The Non-Heme Diiron Alkane Monooxygenase of *Pseudomonas oleovorans* (AlkB) Hydroxylates via a Substrate Radical Intermediate

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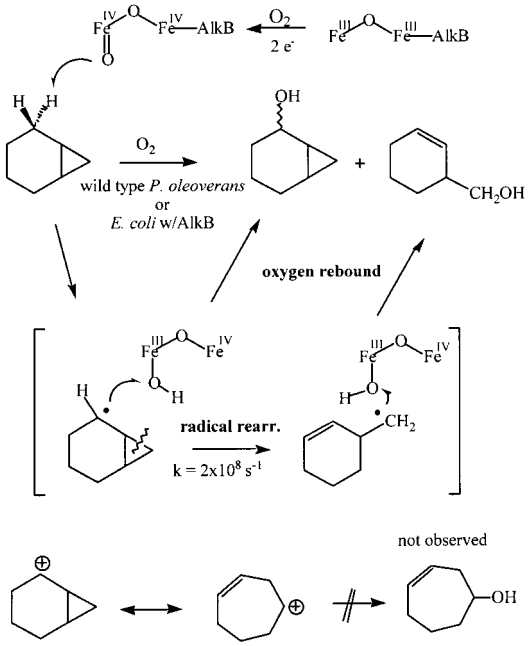
One of the most remarkable oxidations found in nature is the insertion of oxygen into a carbon−hydrogen bond. Although the chemical inertness of paraffins has been well-known to chemists for centuries, the hydroxylation of these materials is a common biological strategy. The soil organism *Pseudomonas oleovorans* TF4-1L (ATCC 29347)[1] can grow on octane as its sole source of carbon,[2] a facility that has been successfully directed toward the large-scale production of octanol from octane.[3] The monooxygenase AlkB from TF4-1L is a dinuclear non-heme iron enzyme[4] that catalyzes the terminal hydroxylation of simple alkanes (C_5−C_{12}). This is the initial step in the metabolic process by which TF4-1L obtains energy from alkanes. Recent genetic experiments have suggested that enzymes with high homology to AlkB, especially in the histidine-rich region thought to be essential for iron binding, are widely distributed in nature.[5] Hence, mechanistic insights into AlkB hydroxylation may shed light on a significant portion of the alkane transformations that take place in the environment and also on the differences and similarities between this enzyme and two, more extensively studied alkane hydroxylating enzymes, cytochrome P450 and soluble methane monooxygenase (sMMO).

We have investigated the mechanism of alkane hydroxylation catalyzed by AlkB using the diagnostic substrates norcarane (bicyclo[4.1.0]heptane) and 2-methyl-1-phenylcyclopropane, both in wild-type TF4-1L and *Escherichia coli* expressing the cloned alkane hydroxylase genes. The results give conclusive evidence for a carbon-centered radical intermediate with a lifetime of approximately 1 ns in the hydroxylation process.

The oxidations of both substrates were performed using growing cells from both TF4-1L and *E. coli* TOP10-(pGJZ1371). The latter plasmid was constructed by cloning an *alkBFG* PCR product encoding the alkane hydroxylase and its associated rubredoxins along with an *alkT* PCR product encoding the rubredoxin reductase into the expression vector pTrcHis2-TOPO. The use of TF4-1L, *E. coli* expressing the alkane hydroxylase, and a negative control of *E. coli* with the expression vector only allow us to unequivocally attribute the chemistry reported here to the AlkB protein.

Wild-type TF4-1L and *E. coli* TOP10-(pGJZ1371) were grown in mineral salts basal (MSB) medium[6] with octane (TF4-1L) or

Scheme 1. Mechanism of Norcarane Hydroxylation by AlkB



nutrients, glucose, and ampicillin (TOP10). *E. coli* was induced with IPTG. Both organisms were incubated with substrates for 23 h. After transformation, the supernatant was extracted and the products assayed directly by GC−MS. The retention times and fragmentation patterns of authentic products were compared to those of the identified peaks in the GC−MS spectra.

Diagnostic substrates that undergo characteristic structural changes in response to forming specific reaction intermediates have been very useful in elucidating key features of a number of metalloenzymes and model compounds.[7−10] 2-Methyl-1-phenylcyclopropane, which has been used previously as a probe for AlkB reactivity,[11] can undergo very rapid cyclopropyl carbinyl−homoallyl radical rearrangement ($k_r = 2 \times 10^{11}$ s^{-1}).[11] However, this probe cannot differentiate unambiguously between cation and radical intermediates. Norcarane can conclusively distinguish between radical and cationic pathways since the 2-norcaranyl *radical* is known to open so as to afford the 3-cyclohexenylmethyl radical.[26a] By contrast, the 2-norcaranyl *cation* is a resonance hybrid with the 3-cyclohepten-1-yl cation, giving products from both systems, depending upon the nucleophile (Scheme 1).[12] Thus, norcarane allowed the determination that oxomanganese porphyrins, as models of cytochrome P450, hydroxylated with discreet radical intermediates.[8]

While diagnostic probes have been widely used, recent experiments have demonstrated that subtle considerations can obscure the information they yield.[13−19] For this reason, we reexamined 2-methyl-1-phenylcyclopropane as a substrate with TF4-1L. We

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(1) Recent 16S rRNA sequencing by van Beilen and Witholt has reclassified *Pseudomonas oleovorans* TF4-1L as *P. putida* (GenBank accession AJ249825). To facilitate further communication, we have elected to use the older name and acknowledge the nomenclature issue here.

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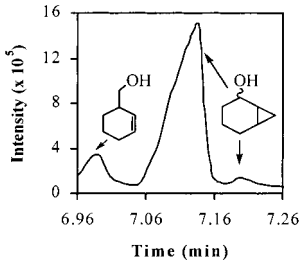

Figure 1. GC−MS total ion trace in the product region for the oxidation of norcarane by *E. coli* TOP10(pGJZ1371).

also used it as a substrate for the first time with a cloned organism, *E. coli* TOP10(pGJZ1371). Control experiments showed 1-phenyl-3-buten-1-ol, *trans*-2-phenylcyclopropylmethanol, and *trans*-2-phenylcyclopropyl carboxylic acid were not further transformed. 2-Methyl-1-phenylcyclopropane yielded *only* 1-phenyl-3-buten-1-ol, with both TF4-1L and TOP10(pGJZ1371).[20]

Norcarane was found to be a good substrate for AlkB both in TF4-1L and in *E. coli* TOP10(pGJZ1371), affording a mixture of products. Approximately 4% of the starting material was metabolized to products over the course of the reaction giving strong, well-resolved peaks in the GC−MS (Figure 1). For both the cloned and wild-type organism, of the total amount of norcarane-derived products, approximately 85% were *cis*- and *trans*-2-norcaranol and 15% were 3-(hydroxymethyl)-cyclohexene, *the ring-opened radical product*. In the longer incubations with TF4-1L, a small amount of norcaranone was detected. When present, it was included in estimates of radical lifetimes. *E. coli* TOP10(pGJZ1371), which does not have an overexpressed alcohol dehydrogenase and has only an inefficient native alcohol dehydrogenase[21] did not generate further oxidation products.[22] Neither the cationic ring-opened product cycohept-3-ene-1-ol nor its corresponding ketone was detected. A series of control experiments with both observed and non-observed products produced only a small amount of norcaranone from incubations of TF4-1L with norcaranol. No further oxidation products were detected with *E. coli*.

Taken together, the products of these oxidations are consistent with an *oxygen rebound* scenario[23] for hydroxylation by AlkB as shown in Scheme 1. Since the stoichiometry of AlkB is the same as those of cytochrome P450 and sMMO, it is reasonable to presume that oxygen binding and reduction can lead to a reactive ferryl species, O═Fe(IV)-Fe(IV),[24] a two-electron oxidant with respect to the resting ferric enzyme. Hydrogen abstraction from the alkane will lead to the intermediate substrate radical. The rate of the norcaranyl radical rearrangement is relatively slow on the scale of well-characterized radical clocks,[25] $\sim 2 \times 10^8$ s^{-1}.[26] Thus, the observation of both ring-opened and ring-closed products provides an opportunity to estimate the lifetime of the intermediate radical, ~1 ns. This estimate comes from consistent results of 15 experiments involving both organisms (see Supporting Information). This lifetime is consonant with the observation of only ring-opened products in the case of AlkB oxidation of 2-methyl-1-phenylcyclopropane.

The nanosecond lifetime for the substrate intermediate observed here is certainly long enough to qualify this species as a distinct intermediate and supports the hypothesis that non-heme iron enzymes can participate in the oxygen rebound mechanism.[23] The unambiguous presence of the radical ring-opened product in the hydroxylation of norcarane enables us to conclusively identify a radical pathway. We note that a stepwise radical process would also explain the unusual stereochemical outcome for the epoxidation of 1-octene mediated by AlkB described in the pioneering work on *P. oleovorans* by May.[27]

It is interesting to speculate on the observed differences between the conclusions presented here and results obtained with sMMO.[15,28] Such variables as the iron coordination sphere,[4] the strength of the scissile C−H bond, and possible spin state crossing effects are in need of careful scrutiny.[29]

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Supporting Information Available: Figure showing estimates of radical lifetimes with norcarane as substrate (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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