other two evolutionarily distinct classes being typified by hemerythrin (class I) and soluble methane monooxygenase (sMMO) (class II) [3]. A genetic survey of aerobic alkane-degrading bacteria showed extensive homology to the eight-histidine motif of AlkB and XylM, further indicating a widespread role for this motif in the environment [4]. XylM has been used both in the native organism for the commercial scale production of heteroaromatic acids [5] and in an alkane-responsive expression system in *Escherichia coli* for the moderate scale conversion of styrene to (*S*)-styrene oxide [6].

The insertion of oxygen into a C–H bond is a chemical reaction that has long fascinated chemists both because C–H bonds are difficult to disrupt and because

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oxygen is difficult to selectively activate. Hydrocarbons represent an energy-rich fuel for microorganisms. The reaction between molecular oxygen and hydrocarbons to yield an alcohol and water is a thermodynamically favorable reaction, generating products that can ultimately be fed into aerobic respiratory pathways. In spite of the thermodynamically favorable nature of hydrocarbon oxygenation, kinetic barriers and other challenges (e.g. the risk to the organism of producing reactive oxygen species) constrain this strategy. Thus, a limited number of organisms exist that can grow on hydrocarbons as their sole source of carbon and energy. Just how limited that number of organisms is not known. Approximately 100 different species of bacteria and 100 different species of fungi that can metabolize alkanes [7, 8] have been identified. Whether that number represents the majority of hydrocarbon transforming species or a minuscule culturable fraction is not clear. Even less is known about the diversity of metalloenzymes that can catalyze the monooxygenation of hydrocarbons. The growing influx of genomic data pressures chemists to ask, and find ways to address, questions regarding the diversity of hydroxylases by rapid and efficient procedures.

Until recently, the two paradigms for biological hydrocarbon oxidation have been cytochrome P450 (CYP), a heme-containing protein [9], and sMMO, a non-heme, diiron enzyme [10]. More than two decades of mechanistic studies on CYP and metalloporphyrin models have led to a consensus mechanism involving the binding and reduction of dioxygen to form first a peroxo-Fe(III) state and ultimately an oxo-iron(IV) porphyrin π -cation radical (compound I) as the active oxygen species (see, for example [11, 12, 13, 14]). This ferryl species was recently observed in Cyp 119 (a thermophile) [15]. While stopped-flow, freeze-quench experiments with P450_{cam} failed to detect a Cpd I intermediate [16], the substrate-derived alcohol has been found at low temperature to be formed with the hydroxyl oxygen bound to the heme iron [17]. Hydroxylation is believed to occur via homolytic bond cleavage of the C–H bond followed by OH rebound to the carbon-centered substrate radical [18, 19, 20]. Cationic processes [21] and electrophilic reactivity of the ferric peroxo species [21] have also been suggested.

Recently, attention has focused on sMMO, which catalyzes the conversion of methane to methanol. The crystal structure of sMMO reveals an active site with two iron atoms in close proximity ligated by four glutamates and two histidines [22]. Despite substantial research, debate remains about the mechanism of alkane hydroxylation by sMMO [23, 24, 25, 26, 27]. We have recently reported that sMMO can react to form both a short-lived substrate radical and a substrate cation. We attribute the presence of both products to an initial homolytic bond cleavage of the C–H bond by the reactive high-valent iron-oxo species termed compound Q. We propose that electron transfer from the substrate radical to the one-electron reduced form of Q [an hydroxo-Fe(III)Fe(IV)

enzyme species termed intermediate R] is kinetically competitive with OH rebound, yielding products indicative of both radical and cationic substrate intermediates [28].

There are also a few reports of mechanistic studies of particulate methane monooxygenase (pMMO) [29, 30, 31, 32]. This membrane-spanning, multi-copper metalloenzyme is thought to be expressed by most methanotrophs, except under copper-limited conditions. Reportedly very difficult to purify while retaining activity, work on this important system has been relatively limited.

AlkB is a membrane-spanning, non-heme diiron enzyme with a histidine-rich active site that catalyzes the terminal hydroxylation of alkanes [4, 33, 34, 35]. Recently, we have reported data with the diagnostic substrate norcarane that point to an oxygen-rebound mechanism for AlkB with a relatively long-lived (1 ns) substrate radical intermediate [36], consistent with previously published work on this enzyme [37]¹. We now report mechanistic information on another hydrocarbon-metabolizing metalloenzyme, XylM, extending our survey of the relationship between active site structure and function. XylM normally catalyzes the hydroxylation of the methyl group of *meta*-xylene.

We have investigated the mechanism of C–H bond hydroxylation catalyzed by xylene monooxygenase using the diagnostic substrate norcarane (bicyclo[4.1.0]heptane) both in a wild-type organism, *Pseudomonas putida* mt-2, and in two separate *E. coli* clones expressing XylM genes. One clone makes XylM from *Pseudomonas putida* mt-2 and the other makes XylM from *Sphingomonas yanoikuyae* Bl. The results give conclusive evidence for a carbon-centered radical intermediate with a lifetime of approximately 0.2 ns in the hydroxylation process.

Materials and methods

Substrates and product standards

Norcarane was synthesized according to a published procedure [38] and distilled before use. Purity was checked by NMR and GC-MS. *endo*-2-Norcaranol was synthesized by carrying out the Simmons–Smith reaction on the corresponding cyclohexenol [39]. It was then converted to the ketone by oxidation with chromic acid and then reduced with LiAlH₄ to produce a 50/50 mixture of *endo*- and *exo*-2-norcaranols. 3-(Hydroxymethyl)cyclohexene was synthesized according to a published procedure [40]. The corresponding aldehyde was synthesized by oxidation with pyridinium chlorochromate [41]. *endo*-3-Norcaranol was synthesized according to a published procedure [20]. It was oxidized to the corresponding ketone with chromic acid and then reduced with LiAlH₄ to produce a 50/50 mixture of *endo*- and *exo*-3-norcaranols (personal communication, Dr. Zhengbo Hu). 3-Cycloheptenol was synthesized according to a published procedure [42]. All chemicals used in synthesis were reagent grade and used without further purification. Solvents used for GC-MS analysis were analytical grade.

¹ This paper reported 100% rearrangement of methyl (phenyl)cyclopropane with partially purified AlkB. While these results are consistent with a radical mechanism, a cationic pathway would have also produced the same product distribution

Clone construction

Clones were constructed to express the xylene monooxygenase genes in *E. coli* for activity analysis. The *xylMA* genes from *Sphingomonas yanoikuyae* B1 were PCR amplified from genomic DNA using the primers 5'-ATGGATGCTCTGCGC-3' and 5'-TCAGCGCATCGTGCT-3', corresponding to the 5' end of the *xylM* gene and the 3' end of the *xylA* gene. The PCR product was cloned into the pCRT7/CT-TOPO vector (Invitrogen, Carlsberg, Calif.) and transformed into *E. coli* TOP10 cells. Several clones were sequenced to determine the orientation of the inserted PCR product and to verify that no sequence errors were introduced by *taq* polymerase. One clone in the correct orientation and with the correct sequence was transferred to the T7 expression strain BL21(DE3)pLysS. In like manner, the *xylMA* genes from *Pseudomonas putida* mt-2 were PCR amplified from genomic DNA using the primers 5'-ATGGACACGCTTCGT-3' and 5'-AGCTTCTGCTACTGC-3'.

Whole-cell incubations

Wild-type mt-2 was grown in mineral salts basal medium [43] with *meta*-xylene delivered in a hanging glass bulb overnight, subcultured into fresh medium, and allowed to grow until reaching an OD at 600 nm of 0.5. Four different concentrations of norcarane were added: 50, 100, 200 μ L, and norcarane via a hanging glass bulb to 20 mL of cell culture. In all cases, xylene was still present and incubation continued for 15 h. The cloned organisms were grown in a similar fashion on mineral salts basal medium supplements with yeast extract (0.1%), casamino acids (0.1%), glucose (20 mM), and ampicillin (100 μ g/mL). The cells were induced with 1 mM IPTG when the OD of the culture reached 0.5 at 600 nm. One and a half hours after induction, the norcarane (same volume-to-volume ratio as described above) was added to the culture and the cells were incubated overnight. The supernatant was collected by centrifugation (8000 \times g, 15 min) and extracted three times with ethyl acetate, concentrated, and the products assayed directly by GC-MS. Authentic products were synthesized and their retention times and fragmentation patterns compared to those of the identified peaks in the GC-MS spectra. A set of experiments was also done with resting cells, as resting cell experiments sometimes yield cleaner baselines, in which the cells were centrifuged, rinsed three times with buffer, resuspended in a 50 mM phosphate buffer (pH 7), and incubated for 12 h. There were no differences in the results from resting cell experiments in comparison to growing cell experiments.

Control experiments in which potential products were provided to growing cells were done. Control experiments with the cloned organisms in which the plasmid (without the gene of interest) was inserted into *E. coli* were done. Control experiments in which all the reaction components were added to the media except for the organisms and the media incubated at the selected incubation temperature (30 $^{\circ}$ C for wild-type organism, 37 $^{\circ}$ C for *E. coli*) were also done.

Analytical methods

The GC-MS used was a HP GC 6890/MS 5973 with a HP-5MS crosslinked 5% PH ME siloxane capillary column (30 m \times 0.25 mm \times 0.25 μ m) run with an initial oven temperature of 50 $^{\circ}$ C, ramping to 220 $^{\circ}$ C at a ramp rate of 10 $^{\circ}$ C/min.

Results

Catalytic activity of XylM towards norcarane

XylM metabolized norcarane, yielding a distribution of ring-closed and ring-open products. Generally, 2–4% of

the norcarane was metabolized to products over the course of the reaction, as determined by quantifying the moles of products, knowing the moles of substrate added, and taking into account the extraction efficiency of the alcohols from the reaction mixture. This conversion rate is consistent with rates seen in other whole-cell assays. Unreacted norcarane is difficult to quantify after the reaction because the volatility of norcarane causes it to be lost during workup. For both the wild-type organism and the mt-2 XylMA and B1 XylMA clones, approximately 87–94% of the norcarane-derived products are *endo*- and *exo*-2-norcaranol (compounds **4** and **5**, respectively) and 2-norcaranone (compound **9**). Approximately 3.9–4.5% of the norcarane-derived products are 3-(hydroxymethyl)cyclohexene (compound **3**), the ring-open product arising from a radical precursor. In many experiments, small (1–2%) amounts of 3-cycloheptenol (compound **2**), the hydroxylation product from the ring-opened cationic resonance form, were seen. Failure to detect it in all experiments may represent the fact that levels in some experiments may have been near the detection limits of the assay. Small amounts of products from oxidation at the mechanistically uninformative 3-position were seen with some organisms. A chromatogram from a typical reaction is shown in Fig. 1. Table 1 shows the average product distributions from all experiments for each organism and the standard error of the values obtained. The data represent the average of four or five different experiments with each strain. When the wild-type organisms were grown with *meta*-xylene, *ortho*-tolylmethanol was

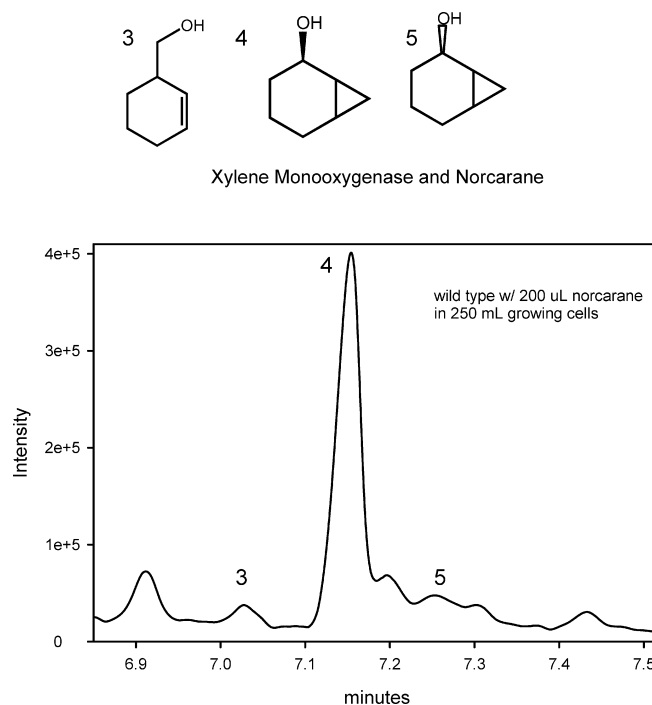


Fig. 1 Representative GC-MS trace for XylM oxidation of norcarane

Table 1 Distribution of products from XylM oxidation of norcarane (average + SE). Data given in terms of total norcarane-derived products

	2	3	4	5	9	7 ^a	8
wt mt-2	2.7 (0.05)	3.9 (0.45)	73.1 (0.15)	17.1 (2.65)	3.2 (2.75)	0	0
mt-2 clone	1.1 (0.65)	4.3 (1.25)	63.3 (5.8)	18.6 (3.35)	12.1 (3.3)	0	1.0 (0.6)
B1clone	2.2 (0.8)	4.5 (0.5)	62 (5.8)	19 (4.4)	5.6 (1.8)	1.6 (1.6)	1.5 (0.5)

^aCompound **6** (*endo*-3-norcaranol) co-elutes with **5** and cannot be precisely resolved, although the fragmentation pattern is clearly dominated by **5**; **7** (*exo*-3-norcaranol) can be resolved

produced. Figure 2 shows the structures of the various compounds and Fig. 3 the product distribution.

Control experiments confirmed that the chemistry detected was due to the catalytic activity of xylene monooxygenase and not due to spurious abiotic or biotic oxidations. No products were seen when norcarane was incubated for 12–15 h in buffer. No products were seen when norcarane was incubated with a negative control of *E. coli* containing only the expression vector with no monooxygenase gene. Abiotic experiments were also done to test the stability of products to the experimental workup conditions and all products were found to remain unmodified by incubation in buffer followed by extraction and GC-MS analysis.

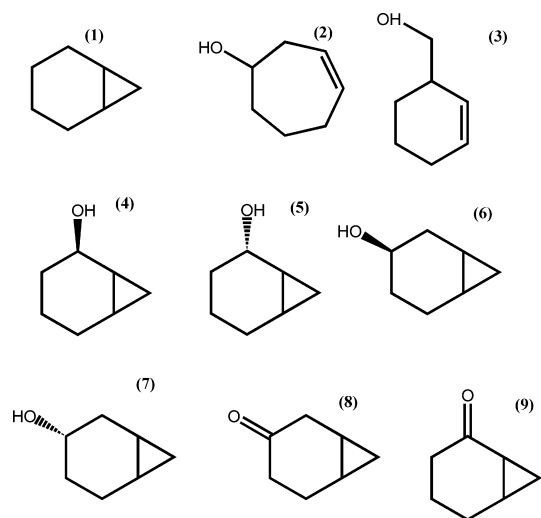
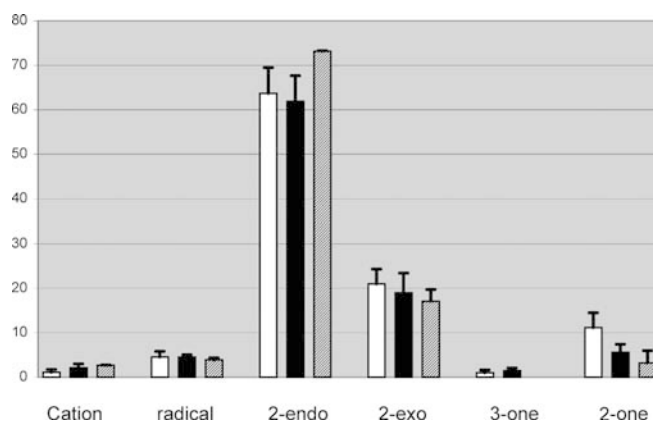
To ensure that product quantification was not compromised by further metabolism of the initial products, we synthesized 2-norcaranone (the corresponding ketone for 2-norcaranol), 3-norcaranone (the corresponding ketone for 3-norcaranol), 3-cycloheptenone (the corresponding ketone for 3-cycloheptenol), and 2-cyclohexene-1-carbaldehyde [the corresponding aldehyde for 3-(hydroxymethyl)cyclohexene] and looked for those molecules as well in the GC-MS chromatograms. When detected, they were included in calculation of the ratio of ring-closed to ring-open products used to determine the radical lifetime of the substrate. In addition, potential products were incubated with the cells as substrates. Alcohols were seen to be transformed gradually and

partially to the expected ketones over the course of a 15-h incubation. The use of two *E. coli* hosts expressing xylene monooxygenase (whose results are the same as the wild-type organism) allow us unequivocally to assign the chemistry being observed to XylM.

The rearrangement rate for norcarane has been determined to be $2 \times 10^8 \text{ s}^{-1}$ [36]. For XylM, we calculate a radical lifetime of $0.2 \times 10^{-9} \text{ s}$ for each of the three organisms tested (the formula for calculating the radical lifetime from the metabolism of a radical clock is

$$\text{radical lifetime} = \left\{ \left(\frac{[\text{ring closed}]}{[\text{ring open}]} \right) (k_{\text{rearrangement}}) \right\}^{-1}.$$

Quantification was based on manual integrations of all product peaks and crosschecked with the intensity of key ions for each product. Standards samples of all products were synthesized. Chromatograms from whole-cell extract often contain multiple peaks; occasionally co-elution of peaks presents a problem. Comparison of both quantification approaches showed that differences in results in the two approaches were small. For all three organisms, the average radical lifetime of 0.2 ns had a standard error of 0.04 ns (organism specific standard errors are as follows: mt-2 clone 0.075 ns, B1m 0.1 ns, wt 0.085 ns). Since the primary question being posed in this work is whether XylM hydroxylates hydrocarbons via an oxygen rebound mechanism similar to that used by the presumably structurally similar enzyme AlkB, the

**Fig. 2** Structures of norcarane and norcarane oxidation products**Fig. 3** Distribution of products from all three organisms. First block in each set is mt-2 clone (white); second block is B1M clone (black); third block is wild type (diagonal black and white stripes). Error bars represent standard errors

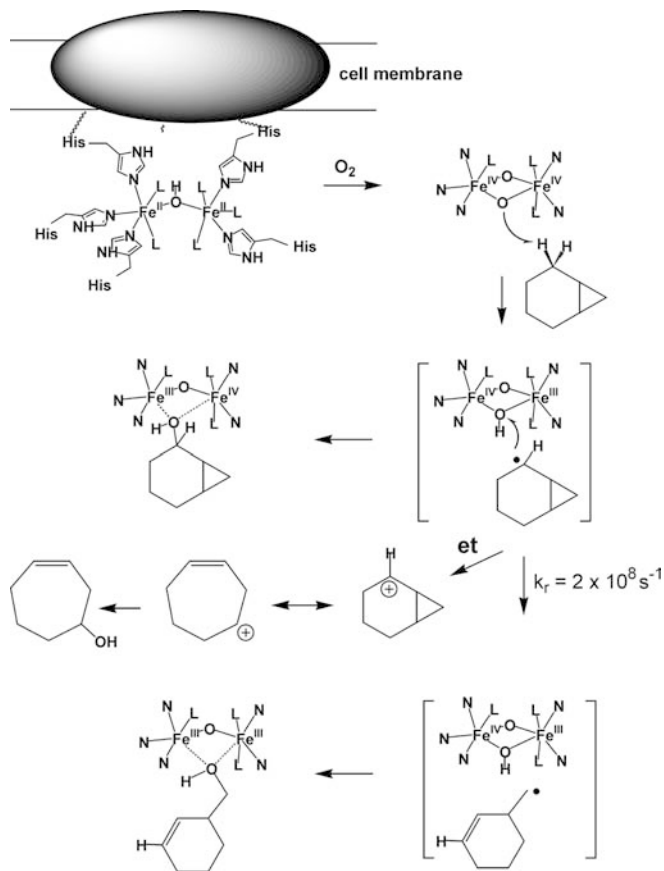


Fig. 4 Proposed reaction mechanism for xylene monooxygenase

resolution of the data is more than adequate to answer that question affirmatively.

A scheme for the reaction between XylM and norcarane is represented in Fig. 4. According to this model, the diiron site on XylM reacts to form a high-valent intermediate analogous to compound Q in sMMO. This species is then capable of abstracting a hydrogen atom from norcarane, forming a carbon-centered radical whose strain-relieving ring opening takes place in concert with the enzyme-mediated OH rebound step. Cationic products are proposed to arise from the abstraction of a second electron from the transient carbon-centered radical.

Discussion

The huge influx of genetic data and the urgency to understand chemical processing in the environment requires that chemists develop methods to elucidate functional data on enzymes that are less time and labor intensive than traditional biochemical protein purification and characterization techniques. If hundreds of new gene products are being reported per year and the average time that it takes to purify a protein and reconstitute activity is two years (if fortunate), the

dilemma is obvious. Retaining the activity required to probe mechanisms of purified membrane-spanning proteins continues to challenge biochemists. We hypothesize that diagnostic substrates, whose use represents a well-established technology in bioinorganic chemistry [44, 45], will provide insightful mechanistic information about enzyme mechanisms when used in whole cells. The ability to clone a metalloenzyme of interest into a host organism, thereby eliminating enzymes found in the wild-type organism that catalyze subsequent metabolic transformations and simplifying growth conditions, further empowers the whole-cell system approach [46, 47]. This paper provides the first detailed report of our efforts to test this hypothesis. The evidence obtained thus far with both AlkB [36] and XylM indicates that whole-cell *in vivo* studies of metalloenzyme mechanisms can reveal mechanistically informative information.

While diagnostic substrates have been widely used in bioinorganic chemistry, there has been, however, some controversy in the field regarding whether or not the substrates are passive reporters of the active site chemistry or whether the chemical nature of the probe molecule affects the results that are “reported back” to the observer. With both sMMO and P450, different probe molecules do yield different mechanistic information, implying some effect of substrate structure on detected chemistry. For example, CYP shows significant substrate rearrangement in some studies [20, 48, 49, 50, 51, 52], consistent with a discrete radical mechanism, but with other substrates very little rearrangement is seen [52, 53, 54], consistent with a concerted mechanism. Likewise, sMMO shows evidence for a discrete radical mechanism with some substrates [28], cationic rearrangements with at least some substrates [28, 55], and evidence for a concerted mechanism with only a radical-like transition state with other substrates [25, 26].

Norcarane is a useful probe molecule [36, 56, 57] for several reasons and its use in whole-cell studies has helped elucidate its utility. Its compact shape and relatively unhindered α -carbon (relative to the cyclopropyl group) make it an innocuous molecule, similar in size and chain length in many cases to the natural substrates. Its ability to distinguish between cationic and radical precursors is also important. Norcarane can distinguish between radical and cationic pathways because the 2-norcaranyl radical rearranges to form predominantly the 3-cyclohexenylmethyl radical [58] with a rearrangement rate constant of $2 \times 10^8 \text{ s}^{-1}$. In contrast, the 2-norcaranyl cation is a resonance hybrid with the 3-cyclohepten-1-yl cation, giving both *endo*- and *exo*-2-norcaranol (from the ring-closed cation) and 3-cycloheptenol (from the ring-opened cation) as hydroxylation products [59]. Cationic processes can result in small amounts of (3-hydroxymethyl)cyclohexene, but only as a very minor product compared to cycloheptenol [60]. In a monooxygenase where homolytic C–H bond abstraction occurs, both the ring-opened and ring-closed radicals will experience “•OH rebound” to yield hydroxylated products.

Therefore, depending on product yield and GC detection sensitivity, production of a norcaranyl radical with a lifetime greater than 0.01 ns should yield both norcaranol (possibly both *exo* and *endo* isomers) and (hydroxymethyl)cyclohexene. The exact product distribution will depend on steric constraints at the active site and enzyme rebound rate. In contrast, production of a cation on norcarane, either through ionization (loss of water) of a protonated alcohol or from the one-electron oxidation of the radical, will produce the corresponding norcaranyl cation and the cycloheptenol cation. Production of cycloheptenol from the oxidation of norcarane is therefore clear evidence of a transient substrate cation (albeit one that could have been preceded by a transient substrate radical), while production of (3-hydroxymethyl)cyclohexene from the oxidation of norcarane is clear evidence of a transient substrate radical. Our use of norcarane as a probe of reaction mechanisms in different classes of monooxygenases is yielding interesting information on the connections between active site structure and function.

Table 2 compares results on reaction mechanism studies using norcarane as the probe substrate for a series of monooxygenases [AlkB, XylM, sMMO (two strains), and P450 (two bacterial P450s and two mammalian P450s)]. These data suggest that monooxygenases do vary in the precise timing of C–H hydroxylation, which implies there are subtle differences in the electronic structure of the active sites.

Shanklin et al. [3] have separated diiron enzymes into three categories, based upon amino acid sequence alignment. In class I the diiron axis is oriented perpendicular to the long axis of the four-helix bundle. The iron atoms are coordinated by five histidines, two bridging carboxylates from two glutamates, a bridging oxo ligand, and an unidentified ligand. The best characterized class I diiron-oxo protein is hemerythrin. Class II is characterized by the R2 component of ribonucleotide reductase and the hydroxylase component of sMMO. In the class II proteins the diiron axis is oriented parallel to the long axis of the four-helix bundle, making it unlikely that class I and class II proteins are evolutionarily related. The iron ligands include two histidines,

four glutamates (two that are bidentate), an oxo or hydroxo bridge, and two waters. The class III proteins have a distinct consensus sequence: HX(3 or 4)HX(20–50)HX(2 or 3)HHX(100–200)HX(2 or 3)HH. All the class III proteins are membrane-spanning proteins. This class includes stearoyl-CoA desaturase, AlkB, and XylM. The exact nature and identity of the iron ligands in this class are not known. Fox et al. [61] speculate that there may be six histidines, one bridging glutamate, a bridging atom, and several other ligands. This third class appears to be evolutionarily distinct from the other two.

There is increasing evidence that the class III proteins may be widely distributed among bacteria that degrade hydrocarbons. A genetic survey by van Beilen and co-workers [4] indicated that the majority of alkane-degrading bacteria in their collection contained a gene with significant homology to AlkB. A survey of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils found that AlkB was generally detected in contaminated soils in these environments [62].

The two xylene monooxygenases tested here represent two distinct members of the xylene monooxygenase class of enzymes [2, 63, 64]. Here they are only 50% identical at the amino acid sequence level. They have distinct substrate specificities, most noticeable by the fact that xylene monooxygenase from *P. putida* mt-2 is capable of hydroxylating indole to 3-hydroxyindole, which spontaneously dimerizes to indigo [65], while xylene monooxygenase from *S. yanoikuyae* B1 does not perform this reaction (E. Kim and G.J. Zylstra, unpublished data). The two *xylMA* clones described in this paper behave similarly: the mt-2 clone produces indigo when indigo is present while the B1 clone does not.

XylM hydroxylates norcarane by initially cleaving the C–H bond homolytically. The carbon-centered radical that is generated persists for about 0.2 ns before the enzyme rebounds with Fe–OH to form an iron-coordinated alcohol. The detection of small amounts of products derived from a cationic precursor is most likely explained by a process of electron transfer from the radical to the Fe(IV)–OH enzyme active site that competes with the oxygen atom transfer rebound process. In this instance, the substrate would have a transient cation and the enzyme would capture oxygen from the active site iron cluster or near by water to afford the rearranged alcohol (Fig. 4) [20, 72]. Our results do not permit us to rule out the possibility of a electrophilic Fe(III)OOH species that could insert OH⁺ into a C–H bond, as has been suggested [21], although the lack of a precedent for this kind of chemistry in model systems and the contrary perspective provided by high-level DFT calculations [66, 70, 71] cause us to view that alternative as less likely [67].

The identification of two non-heme, diiron membrane-spanning enzymes with a histidine-rich metal binding site and the determination of a similar hydroxylation mechanism for both further challenges us to think about the influence of the active site on the reaction mechanism. Recent theoretical development on

Table 2 Comparison of radical lifetimes determined using norcarane as the probe substrate for several different monooxygenases

	Radical lifetime with norcarane (ns)
AlkB (clone) [36]	1
AlkB (wt <i>P. oleovorans</i>) [36]	1
XylM (wt Mt-2)	0.2
XylM (Mt-2 clone)	0.2
XylM (B1clone)	0.2
sMMO (Bath) [52]	0.17
sMMO (OB3b) [28]	0.02
Cytochrome P450 _{cam} [20]	0.052
Cytochrome P450 _{BM3} [20]	0.044
CYP2E1 [20]	0.035
CYP2B1 [20]	0.016

spin-state crossing effects provide a potential explanation for subtle mechanistic differences among monooxygenases [68, 69, 70, 71], although detailed considerations of the relationship between electronic structure and reaction mechanism in non-heme, diiron enzymes are still in their infancy. Whether the presence of a more nitrogen-rich ligation environment has a direct effect on the radical lifetime of substrates is an as yet unanswered question. Additional work that considers the forces affecting the dynamics of radical cage behavior is warranted.

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