Chemistry & Biology
Article

Profiling Mechanisms of Alkane Hydroxylase Activity In Vivo Using the Diagnostic Substrate Norcarane

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DOI 10.1016/j.chembiol.2006.12.007

SUMMARY

Mechanistically informative chemical probes are used to characterize the activity of functional alkane hydroxylases in whole cells. Norcarane is a substrate used to reveal the lifetime of radical intermediates formed during alkane oxidation. Results from oxidations of this probe with organisms that contain the two most prevalent medium-chain-length alkane-oxidizing metalloenzymes, alkane ω-monooxygenase (AlkB) and cytochrome P450 (CYP), are reported. The results—radical lifetimes of 1–7 ns for AlkB and less than 100 ps for CYP—indicate that these two classes of enzymes are mechanistically distinguishable and that whole-cell mechanistic assays can identify the active hydroxylase. The oxidation of norcarane by several recently isolated strains (*Hydrocarboniphaga effusa* AP103, rJ4, and rJ5, whose alkane-oxidizing enzymes have not yet been identified) is also reported. Radical lifetimes of 1–3 ns are observed, consistent with these organisms containing an AlkB-like enzyme and inconsistent with their employing a CYP-like enzyme for growth on hydrocarbons.

INTRODUCTION

Alkanes are ubiquitous, entering the environment through natural oil seepages, oil spills, and biological production. Microorganisms have been exposed to alkanes for eons and have evolved methods for extracting metabolic energy and cell-building material from this energy-rich class of compounds. More than 200 different species of fungi and bacteria that are capable of oxidizing alkanes have been identified [1–3], but in relatively few cases has the enzyme responsible for the chemistry been determined [4]. Six enzyme families are known to be capable of oxidizing unactivated alkanes, or, more generally, to hydroxylate unactivated C-H bonds. These families are the alkane ω-monooxygenases (AlkB) [5], the soluble methane monooxygenases (sMMO)/toluene monooxygenases (TMO) [1, 6–8], the particulate methane monooxygenases (pMMO) [9], cytochrome P450 (CYP) [10], napthalene dioxgenase (NDO) [11], and TauD and related α-ketoacid-dependent hydroxylases [12]. While all families reductively activate dioxygen to hydroxylate their cognate substrates, they differ markedly in their active site coordination chemistry, cellular location, and cofactor content.

The majority of medium- and long-chain alkanes are oxidized either by AlkB or CYP [1, 13–15]. CYPs contain a thiolate-ligated heme active site and can be either membrane spanning or soluble [10]. AlkB contains a diiron active site with a histidine-rich coordination environment [16] and is predicted by sequence analysis and gene fusion studies to have six membrane-spanning helices that hold the diiron active site in place near the inner membrane-cytoplasm interface [17]. Although AlkB can be purified, maintaining activity in the purified state is difficult.

The molecular mechanisms of oxygen activation for some of these metalloenzymes are well studied. Heme-oxygenases, such as CYP, hydroxylate inert hydrocarbon substrates by using a high-valent oxoiron(IV) porphyrin π-cation-radical intermediate similar to peroxidase compound I [10]. The consensus mechanism for oxygen activation and transfer involves a hydrogen atom abstraction-oxygen rebound pathway [10, 18]. Hydroxylation of the very unreactive C-H bond of methane by the non-heme diiron enzyme sMMO has many similarities to the P450 mechanism [19–22], in part because the active oxidant, a μ-oxo-diiron(IV) intermediate called compound Q, is poised at the same net oxidation state as the oxoiron(IV) porphyrin π-cation-radical species produced by the heme-oxygenases. AlkB has been postulated to follow an sMMO-like reaction mechanism [23–25]; however, the details of the reaction cycle for AlkB have not yet been elucidated.

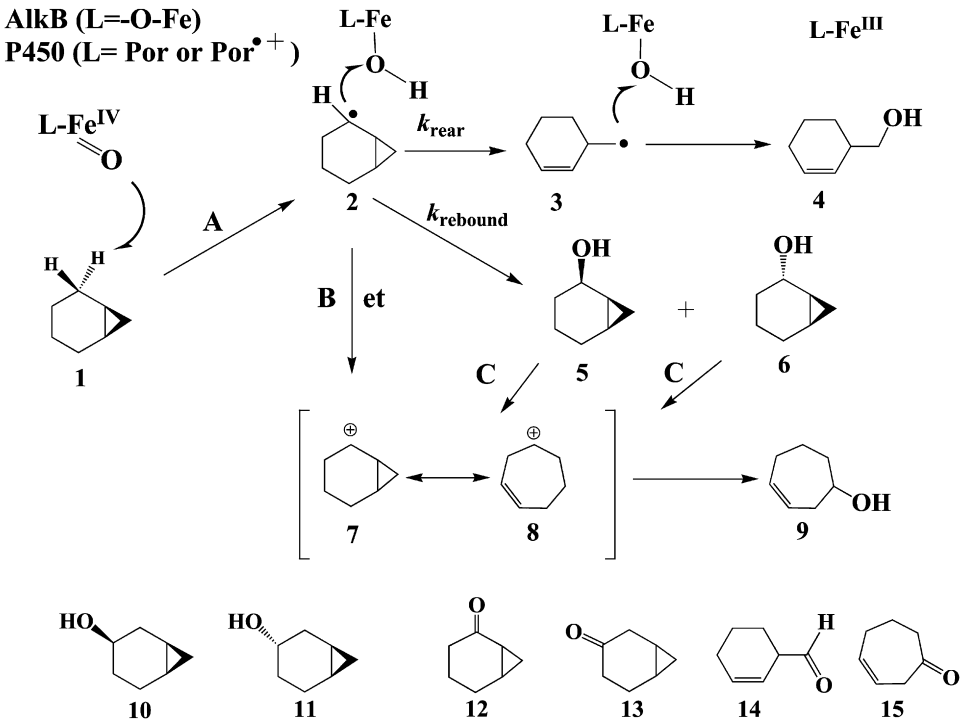

Figure 1. Mechanisms of Oxygenation of the Probe Norcarane by the Reactive Oxo-Iron Intermediate, L-Fe^{IV} = O

Pathway A, which dominates the chemistry reported in this paper, represents C-H bond homolysis to generate a substrate-based radical. Pathways B and C, which are not significant for the oxo-iron intermediate studies in this paper, involve either electron transfer (et) from the substrate to the iron complex to give a substrate-based cation (**7** and **8**), or reionization of product alcohols **5** and **6**. Both pathways could produce the minor product **9**.

In principle, reaction mechanisms are characteristic of specific active sites; thus, mechanistic analysis should provide information on active site structure. To this end, the reactivity of the chemical probe bicyclo[4.1.0]heptane (norcarane) was examined with whole cells harboring various alkane hydroxylase enzymes. This study was designed to test the hypothesis that hydroxylases that oxidize medium-chain alkanes can be identified by their reaction mechanisms, that previously unidentified alkane hydroxylases can be characterized based on their respective mechanistic features, and that, furthermore, these identifications can take place when whole cells are used.

Norcarane (**1**) is a mechanistically diagnostic radical clock substrate that is useful for distinguishing between radical and cationic intermediates formed during hydroxylation reactions. Furthermore, based on product distribution and the known intramolecular rearrangement rate, the lifetime of a putative substrate radical can be determined. An overview of the rearrangement chemistry of norcarane is provided in Figure 1. Norcarane has been applied successfully as a probe of reaction mechanisms in both heme and non-heme oxygenases [19, 20, 22, 23, 25, 26]. Well-established conditions for the analysis of regio- and stereoisomers of norcaranols and rearranged products are an additional advantage of this substrate. Because these product profiles can distinguish between AlkB homologs and CYP homologs that are present, they provide a useful tool for determining which enzyme is predominantly active in vivo.

In the present study, the hydroxylation of norcarane by several different alkane-oxidizing organisms was examined. Of these, five organisms are known to harbor an AlkB-like metalloenzyme: the γ-proteobacterium *Acinetobacter venetianus* RAG-1 (ATCC 31012) [27], the β-proteobacterium *Burkholderia cepacia* UCB 717 (ATCC 25416) [5], *Rhodococcus erythropolis* NRRL B-16531 (ATCC 15960) [5, 28], a *Pseudomonas putida* clone that expresses AlkB from *P. putida* GPo1 [29], and a *P. putida* clone that expresses an AlkB homolog from the marine organism *Alcanovorax borkumensis* AP1 [29]. Three of the organisms are known to harbor a CYP metalloenzyme: *Curvularia lunata* (ATCC 12017) [30], a *P. putida* clone expressing CYP153A6 [14], and *R. erythropolis*, which contains genes for both enzyme types. Three organisms examined represent recently identified species whose alkane-hydroxylating mechanisms have yet to be determined: the γ-proteobacterium *Hydrocarboniphaga effusa* AP103 [31] and the taxonomically related strains, rJ4 and rJ5 [32]. The substrate radical lifetimes obtained with AlkB- and CYP-containing strains are consistent within each enzyme class, but they are dramatically distinct between the two enzyme classes. The differences between the enzyme classes are sufficiently pronounced that the hydroxylases active in the three recently identified species can be predicted to be

Table 1. Representative Product Distribution for Whole-Cell Bacterial Oxidation of Norcarane and Radical Lifetime

Product[a]	GPo1 Cell-free Extract	AlkB from GPo1 in GPo12	AlkB from AP1 in GPo12	*A. venetianus* RAG-1	*B. cepacia*	*R. erythropolis*	AP103	rJ4	rJ5	CYP 153A6	CYP 153A6 Cell-free Extract	*C. lunata*
5	21	12	11	14.5	9.9	21	15	6.4	5.2	72.5	79	50
6[b]	0	0	0	0.6	0	0	0.6	0.5	0.1	4.0	0	29
10	43	57	48	3.0	0	0	26.5	26	7.1	17	16	16
11	2.4	7.5	9.8	27.5	28	42	6.1	16	4.3	4.0	3.0	0
12	0	0.5	0	11	24	14	9.8	21	20.5	2.3	1.3	3
13	2.6	11	8.8	32	11	16	27	19	50.4	0	0	0
4	31	12	21.5	11	26	7	15	11	12.4	0.20	0.1	1
9	0	0	0.5	0	0	0	0	0	0	0.20	0.8	0
Radical lifetime, ns[c]	7	4	9	2	3	1	3	2	2	0.01	0.006	0.06

Values reported are for all norcarane-derived products and are given in percent.
[a] Product yields (percent) are reported relative to the total amount of norcarane-dervived products and were determined by digital integration of the total ion current signal of the gas chromagraph mass spectrometer by using the resident HP ChemStation software. Product identity was confirmed by retention time and fragmentation pattern compared to authentic standards. Products reported in less than 1% relative yield were determined by comparing ion chromatograms of the characteristic ions to those of dilute standard mixtures.
[b] *Endo*-3-norcaranol (**10**) and *exo*-2-norcaranol (**6**) peaks overlapped; their relative contributions were estimated based on intensities of m/z 111 and 112.
[c] Uncertainty in the radical lifetime is generally ±1 ns for AlkB and ±10 ps for CYP.

mechanistically and structurally like AlkB, even though there is very little else known about these isolates.

RESULTS AND DISCUSSION

In Vivo Detection of AlkB

Previous work [23, 25] has shown that the archetypal AlkB from *P. putida* GPo1, under a range of tested conditions, catalyzed the oxidation of norcarane via H atom abstraction to generate a substrate-based radical that persists for 1–18 ns, as indicated by the distribution of products. A characteristic distribution of products includes 6%–35% of the ring-opened product, 3-hydroxymethylcyclohexene (**4**), 9%–20% of the ring-closed product *endo*-2-norcaranol (**5**), and 20%–60% of the mechanistically uninformative product *exo*-3-norcaranol (**10**). The cationic product 3-cyclohepten-1-ol (**9**) was rarely seen. Would other bacterial strains known to contain an AlkB enzyme also show the same mechanistic behavior when given norcarane as a substrate? If so, the use of diagnostic substrates could provide the basis for a mechanistic profiling screen. Known AlkB enzyme systems from two separate bacterial hosts—*P. putida* GPo1 and *Alcanovorax borkumensis* AP1 (a marine γ-proteobacterium that grows almost exclusively on *n*-alkanes)—were tested in vivo to ascertain whether they give the same or similar product ratios and radical lifetimes when oxygenating norcarane as does AlkB in cell-free assays. These AlkBs were studied in *P. putida* GPo12, which is a *P. putida* GPo1 knockout that lacks the ability to use octane as its sole carbon source, but can express alkane hydroxylases from a variety of sources [29].

Table 1 presents results from the oxidation of norcarane by these two enzymes. With *P. putida* hosting AlkB from GPo1, 12% of all norcarane-derived products were 3-hydroxymethylcyclohexene (**4**), which is clear evidence of a radical-based mechanism with an average radical lifetime with norcarane of 4 ns. With *P. putida* hosting the AlkB from AP1, the rearranged product (**4**) accounted for more than 20% of the norcarane-derived products, resulting in an average radical lifetime of 9 ns. Small amounts, less than 1%, of the cationic product 3-cyclohepten-1-ol (**9**) were also detected, which may reflect subtle differences in the active site structures of the AlkB homologs [25].

To test whether assay conditions in the cell differed from those in extracellular assays, a norcarane oxidation experiment was done by using cell lysate from *P. putida* GPo12 expressing AlkB from *P. putida* GPo1. Under the conditions of this assay, the radical product 3-hydroxymethylcyclohexene (**4**) comprised 31.2% of the products, leading to an estimated radical lifetime of 7 ns. This result is similar to that obtained in the in vivo assay and confirms the comparability of in vivo and in vitro approaches.

Both the mechanisms and radical lifetimes of these two enzymes, as assayed by the in vivo screen, are completely consistent with AlkB-like behavior in cell-free extracts (Table 1). This result suggests that the in vivo screening method can be useful for identifying active AlkB hydroxylases.

In Vivo Detection of CYP

Very little work exists characterizing the reaction mechanisms of CYPs in vivo [33]. From prior studies with purified CYP isozymes, it is known that the mechanism of CYP-catalyzed alkane oxidation proceeds through a very short-lived substrate radical—a radical whose lifetime is in the range of tens of picoseconds, not nanoseconds as has been seen with AlkB [22, 34].

To determine whether CYPs in vivo would show the same mechanistic behavior with norcarane as in purified systems, CYP153A6 from *Mycobacterium* sp. strain HXN-1500 as well as its required electron transfer partners, ferredoxin and a ferredoxin reductase, were cloned into *P. putida* GPo12. Norcarane was metabolized, yielding primarily (72.5%) 2-endo-norcaranol (**5**) (Table 1). Small amounts of the ring-opened product, 3-hydroxymethylcyclohxene (**4**), and the cation product (**9**) were detected. Product **4** appeared as a small shoulder on the 2-*endo*-norcaranol peak in the GCMS spectrum, making quantification difficult, although characteristic ions (m/e 94, 81, and 79; 81 is more intense than 79) make identification straightforward. The peak areas were used to quantify the regions delineated by these key ions. Based on the product yields, an estimation of the lifetime of the norcarane radical was determined to be ~10 ps in the catalysis by CYP153A6 in *P. putida* GPo12. Small amounts of cationic products are frequently seen with purified CYP reactions and may indicate electron transfer that competes with OH rebound or reionization of the product alcohols [22].

A norcarane oxidation experiment was done with cell lysate from *P. putida* GPo12 expressing CYP153A6 from *Mycobacterium* sp. strain HXN-1500. Under the conditions of this assay, the radical product 3-hydroxymethylcyclohexene (**4**) comprised ~0.1% of the products, leading to an estimated radical lifetime of 6 ps. This result is similar to that obtained in the in vivo assay and also to recent studies from our laboratories with another diagnostic substrate, bicyclohexane, and CYP-expressing cells and cell-free extracts [33].

Identifying Active Hydroxylases in In Vivo Assays in Cases in which Identifications Based on Genetic Data Have Been Made

Having successfully demonstrated that AlkB and CYP in in vivo assays give product yields consistent with cell-free extracts or purified systems, work was undertaken to identify the metalloenzymes functional in oxygenating alkanes in microorganisms.

A. venetianus RAG-1 has an *alkB* gene that is closely related to the *alkB* gene of *Acinetobacter* sp. M1 and is distantly related to *alkB* from *P. putida* GPo1. The growth of *A. venetianus* RAG-1 on hydrocarbons has been extensively characterized [35–37]. The oxidation of norcarane by *A. venetianus* RAG-1 resulted in 3-hydroxymethylcyclohexene (**4**) and did not result in the cationic ring-opened product cyclohept-3-ene-1-ol (**9**), nor the corresponding ketone (**15**), confirming the radical-centered pathway of this oxygenation reaction with a radical lifetime of ~2 ns. Results from the oxidation of norcarane by *A. venetianus* are summarized in Table 1.

B. cepacia UCB 717 (ATCC 25416) is a food- and waterborne human pathogen [38]. It is known to degrade *n*-alkanes, polycyclic aromatic hydrocarbons, and other environmental contaminants, including tetrachloroethylene and 2,4,5-trichlorophenoxyacetic acid, the main component in Agent Orange [38]. It contains an *alkB* homolog, distantly related to that of *P. putida* GPo1 [39]. Norcarane was hydroxylated by *B. cepacia*. More than 25% of all norcarane-derived products were the rearranged alcohol (**4**), leading to an estimated radical lifetime of 3 ns. No ring-opened cationic products were detected. Results from the oxidation of norcarane by *B. cepacia* are outlined in Table 1.

R. erythropolis NRRL B-16531 (ATCC 15960) is a Gram-positive soil bacterium [39] known to degrade halogenated monocyclic aromatic hydrocarbons as well as medium-chain-length alkanes [5]. It contains at least four *alkB* homologs [28, 39] that are distantly related to that of *P. putida* GPo1 [29]. Protein expression data for this organism have been equivocal. While initial reports indicated that only one AlkB-like enzyme is active [28], a more recent report suggests that none were active and, instead, a CYP was responsible for the alkane oxidation activity detected [15]. Our results are consistent with alkane hydroxylation by an AlkB homolog. 3-hydroxymethylcyclohexene (**4**) was a clear product from the oxidation of norcarane, comprising 7% of the total norcarane-derived products and resulting in a radical lifetime of 1 ns. No cationic ring-opened products were detected. Results from the oxidation of norcarane by *R. erythropolis* are shown in Table 1.

C. lunata is a CYP-containing fungus that hydroxylates alkanes [40]. Under the assay conditions, primarily unrearranged norcaranols and only a small amount of 3-hydroxymethylcyclohexene, **4**, were detected. Because 3-hydroxymethylcyclohexene elutes near 2-*endo*-norcaranol, quantification of 3-hydroxymethylcyclohexene was difficult, although the characteristic ions (m/e 94, 81, and 79; 81 is more intense than 79) were clearly visible. The radical lifetime for *C. lunata* was estimated as 60 ps, which is fully consistent with that for a CYP system.

Thus, in all of these cases, the enzyme identified by the mechanistic screen as transforming the probe molecule matched the available genetic information.

Identifying Active Hydroxylases in In Vivo Assays in Cases in which No Identifications Have Been Made

Can the in vivo approach be useful for identifying enzymes and reaction mechanisms of hydroxylases for which biochemical or genetic information has not yet been obtained or has proven difficult to obtain? To test this proposal, norcarane oxidation was examined with three strains of the recently identified genus *Hydrocarboniphaga* (specifically *H. effusa* AP103 [31], rJ4, and rJ5 [32]), isolated from geographically distant areas. These strains are able to grow on medium-length *n*-alkanes and are taxonomically close to *Nevskia ramosa*, a

freshwater bacterium that was formerly the sole representative of an independent deep branch of the γ subclass of *Proteobacteria* [41, 42], reportedly capable of aerobically degrading toluene [41]. However, the metabolic pathways responsible for *n*-alkane degradation have not yet been characterized in these bacteria.

All three strains were capable of transforming norcarane. The data show norcarane-derived products, notably *endo*- and *exo*-2-norcaranols, **5** and **6**, and their C-3 regioisomers, **10** and **11**. Ketones **12** and **13** were also identified. Overoxidation of the rearranged alcohol (**4**) with the rJ5 strain resulted in cyclohex-2-en-carboxaldehyde (**14**). The yield of the aldehyde (**14**) was added to the total rearrangement product for the radical lifetime evaluation, assuming that peak area was proportional to concentration. The observed radical lifetimes were 3 ns for *H. effusa* AP103, 2 ns for rJ4, and 2 ns for the rJ5 strain (Table 1). Neither the cationic ring-opened product cyclohept-3-ene-1-ol (**9**) nor the corresponding ketone was detected with any of the strains tested. Thus, all three of these organisms show behavior that is consistent with the presence of an AlkB-like enzyme and inconsistent with the presence of an active CYP-like enzyme that can oxygenate norcarane. Figure 2 illustrates the mechanistic differences between CYP and AlkB and supports our assertion that these differences are large enough that AlkB and CYP can be distinguished by this assay.

Conclusions

From the results presented above the following significant conclusions are drawn:

(1) In vivo oxidation of the diagnostic substrate norcarane is consistent with an oxygen-rebound scenario via a substrate-centered radical mechanism for all of the hydroxylases examined.
(2) CYPs behave differently than AlkB in the mechanism of norcarane hydroxylation, showing a 3–4 orders of magnitude shorter lifetime for a substrate-based radical than for AlkB-containing proteins. This difference may reflect fundamental differences in the reactivity of the catalytically competent intermediate. CYP, with its two electrons removed from an iron porphyrin complex, may be effectively more electron deficient than AlkB, which is presumed to have the two oxidizing equivalents removed from spatially separated iron ions.
(3) The behavior of the *Hydrocarboniphaga* strains suggests that they express a hydroxylase that is similar in mechanism to that of AlkB.
(4) The organism *R. erythropolis*, which contains both an AlkB and a CYP, is apparently using AlkB to hydroxylate norcarane.
(5) When norcarane is a substrate for alkane-degrading microorganisms, it functions as an activity-based chemical marker for the qualitative and quantitative comparisons of hydroxylase reactions in vivo.

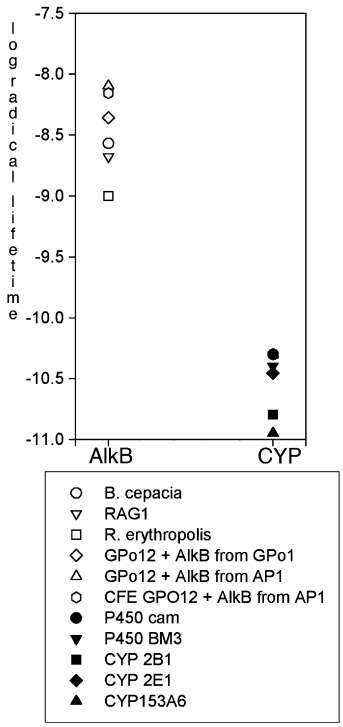

Figure 2. Comparison of Norcarane Oxidation for Various Hydroxylating Systems
Data are given in log radical lifetime (s). Data include results from this paper and from published work [22, 23, 25].

This work, and the approach outlined in it, may provide useful information about new hydroxylases independent from or highly complementary to genetic or biochemical studies.

SIGNIFICANCE

We describe a method for the functional and mechanistic characterization of the active hydroxylases within hydrocarbon-metabolizing organisms. Four bacterial strains known to express AlkB (*B. cepacia*, RAG1, *P. putida* GPo12 with AlkB from *P. putida* GPo1, and *P. putida* GPo12 with AlkB from AP1) were shown to display substrate rearrangement behavior consistent with predictions based on the whole-cell mechanistic analysis of the AlkB hydroxylase from the archetypal *P. putida* GPo1. Two of the strains are clones, permitting unequivocal assignment of the hydroxylase activity. This result indicates that whole-cell mechanism-based profiling can successfully identify the active hydroxylase enzyme. A fifth strain, *R. erthyropolis*, known to contain the gene for both an AlkB and a CYP, showed behavior consistent only with activity deriving from AlkB, indicating that the in vivo assay is capable of distinguishing between genes and active, expressed proteins. This conclusion is made possible by the very distinctive mechanistic behavior of CYPs reported here for whole-cell

incubations with two microbial strains (the fungus *C. lunata* and the *P. putida* GPo12 with CYP153A6). Again, the use of a clone makes the assignment of the chemistry observed for CYP unequivocal. The chemistry assigned to CYP is also consistent with the mechanistic behavior observed in purified enzymes. Finally, for the novel and as yet uncharacterized organisms, the mechanistic in vivo assay enables one to predict that the hydroxylases they use for hydrocarbon metabolism are AlkB like and are not CYPs.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise specified, all reagents were from Sigma-Aldrich. LB, TSB, EDTA, and chloramphenicol were obtained from Fisher Scientific (Fair Lawn, NJ). All chemicals and solvents were *Optima* grade and were used without further purification.

GC-MS analyses were performed on an HP GC-MS (5890/ 5989B) with an Rtx^{R}-5Sil MS capillary column (Restek Corp.) or an HP GC-MS (6890N/5973) with an HP-5MS Agilent column.

Norcarane, bicyclo[4.1.0]heptane (**1**), was synthesized by the Simmons-Smith reaction as previously described [43]. Authentic samples of the product C2 (**5** and **6**) and C3 norcaranols (**10** and **11**), the corresponding ketones (**12** and **13**), and the rearrangement products 3-cyclohepten-1-ol (**9**) and 3-hydroxymethylcyclohexene (**4**) were prepared according to published procedures [44–47]. The purity of each product was assessed by ^{1}H NMR and GC-MS.

Bacterial Strains: Growth Conditions, Alkane Induction, and In Vivo Activity Assay

H. effusa AP103 was previously isolated by our laboratory [31]. *H. effusa* rJ4 and rJ5 strains were generous gifts from Dr. K. Watanabe. *B. cepacia* UCB 717 (ATCC 25416), *R. erythropolis* NRRL B-16531 (ATCC 15960), *A. venetianus* RAG-1 (ATCC 31012), and *C. lunata* were obtained from the American Type Culture Collection. *P. putida* with appropriate plasmids was generated according to previously published procedures [5, 29].

B. cepacia UCB 717, *R. erythropolis* NRRL B-16531, and *A. venetianus* RAG-1 were cultivated in LB medium at 30°C. *H. effusa* strains were grown in TSB medium at 30°C. In the case of the RAG-1 strain, the medium was supplemented with chloramphenicol at a final concentration of 25 μg/ml. After 20–40 hr of growth in shake flasks at 200 rpm, cells were harvested by centrifugation, resuspended in MSB (minimum salt basal) [48] medium, and supplied with an appropriate *n*-alkane (hexadecane for *B. cepacia* UCB 717 and dodecane for other strains) via a suspended glass bulb. Cultures were allowed to grow while being shaken for 24 hr at 30°C.

P. putida Gpo12 with plasmids containing AlkB homologs from *P. putida* GPo1, AP1, and the CYP153A6 from *Mycobacterium* sp. strain HXN-1500 were grown in MSB medium supplemented with octane introduced through the vapor phase by placing the substrate in a glass bulb. When the optical density reached 1.0 at 600 nm, the octane was removed and diagnostic substrate (75 μl) was added to the medium via a fresh glass bulb.

After induction with the appropriate *n*-alkane, the cultures were harvested by centrifugation, washed twice, and resuspended in the assay buffer (50 mM sodium-phosphate [pH 7.4]). The cell density was adjusted to between an optical density of 1.2 and 1.5 at 600 nm. A 2.5 ml portion of the cell suspension was transferred to a Falcon tube, 8 μl of the diagnostic substrate was added, and the resulting suspension was mixed well by vortexing. The tubes were gently shaken at 85 rpm at 25°C. After 18 hr of incubation, 0.5 ml aliquots were taken, cells were separated by centrifugation, and resulting supernatant was extracted by vortex mixing for 1 min with 300 μl ethyl acetate. The phases were separated by centrifugation for 1 min at 14,000 rpm. Combined organic phases were dried by anhydrous sodium sulfate and were analyzed by GC-MS. Products were identified by comparing the observed peaks to the retention times and fragmentation patterns of authentic standards. Alternatively, 75 μl of the substrate was placed in a glass bulb hanging over 50 ml of cells suspended in buffer after induction with hydrocarbons. The larger volume facilitated product identification, and the shorter incubation time decreased the production of overoxidation products. Technical constraints largely dictated the method used. The cells were incubated for 6–8 hr, centrifuged for 15 min at 8000 × g, and extracted three times with an equal volume of ethyl acetate. The organic phases were combined and dried through anhydrous sodium sulfate. The organic phase was concentrated on a rotary evaporator and was analyzed by GC-MS.

Norcarane was found to be a good substrate for all enzyme systems tested, generally yielding more than 25% conversion of substrate to products over the course of the experiment. Small amounts of secondary oxidation products resulting from norcarane desaturation were detected in a minority of samples. These alcohols and epoxides were chromatographically distinct from the radical alcohol and did not affect the results.

C. lunata: Growth Conditions, Alkane Induction, and In Vivo Activity Assay

C. lunata was grown in rabbit food medium. The medium was prepared by combining 1.25 g rabbit food pellets with 50 ml Milli-Q water and heating the solution until boiling. The solution steeped for 30 min and was vacuum filtered before autoclaving. *C. lunata* was inoculated into 50 ml rabbit food broth medium and was grown at 25°C and 300 rpm. Experiments were carried out after ~2–3 days of growth.

Substrate (200 μl) and additional rabbit food medium (10 ml) were added directly to 50 ml growing culture. The culture was incubated with substrate at 25°C and 300 rpm overnight, and it was then filtered through cheese cloth. This filtrate was then extracted three times with ethyl acetate, concentrated, assayed by GC-MS, and analyzed as described above. Control experiments were also done for each experiment with *C. lunata*; all reaction components, except for the organism, were added to the media and incubated.

Cell-free Extract: Preparation of Extract and In Vivo Activity Assay

P. putida GPo12 cells expressing AlkB from *P. putida* GPo1 were grown on octane to an optical density of 2 at 600 nm and were centrifuged at 8000 rpm for 15 min. The pellet was sonicated in a buffer that contained 50 mM potassium phosphate (pH 7.4), 5% glycerol, 1 mM DTT (dithiothreitol), and 200 μM PMSF (phenylmethylsulfonyl fluoride) for 1 min total in 5 s bursts, followed by centrifugation at 4200 rpm for 25 min. The supernatant was decanted and used for assays.

Cell-free assays were done by adding 1 ml supernatant to a glass vial with Teflon-lined screw-top caps with 1 μl of 1 M DTT and 2 μl substrate. The vial was capped with a Teflon seal and shaken at 30°C briefly. The reaction was initiated by the addition of 12 μmol NADH and was incubated with shaking for 2 hr. After 2 hr, the reaction was quenched and extracted with an equal volume of chloroform.

Radical Lifetime Determinations

The radical lifetimes were calculated from the following equation:

$$\text{radical lifetime} = \left(\frac{1}{k_{\text{rearrangement}} \frac{[\text{unrearranged products}]}{[\text{radical rearranged products}]}} \right). \quad (1)$$

The rebound rate is the inverse of the radical lifetime. The rate constant $2 \times 10^{8}\ s^{-1}$ for the norcaran-2-yl radical ring opening was calculated from product ratios for the reaction of 2-chlorononcarane with tri-*n*-butyl-tin hydride as the radical trap [49, 50]. Unrearranged products include both isomers of 2-norcarnol (**5** and **6**) and 2 norcaranone (**12**) when present. Hydroxymethylcyclohexene (**4**) is the primary rearranged product; however, for rJ5, cyclohex-2-en-carbaldehyde (**14**) was detected and included as a rearranged product.

General Comments

Whole-cell oxidation experiments were repeated at least three times for each strain. GC-MS runs were at least duplicated. Control experiments in which potential products were provided to cultures as substrates were routinely done to ensure that assays would permit detection and quantification of all norcarane-derived products. In cases in which secondary oxidation occurred, the products (ketones or aldehydes) were included in the calculation of the radical lifetime.

ACKNOWLEDGMENTS

Financial support from the National Science Foundation through the Environmental Molecular Science Institute CEBIC (Center for Environmental Bioinorganic Chemistry at Princeton University) CHE-0221978 (G.J.Z., R.N.A., and J.T.G.), CHE-0316301 (J.T.G.), MCB-0078465, and CHE-0116233 (R.N.A.); the Camille and Henry Dreyfus Foundation (R.N.A.); and the National Institutes of Health GM-32698 (J.T.G.) and GM072506 (R.N.A.) is gratefully acknowledged. We also thank Dr. John Eng and Dr. Dorothy Little for expert technical assistance with mass spectrometry and Charlotte Lehmann for technical assistance with cell culturing and experiments.

Received: August 11, 2006
Revised: December 5, 2006
Accepted: December 6, 2006
Published: February 23, 2007

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