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MINI-REVIEW

Alkane hydroxylases involved in microbial alkane degradation

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Abstract This review focuses on the role and distribution in the environment of alkane hydroxylases and their (potential) applications in bioremediation and biocatalysis. Alkane hydroxylases play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. Environmental studies demonstrate the abundance of alkane degraders and have lead to the identification of many new species, including some that are (near)-obligate alkanotrophs. The availability of a growing collection of alkane hydroxylase gene sequences now allows estimations of the relative abundance of the different enzyme systems and the distribution of the host organisms.

The microbiology of alkane degradation

Research on microbial alkane degradation started close to a century ago, with a publication by Söhngen (1913) on microbes responsible for the disappearance of oil slicks on surface waters. This work was driven by a concern that the bacteria involved (identified as pseudomonads and mycobacteria) could be pathogenic. During the 20th century, the research focus shifted to topics related to oil production and use (detection of oil-fields, corrosion of steel in contact with oil, blockage of oil-filters by microbial growth), using oil to produce microbial biomass (single cell protein), using bacteria and yeast to convert oil components to more valuable oxygenated derivatives, tackling oil-pollution, and as a tool to understand the power and specificity of enzymes that handle oxygen. For more information on some of the important roadmarks in this development, it is worth to consult a recent review by Coon (2005), who played a crucial role in the characterization of enzymes involved in terminal hydrocarbon hydroxylation.

Research carried out in the first half of the 20th century has provided a solid basis for our present extensive knowledge on the microbiology of alkane degradation, and many microorganisms capable of thriving on these highly reduced organic compounds were identified. In the case of eubacteria, almost all of these belong to the α-, β-, and γ-Proteobacteria and the Actinomycetales (high G + C Gram-positives; van Beilen et al. 2003b; Coleman et al. 2006). More recently, *Bacillus*, *Geobacillus* (phylum Firmicutes), and *Thermus* (phylum Deinococcus-Thermus) isolates were found to degrade alkanes (Marchant et al. 2006; Meintanis et al. 2006). In addition, Flavobacteria and Sphingobacteria (phylum Bacteroidetes-Chlorobi) were found in oil-contaminated environments (Brakstad and Lodeng 2005). Among the eukaryotes, many yeasts and fungi, and some algae, are known to thrive on alkanes (van Beilen et al. 2003b). New genera containing alkane degraders are constantly being identified, leading to a better understanding of ecosystems. Some new marine isolates appear to be (near)-obligate alkanotrophs: They only grow on alkanes and few related compounds. Illustrative examples are *Alcanivorax borkumensis*, a species now found as the predominant organism in oil-impacted marine environments all over the world but below the detection limit in pristine environments (Sabirova et al. 2006) and *Thalassolituus oleivorans* (Yakimov et al. 2004).

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Alkane hydroxylases

Depending on the chain-length of the alkane substrate, different enzyme systems are required to introduce oxygen in the substrate and initiate biodegradation. For simplicity, we distinguish three categories: C_1–C_4 (methane to butane, oxidized by methane monooxygenase-like enzymes), C_5–C_{16} (pentane to hexadecane, oxidized by integral membrane non-heme iron or cytochrome P450 enzymes), and C_{17+} (longer alkanes, oxidized by essentially unknown enzyme systems). The composition of the main groups of alkane hydroxylases is shown in Table 1. The substrate ranges of the enzyme systems sometimes overlap and may cover more than one group of alkanes (Fig. 1).

C_1–C_4

Methane monooxygenases play a key role in the cycling of carbon in the biosphere, are useful biocatalysts, and play a major role in the degradation of xenobiotics in the environment (Murrell et al. 2000). Aerobic methane degraders typically contain membrane-bound particulate copper-containing enzymes (pMMO), whereas under copper-limitation, some methanotrophs such as *Methylococcus capsulatus* (Bath), express soluble non-heme di-iron monooxygenases (sMMO). The latter enzyme system consists of a hydroxylase (MMOH, a 251-kDa $\alpha_2\beta_2\gamma_2$ heterodimer containing the carboxylate-bridged diiron center in the α-subunit), a regulatory component (MMOB), and an NADH-dependent reductase (MMOR; Merkx et al. 2001). As the soluble enzyme was much easier to handle than the membrane-bound enzyme, it initially received more attention, and a 3-D structure has been available since 1993 [see references cited in Lieberman and Rosenzweig (2005)].

The sMMO enzyme belongs to a large family of soluble diiron monooxygenases, which have numerous applications in bioremediation and biocatalysis (Shennan 2006) and can be distinguished in five subgroups based on phylogenetic analyses and gene arrangements (Leahy et al. 2003; Coleman et al. 2006). Most of these enzymes act on small hydrophobic molecules, ranging from (halogenated)-alkanes and alkenes to aromatics such as phenol and tetrahydrofuran. The selectivity of sMMO for methane is controlled by the regulatory protein MMO-B; binding of this subunit to the MMO-H subunit, which contains the active site, appears to open a channel with the size of methane into the active site (Zhang et al. 2006).

Until recently, structure, location, and mechanism of action of the active site of pMMO have remained elusive. However, recent work by two independent groups has now revealed the structure of the elusive pMMO (Kitmitto et al. 2005; Lieberman and Rosenzweig 2005). It was shown to consist of a hydroxylase (pMMO-H, comprised of the three subunits PmoA, PmoB, and PmoC) and an additional component pMMO-R, formed by two polypeptides with molecular masses of 63 and 8 kDa. This component, which was first proposed to be the reductase, is now thought to be the methanol dehydrogenase, the subsequent enzyme in the methane–oxidation pathway (Myronova et al. 2006). The natural electron donor and electron–transfer pathway have

Table 1 Enzyme classes involved in the oxidation of alkanes

Enzyme class	Composition and cofactors	Substrate range	Presence shown in	Reference for GenBank entries
Soluble methane monooxygenase (sMMO)	$\alpha_2\beta_2\gamma_2$ hydroxylase; dinuclear iron reductase, [2Fe–2S], FAD, NADH regulatory subunit	C_1–C_8 (halogenated)-alkanes, alkenes, cycloalkanes	*Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylocella*	McDonald et al. (2006)
Particulate methane monooxygenase (pMMO)	$\alpha_3\beta_3\gamma_3$ hydroxylase trimer composed of PmoA, PmoB, PmoC; mononuclear copper and dinuclear copper in PmoB	C_1–C_5 (halogenated)-alkanes, alkenes	*Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylobacter*, *Methylomonas*, *Methylomicrobium*, etc.	McDonald et al. (2006)
AlkB-related alkane hydroxylases	Membrane hydroxylase; dinuclear iron rubredoxin; mononuclear iron rubredoxin reductase, FAD, NADH	C_5–C_{16} alkanes, fatty acids, alkylbenzenes, cycloalkanes, etc.	*Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, etc.	van Beilen et al. (2003b)
Eukaryotic P450 (CYP52, Class II)	Microsomal oxygenase; P450 heme reductase; FAD, FMN, NADPH	C_{10}–C_{16} alkanes, fatty acids	*Candida maltosa*, *Candida tropicalis*, *Yarrowia lipolytica*	Iida et al. (2000)
Bacterial P450 oxygenase systems (CYP153, class I)	P450 oxygenase; P450 heme ferredoxin; iron–sulfur ferredoxin reductase, FAD, NADH	C_5–C_{16} alkanes, (cyclo)-alkanes, alkylbenzenes, etc.	*Acinetobacter*, *Alcanivorax*, *Caulobacter*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas*, etc.	van Beilen et al. (2006)
Dioxygenase	Homodimer; copper, FAD	C_{10}–C_{30} alkanes	*Acinetobacter* sp. M-1	Maeng et al. (1996)

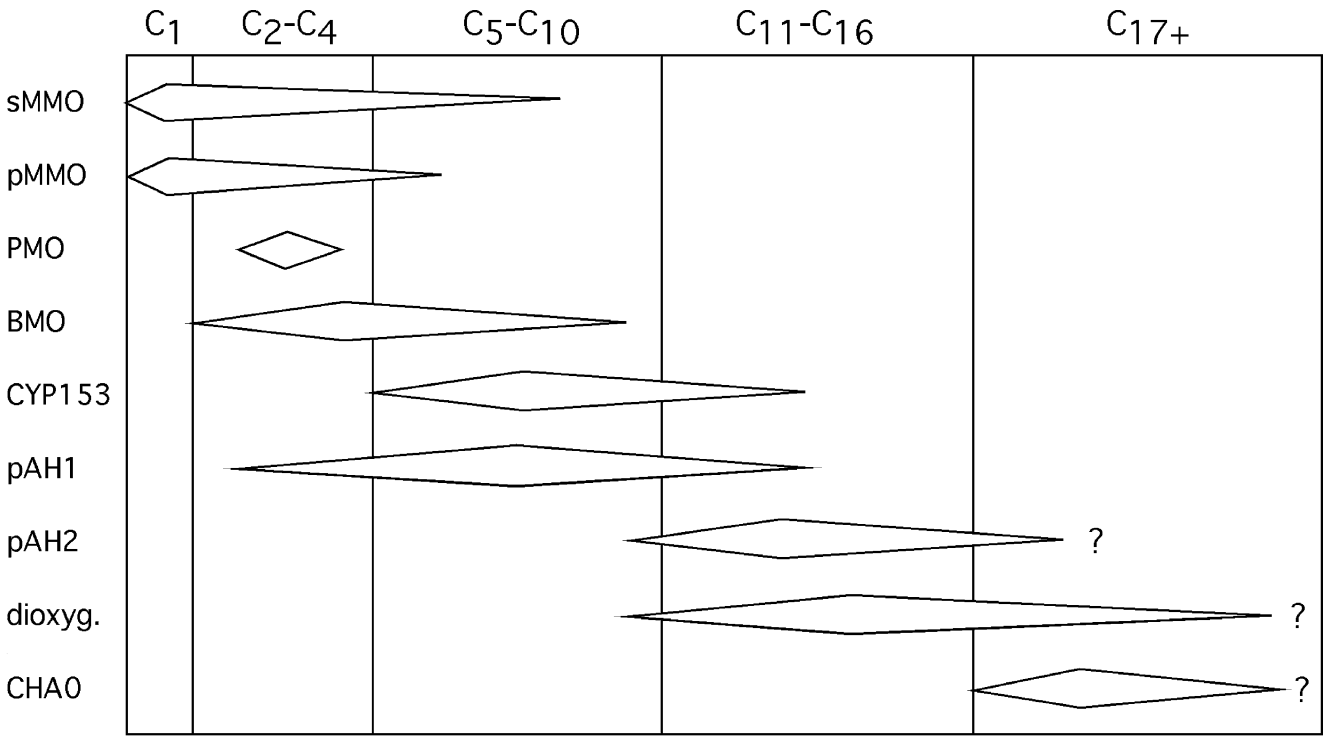

Fig. 1 Overview of the substrate range of alkane hydroxylase with respect to alkanes. *sMMO* Soluble methane monooxygenase (Colby et al. 1977); *pMMO* particulate methane monooxygenase (Elliott et al. 1997); *PMO* propane monooxygenase (Kotani et al. 2003); *BMO* butane monooxygenase (Doughty et al. 2006); CYP153 (van Beilen et al. 2006); *pAH1* medium-chain-length integral membrane alkane hydroxylase (van Beilen et al. 2005b; Johnson and Hyman 2006); *pAH2* long-chain-length integral membrane alkane hydroxylase (van Beilen et al. 2005b); *dioxyg Acinetobacter* sp. M-1 dioxygenase (Maeng et al. 1996); *CHA0* inferred alkane oxygenase in *P. fluorescens* CHA0 (Smits et al. 2002)

still not been identified. The pMMO enzymes are more restricted in their substrate range than the sMMOs (Elliott et al. 1997), but they also act on longer alkanes as well as on several other hydrophobic compounds (Fig. 1).

It appears that enzymes involved in the degradation of ethane, propane, and butane belong to several distinct enzyme classes with similarity to sMMO and pMMO. A three-component non-heme iron butane monooxygenase (BMO) in '*Pseudomonas butanovora*' was found to be similar to sMMO and hydroxylates C_2 to C_9 alkanes on the terminal position but appeared hardly to be active with methane as a substrate. The basis for this selectivity was studied by site-directed mutagenesis, revealing a striking sensitivity for inhibition of BMO by methanol. Due to geometric constraints of the active site, the product release is believed to be rate-limiting, and in a mutant, this restriction was partly abolished leading to a less methanol-sensitive enzyme (Halsey et al. 2006). BMO expression is up-regulated by the oxidation products butyraldehyde and 1-butanol but repressed by propionate, resulting in a difference in the response to even- versus odd-chain-length alkanes (Doughty et al. 2006).

Kotani et al. (2003) have characterized the *Gordonia* sp. TY-5 propane monooxygenase, which is also similar to sMMO based on sequence comparisons, but unlike BMO, it appears to have a very narrow substrate range; it only oxidizes propane at the 2-position. The propane and butane degrader *Mycobacterium vaccae* JOB5 is known for its ability to co-metabolically degrade trichloroethylene (Hamamura et al. 1999) and the gasoline additive methyl-*t*-butyl-ether (Johnson et al. 2004), and inhibition patterns indicate that it contains a non-heme iron monooxygenase. *Nocardioides* CF8 is likely to contain a copper-containing BMO similar to pMMO (Hamamura et al. 1999).

C_5–C_{16}

After Söhngen's pioneering work (Söhngen 1913), many organisms were found to grow on or co-metabolically oxidize alkanes. Early biochemical studies pointed to the involvement of two main classes of enzymes; the first related to the ubiquitous cytochrome P450 enzymes (P450s) found in mammalian liver (Coon 2005; Bernhardt 2006). These alkane-hydroxylating P450s were found in yeasts and in few bacteria (more about these enzymes below).

In several *Pseudomonas* isolates, particulate alkane hydroxylases (pAHs) were detected, which were clearly unrelated to P450 enzymes. The alkane hydroxylase of one particular *Pseudomonas* isolate, now known as *P. putida*

GPo1, was characterized in great detail. It was shown to be an integral-membrane non-heme diiron monooxygenase (AlkB), requiring two electron transfer proteins named rubredoxin and rubredoxin reductase. For a long time, this enzyme system remained as the only well-characterized enzyme of this class (van Beilen et al. 1994). However, short conserved sequence motifs discovered using additional pAH sequences from *Acinetobacter* sp. ADP1 (*alkM*) and *Mycobacterium tuberculosis* H37Rv (Smits et al. 1999), allowed the amplification of related genes from a wide range of bacteria from α-, β-, and γ-Proteobacteria and the Actinomycetales (van Beilen et al. 2003b).

Only a few of these pAHs (those found in *P. putida* GPo1, other pseudomonads, and in related γ-Proteobacteria such as *A. borkumensis*) oxidize C_5–C_{12} alkanes (indicated as pAH1 in Fig. 1), whereas most members of this enzyme family oxidize alkanes longer than C_{10} (see below). As the GPo1 enzyme was recently shown to act on propane and butane as well (Johnson and Hyman 2006), it is likely that other members of the pAH1 group also act on these shorter alkanes. In a GenBank search, new pAH1 sequences were also found in γ-Proteobacteria such as *Marinobacter aquaeolei*, and in a few α-Proteobacteria, such as *Xanthobacter* sp. and *Oceanicaulis alexandrii*, but the encoded enzymes have not been studied with respect to substrate range and other properties (van Beilen, unpublished observations).

Functional expression and knockout studies showed that by far most pAHs, indicated collectively as pAH2 in Fig. 1, are not very active with alkanes shorter than C_{10} and prefer longer alkanes (Smits et al. 2002; van Beilen et al. 2005b). The upper end of the substrate range of these enzymes could not be determined because the host that was used to test the substrate range of these enzymes (an *alkB* knockout of *Pseudomonas fluorescens* CHA0) was still able to grow on alkanes ranging from C_{18}–C_{28} (Smits et al. 2002).

Although the pAHs belong to an important superfamily of enzymes, which includes integral-membrane desaturases, decarbonylases, and epoxidases, a detailed 3-D structure is not available. All pAHs contain six hydrophobic stretches, which are thought to cross the membrane as α-helices; they contain eight to nine histidines that are essential for activity, probably as ligands to the non-heme dinuclear iron center that participates in catalysis (Shanklin and Whittle 2003). One particular amino acid position, located in the middle of transmembrane helix 2, was found to determine whether pAHs are able to hydroxylate alkanes longer than C_{12} (van Beilen et al. 2005b).

Whereas yeasts are well-known for their ability to grow on alkanes by virtue of their microsomal P450s of the CYP52 family, only limited evidence pointed to the presence of P450 alkane hydroxylases in bacteria (van Beilen et al. 2003b). In 2001, Asperger et al. published the sequence of a cytochrome P450 belonging to a new family (CYP153A1) implicated in hexane oxidation (Maier et al. 2001). More recently, we showed, using highly degenerate PCR primers, that many isolates selected for their ability to grow on C_5–C_{10} alkanes contain CYP153 enzymes. Several of these were functionally expressed in *P. putida* GPo12 to demonstrate that these enzymes are indeed responsible for the ability of the isolates to grow on alkanes (van Beilen et al. 2006). The very high affinity of CYP153 enzymes for aliphatic alkanes (K_d in the nanomolar range) added additional evidence to the proposal that alkanes are the physiological substrates of the soluble CYP153 enzymes. In-silico docking of alkanes in a homology model of CYP153A6 showed that alkanes were positioned such that hydroxylation would take place at the terminal methyl-groups, as indeed observed in in vivo and in vitro assays (Funhoff et al. 2006a).

C_{16+}

Alkanes longer than C_{16} support growth of many micro-organisms. For example, many *Rhodococcus* isolates grow well on purified alkanes up to C_{32} by virtue of unknown enzyme systems (van Beilen et al. 2002), whereas an uncharacterized alkane oxygenase allow *P. fluorescens* to grow on C_{18}–C_{28} alkanes (Smits et al. 2002). Because these alkanes are solids, it has proven difficult to screen for mutants lacking this enzyme system. However, cloning of these enzyme systems by complementation or transposon mutagenesis should be possible.

In 1996, Maeng et al. (1996) reported on the isolation of a flavin-containing alkane-dioxygenase in *Acinetobacter* sp. M-1, having a substrate range of C_{10}–C_{30}. Unfortunately, this enzyme has not been cloned yet. It should be noted that the same authors later reported the cloning and characterization of two integral membrane alkane hydroxylases from *Acinetobacter* sp. M-1 that cover the same substrate range (Tani et al. 2001).

Individual alkane degraders frequently contain multiple alkane hydroxylases

Many alkane degraders are able to grow on a wide range of alkanes. Indeed, a common feature of many alkane degraders is that they contain multiple alkane hydroxylases with overlapping substrate ranges. This was first shown for the integral membrane alkane hydroxylases, as discussed earlier (van Beilen et al. 2003b), but was later extended to the CYP153 enzymes (van Beilen et al. 2006). In fact, while several *P. aeruginosa* strains contain one pAH1 and two pAH2 genes, and *Sphingomonas* sp. HXN-200 contains five CYP153 genes, other strains contain both

pAHs and CYP153s. *R. erythropolis* isolates may contain up to five pAH2s and three CYP153s (van Beilen et al. 2006), while *A. borkumensis* isolates contain two pAHs and two CYP153s (Sabirova et al. 2006). A systematic investigation of other alkane hydroxylase systems and alkane degrading microorganisms will probably reveal similar arrangements. For example, the propane-metabolizing *Gordonia* isolate studied by Kotani et al. (2003) grows on C_{13}–C_{22} alkanes in addition to propane, indicating that it is likely to contain additional alkane hydroxylases as well.

Applications of alkane hydroxylases

Oxidative biotransformations of hydrocarbons have attracted sustained attention over the last decades. Alkane oxidation is of special interest as C–H bond cleavage is intrinsically difficult, especially because specific and generally applicable synthetic methods to introduce hydroxy-groups on unactivated hydrocarbons do not exist (Groves 2006). Most alkane oxygenases are relatively complex and difficult to use in vitro, as they consist of multiple components (Fig. 2), leading to low electron transfer rates. They are co-factor dependent, sensitive to inactivation by activated oxygen species, and sensitive to product inhibition. Moreover, the substrates as well as the products of these enzymes tend to be quite hydrophobic and toxic to the host cell. This calls for careful substrate feeding protocols to keep the substrate concentration below toxic limits and in situ extraction set-ups consisting of a second hydrophobic but non-toxic liquid phase or a solid extractant. These hurdles and their consequences for the development of biocatalytic processes utilizing alkane hydroxylases have been discussed previously (van Beilen et al. 2003a; Ayala and Torres 2004).

Although technical solutions exist for most of the identified problems, biocatalytic processes become rather sophisticated, especially if high volumetric productivities are required for an economical process. As a consequence, large-scale applications of alkane hydroxylases and other oxygenases are restricted to high-value compounds such as pharmaceutical intermediates (van Beilen et al. 2003a; Urlacher and Eiben 2006).

Most alkane hydroxylases tend to have a wide substrate range and can be used to synthesize a wide range of alcohols, aldehydes, carboxylic acids, and epoxides. Several recent reviews list substrates and substrate families that can be oxidized by alkane hydroxylases (van Beilen and Witholt 2004; van Beilen and Funhoff 2005; Funhoff and van Beilen 2006). A representative sample of conversions is shown in Fig. 3.

The most useful alkane hydroxylases appear to be the heme-containing cytochrome P450 enzymes. A recombinant *P. putida* strain expressing CYP153A6 (cloned from *Mycobacterium* sp. HXN-1500) was used to convert limonene (a cheap waste product of the orange juice industry) to perillyl alcohol (a valuable flavor compound, which has shown promise as a chemopreventative for certain types of cancer; van Beilen et al. 2005a). The CYP153s in *Sphingomonas macrogoltabida* HXN-200 collectively carry out stereo- and regioselective hydroxylation of a wide range of four-, five-, and six-ring alicyclic compounds with rates of up to 10–20 μmol min^{-1} g^{-1} cell dry weight (Li et al. 2002). Other CYP153 family members, functionally expressed either as chimeric proteins in *Escherichia coli* or with CYP153-associated electron-transfer proteins in *P. putida*, were studied for their ability to synthesize a range of (di)alcohols and epoxides (Kubota et al. 2005; Fujii et al. 2006; Funhoff et al. 2006b; Nodate et al. 2006).

In general, cytochrome P450 enzymes are used for the synthesis of drugs, fine chemicals and fragrances, and in bioremediation. Interestingly, two of these enzymes (*Bacillus megaterium* CYP102 or P450bm-3 and *P. putida* CYP101 or P450cam) were engineered to catalyze the activation of alkanes in addition to their natural substrates [see recent discussions on this enzyme family Funhoff and van Beilen (2006); Urlacher and Eiben (2006)]. These studies illustrated the difficulties to obtain terminal alkane hydroxylation, as the mutant enzymes preferably oxidized

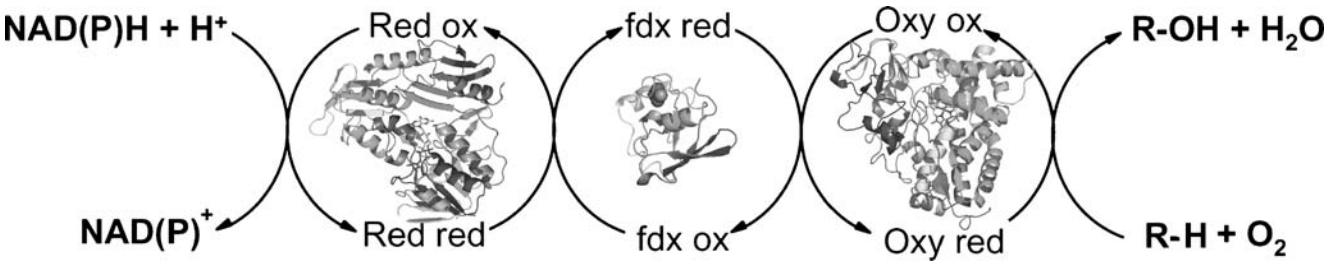

Fig. 2 Schematic representation of the various compounds of an alkane hydroxylase system and its electron transfer system. A reductase protein (*red*) transfers electrons from NAD(P)H via a small electron transfer protein (*fdx*) to the hydroxylase (*oxy*) protein where the substrate is oxidized to an alcohol, aldehyde, carboxylic acid, or epoxide. In integral-membrane alkane hydroxylases (pAHs), a rubredoxin replaces the ferredoxin, whereas in the microsomal P450s, functions of the reductase and the ferredoxin are combined in one protein

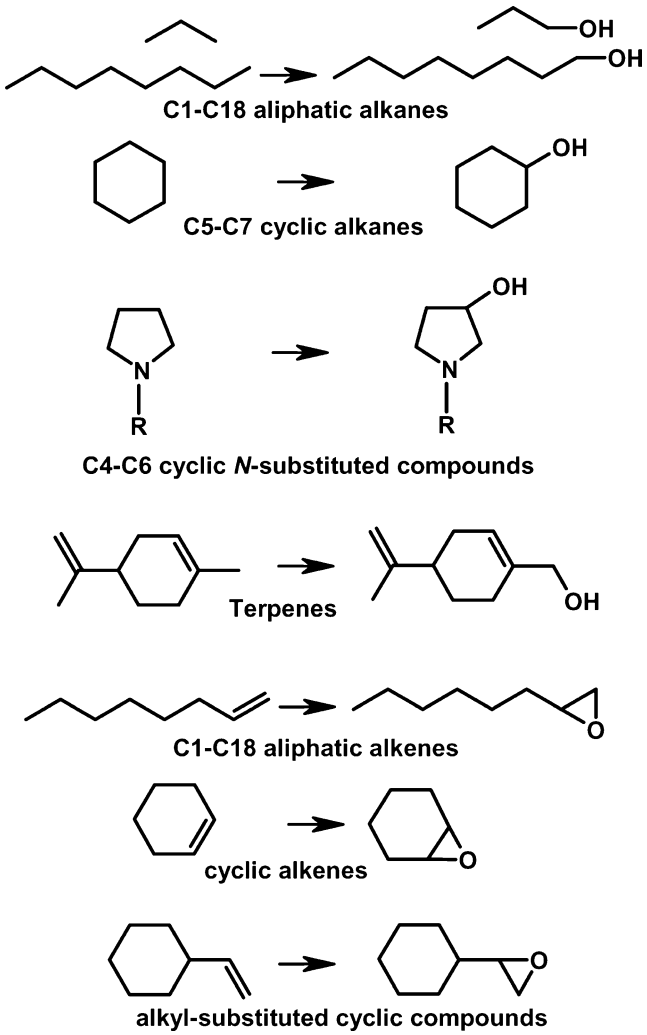

Fig. 3 Examples of oxidation reactions catalyzed by several families of alkane hydroxylases (van Beilen and Witholt 2004; van Beilen and Funhoff 2005; Funhoff and van Beilen 2006). Depending on the host, the products are converted to aldehydes and acids by alcohol and aldehyde dehydrogenases

subterminal positions even after extensive mutagenesis (Meinhold et al. 2006). Another aim of these protein engineering studies is the construction of a soluble, single component, co-factor independent (obtaining its reducing equivalents from hydrogenperoxide or electrochemically), highly active alkane hydroxylase for biocatalytic applications in vitro. Besides the many potential pharmaceutical targets, the conversion of methane to methanol could be useful because the latter is much easier to transport than methane. This conversion would constitute a potential solution to get "stranded gas" (smaller natural gas finds, where building pipelines is uneconomical) to the customer. Attempts to use methane monooxygenase for this purpose indicate that product inhibition is a major problem (Lee et al. 2004; Xin et al. 2004).

Especially the C_1–C_4, alkane hydroxylases are known to carry out co-metabolic oxidations of pollutants including fuel additives such as methyl-*t*-butylether (Johnson et al. 2004), and halogenated alkanes and alkenes, such as trichloroethylene, terpenes, and many other hydrocarbons (Connon et al. 2005; Halsey et al. 2005; Coleman et al. 2006; Kim et al. 2006). For a thorough overview of the applications of these enzymes in bioremediation and biocatalyis, please consult Shennan (2006).

The integral membrane non-heme monooxygenases (pAH1) convert linear, branched, and cyclic alkanes as well as alkenes and alkyl-substituted aromatics to the corresponding alcohols, aldehydes, carboxylic acids, and epoxides. Their application on an industrial scale has been tested by Exxon, DSM, and Shell (van Beilen et al. 2003b; van Beilen and Witholt 2004; van Beilen and Funhoff 2005; Funhoff and van Beilen 2006).

Alkane hydroxylases also serve as models for chemical catalysts and mechanistic studies; robust chemical catalysts for regio- and stereoselective hydrocarbon activation, equaling or surpassing the selectivity and activity of biological catalysts would indeed be very valuable tools (Groves 2006). Several alkane hydroxylases (the integral membrane enzyme from *P. putida* GPo1, and two CYP153 enzymes) have now been tested with diagnostic substrates (the radical clock substrates norcarane and bicyclohexane) revealing interesting differences in the mechanism of these enzymes. Although both enzyme types produce radical intermediates, the radical lifetimes differ significantly (Bertrand et al. 2005; Austin et al. 2006).

The ecology of alkane hydroxylation

Alkane degraders are widespread in oil-polluted environments. However, it is clear that alkane-degraders occur in pristine environments as well (van Beilen et al. 2003b; Harayama et al. 2004). In fact, many organisms (yeasts, plants, insects, and microorganisms) produce alkanes as a moisture barrier, as a reserve material, as a pheromone, and as a metabolic overflow product (Park 2005). Accordingly, many microorganisms have evolved to use this resource and live in close association with the producing organisms, for example on the leaf and root surface of plants. An overview of ecological studies using pAH gene probes shows that these sequences are abundant in many environments (van Beilen et al. 2003b). A general conclusion of this study is that pAH genes are often detected in pristine environments, sometimes at relatively high levels, and that oil or fuel pollution clearly promote this genotype.

In a recent PCR screening study using primers specific for the soluble diiron monooxygenases, 75 novel sequences belonging to six groups of soluble non-heme diiron

monooxygenases (all similar to sMMO but with various numbers of subunits, gene arrangements, and phylogenetic distances) were found in environmental samples and ethene enrichments (Coleman et al. 2006). Primers specific for the *pmoA* gene, encoding the α-subunit of the pMMO-H hydroxylase have also been developed (Steinkamp et al. 2001) and applied in various environments, for example in a trichloroethylene-contaminated aquifer (Baker et al. 2001), in artic soils (Pacheco-Oliver et al. 2002), or with isolates from contaminated soils in Canada and Germany (McDonald et al. 2006).

In marine environments, natural and human-made oil spills provide a constant source of alkanes (Head et al. 2006). Furthermore, algae and other organisms are likely to contribute significantly to alkane production. Harayama et al. (2004) have recently reviewed the state of the art in the analysis of microbial communities in oil-polluted environments. A very interesting finding is that some new isolates are nearly obligate alkanotrophs (they grow on few carbon sources other than alkanes). Illustrative examples are *T. oleivorans* (Yakimov et al. 2004) and *A. borkumensis*, a species now found as the predominant organism in oil-impacted marine environments all over the world but below the detection limit in pristine environments (Sabirova et al. 2006).

Although the 'extreme' environments have been rather neglected with respect to hydrocarbon degradation, several *Bacillus*, *Geobacillus*, *Thermus*, *Thermococcus*, and *Thermotoga* species have been shown to degrade alkanes at elevated temperatures (Meintanis et al. 2006). Whereas in most cases, enzymes involved in alkane degradation by these strains have not been characterized; a *Geobacillus* isolate appears to contain a gene very similar to one of the *Rhodococcus alkB* genes (Marchant et al. 2006). One report concerns the presence of alkanes and alkane-degraders in acidic mud springs; an *Acidisphaera* isolate (an α-Proteobacterium) contains an pAH1 gene that is close to 80% identical to the *P. putida* GPo1 AlkB (Hamamura et al. 2005).

The availability of gene probes and PCR primers for a wide range of pAHs now allows studies to quantify the relative importance and the spatial and temporal distribution of specific alkane hydroxylase genes and their hosts (van Beilen et al. 2003b; Heiss-Blanquet et al. 2005; Coleman et al. 2006). Another exciting development is the metagenome sequencing of DNA from certain environmental niches, which provides an overview of the presence and relative abundance of known and unknown organisms and also reveals the presence or absence of catabolic genes such as those involved in the degradation of alkanes. For example, the Sargasso Sea environmental DNA sequences of Craig Venter (Venter et al. 2004) contain 7 pAHs and 15 CYP153 sequences (van Beilen, unpublished observations). Citations for lists of accession numbers can be found in Table 1.

Conclusions

Until a few years ago, only a limited number of alkane hydroxylases were known and even fewer were characterized in some detail [the membrane-bound alkane hydroxylase of *P. putida* GPo1, the yeast CYP52 enzymes, and the soluble and particulate methane monooxygenases (sMMO and pMMO) of *M. capsulatus* (Bath)]. Recent developments have shown that many microorganisms contain alkane hydroxylases that are related to the above enzymes. Moreover, new alkane hydroxylase families were discovered, such as the CYP153 cytochrome P450s and the short-chain alkane hydroxylases related to sMMO and pMMO. In most cases, the use of molecular tools (degenerate primers for PCR and probes for Southern blotting, colony blotting, and gene chips) has greatly extended our knowledge on the occurrence and distribution of these enzymes in various microorganisms and ecosystems. In some cases (sMMO and pMMO), a 3-D structure is now available, whereas in most other cases, structural information can be obtained by calculating 3-D models (P450 alkane hydroxylases and butane and propane monooxygenases) or rough folding models (integral-membrane non-heme diiron enzymes). In the case of CYP153A6, in-silico docking of alkane molecules on the calculated models shows that the terminal position is available for hydroxylation. Nevertheless, the structural basis of the exquisite specificity of most AHs for terminal positions is unknown. The identity of enzymes involved in the oxidation of alkanes longer than C_{16} is still an open question as well.

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