ELSEVIER

Expanding the alkane oxygenase toolbox: new enzymes and applications

Jan B van Beilen and Enrico G Funhoff

As highly reduced hydrocarbons are abundant in the environment, enzymes that catalyze the terminal or subterminal oxygenation of alkanes are relatively easy to find. A number of these enzymes have been biochemically characterized in detail, because the potential of alkane hydroxylases to catalyze high added-value reactions is widely recognized. Nevertheless, the industrial application of these enzymes is restricted owing to the complex biochemistry, challenging process requirements, and the limited number of cloned and expressed enzymes. Rational and evolutionary engineering approaches have started to yield more robust and versatile enzyme systems, broadening the alkane oxygenase portfolio. In addition, metagenomic approaches provide access to many novel alkane oxygenase sequences.

Addresses
Swiss Federal Institute of Technology Zürich, Institute of Biotechnology, Wolfgang-Pauli Strasse 16, CH-8093, Zürich, Switzerland

Corresponding author: van Beilen, Jan B (vanbeilen@biotech.biol.ethz.ch)

Current Opinion in Biotechnology 2005, **16**:308–314

This review comes from a themed issue on
Environmental biotechnology
Edited by Gerben J Zylstra and Jerome J Kukor

Available online 27th April 2005

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2005.04.005

Introduction

Oxygenases are enzymes that catalyze the chemo-, regio- and stereoselective oxygenation of alkanes and many other compounds [1,2], producing alcohols, aldehydes, epoxides and carboxylic acids. Oxygenases have already enabled new industrial synthetic routes for difficult-to-synthesize complex molecules, such as secondary metabolites (steroids, polyketides, terpenes, etc.) and pharmaceutical and agrochemical intermediates [3–5]. The use of oxygenases to convert petroleum compounds into activated intermediates for the polymer and bulk chemical industries is attractive, because the functionalization of hydrocarbons by chemical means is difficult, energy intensive, and environmentally unfriendly. However, large-scale applications are not yet feasible owing to the intrinsic properties of oxygenases and the requirement for oxygen and cofactors. In general, biotransformations can be carried out either with whole cells or with (partly) purified enzymes, depending on the process requirements and price. The practical application of oxygenases is complicated by factors that are not a concern for hydrolases, lyases or isomerases. If whole cells are used, the toxicity of substrates and products, oxygen mass transfer, and the uptake of lipophilic substrates present significant engineering challenges. *In vitro* enzyme applications are restricted by cofactor requirements and the sensitive nature of many oxygenases, which are often multicomponent systems with oxidizing and electron-shuttling subunits. In recent years, many of these issues have been explored in detail (for reviews see [5,6••,7]). For example, product toxicity can be prevented through the use of two-phase liquid systems or solid-phase extraction, and direct electrochemical methods with immobilized or covalently linked oxygenases could eventually replace expensive cofactor regeneration systems [8••,9••]. In addition, genetic engineering might help to overcome instability or selectivity limitations, thus enabling the exploitation of the unique catalytic power of alkane oxygenases for both high added-value and bulk applications.

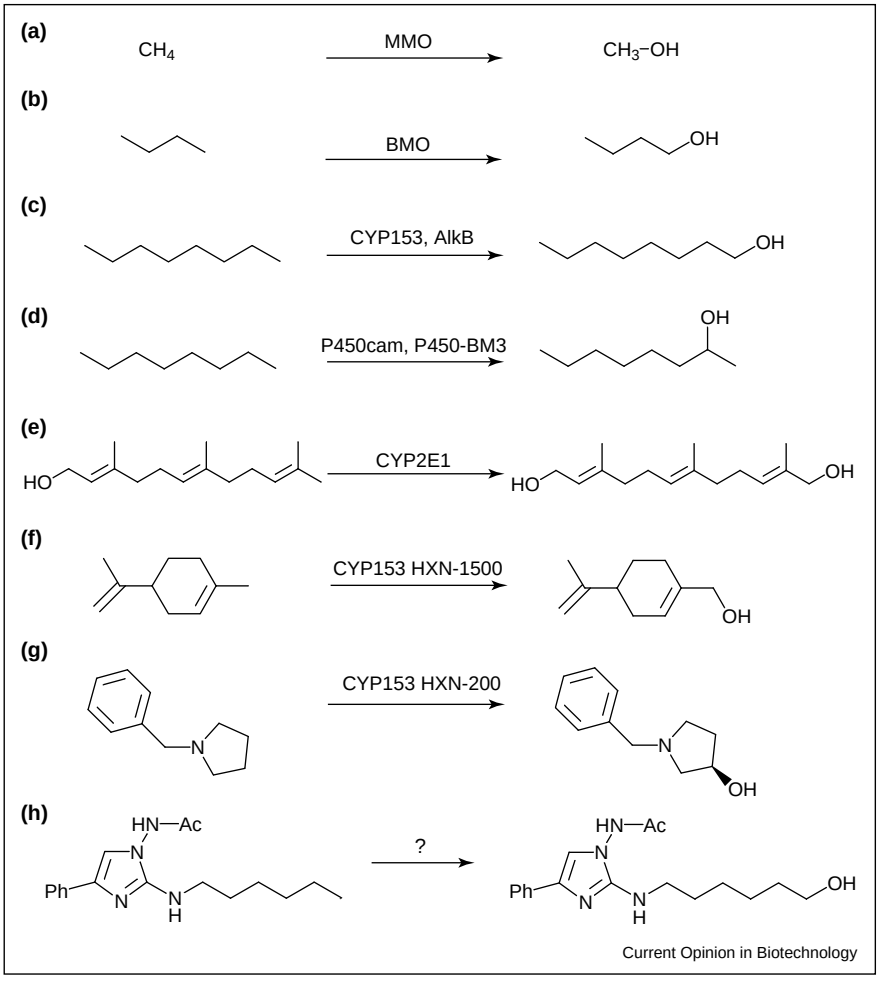
The diversity of alkane oxygenase systems

In prokaryotes and eukaryotes several enzyme systems have evolved to activate aliphatic hydrocarbons under aerobic conditions: cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (e.g. AlkB), soluble di-iron methane monooxygenases (sMMO), and membrane-bound copper-containing (and possibly iron-containing) methane monooxygenases (pMMO) (Figure 1; Table 1). It is now recognized that these enzyme systems are ubiquitous in nature. Recent progress in the identification and characterization of these enzymes, in the elucidation of their diversity, and in the use of these oxygenases in biocatalysis are discussed in the following sections.

Cytochrome P450 alkane hydroxylases

More than 4000 P450 enzymes are known to date, approximately 10–15% of which are found in prokaryotes. The many new P450s discovered in genome sequences illustrate that the surface has barely been scratched: bacteria such as *Mycobacterium tuberculosis* and *Streptomyces coelicolor* contain up to 20 different cytochrome P450s [10]. The most thoroughly researched P450s are P450cam, an enzyme from *Pseudomonas putida* that initiates the metabolism of camphor, and P450BM-3, an enzyme from *Bacillus megaterium* thought to be involved in fatty acid metabolism. Only a few alkane-hydroxylating

Figure 1



Representative reactions catalyzed by alkane hydroxylases. **(a)** Methane is oxidized to methanol, **(b)** butane to 1- or 2-butanol, **(c)** octane to 1-octanol, **(d)** octane to 2-octanol, **(e)** farnesol to 1-hydroxyfarnesol, **(f)** limonene to perillyl alcohol, **(g)** *N*-benzyl-pyrrolidine to *N*-benzyl 3-hydroxy-pyrrolidine, and **(h)** *N*-(2-hexylamino-4-phenylimidazole-1-yl)-acetamide to 1-acetylamino-4-phenylimidazol-2-yl-6-aminohexanol. The enzymes responsible are mentioned in the figure above the arrows: BMO, butane monooxygenase; MMO, methane monooxygenase. More extensive overviews on the substrate range of the discussed enzymes are available elsewhere [13,42,56].

P450 enzymes have been identified and characterized thus far. These belong to both the class I P450s (cytoplasmic three-component systems comprising cytochrome P450, ferredoxin and ferredoxin reductase subunits; Figure 2a) and the class II P450s (microsomal two-component systems comprising a membrane-bound cytochrome P450 and a reductase).

Class I P450 enzymes

Screening of a collection of C_5–C_{10} alkane-degrading strains for the regio- and stereoselective hydroxylation of *N*-benzylpyrrolidine showed that 25–30% of the strains oxidized this substrate to *N*-benzyl-3-hydroxypyrrolidine with varying enantiomeric excess (Figure 1g). The most active and stereospecific strain in this study was *Sphingomonas* sp. HXN-200, isolated from a trickling-bed bioreactor. Subsequently, this strain was shown to hydroxylate many 4-, 5- and 6-ring alicyclic compounds with rates of up to 10–20 μmol min^{-1} g^{-1} cell dry weight [11–13]. Another strain from the same collection, *Mycobacterium* sp. HXN-1500, converted limonene to perillyl alcohol (Figure 1f), a compound that was in clinical trials for the treatment of several types of cancer (inconclusive results). In both cases, cytochrome P450 enzymes of the CYP153 family were shown to be responsible for the hydroxylation reactions [14] (JB Van Beilen, unpublished). The first member of this class I family was cloned from *Acinetobacter calcoaceticus* EB104 and characterized as a likely alkane hydroxylase by Maier *et al.* [15]. PCR screening and sequence databases searches subsequently revealed other strains (35 at present count) that possess CYP153 homologs, several of which could be functionally

Table 1

Enzymes that are able to oxidize alkanes.

Enzyme	Composition and cofactors	Examples of host organisms	Substrate range	Ref.
Class I P450 (CYP153)	P450 oxygenase: P450 heme Ferredoxin: [2Fe–2S] Ferredoxin reductase: FAD, NADH	*Sphingomonas* sp. HXN-200, *Mycobacterium* sp. HXN-1500, *Acinetobacter* sp. EB104	C_4–C_{16}	[15]
Class II P450 (CYP52)	Microsomal oxygenase: P450 heme Reductase: FAD, FMN, NADPH	*Candida maltosa, Candida tropicalis, Yarrowia lipolytica*	C_{10}–C_{16}	[19]
Class II P450 (CYP2E, CYP4B)	Microsomal oxygenase: P450 heme Reductase: FAD, FMN, NADPH	Humans and rabbits	C_6–C_{10}	[20,22,23]
Integral membrane alkane hydroxylase	Membrane hydroxylase: dinuclear iron Rubredoxin: iron Rubredoxin reductase: FAD, NADH	*Acinetobacter, Alcanivorax, Burkholderia, Mycobacterium, Pseudomonas, Rhodococcus*	C_5–C_{16}	[32]
Soluble methane monooxygenase	$\alpha_2\beta_2\gamma_2$ structure Hydroxylase: dinuclear iron Reductase: [2Fe–2S], FAD, NADH Regulatory subunit	*Methylisinus trichosporium* OB3b, *Methylococcus capsulatus* (Bath)	C_1–C_{10}	[42]
Particulate methane monooxygenase	Putative $\alpha_2\beta_2\gamma_2$ structure	All known methanotrophs	C_1–C_5	[45•]
Propane monooxygenase	Putative $\alpha_2\beta_2\gamma_2$ structure Reductase: NADH Regulatory subunit	*Gordonia* sp. TY-5	C_3 and C_{13}–C_{22}	[50]
Butane monooxygenase	$\alpha_2\beta_2\gamma_2$ structure Hydroxylase: dinuclear iron Reductase: [2Fe–2S], FAD, NADH Regulatory subunit	*Pseudomonas butanovora* ATCC 43655	C_2–C_8	[51]
Engineered P450cam	P450 oxygenase: P450 heme Putidaredoxin: [2Fe–2S] Putidaredoxin reductase: FAD, NADH	*Pseudomonas putida* ATCC 29607	C_3–C_{10}	[17••]
Engineered P450BM-3	Single polypeptide: FAD, FMN, NADPH	*Bacillus megaterium* ATCC 14581	C_3–C_8	[24••]

Table adapted from [18].

expressed in *P. putida*. The available data suggest that most, if not all, CYP153 enzymes oxidize C_5–C_{12} *n*-alkanes (JB Van Beilen, unpublished; Figure 1c).

One of the 'work horses' in cytochrome P450 enzyme research, CYP101 (better known as P450cam, a camphor oxidizing enzyme), has recently been engineered to hydroxylate alkanes. Rational design to reduce the volume of the substrate-binding pocket improved the enzyme–substrate fit such that one of the mutant enzymes had a 1900-fold improved activity towards the oxidation of butane to 2-butanol (turnover approximately 1000 min^{-1}) [16,17••], while the activity towards propane increased to a turnover frequency of 170 min^{-1} [17••].

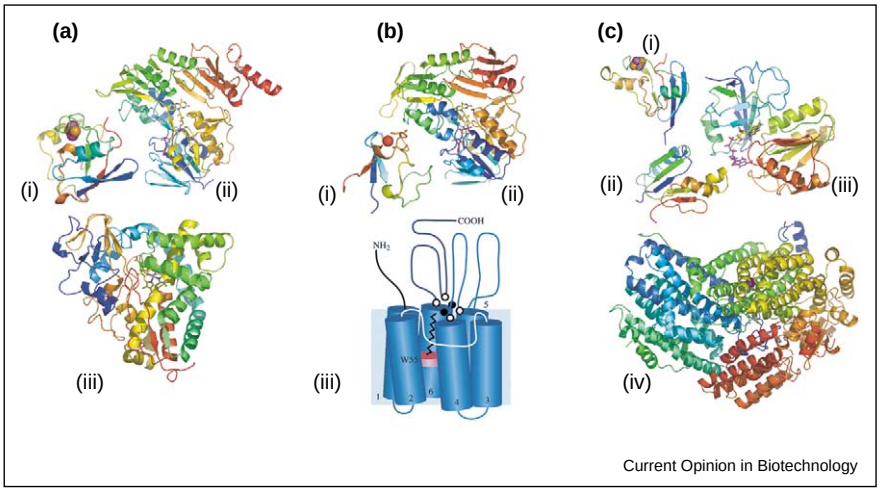
Class II P450 enzymes

Several microsomal class II cytochrome P450s are involved in the metabolism of alkanes or act on alkanes. Most of these belong to the CYP52 family that are found, usually in multiple copies, in various yeast strains [18]. As a recent example, ten CYP52 genes were cloned from the yeast strain *Candida tropicalis* ATCC 20336, which oxidizes *n*-alkanes at the α- and ω-positions to yield fatty acids and dicarboxylic acids [19].

In mammals, CYP2E1 is involved in the metabolism of endogenous compounds and xenobiotics [20] and is the key enzyme in the microsomal pathway for ethanol oxidation. Its substrate range includes hexane, farnesol (Figure 1e) and ethylbenzene, which are hydroxylated at subterminal positions [21,22]. The CYP4B P450 enzymes hydroxylate several structurally diverse compounds, including linear and branched alkanes, in this case predominantly at terminal methyl groups [23].

P450BM-3 is a bacterial (soluble) class II P450 that was cloned from *B. megaterium*. It is the most active of all P450 enzymes, probably because the hydroxylating- and electron-transfer subunits are joined in a single polypeptide. This means that the subunit docking step, which is possibly rate-limiting in catalysis by P450s [7], is eliminated. To change the specificity of P450BM-3 from fatty acids (substrates of the wild-type enzyme) to short-chain alkanes, Arnold and coworkers subjected P450BM-3 to several rounds of directed evolution. One of the mutants, 139-3, which had 11 amino acid substitutions, hydroxylated octane and other alkanes at the 2-, 3- and 4-positions with initial rates of up to 4000 min^{-1} [24••]. Alkenes were converted to epoxides with rates up to 385 min^{-1} [25,26].

Figure 2



Structural representations of the alkane-hydroxylating enzyme systems discussed. **(a)** Soluble class I P450 systems: i) electron shuttling HXN-1500 ferredoxin modeled on putidaredoxin; ii) model of HXN-1500 ferredoxin reductase using putidaredoxin reductase as the template; iii) model of heme-containing oxygen-activating CYP153A6 enzyme using the structure of P450cam as a template. **(b)** Integral membrane non-heme di-iron alkane hydroxylase systems: i) crystal structure of the C-terminal domain of rubredoxin; ii) rubredoxin reductase modeled on the structure of putidaredoxin reductase; and iii) a model for the membrane-bound oxygenase AlkB, the W55 residue that blocks the active site for longer alkanes is indicated (adapted from [39••] with permission). **(c)** Soluble methane monooxygenase systems: i) the N-terminal FeS cluster part of methane monooxygenase reductase (MMOR); ii) crystal structure of the regulatory protein B subunit; iii) crystal structure of the C-terminal domain of MMOR; iv) the crystal structure of the di-iron-containing catalytic subunit of sMMO. Protein alignments were performed with ClustalW. After manual alignment in Deep View/Swiss PdBViewer, models were obtained with Swiss-Model and figures were generated using the program Pymol.

Subsequent rounds of error-prone PCR and DNA shuffling yielded enzymes with a higher activity towards shorter alkanes, such as propane. By rational design, mutant 9-10A-A328V was generated, which primarily formed 2-alkanols (Figure 1d) but also small amounts of 1-alkanols from longer alkanes [27••]. This approach demonstrated the strength of evolutionary design to develop a highly active catalyst for future applications in (sub-)terminal alkane hydroxylation. Two homologs of P450BM-3 (CYP102A2 and CYP102A3) were recently cloned [28] and a double-mutant was engineered that also showed hydroxylating activity towards medium-chain alkanes and aromatic compounds [29].

Integral membrane non-heme iron alkane hydroxylases

Many alkane hydroxylases are related to the integral membrane non-heme iron protein AlkB, first discovered in a hexane-degrading fluorescent pseudomonad now known as *P. putida* GPo1 [30,31] (Figure 2b). To supply electrons to the integral membrane monooxygenase (AlkB), the enzyme requires one or two rubredoxins and the electron-providing NADH-dependent flavoprotein rubredoxin reductase. PCR with highly degenerate primers was used to show that genes encoding AlkB homologs are present in many alkane-degrading α-, β- and γ-Proteobacteria and high G+C content Gram-positive bacteria (Actinobacteria). These strains included *M. tuberculosis*, *Prauserella rugosa*, *Rhodococcus erythropolis*, *Burkholderia cepacia*, *Pseudomonas aeruginosa*, several *Acinetobacter* sp. and *Alcanivorax borkumensis*. Many of the cloned AlkB homologs were subsequently shown to be functional by heterologous expression [32–35]. Similar to the CYP52 enzymes found in yeasts, many bacterial strains contain multiple — quite divergent — integral membrane AlkBs [31,33,36]. There are a number of possible explanations for this apparent redundancy. Firstly, the enzymes have different substrate ranges: some oxidize alkanes from C_5 to C_{12}, whereas others oxidize C_{10}–C_{16} *n*-alkanes [31,37] (Figure 1c). Different AlkBs might also be active during different growth phases [38]. Another explanation is that AlkBs could have different affinity constants for different alkanes or some AlkBs might preferentially oxidize non-linear, branched, cyclic aliphatic or aromatic compounds. Lastly, it is possible that some *alkB* genes could be pseudogenes. Some of these issues are still waiting to be explored, because effective *in vitro* assays are only available for short- and medium-chain length alkane hydroxylases and because the higher molecular weight hydrocarbon substrates are often insoluble. In addition, integral membrane AlkBs are not very stable *in vitro*. However, a recent protein engineering and selection experiment study provides insights into AlkB substrate specificity: as a tryptophan residue located in the middle of one of six transmembrane helices limits the length of the alkane substrates [39••] (W55 in Figure 2b). Moreover, a conserved eight-histidine motif indicates that AlkBs are part of a larger family, which includes desaturases, epoxidases,

decarbonylases and methyl oxidases [40], providing more insight into the structure–function relationship of this class of enzymes.

Methane, propane and butane monooxygenases

Two types of enzyme systems are known to oxidize methane (Figure 1a): the thoroughly characterized, but relatively rare, soluble di-iron methane monooxygenase (sMMO; Figure 2c) [41•,42] and the poorly understood but ubiquitous copper-containing membrane-bound particulate methane monooxygenase (pMMO) [43,44]. Recent reviews give excellent overviews on the occurrence, structure and mechanism of the two different enzyme systems [41•,45•]. Although the heterologous expression of all three components of the sMMO system has been achieved [46,47], this has not yet been possible for the highly unstable pMMO complex. Besides the activation of methane, sMMO is able to oxidize saturated and unsaturated alkanes and halogenated, aromatic and heterocyclic compounds [42]. By contrast, pMMO has a much narrower substrate range, which appears to be restricted to alkanes and alkenes with lengths up to C_5.

Biotechnological applications of the MMOs are restricted to the biodegradation of chlorinated aliphatics and other compounds. The application of these enzymes for synthetic purposes is limited owing to the low regio- and stereoselectivity of these enzymes. A potentially huge market would be opened up if the conversion of methane and ethane to methanol and ethanol, respectively, became feasible (a holy grail of the petrochemical industry). By inhibiting methanol dehydrogenase in *Methylosinus trichosporium* OB3b with sodium chloride, methanol production from methane was enhanced and a methanol concentration of 7 mM was attained in a 36 h batch reaction [48]. In another potential application of MMO, Xin *et al*. [49] immobilized a mixture of *Methylomonas* sp., *Methylococcus capsulatus* and *Methylosinus trichosporium* cells on diatomite particles for the continuous production of epoxypropane. This biofilm reactor was run for 53 days without loss of activity.

Other gaseous alkanes are metabolized by strains expressing propane or butane monooxygenases that are related to pMMO or sMMO. *Gordonia* sp. TY-5 is able to use propane as the sole carbon source, but cannot use any other gaseous alkane. A complete operon encoding a putative di-iron-containing multicomponent monooxygenase — including large and small hydroxylase subunits, an NADH-dependent reductase and a regulatory protein — was cloned and sequenced. Deletion of one of the subunits abolished the ability to grow on propane, confirming its role in propane oxidation [50]. The hydroxylase subunits of propane monooxygenase show relatively high sequence similarity with butane monooxygenase isolated from *Pseudomonas butanovora*, which oxidizes butane to 1-butanol (Figure 1b). This butane monooxygenase was recently cloned and turned out to be quite similar to sMMO: the hydroxylase subunits α and β and the regulatory protein B show more than 60%, 50% and 40% amino acid sequence identity, respectively, to the corresponding subunits of sMMOs [51].

Evidence for other alkane-oxidizing enzyme systems

A crucial step in the synthesis of imidazol-2-yl amino acids was accomplished with alkane-grown bacteria [52]. The biotransformation involved oxidation of the alkyl sidechain of *N*-(2-hexylamino-4-phenylimidazole-1-yl)-acetamide to produce 1-acetylamino-4-phenylimidazol-2-yl-6-aminohexanol and the butanoic acid derivative (Figure 1h). In this study, 15 out of 61 strains were found to have the desired activity, however, the identity of the enzymes involved is not known.

Outlook

Metagenomic profiling now provides access to a multitude of genes belonging to known alkane hydroxylase families that can be screened for the desired stereo-, regio- and enantioselectivity. Completely novel alkane hydroxylase systems might be identified by focussing research on alkane-degrading strains that do not contain known systems. The structural characterization and engineering of these enzymes will provide more insight into enzymatic alkane activation and will support the development of alkane oxygenases for industrial applications.

The 'ideal' alkane hydroxylase for industrial applications is soluble, single-component, stable, has no cofactor requirements, and is highly stereo-, regio- and enantioselective. One particular enzyme, P450BM-3, is already approaching these goals. This soluble, single-component enzyme has been engineered for higher temperature stability [53] and the ability to function as a H_2O_2-driven hydroxylase, in theory eliminating the requirement for NAD(P)H. The instability of this enzyme in the presence of H_2O_2 [54] might be remedied by investigating recently discovered natural P450 'peroxygenases' [55], while further engineering could transfer the excellent regio- and stereoselectivity of the CYP153 enzymes to P450BM-3. The resulting enzyme would meet all of the above-mentioned criteria.

Conclusions

Studies on alkane hydroxylases are no longer limited to a very small number of well-characterized enzyme systems, as shown by the discovery of a new and promising P450 alkane hydroxylase family and the conversion of P450cam and P450BM-3 into alkane hydroxylases by protein engineering. At the same time, more structural information has become available for the integral membrane alkane hydroxylase, sMMO and, very recently, pMMO enzyme systems. The combination of new sequence diversity,

new structures, heterologous expression, and directed evolution, should speed up the development of these alkane hydroxylases for industrial biocatalysis.

Update

Lieberman and Rozenzweig recently elucidated the first crystal structure of pMMO. The structure showed a trimer with a mononuclear and dinuclear copper center and a zinc site, providing a solid foundation for further studies on this intriguing enzyme [57••].

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Hayaishi O: *Molecular Mechanisms of Oxygen Activation*. Edited by Hayaishi O: Academic Press, New York; 1974.

2. Ayala M, Torres E: **Enzymatic activation of alkanes: constraints and prospective**. *Appl Catal A-Gen* 2004, **272**:1-13.

3. Straathof AJJ, Panke S, Schmid A: **The production of fine chemicals by biotransformations**. *Curr Opin Biotechnol* 2002, **13**:548-556.

4. Schmid A, Hollmann F, Park JB, Buhler B: **The use of enzymes in the chemical industry in Europe**. *Curr Opin Biotechnol* 2002, **13**:359-366.

5. van Beilen JB, Duetz WA, Schmid A, Witholt B: **Practical issues in the application of oxygenases**. *Trends Biotechnol* 2003, **21**:170-177.

6. •• Buhler B, Schmid A: **Process implementation aspects for biocatalytic hydrocarbon oxyfunctionalization**. *J Biotechnol* 2004, **113**:183-210.
An extensive discussion of the application of oxygenases with many examples of the associated problems and possible solutions.

7. Duetz WA, van Beilen JB, Witholt B: **Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis**. *Curr Opin Biotechnol* 2001, **12**:419-425.

8. •• Hollmann F, Schmid A: **Electrochemical regeneration of oxidoreductases for cell-free biocatalytic redox reactions**. *Biocat Biotrans* 2004, **22**:63-88.
An excellent review of the integration of biocatalysis and inorganic chemistry; the use of metal complexes to directly regenerate cofactors and coenzymes in a cell-free environment is discussed.

9. •• Udit AK, Hill MG, Bittner VG, Arnold FH, Gray HB: **Reduction of dioxygen catalyzed by pyrene-wired heme domain cytochrome P450BM-3 electrodes**. *J Am Chem Soc* 2004, **126**:10218-10219.
The hydroxylase domain of P450BM-3 was covalently coupled to an electrode, providing electrons directly to the heme without strong uncoupling or enzyme inactivation by surface adsorption.

10. Lamb DC, Skaug T, Song HL, Jackson CJ, Podust LM, Waterman MR, Kell DB, Kelly DE, Kelly SL: **The cytochrome P450 complement (CYPome) of *Streptomyces coelicolor* A3(2)**. *J Biol Chem* 2002, **277**:24000-24005.

11. Chang D, Feiten HJ, Witholt B, Li Z: **Regio- and stereoselective hydroxylation of N-substituted piperidin-2-ones with *Sphingomonas* sp. HXN-200**. *Tetrahedron Asymm* 2002, **13**:2141-2147.

12. Chang D, Feiten H-J, Engesser K-H, van Beilen JB, Witholt B, Li Z: **Practical syntheses of *N*-substituted 3-hydroxyazetidines and 4-hydroxypiperidines by hydroxylation with *Sphingomonas* sp. HXN-200**. *Org Lett* 2002, **4**:1859-1862.

13. Li Z, Chang D: **Recent advances in regio- and stereoselective biohydroxylation of non-activated carbon atoms**. *Curr Org Chem* 2004, **8**:1647-1658.

14. van Beilen JB, Lüscher D, Holtacker R, Witholt B, Duetz WA: **Biocatalytic production of perillyl alcohol from limonene using a novel *Mycobacterium* cytochrome P450 alkane hydroxylase expressed in *P. putida***. *Appl Environ Microbiol* 2005, **71**:1737-1744.

15. Maier T, Foerster H-H, Asperger O, Hahn U: **Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp EB104**. *Biochem Biophys Res Commun* 2001, **286**:652-658.

16. Bell SG, Stevenson JA, Boyd HD, Campbell S, Riddle AD, Orton EL, Wong LL: **Butane and propane oxidation by engineered cytochrome P450(cam)**. *Chem Comm* 2002: 490-491.

17. •• Bell SG, Orton E, Boyd H, Stevenson J-A, Riddle A, Campbell S, Wong L-L: **Engineering cytochrome P450cam into an alkane hydroxylase**. *Dalton Trans* 2003:2133-2140.
This paper shows the strength of rational engineering to improve enzyme properties. Four mutations in the P450cam active site increased the rate of butane hydroxylation to a level comparable with camphor hydroxylation by the wild-type enzyme.

18. van Beilen JB, Li Z, Duetz WA, Smits THM, Witholt B: **Diversity of alkane hydroxylase systems in the environment**. *Oil Gas Sci Technol* 2003, **58**:427-440.

19. Craft DL, Madduri KM, Eshoo M, Wilson CR: **Identification and characterization of the CYP52 family of *Candida tropicalis* ATCC 20336, important for the conversion of fatty acids and alkanes to α,ω-dicarboxylic acids**. *Appl Environ Microbiol* 2003, **69**:5983-5991.

20. Bolt HM, Roos PH, Thier R: **The cytochrome P-450 isoenzyme CYP2E1 in the biological processing of industrial chemicals: consequences for occupational and environmental medicine**. *Int Arch Occup Environ Health* 2003, **76**:174-185.

21. DeBaber AE: **ω-Hydroxylation of farnesol by mammalian cytochromes P 450**. *Biochim Biophys Acta* 2004, **1682**:18-27.

22. Iba MM, Fung J, Gonzalez FJ: **Functional Cyp2E1 is required for substantial *in vivo* formation of 2,5-hexanedione from *n*-hexane in the mouse**. *Arch Toxicol* 2000, **74**:582-586.

23. Fisher MB, Zheng Y-M, Rettie AE: **Positional specificity of rabbit CYP4B1 for ω-hydroxylation of short-medium chain fatty acids and hydrocarbons**. *Biochem Biophys Res Commun* 1998, **248**:352-355.

24. •• Glieder A, Farinas ET, Arnold FH: **Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase**. *Nat Biotechnol* 2002, **20**:1135-1139.
Directed evolution yielded a mutant of P450BM-3 with the highest activity of all hydroxylases towards hexane.

25. Farinas ET, Alcalde M, Arnold F: **Alkene epoxidation catalyzed by cytochrome P450BM-3 139-3**. *Tetrahedron* 2004, **60**:525-528.

26. Alcalde M, Farinas ET, Arnold FH: **Colorimetric high-throughput assay for alkene epoxidation catalyzed by cytochrome P450BM-3 variant 139-3**. *J Biomol Screen* 2004, **9**:141-146.

27. •• Peters MW, Meinhold P, Glieder A, Arnold FH: **Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3**. *J Am Chem Soc* 2003, **125**:13442-13450.
This paper demonstrates the strength of the combination of directed evolution and rational design to generate a mutant of P450BM-3 with improved regioselectivity.

28. Gustafsson MCU, Roitel O, Marshall KR, Noble MA, Chapman SK, Pessegueiro A, Fulco AJ, Cheesman MR, von Wachenfeldt C, Munro AW: **Expression, purification, and characterization of *Bacillus subtilis* cytochromes P450 CYP102A2 and CYP102A3: flavocytochrome homologues of P450BM3 from *Bacillus megaterium***. *Biochemistry* 2004, **43**:5474-5487.

29. Lentz O, Urlacher V, Schmid RD: **Substrate specificity of native and mutated cytochrome P450 (CYP102A3) from *Bacillus subtilis***. *J Biotechnol* 2004, **108**:41-49.

30. van Beilen JB, Panke S, Lucchini S, Franchini AG, Röthlisberger M, Witholt B: **Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk*-genes**. *Microbiology* 2001, **147**:1621-1630.

31. Smits THM, Witholt B, van Beilen JB: **Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa***. *Antonie Van Leeuwenhoek* 2003, **84**:193-200.

32. Smits THM, Balada SB, Witholt B, van Beilen JB: **Functional analysis of alkane hydroxylases from Gram-negative and Gram-positive bacteria**. *J Bacteriol* 2002, **184**:1733-1742.

33. Whyte LG, Smits THM, Labbe D, Witholt B, Greer CW, Van Beilen JB: **Gene cloning and characterization of multiple alkane hydroxylase systems in Rhodococcus strains Q15 and NRRL B-16531**. *Appl Environ Microbiol* 2002, **68**:5933-5942.

34. van Beilen JB, Marin MM, Smits THM, Roethlisberger M, Franchini AG, Witholt B, Rojo F: **Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis***. *Environ Microbiol* 2004, **6**:264-273.

35. Fujii T, Narikawa T, Takeda K, Kato J: **Biotransformation of various alkanes using *Escherichia coli* expressing an alkane hydroxylase system from *Gordonia* sp TF6**. *Biosci Biotechnol Biochem* 2004, **68**:2171-2177.

36. van Beilen JB, Smits THM, Whyte LG, Schorcht S, Röthlisberger M, Plaggemeier T, Engesser K-H, Witholt B: **Alkane hydroxylase homologues in Gram-positive strains**. *Environ Microbiol* 2002, **4**:676-682.

37. van Beilen JB, Witholt B: **Alkane degradation by pseudomonads**. In *The Pseudomonads* vol 3. Edited by Ramos JL: Kluwer Academic Publishers; 2004.

38. Marin M, Yuste L, Rojo F: **Differential expression of the components of the two alkane hydroxylases from *Pseudomonas aeruginosa***. *J Bacteriol* 2003, **185**:3232-3237.

39. •• van Beilen JB, Smits THM, Balada SB, Roos F, Brunner T, Witholt B: **Identification of an amino acid position that determines the substrate range of integral-membrane alkane hydroxylases**. *J Bacteriol* 2005, **187**:85-91.

An elegant model is proposed that explains how members of the family of integral membrane alkane hydroxylases can distinguish the lengths of their alkane substrates.

40. Shanklin J, Whittle E: **Evidence linking the *Pseudomonas oleovorans* alkane omega-hydroxylase, an integral membrane diiron enzyme, and the fatty acid desaturase family**. *FEBS Lett* 2003, **545**:188-192.

41. • Kopp DA, Lippard SJ: **Soluble methane monooxygenase: activation of dioxygen and methane**. *Curr Opin Chem Biol* 2002, **6**:568-576.

A review covering dioxygen activation by sMMO. A detailed discussion including theoretical investigations, kinetic isotope studies, and the use of probe substrates, summarizes current knowledge on the mechanism of sMMO.

42. Baik MH, Newcomb M, Friesner RA, Lippard SJ: **Mechanistic studies on the hydroxylation of methane by methane monooxygenase**. *Chem Rev* 2003, **103**:2385-2419.

43. Lieberman RL, Shrestha DB, Doan PE, Hoffman BM, Stemmler TL, Rosenzweig AC: **Purified particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a dimer with both mononuclear copper and a copper-containing cluster**. *Proc Natl Acad Sci USA* 2003, **100**:3820-3825.

44. Chan SI, Chen KH-C, Yu S-F, Chen C-L, Kuo S-J: **Toward delineating the structure and function of the particulate methane monooxygenase from methanotrophic bacteria**. *Biochemistry* 2004, **43**:4421-4430.

45. • Lieberman RL, Rosenzweig AC: **Biological methane oxidation: regulation, biochemistry, and active site structure of particulate methane monooxygenase**. *Crit Rev Biochem Mol Biol* 2004, **39**:147-164.

An extensive and critical review on the knowns and unknowns of pMMOs.

46. Smith TJ, Slade SE, Burton NP, Murrell JC, Dalton H: **Improved system for protein engineering of the hydroxylase component of soluble methane monooxygenase**. *Appl Environ Microbiol* 2002, **68**:5265-5273.

47. Blazyk JL, Lippard SJ: **Expression and characterization of ferredoxin and flavin adenine dinucleotide binding domains of the reductase component of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath)**. *Biochemistry* 2002, **41**:15780-15794.

48. Lee SG, Goo JH, Kim HG, Oh J-I, Kim YM, Kim SW: **Optimization of methanol biosynthesis from methane using *Methylosinus trichosporium* OB3b**. *Biotechnol Lett* 2004, **26**:947-950.

49. Xin J-Y, Cui J-R, Chen J-B, Li S-B, Xia C-G, Zhu L-M: **Continuous biocatalytic synthesis of epoxypropane using a biofilm reactor**. *Process Biochem* 2003, **38**:1739-1746.

50. Kotani T, Yamamoto T, Yurimoto H, Sakai Y, Kato N: **Propane monooxygenase and NAD(+)-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5**. *J Bacteriol* 2003, **185**:7120-7128.

51. Sluis MK, Sayavedra Soto LA, Arp DJ: **Molecular analysis of the soluble butane monooxygenase from '*Pseudomonas butanovora*'**. *Microbiology* 2002, **148**:3617-3629.

52. Mikolasch A, Hammer E, Schauer F: **Synthesis of imidazol-2-yl amino acids by using cells from alkane-oxidizing bacteria**. *Appl Environ Microbiol* 2003, **69**:1670-1679.

53. Salazar O, Cirino PC, Arnold FH: **Thermostabilization of a cytochrome P450 peroxygenase**. *ChemBioChem* 2003, **4**:891-893.

54. Cirino PC, Arnold FH: **A self-sufficient peroxide-driven hydroxylation biocatalyst**. *Angew Chem Int Ed Engl* 2003, **42**:3299-3301.

55. Matsunaga I, Yamada A, Lee DS, Obayashi E, Fujiwara N, Kobayashi K, Ogura H, Shiro Y: **Enzymatic reaction of hydrogen peroxide-dependent peroxygenase cytochrome P450s: kinetic deuterium isotope effects and analyses by resonance Raman spectroscopy**. *Biochemistry* 2002, **41**:1886-1892.

56. van Beilen JB, Witholt W: **Diversity, function, and biocatalytic applications of alkane oxygenases**. In *Petroleum Microbiology*. Edited by Magot M, Ollivier B: ASM Press; 2005: in press.

57. •• Lieberman LL, Rosenzweig AC: **Crystal structure of a membrane-bound metalloenzyme that catalyzes the biological oxidation of methane**. *Nature* 2005, **434**:177-182.

A crystal structure of pMMO refined to 2.8 Å reveals a $\alpha_3\beta_3\gamma_3$ cylindrical trimer with three metal centers per protomer: a mononuclear and dinuclear coppersite in the soluble region with preferentially histidines as active site residues, and a zincsite in the membrane-spanning region with two histidines, one aspartate, and one glutamate residue.