



An Overview of the Electron-Transfer Proteins That Activate Alkane Monooxygenase (AlkB)

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Alkane-oxidizing enzymes play an important role in the global carbon cycle. Alkane monooxygenase (AlkB) oxidizes most of the medium-chain length alkanes in the environment. The first AlkB identified was from *P. putida* GPo1 (initially known as *P. oleovorans*) in the early 1970s, and it continues to be the family member about which the most is known. This AlkB is found as part of the OCT operon, in which all of the key proteins required for growth on alkanes are present. The AlkB catalytic cycle requires that the diiron active site be reduced. In *P. putida* GPo1, electrons originate from NADH and arrive at AlkB via the intermediacy of a flavin reductase and an iron-sulfur protein (a rubredoxin). In this Mini Review, we will review what is known about the canonical arrangement of electron-transfer proteins that activate AlkB and, more importantly, point to several other arrangements that are possible. These other arrangements include the presence of a simpler rubredoxin than what is found in the canonical arrangement, as well as two other classes of AlKBs with fused electron-transfer partners. In one class, a rubredoxin is fused to the hydroxylase and in another less well-explored class, a ferredoxin reductase and a ferredoxin are fused to the hydroxylase. We review what is known about the biochemistry of these electron-transfer proteins, speculate on the biological significance of this diversity, and point to key questions for future research.

Keywords: alkane monooxygenase, AlkB, rubredoxin, fusion-protein, hydrocarbon oxidation

INTRODUCTION

Alkane monooxygenase (AlkB) is a non-heme diiron integral membrane protein widely found in bacteria capable of growing on medium- to long-chain alkanes as their sole source of carbon and energy (alkanotrophs). Its ability to selectively oxidize alkanes has led to it being the target of numerous biotechnology explorations (McKenna and Coon, 1970; Bosetti et al., 1992; Li et al., 1999; Staijen et al., 2000; van Beilen et al., 2001, 2003; van Beilen and Funhoff, 2005, 2007; Austin and Groves, 2011; Gross et al., 2013; Schrewe et al., 2014; Ladkau et al., 2016; Hoschek et al., 2017; van Nuland et al., 2017). While the three-dimensional structure of the enzyme remains elusive (Xie et al., 2011; Alonso and Roujeinikova, 2012; Alonso et al., 2014), many details of the structure and reaction mechanism have been

determined (May and Abbott, 1972; May et al., 1977; May and Katopodis, 1986; van Beilen et al., 1992, 2005; Shanklin et al., 1997; Austin et al., 2000, 2008; Bertrand et al., 2005; Rozhkova-Novosad et al., 2007; Xie et al., 2011; Alonso and Roujeinikova, 2012; Cooper et al., 2012; Alonso et al., 2014). Common to many monooxygenases, the protein starts the catalytic cycle in the diferric state and requires reduction by two electrons to bind and activate oxygen. This Mini Review will focus on the proteins that transfer electrons from the biological electron source [NAD(P)H] to AlkB. It will begin by describing the canonical electron-transfer partners found as part of the OCT operon, which contains all the proteins required to transform alkanes to fatty acids (van Beilen et al., 1994). Fatty acids can be used for metabolism by many bacteria (Jimenez-Diaz et al., 2017). It will then point out the relatively rare occurrence of the OCT operon in sequenced microbial genomes that contain AlkB. Next, it will explore other arrangements of electron-transfer proteins, including two different arrangements in which one or both electron-transfer proteins are found as part of a gene fusion with the hydroxylase. Common themes with other similar proteins will be examined. Finally, the Mini Review will end with a discussion of the possible functional relevance of this diversity of electron-transfer protein arrangements and point to critical unanswered questions for future work.

THE OCT PLASMID, WHICH CONTAINS THE CANONICAL ELECTRON-TRANSFER PARTNERS ALKG AND ALKT

P. putida GPo1 (van Beilen et al., 1994) is the organism whose genetics and biochemistry of liquid alkane metabolism are best characterized. *P. putida* GPo1 harbors an OCT plasmid (van Beilen et al., 1994). This plasmid contains the alkane hydroxylase component, as well as all of the other proteins required to convert alkanes to carboxylic acids (van Beilen et al., 1994). The alkane hydroxylase component consists of an integral membrane protein (AlkB), a rubredoxin (AlkG), and a reductase (AlkT) (van Beilen et al., 1994). The OCT plasmid is a member of the IncP-2 family of plasmids (van Beilen et al., 1994). It can be transmitted to other *Pseudomonads*, although with low frequency. It also encodes proteins for mercury resistance and D-lysine catabolism (van Beilen et al., 1994). In addition to the alkane hydroxylase components, the alkane-oxidizing component of the plasmid, known as *alkBFGHJKL*, encodes another rubredoxin AlkF, which appears to be a duplication of the inactive N-terminal region of AlkG (Kok et al., 1989). No function for AlkF has been found (Kok et al., 1989). AlkJ is a membrane-bound alcohol dehydrogenase with high homology to choline dehydrogenase, another flavin protein that has the potential to direct electrons to the respiratory chain of *Pseudomonas* (Kirmair et al., 2014). AlkH is an aldehyde dehydrogenase, which oxidizes the aldehyde produced by AlkJ to a carboxylic acid (Kok et al., 1989).

AlkK is an acyl-CoA synthetase, which activates fatty acids so they can enter the β oxidation cycle (van Beilen et al., 2001; Smits et al., 2002). AlkL is an outer membrane transporter (Julsing et al., 2012; Schrewe et al., 2014). In a slightly different region of the plasmid, two additional genes associated with alkane utilization have been identified (*alkS* and *alkT*). AlkS is a DNA regulator that controls AlkB expression (van Beilen et al., 1994, 2001; Procopio et al., 2013). AlkT is a rubredoxin reductase (Eggink et al., 1990). The easy-to-recognize presence of an entire suite of genes that code for the proteins necessary to convert octane to energy and carbon-building blocks has cemented the notion that AlkB exists solely for this purpose.

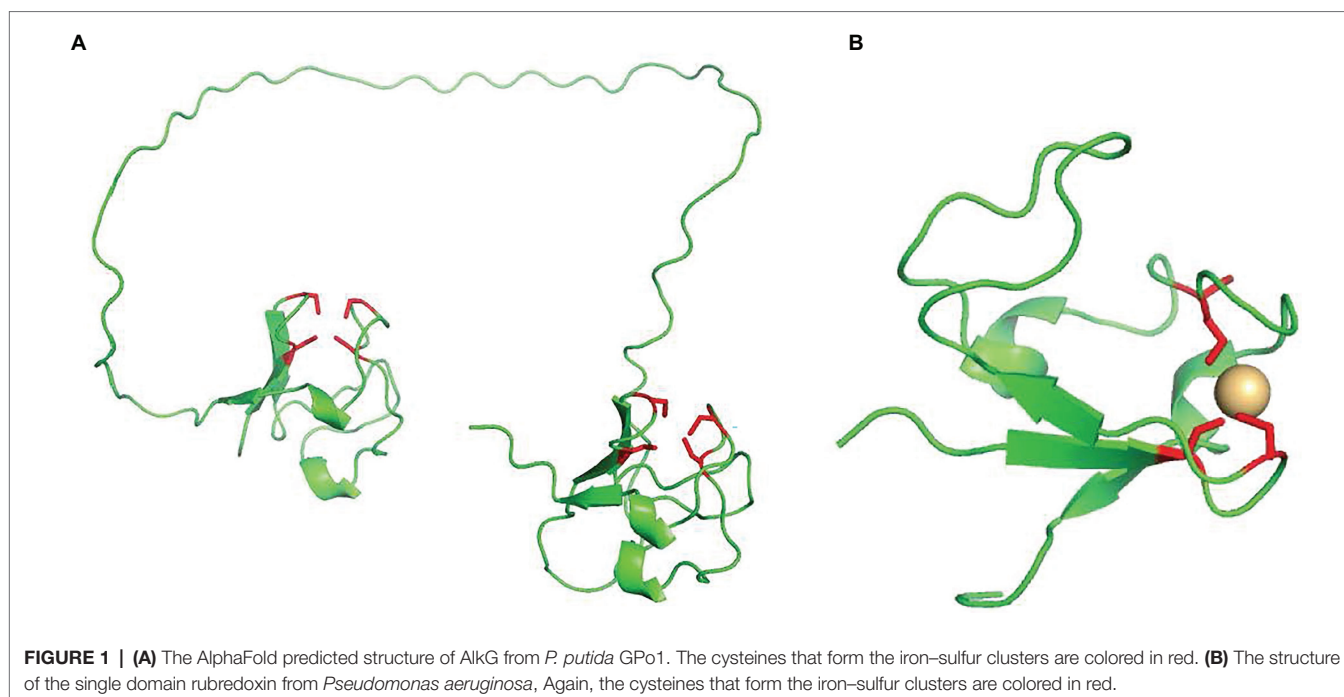
Like soluble methane monooxygenase (sMMO; Wang et al., 2014) and cytochrome P450 (CYP; Groves, 1985), AlkB requires two electrons to bind and activate oxygen. NADH is the ultimate electron source for AlkB (van Beilen et al., 2002). NADH reduces the flavin-based rubredoxin reductase AlkT, which in turn reduces AlkG.

AlkG is an unusual rubredoxin, containing two domains, each with an Fe-S cluster (van Beilen et al., 2002). **Figure 1A** provides an AlphaFold prediction of the structure, which demonstrates the bi-lobal structure (Jumper et al., 2021). The iron ions in the N-terminus (AlkG1) are loosely bound and the rubredoxin in this domain is not essential for activity (van Beilen et al., 2002). The C-terminal rubredoxin AlkG2 is responsible for the redox activity. The two rubredoxins can be distinguished at the sequence level by two CXXCG regions in AlkG2 and CXXCG and CXXCX clusters in AlkG1 (van Beilen et al., 2002).

ALTERNATIVES TO THE OCT PLASMID AND SPECIFICALLY ALTERNATIVES TO ALKG AND ALKT

The structure of the OCT plasmid is so logical that it often dominates the way people think about alkane oxidation in bacteria in spite of the fact that other arrangements of genes are very common (Smits et al., 2002, 2003). In fact in most alkane-oxidizing strains, the genes involved in alkane degradation are distributed over the genome (Smits et al., 2002).

While *P. putida* GPo1 contains this unusual two-domain AlkG, many other AlkB-containing strains have an isolated AlkG2-like rubredoxin, a structure of which is shown in **Figure 1B**. Organisms that do not have AlkG but have instead an isolated AlkG2-like rubredoxin, often also have a highly conserved AlkG1-like rubredoxin that is not required for activity (Kok et al., 1989; van Beilen et al., 2002). Both a crystal structure (Hagelueken et al., 2007) and an NMR structure (Perry et al., 2001) of a reductase/rubredoxin complex with a single domain rubredoxin exist and point to key amino acids that facilitate contact between the two. The importance of the charged residues on the rubredoxin suggests that oppositely charged residues on AlkB may be important in rubredoxin hydroxylase recognition.



AlkBs have been shown capable of using non-native rubredoxins, although with some limitations (Smits et al., 2002; van Beilen et al., 2002). AlkT can be replaced with other reductases, and even other sources of electrons (Eggink et al., 1987), both *in vivo* (Benson et al., 1977) and *in vitro* (Shanklin et al., 1997; Naing et al., 2013). The ability to use other electron-transfer proteins has simplified efforts to activate AlkB and raises the possibility that non-native electron-transfer partners can be optimized for biotechnology applications (Bernhardt and Urlacher, 2014; Li et al., 2020).

There are other AlkBs that differ even more dramatically in the organization of their electron-transfer partners. A class of AlkBs exists in which the rubredoxin is C-terminally fused to the hydroxylase. These rubredoxin-fused AlkBs appear in a number of different genera. All of these rubredoxins are single-domain rubredoxins and cluster into a third cluster that is distinct from AlkG2 and AlkG1 (Nie et al., 2011).

It is not yet clear what the functional effect, if any, the fused rubredoxin has on the overall alkane hydroxylase activity. The presence of a rubredoxin-fused AlkB enabled a *Dietzia* strain to grow on very long-chain alkanes, considerably longer than in its absence (Nie et al., 2011). However recent *in vitro* work in our lab comparing the activity of the fused rubredoxin AlkB to a rubredoxin-deletion mutant showed no difference in substrate preference (Williams et al., 2021). Differences in what these two studies measured—in one case growth of an organism and, in the other case, the ability of an enzyme to hydroxylate alkanes of a given chain length *in vitro*—make clear the gaps that remain in linking our understanding of molecular-level processes to an understanding of how these processes are orchestrated at the cellular level, a point we will return to at the end of this Mini Review. Furthermore, the

in vivo study showed that rubredoxin-fused AlkBs facilitate growth on C32 (Nie et al., 2011), a length of carbon chain outside an easy analytical window for *in vitro* work (Smits et al., 2002). The rubredoxin-fused AlkB of *Prauserella rugosa* NRRL B-2295 could not complement the rubredoxin-deletion mutant, suggesting that this fused rubredoxin was functional ONLY when fused to the hydroxylase (Nie et al., 2011). In contrast, however, the rubredoxin-deletion mutant of AlkB from *D. cinnamomea* can accept electrons from an exogenous AlkG (the AlkG from *P. putida* GPo1, in an assay containing NADH, AlkT, and AlkG; Williams et al., 2021).

A bioinformatics analysis (Nie et al., 2014) suggested the existence of a three-domain AlkB in which a ferredoxin reductase and ferredoxin are N-terminally fused to a hydroxylase (ferr_ferrR_AlkB). We recently expressed the protein and showed it to be active (Williams et al., 2021). We also identified the genera in which this protein can be found (Williams et al., 2021). All current annotations in IMG and NCBI mis-identify the protein as either a fatty acid desaturase or a ferredoxin reductase, neglecting the clear presence of the hydroxylase domain.

COMMONALITIES WITH RELATED PROTEINS

The significance of the arrangement of electron-transfer partners for AlkB may be better appreciated by comparing them to other related proteins. AlkB is a member of the class III diiron proteins, which also includes the mammalian membrane-spanning fatty acid desaturases and fatty acid hydroxylases (Fox et al., 1994). We will examine the electron-transfer proteins

associated with these metalloenzymes as well as cytochromes P450 (CYPs), which perform similar chemistry.

Fatty Acid Desaturases

Some membrane-bound desaturases use a membrane-bound cytochrome b_5 , which receives electrons from NADH or NADPH through a NADH or NADPH cytochrome oxidoreductase. Some of these desaturases are fused to the cytochrome b_5 domain, either at the N- or C-terminal end or even as an internal fusion (Sperling and Heinz, 2001; Zäuner et al., 2012). In some fatty acid desaturases, the presence of the fused cytochrome b_5 domain has been shown to be essential for activity, even *in vivo* (Mitchell and Martin, 1995; Sayanova et al., 1999). No examples of ferredoxin-fused desaturases have been found (Sperling and Heinz, 2001). The most obvious benefit of fusing the electron-transfer partner directly to the enzyme is to improve the rate of electron transfer between two proteins, which is typically slow.

Fatty Acid Hydroxylases

A fatty acid hydroxylase found in yeast (Fah1p) is found fused to cytochrome b_5 , its electron-transfer partner (Mitchell and Martin, 1997). It is postulated to play a role in hydroxylating very long-chain (C20-C28) fatty acids (Mitchell and Martin, 1997). The mammalian fatty acid hydroxylases also have an N-terminal fused electron-transfer partner (Alderson et al., 2004), although in the crystal structure of this protein, that region was disordered (Zhu et al., 2015). An *in vivo* truncation of the N-terminal cytochrome b_5 was still functional (Zhu et al., 2015). In humans, mutations in the cytochrome b_5 domain caused a mild form of hereditary leukodystrophy, in contrast with humans who had mutations in the iron-binding ligands, who had a severe form of the disease (Hama, 2010). This result suggests that the fused electron-transfer partner plays an important, albeit not essential, role in fatty acid hydroxylation.

Cytochrome P450s (CYPs)

CYP fusion proteins with different architectures are common in bacteria (De Mot and Parret, 2002; Munro et al., 2007). Unlike AlkB, bacterial CYPs usually utilize ferredoxin and ferredoxin reductase for activity (Li et al., 2020). Three bacterial CYP153s (van Beilen et al., 2006), which are the CYPs that oxidize alkanes in the environment, have been shown in a bioinformatics analysis to have an N-terminal CYP domain with a ferredoxin reductase domain and a C-terminal ferredoxin domain (Nie et al., 2014). These genes were found in *G. polyisoprenivorans* VH2, *Gordonia araii* NBRC 100433, and *G. polyisoprenivorans* NBRC 16320. A BLAST search indicates numerous other CYPs with a similar three-domain structure in classes of bacteria known to metabolize alkanes.

CYPs fused to electron-transfer partners are characterized by their high activity, leading to the suggestion that gene fusion of electron-transfer partners increases catalytic activity and decreases uncoupling of reducing agents (Munro et al., 2007). In CYP, where transfer of two sequential electrons is required

to generate the catalytically competent reactive intermediate compound 1 (cpd1), it is thought that rapid electron transfer may help to ensure efficient cpd 1 formation (Munro et al., 2007; Li et al., 2020). Fusing the electron-transfer protein to CYP is also thought to aid transcriptional regulation (Munro et al., 2007). There is also evidence that redox partners can alter the profile of reaction products, perhaps by direct protein-protein interactions that influence the active site conformation (Li et al., 2020). Fusion proteins may lock in one particular activity, while having separate electron-transfer proteins may provide more versatility to CYPs *in vivo* (Li et al., 2020).

Intriguingly, however, the ability to deliver electrons rapidly may lead to an increase in the generation of overoxidation products. Fusion proteins have been shown to catalyze more overoxidation of products (Boddupalli et al., 1992; van Beilen et al., 2003).

DISCUSSION

One point that emerges from this effort to understand the functional role of AlkB's diverse electron-transfer partners is the disconnections that exist between the different methods of investigation. Some groups have studied the *in vivo* effects of various gene deletions. Other groups have studied what is biochemically feasible *in vitro* at various stages of protein purification. Alkane metabolism requires the transport of substrates from outside the organism to the active site, as well as the ability to use the product to extract energy and carbon. Therefore, studying growth on alkanes encapsulates multiple distinct biochemical processes. Biochemical studies can reveal what chemistry a protein is capable of, but as we and others have shown, the membrane milieu can change the chemistry of an enzyme such that experiments done on highly purified enzymes might not capture even the fundamental biochemistry *in vivo* (van Beilen et al., 1994; Ro et al., 2018).

In spite of more than 50 years of work on microbial alkane metabolism, many questions remain. Some of the questions are brought into sharper focus by the wealth of genomic and metagenomic data that exists. Below, we outline a few of the pressing questions and suggest approaches to answer them.

We remain puzzled by the functional role, if any, of the second rubredoxin in the canonical AlkG. We are also puzzled by the presence of AlkF and other AlkG1-like rubredoxins that occur frequently in the genome of alkane-oxidizing organisms. The highly conserved sequence suggests a functional role, although we and others have not been successful in endeavors to identify that role. Effector proteins have been shown to be important in other hydroxylases (Lountos et al., 2005; Mitić et al., 2008), where their effects are often subtle. More *in vitro* studies may shed light on conditions where AlkF impacts the chemistry of AlkB. Functional roles for AlkF might emerge from additional whole cells screens.

We see several possible roles for fusion of electron-transfer partners directly to AlkB. The most obvious role is that electron transfer rates in fusion proteins are much faster than in systems where the electron-transfer proteins need to find their redox partners in space, facilitating alkane oxidation. Fusion of the electron-transfer partners also simplifies regulation of alkane

oxidation as only one protein needs to be upregulated in response to the presence of alkanes in this system. Commonly, in systems where the redox partners are not fused, the electron-transfer proteins are differentially regulated (Hagelueken et al., 2007). They may occur on different parts of the chromosome and possibly serve as electron-transfer proteins for other enzymes. Another suggestion is that longer chain alkanes interact with more sites within AlkB, which disrupt rubredoxin binding, thereby necessitating the sort of pre-formed structures that could exist with a fusion protein (Lee et al., 1996; Nie et al., 2011). Intriguingly, prediction of the three-dimensional structure of ferr_ferrR_AlkB by AlphaFold (Jumper et al., 2021) shows all of the key redox active partners in close proximity, even in the absence of metal binding (which AlphaFold cannot, at this point, predict).

It is possible that the presence of electron-transfer proteins fused to the hydroxylase changes the chemistry of the enzyme. It is not yet clear whether AlkB substrates, once transformed to products, exit the substrate channel in the same way they entered it. If so, then the covalent association of electron-transfer proteins could alter the channel structure in ways that could slow product release. AlkBs have been shown capable of oxidizing alkanes to fatty acids; perhaps the presence of the fused rubredoxin slows the rate of product release and facilitates the accumulation of neutral lipids (van Beilen et al., 2003), as has been suggested for fused CYPs (Boddupalli et al., 1992).

We also note that ferr_ferrR_AlkB does not epoxidize alkenes, unlike all of the other AlkBs that have been characterized (Williams et al., 2021). Alkene epoxidation requires some substrate mobility to allow the diiron active site to access the terminal carbon for initial O-atom insertion and then the adjacent carbon to form the epoxide ring. While much work remains to be done to study this phenomenon, it suggests a structural change resulting from the fusion. Understanding the functional impacts of electron-transfer partners is therefore important for biotechnology applications of AlkB.

To what extent, if any, is the presence of other OCT operon genes indicative of alkane mineralization? And, conversely, are *alkBs* functional when they are not near genes encoding other proteins required for alkane oxidation? If so, what are their biological roles? While most *alkBs* are upregulated by the presence of alkanes, not all are (Nie et al., 2011), which again might suggest a functional role outside of alkane oxidation. It has been suggested that the OCT plasmid might be valuable for organisms that have few carbon sources outside of alkanes, while organisms—like most of the pathogens that encode an active AlkB—that can use a variety of carbon sources instead activate AlkB from electron-transfer partners found elsewhere on the genome and hence do not find the OCT plasmid to be advantageous (Smits et al., 2002). We (Williams et al., 2021) and others (Koch et al., 1991) have noted a connection between AlkB and rhamnolipids, but the nature of this connection is not clear.

FUTURE DIRECTIONS

There are many future directions that an interest in the electron-transfer proteins associated with AlkB may take.

More structural information is needed on the nature of the protein–protein interactions that mediate electron transfer. A three-dimensional structure of AlkB would be the starting point for this work. A structure would make it easier to identify possible sites of association for the rubredoxin. We are hopeful that a non-disordered structure of the two- and three-domain AlkB-fusion proteins will also provide information about the structural determinants of electron transfer. We are carrying out mutational studies on both rubredoxins and AlkB to try to pinpoint residues that are critical in protein–protein interactions and electron transfer.

There is interest in making synthetic “self-sufficient” monooxygenases by using tools from molecular biology to fuse electron-transfer partners directly to the catalyst. These synthetic fusion proteins could have more highly specialized functions, simpler transcriptional regulation, and greater ease in biotechnological uses. The ones that already exist in nature that we point to in this Mini Review might be useful “as-is” in various synthetic processes, or after some directed or random evolutionary pressure has been applied to them to alter their substrate scope.

More experiments are required to probe whether AlkBs have functions other than simply activating alkanes for degradation, for example, whether they play any biosynthetic roles.

In conclusion, AlkB is a critical enzyme in the global carbon cycle, whose activity requires that the active site be reduced. Awareness of the unusual structure of AlkG from *P. putida* GPo1 has existed for many years. However, the role of the N-terminal rubredoxin domain in some AlkGs is still unknown. The role of AlkF is also unknown. The dramatic increase in genomic information makes it possible to more broadly interrogate gene neighborhoods, a project which has made clear that the OCT operon represents a minority location for *alkB* genes. Bioinformatic studies have made visible the frequency of two- and three-domain AlkBs, work we have followed up with by biochemically characterizing those proteins. More work remains to be done to connect their biochemistry to their functions in biology and the environment.

AUTHOR CONTRIBUTIONS

RNA and SCW both wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

- Alderson, N. L., Rembiesa, B. M., Walla, M. D., Bielawska, A., Bielawski, J., and Hama, H. (2004). The human FA2H gene encodes a fatty acid 2-hydroxylase. *J. Biol. Chem.* 279, 48562–48568. doi: 10.1074/jbc.M406649200
- Alonso, H., Kleifeld, O., Yeheskel, A., Ong, P. C., Liu, Y. C., Stok, J. E., et al. (2014). Structural and mechanistic insight into alkane hydroxylation by *Pseudomonas putida* AlkB. *Biochem. J.* 460, 283–293. doi: 10.1042/BJ20131648
- Alonso, H., and Roujeinikova, A. (2012). Characterization and 2D crystallization of the membrane component AlkB of the medium-chain alkane hydroxylase system from *Pseudomonas putida* GPo1. *Appl. Environ. Microbiol.* 78, 7946–7953. doi: 10.1128/AEM.02053-12
- Austin, R. N., Chang, H.-K., Zylstra, G., and Groves, J. T. (2000). The non-heme diiron alkane monooxygenase of *Pseudomonas oleovorans* (AlkB) Hydroxylates via a substrate radical intermediate. *J. Am. Chem. Soc.* 122:11747. doi: 10.1021/ja001500v
- Austin, R. N., and Groves, J. T. (2011). Alkane-oxidizing metalloenzymes in the carbon cycle. *Metalomics* 3, 775–787. doi: 10.1039/c1mt00048a
- Austin, R. N., Luddy, K., Erickson, K., Pender-Cudlip, M., Bertrand, E. M., Deng, D., et al. (2008). Cage escape competes with geminate recombination during alkane hydroxylation by the diiron oxygenase AlkB. *Angew. Chem. Int. Ed. Engl.* 47, 5232–5234. doi: 10.1002/anie.200801184
- Benson, S., Fennewald, M., Shapiro, J., and Huettner, C. (1977). Fractionation of inducible alkane hydroxylase activity in *Pseudomonas putida* and characterization of hydroxylase-negative plasmid mutations. *J. Bacteriol.* 132, 614–621. doi: 10.1128/jb.132.2.614-621.1977
- Bernhardt, R., and Urlacher, V. B. (2014). Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. *Appl. Microbiol. Biotechnol.* 98, 6185–6203. doi: 10.1007/s00253-014-5767-7
- Bertrand, E. M., Sakai, R., Rozhkova-Novosad, E. A., Moe, L. A., Fox, B. G., Groves, J. T., et al. (2005). Reaction mechanisms of non-heme diiron hydroxylases characterized in whole cell. *J. Inorg. Biochem.* 99, 1998–2006. doi: 10.1016/j.jinorgbio.2005.06.020
- Boddupalli, S. S., Pramanik, B. C., Slaughter, C. A., Estabrook, R. W., and Peterson, J. A. (1992). Fatty acid monooxygenation by P450BM-3: product identification and proposed mechanisms for the sequential hydroxylation reactions. *Arch. Biochem. Biophys.* 292, 20–28. doi: 10.1016/0003-9861(92)90045-X
- Bosetti, A., van Beilen, J. B., Preusting, H., Lageveen, R. G., and Witholt, B. (1992). Production of primary aliphatic alcohols with a recombinant *Pseudomonas* strain, encoding the alkane hydroxylase enzyme system. *Enzym. Microb. Technol.* 14:702. doi: 10.1016/0141-0229(92)90109-2
- Cooper, H. L. R., Mishra, G., Huang, X., Pender-Cudlip, M., Austin, R. N., Shanklin, J., et al. (2012). Parallel and competitive pathways for substrate desaturation, hydroxylation, and radical rearrangement by the non-heme diiron hydroxylase AlkB. *J. Am. Chem. Soc.* 134, 20365–20375. doi: 10.1021/ja3059149
- De Mot, R., and Parret, A. H. A. (2002). A novel class of self-sufficient cytochrome P450 monooxygenase in prokaryotes. *Trends Microbiol.* 10, 502–508. doi: 10.1016/s0966-842x(02)02458-7
- Eggink, G., Engel, H., Vriend, G., Terpstra, P., and Witholt, B. (1990). Rubredoxin reductase of *Pseudomonas oleovorans*: structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *J. Mol. Biol.* 212, 135–142. doi: 10.1016/0022-2836(90)90310-I
- Eggink, G., Lageveen, R. G., Altenburg, B., and Witholt, B. (1987). Controlled and functional expression of the *Pseudomonas oleovorans* alkane utilizing system in *Pseudomonas putida* and *Escherichia coli*. *J. Biol. Chem.* 262, 17712–17718. doi: 10.1016/S0021-9258(18)45437-3
- Fox, B. G., Shanklin, J., Ai, J., Loehr, T. M., and Sanders-Loehr, J. (1994). Resonance raman evidence for an Fe-O-Fe center in stearoyl-ACP desaturase. Primary sequence identity with other diiron-oxo proteins. *Biochemistry* 33, 12776–12786. doi: 10.1021/bi00209a008
- Gross, R., Buehler, K., and Schmid, A. (2013). Engineered catalytic biofilms for continuous large scale production of n-octanol and (S)-styrene oxide. *Biotechnol. Bioeng.* 110, 424–436. doi: 10.1002/bit.24629
- Groves, J. T. (1985). Key elements of the chemistry of cytochrome P-450. *J. Chem. Ed.* 62:928. doi: 10.1021/ed062p928
- Hagelueken, G., Wiehlmann, L., Adams, T. M., Kolmar, H., Heinz, D. W., Tümmler, B., et al. (2007). Crystal structure of the electron transfer complex rubredoxin–rubredoxin reductase of *Pseudomonas aeruginosa*. *Proc. Acad. Nat. Sci.* 104, 12276–12281. doi: 10.1073/pnas.0702919104
- Hama, H. (2010). Fatty acid 2-hydroxylation in mammalian sphingolipid biology. *Biochim. Biophys. Acta* 1801, 405–414. doi: 10.1016/j.bbali.2009.12.004
- Hoschek, A., Bühler, B., and Schmid, A. (2017). Overcoming the gas–liquid mass transfer of oxygen by coupling photosynthetic water oxidation with biocatalytic oxyfunctionalization. *Angew. Chem. Int. Ed.* 56, 15146–15149. doi: 10.1002/anie.201706886
- Jimenez-Diaz, L., Cabellero, A., and Segura, A. (2017) in *In Aerobic Utilization of Hydrocarbons, Oils, and Lipids, Handbook of Hydrocarbons and Lipid Microbiology*. ed. F. Frojo (Heidelberg, Germany: Springer International Publishing).
- Julsing, M. K., Schrewe, M., Cornelissen, S., Hermann, I., Schmid, A., and Bühler, B. (2012). Outer membrane protein AlkL boosts biocatalytic oxyfunctionalization of hydrophobic substrates in *Escherichia coli*. *Appl. Environ. Microbiol.* 78, 5724–5733. doi: 10.1128/AEM.00949-12
- Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., et al. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589. doi: 10.1038/s41586-021-03819-2
- Kirmair, L., Skerra, A., and Spormann, A. M. (2014). Biochemical analysis of recombinant AlkI from *Pseudomonas putida* reveals a membrane-associated, flavin adenine dinucleotide-dependent dehydrogenase suitable for the biosynthetic production of aliphatic aldehydes. *Appl. Environ. Microbiol.* 80, 2468–2477. doi: 10.1128/AEM.04297-13
- Koch, A. K., Käppeli, O., Fiechter, A., and Reiser, J. (1991). Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J. Bacteriol.* 173, 4212–4219. doi: 10.1128/jb.173.13.4212-4219.1991
- Kok, M., Oldenhuis, R., van der Linded, M. P. G., Meulenber, C. H. C., Kingma, J., and Witholt, B. (1989). The *Pseudomonas oleovorans* alkBAC operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase. *J. Biol. Chem.* 264, 5442–5451. doi: 10.1016/S0021-9258(18)83565-7
- Ladkau, N., Assmann, M., Schrewe, M., Julsing, M. K., Schmid, A., and Bühler, B. (2016). Efficient production of the nylon 12 monomer ω-aminododecanoic acid methyl ester from renewable dodecanoic acid methyl ester with engineered *Escherichia coli*. *Metab. Eng.* 36, 1–9. doi: 10.1016/j.mben.2016.02.011
- Lee, N.-R., Hwang, M.-O., Jung, G.-H., Kim, Y.-S., and Min, K.-H. (1996). Physical structure and expression of alkBA encoding alkane hydroxylase and rubredoxin reductase from *Pseudomonas maltophilia*. *Biochem. Biophys. Res. Commun.* 218, 17–21. doi: 10.1006/bbrc.1996.0004
- Li, S., Du, L., and Bernhardt, R. (2020). Redox partners: function modulators of bacterial P450 enzymes. *Trends Microbiol.* 28, 445–454. doi: 10.1016/j.tim.2020.02.012
- Li, Z., Feiten, H.-J., van Beilen, J. B., Duetz, W. A., and Witholt, B. (1999). Preparation of optically active N-benzyl-3-hydroxypropanolamine by enzymatic hydroxylation. *Tetrahedron Asymmetry* 10:1323
- Li, Z., Jiang, Y., Guengerich, F. P., Ma, L., Li, S., and Zhang, W. (2020). Engineering cytochrome P450 enzyme systems for biomedical and biotechnological applications. *J. Biol. Chem.* 295, 833–849. doi: 10.1016/S0021-9258(17)49939-X
- Lountos, G. T., Mitchell, K. H., Studts, J. M., Fox, B. G., and Orville, A. M. (2005). Crystal structures and functional studies of T4moD, the toluene 4-monooxygenase catalytic effector protein. *Biochemistry* 44, 7131–7142. doi: 10.1021/bi047459g
- May, S. W., and Abbott, B. J. (1972). Mechanistic studies on non-heme iron monooxygenase systems of *Pseudomonas oleovorans*. *Biochem. Biophys. Res. Commun.* 48:1230
- May, S. W., Gordon, S. L., and Steltenkamp, M. S. (1977). Enzymic epoxidation of trans,trans-1,8-dideuterio-1,7-octadiene. Analysis using partially relaxed proton Fourier transform NMR. *J. Am. Chem. Soc.* 99, 2017–2024. doi: 10.1021/ja00449a001
- May, S. W., and Katopodis, A. G. (1986). Oxygenation of alcohol and sulphide substrates by a prototypical non-haem iron monooxygenase: catalysis and biotechnological potential. *Enzym. Microb. Technol.* 8:17. doi: 10.1016/0141-0229(86)90004-9

- McKenna, E. J., and Coon, M. J. (1970). Enzymatic ω -oxidation: IV purification and properties of the ω -hydroxylase of *Pseudomonas oleovorans*. *J. Biol. Chem.* 225, 3882–3889.
- Mitchell, A. G., and Martin, C. E. (1995). A novel cytochrome b5-like domain is linked to the carboxyl terminus of the *Saccharomyces cerevisiae* delta-9 fatty acid desaturase. *J. Biol. Chem.* 270, 29766–29772. doi: 10.1074/jbc.270.50.29766
- Mitchell, A. G., and Martin, C. E. (1997). Fah1p, a *Saccharomyces cerevisiae* cytochrome b5 fusion protein, and its *Arabidopsis thaliana* homolog that lacks the cytochrome b5 domain both function in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids. *J. Biol. Chem.* 272, 28281–28288.
- Mitić, N., Schwartz, J. K., Brazeau, B. J., Lipscomb, J. D., and Solomon, E. I. (2008). CD and MCD studies of the effects of component B variant binding on the biferrrous active site of methane monooxygenase. *Biochemistry* 47, 8386–8397. doi: 10.1021/bi800818w
- Munro, A. W., Girvan, H. M., and McLean, K. J. (2007). Cytochrome P450–redox partner fusion enzymes. *Biochim. Biophys. Acta* 1770, 345–359. doi: 10.1016/j.bbagen.2006.08.018
- Naing, S.-H., Parvez, S., Pender-Cudlip, M., Groves, J. T., and Austin, R. N. (2013). Substrate specificity and reaction mechanism of purified alkane hydroxylase (AlkB) from the hydrocarbonoclastus bacterium *Alcanivorax borkumensis*. *J. Inorg. Biochem.* 121, 46–52. doi: 10.1016/j.jinorgbio.2012.12.012
- Nie, Y., Chi, C.-Q., Fang, H., Liang, J.-L., Lu, S.-L., Lai, G.-L., et al. (2014). Diverse alkane hydroxylase genes in microorganisms and environments. *Sci. Rep.* 4:4968. doi: 10.1038/srep06634
- Nie, Y., Liang, J., Fang, H., Tang, Y.-Q., and Wu, X.-L. (2011). Two novel alkane hydroxylase-rubredoxin fusion genes isolated from a dietzia bacterium and the function of fused rubredoxin domains in long-chain n alkane degradation. *Appl. Environ. Microbiol.* 77, 7279–7288. doi: 10.1128/AEM.00203-11
- Perry, A., Lian, L.-Y., and Scrutton, N. S. (2001). Two-iron rubredoxin of *Pseudomonas oleovorans*: production, stability, and characterization of the individual iron-binding domains by optical, CD and NMR spectroscopies. *Biochem. J.* 354, 89–98. doi: 10.1042/0264-6021:3540089
- Procopio, L., de Cassia Pereira e Silva, M., Dirk van Elsland, J., and Seldin, L. (2013). Transcriptional profiling of genes involved in n-hexadecane compounds assimilation in the hydrocarbon degrading *Dietzia cinnamea* P4 strain. *Braz. J. Microbiol.* 44, 639–641. doi: 10.1590/S1517-83822013000200044
- Ro, S. Y., Ross, M. O., Deng, Y. W., Batelu, S., Lawton, T. J., Hurley, J. D., et al. (2018). From micelles to bicelles: effect of the membrane on particulate methane monooxygenase activity. *J. Biol. Chem.* 293, 10457–10465. doi: 10.1074/jbc.RA118.003348
- Rozhkova-Novosad, E. A., Chae, J.-C., Zylstra, G. J., Bertrand, E. M., Alexander-Ozinskas, M., Deng, D., et al. (2007). Profiling mechanisms of alkane hydroxylase activity *in vivo* using the diagnostic substrate norcarane. *Chem. Biol.* 14, 165–172. doi: 10.1016/j.chembiol.2006.12.007
- Sayanova, O., Shewry, P. R., and Napier, J. A. (1999). Histidine-41 of the cytochrome b5 domain of the borage delta6 fatty acid desaturase is essential for enzyme activity. *Plant Physiol.* 121, 641–646. doi: 10.1104/pp.121.2.641
- Schrewe, M., Julsing, M. K., Lange, K., Czarnotta, E., Schmid, A., and Bühler, B. (2014). Reaction and catalyst engineering to exploit kinetically controlled whole-cell multistep biocatalysis for terminal FAME oxyfunctionalization. *Biotechnol. Bioeng.* 111, 1820–1830. doi: 10.1002/bit.25248
- Shanklin, J., Achim, C., Schmidt, H., Fox, B. G., and Münck, E. (1997). Mossbauer studies of alkane omega-hydroxylase: evidence for a diiron cluster in an integral-membrane enzyme. *Proc. Natl. Acad. Sci.* 94, 2981–2986.
- Smits, T. H. H., Balada, S. B., Witholt, B., and van Beilen, J. B. (2002). Functional analysis of alkane hydroxylases from gram-negative and gram-positive bacteria. *J. Bacteriol.* 184, 1733–1742. doi: 10.1128/JB.184.6.1733-1742.2002
- Smits, T. H., Witholt, B., and van Beilen, J. B. (2003). Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*. *Antonie Van Leeuwenhoek* 84, 193–200. doi: 10.1023/A:1026000622765
- Sperling, P., and Heinz, E. (2001). Desaturases fused to their electron donor. *Eur. J. Lipid Sci. Technol.* 103:158. doi: 10.1002/1438-9312(200103)103:3<158::AID-EJLT158>3.0.CO;2-1
- Staijen, I. E., van Beilen, J. B., and Witholt, B. (2000). Expression, stability and performance of the three-component alkane mono-oxygenase of *Pseudomonas oleovorans* in *Escheria coli*. *Eur. J. Biochem.* 267, 1957–1965. doi: 10.1046/j.1432-1327.2000.01196.x
- van Beilen, J. B., Duetz, W. A., Schmid, A., and Witholt, B. (2003). Practical issues in the application of oxygenases. *Trends Biotechnol.* 21, 170–177. doi: 10.1016/S0167-7799(03)00032-5
- van Beilen, J. B., and Funhoff, E. G. (2005). Alkane oxygenase diversity and applications. *Curr. Opin. Biotechnol.* 16:3. doi: 10.1016/j.copbio.2005.04.005
- van Beilen, J. B., and Funhoff, E. G. (2007). Alkane hydroxylases involved in microbial alkane degradation. *Appl. Microbiol. Biotechnol.* 74, 13–21. doi: 10.1007/s00253-006-0748-0
- van Beilen, J. B., Funhoff, E. G., van Loon, A., Just, A., Kaysser, L., Bouza, M., et al. (2006). Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. *Appl. Environ. Microbiol.* 72, 59–65. doi: 10.1128/AEM.72.1.59-65.2006
- van Beilen, J. B., Kingma, J., and Witholt, B. (1994). Substrate specificity of the alkane hydroxylase system of *Pseudomonas oleovorans* GPo1. *Enzym. Microb. Technol.* 16:904
- van Beilen, J. B., Neuenschwander, M., Smits, T. H. M., Roth, C., Balada, S. B., and Witholt, B. (2002). Rubredoxins involved in alkane oxidation. *J. Bacteriol.* 184, 1722–1732. doi: 10.1128/JB.184.6.1722-1732.2002
- van Beilen, J. B., Panke, S., Lucchini, S., Franchini, A. G., Röthlisberger, M., and Witholt, B. (2001). Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology* 147, 1621–1630. doi: 10.1099/00221287-147-6-1621
- van Beilen, J. B., Penninga, D., and Witholt, B. (1992). Topology of the membrane-bound alkane hydroxylase of *Pseudomonas oleovorans*. *J. Biol. Chem.* 267, 9194–9201
- van Beilen, J. B., Smits, T. H., Roos, F., Brunner, T., Balada, S. B., Röthlisberger, M., et al. (2005). Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases. *J. Bacteriol.* 187, 85–91. doi: 10.1128/JB.187.1.85-91.2005
- van Beilen, J. B., Wubbolts, M. G., and Witholt, B. (1994). Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation* 5, 161–174. doi: 10.1007/BF00696457
- van Nuland, Y. M., de Vogel, F. A., Eggink, G., and Weusthuis, R. A. (2017). Expansion of the ω -oxidation system AlkBGT of *Pseudomonas putida* GPo1 with AlkJ and AlkH results in exclusive mono-esterified dicarboxylic acid production in *E. coli*. *Microb. Biotechnol.* 10, 594–603. doi: 10.1111/1751-7915.12607
- Wang, W., Jacob, R. E., Luoh, R. P., Engen, J. R., and Lippard, S. J. (2014). Electron transfer control in soluble methane monooxygenase. *J. Am. Chem. Soc.* 136, 9754–9762. doi: 10.1021/ja511373g
- Williams, S. C., Forsberg, A. P., Lee, J., Vizcarra, C. L., Lopatkin, A. J., and Austin, R. N. (2021). Investigation of the prevalence and catalytic activity of rubredoxin-fused alkane monooxygenases (AlkBs). *J. Inorg. Biochem.* 219:111409. doi: 10.1016/j.jinorgbio.2021.111409
- Williams, S. C., Luongo, D., Orman, M., Vizcarra, C. L., and Austin, R. N. (2021). An alkane monooxygenase (AlkB) family in which all electron transfer partners are covalently bound to the oxygen-activating hydroxylase. *J. Inorg. Biochem.* 228:111707. doi: 10.1016/j.jinorgbio.2021.111707 [Epub ahead of print]
- Xie, M., Alonso, H., and Roujeinikova, A. (2011). An improved procedure for the purification of catalytically active alkane hydroxylase from *Pseudomonas putida*. *Appl. Biochem. Biotechnol.* 165, 823–831. doi: 10.1007/s12010-011-9300-5
- Zäuner, S., Jochum, W., Bigorowski, T., and Benning, C. (2012). A cytochrome b5-containing plastid-located fatty acid desaturase from *Chlamydomonas reinhardtii*. *Eukaryot. Cell* 11, 856–863. doi: 10.1128/EC.00079-12
- Zhu, G., Koszelak-Rosenblum, M., Connelly, S. M., Dumont, M. E., and Malkowski, M. G. (2015). The crystal structure of an integral membrane fatty acid alpha hydroxylase. *J. Biol. Chem.* 290, 29820–29833. doi: 10.1074/jbc.M115.680124

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