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Substrate specificity and reaction mechanism of purified alkane hydroxylase from the hydrocarbonoclastus bacterium *Alcanivorax borkumensis* (AbAlkB)

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Abstract

An alkane hydroxylase from the marine organism *Alcanivorax borkumensis* (AbAlkB) was purified. The purified protein retained high activity in an assay with purified rubredoxin (AlkG), purified maize ferridoxin reductase, NADPH, and selected substrates. The reaction mechanism of the purified protein was probed using the radical clock substrates bicyclo[4.1.0]heptane (norcarane), bicyclo[3.1.0]hexane (bicyclohexane), methylphenylcyclopropane and deuterated and non-deuterated cyclohexane. The distribution of products from the radical clock substrates supports the hypothesis that purified AbAlkB hydroxylates substrates by forming a substrate radical. Experiments with deuterated cyclohexane indicate that the rate-determining step has significant C-H bond breaking character. The products formed from a number of differently shaped and sized substrates were characterized to determine the active site constraints of this AlkB. AbAlkB can catalyze the hydroxylation of a large number of aromatic compounds and linear and cyclic alkanes. It does not catalyze the hydroxylation of alkanes with a chain length longer than 15 carbons, nor does it hydroxylate sterically hindered C-H bonds.

Keywords

alkane hydroxylase; alkane oxidation; hydrocarbonoclastic; AlkB

1. Introduction

Catastrophic oil spills stimulate the growth of *hydrocarbonoclastic* bacteria—bacteria that use hydrocarbons as their preferred source of carbon and energy [1]. If temperatures are warm enough and oxygen and nutrients plentiful, indigenous hydrocarbonoclastic bacteria

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consume substantial amounts of most components of petroleum, resulting in measureable decreases in the amount of crude oil in impacted environments [2, 3]. A significant amount of oil enters the environment each year through natural seeps as well, providing ecological niches that support the growth of hydrocarbonoclastic bacteria [4].

Detailed biochemical information about the key enzymes that hydrocarbonoclastic bacteria use to oxidize alkanes in the environment is just emerging [4]. Evidence points to Ω -alkane hydroxylase (AlkB)¹, first identified in a soil bacterium by Coon and coworkers in the late 1960s [5], as the enzyme responsible for oxidizing the majority of medium (C5–C32) chain-length linear alkanes in the environment. This evidence includes: (1) the fact that genes for AlkB have been found in the majority of cultured organisms that can grow on alkanes of these chain lengths [6–12]; (2) the presence of DNA that codes for AlkB genes in a number of oil-contaminated environments [13–21]; (3) molecular biology experiments in which AlkB genes have been either disrupted or expressed resulting in either loss or gain of alkane-oxidizing abilities [22–26].

AlkB belongs to a class of enzymes known as alkane monooxygenases for their ability to utilize molecular oxygen to insert a single oxygen atom into alkanes to generate alcohols. Alkane monooxygenases are of great interest because they carry out a thermodynamically and kinetically challenging conversion of substantial technological importance [27–30]. Relatively little biochemical work has been published on AlkB, in spite of both its apparent role in the global cycling of hydrocarbons and its identity as a non-heme monooxygenase. What work has been published has utilized the AlkB from the soil bacterium *Pseudomonas putida* GPo1 (*PpAlkB*) [30–33].

AlkB's are identified by having a set of eight essential and absolutely conserved histidines (HX_(3 or 4)H, HX_(2 or 3)HH, and HX_(2 or 3)HH) that coordinate two iron ions in the active site [34, 35]. There are two other histidines and a glutamine that when mutated lead to a loss of catalytic activity, suggesting that they too might be part of the coordination sphere of the iron ions [35]. Homology modeling suggests that AlkB has a substrate channel that leads from the cytoplasm to the inner periplasmic space where the active site is thought to reside [36]. In at least some AlKBs, the position of a single R group in the substrate channel modulates substrate specificity [37]. However, a detailed understanding of the structural factors that control substrate specificity across this large family of enzymes does not exist. Each AlkB probably has a relatively limited substrate range, with perhaps an optimal carbon chain length and then the ability to oxidize linear alkanes that are 2–3 carbon chains longer and shorter than the optimal chain length. The overall ability of hydrocarbonoclastic bacteria to metabolize so many different linear alkanes probably depends on organisms possessing a suite of AlKBs, along with other alkane-oxidizing enzymes [4].

AlkB requires two electron-transfer proteins, a reductase (AlkT) and a rubredoxin (AlkG)] for activity² [5]. Together, these two proteins transfer reducing equivalents from NADH to AlkB to activate the protein and facilitate O-O bond cleavage. Ultimately one atom of oxygen is transferred from molecular oxygen to the substrate and the other atom is released as water.

In this paper, we report a purification procedure that yields highly active and pure *AbAlkB* and we report as well a detailed characterization of its reaction mechanism and substrate

¹Note there is also a mammalian mononuclear iron DNA repair enzyme known as AlkB, which is completely different from the bacterial alkane monooxygenase reported here.

²Note, there is a recent report of two AlKBs from a Gram positive organism in which the rubredoxin domain is fused to the hydroxylase and genomic evidence that this may be another motif.[10] Y. Nie, J. Liang, H. Fang, Y.-Q. Tang, X.-L. Wu, Appl. Environ. Microbiol 77 (2011) 7279-7288

range. *Alcanovorax borkumensis* is an archetypal member of the hydrocarbonoclastic bacterial family and has been shown, along with other members of the *Alcanovorax* family, to play a dominant role in the degradation of alkanes in the marine environment [1, 23, 38–40]. Mesocosm experiments designed to study the changes in the bacterial community after an oil-spill have shown that microorganisms of the *Alcanivorax* genus dominate petroleum-contaminated seawater, after a brief induction period [13, 41]. Its genome has also been sequenced and hence it potentially provides a wealth of information about the cellular biology of alkane metabolism [1, 38, 42, 43].

AbAlkB is a 404 amino acid protein with a calculated molecular weight of 46,448 daltons and a calculated pI of 6.44. It has 78% sequence identity and 90% sequence similarity to *PpAlkB*. We find its reactivity to be similar to that of *PpAlkB*.

2. Experimental

2.1 Materials and methods

Chemicals and solvents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) or BioRad (Hercules, CA). The diagnostic substrates bicyclo[4.1.0]heptane (norcarane), bicyclo[3.1.0]hexane, and methylphenyl cyclopropane were synthesized and purified following published procedures. Product standards were characterized on a Bruker Advance™ 400 MHz NMR Spectrometer at ambient temperature. Rubredoxin from *Pseudomonas putida* GPo1 and maize ferridoxin reductase (reductase) were previously purified following published procedures [44, 45].

Gas chromatography mass spectrometry (GC-MS) analyses were performed on an Agilent 689N Network GC system with a 6890N Series Injector and 5973N Network Mass selective detector with a HP-5MS crosslinked 5% PH ME Siloxane capillary column (dimensions of 30m × 0.25mm × 0.25 µm). Various methods were used on the GC-MS. Generally the injection temperature was 225 °C, and the initial oven temperature was 50 °C. Both split and splitless injections were done to optimize peak shape and product detection respectively. The typical GC oven method ramped the oven to a final temperature of 220 °C with a ramp rate of 10 °C/min but bicyclohexane samples were also analyzed with a method that ramped the oven at 5 °C/min to a final temperature of 250 °C.

Low speed spins were done with a Sorvall RC 5c Plus with SLA-3000 and SLA-600TC rotors. Ultracentrifugation was done with a Beckman Optima LE-80K Ultracentrifuge with a 50.2 Ti rotor. Cells were lysed using a Misonix Microson Ultrasonic Cell Disrupter. Protein purification utilized an Akta UPC10 fast protein liquid chromatography instrument (FPLC).

Inductively coupled plasmic optical emission spectroscopy (ICP-OES) analyses were done on a Thermo Scientific iCAP 600 ICP. Samples for ICP-OES were prepared by digesting protein samples in 5% trace metal grade HNO₃. Metal content of purified protein was determined by using the concentration of protein (determined by a detergent-modified Bradford assay) and the concentration of iron from ICP and converting both to moles of protein and moles of iron respectively. It was assumed that a fully metallated protein contains two iron ions per protein monomer.

2.2 Cell growth

The cells were a generous gift of Dr. Jan van Beilen (ETH Hönggerberg, Zürich, Switzerland). They were constructed according to a published procedure in which the OCT-plasmid from *P putida* GPo1 was removed (the resulting bacterium is *P putida* GPo12) and then equipped with pGec47ΔB, a plasmid that encodes for gentamicin and tetracycline resistance as well as all of the proteins required for growth on medium-chain-length alkanes

except a functional alkane hydroxylase. A second plasmid, containing the *alkB1* gene from *A. borkumensis* AP1(pCom7alkB1) was inserted into the organism to create the microorganism we utilized [26]. The cells can utilize octane as their sole source of carbon, facilitating the production of cells with active AlkB. Experiments done with the cells that did not contain a functional AlkB showed no ability to transform alkanes [26].

The cells described above were grown initially on plates containing LB medium and the antibiotics tetracycline (0.0125 mg/mL) and gentamicin (0.025 mg/mL). All cells were plated from freezer stock (stored at -80°C) and carefully replated about once a month to prevent contamination. Once streaked on the plate, the cells were incubated at 30°C for 12–24 h. They were then used to inoculate Erlenmeyer flasks of Stanier's basal medium (MSB) medium. Small-scale growth involved delivering octane as the carbon source via a 0.3 mL hanging bulb to 50 mL of MSB medium. Large-scale growth involved directly adding 2 mL of octane to 400 mL of MSB medium in a 2.8 L Erlenmeyer flask. The cells were shaken at 200 rpm at 30°C in an incubator shaker and were provided with a daily 2 mL supply of octane.

2.3 Protein purification procedure

All procedures were done on ice or at 4°C unless otherwise indicated. Once octane-grown cells reached an optical density of approximately 1.0 at 600 nm, they were centrifuged at 8,000 rpm ($5,712 \times g$) for 15 min at 4°C . To each gram of cell pellet, the following reagents were added: 1 mL of 20 μM Tris-HCl buffer (pH = 7.4), 1 μL of 1 M dithiothreitol (DTT), and 4 μL of 100 mM (in ethanol) phenylmethanesulfonylfluoride (PMSF). The cells were frozen and thawed three times using a mixture of dry ice and ethanol. The mixture was sonicated on ice (3×1 min at 30 watts, 1 min resting period between each sonication). After sonication, the solution was centrifuged at 11,000 rpm ($10,800 \times g$) for 30 min at 4°C . The supernatant was collected and re-sonicated on ice (3×1 min, 8 watts, 1 min resting period between pulses). Material at this step of the purification was used for cell-free assays.

Further purification involved centrifuging the supernate from the prior step at 48,000 rpm ($136,400 \times g$) for 1 h at 4°C and collecting the amber-colored gelatinous pellet. The pellet was re-suspended in a 20 μM Tris-HCl buffer of pH = 7.4 (5 mL buffer/g pellet) and homogenized in the tissue grinder with 20 passes of the tight grinder. After centrifuging at 48,000 rpm ($136,400 \times g$) for 3 h at 4°C , the supernatant was discarded and the protein was re-suspended in Tris-HCl buffer (5 mL buffer/g protein), homogenized in the tissue grinder with 20 passes of the tight grinder, and re-centrifuged at 48,000 rpm ($136,400 \times g$) for 3 h at 4°C . 1 mg of N-dodecyl- β -D-maltopyranoside detergent (DDM) was added per mg of protein to solubilize the protein. The protein was stirred at 4°C for 1 h and then centrifuged at 48,000 rpm ($136,400 \times g$) for 30 min at 4°C . The supernatant was decanted and saved as the solubilized protein.

The buffer for the solubilized protein was exchanged with a 20mM Tris-HCl, 0.04% DDM, pH 7.9 filtered and degassed buffer and filtered through a 0.2 μM regenerated cellulose filter. The sample was injected onto a HiTrap Q FF anion exchange column at 4°C and then eluted with a salt gradient of using a 20mM Tris-HCl, 500 mM NaCl, 0.04% DDM, pH 7.9, filtered and degassed buffer. The AlkB fraction eluted from the column around 225 mM NaCl and was concentrated using a 30K concentrator. Gels of the purified protein indicate the presence of primarily a single protein after the anion exchange column.

Further purification was done using a size exclusion column. The post-anion exchange fraction containing AlkB was buffer exchanged into phosphate buffered saline (PBS) and injected onto a HiLoad 26/200 Superdex 75 320 mL size exchange column. AlkB eluted as a

single sharp peak after 110 mL (shown in Fig. 1). Calibration standards indicate that the approximate molecular weight of this peak is greater than 150 kDa.

A western antibody assay was done by using purified protein and running a 10% SDS-page gel. After electrophoresis the gel was electroblotted onto a nitrocellulose membrane using a constant current of 100 mA for 1 h. The primary antibody, rabbit antiAlkB had been generously supplied by Dr. Jan van Beilen and was used at a 1:5000 dilution. The primary antibody solution was preincubated with a nitrocellulose membrane that had been blotted with protein samples from *P putida* GPo12 (*P putida* without the OCT plasmid and no AlkB, see description above). The chemiluminescence was detected using X-MAT AR Film (Kodak, Rochester, NY)

2.4 Protein activity assays

Cell free activity assays were done with 1 mL of material from the purification before the cellular membranes were isolated to which 1 μ L 1 M DTT, 2 μ L organic substrate (approximately 20 μ mol, although the exact number of moles added varied slightly depending on the density and molecular weight of the substrate) and 33 μ L of a 0.36M (11.8 μ mol) NADH solution were added. Controls were done for all experiments with a buffer solution, DTT, organic substrate, and NADH. After incubation in a sealed centrifuge tube for 15 min (activity assays) or 2 h (substrate profiling) at 37 °C and 200 rpm, the samples were extracted with 0.5 mL CDCl_3 (stabilized by 0.5 wt.% silver foil), vortexed for 1 min, and centrifuged in a tabletop microcentrifuge for 5 min. The organic layer was extracted, dried over anhydrous sodium sulfate, and injected onto the GC-MS for analysis. Products were identified by comparison with synthesized standards (for all norcarane, bicyclohexane, and methylphenylcyclopropane analyses), purchased standards where readily commercially available, or by careful comparison with the NIST spectral library. Activity was assessed both by comparing the area of the product peak to the baseline observed with the control experiments (see supplemental material for GC traces) and the area of the substrate peak to the area of the substrate peak in the control experiments to account for any abiotic losses. All experiments were done at least twice in duplicate.

The purified protein assay contained approximately 1 mg (22 nmol) purified protein in 1 mL 20mM Tris-HCl 0.04% DDM, pH 7.4 buffer (22 μ M hydroxylase) with 0.2 nmol reductase (0.2 μ M final concentration, 290 μ M stock), 0.2 nmol rubredoxin (0.2 μ M final concentration, 200 μ M stock), 1 μ L 1M DTT, 2 μ L 100 mM PMSF, 2 μ L substrate, and 11.8 μ mol NADPH. The substrate was added after all the reagents except the NADPH solution were added. The reaction mixture was stirred and then the NADPH solution was added. A control was set up using 1 mL of 20 mM Tris-HCl buffer (pH= 7.4) and all other reagents except for the purified protein. Samples were incubated in a sealed centrifuge tube for 15 min (activity assays) or 2 h (diagnostic substrates) at 200 rpm at 37 °C. The samples were then extracted with 0.5 mL of CDCl_3 (stabilized by 0.5 wt.% silver foil), vortexed for 1 min, centrifuged for 5 min in a tabletop microcentrifuge and injected onto the GC-MS for product analysis. Yields were quantified by integrating the area under the GC-MS peak for the product and comparing it to the area under the GC-MS peak for the substrate. All experiments were done at least twice in duplicate.

The specific activity of AlkB was routinely quantified by monitoring the epoxidation of 1,7-octadiene and quantifying the concentration of the mono and di-epoxidized products from calibration curves generated previously. Specific activity was also measured spectrophotometrically by following the consumption of NADH or NADPH at 340 nm. Protein concentration was determined by Bradford assay modified for membrane-spanning proteins that contain detergents, using bovine serum albumin to create a standard curve, which was generated prior to every measurement.

3. Results

The purification procedure reported in this paper yields highly active protein and represents the first reported purification of *AbAlkB*. Fig. 1. shows an SDS-Page gel of the purified AlkB along with results from a western assay with an AlkB antibody confirming the identity of the band on the gel as AlkB. Fig. 1. also shows the UV absorption spectrum from a size exclusion column done to purify *AbAlkB*. The protein elutes as a single sharp band, coming off the column 110 mL after sample injection. A typical purification procedure begins with whole cells that catalyze the epoxidation of octadiene yielding 16.2 nanomoles of epoxide per mg of cell (15 min assay). After sonication, 15 g of cells yields 300–400 mg of total protein with an activity of 280 nanomoles of epoxide per mg of protein. Typically 60–100 mg of total protein remains after solubilization in the DDM buffer with an activity of 290 nanomoles of epoxide per mg of protein. After further purification on the anion exchange column, typically 10 mg of protein is collected with an activity of 4.5 micromoles of epoxide produced per mg of protein.

Iron ICP-OES analysis of the purified protein suggest that 97% of the protein is fully metallated, although the purified protein often contains a small amount of a heme contaminant that contributes to the total iron content. Others have reported the presence of a heme contaminant in the purification of *PpAlkB* as well [46] and other membrane-spanning enzymes. Using the molar absorptivity for heme of $106.1 \text{ mM}^{-1} \text{ cm}^{-1}$ (410 nm) [47], we estimate that no more than 10% of the iron detected is from the heme contaminant, leading to 85–90% of the AlkB containing two iron ions per monomer. The host organism does not contain an alkane-oxidizing CYP (cytochrome P450) [26] and the reactivity detected is inconsistent with CYP reactivity [48].

AbAlkB reaction mechanism was characterized by using diagnostic radical clock substrates and a deuterated substrate. The two diagnostic radical clock substrates used were norcarane and bicyclohexane, which have been widely used to characterize the reaction mechanisms of monooxygenases [48–54]. The commonly observed products from the oxidation of norcarane are identified in Fig. 2 and the commonly observed products from the oxidation of bicyclohexane are identified in Fig. 3. Norcarane and bicyclohexane were both reasonably good substrates for *AbAlkB* showing 21 and 19% conversion of substrate to products in these assays.

The pathways that generate these compounds have previously been established [55, 56]. Compounds 1 and 10 are clear indicators of the presence of a substrate radical. Tables 1 and 2 present the distribution of norcarane and bicyclohexane products respectively detected after the *AbAlkB*-catalyzed oxidation of the radical clock substrates for both the cell-free extract and the purified enzyme. Products are listed in the table in the order in which they elute from the GC column and percentages of total products with the accompanying standard error are also provided. The ratio of rearranged (R) to unrearranged (U) products are listed in the table as is the ratio of compounds where oxidation occurred at the 2 position (the weaker C-H bond but more sterically hindered position) relative to those where oxidation occurred at the 3 position (the stronger C-H bond but sterically more accessible position).

For the larger substrate, norcarane, oxidation preferentially occurs at the less sterically hindered 3-position while for the smaller substrate, bicyclohexane, that distinction is not seen. Overall, the results for the purified protein are similar to the results obtained for less pure preparations of the protein. The one exception, however, is that significantly more norcarane (56.5% v. 23.0% of total products), and concomitantly less 3-norcaranol (14.3% of v. 32.9% total products) was seen with the purified protein than the less pure preparation. Prior work with *PpAlkB* lead us to speculate that the location of the enzyme within the

membrane might affect the reaction mechanism [48, 49, 57]. Desaturation vs. hydroxylation is thought to be very sensitive to the position of the substrate relative to the active site [58, 59]. The difference in the relative amounts of desaturation and hydroxylation in the purified protein in comparison to the protein still presumably embedded in the cellular membrane may reflect subtle differences in active site structure [58, 59].

In addition, methylphenylcyclopropane (**16**) was oxidized by *AbAlkB*. The only detectable product was 1-phenylbut-3-en-1-ol (**17**) as shown in Fig. 4. The results from all three radical clock substrates (norcarane, bicyclohexane, and methyl phenylcyclopropane) are consistent with the formation of a substrate radical that persists in the active site. In a recent paper we hypothesized that the *AlkB* substrate channel might facilitate diffusion of the substrate radical away from the reactive intermediate, thereby extending its apparent lifetime beyond that seen in most other alkane oxidizing enzymes [53]. The results presented here are consistent with that model.

To further probe the hypothesis that alkane hydroxylation in *AbAlkB* utilizes an oxygen-rebound mechanism in which a substrate radical is transiently generated [60], cyclohexane (C_6H_{12}) and deuterated cyclohexane (C_6D_{12}) were both used as substrates under identical conditions in separate reactions. *AbAlkB* catalyzed the hydroxylation of both molecules, producing cyclohexanol and deuterated cyclohexanol. The area under both peaks was integrated and assumed to be proportional to the concentration of the alcohol, with the same proportionality constant. The area under the cyclohexanol peak was 3.81 times larger than the area under the deuterated cyclohexanol peak (see Fig. 5). Thus, we conclude that *AbAlkB* catalyzes the hydroxylation of the non-deuterated version 3.8 times faster than it catalyzed the hydroxylation of the fully-deuterated form. These results are consistent with a rate-determining step for the reaction that has considerable C-H(D) bond breaking character. Mass spectra for both products are included in the supplemental material.

To learn more about the active site constraints of *AbAlkB*, and to compare those active site constraints to those observed with *PpAlkB*, a series of cyclic and linear alkanes and alkyl substituted aromatic molecules were studied for their potential to be oxidized by *AbAlkB*. Molecules were assessed as to whether they were poor substrates with no more than 1–2% of the total substrate being transformed over the course of the reaction or excellent substrates where 15–26% of the substrate was transformed during the catalytic reaction. Hexadecane showed no detectable oxidation over the course of the reaction. Adding an alcohol at the terminal carbon of an alkylbenzene (phenylmethanol) resulted in no products. In all cases control experiments were done to control for any abiotic losses or oxidations. These results are summarized in Fig. 6. The GC-MS data used to generate Fig. 6. is provided in the supplemental material.

4. Discussion

Experimental evidence, including evidence presented in this paper, indicates that *AbAlkB* has a reaction mechanism in which a very long-lived substrate radical is generated, a substrate channel with a single bulky amino acid that controls substrate specificity [37], a histidine-rich coordination environment that coordinates two iron ions [34], and, perhaps, activity that depends on some sort of oligomeric structure [61]. *AbAlkB* may offer new insights into the chemistry of alkane oxidation.

In this paper, we report an effective method for purifying *AbAlkB* that yields a protein with high activity. Utilizing this pure protein to catalyze the hydroxylation of a series of diagnostic substrates leads to the conclusion that *AbAlkB* also utilizes the “oxygen-rebound” mechanism seen with many other monooxygenases [60, 62]. The substrate-radical

generated during the *AbAlkB*-catalyzed reaction persists for an unusually long time in the active site. This is consistent with our hypothesis, formed based on data collected with whole cells and cell free extracts containing *PpAlkB*, that the substrate radical can dissociate from the enzyme active site and migrate down the substrate channel before being recaptured in the rebound step [49]. The observation that a substrates with C-D bonds is hydroxylated more slowly than the same substrate with C-H bonds is also consistent with the proposed mechanism in which C-H bond breaking is postulated to occur.

PpAlkB and *AbAlkB* both hydroxylate the terminal methyl group of medium chain alkanes, where octane is apparently close to the optimal chain length. In a remarkable paper published in 2005 [37], van Beilen and co-workers showed that this substrate specificity is linked to a single bulky amino acid that they predicted, based on homology modeling, would be approximately 15–21 Å from the conserved histidines that presumably ligate the iron ions. Organisms that contain either *PpAlkB* or *AbAlkB* do not oxidize alkanes longer than 12–13 carbon atoms. Van Beilen *et al* showed that when this conserved amino acid was mutated (W55 in *PpAlkB* and W58 in *AbAlkB*), organisms containing these mutated AlkBs were then capable of hydroxylating longer chain alkanes. Van Beilen's work on the substrate specificity of *PpAlkB* [32] coupled with work from our own lab [57] has led us to further speculate a restricted active site at the end of a substrate channel that leads the enzyme to, for example, preferentially hydroxylate the sterically less hindered 3-position of norcarane even though it contains a strong C-H bond than the 2- position, while showing less selectivity with the slightly smaller, but structurally quite similar molecule, bicyclohexane. Spirooctane is another molecule that is selectively hydroxylated at the sterically less hindered 6-position; none of the other C-H bonds show any reactivity. We also note that product yields were lower from smaller chain alkane substrates (4 or 5 carbon). Medium length alkanes/alkenes (7–10 carbon) generated the most product. Very long chain alkanes such as hexadecane were not oxidized. This suggests that smaller substrates may also not fit optimally in the substrate channel. Perhaps this bulky amino acid is important in positioning the substrate in the channel in close proximity to the iron active site.

Very recently, a paper was published that reports the characterization of two dimensional crystals from *PpAlkB* [61]. In this paper, they report seeing no activity when *PpAlkB* was purified with DDM, in contrast to what we observe here. They also have evidence that in detergents that generate active AlkB, AlkB appears to form a trimeric structure. Our own SEC data (shown in Fig. 1) is consistent with an oligomeric structure for active *AbAlkB* purified in DDM although it is also consistent with a monomer and a DDM micelle. A more definitive structural characterization is required before we can assert that *AbAlkB* is not a monomer when active.

AlkBs have been found associated with more than 700 different taxonomic groups of bacteria and almost 1500 different sequences exist in the protein data bank. As a class they are capable of catalyzing the hydroxylation of alkanes that range in size from four to 32 carbons. They also appear to be responsible for oxidizing the majority of alkanes that enter the environment in this size range. Understanding the structural determinants that enable this family of enzymes to catalyze the hydroxylation of this range of molecules with apparent selectivity remains an unsolved challenge. Expanding the range of characterized AlkB's beyond the well-studied *PpAlkB* is a step towards developing a better understanding of the connection between structure and function for these critical players that facilitate one part of the global carbon cycle and whose ability to selectively active inert C-H bonds and utilize molecular oxygen as the oxygen atom source continues to fascinate chemists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Alkane hydroxylase from the marine organism *Alcanivorax borkumensis* is purified
- The purified protein is highly active
- The purified protein oxidizes a wide range of substrates, showing a preference for sterically unhindered C-H bonds
- The enzyme utilizes the oxygen rebound mechanism and generates a substrate radical during the reaction

Fig. 1. (a) SDS page gel of purified *AbAlkB* (b) Western blot image from *AlkB* antibody (c) UV trace of *AlkB* peak eluted from SEC

Fig. 2.

Products from the oxidation of norcaradiene. Path (a) shows the distinct product from a substrate radical, (b) products from all paths, (c) distinct cationic product.

Fig. 3. Products from oxidation of bicyclohexane. The labeling scheme is the same as in Fig. 2.

Fig. 4.
The radical clock substrate methylphenyl cyclopropane is converted to 2-phenylbut-3-en-1-ol by *AbAlkB*

Fig. 5. Chromatograms of reaction products from AlkB-catalyzed reactions of cyclohexane (red trace, retention time 4.1 min) and deuterated cyclohexane (blue trace, retention time 4.025 min)

Fig. 6. Picture of AlkB with presumed substrate channel identified and substrates sorted by efficiency with which they are oxidized by AlkB

Table 1

Distribution of products for the *AbAlkB* catalyzed oxidation of norcarane. See Fig. 2. for compound identities

	(6)	(7)	(8)	(9)	(5)	(1)	(2)	(3)	(4)	R/U	[2]:[3]
CFE	0	3.9 (0.2)	23.0 (1.7)	2.2 (0.3)	0	20.9 (0.6)	10.2 (0.3)	7.0 (0.3)	32.9 (1.0)	2.1 (0.1)	1:2.2 (0.1)
purified	0	0	56.5 (2.9)	0	0	15.2 (2.0)	9.5 (1.2)	4.5 (0.5)	14.3 (1.6)	1.7 (0.4)	1:3.1 (0.1)

Table 2

Distribution of products from the *AbAlkB*-catalyzed reaction of bicyclohexane. See Fig. 3. for compound identities.

	(10)	(11)	(12)	(13)	(14)	(15)	R/U	[2]:[3]
CFE	79.2 (0.4)	4.5 (0.1)	3.8 (0.2)	8.0 (0.1)	4.5 (0.2)	0	6.3 (0.1)	21.5:1 (0.9)
Purified	76.2 (1.6)	6.9 (1.1)	4.6 (0.6)	7.9 (0.2)	4.4 (0.8)	0	5.2 (0.4)	21.7:1 (4.2)