

Reaction mechanisms of non-heme diiron hydroxylases characterized in whole cells

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Abstract

Whole cells expressing the non-heme diiron hydroxylases AlkB and toluene 4-monooxygenase (T4MO) were used to probe enzyme reaction mechanisms. AlkB catalyzes the hydroxylation of the radical clock substrates bicyclo[4.1.0]heptane (norcarane), spirooctane and 1,1-diethylcyclopropane, and does not catalyze the hydroxylation of the radical clocks 1,1-dimethylcyclopropane or 1,1,2,2-tetramethylcyclopropane. The hydroxylation of norcarane yields a distribution of products consistent with an “oxygen-rebound” mechanism for the enzyme in both the wild type *Pseudomonas putida* GPo1 and AlkB from *P. putida* GPo1 expressed in *Escherichia coli*. Evidence for the presence of a substrate-based radical during the reaction mechanism is clear. With norcarane, the lifetime of that radical varies with experimental conditions. Experiments with higher substrate concentrations yield a shorter radical lifetime (≈ 1 ns), while experiments with lower substrate concentrations yield a longer radical lifetime (≈ 19 ns). Consistent results were obtained using either wild type or AlkB-equipped host organisms using either “resting cell” or “growing cell” approaches. T4MO expressed in *E. coli* also catalyzes the hydroxylation of norcarane with a radical lifetime of ≈ 0.07 ns. No radical lifetime dependence on substrate concentration was seen. Results from experiments with diethylcyclopropane, spirooctane, dimethylcyclopropane, and diethylcyclopropane are consistent with a restricted active site for AlkB.

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1. Introduction

Oxygen activation and O-atom insertion play a critical role in many biosynthetic and biodegradative pathways, as well as in the metabolism of xenobiotics. Cytochromes P450 (CYP) are widely distributed in nature and are the primary family of monooxygenases linked to oxygen activation and insertion in higher organisms [1,2]. Their heme active site, with its relatively

well characterized reaction mechanism, provides the paradigmatic model of biological oxygen activation.

Non-heme diiron monooxygenases also activate oxygen and mediate oxygen atom transfer into organic substrates. These proteins include soluble methane monooxygenase (sMMO), alkane monooxygenase (AlkB), xylene monooxygenase (XylM), and toluene monooxygenase (TMO). The soluble diiron enzymes like sMMO [3,4] and TMO [5] are relatively well studied, while the membrane-spanning systems such as AlkB and XylM [6] are less well characterized.

AlkB is the alkane ω -hydroxylase first characterized from *Pseudomonas putida* GPo1 (formerly known as

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Pseudomonas oleovorans). AlkB homologues (or the genetic precursors) have been found in all environments studied, including pristine and polluted soils and oceans [7–10] as well as in several human pathogens [11]. Alkane hydroxylation requires a soluble NADH reductase (AlkT) and a soluble rubredoxin (AlkG) in addition to the membrane-spanning monooxygenase AlkB. The active site structure of AlkB is not known. Spectroscopic and genetic evidence points to a nitrogen-rich coordination environment [12] located in the cytoplasm with as many as eight [13] or nine [14] histidines coordinating the two iron ions and a carboxylate residue bridging the two metals [14].

AlkB utilizes the prototypical “oxygen rebound” [1,2,25] mechanism to hydroxylate alkanes. This mechanism involves homolytic cleavage of the C–H bond by an electrophilic metal-oxo intermediate to generate a substrate-based radical. Alternatives to this mechanism include C–H bond heterolysis, H atom abstraction followed by electron transfer, and direct O-atom insertion. Diagnostic substrates – substrates that undergo structural changes *diagnostic* of a particular reaction pathway – can be used to distinguish between the possible mechanisms for alkane hydroxylation. They have been used to probe the reaction mechanisms of six alkane-hydroxylating enzymes, sMMO [15–18], pMMO [19], CYP [20–24], AlkB [25–27], T4MO [28], and XylM [29].

Norcarane is a particularly useful diagnostic substrate for probing reaction mechanisms because it can distinguish between radical and cationic pathways. The 2-norcaranyl cation is a resonance hybrid with the 3-cyclohepten-1-yl cation, giving 3-cycloheptenol (1) (from the ring opened cation) and both *endo*-2-norcaranol (3) (from the ring-closed cation) as hydroxylation products [30]. The 2-norcaranyl radical

rearranges to form predominantly the cyclohexenyl-methyl radical [31] (rearrangement rate constant of $2 \times 10^8 \text{ s}^{-1}$), ultimately producing cyclohex-2-enyl methanol (2) and *endo* and *exo*-2-norcaranol (3) (see Fig. 1 for an overview of norcarane chemistry). The distribution of ring-opened vs. ring-closed products will depend on the rebound rate of the enzyme and the distribution of *endo* and *exo* isomers will depend on active site constraints and thermodynamics. Cationic processes can result in small amounts of cyclohex-2-enyl methanol (2), but only as a very minor product compared to cycloheptenol [32]. 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 cyclohex-2-enyl methanol from the oxidation of norcarane is clear evidence of a transient substrate radical. Hydroxylation at the 3-position to form *endo*-3 and *exo*-3 norcaranol (4) is possible, but mechanistically uninformative. *Endo*-3 and *exo*-3 norcaranol can be further oxidized to form 3-norcaranone (5), while *endo*-2 and *exo*-2-norcaranol can be oxidized to form 2-norcaranone (6). The relatively clean chromatography of norcarane-derived products (with the exception that *exo*-2-norcaranol and *endo*-3-norcaranol coelute) facilitates quantification.

A limitation, however, of probing enzyme reaction mechanisms has historically been the requirement of maintaining stability and activity in a purified system. This challenge has restricted work on some enzyme systems, particularly membrane-spanning systems like AlkB. To overcome these limitations, in this and previous papers [25,29] we describe the behavior of diiron hydroxylases as they catalyze the hydroxylation of a series of diagnostic substrates within the wild type

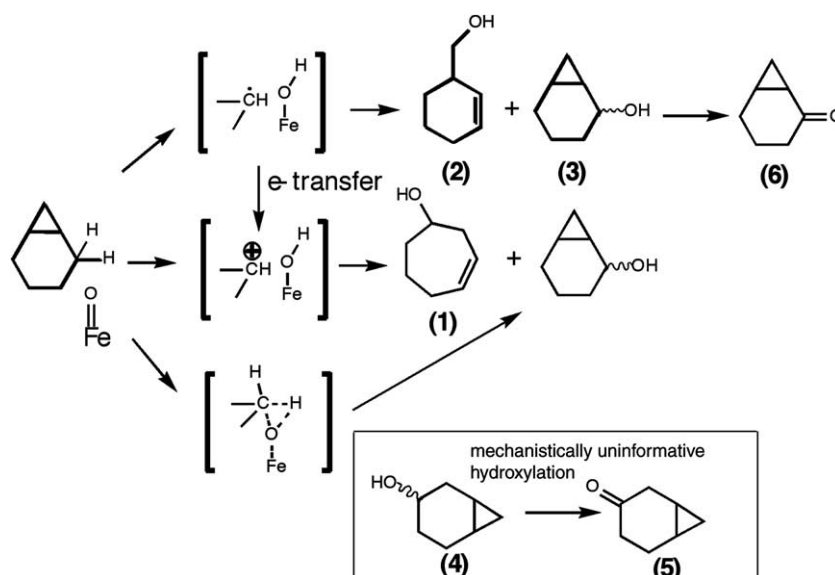


Fig. 1. Potential products and reaction pathways from metalloenzyme-catalyzed oxidation of norcarane.

organisms and *Escherichia coli* clones containing an inducible plasmid. Notably, this paper reports conditions under which the substrate-derived radical, generated in the AlkB-catalyzed hydroxylation process, persists in the active site for an unprecedented 19 ns. These results shed light on how substrate interactions with the enzyme active site affect reactivity. This paper also reports on the chemistry of T4MO in an *E. coli* clone containing an inducible T4MO-containing plasmid and compares those results to results recently obtained using purified T4MO [28] to show that the in vivo approach used here gives comparable results to those obtained with in vitro assays.

2. Experimental

2.1. Substrates and product standards

Norcarane was synthesized according to the published procedure and distilled before use [33]. Cyclohex-2-enyl methanol was synthesized according to a published procedure [34]. Diethylcyclopropane and spirooctane were purchased from Chemsampco. Dimethylcyclopropane was purchased from Pfaltz & Bauer Chemicals Catalog. Tetramethyl cyclopropane and 2,4-dimethyl-4-penten-2-ol were purchased from Alfa Aesar. All other reagent grade chemicals were purchased from Sigma–Aldrich and used without further purification. Solvents used for Gas Chromatograph–Mass Spectroscopy (GC–MS) analysis were of analytical grade.

2.2. Oxygenase expression systems

AlkB activity was transferred to *E. coli* by cloning genes for *alkB*, *alkT*, and *alkG* from *P. putida* GPO1 into a vector that confers ampicillin resistance and transforming them into a suitable *E. coli* host as described in a previous paper [25]. The toluene-4-monooxygenase plasmid containing *tmoA*, *tmoB*, *tmoC*, *tmoD*, *tmoE* and *tmoF* genes from *Pseudomonas mendocina* KR1 and a gene for ampicillin resistance was inserted into *E. coli* DH5 α competent cells (Invitrogen) as described in a previous paper [35].

2.3. Whole cell incubations

Wild type *P. putida* GPO1 was grown overnight in mineral salts basal medium [36] at 30 °C with octane delivered in a sterile glass bulb hanging over the culture so that substrate vapors were introduced into the liquid medium, subcultured into fresh medium, and allowed to grow until reaching an optical density (O.D.) at 600 nm of 0.5. The substrate was either added directly to the medium (hereafter referred to as the “direct addition method”) using either 200 μ L of substrate in 50 mL of

medium (0.4% v/v), 100 μ L of substrate in 50 mL of medium (0.2% v/v), 50 μ L of substrate in 50 mL of medium (0.1% v/v), or 20 μ L of substrate in 50 mL of medium (0.04% v/v) or added to a hanging glass bulb (75 μ L in the bulb hanging over 50 mL of medium, henceforth called “vapor phase method”). The culture was incubated with substrate at 30 °C and 300 rpm for 6–8 h.

The cloned organisms were grown in a similar fashion on Luria Bertani (LB) medium with ampicillin (100 μ g/mL) at 37 °C. The cells were induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) when the O.D. of the culture reached 0.5 at 600 nm. One and a half hour after induction, the substrate was added using either the direct addition method or the vapor phase method and the cells were incubated at 37 °C and 300 rpm for 6–8 h. Resting cell experiments were done by centrifuging the growing cells when they reached an O.D. at 600 nm of 1.0, rinsing the cells twice with a phosphate buffer (pH 7.3, 50 mM), and resuspending them in a phosphate buffer so that the final optical density at 600 nm was 2. Substrate was added via the vapor phase method or the direct addition method (0.4% v/v only) and the flask returned to the incubator for 5–6 h. In all cases, after the incubation was completed, the supernatant was collected by centrifugation (8000g, 15 min), extracted three times with ethyl acetate, concentrated, and the products assayed directly by GC–MS.

In reactions with more volatile substrates (e.g., dimethyl and tetramethyl cyclopropane), the substrates were introduced only via the vapor phase method and were extracted with chloroform (1 mL CHCl₃ for each 10 mL of supernatant). Extracts were analyzed by GC–MS after being extracted and after being concentrated by a gentle stream of argon blown across the top of the vial.

Control experiments in which cyclohex-2-enyl methanol was provided to growing cells both by direct addition (20 μ L in 50 mL culture) and by the vapor phase method were done. Control experiments where all the reaction components were added to the medium except for the organisms, and the medium was incubated at the selected temperature (30 °C for wild type organism, 37 °C for *E. coli*) were also done for each experiment. Control experiments in which the cells (after incubation with substrate) were sonicated for 10 min, before being centrifuged and the supernatant extracted, were done. All data reported represents the average of at least two and as many as ten independent experiments.

2.4. 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 °C, ramping to 220 °C at a ramp rate of 10 °C/min. Authentic products were synthesized

and their retention times and fragmentation patterns compared to those of the identified peaks in the GC–MS spectra.

2.5. Radical lifetime calculation

The lifetime of the substrate-based radicals were calculated using the following equation [37,20]:

$$k_{\text{rebound}} = k_{\text{rearrangement}} \left\{ \frac{[\text{ring closed}]}{[\text{ring opened}]} \right\}, \quad (1)$$

$$\text{radical lifetime} = (k_{\text{rebound}})^{-1},$$

where [ring closed] and [ring opened] represent the concentrations of the ring-closed (unrearranged) and ring-opened (rearranged) products, k_{rebound} is the rate at which the enzyme hydroxylates a substrate-based radical, and $k_{\text{rearrangement}}$ is the rate at which a substrate-based radical rearranges to relieve ring strain.

3. Results

Using a suite of diagnostic substrates to probe the reaction mechanism of the non-heme diiron enzyme AlkB expressed within its native host and inside an *E. coli* clone provides information about AlkB's active site structure and function. Substrates used in this study include norcarane, with a radical rearrangement rate of $2 \times 10^8 \text{ s}^{-1}$ [25], dimethylcyclopropane, with a radical rearrangement rate of $0.8 \times 10^8 \text{ s}^{-1}$ [38], tetramethylcyclopropane, with reported radical rearrangement rates of 20×10^8 and $2.05 \times 10^8 \text{ s}^{-1}$ [38] (tertiary radical and primary radical, respectively), diethylcyclopropane, with a rearrangement rate of $0.54 \times 10^8 \text{ s}^{-1}$ [38], and spirooctane, with a rearrangement rate of $0.5 \times 10^8 \text{ s}^{-1}$ [23]. Only norcarane was used with T4MO.

AlkB catalyzed the hydroxylation of diethylcyclopropane and spirooctane but only at the mechanistically uninformative positions not adjacent to the cyclopropyl ring. When diethylcyclopropane was used as the substrate, the only product detected was identified as 2-(1-ethyl-cyclopropyl)-ethanol. When spirooctane was used as a substrate, spiro[2.5]octan-6-ol was the only product detected.

Fig. 2 presents a chromatogram typical of those obtained from experiments with both the AlkB-expressing wild type and clone using the vapor phase method, along with a sterile control for comparison, and shows that norcarane is effectively hydroxylated by AlkB. Total ion current in these experiments is large – in contrast to the control, which shows a flat baseline in this region. The baseline separation of the various norcarane-derived products has been well established and the high product yields in these experiments make quantification of products straightforward.

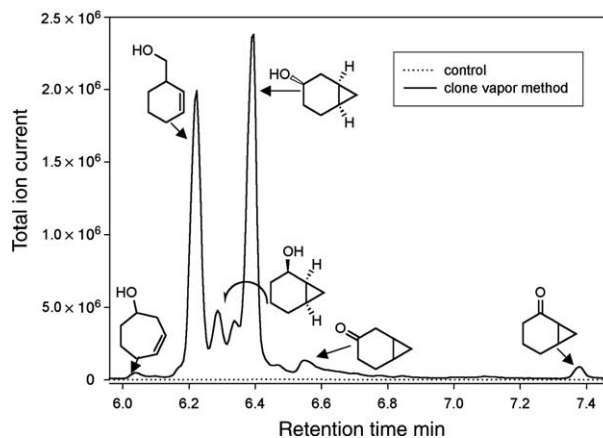


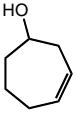
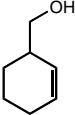
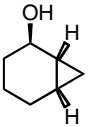
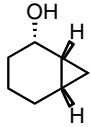
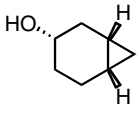
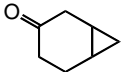
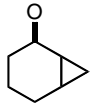
Fig. 2. Plot of total ion current in a typical chromatogram for AlkB-catalyzed hydroxylation of norcarane at low substrate concentrations using the vapor phase method. Endo-3-norcaranol forms a small shoulder on exo-3-norcaranol at 6.34 min. The small peak at 6.48 min corresponds to a cellular metabolite found, through mass spectrometry data, not to be derived from norcarane.

No differences in radical lifetimes were seen between resting cell and growing cell experiments for both the wild type *P. putida* GPo1 organism incubated at 30 °C and the AlkB-expressing cloned organism incubated at 37 °C. Higher product yields were seen with growing cell experiments; therefore, the majority of experiments were conducted with growing cells.

The distribution of AlkB-catalyzed norcarane-derived products from the two different organisms under the range of substrate concentration tested is provided in Table 1. When the concentration of substrate is low, relatively more of the ring-opened product, cyclohex-2-enyl methanol (2), is formed. The total amount of hydroxylation at the mechanistically uninformative 3-position is greater with the wild type than with the clone at all concentrations. Also, as expected, the wild type organism produces more ketone than the clone. This is expected because the clone contains only the hydroxylase and not the gene for the alcohol dehydrogenase (AlkJ) and *E. coli* contains only a very ineffective native alcohol dehydrogenase. Finally, small amounts (generally less than 0.5%) of the ring opened cationic product are occasionally detected in both the wild type and the clone, generally at low substrate concentrations.

The average substrate-based radical lifetime generated by AlkB in the wild type organism at high substrate concentration (0.4% v/v) is $1.7 \pm 0.4 \text{ ns}$ and for AlkB in the clone, under the same conditions, the average radical lifetime is $2.6 \pm 0.9 \text{ ns}$. In contrast, at the lowest concentrations tested (direct addition method 0.04% v/v) with AlkB in the wild type organism, the average radical lifetime is $19.2 \pm 3.9 \text{ ns}$ and for AlkB in the clone, under the same conditions, the average radical lifetime is $18.6 \pm 1.2 \text{ ns}$. The average radical lifetimes at intermediate

Table 1
Distribution of norcarane-derived products, reported as a percentage of total norcarane-derived products

							
<i>Wild type</i>							
0.4% v/v	0 (0)	5.29 (0.93)	17.67 (4.8)	0	50.96 (7.07)	18.15 (3.87)	2.09 (0.54)
0.2% v/v	0.005 (0.004)	15.48 (2.15)	8.64 (0.23)	0	64.71 (1.98)	8.96 (2.65)	1.17 (0.21)
0.1% v/v	0.27 (0.14)	18.96 (0.91)	7.70 (0.34)	0	61.91 (2.97)	9.82 (3.53)	1.34 (0.31)
0.04% v/v	0.30 (0.18)	29.36 (3.13)	6.98 (0.38)	0	52.94 (4.38)	9.40 (2.03)	1.038 (0.47)
Vapor	0.09 (0.09)	26.58 (1.47)	8.46 (0.27)	0	58.55 (1.71)	5.69 (0.75)	0.73 (0.24)
Vapor resting	0.65	27.3	10.2	0	57.4	4.2	0.2
<i>Clone</i>							
0.4% v/v	0 (0)	24.51 (4.25)	50.43 (5.79)	0	21.22 (6.4)	1.67 (0.97)	2.56 (1.33)
0.2% v/v	0 (0)	31.01 (1.43)	20.14 (3.81)	0	46.80 (2.19)	1.87 (0.086)	0.18 (0.076)
0.1% v/v	0.15 (0.15)	39.09 (1.7)	9.18 (3.94)	0	44.41 (5.0)	0 (0)	7.16 (7.16)
0.04% v/v	0.09 (0.072)	33.47 (1.99)	5.18 (1.71)	0	49.70 (2.60)	7.70 (0.07)	3.85 (1.90)
Vapor	0.33 (0.16)	39.15 (2.42)	9.47 (0.31)	0	49.60 (2.24)	1.72 (0.51)	0.13 (0.098)
Vapor resting	0	31.6	13.2	0	55.2	0	0
<i>T4MO</i>							
0.4%	0	1.67 (0.2)	49.05 (1.1)	43.84 (1.4)	2.26 (1.3)	0.7 (0.35)	2.32 (1.15)
Vapor	0	0.93 (0.16)	47.50 (1.1)	47.85 (2.7)	0.96 (0.7)	0.42 (0.4)	2.57 (1.1)

Standard errors given in parentheses.

concentrations fall between these extremes; the general trend is that radical lifetimes are inversely proportional to concentration. Fig. 3 illustrates the difference in the substrate-based radical lifetime generated by AlkB as a function of substrate concentration and also the similarity between the behavior of AlkB in the wild type and the cloned organisms.

T4MO, in *E. coli* clones induced to express T4MO, catalyzes the hydroxylation of norcarane. *Endo-2-norcaranol* represents 48% of the norcarane-derived prod-

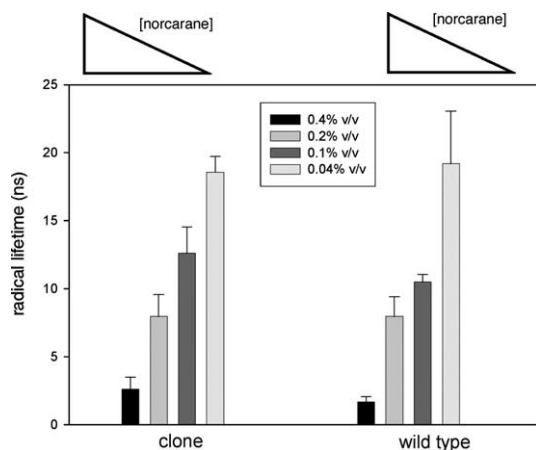


Fig. 3. Average radical lifetimes for AlkB-catalyzed oxidation of norcarane by wild type and clone as a function of substrate concentration.

ucts, while 45% of the norcarane-derived product are assigned as *exo-2-norcaranol* (although it is difficult to resolve *exo-2-norcaranol* from *endo-3-norcaranol*). No cationic products are detected. The average radical lifetime is 0.066 ± 0.025 ns. When norcarane is added to T4MO-expressing cells directly (200 μ L in 50 mL of culture), the average radical lifetime is 0.087 ± 0.029 ns. When norcarane is added to T4MO-expressing cells via the vapor phase method, the average radical lifetime is 0.052 ± 0.016 ns.

Control experiments were done where norcarane-derived products were provided to growing and resting cells at both high and low concentrations. The cloned organisms (both the clone expressing AlkB and the clone expressing T4MO) show essentially no transformation of the norcarane-derived products, while the wild type organism (only the AlkB-expressing wild type organism was tested) convert alcohols to expected ketones and carboxylic acids. As authentic standards of all post-hydroxylation products have been synthesized and/or purchased and used as calibration standards, the presence of these compounds is easily quantified and included in calculations of radical lifetimes where relevant. Of particular interest for this study is the potential transformation of the radical ring-opened product cyclohex-2-enyl methanol (**2**). When cyclohex-2-enyl methanol (**2**) is added directly to growing cultures of the wild type organism, transformation is detected.

Consistent with reports by May et al. [39], very little aldehyde is detected. Instead, significant amounts of the corresponding carboxylic acid are detected. In contrast, experiments where cyclohex-2-enyl methanol (**2**) is provided as the substrate for the AlkB- and T4MO-containing clones showed essentially no transformation; data from the control and live experiments are superimposable in both instances. Control experiments where the cells were incubated with substrate and then lysed prior to extraction show no difference with standard experiments where cells are not lysed.

4. Discussion

We have reported initial findings on the hydroxylation of norcarane and methylphenylcyclopropane by AlkB contained within both wild type and *E. coli* cells. The results indicated that AlkB uses an oxygen rebound reaction mechanism similar to the consensus mechanism for CYP and that the substrate-based radical persisted for ≈ 1 ns in the active site of AlkB [25], consistent with previously published work [27]. In this full accounting, conditions are presented under which an even longer substrate-based radical lifetime is detected, and the whole cell method is tested by providing data that can be used to compare the results from the whole cell approach with results from a purified enzyme.

The diagnostic substrates used in this study are all based on the cyclopropyl radical clock motif. In a cyclopropyl-based diagnostic substrate, the C–H bonds at the carbons adjacent to the cyclopropyl ring generally have the weakest bond strength so hydrogen abstraction of the mechanistically significant hydrogen is the favored abstraction. With AlkB, hydroxylation at the mechanistically informative carbon occurs only with the least sterically hindered substrate norcarane (and even with this substrate hydroxylation occurs at mechanistically uninformative positions as well). Diethylcyclopropane and spirooctane are hydroxylated only at the least sterically hindered (and mechanistically uninformative) carbons, while di- and tetramethyl cyclopropane, which offer only quite sterically hindered methyl groups, are not hydroxylated at all.

In vivo approaches have been used by others to surmount obstacles presented by enzymes whose activities are difficult to maintain in vitro. In vivo experiments, in addition to facilitating the analysis, offer the benefit of probing the enzyme mechanism in its native environment. In general, when results obtained in purified, or partially purified, enzyme systems have been compared, they have agreed with results obtained with in vivo assays [27,40]. The T4MO results presented here do as well. In the in vivo experiments T4MO catalyzes the hydroxylation of norcarane, providing evidence for a substrate-based radical in the process. The radical life-

time detected in the in vivo experiments was comparable to that detected with purified enzyme, with small differences being attributable to chromatographic differences between GC–MS instruments at different laboratories and the difficulties in resolving small amounts of the mechanistically uninformative 3-*endo*-norcaranol from the mechanistically important 2-*exo*-norcaranol.

The results reported here for AlkB oxidation are unprecedented in two ways. First, a substrate-based radical lifetime of 19 ns is the longest lifetime ever reported for a monooxygenase. It is three orders of magnitude longer than the radical lifetimes detected with P450s, which are between 10 and 50 ps [23]. It is longer than the 20–350-ps radical lifetime detected with sMMO [18] and T4MO [28] as well. Second, it is the first report where a change in conditions changes the radical lifetime detected in an enzyme.

These results do not permit conclusions to be drawn about what factors lead to the long radical lifetime observed, but they do point to some intriguing possibilities. Below the factors that might enable such a long radical lifetime to exist are considered below and the evidence for or against each factor is considered.

An effector protein, protein B, has been postulated to play an important role in shaping the active site of sMMO and modulating its overall reactivity by affecting the redox potential of the diiron site [41], substrate specificity, and product distribution [42]. Such an effector protein, were it normally involved in AlkB functioning, might be implicated in the chemistry detected. The OCT plasmid that contains AlkB has been well characterized and does not contain an effector protein [43]. Furthermore, the *E. coli* clone is constructed from only the hydroxylase, a reductase and a rubredoxin. Since the *E. coli* clone and wild type organism behave very similarly in our assay, it is unlikely that the unusual effects we see are due to the presence of an unidentified effector protein.

The possibility that the results represent an artifact of our intact cell assay conditions has been considered. For several reasons, however, this explanation is not likely. First, the same results are observed with two very different organisms – the wild type *P. putida* GPO1 and the *E. coli* clone. Second, we – and others – have done experiments comparing results obtained with whole cells to results obtained with purified enzymes and they generally agree [27,39,40]. This does not rule out the possibility that there could be some effect that will differentiate the results obtained with purified enzymes from those obtained with intact cells, but it does indicate that the approach of using whole cells to investigate enzyme mechanisms is inherently sound. Third, the cells have been lysed after incubation with substrate and the distribution of products studied. No differences are seen between the products observed when cells are lysed and not lysed, ruling out the possibility that products

are differentially released from the cells under some conditions. Finally, careful control experiments have been done with potential products and no unexpected transformation of products is seen. Because the analysis that leads to the determination of radical lifetimes relies on the relative amounts of ring-opened and ring-closed products, any failure to account for all of the products could introduce an error. The use of GC–MS and sterile controls for every experiment permits identification of all norcarane-derived products. If a radical-containing intermediate were to diffuse from the active site and react or dimerize, the resulting product would be detectable by GC–MS. Furthermore, the presence of data from both the clone and the wild type organism are helpful in this regard in that the clone has only a relatively ineffective native alcohol dehydrogenase (not one that is being over-expressed as part of the over-expression system) and so conversion of alcohols to ketones and aldehydes is slow. The compatibility of the data from all of these different organisms and experimental approaches supports the claim that the results reported here are robust. In addition, the direct comparison experiment between purified T4MO oxidation of norcarane and whole cell T4MO oxidation of norcarane showed very little difference between the whole cell and purified enzyme experiments, confirming the general validity of using in vivo approaches to determining enzyme reaction mechanisms.

These results suggest relatively restricted substrate access to the metal site, consistent with other published reports on the regioselectivity of AlkB [39,44,45]. Diethyl cyclopropane is transformed effectively by AlkB but is hydroxylated exclusively at the terminal position, despite the fact that the C–H bond adjacent to the cyclopropyl group should be weaker than other C–H bonds, especially a terminal methyl group. With norcarane, significant hydroxylation (between 23% and 72% of the total norcarane-derived products) occurs at the C-3 position, which is again the energetically disfavored position although sterically less hindered. This is in striking contrast to results seen with other enzymes where a very small percentage of the norcarane hydroxylation occurs at the 3-position. With T4MO, approx 10% of the norcarane hydroxylation occurs at the 3-position [28], with XylM between 0% and 3% of the norcarane hydroxylation occurs at the 3-position [29], with sMMO 9% of the hydroxylation occurs at the 3-position [18], and with P450 between 8% and 13% of the norcarane hydroxylation occurs at the 3-position [23].

These results suggest another hypothesis for the presented data. Perhaps the substrate affects the enzyme active site in a manner that changes the rate of radical capture by the enzyme. Recent results with T4MO confirm that active site structure can affect radical lifetime [28]. In those experiments, three single amino acid mutants were studied in addition to the native enzyme.

While all four isoforms catalyzed the hydroxylation of norcarane with similar coupling efficiency and catalytic parameters, they yielded different values for the radical lifetime, ranging from 0.08 ± 0.011 to 0.343 ± 0.055 ns [28].

We can envision two possible routes by which the presence of substrate might change the active site. Because the active site of AlkB is so near the cell membrane, the presence of hydrophobic compounds in the cell membrane might affect the structure and hence the reactivity of the enzyme. Due to their lipophilic nature, hydrocarbons accumulate in the lipid bilayer. This affects both membrane structure and function [46,47]. Expression of AlkB in both the native host and in several *E. coli* strains leads to the formation of vesicle-like structures in the cytoplasm [48]. In *E. coli* W3110 where these vesicles were analyzed, they were enriched in AlkB and contained a higher phospholipid/protein ratio than the cytoplasmic membrane [48], pointing to the possibility that AlkB in both native and non-native hosts experiences an unusual molecular environment. There is evidence from abiotic systems that the nature of the environment surrounding a radical pair can affect the radical lifetime. In a paper exploring the chemistry of photoinitiated radical pairs generated inside the cages of porous polymeric materials, wall stiffness (which is related to relaxation rates of the polyolefinic chains) and cage shape anisotropy affected the lifetime of the radical pairs and allowed some radicals to persist for much longer than they would in other environments [49].

Alternatively, there might be a substrate accessible channel attached to the active site that could affect the active site differently when filled with water or with substrate molecules. A recent crystal structure of the soluble toluene monooxygenase hydroxylase from *Pseudomonas stutzeri* provides clear evidence for a substrate channel that can simultaneously accommodate multiple substrate molecules [50]. Organisms that metabolize hydrocarbons have evolved strategies to regulate hydrocarbon concentrations inside cells to avoid toxic effects [46]. Whether substrate channels turn out to reflect part of a protective strategy widely incorporated into hydrocarbon-transforming enzymes remains to be seen, but it is reasonable to hypothesize that they may.

A recent paper by van Beilen et al. [14] presents data that are intriguing in light of the findings presented here. In this paper, a single amino acid was identified that controlled the alkane chain length that AlkB-like homologs could hydroxylate. In the prototypical AlkB from *P. putida* GPo1, when the tryptophan at position 55 was mutated (either deliberately or through selection experiments) to a less bulky amino acid, the enzyme could hydroxylate alkanes with chain lengths that exceed its normal substrate range. Conversely when the AlkB from *Mycobacterium tuberculosis* H37RV was mutated to introduce a bulky amino acid into the corre-

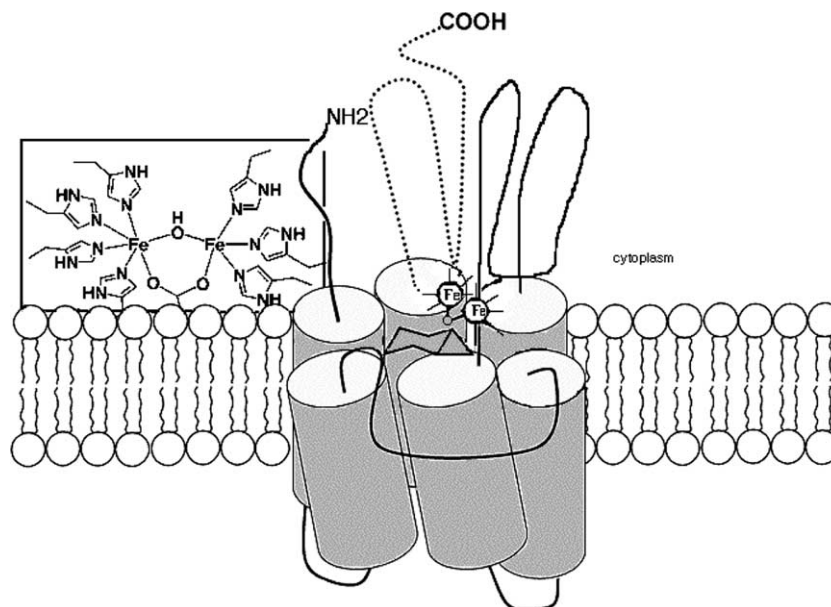


Fig. 4. Presumed structure of membrane-bound AlkB (adapted from [14]). Six trans-membrane helices form a substrate channel to the diiron site on the cytoplasmic side. Norcarane is depicted at the active site end of the substrate channel. The histidine-rich coordination environment of the diiron site is depicted in the inset.

sponding site, it was no longer able to hydroxylate alkanes with chain lengths greater than 11 carbons. The key amino acid in these experiments is predicted to lie approximately half way through a trans-membrane helix, between 15 and 19.5 Å from the conserved histidines thought to bind the diiron motif. These results are consistent with the presence of a substrate channel inside the trans-membrane spanning portion of the protein where this amino acid acts as a plug, which, when bulky, limits the substrate length that can fill the channel and still reach the active site. This work again points to a restricted active site for GPO1 AlkB and suggests that minor changes in the scaffolding around the active site can yield large changes in enzyme reactivity. A picture illustrating the key structural features of AlkB that may affect its reactivity is provided in Fig. 4.

Detailed explorations of the reaction mechanisms of membrane spanning proteins are just beginning as the difficulties associated with purifying them and maintaining activity are substantial. However, it is clear that subtle changes in the active site structure can alter radical lifetimes. Further experiments to explore the nature of the structural changes at play in this work are underway.

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References

- [1] J.T. Groves, in: P.R. Ortiz de Montellano (Ed.), *Cytochrome P450: Structure, Mechanism, and Biochemistry*, third ed., Kluwer Academic/Plenum Publishers, New York, 2004, pp. 1–44.
- [2] J.T. Groves, *Proc. Nat. Acad. Sci. USA* 100 (2003) 3569–3574.
- [3] M.-H. Baik, M. Newcomb, R.A. Friesner, S.J. Lippard, *Chem. Rev.* 103 (2003) 2385–2419.
- [4] M. Merx, D.A. Kopp, M.H. Sazinsky, J.L. Blazyk, J. Muller, S.J. Lippard, *Angew. Chem., Int. Ed.* 40 (2001) 2782–2807.
- [5] J.D. Pikus, J.M. Studts, C. Achim, K.E. Kaufmann, E.M. Münck, R.J. Steffan, K. McClay, B.G. Fox, *Biochemistry* 35 (1996) 9106–9119.
- [6] M. Susuki, T. Hayakawa, J. Shaw, M. Rekik, S. Harayama, *J. Bacteriol.* 173 (1991) 1690–1695.
- [7] T.H.H. Smits, M. Röthlisberger, B. Witholt, J.B. van Beilen, *Environ. Microbiol.* 1 (1999) 307–317.
- [8] L.G. Whyte, T.H.M. Smits, D. Labbe, B. Witholt, C.W. Greer, J.B. van Beilen, *Appl. Environ. Microbiol.* 68 (2002) 5933–5942.
- [9] R. Margesin, D. Labbé, F. Schinner, C.W. Greer, L.G. Whyte, *Appl. Environ. Microbiol.* 69 (2003) 3085–3092.
- [10] J.B. van Beilen, M.M. Marin, T.H.M. Smits, M. Roethlisberger, A.G. Franchini, B. Witholt, F. Rojo, *Environ. Microbiol.* 6 (2004) 264–273.
- [11] T.H.M. Smits, S.B. Balada, B. Witholt, J.B. van Beilen, *J. Bacteriol.* 184 (2002) 1733–1742.
- [12] J. Shanklin, C. Achim, H. Schmidt, B.G. Fox, E. Münck, *Proc. Natl. Acad. Sci. USA* 94 (1997) 2981–2986.

- [13] J. Shanklin, E. Whittle, FEBS Lett. 545 (2003) 188–192.
- [14] J.B. van Beilen, T.H.M. Smits, F.Z. Roos, T. Brunner, S.B. Balada, M. Röhlsberger, B. Witholt, J. Bacteriol. 187 (2005) 85–91.
- [15] A.M. Valentine, B. Wilkinson, K.E. Liu, S.K. Panicucci, N.D. Priestley, P.G. Williams, H. Morimoto, H.G. Floss, S.J. Lippard, J. Am. Chem. Soc. 119 (1997) 1818–1827.
- [16] Y. Jin, J.D. Lipscomb, Biochim. Biophys. Acta 1543 (2000) 47–59.
- [17] S.-Y. Choi, P.E. Eaton, D.A. Kopp, S.J. Lippard, M. Newcomb, R. Shen, J. Am. Chem. Soc. 121 (1999) 12198–12199.
- [18] B.J. Brazeau, R.N. Austin, C. Tarr, J.T. Groves, J.D. Lipscomb, J. Am. Chem. Soc. 123 (2001) 11831–11837.
- [19] B. Wilinon, M. Zhu, N.D. Priestley, H.H.T. Nguyen, H. Morimoto, P.G. Williams, S.I. Chan, H.G. Floss, J. Am. Chem. Soc. 118 (1996) 921–922.
- [20] V.W. Bowry, K.U. Ingold, J. Am. Chem. Soc. 113 (1991) 5699–5707.
- [21] P.R. Ortiz de Montellano, R.A. Stearns, J. Am. Chem. Soc. 109 (1987) 3415–3420.
- [22] M. Newcomb, R. Shen, S.-Y. Choi, P.H. Toy, P.F. Hollenberg, A.D.N. Vaz, M.J. Coon, J. Am. Chem. Soc. 122 (2000) 2677–2686.
- [23] K. Auclair, Z. Hu, D.M. Little, P.R. Ortiz de Montellano, J.T. Groves, J. Am. Chem. Soc. 124 (2002) 6020–6027.
- [24] J.K. Atkinson, K.U. Ingold, Biochemistry 32 (1993) 9209–9214.
- [25] R.N. Austin, H.-K. Chang, G.J. Zylstra, J.T. Groves, J. Am. Chem. Soc. 122 (2000) 11747–11748.
- [26] E. Caspi, S. Shapiro, J. Piper, Tetrahedron 37 (1981) 3535–3543.
- [27] H. Fu, M. Newcomb, C.H. Wong, J. Am. Chem. Soc. 113 (1991) 5878–5880.
- [28] L. Moe, Z. Hu, D. Deng, R.N. Austin, J.T. Groves, B.G. Fox, Biochemistry 43 (2004) 15688–15701.
- [29] R.N. Austin, K. Buzzi, E. Kim, G.J. Zylstra, J.T. Groves, J. Biol. Inorg. Chem 8 (2003) 733–740.
- [30] E.C. Friedrich, J.D.C. Jassawalla, J. Org. Chem. 44 (1979) 4224–4229.
- [31] E.C. Friedrich, R.L. Holmstead, J. Org. Chem. 37 (1972) 2546–2550.
- [32] E.C. Friedrich, J.D.C. Jassawalla, Tetrahedron Lett. (1978) 953–956.
- [33] E.J. LeGoff, Org. Chem. 29 (1964) 2048–2050.
- [34] M. Chini, P. Crotti, L.A. Flippin, C. Gardelli, F. Macchia, J. Chem. Org 57 (1992) 1713–1718.
- [35] J.M. Studts, K.H. Mitchell, J.D. Pikus, K. McClay, R.J. Steffan, B.G. Fox, Protein Express. Purif. 20 (2000) 58–65.
- [36] R.Y. Stanier, N.J. Palleroni, M.J. Duodoroff, Gen. Microbiol. 43 (1966) 159–271.
- [37] D. Giller, K.U. Ingold, Accounts Chem. Rev. 13 (1980) 317–323.
- [38] V.M. Bowry, J. Luszytk, K.U. Ingold, J. Am. Chem. Soc. 113 (1991) 5687–5698.
- [39] A.G. Katopodis, K. Wimalasena, J. Lee, S.W. May, J. Am. Chem. Soc. 106 (1984) 7928–7935.
- [40] J.B. van Beilen, J. Kingma, B. Witholt, Enzyme Microb. Technol. 16 (1994) 904–911.
- [41] J. Kazlauskaite, H.A.O. Hill, P.C. Wilkins, H. Dalton, Eur. J. Biochem. 241 (1996) 552–556.
- [42] W.A. Froland, K.K. Anderson, S.K. Lee, Y. Liu, J.D. Lipscomb, J. Biol. Chem. 267 (1992) 17588–17597.
- [43] J.B. van Beilen, M.G. Wubbolts, B. Witholt, Biodegradation 5 (1994) 161–174.
- [44] J.B. van Beilen, D. Penninga, B. Witholt, J. Biol. Chem. 267 (1992) 9194–9201.
- [45] J.A. Peterson, D. Basu, M.J. Coon, J. Biol. Chem. 241 (1966) 5162–5164.
- [46] J. Sikkema, J.A.M. De Bont, B. Poolman, Microbiol. Rev. 59 (1995) 201–222.
- [47] J. Kieboom, J.J. Dennis, J.A.M. De Bont, G. Zylstra, J. Biol. Chem. 273 (1998) 85–91.
- [48] M. Nieboer, J. Kingma, B. Witholt, Mol. Microbiol. 8 (1993) 1039–1051.
- [49] W. Gu, R.G. Weiss, Tetrahedron 56 (2000) 6913–6925.
- [50] M.H. Sazinsky, J. Bard, A.D. Donato, S.J. Lippard, J. Biol. Chem. 279 (2004) 30600–30610.