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Valorization of Small Alkanes by Biocatalytic Oxyfunctionalization

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The oxidation of alkanes into valuable chemical products is a vital reaction in organic synthesis. This reaction, however, is challenging, owing to the inertness of C–H bonds. Transition metal catalysts for C–H functionalization are frequently explored. Despite chemical alternatives, nature has also evolved powerful oxidative enzymes (e.g., methane monooxygenases, cytochrome P450 oxygenases, peroxygenases) that are capable of transforming C–H bonds under very mild conditions, with only the use of molecular oxygen or hydrogen peroxide as electron acceptors. Although progress in alkane oxidation has been reviewed extensively, little attention has been paid to small alkane oxidation. The latter holds great potential for the manufacture of chemicals. This Minireview provides a concise overview of the most relevant enzyme classes capable of small alkanes ($C_{<6}$) oxyfunctionalization, describes the essentials of the catalytic mechanisms, and critically outlines the current state-of-the-art in preparative applications.

1. Introduction

Alkanes (saturated alkanes of the general formula C_nH_{2n+2}) are major constituents of natural gas, oil, coal and are still produced on a very large scale every year. For instance, the estimated annual global methane emissions are approximately 570 Million tonnes (Mt),[1] and butane demand was 13823 Mt in 2015.[2] The historical trivial name of an alkane is paraffin (from Latin: parum affinis = showing little affinity), indicating the major challenge of chemical transformation into value-added products. Alkanes are nonpolar molecules offering no possibility for nucleophilic or electrophilic reaction pathways. Furthermore, the C–H and C–C bonds in alkanes are very stable necessitating very strong reagents such as radicals, activated (hydro)peroxides, or highly oxidized metal–oxo complexes for their activation. The radical nature of these reactions causes major issues in selectivity and often complex product mixtures are obtained. Therefore, it is unsurprising that the majority of alkanes are used thermally, being burned to generate heat or propulsion. Economically, converting alkanes into functionalized derivatives represents a value-adding reaction, especially considering that global demand for products such as alkanols is rapidly growing (Figure 1).

Life has been facing the above-mentioned challenges for eras, giving it ample time to develop catalytic strategies to transform alkanes for either degradation/detoxification purposes or to utilize alkanes as a source of carbon and energy. Especially microbes have developed various catalysts to transform alkanes. As early as 1905, Söhngen and coworkers reported bacteria metabolizing methane and some higher alkanes.[3] Ever since then, the repertoire of known alkane-degrading microorganisms has grown considerably.[4] Especially industrial waste streams and environmental disasters such as

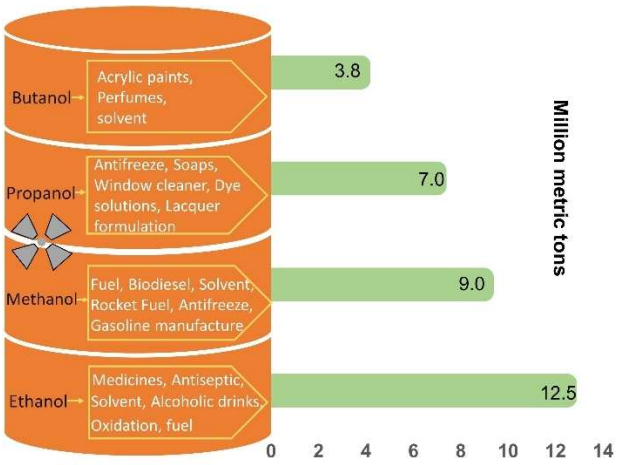

Figure 1. Various applications of some prominent small alcohols (butanol, propanol, methanol, and ethanol) and approximate demand for respective alcohols up to 2019 presented in million metric tons.

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the oil spill of the Exxon Valdez[5] or Deepwater Horizon,[6] to mention just a few, offer environments for the discovery of alkane degrading microbes.

Metal-oxo complexes dominate the chemical repertoire of C–H activating catalysts.[7] Amongst the bioavailable metals such as Fe, Cu, Mn, and Co especially iron-oxo complexes are predominantly used by enzymatic systems for the functionalization of nonactivated C–H bonds as found in alkanes.[8] The vast diversity of Fe-dependent oxygenases (enzymes inserting oxygen atoms into organic starting materials) can be divided into heme-iron and nonheme-iron complexes as prosthetic groups (Figure 2). Monooxygenases utilize molecular oxygen as a source of the O-atom incorporated; for the reductive activation of O_2, oxygenases rely on the supply with reducing equivalents. Peroxygenases utilize (already reduced) H_2O_2 as O-source.

2. Oxygenases for Selective Alkane Oxidation

2.1. Methane monooxygenase

Methanotrophs are microorganisms (bacteria or archaea) capable of utilizing methane as a carbon and energy source. Until now, 130 methanotrophs have been isolated, which were found in wetlands, soils, marshes, rice paddies, landfills, and aquatic systems.[10,11] To activate the C–H bond in methane ($\Delta H_{298} =$ 105 kcal mol^{-1} for CH_3–H),[12] these organisms utilize methane monooxygenases (MMOs) to transform methane into methanol. While this first step is energy consuming (NAD(P)H-dependent), the next mineralization steps are catalyzed by dehydrogenases yielding one equivalent of NAD(P)H in each oxidation step[13] resulting in a net NAD(P)H balance of 2 for the mineralization of methane. Furthermore, the intermediate formaldehyde is a versatile building block for other metabolic pathways such as in the serine pathway or the ribulose monophosphate pathway (Figure 3).

Two types of methane monooxygenases are known: the soluble methane monooxygenase (sMMO) and the membrane-located, particulate methane monooxygenase (pMMO). sMMO and pMMO not only differ in their cellular localization but also their catalytic mechanisms. While sMMO contains a binuclear nonheme diiron active site, pMMO is a copper enzyme.

Dr Wuyuan Zhang studied at the Delft University of Technology (TU Delft) and obtained his Ph.D. degree under the supervision of Prof. Isabel Arends and Dr. Kristina Djanashvili. He then spent four years as a postdoctoral researcher at TU Delft with Prof. Frank Hollmann. Later, he worked shortly at Xi'an Jiaotong University as a principal investigator. In 2020, he moved to Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences (CAS). Currently he is leading the group of biocatalysis, with research interests focusing on photobiocatalysis, redox biocatalysis, and the industrial application of biocatalysts.

2.1.1. Soluble methane monooxygenase

sMMO is a heterotrimeric enzyme consisting of an oxygenase subunit (catalyzing the actual methane conversion), a reductase (delivering electrons needed by the oxygenase subunit from NADH), and a regulatory subunit whose exact role is not yet fully understood.[14] Crystal structures of sMMO are available (Figure 4).[15]

The resting state of sMMO contains a di-Fe^{II} core. In the first step of the catalytic mechanism, both Fe^{III} centers are reduced via single electron transfers from the reductase subunit of sMMO. The reduced Fe-core binds O_2 as μ-(1,2)-peroxo complex thereby reoxidizing the Fe^{II} centers back to Fe^{III}. Depending on the geometry of the peroxo compound, the *cis*- and *trans*-isomers are generally abbreviated as intermediate P* and P, respectively.[18,19] In the next step of the catalytic mechanism, the peroxo-O–O bond is reductively cleaved and the two Fe^{III} are formally oxidized to Fe^{IV}.[20] The resulting compound Q, also called Diamond Core,[21] then performs the hydroxylation of methane. Possibly, this reaction occurs via H-atom abstraction from the bound methane substrate followed by rapid rebounding of the transient CH_3-radical with the Fe^{IV}–Fe^{III} bridging OH group (Figure 5). However, alternative mechanisms have been proposed.[22] In any case, methanol is formed and the resting di-Fe^{III} configuration is restored.

MMOs catalyze the reductive activation of molecular oxygen, which also implies that for this reaction two electrons are needed. The stoichiometric electron donor for sMMO is NADH, which as an obligate hydride donor is mechanistically incompatible with the reduction of Fe^{III} to Fe^{II}, thereby necessitating single electron transfer steps. Therefore, sMMO (and other enzyme systems discussed later in this contribution) needs an enzymatic relays system converting the NADH-related hydride transfer into two single electron transfer steps. For this, the reductase utilizes a flavin prosthetic group which oxidizes NADH by hydride abstraction and shuttles the two reducing equivalents as single electrons to the monooxygenase subunit (Figure 6).

It is worth noting that, despite the name suggesting a high preference for methane, sMMOs have also been shown to hydroxylate other small hydrocarbons such as ethane, propane, butane, pentane, and hexane (Table 1).

2.1.2. Particulate methane monooxygenase

Compared to sMMO, pMMO is far less understood. This is partially due to the fact that as a membrane protein pMMO has for a long time withstood purification and characterization efforts and was first purified only in 1995[34] and the first crystal structure was reported in 2005 (Figure 7).[35] The oxygen- and methane-activating mechanism of the multi-copper-containing pMMO is still under debate. In contrast to sMMO, pMMO utilizes hydroquinones as sacrificial electron donors for the reductive activation of O_2.

Attempts have been made to understand the chemistry of sMMO and pMMO for CH_4 oxidation. Some hidden character-

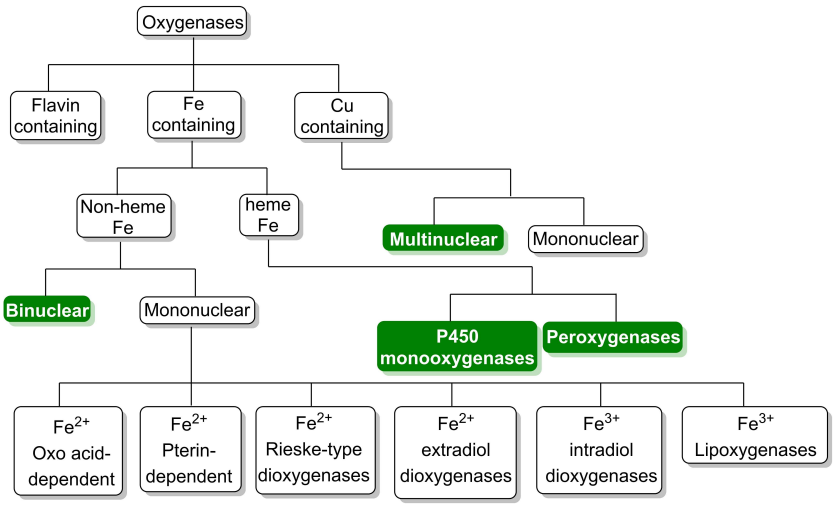

Figure 2. Classification of oxygenases. Adapted from ref. [9]. Subclasses discussed herein are marked in green.

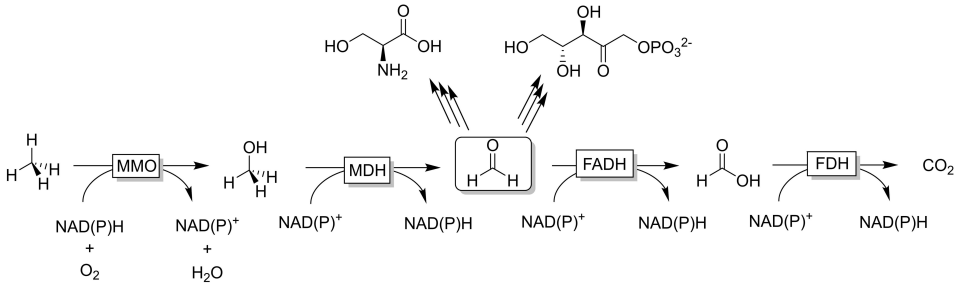

Figure 3. Methane activation and metabolization by methanotrophs. The full mineralization yields net two NAD(P)H equivalents. Formaldehyde is also used in various anabolic pathways such as in serine- or ribulose-1-phosphate synthesis. MMO = methane monooxygenase; MDH = methanol dehydrogenase; FADH = formaldehyde dehydrogenase; FDH = formate dehydrogenase.

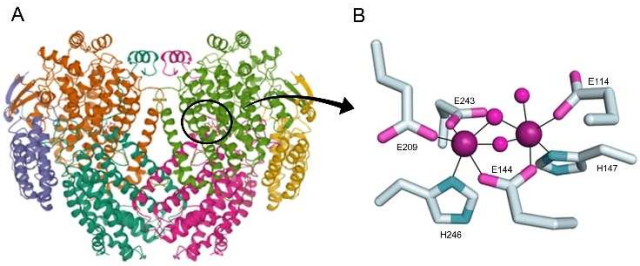

Figure 4. A) Crystal structure of MMOH component of *Methylococcus capsulatus* (adopted from PDB ID 1FZ1): MMOH is $\alpha2\beta2\gamma2$ homodimer containing three subunits (oxygenase subunit, reductase subunit, and regulatory protein). Reproduced with permission from ref. [16]; copyright American Chemical Society 2001. B) The active site architecture of sMMO where Fe coordinated with 2 histidine and 4 glutamic acid residues. Reproduced from ref. [17]; copyright Wiley Periodicals LLC 1997.

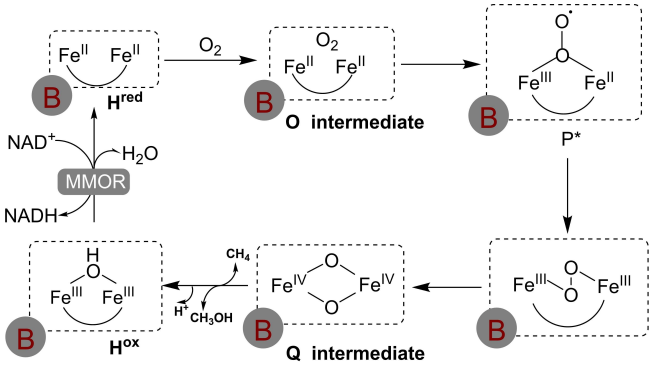

Figure 5. The catalytic cycle of sMMO. **B** represents a regulatory component.

istics of enzymes are still under investigation such as the copper active site, structural core of substrate and product access,[37] electron source for catalytic reaction of pMMO, and characterization of intermediate Q of sMMO.[38] Despite these gaps, significant information has been extracted of biological CH_4 oxidation and implemented in designing biomimetic catalyst (tricopper complex) for the oxidation of CH_4 under ambient conditions.[39] Although, there is room for improvement (identification of new species,[40] cost-effective, environment friendly) to acquire a robust catalyst. Further deep understanding of enzyme catalysis and structural architecture will be an important step towards oxidation of CH_4 under ambient conditions.

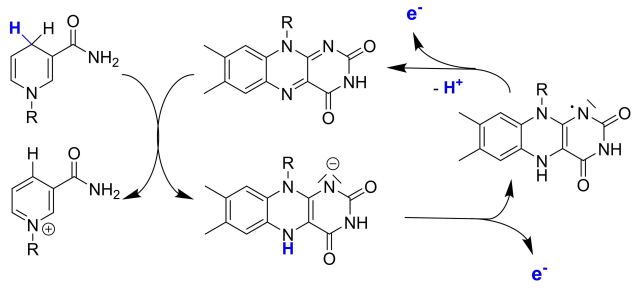

Figure 6. Oxidoreductases using NADH-related hydride transfer pathways.

Table 1. Enzymes used for small alkane oxyfunctionalization.

Enzyme	Substrate	Catalytic activity [s^{-1}]	Ref.
sMMO (Methylosinus trichosporium OB3b)	Methane	3.7	[23]
sMMO (M. capsulatus (bath))	Methane	0.2–1.0	[24]
	Methane	0.376[a]	[25]
	Ethane	0.304[a]	
	Propane	0.309[a]	
	Butane	0.345[a]	
	Pentane	0.327[a]	
	Hexane	0.179[a]	
Bacterium methylocystis Sp. Strain WI14	Propane	1.60	[26]
pMMO (M. capsulatus (bath))	Methane	0.05–0.27	[27]
sBMO	Methane	1.3	[28]
	Ethane	0.76	
	Propane	0.55–0.98	
	Butane	0.12–0.48	
	Pentane	0.008–0.34	
Cyt153 A6 (A6)	Methane	0.00001	[29]
P450 (BM3) with $CF_3(CF_2)_nCO_2H$	Methane (n = 8)	41.2	[30]
	Propane (n = 9)	17.01	
	Butane (n = 5)	60.5	
A6 BMO-I	Methane	0.000003	[29]
P450 propane monooxygenase ($P450_{PMO}$)	Ethane	40.8 ± 4.1	[31]
	Propane	556.6 ± 48.3	
	Butane	256.6 ± 30	
	Pentane	25.8 ± 2	
	Hexane	36.6 ± 3	
$CytP450_{cam}$ F87W/Y96F/T101 L/V247L	Butane	12.5	[32]
	Propane	1.8	
Cyt P450 BM3 (35-E11)	Ethane	4.1	[33]
	Propane	100	

[a]Molecular weight of 269000 Da, M_w has been assumed to calculate the turnover number from specific activity.[24]

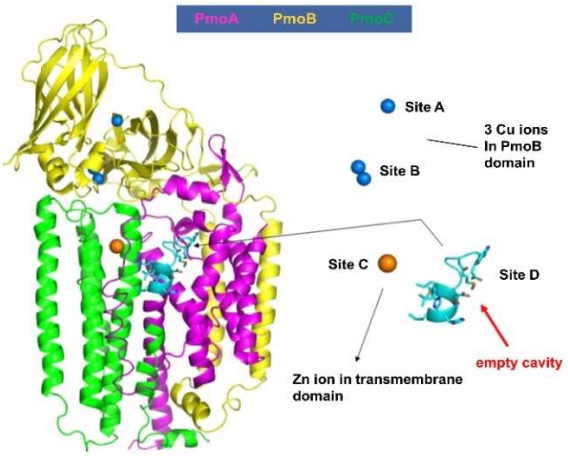

Figure 7. Crystal structure of pMMO from *Methylococcus capsulatus* (bath) defining the different sites. Sites A and B contain copper(I) and dicopper, respectively. Site C of $pMMO_{Mc}$ contains Zn atom as a buffer required for crystallization. Site D is considered as a putative catalytic center with $Cu^{II}Cu^{II}Cu^{II}$ tricopper cluster. Reproduced with permission from ref. [35]; copyright NPG 2005.

2.2. Alkane monooxygenases

In addition to the above-discussed MMOs, a range of alkane monooxygenases have also been described. Particularly, butane monooxygenases (BMO) and, more generally, alkane monooxygenases have been described.

Soluble butane monooxygenase (sBMO) has received some attention in the past few years. One of the best-studied sBMO from *P. butanovora* utilizes the C_2–C_9 alkanes and its oxygenated derivates such as alcohols and acids.[41,42] The overall molecular architecture of BMO is very similar to sMMO, comprising a monooxygenase subunit, a reductase, and a regulatory protein. The monooxygenase subunit contains, similar to sMMO, a diiron active site, suggesting a similar catalytic mechanism.[43] Functionally, both enzymes differ to some extent with respect to their product scope: with higher alkanes sMMO preferentially forms secondary alcohol products[44] whereas BMO forms primary alcohols (exclusive terminal hydroxylation).[45]

The most prominent and widely used alkane monooxygenases are AlkBs from *Pseudomonas strains*.[46] More than 250 homologs from 45 different bacterial species have been reported.[47–49] AlkB constitutes the monooxygenase subunit of a multienzyme complex comprising rubredoxin reductase (AlkT) and two rubredoxins (AlkF and AlkG) which mediate the electron transfer from NADH to AlkB.[50] The amino acids coordinating the two catalytically active iron ions in AlkB are exclusively histidines, and the amino acid motif $HX_{(3or4)}HX_{(n)}$-$HX_{(2or3)}HHX_{(n)}HX_{(2or3)}HH$ is highly conserved within all known alkane monooxygenases. AlkB is a membrane protein which also to some extent explains why so far, mechanistic and structural studies are scarce.[50b,51] It is worth mentioning that the *alk* operon also encodes for a fatty alcohol dehydrogenase and an aldehyde dehydrogenase, suggesting that its natural function is to detoxify and energetically valorize xenobiotic alkanes by activating and channeling them into β-oxidation.[52] In line with this assumption, AlkB exhibits the highest activities towards medium and long-chain *n*-alkanes.[47] Additionally, small alkanes, cycloalkanes, and alkylbenzenes have also been reported as substrates.[53,54]

2.3. Cytochrome P450

P450 monooxygenases are heme-iron enzymes capable of oxyfunctionalizing nonactivated C–H bonds in alkanes. This vast enzyme class (more than 300000 P450 MO sequences have been deposited[55]) has been in focus as catalysts for the selective transformation of natural and xenobiotic hydrocarbons, fatty acids, and steroids for decades.[56]

Similar to the above-mentioned nonheme monooxygenases, the P450 MO mechanism is based on the reductive activation of molecular oxygen promoted by the sequential single electron transfer to the Fe active site. Again, NAD(P)H serves as a stoichiometric reductant necessitating relays systems similar to the ones described above. The actual catalytic mechanism comprises of reduction of the resting state Fe^{III} to Fe^{II}, followed by O_2 binding, a second reduction step, and protonation, resulting in a hydroperoxo-Fe^{III}-heme species (compound **0**). After water elimination, a formal Fe^{IV}–oxo complex (compound **I**) is formed (Figure 8). Compound **I** resembles a Fe^{IV} oxo species with a delocalized radical cation-heme ligand (thereby avoiding extremely high Fe oxidation states). Compound **I** performs a H-atom abstraction from the bound alkane starting material. The resulting alky radical rapidly recombines with the Fe-coordinated OH group (rebound mechanism) to form the hydroxylated product. Finally, the alcohol product is substituted by incoming water and releases the product and regenerates the resting state of the enzyme.

Amongst the many P450 MOs potentially available, the enzyme from *Bacillus megaterium* (P450 BM3) has been receiving particular attention as this enzyme is 1) soluble (not membrane-bound) and 2) comprises a natural fusion protein of the oxygenase subunit, the reductase, and the electron transfer protein resulting in a 'self-sufficient' NADPH-dependent monooxygenase (Figure 9). The best substrate for wild-type P450BM3 appears to be arachidonic acid, rendering its application for the oxyfunctionalization of small alkanes rather challenging. Protein engineering, however, proved to be a very successful approach to generate more active P450BM3 variants by reducing the voluminous active site and facilitating binding of smaller alkanes.[57]

Reduction of the active site volume to facilitate positioning of smaller alkanes can also be achieved by the so-called decoy molecule approach. Here, larger, nonreactive compounds can be used to fill up the enzyme active site. Watanabe and co-workers, for example, used perfluorinated carboxylic acids to facilitate the conversion of small alkanes such as propane or butane.

This 'decoy molecule' approach has also been proven successful in promoting the so-called peroxide shunt pathway of P450 MOs. H_2O_2 can principally be used to generate Compound **I** directly from the resting state of the heme enzyme. In principle, this would offer a highly simplified reaction scheme compared to the NAD(P)H-dependent reductive regeneration of the P450 MOs. Unfortunately, however, H_2O_2 is a notoriously poor substrate of most P450 MOs and predominantly leads to oxidative, irreversible inactivation of the heme prosthetic group. Cong and co-workers argued that deprotonation of the primary H_2O_2 coordination adduct to the heme iron may be the overall rate-limiting state and therefore designed next-generation decoy molecules containing a general base to facilitate the deprotonation step.[59] Indeed, some drastic improvements in the H_2O_2-driven hydroxylation using P450 MOs have been achieved.[60]

2.4. Peroxygenases

Peroxygenases are microsomal, secreted enzymes metalloenzymes for alkane oxidation. In 2004, a new heme thiolate peroxidase (HTP) having characteristics of peroxidases and P450 enzymes, which was known as unspecific peroxygenase (UPO). Peroxygenase is a heme-containing enzyme having a similar catalytic moiety as P450 monooxygenase and consumes H_2O_2.

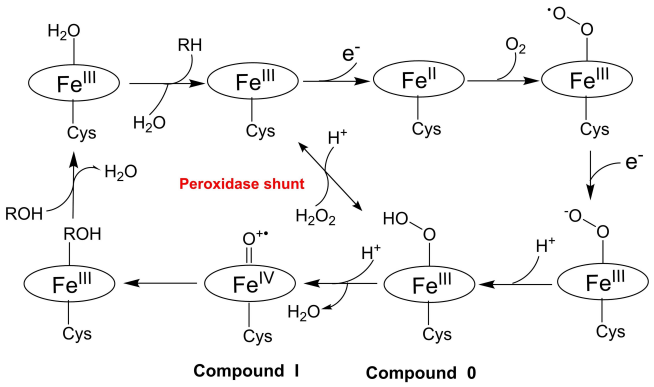

Figure 8. The catalytic cycle of CytP450 with substrate (R–H) hydroxylation is shown: Displacement of axial H_2O molecule and conversion of low spin (LS) heme iron to high spin (HS) state. Fe^{III} is reduced to Fe^{II} via electron transfer and O_2 rapidly binds with reduced Fe^{II} forming Fe^{II}–O_2. This complex transformed into a more stable Fe^{III}–O_2^- form. The second reduction and protonation form ferric hydroperoxo intermediate known as compound **0** and assigned as a rate-determining step. An alternative route is also governed by an enzyme in which oxygen atom donates from oxidizing agents like peroxides and hypochlorite known as peroxide shunt and forms compound **0**. Dehydration of compound **0** forms ferryl (Fe^{IV})–oxo, porphyrin radical cation (compound **I**) intermediate. Hydroxylated form of substrate is released and enzyme returns to the initial state.

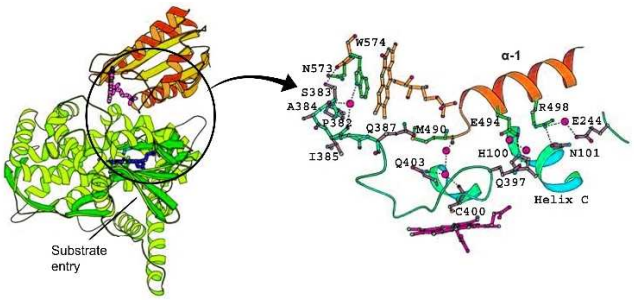

Figure 9. Crystal structure of CytP450 BM3 representing the heme (green)-flavin (yellow-orange) domain, heme (blue), and FMN (magenta) complex. The enlarged part of the figure shows the heme-FMN interface. Reproduced with permission from ref. [58]; copyright National Academy of Sciences of the United States of America 1999.

As H_2O_2 is liquid so the mass transfer compared to aeration is easy and quicker, and no need for co-reductant, stability, solubility in an aqueous environment makes the peroxygenase more promising for applicative purposes.[61] One of the best described UPOs is the enzyme from *Agrocybe aegerita* (*Aae*UPO) capable of selective alkane hydroxylation. *Aae*UPO has a broad substrate range and able to catalyze reactions such as hydroxylation, epoxidation,[62] sulfoxidation,[63] phenol oxidation[64] and ether cleavage.[65] In 2011 Hofrichter and his team described the hydrogen abstraction and oxygen rebound mechanism for *Aae*UPO.[66] This mechanism is similar to the P450's hydroxylase activity, which expands the scope of peroxygenases for oxygen incorporation into saturated C–H bonds of alkanes to give respective alcohols with an enantiomeric excess (*ee*) up to 99%.

It had been experimentally proven that *Aae*UPO hydroxylates 2,4- dimethylpentane at the C3 position to 2,4-dimethylpentan-3-ol and 2,3,4-trimethylpentane to 2,3,4-trimethylpentan-3-ol.[66] Furthermore, it was reported that *Aae*UPO is capable of hydroxylating linear, cyclic and branched alkanes. *Aae*UPO hydroxylates the linear alkanes (C_3–C_{16}) with preferable regioselectivity for secondary and tertiary carbons instead of primary carbon. For instance, gaseous alkanes such as *n*-propane and *n*-butane were converted into 2-propanol and 2-butanol. It has been shown that *Aae*UPO can oxidize small alkanes like ethane, which is important for fuel applications.[67] It has also been demonstrated that *Aae*UPO hydroxylates other small alkanes as reflected in Figure 10.

*Aae*UPO also hydroxylates the cyclic and branched alkanes, exemplified by 2,3-dimethyl butane to 2,3-dimethyl butane-2-ol, cyclohexane to cyclohexanol with an insignificant amount of cyclohexanone, and isobutane to 2-methyl-propan-2-ol.[67] In the above cases, the *Aae*UPO exhibited higher *ee* values for 2° alcohol in comparison to 3° alcohol products.[66] To reflect the enzyme for industrial and specific purposes, the *Aae*UPO crystal structure was solved at 2.2 Å[68b] (Figure 11).

The described reaction mechanism is similar to the peroxide-shunt pathway of P450 enzymes (Figure 12).[66] 1) In the resting state, Fe exists in the ferric state with a water molecule at the distal side. Kinne and co-workers suggested that H_2O_2 binds first to form an extremely short-lived Fe^{III}-

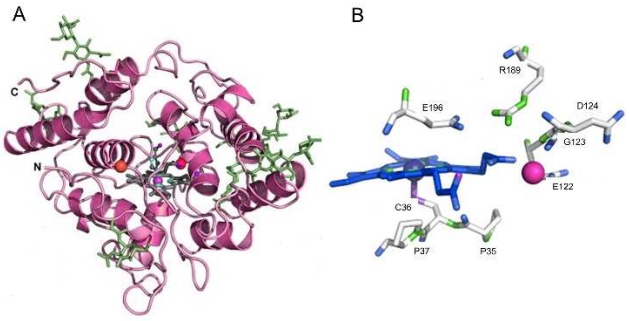

Figure 11. A) Crystal of *Aae*UPO revealed structural details of the enzyme with heme, 4(5)-(hydroxymethyl)imidazole (MZ0) as ball and sticks, carbohydrate, and chlorite (red), and disulfide bridge. It revealed ten α helices and five short β sheets with cone frustam shaped heme channel, consisting of hydrophobic aromatic residues to direct the substrate towards the active site. B) The active site with the heme-binding region contains the PCP-motif (Pro35-Cys36-Pro37) with the axial ligand and the EGD-motif (Glu122-Gly123-Asp124). Blue and magenta balls are heme and magnesium, respectively. Reproduced with permission from ref. [68]; copyright American Society for Biochemistry and Molecular Biology 2013.

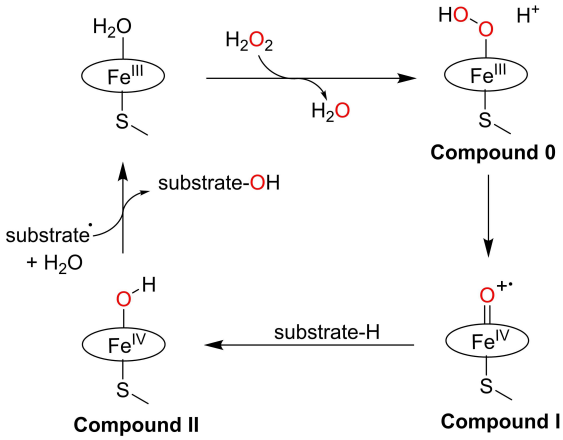

Figure 12. UPO contains Fe^{III} and distal water at the resting stage and reacts with peroxide and forms a ferric peroxo complex known as compound **0**. Negatively charged compound **0** cleaved heterolytically and transformed key intermediate oxo-ferryl cation radical complex (compound **I**). Compound **I** remove a hydrogen atom from the substrate and form compound **II** and an alkyl radical. Alkyl radical combines with oxygen and forms a hydroxylated product.

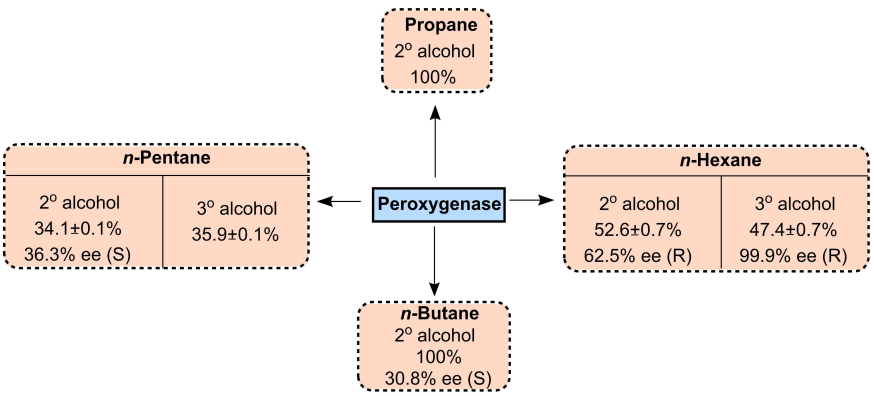

Figure 10. Distribution of products and % enantiomeric excess (ee) of small alkanes by peroxygenase. Data are taken from ref. [66].

peroxide complex known as compound **0**. However, it remains elusive either the substrate or H_2O_2 binds first to the enzyme. 2) Compound **0**, heme(Fe^{III}–O–OH)] heterolytically cleaves the O–O bond under loss of water and a highly reactive (heme (Fe^{IV}=O)$^{\bullet}$+) cation radical (compound **I**). Compound **I** has a specific UV/Vis spectrum and is captured by stopped-flow measurements. 3) Compound **I** is able to abstract a hydrogen from the substrate and form the radical intermediate and protonated compound **II** (heme Fe^{IV}–OH). In contrast to compound **I**, compound **II** measurements were not possible due to a short lifetime thus far. 4) The radical intermediate has a short lifetime of around 10 ps and readily recombines with the hydroxide from compound **II** by a rebound mechanism to form the hydroxylation product and recover the enzyme in resting-state Fe^{III}.[69]

To date, more than 2500 fungal peroxygenases genome contains thousands of undescribed UPO have been deposited in the NCBI database.[70] However, owing to their posttranslational modifications, the heterologous production in prokaryotic hosts is challenging. Recently, substantial efforts were made towards the heterologous expression of a range of UPOs in *S. cerevisiae* and *P. pastoris*. A modular system was developed, which permitted the rapid screening of different signal peptides and led to the expression of six UPOs for the first time in yeast and the characterization of four of them.[71] In an expanded approach, an episomal *P. pastoris* expression system allowing the variation of the promoter resulted in two further new UPOs.[71]

Besides the vast pool of available putative UPO sequences, protein engineering of UPOs towards increased activities has also been achieved for *Aae*UPO.[72] By an *E. coli* expression system and computational models the *Marasmius rotula* enzyme (*Mro*UPO)[73] and *Collariella virescens* (*Cvi*UPO) have been shown to be able to be engineered.[74] The promising advances made in the heterologous production of UPOs and their optimization by protein engineering will further boost the development of the peroxygenase field and its application for industrial relevant substrates and processes.

2.5. Other enzymes

On the one hand alkanes are toxic to many microorganisms[75] but on the other hand, various microorganisms can utilize alkanes as carbon and energy sources. Gram-negative and gram-positive bacterial classes, some yeasts, filamentous fungi, and algal prototheca genus can consume alkanes.[76] Apart from the very relevant enzymes mentioned above, more than 500 species of bacteria and fungi can utilize the alkanes.[47] The different attributes of aerobic and anaerobic degradation via bacteria are well outlined in review[77] and mentioned in Table 2. For example, distinct aspects of methane utilizing MMOs have been described in some excellent reviews.[14a,78] Industrial important native and engineered CytP450 and peroxygenase are well summarized in recent excellent articles.[7b,59a,66,79] Together with these, for a convenient overview we have briefed enzymes having an active site for C–H bond activation in Table 2.

3. Practical Considerations and Concepts

From the application point of view, the purification, stability, activity, and substrate scope, etc. of an enzyme are key factors to consider. For instance, sMMO is stable and easy to purify in comparison to pMMO.[95] pMMO is unstable in crude extract although the addition of copper ions improves the stability of exfoliated pMMO.[96] In the case of chemical specificity, regioselectivity, and stereoselectivity, pMMO exhibits narrow window in response to sMMO. The specific activity of sMMO and pMMO varies for the same substrate.[97] sBMO shows slow turnover frequency for butane, ethane, methane, propane, and pentane (from 0.12, 0.76, 1.3, 0.55, and 0.008 s^{-1}, respectively).[43b] Thus, improving the enzyme activity of sMMO and pMMO and the solubility of pMMO remain as key issues of methane monooxygenases.

The pioneering work of direct evolution introduced by Arnold group reported the engineered Cytp450BM3 by implication of the same approach.[98] Variants such as 35E11, 19A12, 11–3, and 1–3 displayed C_2–C_{10} (ethane to decane) oxidation with NADPH cofactor with high turnover numbers.[82a]

The dummy substrate (perfluorocarboxylic acid) is another inspiring approach to enhancing the activity of the CytP450BM3. For instance, PFC10 and PFC 9 promoted the hydroxylation of butane to 2-butanol with a product rate of 100 and 113 min^{-1} whereas PFC9 formed cyclohexane with a rate of 110 min^{-1} and coupling efficiency of 35%.[99] Moreover, the strategy of dummy substrate allowing for a switch of P450 monooxygenases into peroxygenases is worthy considering to enhance the enzyme turnover numbers in small alkane oxidation.[59a,100]

Like all heme-containing enzymes, peroxygenases display sensitivity towards H_2O_2.[101] To sustain the peroxygenase-catalyzed reactions, an optimal concentration of H_2O_2 that complies with its consumption by peroxygenases is essential.[102] A number of methodologies have been developed for in situ H_2O_2 generation to circumvent this problem.[103] Amongst them is the well-known glucose oxidase system which generates H_2O_2 from O_2 by glucose oxidation.[104] Alternative methods with higher atom-efficiency have been developed such as electrochemical oxidation of H_2O,[105] enzymatic oxidation of co-substrates,[106] generation of H_2O_2 using H_2 and O_2,[107] coupling methods based on heterogeneous TiO_2,[107–108] and the utilization of polymerized inorganic g-C_3N_4 and irradiation.[106c,109] With these systems, total turnover numbers close to 300000 were achieved, revealing the synthetic potential of these catalysts.[106] The combination of immobilized enzyme and organic *tert*-butyl hydroperoxide[110] is also useful solution to address the peroxide inactivation.

AlkB is a moderately stable enzyme from days to months upon storage between 4 and −20 °C.[111] The reported specific activity of AlkB differs significantly. AlkB from *Pseudomonas oleovorans* wild type and mutants showed specific activity in a range of 0.57 to 596 μmol min^{-1} mg^{-1},[112] whereas the AlkB from *Pseudomonas putida* is a few orders of magnitude lower (0.00012 to 0.00773 μmol min^{-1} mg^{-1}),[113] leaving space of improvement of the enzyme activity in future.

Table 2. Different metalloenzymes involved in C–H bond activation and oxidation of the respective alkanes.[a]

Enzymes	Organism	Metal ions	Substrate	Ref.
Cytochrome P450 (CYP52, Class II)	*Candida maltosa*, *Candida tropicalls*, *Yarrowia lipolytica*	Heme-thiolate	C_{10}–C_{16} *n*-alkanes	[80]
Bacterial P450 oxygenase system (CYP153, Class I)	*Sphingomonas* sp. HXN-200, *Mycobacterium* sp. HXN1500 *Acinetobacter* sp. EB104		C_4–C_{16} *n*-alkanes	[81]
Engineered P450cam, P450BM-3, DSFM	*Pseudomonas putida*, **Bacillus** *megaterium*		C_3–C_{10} *n*-alkanes	[59a, 82]
Fungal peroxygenase	*Agrocybe aegerita*		linear, cyclic and branched alkanes	[66]
pMMO	Methanotrophs	Dicopper	C_1–C_5	[83]
Dioxygenase	*Acinetobacter* sp.		C_{10}–C_{30} *n*-alkanes	[84]
NDO	*Pseudomonas putida*	Mono-iron (His2Asp)	naphthalene derivatives, monooxygenase reactions	[85]
TauD	*Escherichia coli*		taurine to 2-amino-2-hydroxyethanesulfonic acid	[86]
sMMO	*Methylococcus Capsulatus*, *Methylisinus Trichosporum OB3b*	Diiron (His2-Glu2)	C_1–C_{10} *n*-alkanes	[22]
pMMO	All Methanotrophs		C_1–C_5 alkane	[83]
BMO	*Pseudomonas butanovora* ATCC 43655)		C_3 and C_{13}–C_{22} *n*-alkanes	[43b]
AlkB	*Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* etc.		medium to long-chain alkanes to alcohols C_5–C_{16} *n*-alkanes	[49]
Propane monooxygenase	*Gordonia* sp.		C_2–C_8 *n*-alkanes	[87]
Ammonia monooxygenase	*Nitrosomonas europaea*		C_1–C_8 *n*-alkanes	[88]
TMO	**Burkholderia cepacia** G4		hydroxylation of the methyl group on toluene	[89]
XylM	*Pseudomonas putida*	Diiron (HXXXH)4	hydroxylation of the methyl group on xylene	[90]
Desaturases	Fungus, Bacteria, *Chlorella vulgaris*,		desaturation of fatty acids	[91]
Alkyl succinate synthase	*Desulfatibacillum alkenivorans* AK-01	Fe–S cluster + glycyl radical	alkanes and fumarate to form (1-methyl alkyl) succinate	[92]
LadA	*Geobacillus thermodenitrificans* NG80-2	Flavin	C_{15}–C_{36} *n*-alkanes	[93]
AlmA	*Acinetobacter* strain, DSM 17874,			
MCR	**Methanothermobacter marburgensis**	Ni F430	conversion of methane and the heterodisulfide of coenzymes M and B to methyl coenzyme M and coenzyme B	[94]

[a] pMMO = particulate methane monooxygenase; NDO = mono-iron naphthalene-1,2-dioxygenases; TauD = taurine:α-ketoglutarate dioxygenase; sMMO = soluble methane monooxygenase; BMO = butane monooxygenase; TMO = toluene monooxygenase; XylM = xylene monooxygenase hydroxylase; LadA = flavin-dependent oxidoreductase; AlmA = flavin-binding monooxygenase.

Apart from the performance of the enzymes themselves determine their usefulness in small alkane valorization, the poor solubility of gaseous alkanes remains also a big challenge limiting the enzyme activity and product titers. To address this issue, recently a bubble column reactor (2 L scale) was designed to allow peroxygenase catalyzing butane into 2-butanol with an overall TTN of 15000.[114] Moreover, an optimized production process of pMMO in a hollow-fiber membrane bioreactor has been used to give better control over concentration of copper (associated to specific enzyme activity and salt resistance).[115] Thus, the process engineering combined with reactor design holds great potential for preparative oxidation of inert and gaseous alkanes.

4. Concluding Remarks

It is well known that alkanes are very inert molecules in nature. Alkanes undergo oxidation and lead to valuable alcohols, aldehydes, ketones, and acids which have been used widely in chemical industry. However, alkanes do not easily react with oxygen, even in the presence of chemical catalysts, at room temperature. Selective C–H activation and functionalization is a class of reactions that could lead to a paradigm shift in organic synthesis. Metalloenzymes have shown good stereo- and regioselectivity in C–H bond functionalization reactions. This is promising not only in expanding general organic synthesis but also in converting small alkanes into value-added chemicals under ambient conditions approaching economic viability.

Although the integration of metalloenzymes for alkane oxidation shows high potential for future applications, several challenges remain, such as the need for cofactors, byproduct formation, poor in vivo performance, and small-scale reaction. The active sites of some enzymes are delineated and reaction mechanisms have been resolved but a detailed study of every key intermediate must be done, and the chemistry of the catalytic mechanism still needs to be defined. Detailed characterization will be helpful in the rational design of strategies for protein engineering. Substantial progress has been made in this regard; several enzymes have been reported in uniport but few have been characterized. Methods of protein engineering, such as structural prediction and directed evolution of natural and mutant enzymes have been reported.

Some successful developments, however, need to be further optimized for preparative-scale use of enzymes in alkane oxidation, where several enzymes are still often required for similar applications with high selectivity and efficiency. We can anticipate the genomic analysis of enzymes from different sources and structural comparison for some potential enzyme sources. Furthermore, bioinformatic analyses will help to reduce the experimental efforts and hit some potential targets with great performance. The characterization of selected enzymes combined with direct evolution and new methods of protein engineering will enable the development of promising biocatalysts. There is still the question of whether we can combine two different metalloenzymes into a single moiety for potential tandem and cascade reactions. Reaction engineering should receive great attention to bring the biocatalytic valorization of small alkanes forward to large-scale application. Despite the successes achieved to date, there is still much room for improvement to achieve the ideal biocatalysts for optimized reactions under ambient conditions.

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Conflict of Interest

The authors declare no conflict of interest.

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[1] IEA, Methane Tracker 2020, Paris, **2020**
[2] Butane Market Size, Share & Trends Analysis Report By Application (LPG (Residential/Commercial, Chemical/ Petrochemical, Industrial, Auto fuel, Refinery), Petrochemicals, Refineries),By Region, And Segment Forecasts, 2018–2025, **2016**.
[3] a) N. L. Söhngen, *K. Ned. Akad. Wet. Proc. Ser. B* **1905**, *8*, 327–331; b) N. L. Söhngen, *K. Ned. Akad. Wet. Proc. Ser. B* **1913**, *15*, 1145–1151.
[4] a) A. B. Guerra, J. S. Oliveira, R. C. B. Silva-Portela, W. Araujo, A. C. Carlos, A. T. R. Vasconcelos, A. T. Freitas, Y. S. Domingos, M. F. de Farias, G. J. T. Fernandes, L. F. Agnez-Lima, *Environ. Pollut.* **2018**, *235*, 869–880; b) D. J. Lea-Smith, S. J. Biller, M. P. Davey, C. A. Cotton, B. M. Perez Sepulveda, A. V. Turchyn, D. J. Scanlan, A. G. Smith, S. W. Chisholm, C. J. Howe, *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 13591–13596; c) R. Margesin, D. Labbe, F. Schinner, C. W. Greer, L. G. Whyte, *Appl. Environ. Microbiol.* **2003**, *69*, 3085–3092; d) E. Z. Ron, E. Rosenberg, *Curr. Opin. Biotechnol.* **2014**, *27*, 191–194.
[5] R. J. W. Brooijmans, M. I. Pastink, R. J. Siezen, *Microb. Biotechnol.* **2009**, *2*, 587–594.
[6] N. Dombrowski, J. A. Donaho, T. Gutierrez, K. W. Seitz, A. P. Teske, B. J. Baker, *Nat. Microbiol.* **2016**, *1*, 16057.
[7] a) A. Bakac, *Inorg. Chem.* **2010**, *49*, 3584–3593; b) M. Costas, *Coord. Chem. Rev.* **2011**, *255*, 2912–2932.
[8] J. Münch, P. Püllmann, W. Zhang, M. J. Weissenborn, *ACS Catal.* **2021**, 9168–9203.
[9] L. M. Blank, B. E. Ebert, K. Buehler, B. Bühler, *Antioxid. Redox Signal.* **2010**, *13*, 349–394.
[10] N. L. Söhngen, *Centralbl. Bakteriol. Parasitenkd. Infektionskrankh. Hyg. Abt.* **1906**, 513–517.
[11] a) J. D. Semrau, A. A. DiSpirito, S. Yoon, *FEMS Microbiol. Rev.* **2010**, *34*, 496–531; b) Y. A. Trotsenko, J. C. Murrell, *Adv. Appl. Microbiol.* **2008**, *63*, 183–229.
[12] a) S. J. Blanksby, G. B. Ellison, *Acc. Chem. Res.* **2003**, *36*, 255–263; b) Y.-R. Luo, *Handbook of Bond Dissociation Energies in Organic Compounds*, CRC Press, Boca Raton, FL, **2002**.
[13] a) M. Kanehisa, *Protein Sci.* **2019**, *28*, 1947–1951; b) M. Kanehisa, M. Furumichi, Y. Sato, M. Ishiguro-Watanabe, M. Tanabe, *Nucleic Acids Res.* **2021**, *49*, D545-D551; c) M. Kanehisa, S. Goto, *Nucleic Acids Res.* **2000**, *28*, 27–30.
[14] a) C. E. Tinberg, S. J. Lippard, *Acc. Chem. Res.* **2011**, *44*, 280–288; b) B. J. Wallar, J. D. Lipscomb, *Chem. Rev.* **1996**, *96*, 2625–2658.
[15] a) V. Izzo, G. Leo, R. Scognamiglio, L. Troncone, L. Birolo, A. Di Donato, *Arch. Biochem. Biophys.* **2010**, *505*, 48–59; b) M. H. Sazinsky, M. Merkx, E. Cadieux, S. Tang, S. J. Lippard, *Biochemistry* **2004**, *43*, 16263–16276; c) V. Srinivas, R. Banerjee, H. Lebrette, J. C. Jones, O. Aurelius, I.-S. Kim, C. C. Pham, S. Gul, K. D. Sutherlin, A. Bhowmick, J. John, E. Bozkurt, T. Fransson, P. Aller, A. Butryn, I. Bogacz, P. Simon, S. Keable, A. Britz, K. Tono, K. S. Kim, S.-Y. Park, S. J. Lee, J. Park, R. Alonso-Mori, F. D. Fuller, A. Batyuk, A. S. Brewster, U. Bergmann, N. K. Sauter, A. M. Orville, V. K. Yachandra, J. Yano, J. D. Lipscomb, J. Kern, M. Högbom, *J. Am. Chem. Soc.* **2020**, *142*, 14249–14266.
[16] D. A. Whittington, S. J. Lippard, *J. Am. Chem. Soc.* **2001**, *123*, 827–838.
[17] A. C. Rosenzweig, H. Brandstetter, D. A. Whittington, P. Nordlund, S. J. Lippard, C. A. Frederick, *Proteins* **1997**, *29*, 141–152.
[18] Y. Liu, J. C. Nesheim, S. K. Lee, J. D. Lipscomb, *J. Biol. Chem.* **1995**, *270*, 24662–24665.
[19] a) K. E. Liu, A. M. Valentine, D. Wang, B. H. Huynh, D. E. Edmondson, A. Salifoglou, S. J. Lippard, *J. Am. Chem. Soc.* **1995**, *117*, 10174–10185; b) L. Shu, J. C. Nesheim, K. Kauffmann, E. Münck, J. D. Lipscomb, L. Que, Jr., *Science* **1997**, *275*, 515–518; c) A. M. Valentine, S. S. Stahl, S. J. Lippard, *J. Am. Chem. Soc.* **1999**, *121*, 3876–3887.
[20] a) C. E. Tinberg, S. J. Lippard, *Biochemistry* **2009**, *48*, 12145–12158; b) R. Banerjee, K. K. Meier, E. Münck, J. D. Lipscomb, *Biochemistry* **2013**, *52*, 4331–4342; c) R. Banerjee, Y. Proshlyakov, J. D. Lipscomb, D. A. Proshlyakov, *Nature* **2015**, *518*, 431–434.
[21] S. K. Lee, B. G. Fox, W. A. Froland, J. D. Lipscomb, E. Munck, *J. Am. Chem. Soc.* **1993**, *115*, 6450–6451.
[22] M. H. Baik, M. Newcomb, R. A. Friesner, S. J. Lippard, *Chem. Rev.* **2003**, *103*, 2385–2419.
[23] B. G. Fox, W. A. Froland, D. R. Jollie, J. D. Lipscomb, *Methods Enzymol.* **1990**, *188*, 191–202.
[24] J. Green, H. Dalton, *J. Biol. Chem.* **1985**, *260*, 15795–15801.
[25] J. Colby, D. I. Stirling, H. Dalton, *Biochem. J.* **1977**, *165*, 395–402.
[26] S. Grosse, L. Laramee, K. D. Wendlandt, I. R. McDonald, C. B. Miguez, H. P. Kleber, *Appl. Environ. Microbiol.* **1999**, *65*, 3929–3935.
[27] S. Sirajuddin, D. Barupala, S. Helling, K. Marcus, T. L. Stemmler, A. C. Rosenzweig, *J. Biol. Chem.* **2014**, *289*, 21782–21794.
[28] R. B. Cooley, B. L. Dubbels, L. A. Sayavedra-Soto, P. J. Bottomley, D. J. Arp, *Microbiology* **2009**, *155*, 2086–2096.
[29] M. M. Chen, P. S. Coelho, F. H. Arnold, *Adv. Synth. Catal.* **2012**, *354*, 964–968.

[30] F. E. Zilly, J. P. Acevedo, W. Augustyniak, A. Deege, U. W. Häusig, M. T. Reetz, *Angew. Chem. Int. Ed.* **2011**, *50*, 2720–2724; *Angew. Chem.* **2011**, *123*, 2772–2776.
[31] R. Fasan, Y. T. Meharenna, C. D. Snow, T. L. Poulos, F. H. Arnold, *J. Mol. Biol.* **2008**, *383*, 1069–1080.
[32] S. G. Bell, J.-A. Stevenson, H. D. Boyd, S. Campbell, A. D. Riddle, E. L. Orton, L.-L. Wong, *Chem. Commun.* **2002**, 490–491.
[33] P. Meinhold, M. W. Peters, M. M. Chen, K. Takahashi, F. H. Arnold, *ChemBioChem* **2005**, *6*, 1765–1768.
[34] a) H. H. Nguyen, A. K. Shiemke, S. J. Jacobs, B. J. Hales, M. E. Lidstrom, S. I. Chan, *J. Biol. Chem.* **1994**, *269*, 14995–15005; b) M. Takeguchi, K. Miyakawa, I. Okura, *J. Mol. Catal. A* **1998**, *132*, 145–153.
[35] R. L. Lieberman, A. C. Rosenzweig, *Nature* **2005**, *434*, 177–182.
[36] a) S. I. Chan, S. S. Yu, *Acc. Chem. Res.* **2008**, *41*, 969–979; b) Y. J. Lu, M. C. Hung, B. T. Chang, T. L. Lee, Z. H. Lin, I. K. Tsai, Y. S. Chen, C. S. Chang, Y. F. Tsai, K. H. Chen, S. I. Chan, S. S. Yu, *J. Inorg. Biochem.* **2019**, *196*, 110691.
[37] a) P. P.-Y. Chen, P. Nagababu, S. S.-F. Yu, S. I. Chan, *ChemCatChem* **2014**, *6*, 429–437; b) M. A. Culpepper, A. C. Rosenzweig, *Crit. Rev. Biochem. Mol. Biol.* **2012**, *47*, 483–492.
[38] R. G. Castillo, R. Banerjee, C. J. Allpress, G. T. Rohde, E. Bill, L. Que, J. D. Lipscomb, S. DeBeer, *J. Am. Chem. Soc.* **2017**, *139*, 18024–18033.
[39] a) S. I. Chan, S. S. F. Yu, *Nat. Catal.* **2019**, *2*, 286–287; b) P. Nagababu, S. S. F. Yu, S. Maji, R. Ramu, S. I. Chan, *Catal. Sci. Technol.* **2014**, *4*, 930–935.
[40] N.-L. Nguyen, W.-J. Yu, H.-Y. Yang, J.-G. Kim, M.-Y. Jung, S.-J. Park, S.-W. Roh, S.-K. Rhee, *J. Microbiol.* **2017**, *55*, 775–782.
[41] J. Takahashi, Y. Ichikawa, H. Sagae, I. Komura, H. Kanou, K. Yamada, *Agric. Biol. Chem.* **1980**, *44*, 1835–1840.
[42] D. J. Arp, *Microbiology* **1999**, *145*, 1173–1180.
[43] a) J. G. Leahy, P. J. Batchelor, S. M. Morcomb, *FEMS Microbiol. Rev.* **2003**, *27*, 449–479; b) M. K. Sluis, L. A. Sayavedra-Soto, D. J. Arp, *Microbiology* **2002**, *148*, 3617–3629; c) J. B. van Beilen, E. G. Funhoff, *Appl. Microbiol. Biotechnol.* **2007**, *74*, 13–21.
[44] W. A. Froland, K. K. Andersson, S. K. Lee, Y. Liu, J. D. Lipscomb, *J. Biol. Chem.* **1992**, *267*, 17588–17597.
[45] B. L. Dubbels, L. A. Sayavedra-Soto, D. J. Arp, *Microbiol.* **2007**, *153*, 1808–1816.
[46] J. N. Baptist, R. K. Gholson, M. J. Coon, *Biochim. Biophys. Acta.* **1963**, *69*, 40–47.
[47] L. Wang, W. Wang, Q. Lai, Z. Shao, *Environ. Microbiol.* **2010**, *12*, 1230–1242.
[48] L. G. Whyte, A. Schultz, J. B. Van Beilen, A. P. Luz, V. Pellizari, D. Labbé, C. W. Greer, *FEMS Microbiol. Ecol.* **2002**, *41*, 141–150.
[49] T. H. M. Smits, S. B. Balada, B. Witholt, J. B. Van Beilen, *J. Bacteriol.* **2002**, *184*, 1733–1742.
[50] a) J. A. Peterson, D. Basu, M. J. Coon, *J. Biol. Chem.* **1966**, *241*, 5162–5164; b) J. Shanklin, C. Achim, H. Schmidt, B. G. Fox, E. Münck, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2981–2986.
[51] H. Alonso, A. Roujeinikova, *Appl. Environ. Microbiol.* **2012**, *78*, 7946–7953.
[52] J. B. van Beilen, M. G. Wubbolts, B. Witholt, *Biodegradation* **1994**, *5*, 161–174.
[53] E. L. Johnson, M. R. Hyman, *Appl. Environ. Microbiol.* **2006**, *72*, 950–952.
[54] J. B. van Beilen, J. Kingma, B. Witholt, *Enzyme Microb. Technol.* **1994**, *16*, 904–911.
[55] D. R. Nelson, *Biochim. Biophys. Acta Proteins Proteom.* **2018**, *1866*, 141–154.
[56] a) S. Janocha, D. Schmitz, R. Bernhardt, *Adv. Biochem. Eng./Biotechnol.* **2015**, *148*, 215–250; b) V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2019**, *37*, 882–897.
[57] a) A. Glieder, E. T. Farinas, F. H. Arnold, *Nat. Biotechnol.* **2002**, *20*, 1135–1139; b) M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450.
[58] I. F. Sevrioukova, H. Li, H. Zhang, J. A. Peterson, T. L. Poulos, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1863–1868.
[59] a) J. Chen, F. Kong, N. Ma, P. Zhao, C. Liu, X. Wang, Z. Cong, *ACS Catal.* **2019**, *9*, 7350–7355; b) N. Ma, Z. Chen, J. Chen, J. Chen, C. Wang, H. Zhou, L. Yao, O. Shoji, Y. Watanabe, Z. Cong, *Angew. Chem. Int. Ed.* **2018**, *57*, 7628–7633; *Angew. Chem.* **2018**, *130*, 7754–7759.
[60] S. B. J. P. Willot, F. Tieves, M. Girhard, V. B. Urlacher, F. Hollmann, G. de Gonzalo, *Catalysts* **2019**, *9*, 567.
[61] R. Ullrich, M. Hofrichter, *Cell. Mol. Life Sci.* **2007**, *64*, 271–293.
[62] a) M. Kinne, M. Poraj-Kobielska, E. Aranda, R. Ullrich, K. E. Hammel, K. Scheibner, M. Hofrichter, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3085–3087; b) M. Kinne, C. Zeisig, R. Ullrich, G. Kayser, K. E. Hammel, M. Hofrichter, *Biochem. Biophys. Res. Commun.* **2010**, *397*, 18–21.
[63] R. Ullrich, C. Dolge, M. Kluge, M. Hofrichter, *FEBS Lett.* **2008**, *582*, 4100–4106.
[64] E. Aranda, R. Ullrich, M. Hofrichter, *Biodegradation* **2009**, *21*, 267–281.
[65] G. F. White, N. J. Russell, E. C. Tidswell, *Microbiol. Rev.* **1996**, *60*, 216–232.
[66] S. Peter, M. Kinne, X. Wang, R. Ullrich, G. Kayser, J. T. Groves, M. Hofrichter, *FEBS J.* **2011**, *278*, 3667–3675.
[67] X. Wang in *A Novel Heme-Thiolate Peroxygenase AaeAPO and Its Implications for C–H Activation Chemistry*, Springer International Publishing, Cham, **2016**, pp. 23–40.
[68] a) M. J. Pecyna, R. Ullrich, B. Bittner, A. Clemens, K. Scheibner, R. Schubert, M. Hofrichter, *Appl. Microbiol. Biotechnol.* **2009**, *84*, 885–897; b) K. Piontek, E. Strittmatter, R. Ullrich, G. Gröbe, M. J. Pecyna, M. Kluge, K. Scheibner, M. Hofrichter, D. A. Plattner, *J. Biol. Chem.* **2013**, *288*, 34767–34776.
[69] M. Hofrichter, R. Ullrich, *Curr. Opin. Chem. Biol.* **2014**, *19*, 116–125.
[70] A. Kinner, K. Rosenthal, S. Lütz, *Front. Bioeng. Biotechnol.* **2021**, DOI: 10.3389/fbioe.2021.705630.
[71] P. Püllmann, A. Knorrscheidt, J. Münck, P. R. Palme, W. Hoehenwarter, S. Marillonnet, M. Alcalde, B. Westermann, M. J. Weissenborn, *Commun. Biol.* **2021**, *4*, 562.
[72] a) P. Gomez de Santos, M. Cañellas, F. Tieves, S. H. H. Younes, P. Molina-Espeja, M. Hofrichter, F. Hollmann, V. Guallar, M. Alcalde, *ACS Catal.* **2018**, *8*, 4789–4799; b) D. M. Mate, M. A. Palomino, P. Molina-Espeja, J. Martin-Diaz, M. Alcalde, *Protein Eng. Des. Sel.* **2017**, *30*, 189–196; c) P. Molina-Espeja, M. Cañellas, F. J. Plou, M. Hofrichter, F. Lucas, V. Guallar, M. Alcalde, *ChemBioChem* **2016**, *17*, 341–349; d) P. Molina-Espeja, E. Garcia-Ruiz, D. Gonzalez-Perez, R. Ullrich, M. Hofrichter, M. Alcalde, *Appl. Environ. Microbiol.* **2014**, *80*, 3496–3507.
[73] M. Municoy, A. González-Benjumea, J. Carro, C. Aranda, D. Linde, C. Renau-Mínguez, R. Ullrich, M. Hofrichter, V. Guallar, A. Gutiérrez, A. T. Martínez, *ACS Catal.* **2020**, *10*, 13584–13595.
[74] D. Linde, A. Olmedo, A. González-Benjumea, M. Estévez, C. Renau-Mínguez, J. Carro, E. Fernández, A. Gutiérrez, A. T. Martínez, *Appl. Environ. Microbiol.* **2020**, *86*, e02899-19.
[75] a) J. Kieboom, J. J. Dennis, J. A. de Bont, G. J. Zylstra, *J. Biol. Chem.* **1998**, *273*, 85–91; b) J. Sikkema, J. A. de Bont, B. Poolman, *Microbiol. Rev.* **1995**, *59*, 201–222; c) R. J. Watkinson, P. Morgan, *Biodegradation* **1990**, *1*, 79–92.
[76] C. Schmitz, I. Goebel, S. Wagner, A. Vomberg, U. Klinner, *Appl. Microbiol. Biotechnol.* **2000**, *54*, 126–132.
[77] F. Abbasian, R. Lockington, M. Mallavarapu, R. Naidu, *Appl. Biochem. Biotechnol.* **2015**, *176*, 670–699.
[78] a) M. H. Sazinsky, S. J. Lippard, *Met. Ions Life Sci.* **2015**, *15*, 205–256; b) S. Sirajuddin, A. C. Rosenzweig, *Biochemistry* **2015**, *54*, 2283–2294; c) V. C. Wang, S. Maji, P. P. Chen, H. K. Lee, S. S. Yu, S. I. Chan, *Chem. Rev.* **2017**, *117*, 8574–8621.
[79] a) M. Costas, K. Chen, L. Que, *Coord. Chem. Rev.* **2000**, *200–202*, 517–544; b) S. Kumar, *Expert Opin. Drug Metab. Toxicol.* **2010**, *6*, 115–131; c) M. C. R. Sigmund, G. J. Poelarends, *Nat. Catal.* **2020**, *3*, 690–702.
[80] D. L. Craft, K. M. Madduri, M. Eshoo, C. R. Wilson, *Appl. Environ. Microbiol.* **2003**, *69*, 5983–5991.
[81] T. Maier, H. H. Forster, O. Asperger, U. Hahn, *Biochem. Biophys. Res. Commun.* **2001**, *286*, 652–658.
[82] a) R. Fasan, Y. T. Meharenna, C. D. Snow, T. L. Poulos, F. H. Arnold, *J. Mol. Biol.* **2008**, *383*, 1069–1080; b) M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450.
[83] R. L. Lieberman, A. C. Rosenzweig, *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 147–164.
[84] J. H. Maeng, Y. Sakai, T. Ishige, Y. Tani, N. Kato, *FEMS Microbiol. Lett.* **1996**, *141*, 177–182.
[85] S. M. Resnick, K. Lee, D. T. Gibson, *J. Ind. Microbiol. Biotechnol.* **1996**, *17*, 438–457.
[86] J. M. Bollinger, Jr., J. C. Price, L. M. Hoffart, E. W. Barr, C. Krebs, *Eur. J. Inorg. Chem.* **2005**, 4245–4254.
[87] T. Kotani, T. Yamamoto, H. Yurimoto, Y. Sakai, N. Kato, *J. Bacteriol. Res.* **2003**, *185*, 7120–7128.
[88] M. R. Hyman, I. B. Murton, D. J. Arp, *Appl. Environ. Microbiol.* **1988**, *54*, 3187–3190.
[89] J. D. Pikus, J. M. Studts, C. Achim, K. E. Kauffmann, E. Münck, R. J. Steffan, K. McClay, B. G. Fox, *Biochemistry* **1996**, *35*, 9106–9119.
[90] J. P. Shaw, S. Harayama, *J. Ferment. Bioeng.* **1995**, *79*, 195–199.
[91] J. Shanklin, E. B. Cahoon, *Annu. Rev. Plant Biol.* **1998**, *49*, 611–641.

[92] A. V. Callaghan, B. E. L. Morris, I. A. C. Pereira, M. J. McInerney, R. N. Austin, J. T. Groves, J. J. Kukor, J. M. Suflita, L. Y. Young, G. J. Zylstra, *Environ. Microbiol.* **2012**, *14*, 101–113.
[93] R. N. Austin, J. T. Groves, *Metallomics* **2011**, *3*, 775–787.
[94] S. Scheller, M. Goenrich, R. Boecher, R. K. Thauer, B. Jaun, *Nature* **2010**, *465*, 606–608.
[95] M. Merkx, S. J. Lippard, *J. Biol. Chem.* **2002**, *277*, 5858–5865.
[96] J. Y. Xin, J. R. Cui, X. X. Hu, S. B. Li, C. G. Xia, L. M. Zhu, Y. Q. Wang, *Biochem. Biophys. Res. Commun.* **2002**, *295*, 182–186.
[97] L. Jeske, S. Placzek, I. Schomburg, A. Chang, D. Schomburg, *Nucleic Acids Res.* **2018**, *47*, D542-D549.
[98] E. T. Farinas, U. Schwaneberg, A. Glieder, F. H. Arnold, *Adv. Synth. Catal.* **2001**, *343*, 601–606.
[99] N. Kawakami, O. Shoji, Y. Watanabe, *Angew. Chem. Int. Ed.* **2011**, *50*, 5315–5318; *Angew. Chem.* **2011**, *123*, 5427–5430
[100] O. Shoji, T. Fujishiro, H. Nakajima, M. Kim, S. Nagano, Y. Shiro, Y. Watanabe, *Angew. Chem. Int. Ed.* **2007**, *46*, 3656–3659; *Angew. Chem.* **2007**, *119*, 3730–3733.
[101] a) S. Bormann, A. Gomez Baraibar, Y. Ni, D. Holtmann, F. Hollmann, *Catal. Sci. Technol.* **2015**, *5*, 2038–2052; b) Y. Wang, D. Lan, R. Durrani, F. Hollmann, *Curr. Opin. Chem. Biol.* **2016**, *37*, 1–9.
[102] B. O. Burek, S. Bormann, F. Hollmann, J. Z. Bloh, D. Holtmann, *Green Chem.* **2019**, *21*, 3232–3249.
[103] Y. Li, B. Yuan, Z. Sun, W. Zhang, *Green Synth. Catal.* **2021**, DOI: 10.1016/j.gresc.2021.06.001
[104] B. M. Trost, *Science* **1991**, *254*, 1471–1477.
[105] a) S. Bormann, M. van Schie, T. P. De Almeida, W. Zhang, M. Stöckl, R. Ulber, F. Hollmann, D. Holtmann, *ChemSusChem* **2019**, *12*, 4759–4763; b) D. S. Choi, H. Lee, F. Tieves, Y. W. Lee, E. J. Son, W. Zhang, B. Shin, F. Hollmann, C. B. Park, *ACS Catal.* **2019**, *9*, 10562–10566.
[106] a) Y. Ni, E. Fernández-Fueyo, A. Gomez Baraibar, R. Ullrich, M. Hofrichter, H. Yanase, M. Alcalde, W. J. van Berkel, F. Hollmann, *Angew. Chem. Int. Ed.* **2016**, *55*, 798–801; *Angew. Chem.* **2016**, *128*, 809–812; b) F. Tieves, S. b. J.-P. Willot, M. M. C. H. van Schie, M. C. n. R. e. Rauch, S. H. H. Younes, W. Zhang, J. Dong, P. Gomez de Santos, J. M. Robbins, B. Bommarius, M. Alcalde, A. S. Bommarius, F. Hollmann, *Angew. Chem. Int. Ed.* **2019**, *58*, 7873–7877; *Angew. Chem.* **2018**,*130*,9380–9404; c) S. b. J. P. Willot, E. Fernández-Fueyo, F. Tieves, M. Pesic, M. Alcalde, I. W. C. E. Arends, C. B. Park, F. Hollmann, *ACS Catal.* **2019**, *9*, 890–894.
[107] S. J. Freakley, S. Kochius, J. van Marwijk, C. Fenner, R. J. Lewis, K. Baldenius, S. S. Marais, D. J. Opperman, S. T. L. Harrison, M. Alcalde, M. S. Smit, G. J. Hutchings, *Nat. Commun.* **2019**, *10*, 4178.
[108] W. Zhang, E. Fernández-Fueyo, Y. Ni, M. van Schie, J. Gacs, R. Renirie, R. Wever, F. G. Mutti, D. r. Rother, M. Alcalde, F. Hollmann, *Nat. Catal.* **2018**, *1*, 55–62.
[109] W. Zhang, H. Liu, M. M. C. H. van Schie, P.-L. Hagedoorn, M. Alcalde, A. G. Denkova, K. Djanashvili, F. Hollmann, *ACS Catal.* **2020**, *10*, 14195–14200.
[110] M. C. R. Rauch, F. Tieves, C. E. Paul, I. W. C. E. Arends, M. Alcalde, F. Hollmann, *ChemCatChem* **2019**, *11*, 4519–4523.
[111] a) G. R. Griffith, R. T. Ruettinger, E. J. McKenna, M. J. Coon, *Methods Enzymol.* **1978**, *53*, 356–360; b) S. W. May, A. G. Katopodis, *Methods Enzymol.* **1990**, *188*, 3–9.
[112] a) E. J. McKenna, M. J. Coon, *J. Biol. Chem.* **1970**, *245*, 3882–3889; b) R. T. Ruettinger, G. R. Griffith, M. J. Coon, *Arch. Biochem. Biophys.* **1977**, *183*, 528–537; c) J. Shanklin, E. Whittle, *FEBS Lett.* **2003**, *545*, 188–192.
[113] C. A. Smith, M. R. Hyman, *Appl. Environ. Microbiol.* **2004**, *70*, 4544–4550.
[114] F. Perz, S. Bormann, R. Ulber, M. Alcalde, P. Bubenheim, F. Hollmann, D. Holtmann, A. Liese, *ChemCatChem* **2020**, *12*, 3666–3669.
[115] S. S.-F. Yu, K. H. C. Chen, M. Y.-H. Tseng, Y.-S. Wang, C.-F. Tseng, Y.-J. Chen, D.-S. Huang, S. I. Chan, *J. Bacteriol. Res.* **2003**, *185*, 5915–5924.

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