Oxyfunctionalization of alkanes, alkenes and alkynes by unspecific peroxygenase (EC 1.11.2.1)

Oxyfunktionalisierung von Alkanen, Alkenen und Alkinen durch die Unspezifische Peroxygenase (EC 1.11.2.1)

Vom Institutsrat des Internationalen Hochschulinstitutes Zittau und der Fakultät Mathematik/ Naturwissenschaften der Technischen Universität Dresden genehmigte Dissertation zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) vorgelegt von Dipl.-Ing. (Umwelttechnik) Sebastian Peter geboren am 04. Juni 1982 in Erlenbach am Main (Deutschland)

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Tag der Verteidigung: 26.04.2013
Oxyfunctionalization of alkanes, alkenes and alkynes by unspecific peroxygenase (EC 1.11.2.1)

Approved by the council of International Graduate School of Zittau and the Faculty of Science of the TU Dresden

Academic Dissertation

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(Dr. rer. nat.)

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Day of defense: April 26, 2013
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# List of Abbreviations

- **AaeUPO**: *Agrocybe aegerita* Unspecific peroxygenase
- **AaP**: *Agrocybe aegerita* peroxidase
- **AbMnP**: *Agaricus bisporus* manganese peroxidase
- **ABTS**: 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonate)
- **APO**: aromatic peroxygenase
- **BDE**: bond-dissociation energy
- **BEP**: Brønsted-Evans-Polanyi
- **BSA**: N,O-Bis (trimethylsilyl)acetamide
- **CiP**: *Coprinus cinereus* peroxidase
- **CPO**: chloroperoxidase
- **CPR**: cytochrome P450 reductase
- **CPx**: cytochrome P450 electron transfer protein
- **CrP**: *Coprinellus radians* peroxygenase
- **DBT**: dibenzothiophene
- **DCM**: dichloromethane
- **DMB**: 2,3-dimethyl butane
- **DMP**: 2,6-dimethoxyphenol
- **DMSO**: dimethylsulfoxide
- **ee**: enantiomeric excess
- **HMN**: 2,2,4,4,6,8,8-heptamethylnonane
- **$k_{\text{cat}}$**: turnover number
- **$k_H/k_D$**: intrinsic deuterium isotope effect
- **[(H/D)$_{\text{obs}}$]**: observed deuterium isotope effect
- **$K_m$**: Michaelis-Menten constant
- **LiP**: lignin peroxidase
- **MCD**: 2-chloro-5,5-dimethyl-1,3-cyclohexanedione
- **MMO**: methane monooxygenase
- **MnP**: manganese peroxidase
- **NAD(P)H**: nicotinamide adenine dinucleotide phosphate
- **P450**: cytochrome P450 monooxygenase
- **PEG**: polyethylene glycol
- **pMMO**: particulate methane monooxygenase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PcLiP</td>
<td><em>Phanerochaete chrysosporium</em> lignin peroxidase</td>
</tr>
<tr>
<td>PeVP</td>
<td><em>Pleurotus eryngii</em> versatile peroxidase</td>
</tr>
<tr>
<td>PY</td>
<td>pyridine</td>
</tr>
<tr>
<td>sMMO</td>
<td>soluble methane monooxygenase</td>
</tr>
<tr>
<td>SVD</td>
<td>singular value decomposition</td>
</tr>
<tr>
<td>TBHP</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TCM</td>
<td>tetrachloromethane</td>
</tr>
<tr>
<td>TMCS</td>
<td>trimethylchlorosilane</td>
</tr>
<tr>
<td>TMSI</td>
<td>N-trimethylsilyimidazole</td>
</tr>
<tr>
<td>TTN</td>
<td>total turnover number</td>
</tr>
<tr>
<td>UPO</td>
<td>unspecific peroxygenase</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>Vis</td>
<td>visible</td>
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Abstract

Unspecific peroxygenase (EC 1.11.2.1) represents a group of secreted heme-thiolate proteins that are capable of catalyzing the selective mono-oxygenation of diverse organic compounds using only $\text{H}_2\text{O}_2$ as a cosubstrate. In this study, the peroxygenase from *Agrocybe aegerita* (*Aae*UPO) was found to catalyze the hydroxylation of various linear (e.g. $n$-hexane), branched (e.g. 2,3-dimethylbutane) and cyclic alkanes (e.g. cyclohexane). The size of $n$-alkane substrates converted by *Aae*UPO ranged from gaseous propane ($\text{C}_3$) to $n$-hexadecane ($\text{C}_{16}$). They were mono-hydroxylated mainly at the $\text{C}_2$ and $\text{C}_3$ position, rather than at the terminal carbon, and the corresponding ketones were formed as a result of overoxidation. In addition, a number of alkenes were epoxidized by *Aae*UPO, including linear terminal (e.g. 1-heptene), branched (2-methyl-2-butene) and cyclic alkenes (e.g. cyclopentene), as well as linear and cyclic dienes (buta-1,3-diene, cyclohexa-1,4-diene). Furthermore, the conversion of terminal alkynes (e.g. 1-octyne) gave the corresponding 1-alkyn-3-ol in low yield. Some of the reactions proceeded with complete regioselectivity and - in the case of linear alkanes, terminal linear alkenes and alkynes - with moderate to high stereoselectivity. The conversion of $n$-octane gave ($R$)-3-octanol with 99% enantiomeric excess ($ee$) and the preponderance of the ($S$)-enantiomer reached up to 72% $ee$ of the epoxide product for the conversion of 1-heptene. Catalytic efficiencies ($k_{\text{cat}}/K_m$) determined for the hydroxylation and respectively epoxidation of the model compounds cyclohexane and 2-methyl-2-butene were $2.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. The results obtained in the deuterium isotope effect experiment with semi-deuterated $n$-hexane and the radical clock experiment with norcarane clearly demonstrated that the hydroxylation of alkanes proceeds via hydrogen abstraction, the formation of a substrate radical and a subsequent oxygen rebound mechanism. Moreover, stopped-flow experiments and substrate kinetics proved the involvement of a porphyrin radical cation species (compound I; *Aae*UPO-I) as reactive intermediate in the catalytic cycle of *Aae*UPO, similar to other heme-thiolate enzymes (e.g. cytochrome P450 monoxygenases, P450s).
Zusammenfassung

Die Gruppe der Unspezifischen Peroxygenasen (EC 1.11.1.2) umfasst extrazelluläre Häm-Thiolat-Enzyme, die mittels H$_2$O$_2$ als Cosubstrat die selektive Monooxygenierung unterschiedlicher organischer Verbindungen katalysieren. In der vorliegenden Arbeit konnte gezeigt werden, dass die von *Agrocybe aegerita* sekretierte Peroxygenase (AaeUPO) verschiedene lineare (z. B. *n*-Hexan), verzweigte (z. B. 2,3-Dimethylbutan) und zyklische Alkane (z. B. Cyclohexan) hydroxyliert. Die Größe der von der AaeUPO umgesetzten Substrate reichte vom gasförmigen Propan (C3) bis hin zu *n*-Hexadekan (C16). Die Alkane wurden bevorzugt am zweiten und dritten Kohlenstoffatom (C2 und C3) hydroxyliert; eine Hydroxylierung am terminalen Kohlenstoff konnte nur vereinzelt und in geringem Umfang beobachtet werden. Die Überoxidationen der primär gebildeten, sekundären Alkohole führte außerdem zur Entstehung der entsprechenden Ketonderivate. Darüber hinaus wurde eine Vielzahl linearer terminaler (z. B. *1*-Hepten), verzweigter (z. B. 2-Methyl-2-Buten) und zyklischer Alkene (z. B. Cyclopenten) sowie linearer und zyklischer Diene (1,3-Butadien, 1,4-Cyclohexadien) durch die AaeUPO epoxidiert. Die Umsetzung terminaler Alkine (z. B. *1*-Octin) führte zur Entstehung der jeweiligen 1-Alkin-3-ole. Manche dieser Reaktionen verliefen ausgeprägt regioselektiv und, im Falle der linearen Alkane sowie der linearen terminalen Alkene und Alkine, mit mittlerer bis hoher Stereoselektivität. So ergab beispielsweise die Umsetzung von *n*-Octan einen Enantiomerenüberschuss größer 99% für (R)-3-Octanol; die Epoxidierung von *1*-Hepten lieferte einen Enantiomerenüberschuss (ee) von bis zu 72% für das (S)-Enantiomer. Die katalytischen Effizienzen, die für die Hydroxylierung bzw. Epoxidierung der Modellverbindungen Cyclohexan und 2-Methyl-2-Buten ermittelt wurden, betrugen 2.0 × 10$^3$ M$^{-1}$ s$^{-1}$ und 2.5 × 10$^5$ M$^{-1}$ s$^{-1}$. Der ausgeprägte Deuterium-Isotopen-Effekt, der im Zuge der Umsetzung von semideuteriertem *n*-Hexan beobachtet wurde sowie die Ergebnisse des Radical-Clock-Experiments mit Norcarane als Substrat bestätigten, dass die Hydroxylierung von Alkanen über Wasserstoffabstraktion, die Bildung eines Substratradikals und anschließende direkte Sauerstoffrückbindung verläuft. Die Stopped-Flow-Experimente belegen zudem das Auftreten eines Porphyrin-Kationradikal-Intermediates (Compound I; AaeUPO-I) im katalytischen Zyklus der AaeUPO (vergleichbar mit dem reaktiven Intermediat der P450-Monoxygenasen).
1. Introduction

1.1 The importance of enzymes in living organisms

Life on our planet as such depends on the activities accomplished by biocatalysts. Biocatalysts are either proteins (enzymes) or, in a few cases, nucleic acids (ribozymes) (Lilley 2005). Enzymes are necessary in all living systems to catalyze the chemical reactions required for survival, reproduction and spreading of cells. So far more than 17,000 enzymes are described (Schomburg 2012) and every year thousands of new enzymes are discovered. The enzyme class of oxidoreductases (EC 1.X.X.X) alone contains more than 4,000 different enzymes. Oxidoreductases in general catalyze the electron transfer from one molecule (reductant) to another (oxidant) and are subclassified by the functional group that acts as an electron donor. Among them are important enzyme types such as cytochrome P450s, which, for instance, are responsible for the detoxification processes in human liver cells. Another important group of oxidoreductases is the group of peroxidases, heme proteins secreted from fungi (Lignin Peroxidase, LiP; Manganese Peroxidase, MnP; etc.) that participate in the degradation of wood, as they are able to attack the recalcitrant lignin fraction and therefore make it accessible to further biodegradation and reentry to the carbon cycle (Hatakka 1994).

1.2 *Agrocybe aegerita* peroxynasenase – history and classification over time

An extracellular enzyme secreted by the agric fungus *Agrocybe aegerita* (Figure 1) was mentioned for the first time in 1995 (Upadhyay 1995) and first facts were presented to a broader audience 1996 (Hofrichter et al. 1996). Due to its ability to oxidize veratryl alcohol to veratryl aldehyde at pH 7, it was referred to as “alkaline lignin peroxidase”, as true LiP catalyzes reactions in a pH range from 2 to 5 with an optimum at about pH 2 (Tuisel et al. 1990). After additional tests it was found that the enzyme, in contrast to LiP, does not oxidize nonphenolic lignin moieties (O-4-structures). Although this fact was disproved several years later (Kinne et al.
2011), the enzyme was referred to as an “unusual peroxidase” (Hofrichter and Ullrich 2006) due to this supposed inability.

Initially, it was difficult to study the new enzyme, as the cultivation conditions for the fungus were not yet optimized and only few units of the enzyme per liter medium could be obtained. After further investigation by Ullrich (Ullrich et al. 2004), a more productive strain of the fungus was found and with a new complex growth medium based on soybeans, more than 1,300 units of the enzyme per liter could be yielded. Afterwards, two fractions (I and II) of the enzyme were purified and characterized for the first time, showing six isoforms with a molecular mass of 46 kDa and isoelectric points of 4.6 to 5.4 and 4.9 to 5.6, respectively. Moreover, the enzyme showed more and more differences to LiP. Besides the neutral pH optimum for veratryl alcohol oxidation, the ultraviolet/visible (UV/Vis) spectrum differed from the one reported for LiP. The spectrum of the peroxidase from Agrocybe aegerita showed a Soret band at 420 nm and two additional bands in the visible spectrum at 540 nm and 572 nm, whereas the known maxima for LiP are at 407 nm, 496 nm and 630 nm (Renganathan and Gold 1986). Furthermore, the peroxidase from Agrocybe aegerita catalyzed the conversion of typical peroxidase
substrates like ABTS [2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] or DMP (2,6-dimethoxyphenol), oxidized aryl alcohols to their corresponding aldehydes and acids and was able to halogenate 2-chloro-5,5-dimethyl-1,3-cyclohexanedione (MCD) (strong bromination and very weak chlorination). Based on its ability to catalyze halogentation reactions, it was classified as haloperoxidase and renamed to *Agrocybe aegerita* peroxidase (AaP) (Ullrich et al. 2004). The conversion of aryl alcohols by AaP was considered to proceed via attack of the benzylic carbon and the formation of a benzylic radical, as suggested for chloroperoxidase (CPO). Figure 2 shows a general catalytic cycle of heme-thiolate haloperoxidases.

In the first step the resting enzyme (1) binds H₂O₂ to form compound 0, a transient iron-(III)-peroxide complex, which is transformed to compound I (2) and a water molecule. The reactive oxo-ferryl radical cation complex can now react in three different ways: (I) A classic peroxidase reaction, where compound I reacts with the first substrate molecule to form a substrate radical and compound II (5), which then reacts with a second substrate resulting in a second substrate radical and the native enzyme. (II) A halogenation reaction, where compound I forms a hypothetical ferric hypohalide adduct termed compound X (3) that, in aqueous solution, decomposes to the resting enzyme and hypohalous acid. The latter can now react with a substrate, which most likely happens outside the active site (Manoj 2005). (III) Another possibility is the oxygenation, where the protonated compound II (4) can transfer oxygen to a previously formed substrate radical to yield a hydroxylated product and the ferric enzyme.
In addition to a similar substrate spectrum, examination of the N-terminus showed that AaP shares the first three amino acids with chloroperoxidase from *Caldariomyces fumago* (CPO) but shows almost no homology with other peroxidases (Figure 3).
Nevertheless, AaP also showed clear differences from CPO in terms of substrate spectrum, specificity and pH-behavior. For instance, AaP was able to catalyze the chlorination and bromination of MCD, but the specific activities at pH 2.75 were up to 20-fold lower compared to those of CPO. In contrast, the activity of AaP towards ABTS, DMP and benzyl alcohol was enhanced when the pH was increased, whereas the activity of CPO declined. In addition, the maxima of the UV/Vis spectrum of AaP (420 nm, 540 nm and 572 nm) showed only slight accordance to the reported spectrum of CPO (403 nm, 515 nm, 542 nm and 650 nm; (Dunford 1999)).

In 2005, the halogenating activity of AaP was confirmed, as the enzyme brominated phenol to 2-bromo- and 4-bromophenol in the presence of bromide (Ullrich and Hofrichter 2005). Moreover, AaP showed low chlorinating activity, but only when high AaP amounts were incubated with phenol in the presence of chloride and only traces of the corresponding 2- and 4-chlorophenol could be detected, but instead considerable amounts of p-benzoquinone were yielded. The higher chlorinating activities towards MCD reported in 2004 may be attributed to the oxygenation rather than the chlorination of the substrate.

In addition, a new AaP-catalyzed reaction was discovered. The enzyme was capable of aromatic ring hydroxylation in substrates such as toluene or naphthalene. This reaction is not known for CPO (Miller et al. 1995), but typical for P450-catalyzed monooxygenations, e.g. metabolism in liver cells (Nakajima 1997), and thus separated AaP from CPO. The conversion of toluene gave benzyl...
alcohol and ring hydroxylation products in a ratio of about 1:1 and monohydroxylated products, such as benzyl alcohol, were further oxidized to the corresponding benzaldehyde and benzoic acid. The oxidation of naphthalene proceeded even better than toluene and yielded 1-naphthol as main product as well as smaller amounts of 2-naphthol and 1,4-naphtoquinone (Ullrich and Hofrichter 2005).

Comparison of the UV/Vis spectrum obtained from AaP with that of P450LM2 also showed high similarities. It exhibits a soret band at 418 nm as well as additional maxima at 535 nm and 569 nm. Furthermore, the CO-complex of AaP showed a shift of the soret band to 445 nm. This value is comparable to P450s (Correia 2005, Lewis 2001) and CPO (443-445 nm) (Hollenberg and Hager 1973) and indicates a heme-thiolate in the active site of the enzyme (Lewis 2001). In addition, the N-terminal amino acid sequence of AaP (Figure 3) was found to share 21% identity with CYP55a2 of *Cylindrocarpon tonkinense* (Kudo 1996). Nevertheless, AaP did not show monooxygenase activity when incubated with nicotinamide adenine dinucleotide phosphate (NAD(P)H), O2 and substrate. Due to all these facts, AaP was considered to be the functional “missing link” between heme-thiolate haloperoxidases and P450s.

Later in 2006, AaP was investigated by Pricelius et al. in dye-decolorization tests using the azo-dyes Flame Orange (2-[(4-aminophenyl)diazenyl]-1,3-dimethyl-1H-imidazol-3-iium) and Ruby Red 2-[4-(Dimethylamino)phenylazo]-1,3-dimethyl-1H-imidazol-3-iium (Pricelius et al. 2007). Although complete decoloration by AaP could not be observed, they found that AaP is able to catalyze the two step N-demethylation of Ruby Red to form Flame Orange (Figure 4).

The experiments of Ullrich (Ullrich and Hofrichter 2005) on the aromatic ring hydroxylation of naphthalene were continued by Kluge (Kluge et al. 2007) who developed a new spectrophotometric assay for the enzyme. Monitoring the hydroxylation of naphthalene to 1-naphthol, the assay was used to obtain kinetic data for the catalytic oxygen transfer by AaP.
The apparent kinetic parameters Michaelis-Menten constant ($K_m$), maximum rate ($v_{max}$), turnover number ($k_{cat}$) and $K_m/k_{cat}$ for the hydroxylation of naphthalene were found to be similar to those of other AaP substrates (Ullrich and Hofrichter 2005, Ullrich et al. 2004). Interestingly, the product distribution between the main product 1-naphthol and the minor product 2-naphthol was found to be pH-dependent, as only small amounts of 2-naphthol were detected at pH 7-8 (3%), whereas considerably higher amounts were observed at lower pH (18% at pH 3).

In July 2008, Kinne et al. found that AaP can catalyze the regio- and enantioselective aromatic ring hydroxylation in 2-phenoxypionic acid (Kinne et al. 2008). This compound is an essential precursor in the large scale production of aryloxyphenoxypropionic acid-type herbicides and is hard to synthesize. The hydroxylation proceeded with isomeric purity of almost 98% and yielded (R)-2-(4-hydroxyphenoxy)propionic acid with an enantiomeric excess of 60%.

To prevent undesirable product polymerization, a side reaction caused by the general peroxidase activity of AaP (i.e. oxidation of phenolic compounds), the effect of supplemented radical scavengers in the reaction mixture was tested. Ascorbic acid was found to efficiently inhibit oxidative polymerization by re-reducing phenoxy radical intermediates. In addition, an experiment to determine the origin of the oxygen incorporated during substrate hydroxylation by AaP was performed for the first time. In this experiment, $^{18}$O-labeled H$_2$O$_2$ instead of regular H$_2^{16}$O$_2$ was used as an oxidant (Figure 5). A shift in the mass spectrum of the substrate incubated with labeled H$_2$O$_2$ by m/z 2 compared to the product produced.

Figure 4: Two step demethylation of Ruby Red (a) to Flame Orange (b); modified according to Pricelius (Pricelius et al. 2007).
with unlabeled H₂O₂ clearly proved the origin of the oxygen to be hydrogen peroxide. A further experiment, in which the purging of the reaction mixture with N₂ had no effect on the product formation, excluded O₂ and confirmed H₂O₂ as the source of oxygen.

Figure 5: Regioselective hydroxylation of 2-phenoxypropionic acid with high ee for (R)-2-(4-hydroxyphenoxy)propionic acid by AaP in the presence of ¹⁸O-labeled hydrogen peroxide (Kinne et al. 2008).

Further investigations on the hydroxylation of naphthalene by AaP showed that the reaction proceeds with an epoxide as a primary product (Figure 6) (Kluge et al. 2009).

Figure 6: Proposed reaction scheme for the AaP oxidation of naphthalene (1), to a primary epoxide product (2) and the pH-dependent dissociation to 1-naphthol (3) and 2-naphthol (4); modified according to Kluge (Kluge et al. 2009)
The authors reported that the conversion of naphthalene gave an unknown initial product under alkaline conditions (pH 9), which disappeared after the acidification of the reaction mixture to form the known products 1- and 2-naphthol (Kluge et al. 2007, Ullrich and Hofrichter 2005). An analysis of the m/z values obtained by HPLC-ESI-MS as well as the comparison of UV-spectra with published data confirmed this product to be naphthalene 1,2-oxide.

Two more studies on the catalytic properties of AaP were published in 2008. Aranda et al. examined the conversion of dibenzothiophene (DBT) by the peroxygenase of A. aegerita (Aranda et al. 2008). The product spectrum of this reaction depended on the presence of the radical scavenger ascorbic acid. AaP converted DBT mainly into the corresponding mono-, di-, tri- and tetrahydroxylated products, while the exact position of the hydroxyl groups remained unclear. Sulfoxidation was only observed in the absence of ascorbic acid and even then only traces of the respective products, DBT-sulfoxide and DBT-sulfone, were detected. Ascorbic acid prevented polymerization of hydroxylated DBT products, which were target of the phenol oxidizing activity of AaP otherwise.

In the same year, Ullrich et al. showed that AaP catalyzes the regioselective oxygenation of pyridine (PY) and a variety of substituted PYs (Ullrich et al. 2008). Most of these substrates were oxidized in the N-position and no other oxygenation products were detected, except for 3-methyl-PY and 3,5-dimethyl-PY, in the case of which an attack of the methyl group resulting in the formation of additional nicotinic alcohol and the corresponding overoxidation products was observed. Interestingly, the oxidizability of PY derivatives halogenated in meta-position was not dependent on the atom radius of the substituents, but followed the reverse order of their electronegativity. It was concluded that AaP has a relatively big pocket at the active site. In addition, an effect of the distance between the halogen position to the oxidized nitrogen was noticed, as the conversion followed the order \( \text{para} > \text{meta} > \text{ortho} \). Although monochlorinated PYs were oxidized by AaP, no products were detected for di- and per-chlorinated PYs. Furthermore, kinetic data were determined for the AaP-catalyzed PY N-oxidation and compared with data previously obtained for other substrates; thus the catalytic efficiency \( \frac{k_{\text{cat}}}{K_m} \) of PY N-oxidation was 100-fold lower than that of naphthalene or veratryl alcohol.
oxidation. This fact was attributed to the lower activation of PY compared to the other molecules that explains low $k_{\text{cat}}$ values. In contrast, $K_m$ for PY was rather low (69 µM), which indicated a high affinity of AaP towards PY\(^1\) (Table 1).

Table 1 Kinetic parameters of PY oxidation catalyzed by AaP in comparison with data obtained for other AaP substrates; modified according to Ullrich (Ullrich et al. 2008).

<table>
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<tr>
<th>Substrate</th>
<th>Product</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}$ (s(^{-1}))</th>
<th>$k_{\text{cat}}/K_m$ (s(^{-1})M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine (PY)</td>
<td>PY N-oxide</td>
<td>69</td>
<td>0.21</td>
<td>3.04×10(^3)</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>1-Naphthol</td>
<td>320</td>
<td>166</td>
<td>5.17×10(^5)</td>
</tr>
<tr>
<td>2,6-Dimethoxyphenol</td>
<td>Coerulignone</td>
<td>298</td>
<td>108</td>
<td>3.61×10(^5)</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>Benzaldehyde</td>
<td>1001</td>
<td>269</td>
<td>2.69×10(^5)</td>
</tr>
</tbody>
</table>

The ability of AaP to catalyze the oxygenation of naphthalene and pyridine separated it from other peroxidases, as they do not catalyze these reactions. Therefore all results obtained from the examination of AaP’s catalytic properties and the fact that aromatic peroxygenases were found in other fungi (Anh et al. 2007) indicated that the peroxygenase from \emph{A. aegerita} could represent a new sub-subclass of oxidoreductases. Analyses of the gene sequence of AaP strengthened this assumption. In 2009, Pecyna (Pecyna et al. 2009) identified the first gene (\emph{apo}1; aromatic peroxygenase) of \emph{A. aegerita} peroxygenase on the level of messenger RNA and genomic DNA and found no matches between the sequence and heme-imidazole peroxidases or P450s in BLAST searches, but confirmed that AaP shares some similarities with CPO (27% sequence identity), as previously proposed by (Ullrich et al. 2004). However, these similarities only concern particular regions in the sequence, such as the proximal heme-binding region and part of the distal heme pocket, both part of the \emph{N}-terminal moiety. In contrast, the \emph{C}-terminal part of CPO was found to be totally different from that of AaP. In addition to these observations a comparison of the heme-binding regions showed a conserved cysteine residue (PCP motif), which is known to serve as the fifth heme ligand in CPO. Thus the already assumed affiliation of AaP/AaeAPo to the group of heme-thiolate enzymes (Ullrich and Hofrichter 2005) was confirmed.

\(^1\) Note that PY is known to be a (competitive) inhibitor of other heme enzymes, for instance P450s (Kaul and Novak 1987)
Besides the partial match of AaP/AaeAPO with CPO found in BLAST searches, strong similarities with putative protein sequences from sequencing projects and transcriptome studies were observed. More than 100 DNA sequences from 22 fungal species and 15 genera matched the one of AaP to different extents. Surprisingly, these sequences were not limited to related fungal species like *Coprinopsis cinerea*, but also belonged to saprophytic ascomycetes including several *Aspergillus* species, phytoparasitic fungi and ectomycorrhizhal basidiomycetes. As the occurrence of APO homologous sequences is not only limited to a small group of related fungi, but seems to be spread in a number of taxonomically different organisms, it was concluded that the enzyme may belong to a bigger, phylogenetically old protein (super)family (Hofrichter et al. 2010).

To determine the scope of reactions catalyzed by AaP, many different substrates were now being tested. For instance, Kinne et al. reported the ability of AaP to rapidly and regioselectively hydroxylate the β-adrenergic blocker propranolol as well as the anti-inflammatory drug diclofenac to yield the corresponding human drug metabolites 5-hydroxypropranolol and 4’-hydroxyciclofenac, respectively (Kinne et al. 2009a). Later, Aranda compared the conversion of polyaromatic compounds of various sizes and structures by AaP to that catalyzed by another fungal peroxygenase from *Coprinellus radians* (*CrP*/CraAPO) (Aranda et al. 2009). They found that AaP and *CrP* are able to hydroxylate most of the tested substrates. The conversion rate was influenced by substrate size, as the oxidizability of substrates showed the following order: 1-/2-methylnaphtalene > dibenzofurane/ flourene > phenanthrene > anthracene > pyrene. Perylene, however, was not attacked at all. These results confirmed the relatively large pocket size in the active site of AaP as proposed previously (Ullrich et al. 2008), but also showed limits in substrate size.

Similar results were obtained by Kinne et al. (Kinne et al. 2009b), when they examined a new reaction type - the conversion of ethers by AaP (O-dealkylation). Although the enzyme was able to cleave a broad spectrum of different alkyl and alkyl aryl ethers to form the corresponding aldehyde and alcohol/phenol products, it showed limitations in the conversion of large molecules like 1,4-di-n-
butoxybenzene or 4-nitrophenyl-terminated polyethylene glycol (PEG). In addition, an experiment with symmetrical, semi-deuterated 1-methoxy-4-trideuteromethoxybenzene was performed to investigate the reaction mechanism of ether cleavage. Sample analyses showed a clear preponderance of 4-methoxyphenol-\(d_3\) over 4-methoxyphenol-\(h_3\) and gave an intramolecular isotope effect of \(\sim 12\), which pointed to a hydrogen abstraction mechanism.

All catalytic results obtained so far showed that AaeUPO catalyzes a variety of oxyfunctionalizations including some reactions that are typical for P450s (e.g. ether cleavage, aromatic ring epoxidation/hydroxylation, benzylic hydroxylation). However, it was unclear at the beginning of this PhD work, whether also alkanes and other aliphatics are subject to peroxygenation.

1.3 Aliphatic hydrocarbons

1.3.1 Alkanes

Saturated hydrocarbons, so called alkanes, only consist of carbon and hydrogen atoms. Their basic units are linked \(\text{CH}_2\)-subunits (methylene groups) that can form linear, branched and cyclic alkanes. As alkanes do not possess functional groups, double or triple bonds, the general empirical formula for linear and branched alkanes is \(\text{C}_n\text{H}_{2n+2}\), whereas it is \(\text{C}_n\text{H}_{2n}\) for cyclic alkanes. The main characteristic of alkanes is their chemical inertness, as the C-H bonds are rather stable and hard to break. For example, the bond dissociation energy needed to break a C-H bond in methane is 105 kcal mol\(^{-1}\), making it one of the strongest aliphatic bonds.

The biggest alkane sources nowadays are fossil oil and gas deposits which result from dead organic material from former geological eras transformed by anaerobical biological and geological processes. These deposits are exploited by mankind as energy and chemical sources and as a result of this activity, large amounts of alkanes and other hydrocarbons are continually released into the biosphere through leaking pipelines, oil spills or accidental spilling of fuels. During the biggest oil spill, caused by the sinking of the Deep Water Horizon on April 20, 2010, an estimate of 4.9 million barrels (about 780 million liter) of crude oil and 144,000 to 200,000 tons of methane were released into the Gulf of Mexico (Joye
et al. 2011, Kessler et al. 2011, Robertson 2010). In 2002, the amount of petroleum entering the environment was estimated at 1.3 million tons a year, half of which being released by natural seeps (Austin and Groves 2011).

Apart from these fossil sources, alkanes and alkane structures are widespread in nature, as they are produced by a number of different organisms, including plants, animals, bacteria and algae. For instance, about 74% of the emitted methane is produced by strictly anaerobic prokaryotes, the so-termed methanogenic archaea (Liu and Whitman 2008). Due to their sensitivity to oxygen, these bacteria inhabit anaerobe environments such as marine and freshwater sediments, flooded soils as well as human and animal gastrointestinal tracts. There they use CO$_2$ and different electron donors like H$_2$, formate, methanol, methylamines or acetate to form methane (Liu and Whitman 2008, Thauer et al. 2008). In contrast to the small methane molecules produced by methanogenics as a waste product of their metabolism, plants mainly produce structures comprising long-chain alkanes, so-termed waxes, to protect their leaves and bark against dehydration, abrasive damage and infection by pathogens. In some phototrophic protists, such as *Euglena*, wax is also known to serve as a food reservoir (Kolattukudy 1970).

Alkanes found in plant waxes include linear (C18-C37) (Kolattukudy 1970, Zygadlo et al. 1994) as well as branched (C18-C35) (Carruthers and Johnstone 1959, Kolattukudy 1970, Mold et al. 1963) and cyclic alkanes (Kolattukudy 1970). In addition, it was reported that plants produce gaseous ethane in response to injuries, hypoxia, water deficit and freezing (Kimmerer and Kozlowski 1982). In both photosynthetic and non-photosynthetic bacteria, alkanes range from C15-C31, with a strong preponderance of C17 molecules. Interestingly, the distribution of odd and even numbered alkanes is about equal for bacteria, whereas in higher plants mainly odd-numbered alkyls occur (Han and Calvin 1969). In contrast to plants, bacteria and algae, the occurrence of alkanes in humans and animals has not been well studied. Although there is some evidence for pristane (2,6,10,14-tetramethylpentadecane) in human, bovine and rat tissues, it is unclear, whether this substance is produced in these organisms, provided by bacterial symbiotes or accumulated by ingestion (Avigan et al. 1967, O'Neill et al. 1969).

The general formation of alkanes by living organisms is thought to proceed either via decarboxylation of the corresponding fatty acid (Han and Calvin 1969) or by
head-to-head condensation between two biochemically dissimilar fatty acids and subsequent specific decarboxylation of one of them (Kolattukudy 1970).

Figure 7: Important sources of alkanes and alkenes in nature - anthropogenic exploitation of oil and gas deposits; hydrocarbons as essential part of fuels; pheromones, fragrants and waxes produced by plants; production of methane by methanogenic archaea in anaerobe environment like flooded soils and animal gastrointestinal tracts. Illustrations by Aleksandra Peter.
Although most of the organisms mentioned so far seem to produce either very small (methane, ethane) or long-chain alkanes with a main chain length of about C25-C30, there is evidence for the production of average alkanes, such as tridecane (Zygadlo et al. 1994) and even \( n \)-heptane (Sandermann and Schweers 1960). Furthermore, it is assumed that, for instance in plant waxes, small alkanes are lacking because they evaporate from the plant surface or are lost during isolation procedures due to their volatile nature (Kolattukudy 1970). In view of these facts, it can be expected that most known alkanes can be produced, at least to smaller amounts, by living organisms.

1.3.2 Alkenes

Alkenes differ from alkanes as they exhibit one or more carbon-carbon \( \pi \)-bonds, which make them more reactive and therefore less stable than alkanes. As mentioned for alkanes before, one source of alkenes are fossil oil and gas deposits. Besides these sources, large amounts of alkenes are produced in various living organisms. One of the most important alkenes by number is isoprene, about 500 million tons of which are released into the atmosphere per year. Isoprene is produced by a number of different organisms such as fungi, bacteria, algae, animals, humans and especially plants (Sharkey and Yeh 2001). The production of isoprene by plants was found to be temperature-dependent and seems to provide tolerance towards short high-temperature events, which occur multiple times a day in plant leaves (Sharkey et al. 2001). Apart from isoprene a number of complex alkenes are produced by plants, such as limonene, pinene or cineole (Sharkey 1996). Furthermore, the production of ethylene by vascular plants including trees as a result of environmental stress or wounding (Kimmerer and Kozlowski 1982), as well as its production as a phytohormone in the ripening process of fruits is well known (Lelievre et al. 1997). As mentioned before, bacteria and algae also produce alkenes and in addition to linear alkenes (Youngblood et al. 1971), branched and cyclic alkenes were found, for example, in the photosynthetic species \textit{Rhodospirillum rubrum} and \textit{Rhodomicrobium vannielii}, where they can reach up to 95\% of the total cell carbon (Han and Calvin 1969). In addition, the polyene substance squalene is known to be the main component in
human sebum (Mudiyanselage et al. 2003) and also occurs in high amounts of up to 80\% in shark liver oil (Bakes and Nichols 1995). With regard to all mentioned sources, alkanes, alkenes and derived structures can be considered as widely spread in nature.

1.3.3. Alkynes

Like alkenes, alkynes are unsaturated hydrocarbons with at least one characteristic carbon-carbon triple bond. Although the triple bond is very strong (200 kcal mol\(^{-1}\)), alkynes are known to be even more reactive than alkenes and tend to undergo polymerization and addition reactions. Unlike with alkanes and alkenes, natural occurrence of alkynes is rather rare and only about a thousand different compounds are known so far. Nevertheless, some alkynes, but especially polyynes play a role in different organisms and they exhibit a broad range of interesting biological activities, such as pesticidal, antifungal, antitumor, anticancer and antibacterial properties (Shi Shun and Tykwinski 2006).

In addition, some species produce polyynes as poisonous defence agents against predators or herbivores. For instance, *Ichtyothere terminalis* was shown to produce the triyne compound ichthyothereol and its acetate (Shi Shun and Tykwinski 2006); both are toxic to fish and mammals and have been used as a fish poison by natives of the lower Amazon Basin. Another example is histrionicotoxicin (Figure 8), a toxic diyne secreted from the skin of poison dart frogs from the genus *Dentrobates* (Sinclair and Stockman 2007).

Table 2: Physicochemical data of some alkanes, alkenes and alkynes tested as substrates for AaeUPO. According to the GESTIS database (Smola 2012).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Formula</th>
<th>Melting point (^\circ)C</th>
<th>Boiling point (^\circ)C</th>
<th>Solubility in water (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkanes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propane</td>
<td>C(_3)H(_8)</td>
<td>-188</td>
<td>-42</td>
<td>75 (20 °C)</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>C(_6)H(_14)</td>
<td>-95</td>
<td>69</td>
<td>50 (20 °C)</td>
</tr>
<tr>
<td>n-Decane</td>
<td>C(<em>{10})H(</em>{22})</td>
<td>-29.7</td>
<td>174</td>
<td>0.05 (25 °C)</td>
</tr>
<tr>
<td>n-Tetradecane</td>
<td>C(<em>{14})H(</em>{30})</td>
<td>6</td>
<td>254</td>
<td>0.1 (20 °C)</td>
</tr>
<tr>
<td>Organic Type</td>
<td>Formula</td>
<td>Boiling Point</td>
<td>Melting Point</td>
<td>Solubility</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------</td>
<td>---------------</td>
<td>---------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>n-Hexadecane</td>
<td>C₁₆H₃₄</td>
<td>18</td>
<td>287</td>
<td>practically insoluble</td>
</tr>
<tr>
<td>n-Heptadecane</td>
<td>C₁₇H₃₆</td>
<td>21-23</td>
<td>302</td>
<td>practically insoluble</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>C₆H₁₂</td>
<td>6,6</td>
<td>81</td>
<td>50 (20 °C)</td>
</tr>
<tr>
<td>2,3-Dimethylbutane</td>
<td>C₈H₁₄</td>
<td>-129</td>
<td>58</td>
<td>practically insoluble</td>
</tr>
<tr>
<td>2,3,4-Trimethylpentane</td>
<td>C₈H₁₈</td>
<td>-110</td>
<td>113</td>
<td>practically insoluble</td>
</tr>
</tbody>
</table>

**Alkenes**

<table>
<thead>
<tr>
<th>Organic Type</th>
<th>Formula</th>
<th>Boiling Point</th>
<th>Melting Point</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Propene</td>
<td>C₃H₆</td>
<td>-185,3</td>
<td>-47,7</td>
<td>384 (20 °C)</td>
</tr>
<tr>
<td>1-Hexene</td>
<td>C₆H₁₂</td>
<td>-139,8</td>
<td>63</td>
<td>50-100 (20 °C)</td>
</tr>
<tr>
<td>Cyclohexene</td>
<td>C₆H₁₀</td>
<td>-103,7</td>
<td>83</td>
<td>50 (20 °C)</td>
</tr>
<tr>
<td>2,3-Dimethyl-2-buten</td>
<td>C₈H₁₂</td>
<td>-74,3</td>
<td>73</td>
<td>71 (25 °C)</td>
</tr>
<tr>
<td>Limonene</td>
<td>C₁₀H₁₆</td>
<td>-89</td>
<td>175</td>
<td>slightly soluble</td>
</tr>
</tbody>
</table>

**Alkynes**

<table>
<thead>
<tr>
<th>Organic Type</th>
<th>Formula</th>
<th>Boiling Point</th>
<th>Melting Point</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Octyne</td>
<td>C₈H₁₄</td>
<td>-79</td>
<td>126-128</td>
<td>24 (25 °C)</td>
</tr>
<tr>
<td>2-Hexyne</td>
<td>C₆H₁₀</td>
<td>-90</td>
<td>84-85</td>
<td>slightly soluble</td>
</tr>
<tr>
<td>3-Hexyne</td>
<td>C₆H₁₀</td>
<td>-105</td>
<td>81-82</td>
<td>slightly soluble</td>
</tr>
</tbody>
</table>

Figure 8: Occurrence of alkynes in nature - the toxic diyne histrionicotoxin found in the skin of *Dentrobates* spp. Illustration by Aleksandra Peter.
1.4 Enzymes catalyzing alkane, alkene and alkyne oxidation

Since alkanes, alkenes and alkynes as well as their derivatives are widespread in nature (cf. 1.3), a number of organisms have developed a variety of enzymes to detoxify these structures or to utilize them as a source of energy and carbon. Table 3 shows a summary of different enzymes known to catalyze alkane, alkene and/or alkyne oxidation, such as methane monooxygenase, alkane hydroxylase and diverse cytochrome P450 monooxygenases. In addition to naturally occurring enzymes, there have been attempts to improve the properties of known oxidizing enzymes by genetic engineering, with regard to stability, selectivity and catalytic performance. As these improved enzymes represent the highest standard in biochemical applications, their catalytic and kinetic properties are a good reference point for comparison with newly discovered enzymes including peroxygenases. The following chapter focuses on the different groups of enzymes known to catalyze aliphatic alkane, alkene and alkyne oxidation and illuminates the structures and functions of the most prominent representatives.
Table 3: Enzymes known to catalyze alkane, alkene and/or alkyne oxidation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC number</th>
<th>Examples of host organisms</th>
<th>Substrate range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alkanes</td>
</tr>
<tr>
<td><strong>Heme enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytochrome P450 monoxygenases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I (CYP153)</td>
<td>1.14.15.3</td>
<td>*Spingomonas sp. HXN-200, Mycobacterium sp. HXN-1500 Acinetobacter sp. EB104</td>
<td>C4-C16 (Maier et al. 2001)</td>
</tr>
<tr>
<td>Peroxidases</td>
<td>1.11.1.7</td>
<td>Armoracia rusticana</td>
<td>-</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>------------</td>
<td>---------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>1.11.1.10</td>
<td>Cardariomyces fumago</td>
<td>-</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>1.11.1.13</td>
<td>Phanerochaete chrysosporium</td>
<td>-</td>
</tr>
<tr>
<td>Other Peroxidases (MnP, LiP, VP,…)</td>
<td>1.11.1.14</td>
<td>Pleurotus eryngii</td>
<td>-</td>
</tr>
<tr>
<td>Non-heme enzymes</td>
<td>1.11.1.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diiron/dicopper enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble methane monooxygenase (sMMO)</td>
<td>1.14.13.25</td>
<td>Methylisinus trichosporium OB3b, Methyllococcus capsulatus (Bath)</td>
<td>C1-C10 (Baik et al. 2003)</td>
</tr>
<tr>
<td>Particulate methane Monooxygenase (pMMO)</td>
<td>1.14.18.3</td>
<td>All known methanotrophs</td>
<td>C1-C5</td>
</tr>
<tr>
<td>Enzyme Type</td>
<td>EC Number</td>
<td>Organism/Strain</td>
<td>Substrate Range</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------</td>
<td>-------------------------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Propane monooxygenase</td>
<td>1.14.15.3</td>
<td><em>Gordonia sp.</em> TY-5</td>
<td>C3 and C13-C22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butane monooxygenase</td>
<td>1.14.15.3</td>
<td><em>Pseudomonas butanovora</em> ATCC 43655</td>
<td>C2-C8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia monooxygenase</td>
<td>1.14.99.39</td>
<td><em>Nitrosomonas europaea</em></td>
<td>C1-C8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoiron enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral membrane alkane hydroxylase</td>
<td>1.14.15.3</td>
<td><em>Acinetobacter</em>, <em>Alcanivorax</em>, <em>Burkholderia</em>, <em>Mycobacterium</em>, <em>Pseudomonas</em>, <em>Rhodococcus</em></td>
<td>C5–C16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavin containing enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LadA, AlmA</td>
<td>1.14.15.X</td>
<td><em>Geobacillus thermodenitrificans</em> <em>Acinetobacter sp.</em></td>
<td>C15-C36 ; &gt;C30</td>
</tr>
</tbody>
</table>
1.4.1 Diiron enzymes - methane monooxygenase

Methanotrophic bacteria, such as \textit{Methylococcus capsulatus} or \textit{Methylobacterium trichosporium}, are able to utilize methane as a sole source of carbon and energy, using the so called methane monooxygenase (MMO) to activate this rigid substrate. It is the only known enzyme, capable of efficient methane oxidation, because, with a bond dissociation energy of 105 kcal mol\(^{-1}\), the C-H bond in methane is difficult to attack.

MMOs are nonheme proteins and exhibit two forms, the particular (pMMO) and the soluble form (sMMO). As the former is a membrane-bound enzyme, and therefore not easy to purify and characterize, only little information is available regarding this enzyme. The main difference to sMMO, besides the immobility, is the dicopper center in the active site (Lieberman and Rosenzweig 2005, Rosenzweig et al. 1993). In contrast to pMMO, sMMO has been intensively studied over the last decades. It is generally composed of three independent protein subunits: (I) a 245 kDa hydroxylase, which contains the active site with a hydroxide-ion-bridged diiron cluster and catalyzes the oxidation of methane, (II) component B, a small 15 kDa protein that binds to the hydroxylase unit and, among other things, efficiently influences the velocity of O\(_2\)-binding and other steps in the catalytic cycle, and (III) a 38 kDa reductase component containing FAD and a [Fe\(_2\)S\(_2\)] cluster that transfers electrons to the hydroxylase unit (Basch et al. 1999, Lipscomb 1994, Wallar and Lipscomb 2001). A general catalytic cycle for sMMO is shown in Figure 9.

The cycle begins with the reduced form of the MMO-hydroxylase-unit [Fe\(^{II}\)Fe\(^{II}\)] (H\(_{\text{red}}\) and the binding of molecular oxygen. (H\(_{\text{red}}\)) is first converted to the putative intermediates \textbf{compound O} and \textbf{compound P} (both not shown in Figure 9) and finally decays to the diferric peroxy species, \textbf{compound P}. In the next step, \textbf{P} is spontaneously transformed into \textbf{compound Q} that contains a diferryl-oxo cluster [Fe\(^{IV}\)Fe\(^{IV}\)]. The structure of the cluster has been shown to form a so-called “diamond core”. \textbf{Compound Q} is thought to be the reactive species that finally reacts with the substrate, as for instance its decay rate is dependent on the substrate concentration (Lipscomb and Que Jr 1998, Wallar and Lipscomb 2001). The reaction of \textbf{Q} with the substrate proceeds either via hydrogen abstraction and the formation of a free alkyl radical and \textbf{QH (R)}, as known for P450s, or via a four-
center transition state followed by a “hydrido-alkyl-Q” compound (Basch et al. 1999).

![Catalytic cycle of sMMO](image)

Figure 9: Catalytic cycle of sMMO; in which the enzyme is only illustrated as the diiron cluster in the active site. For further information see text. Modified according to (Basch et al. 1999, Lipscomb 1994, Wallar and Lipscomb 2001)

Although some studies with radical clock substrates confirm the radical pathway, other studies with MMO from different species do not (Valentine et al. 1999). Therefore more research on this part of the reaction cycle of MMOs is needed. After the hydroxylated product is released from the enzyme product complex (T), the oxidized hydroxylase (H<sub>ox</sub>) is reduced by the reductase unit to begin the cycle again.

In the last decades, research has shown that MMOs from different organisms do not only convert methane to methanol, but also oxidize a variety of other substrates, e.g. linear alkanes from methane to octane are hydroxylated, with a distinct preference for the conversion of small linear compounds from C1 to C5 was observed and a decline in activity with an increasing chain length. The products are mainly the corresponding 1- and 2-alcohols; 3- or 4-alcohols were only observed in traces. In addition, cyclic and branched alkanes and alkenes, chlorinated, fluorinated and brominated alkenes, as well as aromatic substrates
are oxidized by MMOs. Furthermore, aliphatic ethers are hydroxylated and cleaved to the corresponding alcohol and aldehyde products (Lipscomb 1994).

1.4.2 Monoiron enzymes – alkane hydroxylases (alkB)

While methanotrophic bacteria mainly utilize short chain alkanes, other microorganisms have focused on alkanes of medium to long chain length. These species, e.g. *Alcanivorax burkumensis* or *Pseudomonas oleovorans*, are often specialized in alkane degradation and represent the dominant species in areas polluted by alkanes, like oil spills, while they remain below detection limit in pristine environments (Sabirova et al. 2006, van Beilen and Funhoff 2007). As these organisms catalyze the hydroxylation of fatty acids and alkanes in the ω- or terminal position, respectively; the enzymes responsible for this reaction are referred to as ω-hydroxylases and alkane hydroxylases (alkB). Most of these specialized organisms are thought to exhibit more than one alkane hydroxylase system to utilize a huge range of alkanes, as shown for some *Rhodococcus* strains (Whyte et al. 2002). To examine the structure and function of alkBs, the enzyme system of *P. oleovorans* has been intensively studied. Similar to pMMO, it consists of three components. The first is a membrane-bound sulfide-containing non-heme iron hydroxylase with a high molecular weight (~500 kDa), which catalyzes the oxygen transfer to the substrate. The second subunit is rubredoxin, a red non-heme iron protein with a molecular weight of 12.8 kDa that functions as an electron carrier. It exhibits one or two iron atoms, in separate active centers of which only one is necessary to remain functioning (Lode and Coon 1971, May et al. 1984). The third component is a diphosphopyridine nucleotide-rubredoxin reductase with a molecular weight of 55 kDa that contains FAD as prosthetic group (McKenna and Coon 1970, Peterson et al. 1967, Ueda and Coon 1972). The general mechanism of alkane hydroxylation by alkane hydroxylases is shown in Figure 10.

The reducing equivalents required for the alkane hydroxylation are supplied by rubredoxin reductase, which transfers electrons from NADH to rubredoxin. In turn, rubredoxin transfers the electrons to the active center of the hydroxylase unit, where, in the main reaction, an alkane substrate is hydroxylated to the corresponding 1-alcohol. A more precise mechanism or reactive intermediates of
the reaction cycle are not known yet and will be hard to achieve in the future, as the isolation of the membrane-bound hydroxylase unit is difficult and the purified enzyme is unstable (McKenna and Coon 1970, Tani et al. 2001).

Figure 10: Structure of the alkane hydroxylase system with three subunits: (I) alkane hydroxylase, (II) rubredoxin and (III) rubredoxin reductase. For more information see text. Modified according to van Beilen (van Beilen et al. 1994).

In addition to their ability to hydroxylate fatty acids and linear alkanes, alkane hydroxylases can catalyze a number of oxidation reactions, such as epoxidation of alkenes, O-demethylation, oxidation of alcohols, aldehydes and alkyl benzenes as well as sulfoxidation (van Beilen and Witholt 2004).

1.4.3 Heme enzymes
1.4.3.1 Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases are common in a large number of organisms from all biological kingdoms (Hannemann et al. 2007). To this day, more than 12,000 different P450s are known (Nelson and Nebert 2011). As their physiological function was unclear in the beginning, cytochrome P450 monooxygenases (P450s) were named after their characteristic absorption maxima near 450 nm (Soret band), which is obtained after treatment of the
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reduced enzyme with carbon monoxide. Unlike other enzymes, P450s are not classified by their physiological function, but according to their structural homology. The general abbreviation CYP is followed by a number indicating the P450-family. Enzymes with a sequence homology of more than 40% belong to the same family and with similarities of more than 55% to the same subfamily. Subfamilies are indicated by a letter and all enzymes in one subfamily are numbered consecutively in the order of their discovery. For example, CYP4B2 is the second enzyme discovered in the subfamily B of family 4 (Nelson et al. 1996, Zehentgruber 2009). Although the peptide chains vary a lot from one P450 to another, the overall tertiary structure is quite conserved (Cirino and Arnold 2003). P450s play an important role in the biosynthesis of secondary metabolites, degradation and detoxification of recalcitrant and toxic substances and many other processes, as they catalyze a lot of different reactions. Among the most important reaction, the selective hydroxylation of inactivated C-H bonds, P450s catalyze for example the epoxidation of C-C double bonds, the oxidation of heteroatoms (S; N) as well as dealkylation reactions (Isin and Guengerich 2007, Sono 1996).

The mechanism of C-H activation by P450s has been studied intensively in the last decades. The catalytic cycle, as shown in Figure 11, starts with a resting state low spin Fe$^{III}$ (1). The iron is six-fold coordinated by four nitrogen atoms of the porphyrin ring in the equatorial level of the iron-porphyrin complex, a cysteine and a water molecule in the axial level. The binding of a substrate activates the enzyme by forming a high-spin ferric P450-substrate complex (2) along with a change in the redox potential from about -300 to -170 mV, which increases the reducibility of the active site. However, for some mammalian P450s, this step is not a prerequisite for the reduction of the heme iron (Guengerich and Johnson 1997, Isin and Guengerich 2008). Furthermore, the binding of the substrate displaces water in the active site of the enzyme. The increase of the reducibility is important in order to prevent uncoupling of the oxygen reduction by NAD(P)H. A high-spin state is prerequisite for an electron transfer from NAD(P)H to the heme iron. In a second step, the high-spin Fe$^{III}$ is reduced to a high-spin ferrous Fe$^{II}$ state (3) by transferring one electron provided by NAD(P)H. The binding of triplet oxygen to this complex is supported by its out-of-plane structure and its four unpaired electrons. With the binding of oxygen, the relatively stable nucleophilic
ferrisuperoxo anion complex is formed (4). The transfer of a second electron forms a ferriperoxo intermediate (5), which contains one negative charge on the oxygen and another one dislocated over the cysteine ligand. In the next step protonation of the distal oxygen atom generates a ferrihydroperoxo intermediate (6) with a negative charge dislocated over the cysteine ligand. In step six, a second proton is added to the distal oxygen atom. The generated transient iron oxo-H$_2$O adduct (not shown in Figure 11) immediately dissociates to water and a porphyrin π cation radical iron$^{IV}$ oxo intermediate, often referred to as compound I (Por••••Fe$^{IV}$=O) (7) in literature.

Nowadays, the structure of this compound I is commonly accepted to be an iron$^{IV}$ oxo (or ferryl) intermediate with an additional oxidizing equivalent delocalized over the porphyrin and thiolate ligands (Rittle and Green 2010). Due to the lack of proof for compound I being the active species, which was finally provided by Rittle (Rittle and Green 2010), two alternative hydroxylating agents had been proposed for a long time: a perferryl [iron(V)oxo] and a ferric hydroperoxo complex (Denisov et al. 2005, Newcomb et al. 2003, Ortiz de Montellano 2009, Wang et al. 2009). However, while experiments have ruled out the involvement of the hydroperoxo species (Davydov et al. 2001), there is also no evidence for the existence of the perferryl complex. In the final step of the cycle that yields a resting ferric heme complex and an oxygenated product, the reactive compound I transfers the oxygen atom to the substrate. This step is thought to proceed via hydrogen abstraction and an oxygen rebound mechanism, which is discussed in more detail in section 4.7.3.

Apart from the described catalytic cycle, there are at least three more possibilities for the activated oxygen species to dissociate from the heme iron, a process known as uncoupling. The peroxide shunt is the best studied among the so-called shunt pathways. In the presence of strong oxidizing agents, such as hydrogen peroxide, the high-spin ferric Fe$^{III}$ (2) is directly converted into the ferrihydroperoxo intermediate (6) and therefore makes the substrate oxygenation cofactor independent. In addition, some P450s, such as P450cam, CYP2B4 and CYP3A4, can react directly with H$_2$O$_2$ by initially forming the protonated ferrihydroperoxo complex.
Furthermore, the reverse reaction from intermediate (6) to intermediate (2) proceeds with formation of $\text{H}_2\text{O}_2$. Other uncoupling possibilities are the autoxidation shunt, when complex (4) dissociates to complex (2) and superoxide,
and the oxidase shunt, when complex (6) is converted to complex (2) and water consuming two molecules NAD(P)H (both not shown in Figure 11). When P450s are used as biocatalysts with the aim of high product yields, these uncoupling reactions are undesirable as NAD(P)H is consumed without product formation. As shown in the catalytic cycle, P450s are dependent on redox equivalents provided by NAD(P)H. Therefore most P450 interact with one or more redox partner enzymes, which transport electrons to the actual P450 monooxygenase. The diversity of these electron transport systems reflects the diversity of P450s itself. In three component systems, as they are common for the most bacterial P450 systems, for instance, two other protein components, a cytochrome P450 reductase (CPR) and an iron-sulfur protein ([2Fe-2S], CPx) are required apart from the monooxygenase unit. In the first step NAD(P)H is oxidized to NAD(P)+ and electrons are subsequently transferred from the FAD containing CPR unit to the CPx and finally to the P450 (Bernhardt 2006, Hannemann et al. 2007, Zehentgruber 2009).

Apart from this example of a three component system, there are a number of different P450-redox partner-systems (Hannemann et al. 2007). Among these, fusion enzymes, such as CYP102A1 from Bacillus megaterium, are of particular interest. In these complex enzymes, the CPR unit and the P450 are coupled in a single polypeptide chain making the enzyme complex self-sufficient and much easier to handle for research or potential industrial applications. Figure 12 shows some of the different P450 systems.

1.4.3.2 Heme peroxidases

Peroxidases are secreted, microsomal or cytosolic enzymes found in all kingdoms of life, the majority being b-type heme proteins (Kinne 2010, Margis et al. 2008). They are related to P450s (Passardi et al. 2007), but, in contrast to the latter, peroxidases are capable of using H2O2 or hydroperoxides to catalyze oxidation reactions. A hypothetical reaction mechanism for a haloperoxidase is shown and explained in section 1.2 (Figure 2). While alkane oxidation was not reported for peroxidases, there is evidence for the peroxidase-catalyzed oxidation of alkenes and alkynes as well as of benzylic carbon. Thus, Duescher reported on the
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Figure 12: Schematic organization of different Cytochrome P450 systems with (a) bacterial system, (b) mitochondrial system, (c) microsomal system and (d) self-sufficient fusion system (e.g. of CYP102, see text).

Modified according to (Bernhardt 2006, Zehentgruber 2009).

oxidation of 1,3-butadiene by chloroperoxidase (CPO) from *Caldariomyces fumago* and human myeloperoxidase, which yielded 3-butenal and butadiene monoxide as well as crotonaldehyde, respectively (Duescher and Elfarra 1992, Duescher and Elfarra 1993).
In addition, Dembitzky showed that CPO catalyzes the oxidation of a number of different alkenes and alkynes (Dembitsky 2003). Moderate hydroxylation and further oxidation of one methyl group of \( p \)-xylene (benzylic hydroxylation) by CPO was reported by Morgan et al. (Morgan et al. 2002).

1.4.4 Biotechnological relevance of hydrocarbon oxygenation and fungal bioremediation

The selective oxidation of hydrocarbons is a desired goal, as the conversion of petroleum compounds to higher value chemicals already had a market of more than 60 billion dollars in 1999 (Boswell 1999) and the demand is still growing with regard to the growing world population. However, although alkanes, such as methane, ethane and propane, are cheap and abundant hydrocarbons, they are not directly used as raw materials for the production of more valuable products (e.g. solvents, pesticides, pharmaceuticals, etc.). This results from their chemical inertness and low reactivity (c.f. section 1.3). Instead of using alkanes directly, the petrochemical industry usually produces the more reactive alkenes from heavier fractions by high-temperature endothermic processes, such as dehydrogenation or cracking, which consume about 5,000 kcal (kg product\(^{-1}\)) as they operate at temperatures of 800 °C. The thus produced alkenes serve as raw material for most of the available petrochemicals, from polymers to solvents, through to plasticizers, adhesives, detergents and additives. Alkanes are used mainly as fuels to produce energy and low-value carbon oxides. Thus, the direct activation of alkanes to value-added petrochemicals would exploit an inexpensive hydrocarbon feedstock in a more efficient fashion (Ayala and Torres 2004).

However, selective oxyfunctionalization of inactivated hydrocarbons is still a challenge in modern chemistry. Methods using chemical catalysts developed over the last decades are, despite recent advances, often characterized by harsh conditions, long reaction times, poor selectivity and overoxidation (Arakawa et al. 2001). Therefore, biocatalysts that are capable to effectively catalyze the selective hydrocarbon oxidation in mild environments are of great interest and have been

In addition, microorganisms able to grow on hydrocarbons in contaminated soils are of interest for bioremediation processes. Among these microorganisms, a number of yeasts and fungi were found to effectively degrade various alkanes and even crude oil. Lowery reported that yeasts from the genera *Candida*, *Rhodotorula* and *Dembaryomyces* as well as filamentous fungi from the genera *Aspergillus*, *Cephalosporium*, *Demantium*, *Epicillium* and *Trichoderma* exhibited the ability to grow on *n*-alkanes as sole carbon source (Lowery et al. 1968). Furthermore, Colombo showed that not only imperfect fungi (e.g. *Fusarium solani*, *Aspergillus terreus*), but also ligninolytic fungi (*Pleurotus ostreatus*, *Trametes villosus* and *Coriolopsis rigida*) are able to degrade aliphatic and aromatic hydrocarbons with a chain length from C15-C30 (26-35% in 90 days) (Colombo et al. 1996). The metabolic pathways and the enzymes involved in hydrocarbon degradation by fungi have not yet been examined to a large extent, but it is likely that besides the known P450s (Črešnar and Petrič 2011, Scheller et al. 1996, van den Brink et al. 1998), other oxidoreductases like peroxygenases or peroxidases may be involved in this process. Multiple hydrocarbon oxidizing enzyme systems, as they have been reported for hydrocarbon degrading bacteria (Liu et al. 2011, van Beilen and Witholt 2004), are also imaginable in fungi.

1.5 Objectives and aims

In the beginning of this study, previous results obtained for *AaeUPO* indicated that some of the catalyzed reactions such as ether cleavage or aromatic ring epoxidation/hydroxylation are typical for P450s (c.f. section 1.2). At this point, the question arose, whether *AaeUPO* is also able to catalyze one of the most important P450 reactions – the oxidation of inactivated C-H bonds as they are found in alkanes.

The following aims were set in the form of questions which we attempted to answers in the course of the PhD thesis:
1. Is the unspecific peroxygenase of *Agrocybe aegerita* (AaeUPO) capable of hydroxylating less activated alkanes including gaseous compounds? And if so, which are the limitations in terms of molecule size?

2. Which are the products of alkane oxidation by AaeUPO?

3. Are alkenes subject to epoxidation reactions?

4. What happens to alkynes?

5. Does alkane, alkene and alkyne conversion by AaeUPO proceed regio- and stereoselectively?

6. Is it possible to kinetically describe these reactions?

7. Does hydroxylation of alkanes proceed via substrate radical and hydrogen abstraction mechanisms as known for other heme-thiolate enzymes?

8. Is a compound I type species formed as reactive intermediate during AaeUPO catalysis?

### 2. Materials and Methods

#### 2.1 Reagents

Commercially available chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany), TCI Europe (Eschborn, Germany) and Chemos GmbH (Regenstauf, Germany), except for H$_2^{18}$O$_2$ (90 atom% $^{18}$O, 2% w/v), which was obtained from Icon Isotopes, n-hexane-1,1,1,2,2,3,3-D$_7$ (99.8%; 99.2 atom% D), which was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and 2,3-epoxy-2-methylbutane, which was purchased from Acros Organics. Authentic standards of the product norcaranol and the rearranged products cyclohept-3-enol.
and cyclohex-2-enyl methanol were prepared according to published procedures (Auclair et al. 2002). Wild-type extracellular peroxygenase of A. aegerita (isoform II, pI 5.6, 46 kDa) was produced in bioreactors with a soybean flour suspension as a growth substrate and purified as described previously (Ullrich et al. 2009, Ullrich et al. 2004). The enzyme preparation was homogeneous by SDS-PAGE and exhibited an A418 nm/A280 nm ratio of 1.86. The specific activity of the peroxygenase was 63 U mg\(^{-1}\), with 1 U representing the oxidation of 1 µmol of 3,4-dimethoxybenzyl alcohol (veratryl alcohol) to 3,4-dimethoxybenzaldehyde (veratraldehyde) in 1 min at a temperature of 23 °C (Ullrich et al. 2004).

2.2 Solvent selection and enzyme stability

2.2.1 The stability of AaeUPO in organic solvents

The diluted peroxygenase (5 µL) was incubated with nonpolar organic solvents (95 µL; n-hexane, 2,3-dimethylbutane, dichloromethane, heptamethylnonane, tetrachloromethane, or n-tridecane) and stirred. The enzyme was then extracted with water (45 µl) by vigorous shaking. A portion (5 µl) of the aqueous phase served as a sample to assay the enzyme activity as described previously (Ullrich et al. 2004). For acetone, the complete incubation mixture (100 µl) served as a sample for enzyme activity measurements.

2.2.2 The optimum acetone concentration

In order to obtain the optimum acetone concentration for alkane and alkene conversion by AaeUPO, peroxygenase (2 U ml\(^{-1}\), 0.76 µM) was incubated with cyclohexane (5 µl) in different acetone/water mixtures (40-90%; v/v). \(\text{H}_2\text{O}_2\) was added four times and the products were extracted from the reaction mixture with 100 µl cyclohexane after 4 min. The cyclohexane extract was analyzed by GC/MS as described below (c.f. 2.4).

2.2.3 Inhibition experiment with DMSO
The veratryl alcohol assay (Ullrich et al. 2004) was modified by addition of different amounts of DMSO (dimethylsulfoxide) to the reaction mixture. This experiment was conducted twice. The first time, DMSO concentrations from 5 µM to 25 mM were used and then increased in the second experiment (0.1% - 10%; v/v).

2.3 Substrate conversion

2.3.1 Alkane conversion

Typical reaction mixtures (total volume: 0.2 ml for liquid and solid alkanes; 1.2 ml for gaseous alkanes) contained purified peroxygenase (2 U ml$^{-1}$), 0.76 µM dissolved in a potassium phosphate buffer (10 mM, pH 7.0), acetone (60%, pH 5.3), and the alkane substrate (10% vol/vol). The reactions were started by the addition of H$_2$O$_2$ via syringe pump (4 mM h$^{-1}$), the mixture was then stirred at room temperature for 60 min. Gaseous propane and n-butane were treated under the same conditions but with continuous bubbling of the pure gas through the reaction vial (~1 l h$^{-1}$). A general setup for the conversion of gaseous substrates is shown in Figure 13. The reaction mixtures were extracted with cyclohexane (0.1 ml) by vigorous shaking.

2.3.2 Alkene conversion

Typical reaction mixtures (total volume: 0.2 ml; 1.2 ml for gaseous alkenes) contained purified peroxygenase (1 U ml$^{-1}$, 0.38 µM) dissolved in a potassium phosphate buffer (10 mM, pH 7.0), acetone (60%, pH 5.3), and the alkene substrate (5% vol/vol; except for +/- limonene, 1-methyl-1-cyclohexene, 4-methyl-1-cyclohexene, 1,3-cyclohexadiene, 1,4-cyclohexadiene and cis-/ trans-2-butene 5 mM of the substrate were used). The reactions were started by the addition of H$_2$O$_2$ via syringe pump (4 mM h$^{-1}$, except for 2-methyl-2-butene and 2,3-dimethyl-2-butene 2 mM h$^{-1}$ were used), the mixture was then stirred at room temperature for 30 min. Gaseous propene and n-butene were treated under the same conditions but by continuously bubbling the pure gas through the reaction vial (approx. 1 l h$^{-1}$). The reaction mixtures were extracted with hexane (0.1 ml) by vigorous shaking.
2.3.3 Alkyne conversion

The conversion of alkynes was performed under the same conditions as described for alkane conversion (cf. 2.5.1), but the products were extracted with \( n \)-hexane instead of cyclohexane.

![General setup for the conversion of gaseous substrates by AaeUPO.](image)

2.4 Product identification

2.4.1 Products of alkane conversion

The reaction products were analyzed by GC with a Hewlett Packard 6890 chromatograph equipped with a Hewlett Packard 5973 mass spectrometer (Waldbronn, Germany) and a DB-5MS UI capillary column (250 \( \mu \)m in diameter, 30 m in length, 0.25 \( \mu \)m film thickness; J&W Scientific, Folsom, CA, USA) and a ZB-Wax plus capillary column (250 \( \mu \)m in diameter, 30 m in length, 0.25 \( \mu \)m film thickness; Phenomenex, Torrance, CA, USA) for the products of gaseous substrates, respectively. For analysis, 1 \( \mu \)L of the cyclohexane extract was injected into the GC system. GC was performed with various temperature profiles, depending on the analyte. \( n \)-Pentane: 40 °C, 1 °C min\(^{-1}\) to 50 °C, 20 °C min\(^{-1}\) to
150 °C; n-hexane: 45 °C, 5 °C min⁻¹ to 100 °C, 20 °C min⁻¹ to 150 °C; n-heptane: 40 °C, 20 °C min⁻¹ to 45 °C, 10 °C min⁻¹ to 150 °C; n-octane and n-nonane: 50 °C, 20 °C min⁻¹ to 60 °C, 10 °C min⁻¹ to 150 °C; n-decane: 50 °C, 20 °C min⁻¹ to 90 °C, 10 °C min⁻¹ to 200 °C; n-undecane and n-dodecane: 50 °C, 20 °C min⁻¹ to 130 °C, 5 °C min⁻¹ to 150 °C, 30 °C min⁻¹ to 280 °C; n-tridecane: 100 °C, 20 °C min⁻¹ to 200 °C, 5 °C min⁻¹ to 230 °C, 30 °C min⁻¹ to 300 °C; n-tetradecane: 100 °C, 10 °C min⁻¹ to 180 °C, 1 °C min⁻¹ to 190 °C, 30 °C min⁻¹ to 300 °C; n-hexadecane: 100 °C, 20 °C min⁻¹ to 300 °C; 2,2,3,3-tetramethylbutane, 2,3-dimethylbutane, 2,3,3-trimethylbutane, 2,4-dimethylpentane, 2,2,4,4-tetramethylpentane and 2,3,4-trimethylpentane: 40 °C, 1 °C min⁻¹ to 50 °C, 20 °C min⁻¹ to 250 °C; cyclopentane: 40 °C, 1 °C min⁻¹ to 50 °C, 30 °C min⁻¹ to 140 °C; cyclohexane: 45 °C, 20 °C min⁻¹ to 160 °C; cycloheptane: 60 °C, 10 °C min⁻¹ to 100 °C, 30 °C min⁻¹ to 200 °C; cyclooctane: 50 °C, 20 °C min⁻¹ to 90 °C, 10 °C min⁻¹ to 200 °C; cyclodecane: 60 °C, 20 °C min⁻¹ to 200 °C, 10 °C min⁻¹ to 260 °C; isobutane and propane: 35 °C for 5 min; n-butane: 40 °C for 5 min; methylcyclohexane, cis/trans-decalin and ethylcyclohexane: 60 °C, 20 °C min⁻¹ to 120 °C, 30 °C min⁻¹ to 200 °C. In all cases, helium was used as carrier gas at a column flow rate of 1.5 mL min⁻¹. The products were identified relative to authentic standards by their retention times and/or by electron impact MS at 70 eV.

2.4.2 Products of alkene and alkyne conversion

The reaction products were analyzed by GC using a Hewlett Packard 6890 chromatograph equipped with a Hewlett Packard 5973 mass spectrometer (Waldbrohn, Germany) and a ZB-Wax plus capillary column (250 µm diameter by 30 m length, 0.25 µm film thickness, Phenomenex, Torrance, California, USA). For analysis, 1 µl of the n-hexane extract was injected into the GC-system. GC was performed using various temperature profiles depending on the analyte. Propene: 30 °C hold 2 min; 1-butene: 35 °C hold 3.5 min, 30 °C min⁻¹ to 100 °C; 1-pentene: 50 °C hold 3.5 min, 40 °C min⁻¹ to 115 °C; 1-hexene: 70 °C hold 3.5 min, 40 °C min⁻¹ to 135 °C; 1-heptene: 90 °C hold 3.5 min, 40 °C min⁻¹ 145 °C; 1-octene: 110 °C hold 3.5 min, 20 °C min⁻¹ to 120 °C; 1-octyne/1-hexyne/2-hexyne: 45 °C hold 2 min, 10 °C min⁻¹ to 200 °C; cyclohexene: 95 °C hold 3.5 min, 40 °C min⁻¹ to 170 °C; 4-methyl-1-cyclohexene: 60 °C, 10 °C min⁻¹ to 160 °C; 1-
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methyl-1-cyclohexene: 60 °C, 10° C min⁻¹ to 160 °C hold 2 min; +/- limonene: 70 °C, 5° C min⁻¹ to 125 °C, 30° C min⁻¹ to 230 °C; 1,3-cyclohexadiene/1,4-cyclohexadiene: 60 °C, 10° C min⁻¹ to 230 °C. In all cases, helium was used as carrier gas at a column flow rate of 1.5 mL min⁻¹. The products were identified relative to authentic standards by their retention times and/or by electron impact MS at 70 eV.

2.4.3 Chiral separation of alcohols, epoxides and alkynols

The chiral separation of alcohols was performed by GC/MS using the above-mentioned apparatus fitted with a Chiraldex GTA capillary column (250 μm in diameter, 30 m in length, 0.12 μm film thickness; Astec, Whippany, NJ, USA). The reaction products were completely derivatized beforehand by the addition of trifluoroacetic anhydride (2% v/v) to the cyclohexane phase according to a method described previously (Mommers et al. 2008). GC was performed using various temperature profiles depending on the analyte. n-Butane: 40 °C for 15 min; n-pentane, n-hexane and n-octane: 50 °C for 5 min, 20 °C min⁻¹ to 110 °C, 5 °C min⁻¹ to 130 °C, 20 °C min⁻¹ to 160 °C; n-heptane: 50 °C for 5 min, 35 °C min⁻¹ to 100 °C, 0.5 °C min⁻¹ to 105 °C, 35 °C min⁻¹ to 160 °C. The reaction products were completely derivatized, as nonderivatized alcohols were not detectable.

The chiral separation of epoxids and alkynols was performed by GC/MS using the above mentioned apparatus fitted with a Beta DEX™ 120 capillary column (250 μm in diameter by 30 m length, 0.25 μm film thickness, Supelco, Bellefonte, PA, USA). GC was performed using various temperature profiles. 1-Hexene: 45 °C hold 20 min; 1-heptene: 45 °C hold 37 min; 1-octene: 55 °C hold 46 min; 1-octyne: 45 °C hold 2 min, 10 °C min⁻¹ to 155 °C.

All products were identified relative to authentic standards by their retention time and by electron impact MS at 70 eV.

2.5 pH-Optimum of AaeUPO-catalyzed cyclohexane conversion

The pH-optimum was determined using a modified standard reaction protocol (2.5.1; total volume: 0.2 ml), with cyclohexane instead of an acetone-water mixture
and 76 nM purified peroxygenase (0.2 U ml\(^{-1}\)). The enzyme was diluted from a stock solution with potassium phosphate buffer (10 mM) at eleven different pH values. The reactions were started by manually adding 2 mM H\(_2\)O\(_2\) to the reaction mixture (2.5 µl; 40 mM H\(_2\)O\(_2\); 4 times) and the mixture was then stirred for four minutes.

### 2.6 \(^{18}\)O-labeling experiments

The reactions were performed as described above, but initiated by manually added H\(_2\)^\(^{18}\)O\(_2\) (90% atom \(^{18}\)O; 40 mM; 5 µl, four times, with a pipette) and analyzed after 5 min by GC/MS. For each \(m/z\) value, the average total ion current within the product peak was used after background correction to generate the ion current used for mass abundance calculations.

#### 2.6.1 Conversion of cyclohexane with H\(_2\)^\(^{18}\)O\(_2\)

For cyclohexane, the incorporation of \(^{18}\)O into the product cyclohexanol was calculated by the ratio of the unlabeled fragment ion \([M-C_3H_7]^+\) (\(m/z\) 57) to the \(^{18}\)O-labeled fragment ion (\(m/z\) 59).

\[
\text{Incorporation(\%) = } \frac{TIC(59)}{TIC(59 + 57)} \times 100\%
\]

#### 2.6.2 Conversion of 2,3-dimethyl-2-butene with H\(_2\)^\(^{18}\)O\(_2\)

The same method was used for 2,3-dimethyl-2-butene and the epoxide product 2,2,3,3-tetramethyl oxirane. The incorporation of \(^{18}\)O was calculated by the ratio of the unlabeled fragment ion \([M-C_4H_9]^+\) (\(m/z\) 43) to the \(^{18}\)O-labeled fragment ion (\(m/z\) 45).

\[
\text{Incorporation(\%) = } \frac{TIC(45)}{TIC(45 + 43)} \times 100\%
\]
2.7 Enzyme kinetics

2.7.1 Cyclohexane conversion

The kinetics of cyclohexane hydroxylation were analyzed in stirred reaction vials (0.20 ml, 23 °C) that contained 0.18 µM peroxygenase, acetone (60% vol/vol), and 0.1-30 mM substrate. Reactions were initiated with 2 mM H₂O₂ and stopped with 20 µl of 10 mM sodium azide solution after 10 s, at which time less than 1% of the cyclohexane had been consumed. The resulting cyclohexanol was quantified by GC/MS as described below, and an apparent value of the $K_m$ for cyclohexane was obtained by nonlinear regression with the Michaelis-Menten model in the ANEMONA program (Hernandez and Ruiz 1998).

2.7.2 2-Methyl-2-butene conversion

The kinetics of 2-methyl-2-butene epoxidation were analyzed in stirred reaction vials (0.2 ml, 23 °C) that contained 0.09 µM of the peroxygenase, acetone (60%), and 0.1-40 mM substrate. The reactions were initiated with 2 mM H₂O₂ and stopped with 20 µl of 10 mM sodium azide solution after 10 s, at which time less than 5% of the substrate had been consumed. The resulting trimethyloxirane was quantified by GC/MS as described below, and an apparent value of the $K_m$ for 2-methyl-2-butene was obtained by nonlinear regression using the Michaelis-Menten model in the ANEMONA program (Hernandez and Ruiz 1998).

2.8 Product quantification

Quantitative analyses of the reaction products were performed by GC/MS as described above, using external standard curves of the respective authentic standards. All standard curves had linear regression values of $R^2 >0.98$.

2.9 Deuterium isotope effect experiments

The reactions were performed as described above, but initiated with manually added H₂O₂ (5 µl of a 40 mM stock solution, four times, with a pipette) and extracted after 4 min. $n$-Hexane-1,1,1,2,2,3,3-D₇ served as a diagnostic substrate.
In the next step, the cyclohexane extracts were silylated with a mixture of N,O-bis(trimethylsilyl)acetamide (BSA), trimethylchlorosilane (TMCS), and N-trimethylsilylimidazole (TMSI) (3 : 2 : 3; Sylon BTZ Kit, Sigma-Aldrich; 1.5% vol/vol). The derivatized reaction mixture was analyzed by GC/MS as described above, with the following temperature profile: 45 °C, 5 °C min\(^{-1}\) to 65 °C for 2 min, 20 °C min\(^{-1}\) to 130 °C. For each \(m/z\) value, the average total ion current within the product peak was used after background correction to generate the ion current used for mass abundance calculations. The observed isotope effect \([\text{obs} \frac{k_H}{k_D}]\) was calculated by dividing the relative abundance of the respective fragment ion of trimethylsilyl-hexanol-D\(_7\) \([\text{M-CH}_3]^+ 166\) by the respective fragment ion of trimethylsilyl-hexanol-D\(_6\) \([\text{M-CH}_3]^+ 165\), as proposed previously (Jones et al. 1986). Nonderivatized alcohols were not detectable. The data represent an average of three independent experiments, in which each completed reaction was measured three times.

### 2.10 Radical clock experiments

The reaction mixtures (0.20 ml, stirred at room temperature) contained 0.4 U of the peroxygenase, potassium phosphate buffer (50 mM, pH 7.0) and 0.5% norcarane. The reaction mixture was incubated in an ultrasonic bath until a stable emulsion had formed. The reaction was then started by adding 2 mM \(\text{H}_2\text{O}_2\); after 10 s of reaction, the mixture was extracted with ethyl acetate as described previously (Rozhkova-Novosad et al. 2007). The reaction mixture was then analyzed by GC/MS with an HP-5MS cross-linked 5% PH ME siloxane capillary column (250 μm in diameter, 30 m in length, 0.25 μm film thickness; Agilent Technologies J&W, Santa Clara, CA, USA) using the following temperature profile: 50 °C for 2 min, 10 °C min\(^{-1}\) to 230 °C. The radical lifetime and rebound rate were calculated as described previously (Auclair et al. 2002).

### 2.11 Heme \(N\)-alkylation by alkenes and alkynes

Enzyme solution [140 μl, containing 1.5 U ml\(^{-1}\) (0.57 μM) in 100 mM potassium phosphate buffer, pH 7] was incubated with 50 μl 1-heptene, 1-octyne, 2-hexyne or 3-hexyne. 2 mM \(\text{H}_2\text{O}_2\) were repeatedly added (4 × 2.5 μl; 40 mM). The aqueous
RESULTS

Phase was extracted after four minutes and the enzyme activity towards veratryl alcohol was measured (Ullrich et al. 2004) using a Cary 50 spectrophotometer (Varian, Darmstadt, Germany). The activities obtained were compared to controls, which were not incubated with alkenes or alkynes.

2.12 Stopped-flow experiments

UV/Vis spectral measurements were made with a Hewlett Packed 8453 diode array spectrophotometer at room temperature. Stopped-flow experiments were performed using a Hi-Tech SF-61 DX2 double mixing instrument (Bradford on Avon, United Kingdom) with 1 cm path length. Unknown conversion products were either identified by GC/MS in comparison to authentic standards or by \(^1\)H-\(^13\)C-NMR. GC/MS analyses were run using an Agilent 7890A GC apparatus coupled to a 5975 Inert MSD (Santa Clara, CA, USA) with a Rtx-5Sil MS column. NMR spectra were recorded on a 500 MHz Bruker Avance II spectrometer (Billerica, MA, USA). Second-order rate constants for the reaction of AaeUPO with mCPBA were obtained by monitoring the conversion of ferric protein at 417 nm and plotting the single exponential decay of ferric enzyme against a range of mCPBA concentrations. Full diode array spectra confirmed that the porphyrin radical cation absorbances at 361 nm and 694 nm grew and decayed with the same kinetics.

2.12.1 Method improvement

In order to find the best conditions for compound I observation, other oxidants than mCPBA, i.e. peracetic acid, H\(_2\)O\(_2\) and tert-butyl hydroperoxide (TBHP), in various concentrations and at different pH (3, 4, 5, 6, 7) were tested.

2.12.2 AaeUPO compound I – kinetics

Kinetic data were collected at 4 °C in 100 mM buffer (citrate buffer for pH 5.0 and phosphate-citrate buffer for pH 3.0-7.0). Each experiment was repeated two or three times. The concentrations presented are the final concentrations after mixing. Kinetic data analysis for substrate hydroxylation was performed in the double-mixing mode using either diode array detection or the single wavelength.
mode at 417 nm. Values of $k_1$ were obtained by fitting of initial rates with a series of $m$CPBA concentrations. Values of $k_{obs}$ were processed by fitting the kinetic profile to a single exponential equation using Kinetic Studio from Hi-Tech. Values of $k_2$ were obtained from the slope of a $k_{obs}$ vs. the [substrate] plot. Singular value decomposition (SVD) and global analyses were performed with ReactLabTM Kinetics from Jplus Consulting.

3. Results

3.1 Solvent selection and enzyme stability

As alkanes, alkenes and alkynes are nonpolar compounds and thus only slightly miscible with water, an appropriate co-solvent had to be found, in which AaeUPO exhibited the smallest possible loss in activity. In the following chapter, the results of the enzyme stability tests in different organic solvents as well as results of the inhibition experiments with DMSO are presented.

3.1.1 Stability of AaeUPO in organic solvents

To optimize the reaction conditions for the peroxygenase-catalyzed alkane hydroxylation, the stability of AaeUPO was tested in different organic solvents for two hours. Table 4 shows that the peroxygenase maintained at least 50% of its activity after 30 min of incubation in all solvents tested. The highest relative activity (108%) was found in an acetone/water mixture (60% vol/vol) after 2 h of incubation. Thus, 60% acetone in water was used in most further experiments due to the optimum ratio of substrate solubility and enzyme activity (Figure 14).

3.1.2 Inhibition of AaeUPO by DMSO

While searching for the best solvent for substrate conversion, an inhibition of AaeUPO was observed in the presence of DMSO, a widely used co-solvent in organic synthesis.
RESULTS

Table 4: Stability of AaeUPO in different organic solvents

TCM Tetrachloromethane, HMN Heptamethylnonane; DMB 2,3-Dimethylbutane; DCM Dichloromethane

*Although the enzyme was still active after aqueous extraction, the conversion of \( n \)-hexane in this solvent was not efficient (data not shown). Enzyme activity was measured with veratryl alcohol (Ullrich et al. 2004).

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<th>Solvent</th>
<th>Relative enzyme activity (%)</th>
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<tr>
<td>Acetone(^a)</td>
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<tr>
<td>HMN(^*)</td>
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<td>( n )-Hexane</td>
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<td>TCM(^*)</td>
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<tr>
<td>( n )-Tridecane</td>
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<tr>
<td>DMB</td>
<td>85.8±13.6</td>
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<tr>
<td>DCM(^*)</td>
<td>51.9±0.2</td>
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</table>

\(^a\)(60% in water, vol/vol)

Figure 14: Conversion of cyclohexane in different acetone/water mixtures. Total ion count (TIC) of the hydroxylation product cyclohexanol against acetone concentration.
In the first experiment, DMSO concentrations between 5 and 2,500 µM were added to the reaction mixture, but only a small decrease of activity was observed (<20%; data not shown). In a second experiment, DMSO concentrations between 0.1 and 10% (vol/vol) were added (Figure 15a). At a concentration of less than 1.5% DMSO (~212 mM), the enzyme activity dropped below 50% and with 7.5% DMSO (~1.056 M), almost no activity was observed. To examine the type of inhibition, a third experiment with a doubled concentration of veratryl alcohol (10 mM) was performed. Figure 15b shows that the relative activity of AaeUPO towards veratryl alcohol in the presence of DMSO is higher when the veratryl alcohol concentration was increased.

![Figure 15: Influence of DMSO concentration on the enzyme activity in the reaction mixture of the veratryl alcohol assay (Ullrich et al. 2004), with a) 5 mM veratryl alcohol and b) 10 mM veratryl alcohol.](image)

### 3.2.1 Alkane conversion

In qualitative experiments done with substrate excess, it was found that the *A. aegerita* peroxigenase hydroxylated diverse alkanes. Table 5 shows an overview
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<th>Substrate</th>
<th>Alcohol products</th>
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</tr>
<tr>
<td><a href="C5-C8">cyclic alkanes</a></td>
<td>![Alcohol product]</td>
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</tbody>
</table>

**Results**
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<td><img src="image9" alt="Structure 9" /></td>
<td><img src="image10" alt="Structure 10" /></td>
</tr>
</tbody>
</table>
RESULTS

of all tested alkane substrates and the identified alcohol products. Only monohydroxylated products were detected. Since the standard reactions were performed with non-limiting H₂O₂ and peroxygenase, further oxidation of the resulting alcohol moieties also occurred, thus generating carbonyl compounds as reported recently (Kinne et al. 2010).

3.2.1.1 Linear alkanes

Notably, linear alkanes (C₃-C₁₆) were hydroxylated either at their 2- or 3-position, yielding the corresponding alcohols. The gaseous alkanes n-propane and n-butane were hydroxylated regioselectively, affording 2-propanol and 2-butanol, whereas longer n-alkanes yielded mixtures of the corresponding 2- and 3-alcohols. No hydroxylation product was observed for linear alkanes longer than C16 (Table 5).

In addition, Table 7 (page 62) shows the product distribution of linear alkane substrates from propane to n-octane. The ratio between 2- and 3-alcohols seems to depend on the substrate size. For example, while it is 1 : 1.9 for n-pentane, it is reversed for n-heptane, namely 1.5 : 1.

3.2.1.2 Branched alkanes

Like linear alkanes, branched alkanes were oxidized regioselectively to the corresponding 2- and 3-alcohols. For example, 2,3-dimethylbutane was oxidized to 2,3-dimethylbutan-2-ol and isobutane gave 2-methylpropan-2-ol as the only product. Interestingly 2,2,4,4-tetramethylpentane was not hydroxylated at the 3-position, whereas 2,4-dimethylpentane and 2,3,4-trimethylpentane gave the corresponding 2- and 3-alcohols. No hydroxylation product was detected for heavily branched 2,2,3,3-tetramethylbutane (Table 5).

3.2.1.3 Cyclic alkanes

Cyclic alkanes (C₅-C₈) yielded monohydroxylated products. For example, cyclohexane gave cyclohexanol and the overoxidation product cyclohexanone. The GC/MS elution profile of the AaeUPO-catalyzed cyclohexane conversion is shown in Figure 16. When methylcyclohexane served as peroxygenase substrate,
a mixture of all possible alcohols (1-, 2-, 3-, and 4-methylcyclohexanol) with a strong preference for 2-methylcyclohexanol (>95%) was formed. The conversion of ethylcyclohexane gave 2-ethylcyclohexanol, 1-cyclohexylethanol, 4-cyclohexanol, 1-ethylcyclohexanol and 3-ethylcyclohexanol (33 : 19 : 13 : 1.6 : 1). In contrast, no hydroxylation product was detected for cyclodecane. Furthermore, a difference in the product spectrum was observed, when the two-ring molecules of cis-decalin and trans-decalin served as substrates. As shown in Table 5 and Table 6, cis-decalin was hydroxylated and gave 1-decalinol and 2-decalinol (ratio 1 : 1.4), whereas the conversion of trans-decalin was less efficient and yielded 2-decalinol as sole product.
RESULTS

Figure 16: GC/MS elution profiles of the AaeUPO-catalyzed cyclohexane conversion with the alcohol and ketone products cyclohexanol and cyclohexanone, respectively. S = sample; C = control without enzyme. Insets show the mass spectra of both products.

Table 6: Product distribution for the conversion of cis-decalin and trans-decalin

<table>
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<th>Substrate</th>
<th>Product</th>
<th>Amount (TIC)</th>
<th>Product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-decalin</td>
<td>1-decalinol</td>
<td>84,975</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>2-decalinol</td>
<td>115,084</td>
<td>58</td>
</tr>
<tr>
<td>trans-decalin</td>
<td>2-decalinol</td>
<td>45,761</td>
<td>100</td>
</tr>
</tbody>
</table>

As the right pH is important for good turnover rates in enzymatic conversions, the pH optimum was determined for the hydroxylation of the model substrate cyclohexane. It was found to be 5.5, with 50% activity occurring at pH 3.6 and pH 7.7 (Figure 17).

Figure 17: Relative rates of cyclohexane hydroxylation expressed in cyclohexanol formation by AaeUPO at various pH values. The highest amount of cyclohexanol (formed at pH 5.5) was set 100%.

Figure 17: Relative rates of cyclohexane hydroxylation expressed in cyclohexanol formation by AaeUPO at various pH values. The highest amount of cyclohexanol (formed at pH 5.5) was set 100%.
3.2.1.4 Stereoselectivity of alkane hydroxylation

In addition to investigations on regioselectivity, the stereoselectivity of the hydroxylation of linear alkanes was examined. As shown in Table 7, peroxxygenase-catalyzed oxidation of \( n \)-alkanes proceeded stereoselectively depending upon the particular substrate. For example, \( n \)-hexane gave \((R)\)- and \((S)\)-enantiomers of 2- and 3-hexanol (Figure 18a), whereas the \((R)\)-enantiomer of 3-octanol was produced with almost complete enantiomeric excess (Figure 18b). Moreover, the substrates \( n \)-butane and \( n \)-pentane yielded the \((S)\)-form of the corresponding 2-alcohol, while \( n \)-hexane, \( n \)-heptane and \( n \)-octane gave the \((R)\)-enantiomers of the alcoholic products. The highest enantiomeric excess was obtained for 3-heptanol and 3-octanol.

Figure 18: GC/MS analysis of enantiomers of 2- and 3-alcohols produced during peroxxygenase-catalyzed hydroxylation of (a) \( n \)-hexane (H) and (b) \( n \)-octane (O). The alcohols were measured as their trifluoroacetic acid esters.

3.2.2 Alkene conversion

As shown in Table 8, a broad range of alkene substrates is epoxidized and/or hydroxylated by \( AaeUPO \). Both monohydroxylated alkenes and alkene epoxides were found as products.
Table 7: Product distributions (% total alcohols) and % ee of selected products

The numbers reported here are the total of all ketones (%) relative to total products (alcohols plus ketones).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2-alcohol (%)</th>
<th>ee (%)</th>
<th>3-alcohol (%)</th>
<th>ee (%)</th>
<th>ketones (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>b</td>
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<td>Propane</td>
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<tr>
<td>n-Butane</td>
<td>100</td>
<td>30.8(S) ± 4.7</td>
<td></td>
<td>trace</td>
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<tr>
<td>n-Pentane</td>
<td>34.1 ± 0.1</td>
<td>36.3(S) ± 4.7</td>
<td>65.9 ± 0.1</td>
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<td>0.13 ± 0.18</td>
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<td>n-Hexane</td>
<td>52.6 ± 0.7</td>
<td>62.5(R) ± 2.5</td>
<td>47.4 ± 0.7</td>
<td>79.5(R) ± 5.1</td>
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<td>n-Heptane</td>
<td>60.5 ± 0.1</td>
<td>62.2(R) ± 2.9</td>
<td>39.5 ± 0.1</td>
<td>99.9(R) ± 0.1</td>
<td>3.0 ± 0.2</td>
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<tr>
<td>n-Octane</td>
<td>55.0 ± 0.3</td>
<td>50.6(R) ± 1.5</td>
<td>45.0 ± 0.3</td>
<td>99.9(R) ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
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</table>

a Product distribution determined as ratio of a specific alcohol product in relation to the total amount of all alcohol products. b Favored enantiomer is listed in parentheses. Sum of the alcohols (µM): n-propane 729; n-butane 918; n-pentane 1,300; n-hexane 1,430; n-heptane 1,090; n-octane 933. 1-and 4-alcohols were not observed. c Product distribution for ketones was similar to that of alcohol product distribution.

3.2.2.1 Terminal linear alkenes

Small terminal linear alkenes gave monohydroxylated and epoxidized products. The yield of this reaction was very low. For example, when AaeUPO (1.0 U ml⁻¹, 0.37 µM) was incubated with n-heptene in the presence of H₂O₂ (2 mM) for 30 min, the yield was just 1 µM 1-hept-3-ol and 9 µM 2-pentyloxirane. For the conversion of propene only an epoxide product was observed.

3.2.2.2 Non-terminal and branched alkenes

The conversion of small non-terminal and branched alkenes like 2-methyl-2-butene gave only epoxidation products; hydroxylated products were not observed. The reaction proceeded with high velocity and good yield. For example, when 2-methyl-2-butene was incubated with AaeUPO (1.0 U ml⁻¹, 0.37 µM) and H₂O₂ (1 mM) for 30 min, the yield of the corresponding 2,2,3-trimethyl oxirane was 989 µM. Under the same conditions, 496 µM 2,2,3,3-tetramethyl-oxirane were obtained, when 2,3-dimethyl-butene served as substrate. In a similar way, the
conversion of cis-2-butene, trans-2-butene and isobutene under slightly modified conditions (2 mM H$_2$O$_2$) gave 900 µM, 1,910 µM and 912 µM of the respective epoxide products.

3.2.2.3 Cyclic alkenes

When AaeUPO (1.0 U ml$^{-1}$, 0.37 µm) was incubated with 4-methyl-1-cyclohexene in the presence of H$_2$O$_2$ (2 mM) for 30 min, 1,333 µM 4-methyl-1,2-cyclohexene oxide and 584 µM 6-methylcyclohex-2-en-1-ol were obtained. The conversion of 1-methyl-1-cyclohexene gave 555 µM 3-methyl-cyclohexanol and 1,279 µM 1-methyl-cyclohexene oxide.

The ratio of alcohol and epoxide products in both cases was about 30 : 70. In contrast, when cyclohexene was used as substrate, 626 µM cyclohex-2-ol-1-en and 772 µM cyclohexene oxide were yielded. The product ratio in this case was 45 : 55.

The main products for the conversion of (+)- and (-)-limonene were 1,2-epoxylimonene, 8,9-epoxylimonene and carveol (mentha-6,8-dien-2-ol). However, while the conversion of (+)-limonene gave a product ratio of 1.9 : 1.25 : 1, the ratio for (-)-limonene was 3.5 : 2.2 : 1. Moreover, the ratio of cis- and trans-products shifted in case of 1,2-epoxylimonene and carveol. Thus, the dominant form of (+)-1,2-epoxylimonene as well as of (+)-carveol was the cis-isomer [cis:trans ratio for (+)-1,2-epoxy limonene, 154 : 1; for (+)-carveol, 6 : 1], whereas in case of (-)-epoxylimonene and (-)-carveol, the trans-isomer was found to be the major form [cis:trans ratio for (-)-1,2-epoxylimonene, 1 : 9; for (-)-carveol, 1 : 3].

3.2.2.4 Dienes

The epoxidation of cyclodienes was observed to proceed less efficiently than the conversion of mono-unsaturated cyclic alkenes. So the conversion of 1,3- and 1,4-cyclohexadiene yielded only traces of 3,4-epoxy-1-cyclohexene and 4,5-epoxy-1-cyclohexene, respectively. The conversion of the linear molecule 1,3-butadiene gave vinyl oxirane (1,2-epoxy-3-butene) as the only product.
Table 8: Alkene substrates and identified epoxidation/hydroxylation products
substrates top-down: propene, 1-butene, 1-pentene, 1-hexene, 1-heptene, 1-octene, 2-methyl-2-butene, 2,3-dimethyl-2-butene, trans-2-butene, cis-2-butene, 2-methylpropene, cyclohexene, 1-methyl-1-cyclohexene, 4-methyl-1-cyclohexene, (R)-(+)−limonene, (S)-(−)-limonene, 1,3-cyclohexadiene, 1,4-cyclohexadiene, 1,3-butadiene, isoprene. nd = not determined

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Alcohol Product</th>
<th>Amount (µM)</th>
<th>Epoxid Product</th>
<th>Amount (µM)</th>
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<td></td>
<td></td>
<td>247</td>
<td></td>
<td>460/309</td>
<td>1,020</td>
<td>76 (46/30)</td>
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<td><img src="image4.png" alt="Chemical Structure 4" /></td>
<td><img src="image5.png" alt="Chemical Structure 5" /></td>
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<td></td>
<td></td>
<td>163</td>
<td></td>
<td>561/360</td>
<td>1,084</td>
<td>85 (52/33)</td>
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<td><img src="image12.png" alt="Chemical Structure 4" /></td>
<td><img src="image13.png" alt="Chemical Structure 5" /></td>
<td><img src="image14.png" alt="Chemical Structure 6" /></td>
<td><img src="image15.png" alt="Chemical Structure 7" /></td>
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<td><img src="image21.png" alt="Chemical Structure 5" /></td>
<td><img src="image22.png" alt="Chemical Structure 6" /></td>
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<td><img src="image28.png" alt="Chemical Structure 4" /></td>
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<td><img src="image30.png" alt="Chemical Structure 6" /></td>
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<tr>
<td><img src="image33.png" alt="Chemical Structure 1" /></td>
<td><img src="image34.png" alt="Chemical Structure 2" /></td>
<td><img src="image35.png" alt="Chemical Structure 3" /></td>
<td><img src="image36.png" alt="Chemical Structure 4" /></td>
<td><img src="image37.png" alt="Chemical Structure 5" /></td>
<td><img src="image38.png" alt="Chemical Structure 6" /></td>
<td><img src="image39.png" alt="Chemical Structure 7" /></td>
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<td></td>
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<td></td>
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<td></td>
<td>nd</td>
</tr>
<tr>
<td><img src="image41.png" alt="Chemical Structure 1" /></td>
<td><img src="image42.png" alt="Chemical Structure 2" /></td>
<td><img src="image43.png" alt="Chemical Structure 3" /></td>
<td><img src="image44.png" alt="Chemical Structure 4" /></td>
<td><img src="image45.png" alt="Chemical Structure 5" /></td>
<td><img src="image46.png" alt="Chemical Structure 6" /></td>
<td><img src="image47.png" alt="Chemical Structure 7" /></td>
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</table>
The reaction was faster than for cyclodienes and the yield comparable to small branched mono-alkenes (e.g. 2,3-dimethyl-2-butene). Similar results were obtained when converting isoprene (2-methyl-1,3-butadiene), which gave 3,4-epoxy-3-methyl-1-butene (2-methyl-2-vinyl oxirane) and 3,4-epoxy-2-methyl-1-butene [2-(1-methylethenyl)oxirane] as products.

3.2.2.5 Stereoselectivity of alkene epoxidation

The epoxidation of linear \( n \)-hexene, \( n \)-heptene and \( n \)-octene proceeded with preponderance for the \((S)\)-enantiomer. The enantiomeric excess for \((S)\)-1,2-epoxyhexane was 67\%, for \((S)\)-1,2-epoxyheptane 72\% and for \((S)\)-1,2-epoxyoctane 66\%.

3.2.3 Alkyne conversion

3.2.3.1 Linear alkynes

The conversion of 1-octyne and 1-hexyne gave 1-octyn-3-ol and 1-hexyn-3-ol, respectively. The reaction proceeded with low efficiency and other products were not observed. 2-Hexyne was not converted at all.

3.2.3.2 Stereoselectivity of alkyne conversion

A high stereoselectivity was observed for the conversion of 1-octyne. The ee reached 67\% for \((S)\)-1-octyn-3-ol. A similar result was obtained for 1-hexyne, but an exact number could not be calculated due to the poor product yield and an inconsistent enantiomeric ratio.

3.3 Source of the oxygen incorporated during substrate peroxygenation

To determine the source of the oxygen incorporated into the product during substrate oxidation by AaeUPO, a hydroxylation and an epoxidation reaction were performed with \(^{18}\text{O}\)-labeled \( \text{H}_2\text{O}_2 \). As shown in Figure 19 and Figure 20, the
RESULTS

Peroxygenase-catalyzed oxidation of cyclohexane and 2,3-dimethyl-2-butene in the presence of 90 atom % H$_2^{18}$O$_2$ resulted in an almost complete incorporation of $^{18}$O into the alcohol group of the resulting cyclohexanol and the oxirane group of tetramethyl-oxirane, respectively. The incorporation of $^{18}$O from H$_2^{18}$O$_2$ was also observed for 2- and 3-hexanol, when n-hexane served as substrate (data not shown).

![Figure 19: Incorporation of $^{18}$O from H$_2^{18}$O$_2$ into the alcohol group of cyclohexanol after hydroxylation of cyclohexane by A. aegerita peroxygenase.](image)

Upper: MS of the product obtained H$_2^{16}$O$_2$. Structural assignments for m/z values are as follows: [M]$^+$, 100; [M – C$_3$H$_7$]$^+$, 57; [M – C$_2$H$_5$-C$_2$H$_4$]$^+$, 44 (Carvalho et al. 2006). Lower: MS of the product obtained with 90 atom % H$_2^{18}$O$_2$. 

67
All these data are apparent, since they refer to two-substrate reactions, in the course of which the concentration of one substrate, the peroxide, was held constant (Bisswanger 2000).

Figure 20: Incorporation of $^{18}$O from $\text{H}_2^{18}\text{O}_2$ into the oxirane group of tetramethyl-oxirane after epoxidation of 2,3-dimethyl-2-butene by AaeUPO. Upper: MS of the product
obtained with $\text{H}_2^{16}\text{O}_2$. Structural assignments for $m/z$ values are as follows: $[\text{M}]^+$, 100; $[\text{M-}\text{C}_4\text{H}_9]^+$, 143. Lower: MS of the product obtained with 90 atom % $\text{H}_2^{18}\text{O}_2$.

3.4 Kinetic data for AaeUPO-catalyzed reactions

Kinetic parameters are important measures to compare different enzymes. This chapter provides kinetic data, such as Michaelis-Menten constants ($K_m$), turnover numbers ($k_{\text{cat}}$) and catalytic efficiencies ($k_{\text{cat}}/K_m$) for the conversion of an alkane substrate (cyclohexane) and an alkene substrate (2,-methyl-2-butene).

3.4.1 Cyclohexane hydroxylation

The $K_m$ of the peroxygenase for cyclohexane was 18.4 mM, $k_{\text{cat}}$ was 37 s$^{-1}$ and the calculated catalytic efficiency, $k_{\text{cat}}/K_m$, was $2.0 \times 10^3$ M$^{-1}$ s$^{-1}$. Figure 21 shows the data of the apparent Michaelis-Menten kinetic with cyclohexane as substrate.

![Figure 21: Apparent Michaelis-Menten kinetic of cyclohexane hydroxylation. Kinetic parameters were calculated by nonlinear regression with the Michaelis-Menten model in the ANEMONA program (Hernandez and Ruiz 1998).](image-url)
3.4.2 Epoxidation of 2-methyl-2-butene

The $K_m$ for the epoxidation of 2-methyl-2-butene to the corresponding trimethyl-oxirane was 4,976 µM, $k_{cat}$ was 1,257 s$^{-1}$ and the calculated catalytic efficiency, $k_{cat}/K_m$ was $2.5 \times 10^5$ M$^{-1}$ s$^{-1}$. Figure 22 shows data of the apparent Michaelis-Menten kinetic with 2-methyl-2-butene as substrate.

![Apparent Michaelis-Menten kinetic of 2-methyl-2-butene epoxidation](image)

Figure 22: Apparent Michaelis-Menten kinetic of 2-methyl-2-butene epoxidation. Kinetic parameters were calculated by nonlinear regression with the Michaelis-Menten model in the ANEMONA program (Hernandez and Ruiz 1998).

3.5 Deuterium isotope effects

GC/MS analysis of trimethylsilyl derivates of the reaction products showed that the peroxygenase-catalyzed hydroxylation of symmetrical $n$-hexane-$1,1,1,2,2,3,3$-$D_7$ resulted in a high preponderance of the 2- and 3-hexanol-$D_7$ trimethylsilyl ether derivatives over the 2- and 3-hexanol-$D_6$ trimethylsilyl ether derivatives (Figure 23). The observed mean intramolecular isotope effects $[(k_H/k_D)_{obs}]$ of three experiments were $16.0 \pm 1.0$ for 2-hexanol and $8.9 \pm 0.9$ for 3-hexanol.
RESULTS

Figure 23: Preferential hydroxylation by AaeUPO of the non-deuterated $\omega$-1 and $\omega$-2 carbon in $n$-hexane-$1,1,1,2,2,3,3$-$\text{D}_7$. **Upper:** MS of the 2-hexanol-$\text{D}_7$ and 2-hexanol-$\text{D}_6$ trimethylsilylether derivative obtained from the oxidation of natural-occurring $n$-hexane-$1,1,1,2,2,3,3$-$\text{D}_7$. **Lower:** MS of the 3-hexanol-$\text{D}_7$ and 3-hexanol-$\text{D}_6$ trimethylsilylether derivative obtained from the oxidation of natural-occurring $n$-hexane-$1,1,1,2,2,3,3$-$\text{D}_7$. Each MS shown is one of three used to calculate the observed mean intramolecular isotope effects.
3.6 Evidence for the presence of a radical intermediate

The GC/MS profile of the peroxygenase-catalyzed hydroxylation of norcarane showed that diverse reaction products were formed (Figure 24). The reactions yielded exo-2-norcaranol (C) as the major product and smaller amounts of endo-2-norcaranol (B) as well as exo- and endo-3-norcaranol (E and F); furthermore, a product expected to derive from the cationic intermediate 3-cyclohepten-1-ol (D) and the radical rearrangement product 4-(hydroxymethyl)-cyclohexane (A) were observed.

Figure 24: Left: Rearrangement pathways and products resulting from the formation of a radical or a cation intermediate upon oxidation of norcarane by the unspecific peroxygenase (UPO). Modified according to Auclair (Auclair et al. 2002). Right: GC/MS total ion current chromatogram in the region of the alcohols produced from the hydroxylation of norcarane by A. aegerita peroxygenase. In addition, an epoxide and traces of desaturation products were detected (data not shown).

On the basis of the ratio of the rearranged radical reaction products cyclohex-2-enyl methanol (A) and norcaranol (sum of B and C), a radical lifetime of 9.4 ps and an oxygen rebound rate of $1.06 \times 10^{11} \text{ s}^{-1}$ was calculated. The amount of the
RESULTS

major product exo-2-norcaranol was about 25-times higher than that of endo-2-norcaranol. Moreover, 3-cyclohepten-1-ol was found, which is indicative of a cationic pathway.

3.7 Enzyme inactivation by heme $N$-alkylation

The incubation of AaeUPO with 1-heptene in the presence of $H_2O_2$ resulted in a complete inactivation of the enzyme, as no activity towards veratryl alcohol was detected in the aqueous phase after 5 min incubation. Similar results were obtained when incubating AaeUPO with 1-octyne, 2-hexyne and 3-hexyne; a recovery of enzymatic activity after several hours was not possible.

3.8 Stopped-flow experiments

To characterize AaeUPO-I (compound I), its photospectrometrical properties as well as its reactivity with a number of different substrates were examined. The following chapter provides the results of stopped-flow method improvement, UV/Vis spectra of AaeUPO-I formation and kinetic data for this transient intermediate.

3.8.1 Method improvement

Stopped-flow measurements were carried out at different pHs and with different oxidants in varying concentrations. As shown in Figure 25 and Table 9, the formation rate of AaeUPO-I was the best at pH 5, and at the same time, its decay rate the slowest. In addition, the investigation of different oxidants showed that $m$CPBA, in the ratio 1:2 (enzyme:oxidant), gave the best results in AaeUPO-I production, as AaeUPO-I did not accumulate with any other oxidant tested, such as peracetic acid, $H_2O_2$ or TBHP (data not shown).

3.8.2 AaeUPO-I – UV/Vis spectrum and properties

Using the best conditions obtained by method improvement (cf. 3.8.1), UV/Vis spectra of a transient intermediate (AaeUPO-I) were obtained (Figure 26). They
displayed a new band at 361 nm and a distinct absorbance at 694 nm. This spectral transient appeared within 30 ms after mixing. The Soret band of the remaining ferric enzyme decreased dramatically during this time.

Figure 25: Absorbance changes measured at 417 nm upon mixing AaeUPO with mCPBA (1 : 2) at different pHs.

Table 9: Formation and decay rates of AaeUPO-I at different pHs.

<table>
<thead>
<tr>
<th>pH</th>
<th>Formation rate (M⁻¹ s⁻¹)</th>
<th>Decay rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>1.6 (± 0.1)×10⁵</td>
<td>2.5 (± 0.03)</td>
</tr>
<tr>
<td>4.0</td>
<td>2.5 (± 0.3)×10⁵</td>
<td>3.9 (± 0.04)</td>
</tr>
<tr>
<td>5.0</td>
<td>4.9 (± 0.3)×10⁵</td>
<td>1.4 (± 0.03)</td>
</tr>
<tr>
<td>6.0</td>
<td>4.0 (± 0.4)×10⁵</td>
<td>3.1 (± 0.03)</td>
</tr>
<tr>
<td>7.0</td>
<td>3.2 (± 0.2)×10⁵</td>
<td>4.1 (± 0.04)</td>
</tr>
</tbody>
</table>

The formation of AaeUPO-I reached a maximum of 70%, 31 ms after mixing. Accordingly, no spectral subtractions were necessary in these analyses. The maximal rate constant observed for AaeUPO-I formation was 1.1 (± 0.5) × 10⁷ M⁻¹ s⁻¹ at pH 6.
3.8.3 Compound I – kinetic data

Using the double-mixing-mode, kinetic data for the reaction of AaeUPO-I with 10 different substrates was obtained. Figure 27 shows the linear slopes of the reaction of AaeUPO-I with different substrate concentrations for each substrate tested. The slopes were used to calculate the apparent rates $appk_2$, $appk_2'$ and $logappk_2'$, where $appk_2'$ is $appk_2$ corrected by the number of equivalent C-H bonds in the substrates. The data obtained are listed in Table 10 together with the corresponding bond dissociation energies (BDEs).

4. Discussion

The unspecific peroxygenase from A.aegerita is able to catalyze the hydroxylation of various linear, branched and cyclic alkanes. In addition, a number of alkenes are epoxidized, including linear terminal, branched and cyclic alkenes as well as linear and cyclic dienes. Furthermore, the conversion of terminal alkynes gave the corresponding 1-alkyn-3-ol in low yield. The conversion of alkanes, alkenes,
alkynes proceeds highly regio- and - in some cases - even stereoselectively and the ee can reach up to 99% (n-octane, n-heptane).

Figure 27: Stopped-flow kinetics with AaeUPO-I and different substrates, obtained in the double mixing mode. AaeUPO and mCPBA were premixed 1:2 and after 200 ms, when accumulation of AaeUPO-I was the highest, different substrate concentrations were added with the second shot.
Table 10: Different substrates used in stopped-flow experiments to obtain kinetic data with their BDE, $appk_2$, $appk_2'$ and $logappk_2'$.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Equivalent C-H Bonds *</th>
<th>BDE (kcal mol⁻¹)</th>
<th>$appk_2$ (M⁻¹s⁻¹)</th>
<th>$appk_2'$ (M⁻¹s⁻¹) **</th>
<th>$logappk_2'$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Isopropylbezoic acid</td>
<td>1</td>
<td>83</td>
<td>2.1 (± 0.1)×10⁵</td>
<td>2.0×10⁵</td>
<td>5.3</td>
</tr>
<tr>
<td>$p$-Ethylbenzoic acid</td>
<td>2</td>
<td>85.5</td>
<td>3.85 (± 0.3)×10⁵</td>
<td>1.9×10⁵</td>
<td>5.3</td>
</tr>
<tr>
<td>$p$-Toluic acid</td>
<td>3</td>
<td>90</td>
<td>2.75 (± 0.3)×10⁵</td>
<td>9.3×10⁴</td>
<td>5.0</td>
</tr>
<tr>
<td>THF</td>
<td>4</td>
<td>92</td>
<td>1.7 (± 0.2)×10⁴</td>
<td>4.3×10³</td>
<td>3.6</td>
</tr>
<tr>
<td>THF-d8</td>
<td>4</td>
<td></td>
<td>3.95 (± 0.3)×10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheptane Carboxylic acid</td>
<td>4</td>
<td>94</td>
<td>3.0 (± 0.2)×10⁴</td>
<td>7.5×10³</td>
<td>3.9</td>
</tr>
<tr>
<td>Cyclopentane Carboxylic acid</td>
<td>8</td>
<td>95.6</td>
<td>1.0 (± 0.1)×10⁴</td>
<td>1.3×10³</td>
<td>3.1</td>
</tr>
<tr>
<td>Cyclohexane Carboxylic acid</td>
<td>6</td>
<td>99</td>
<td>1.0 (± 0.4)×10⁴</td>
<td>1.7×10³</td>
<td>3.2</td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>8</td>
<td>96</td>
<td>3.45 (± 0.1)×10²</td>
<td>4.4×10¹</td>
<td>1.6</td>
</tr>
<tr>
<td>3,3-Dimethylbutanoic acid</td>
<td>9</td>
<td>100</td>
<td>6 (± 0.1)×10¹</td>
<td>6.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*The numbers of equivalent C-H bonds were based on the numbers of sites being hydroxylated. **Second-order rate constants $k_2'$ were adjusted based on the number of equivalent C-H bonds in the substrates.
Kinetic data obtained for the hydroxylation of alkanes and the epoxidation of alkenes from the conversion of the model substrates cyclohexane and 2-methyl-2-butene show a catalytic efficiency \( \frac{k_{cat}}{K_m} \) of \( 2.0 \times 10^3 \text{M}^{-1} \text{s}^{-1} \) and \( 2.5 \times 10^5 \text{M}^{-1} \text{s}^{-1} \), respectively. The results obtained in the deuterium isotope effect experiment and the radical clock experiments clearly demonstrate that the hydroxylation of alkanes proceeds via substrate radical and hydrogen abstraction mechanisms. Moreover, stopped-flow experiments and substrate kinetics prove the involvement of a porphyrin radical cation species (AaeUPO-I) in the catalytic cycle of AaeUPO.

4.1 AaeUPO stability, inhibition and inactivation

4.1.1 AaeUPO stability in organic solvents

Due to the aim to convert alkanes, alkenes and alkynes, which are hydrophobic and thus not miscible with regularly used potassium phosphate buffer (Kinne et al. 2009b, Kluge et al. 2009, Kluge et al. 2012, Ullrich et al. 2004), a new reaction co-solvent had to be found. However, many enzymes are unstable in the presence of organic solvents due to partial denaturation (Klibanov 1997, Takemori et al. 1967). Therefore, the activity and stability of AaeUPO in different organic solvents was tested. AaeUPO was found to be stable in most organic solvents over a relatively long time (section 3.1.1, Table 4) and maintained at least 50% of its activity after 30 min of incubation in all solvents tested. Nevertheless, most solvents were not suitable for substrate conversion, since the conversion of the model compound \( n \)-hexane in these solvents was either not efficient (tetrachloromethane, heptamethylnonane, dichloromethane), or they gradually turned out to be substrates themselves (\( n \)-hexane, 2,3-dimethylbutane, tridecane). So an acetone/water mixture was found to be the solvent of choice because acetone was not further oxidized by AaeUPO and reaction products could be rapidly and almost completely extracted with cyclohexane or \( n \)-hexane forming an easy-to-handle 2-phase system with acetone/water. To figure out the best acetone concentration in terms of enzyme activity, substrate solubility and product extractability, the conversion of cyclohexane into cyclohexanol was studied in different acetone/water mixtures (section 3.1.1, Figure 14). As the amount of product
obtained was similar for 40%, 50% and 60%, 60% acetone in water was chosen as the reaction co-solvent for most of peroxygenase-catalyzed reactions because of the higher solubility of substrates. Beginning with 70% acetone, a decline in enzyme activity was observed, resulting in less cyclohexanol production. Nevertheless, at 90% acetone, still 50% of the product yield at 60% acetone was achieved. The suitability of acetone as co-solvent in enzyme-catalyzed reactions was already shown for other reactions, such as the enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase (Eibes et al. 2006), and can be explained by its good solvent properties and its unlimited miscibility with water (Smola 2012).

4.1.2 AaeUPO inhibition by DMSO

Among other organic solvents, DMSO was tested and found to inhibit substrate conversion (section 3.1.2, Figure 15). At a DMSO concentration of 10%, no more activity of AaeUPO towards veratryl alcohol could be observed. The inhibition of AaeUPO by DMSO is competitive, since a doubling of the veratryl alcohol concentration from 5 mM to 10 mM changed the maximum DMSO concentration at which activity was still observed to more than 20%. DMSO is a widely used co-solvent in enzymatic conversions because of its miscibility with water (Morisseau et al. 1997). The inhibition effect observed here may be attributed to the binding of the sulphoxide group to the active site preventing the access of the assay substrate veratryl alcohol. In this context, it is important to mention that sulphoxides were shown to be subject of overoxidation into sulphones in other studies with peroxygenases (Aranda et al. 2008, Horn 2009).

4.2 Scope of alkane, alkene and alkyne conversion

4.2.1 Alkane conversion

A variety of alkane substrates were hydroxylated by AaeUPO and the conversion proceeded with good yield and velocity. For example, when peroxygenase (2 U ml$^{-1}$, 0.76 µM) was incubated with cyclohexane in the presence of H$_2$O$_2$ (4 mM) over 60 min, $3.286 \pm 0.018$ mM of cyclohexanol and $0.010 \pm 0.007$ mM of
cyclohexanone were obtained (total turnover number, TTN = 4324, 82% efficiency in H₂O₂).

The conversion of linear and branched alkanes proceeded with high regioselectivity and yielded only the corresponding 2- and 3-alcohols as well as small amounts of the corresponding ketones. Terminal hydroxylation products or products that were hydroxylated at the C4 position, as reported for some P450 mutants (Glieder et al. 2002, Meinhold et al. 2006), were not observed. These findings conflict with the results obtained from stopped-flow experiments on the hydroxylation of 3,3-dimethylbutanoic acid, when 4-hydroxy-3,3-dimethylbutanoic acid was found and confirmed by ¹H-NMR analyses, as well as results reported by Gutièrrez (Gutiérrez et al. 2011), where fatty acids were also slightly hydroxylated at the ω- and ω-3 to ω-5 positions. However, the rates for this reaction and the amounts of product found were quite low in both cases. Thus, other hydroxylated terminal alkanes were probably not found here due to moderate reaction rates for terminal C-H activation and, for most substrates, the alternate weaker C-H bond at the C2 and C3 position. When enlarging the curve in the BEP-plot (Brønsted-Evans-Polanyi) to higher BDE (Figure 30; section 4.7.1), one can see a theoretical possibility for ethane to be substrate of AaeUPO. The corresponding rates for methane seem to be around zero so that an effective conversion is not likely. Both substrates were tested, but no formation of the corresponding alcohol, aldehyde or carboxylic acid product could be observed (data not shown).

When methylcyclohexane served as peroxigenase substrate, a mixture of all possible alcohols (1-, 2-, 3-, and 4-methylcyclohexanol) was formed, as also reported for some P450s (White et al. 1979, White and McCarthy 1984), but with a strong preference for 2-methylcyclohexanol (>95%). As White and McCarthy report, methylcyclohexane was converted by P450_{LM2} to all possible alcohols, except 2-methylcyclohexanol. The main products were 3-methylcyclohexanol (~56%) and 4-methylcyclohexanol (~35%). According to the author, the absence of 2-hydroxylation reflects steric hindrance to hydrogen abstraction adjacent to the methyl group, while the methyl group itself is little attacked because it is primary (White et al. 1979). In comparison, no hydroxylation of the methyl group was observed for the AaeUPO-catalyzed conversion of methylcyclohexane.
Similar results were obtained for the conversion of ethylcyclohexane. The main product was 2-ethylcyclohexanol (49%), which is comparable to the conversion of methylcyclohexane, followed by 1-cyclohexylethanol (28%), 4- (19%), 1- (2.4%) and 3-ethylcyclohexanol (1.5%). The attack of the ethyl group and the corresponding product 1-cyclohexylethanol is consistent with results obtained on the conversion of ethylbenzene by Kluge et al. (Kluge et al. 2012), yielding the corresponding (R)-1-phenylethanol, which also applies for P450s (Loida and Sligar 1993).

Another interesting fact is the result obtained for the conversion of cis-decalin and trans-decalin (section 3.2.1.3, Table 6). The differences in the product distribution and the higher conversion rates for cis-decalin can be explained by the higher stability of trans-decalin compared to cis-decalin, which is conformationally more mobile than trans-decalin, but also less stable because it has one axial substituent in each ring (Santikunaporn et al. 2004). Furthermore the bigger molecule size of the plane trans-decalin will prevent an easy access to the active site since the channel width of AaeUPO is 7 Å (Piontek et al. 2010)(Figure 28).

Figure 28: left: cis-decalin (diameter 6.4 Å), right: trans-decalin (diameter 7.3 Å)

Some apparent size and steric limitations of AaeUPO regarding alkane substrates were observed. For example, the enzyme failed to hydroxylate liquid cyclodecane and solid hepta- and octadecane. For P450s, the conversion of cyclodecane with different P450 BM-3 mutants (Weber 2011), the in vitro metabolism of heptadecane by hepatic microsomes (Perdu-Durand and Tulliez 1985) as well as
the conversion of octadecane by liver microsomes was reported (Kosuke et al. 1969). Moreover, although AaeUPO was able to hydroxylate 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, the enzyme did not oxidize 2,2,4,4-tetramethylpentane, perhaps due to steric hindrance at the active site (section 3.2.1, Table 5). However, also no reference was found for the conversion of 2,2,4,4-tetramethylpentane by P450s.

4.2.2 Alkene conversion

As shown in Table 8 (section 3.2.2), a broad range of alkene substrates was epoxidized and/or hydroxylated by the AaeUPO. The products were monohydroxylated alkenes and alkene epoxides. Interestingly, branched and cyclic alkenes seem to be much better substrates than linear n-alkenes. For instance, when 4-methyl-1-cyclohexene was converted by AaeUPO, 1,333 µM 4-methyl-1,2-cyclohexene oxide and 584 µM 6-methylcyclohex-2-en-1-ol were obtained. In contrast, the yield for n-heptene under the same conditions was just 1 µM 1-hept-3-ol and 9 µM 2-pentyloxirane. Therefore, a possible enzyme deactivation by N-alkylation of the heme moiety by linear terminal alkenes, as proposed and reported for other heme enzymes, such as P450s (Correia and Montellano 2005) or chloroperoxidase (Dexter and Hager 1995), is discussed separately in section 4.6.

Nevertheless, the conversion rate of n-heptene and n-hexene by AaeUPO (~ 1,000 nM/ min/ nM enzyme) is comparable to the P450-catalyzed oxidation of n-hexene (~ 1,300 nM/ min/ nM enzyme), whereas the oxidation of propylene and cyclohexene by AaeUPO is 14 and 102 times higher (~ 10,000 and 122,533 nM/ min/ nM enzyme, respectively) than the oxidation catalyzed by P450 BM-3 mutant 139-3 (~ 700 and 1200 nM/ min/ nM enzyme, respectively), respectively (Farinas et al. 2004).

The main products of the conversion of (+)- and (-)-limonene were 1,2-epoxy limonene, 8,9-epoxy limonene and carveol, as previously reported for P450s from liver microsomes (Miyazawa et al. 2002, Watabe et al. 1981) and cells of
Figure 29: Different product distribution for the conversion of (+)-limonene (a) and (-)-limonene (b). Products: 1) cis-carveol, 2) trans-carveol, 3) cis-1,2-epoxy limonene and 4) trans-1,2-epoxy limonene.
*Rhodococcus opacus* (Duetz et al. 2001). However, while the conversion of (+)-limonene gave a product ratio of 1.9 : 1.25 : 1, the ratio for (-)-limonene was 3.5 : 2.2 : 1. Moreover, the ratio of cis- and trans-products differed for 1,2-epoxy limonene and carveol (Figure 29).

While the conversion of (+)-limonene gave the cis-isomer with high preponderance (cis:trans; 1,2-epoxy limonene: 154 : 1; carveol: 6 : 1), the conversion of (-)-limonene yielded mainly the trans-isomer (cis:trans; 1,2-epoxy limonene: 1 : 9; carveol: 1 : 3). These differences are probably caused by the structure of the active site of AaeUPO, as similar results, i.e. high regio- and stereoselectivity of the enzyme, were also observed for some other substrates (e.g. *n*-octane).

Thus, the AaeUPO offers a biochemical potential to selectively prepare hydroxylation and epoxidation products of fragrants like limonene, which are used in industrial products (Duetz et al. 2003). Moreover, carveol and its derivative sobrerol ((1S)-5-(1-hydroxy-1-methylethyl)-2-methylcyclohex-2-en-1-ol) have been shown to be effective, non-toxic chemopreventive agents in mammary and other rodent tumor models. The studies of Crowell et al. investigated structure-activity relationships between limonene and three hydroxylated derivatives in the prevention of dimethylbenz[a]anthracene (DMBA)-induced mammary cancer (Crowell et al. 1992). Therefore, carveol could play a role in human cancer prevention in the near future.

Carveol could be further oxidized by AaeUPO to the corresponding carvone (2-methyl-5-(1-methylethynyl)-2-cyclohexenone). Perillaldehyde ((S)-4-(1-methylethynyl)-1-cyclohexene-1-carboxaldehyde) and carvone have been shown to effectively inhibit the transformation of the relatively benign cellular yeast form of *Candida albicans* to the pathogenic filamentous form, which is responsible for a high death rate among patients with compromised immune systems, such as cancer patients undergoing immunosuppressive therapy or AIDS patients (McGeady et al. 2002).

The conversion of 1-methyl-1-cyclohexene and 4-methyl-1-cyclohexene seems to proceed quite similar. The product ratio of epoxidation vs. hydroxylation products for both substrates was 2.3 : 1. In contrast, the oxidation of the comparable
substrate cyclohexene yielded epoxycyclohexene and cyclohex-2-en-1-ol in the ratio 1.2 : 1. So the absence of the methyl group seems to influence the product distribution. Presumably it increases the mobility of the substrate at the active site causing a more balanced product distribution. Compared to cytochrome P450s the conversion of cyclohexene by AaeUPO proceeds more unspecific. As reported by Farinas (Farinas et al. 2004), the product ratio for the conversion of cyclohexene with P450 BM-3 mutant 139-3 was found to be 5.7 : 1 (epoxycyclohexene:cyclohex-2-en-1-ol).

The epoxidation of cyclodienes was observed to proceed less efficient than the epoxidation/hydroxylation of mono-unsaturated cyclic alkenes. The conversion of 1,3- and 1,4-cyclohexadiene yielded only traces of 3,4-epoxy-1-cyclohexene and 4,5-epoxy-1-cyclohexene, respectively. In contrast, the oxidation of linear dienes, such as isoprene or 1,3-butadiene, gave average amounts of the corresponding epoxides 2-ethenyl-2-methyloxirane, 2-(prop-1-en-2-yl)oxirane (2.5 : 1) and vinyl oxirane, respectively. Similar results for the oxidation of 1,3-butadiene by P450s, mouse liver microsomes and chloroperoxidase were described before (Duescher and Elfarra 1994, Elfarra et al. 1991, Kohn and Melnick 1993), but Elfarra and Duescher also obtained crotonaldehyde, which was not observed with AaeUPO.

The conversion of isoprene by mice and rat liver P450s was reported previously yielding both epoxides obtained with AaeUPO (Watson et al. 2001, Wistuba et al. 1994). Further epoxidation of the monoepoxidized products, as described for P450s, was not observed.

4.2.3 Alkyne conversion

As observed for linear terminal alkenes, the conversion of linear terminal alkynes proceeded inefficiently and the corresponding 1-alkyn-3-ol was only detected in small amounts. In addition, AaeUPO was unable to oxidize non-terminal alkynes, such as 2-hexyne or 3-hexyne, as no oxidation products were detected. A possible reason for the poor conversion rates could be a deactivation of the enzyme by N-alkylation of the heme moiety by alkynes, as proposed and reported for other heme enzymes, such as P450s (Correia and Montellano 2005, Waltham et al. 2011) or chloroperoxidase (Dexter and Hager 1995). This possibility is discussed
separately in section 4.6. In contrast to the poor product yields for AaeUPO, hydroxylation of linear terminal alkynes by P450 BM3 was reported to proceed quite well, whereas conversion of non-terminal linear alkynes was also not observed (Waltham et al. 2011). The difference in alkyne oxidation of P450s and AaeUPO probably results again from the different architecture and amino acid composition of the active sites of both enzyme types.

4.3 Kinetic parameters of AaeUPO-catalyzed reactions

4.3.1 Cyclohexane hydroxylation

The apparent \( K_m \) of AaeUPO for cyclohexane was 18.4 mM and therefore much higher compared to other AaeUPO substrates. Thus, the affinity of the enzyme towards alkanes seems to be lower than towards aromatic compounds, such as naphthalene. In addition, the \( k_{\text{cat}} \) of 37 s\(^{-1}\) is also lower than for most other substrates, except pyridine. The calculated catalytic efficiency (\( k_{\text{cat}}/K_m \)) was \( 1.8 \times 10^3 \) M\(^{-1}\) s\(^{-1}\), which is lower than the values reported for an engineered P450 BM-3 mutant, IX139-3 \( k_{\text{cat}}/K_m = 3.7 \times 10^5 \) M\(^{-1}\) s\(^{-1}\) (Farinas et al. 2008) and the peroxynase-catalyzed ether cleavage \( k_{\text{cat}}/K_m = 5.0 \times 10^5 \) M\(^{-1}\) s\(^{-1}\) (Kinne et al. 2009b). The lower values compared to P450 and other AaeUPO substrates (Table 11) could be a result of acetone in the reaction mixture. Although AaeUPO is stable over a long time in an acetone water mixture, a decrease of enzyme activity under this conditions compared to an aqueous buffer solution has to be considered and seems to be likely. Furthermore, more accurate bisubstrate kinetics with a photospectrometric assay as reported by Kinne (Kinne et al. 2009b) were not possible with alkane substrates and therefore the interfering pseudocatalase activity of AaeUPO has to be considered (Ullrich et al. 2008).

4.3.2 2-Methyl-2-butene epoxidation

The same applies for the kinetic data of 2-methyl-2-butene epoxidation, whereas the catalytic efficiency obtained for this reaction (\( k_{\text{cat}}/K_m = 2.5 \times 10^5 \) M\(^{-1}\) s\(^{-1}\)) is comparable to the peroxynase-catalyzed ether cleavage of ethyl 3,4-dimethoxybenzyl ether (\( k_{\text{cat}}/K_m = 5.0 \times 10^5 \) M\(^{-1}\) s\(^{-1}\)) (Kinne et al. 2009b) and even
higher than the values reported for the epoxidation of 4,5-de-epoxypimaricin by a cytochrome P450 from *Streptomyces natalensis* \((k_{\text{cat}}/K_m = 2.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1})\) (Mendes et al. 2005).

In comparison to cyclohexane, the affinity of AaeUPO towards alkenes or 2-methyl-2-butene in particular seems to be much higher \((K_m = 4,976 \mu\text{M})\) and the \(k_{\text{cat}}\) is also increased by a factor of 30 \((k_{\text{cat}} = 1,257 \text{ s}^{-1})\). Maybe the high number of phenylalanine residues in the heme channel is the reason for this behavior, i.e. the aromatic residues may have a higher affinity to double bonds than to single bonds and thus “guide” alkenes with higher efficiency towards the heme center.

Table 11: Kinetic constants of alkane hydroxylation and alkene epoxidation by AaeUPO in comparison to data obtained for other AaeUPO substrates (Kinne et al. 2009b, Kluge et al. 2009, Peter et al. 2011, Ullrich et al. 2008).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (µM)</th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(k_{\text{cat}}/K_m) (s(^{-1}) M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methyl-2-butene</td>
<td>4,976</td>
<td>1,257</td>
<td>2.5 \times 10^5</td>
</tr>
<tr>
<td>3,4-Dimethoxybenzyl methyl ether</td>
<td>467</td>
<td>351</td>
<td>7.5 \times 10^5</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>320</td>
<td>166</td>
<td>5.2 \times 10^5</td>
</tr>
<tr>
<td>Pyridine</td>
<td>69</td>
<td>0.21</td>
<td>3.0 \times 10^3</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>18,400</td>
<td>37</td>
<td>2.0 \times 10^3</td>
</tr>
</tbody>
</table>

4.4 Stereoselectivity of AaeUPO-catalyzed reactions

4.4.1 Stereoselectivity of alkane hydroxylation

The conversion of linear alkanes from pentane to octane showed a high stereoselectivity. It is interesting to compare the values obtained using a wild-type peroxygenase with those of functionally similar, engineered P450s, which were optimized for the selective oxidation of alkanes (Peters et al. 2003). In general, the \(ee\)-values of the \((R)\)- and \((S)\)-enantiomers obtained are somewhat higher than those of engineered P450s. This applies in particular to the \(ee\) of more than 99% for the 3-alcohols gained after the reaction of AaeUPO with \(n\)-heptane or \(n\)-octane. Moreover, the majority of the tested bacterial P450s yielded additional 1- and 4-alcohols (Peters et al. 2003). It is also interesting that the enantiomeric preference
changed with increased chain length. Thus, \( n \)-butane and \( n \)-pentane yielded the \((S)\)-2-alcohol with relatively low \( ee \) (\(~30%\)) whereas \( n \)-hexane, \( n \)-heptane and \( n \)-octane preferentially gave the \((R)\)-2-alcohol with \( ee \) between 50% and 63%. As the \( ee \) for the \((R)\)-3-alcohol is even higher with up to 99%, there has to be a relation between the neighboring groups of the attacked carbon and the stereoselectivity of the conversion. When, for example, \( n \)-butane is hydroxylated at the C2 position to form 2-butanol, there is only a methyl and an ethyl group next to it, whereas the C3 position in \( n \)-heptane is surrounded by an ethyl and a butyl group. The longer the neighboring groups to the hydroxylated carbon are, the more immobile the substrate in the active site seems to be. Therefore, this immobility results in higher stereoselectivity.

4.4.2 Stereoselectivity of alkene epoxidation

In addition, the stereoselectivity of small linear terminal alkenes was investigated. Although the conversion proceeded with low yield, the \( ee \) for the \((S)\)-enantiomer of the corresponding epoxide was quite high. For example, the conversion of \( n \)-heptene gave \((S)\)-1,2-epoxyheptene with 72% \( ee \), while the ratio of epoxide and alcohol products (1-heptene-3-ol) was 9 : 1 and the TTN for all products was 26. In contrast, the TTN for the conversion of \( n \)-octene was 510, the ratio of the corresponding 1,2-epoxyoctene and 1-octene-3-ol was about 1 : 1 and the \((S)\)-enantiomer of the epoxide was obtained with 66% \( ee \). These results are comparable to P450 BM-3 and some of its mutants, whereas the preference for the epoxide product is somewhat higher for engineered P450 mutants (Kubo et al. 2006).

As aliphatic epoxides can be used to synthesize various chiral intermediates, e.g. for the production of ferroelectric liquid crystals (Liese et al. 2006), this might be of interest for special biotechnological applications.

4.4.3 Stereoselectivity of alkyne hydroxylation

Although the conversion of terminal alkynes, such as 1-hexyne or 1-octyne, proceeded inefficiently, the reaction was stereoselective and predominantly gave the corresponding \((S)\)-1-hexyn-3-ol and \((S)\)-1-octyn-3-ol, respectively. The \( ee \) of
up to 67% for (S)-1-octyn-3-ol corresponds with results obtained for P450 BM3, where a preponderance for the (S)-enantiomer with an ee of up to 90% was obtained (Waltham et al. 2011). Even though the ee obtained for the 1-octyne conversion by AaeUPO is somewhat lower than that reported for P450 BM3, there is an obvious similarity in the preponderance for the (S)-enantiomer.

4.5 Effect of cis-trans isomerism on alkene conversion

Interestingly, the conversion of cis-2-butene and trans-2-butene yielded different amounts of epoxidation products. When cis-2-butene was epoxidized to cis-2,3-dimethyloxirane (cis-2,3-epoxybutane), 1,910 µM product were obtained, whereas, under identical conditions (cis-trans ratio about 2.1 : 1), the conversion of trans-2-butene gave only 900 µM trans-2,3-dimethyloxirane (trans-2,3-epoxybutane). These results are consistent with observations published previously by Kluge on the conversion of cis-β-methylstyrene and trans-β-methylstyrene (Kluge et al. 2012). They found that trans-β-methylstyrene was oxidized only to a small extend at the terminal carbon. In contrast, cis-β-methylstyrene was almost completely converted into (1R,2S)-cis-β-methylstyrene oxide (>99% ee) as the sole product. Comparing these results to the findings for cis/trans-2-butene, it can be concluded that the steric differences caused by cis-trans isomerism strongly influence the oxidation of alkenes. As shown above, the simple shift of a methyl group from trans- to cis-position doubled the conversion rate of the respective 2-butenes. This effect was even more pronounced in case of methylstyrenes bearing larger isomeric groups; thus the ratio for the conversion of cis-β-methylstyrene compared to trans-β-methylstyrene was 5 : 1 (Kluge et al. 2012).

4.6 AaeUPO inactivation by heme N-alkylation

A total enzyme inactivation by N-alkylation of the heme moiety in the presence of alkenes and alkynes has been reported previously for different heme enzymes, such as P450s (Correia and Montellano 2005) or chloroperoxidase (Dexter and Hager 1995). While the inactivation of the latter was reported to be transient and only occured with terminal alkenes and alkynes, the inactivation of P450s is also known for non-terminal alkynes (Waltham et al. 2011). A similar mechanism for
heme $N$-alkylation of AaeUPO is likely, as the active site of the enzyme also exhibits a heme moiety and inactivation experiments with terminal and non-terminal alkenes and alkynes clearly showed a complete enzyme inactivation for 1-heptene, 1-octyne, 2-hexyne and 3-hexyne (section 3.7). A possible mechanism of heme $N$-alkylation by terminal alkenes is shown below (Figure 30).

Figure 30: Upper: Inactivation of heme proteins caused by $N$-alkylation of the heme by terminal alkenes. Lower: Alternate epoxidation pathway. Modified according to Hager and Collman (Collman et al. 1990, Hager et al. 1998)

4.7 Reaction mechanism of AaeUPO

4.7.1 AaeUPO compound I – formation, properties and kinetics

As shown in section 3.8.2 (Figure 26), the formation of AaeUPO compound I results in a dramatic decrease of the Soret band of the ferric enzyme. SVD analysis and the observation of a clear set of multiple isosbestic points (390 nm, 448 nm, 516 nm and 586 nm) indicated that only two major species were present during the transformation. The weak, blue-shifted Soret band and the absorbance
at 694 nm strongly suggest the presence of a porphyrin radical cation species and that the intermediate is AaeUPO compound I. The spectrum of AaeUPO-I, which was obtained by globally fitting the data in Figure 26, recapitulates those of P450 (Egawa et al. 1994, Rittle and Green 2010) and CPO (Egawa et al. 2001), but shows some differences to other heme enzymes, such as catalase or horseradish peroxidase (Table 12). These differences probably occur due to different proximal iron ligands, as catalase and horseradish peroxidase are not heme-thiolate proteins, but exhibit tyrosine and histidine as proximal ligand, respectively. Figure 31 shows UV/Vis spectra of P450 compound I formation, which are almost identical to those obtained for AaeUPO-I (Figure 26).

Figure 31: UV/Vis spectra obtained from the stopped-flow mixing (1:1) of 20 mM ferric CYP119 with 40 mM m-CPBA at 4°C. The blue traces correspond to spectra taken 5, 15, and 35 ms after mixing. Maximum yield of P450-I was ~ 70% at 35 ms. Adapted from Rittle (Rittle and Green 2010).

The only visible difference is the absence of the band at 610 nm (Q band) for AaeUPO-I that occurs in the spectrum of CYP119-I. On the other hand, the Q band seems to be a characteristic of CYP119-I as it is also lacking for other
P450s, such as CYP101-I. An examination of more P450 compound I UV/Vis spectra in the future could give more clarity on that question. The rates of AaeUPO-I decay followed first-order kinetics and were fitted directly from the data in Figure 26. The spontaneous reduction of AaeUPO-I was slowest at pH 5.0, $k_1 = 1.4 \pm 0.03$ s$^{-1}$.

Table 12: Comparison of the absorption maxima of Compounds I for various heme proteins. Modified according to Kellner (Kellner et al. 2002).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Compound I absorption maxima (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaeUPO</td>
<td>362, 694</td>
<td>This work, (Wang et al. 2012)</td>
</tr>
<tr>
<td>CYP101</td>
<td>367, 694</td>
<td>(Egawa et al. 1994)</td>
</tr>
<tr>
<td>CYP119</td>
<td>370, 610, 690</td>
<td>(Kellner et al. 2002)</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>367, 688</td>
<td>(Hager et al. 1972)</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>400, 577, 622, 651</td>
<td>(Davies et al. 1976)</td>
</tr>
<tr>
<td>Catalase</td>
<td>405, 660</td>
<td>(Benecky et al. 1993)</td>
</tr>
<tr>
<td>Lignin peroxidase</td>
<td>408, 550, 608, 650</td>
<td>(Renganathan and Gold 1986)</td>
</tr>
<tr>
<td>Manganese peroxidase</td>
<td>407, 558, 605, 650</td>
<td>(Wariishi et al. 1988)</td>
</tr>
<tr>
<td>Coprinus cinereus peroxidase</td>
<td>402, 658</td>
<td>(Anderson et al. 1991)</td>
</tr>
</tbody>
</table>

The decay rate is faster than that of CPO-I (0.5 s$^{-1}$ at pH 4.7, 25 °C) (Araiso et al. 1981) but slower than that of CYP119-I (9 s$^{-1}$, pH 7.0, 4 °C) (Kellner et al. 2002, Rittle and Green 2010). While the mechanism of spontaneous decay of compound I is unknown, more than 90% ferric protein was recovered in the process.

Furthermore, it is of interest to compare the reactivity observed for AaeUPO-I to the reactivities of CYP119 compound I, as recently reported by Rittle and Green (Rittle and Green 2010), CPO compound I as reported by Zhang (Zhang et al.
DISCUSSION

2006) and the reactive model oxoiron(IV) porphyrin radical cation species, [O=Fe^{IV}–4–TMPyP]^+, as recently described by (Bell and Groves 2009). It is also instructive to consider these comparisons in light of insights derived from computational approaches (Shaik et al. 2009, Shaik et al. 2008, Shaik et al. 2010). The rate constant observed for benzylic C-H hydroxylation of ethylbenzoic acid by AaeUPO-I is 125 times faster than that observed for the model compound I ferryl porphyrin at 4 °C and 250 times faster than that of CPO-I with similar substrates at 22 °C. For CYP119 compound I, rate constants of $10^3 – 10^7$ M$^{-1}$ s$^{-1}$ have been reported for unactivated methylene groups of fatty acids (Rittle and Green 2010). The slower of these rates, which are for hexanoic and octanoic acid, are similar to those observed for AaeUPO-I, while the fastest rate constant for CYP119 was for lauric acid, a tight-binding substrate, and may be an irreversible binding event. Notably, even the fastest C-H hydroxylation reactions found for AaeUPO-I are still more than one order of magnitude slower than the rate of oxidation of the ferric protein by mCPBA. Similarly, the low-spin to high-spin transition upon substrate binding was found to be very fast ($>2 \times 10^6$ M$^{-1}$ s$^{-1}$). Thus, the rate of substrate access to the active site is significantly faster than the rate of reaction and, accordingly, the observed rates are likely to be measures of the intrinsic C-H reactivity toward AaeUPO-I. Consistent with this assumption, the preliminary X-ray structure of AaeUPO shows a shallow, hydrophobic substrate-binding cavity flanked by several phenylalanine residues (Piontek et al. 2010). Further evidence for fast, reversible substrate binding is the observation of an intrinsic deuterium isotope effect ($k_H/k_D$) of 4.3 for THF and THF-$d_8$.

In addition, a plot of second-order rate constants for C-H hydroxylation by AaeUPO-I for all substrates vs. BDE of the scissile C-H bond revealed a very distinct, non-linear correlation (Figure 32). Notably, there was almost no change in $k_2'$ for the substrates with a C-H BDE of less than 90 kcal mol$^{-1}$. By contrast, the slope of log $k_2'$ vs. BDE above 90 kcal mol$^{-1}$ was $\sim 0.4$. Based on the BEP relationship: log($k_H$) = $\alpha \Delta H^0 + c$, where $\Delta H^0$ is related to the C-H BDE and $\alpha$ is a measure of transition state location. Thus, an $\alpha$ of 0.4 for the stronger C-H bonds indicates that these hydrocarbon hydroxylations mediated by AaeUPO-I have a nearly symmetrical [FeO---H---C] transition state. A linear BEP relationship for a model compound I with a series of C-H substrates was recently reported (Bell and
Groves 2009). The observed log $k$ vs BDE plot was rather flat ($\alpha = 0.28$) in that case, intermediate between the high and low values observed for AaeUPO-I (Figure 32).

### 4.7.2 Glutamic acid in the active site supports AaeUPO-I formation

Another interesting result that occurred during the investigation of AaeUPO-I formation was the fact that the formation rate of AaeUPO-I was at its highest at pH 5 and reached $4.9 \times 10^6$ M$^{-1}$ s$^{-1}$, whereas the decay rate was at its lowest ($1.4$ s$^{-1}$). When the pH was changed to more acidic or basic conditions the formation rates declined and the decay rates increased at the same time. This observation can be explained by the amino acid structure of the enzyme. AaeUPO is known to have an active site glutamate close to the heme center (Pecyna et al. 2009, Piontek et al. 2010) (Figure 33), which could play a role in the formation of AaeUPO-I by hydrogen peroxide, apparently its natural co-substrate.

![Figure 32: Evans-Polanyi plot with log$k'_2$ against BDE of various substrates, where $k'_2$ is $k_2$ (C–H hydroxylation rates) corrected by the number of possible hydroxylation products for each substrate. The green and the blue line show the BDE for the C–H bond in ethane and methane, respectively.](image-url)
Thus, *m*CPBA would initially replace a distal water ligand in resting *AaeUPO* with proton transfer to the neighboring glutamate to form an unseen peroxo-adduct similar to peroxidase compound 0. Subsequently, the O-O bond heterolysis and the formation of *AaeUPO-I* would be assisted by proton transfer from the active site glutamate to the product, *mCBA*, as outlined in Figure 34.

![Crystal structure of the active site of AaeUPO with glutamic acid residue. Adapted from Piontek 2011 (unpublished results) and according to Piontek (Piontek et al. 2010).](image)

Figure 33: Crystal structure of the active site of *AaeUPO* with glutamic acid residue. Adapted from Piontek 2011 (unpublished results) and according to Piontek (Piontek et al. 2010).

This role of the distal glutamate is similar to that proposed for the substrate carboxylate group in fatty acid hydroxylation by cytochrome P450 BSβ (Lee et al. 2003, Shoji et al. 2007) and SPα (Fujishiro et al. 2011). Furthermore, it is known that the protonated form of *m*CPBA (*pKₐ* 7.6) is the active species for compound I formation leading to increased rates of compound I formation at lower pH (Blake and Coon 1989, Kellner et al. 2002). Combined with the described possible mechanism of unprotonated glutamic acid (*pKₐ* 4.1) supporting *AaeUPO-I* formation, pH 5 as the best condition for *AaeUPO-I* formation is a logical result. A similar mechanism is also known for CPO, where a glutamic acid residue in the...
active site supports CPO compound I formation (Sundaramoorthy et al. 1998). In classical peroxidases this glutamate is substituted by a histidine, whereas in DyP type peroxidases an aspartic acid and an arginine support the formation of compound I (Sugano et al. 2007). Such an arginine is also present in AaeUPO (Arg189) and acts as a charge stabilizer of the glutamate (see Fig. 33).

![Figure 34](image)

Figure 34: Hypothetical support mechanism of AaeUPO-I formation by glutamic acid residue in the active site of AaeUPO.

4.7.3 Deuterium isotope effect and Evans-Polanyi correlation prove hydrogen abstraction

The final oxygen transfer in the hydroxylation process of P450s is known to proceed via hydrogen abstraction and oxygen rebound mechanism (Groves 1985), which is described in Figure 35. In order to prove whether this mechanism also applies for AaeUPO, experiments with semi-deuterated, symmetrical substrates can be performed and the intramolecular isotope \(\left(\frac{k_H}{k_D}\right)_{\text{obs}}\) effect can be calculated. As the BDE for C-H bonds is lower than for C-D bonds, C-H bonds are
An intramolecular isotope effect of more than 1 indicates the preference for a hydroxylation of the weaker C-H bond and therefore indirectly proves the existence of a hydrogen abstraction mechanism. Recently, a pronounced intramolecular deuterium isotope effect for peroxygenase-catalyzed ether cleavage was observed, which points to hydrogen abstraction from the carbon before oxygen can rebound from the heme iron (Kinne et al. 2009b).

![Figure 35: Hydrogen abstraction and oxygen rebound mechanism for hydroxylation reaction by P450s. Modified according to Maurer (Maurer 2006).](image)

An analogous experiment is difficult to perform with linear alkanes, as suitable substrates are often prochiral for the peroxygenase reaction and have more than one potential hydroxylation site. Eventually, the symmetrical 1,1,2,2,3,3-D$_7$ molecule was selected as the most suitable substrate to determine whether alkane hydroxylation by peroxygenase exhibits an intramolecular deuterium isotope effect. The observed mean intramolecular isotope effects [(k$_H$/k$_D$)$_{obs}$] of three experiments were 16.0 ± 1.0 for 2-hexanol and 8.9 ± 0.9 for 3-hexanol. In general, P450-catalyzed oxidations that occur via hydrogen abstraction at the ω-carbon of a semiterminal-deuterated alkane exhibit intrinsic deuterium intramolecular isotope effects of ~9 and (k$_H$/k$_D$)$_{obs}$ of 6 (Nelson and Trager 2003). Thus, the large values observed here and previous results strongly indicate a hydrogen abstraction mechanism with formation of a radical substrate intermediate in the reaction cycle of A. aegerita peroxygenase (Kinne et al. 2009b). In addition, the continuous decline in AaeUPO-I activity with a simultaneous increase of the substrate C-H-BDEs obtained in stopped-flow kinetic
studies (Evans-Polanyi-plot; section 4.7.1, Figure 32) proves the existence of a hydrogen abstraction mechanism.

4.7.4 Radical clock experiment proves substrate radical existence

To verify whether a substrate radical is formed, the peroxygenation of norcarane (bicyclo[4.1.0]heptane) was studied, a radical clock substrate that has often been used to evaluate the reaction mechanism of P450s and other alkane-oxidizing enzymes (Auclair et al. 2002, Austin et al. 2006, Austin et al. 2008, Groves 2006, Newcomb et al. 2002).
The GC/MS profile of the peroxygenase-catalyzed hydroxylation of norcarane shows that various reaction products were formed (section 3.6, Figure 24). The reactions yielded \textit{exo}-2-norcaranol (C) as the major product, smaller amounts of \textit{endo}-2-norcaranol (B) as well as \textit{exo}- and \textit{endo}-3-norcaranol (E and F), furthermore, a product expected to derive from the cationic intermediate 3-cyclohepten-1-ol (D), and the radical rearrangement product 4-(hydroxymethyl)-cyclohexane (A), which all have been described previously for P450s.

Table 13: Product distribution for the conversion of norcarane with AaeUPO compared to different P450s. Product yields were reported in % relative to \textit{endo}-2-norcaranol for P450s and \textit{exo}-2-norcaranol for AaeUPO, respectively; nd = not detected; table modified according to Auclair (Auclair et al. 2002).

<table>
<thead>
<tr>
<th>Products</th>
<th>AaeUPO</th>
<th>P450cam</th>
<th>P450BM3</th>
<th>CYP2B1</th>
<th>CYP2E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>35</td>
<td>49</td>
<td>59</td>
<td>188</td>
</tr>
<tr>
<td>A</td>
<td>0.2</td>
<td>1.6</td>
<td>1.3</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>D</td>
<td>1.8</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
<td>nd</td>
</tr>
<tr>
<td>Radical lifetime (ps)</td>
<td>9.4</td>
<td>52</td>
<td>44</td>
<td>16</td>
<td>35</td>
</tr>
</tbody>
</table>

The calculated radical lifetime of 9.4 ps and the oxygen rebound rate of $1.06 \times 10^{11}$ s$^{-1}$ is 6-20 times faster than the values observed for functionally similar P450s ($0.62$ to $2 \times 10^{10}$ s$^{-1}$) (Auclair et al. 2002, Cooper and Groves 2011). Interestingly, the major product of peroxygenase catalysis was \textit{exo}-2-norcaranol.
Its amount was about 25 times higher than that of *endo*-2-norcaranol, which differs from P450s (Table 13) (Auclair et al. 2002). Moreover, 3-cyclohepten-1-ol was found, which is indicative of a cationic pathway also described for P450s-catalyzed oxidations, where its formation was attributed to a competing electron-transfer oxidation of the incipient radical (Ortiz de Montellano and Groves 2005).

4.7.5 Hydrogen peroxide serves as oxygen source

It was shown recently by means of $^{18}$O-labeling that $\text{H}_2\text{O}_2$ supplies the oxygen atom, introduced during substrate hydroxylation and epoxidation by *AaeUPO* (Kinne et al. 2009a, Kinne et al. 2009b, Kinne et al. 2008, Kinne et al. 2010, Kluge et al. 2009). In this study, cyclohexane and 2,3-dimethyl-2-butene were chosen as substrates to be tested in this respect. The GC/MS analyses showed that the peroxygenase-catalyzed oxidation of these substrates in the presence of 90 atom% $\text{H}_2^{18}\text{O}_2$ resulted in almost complete $^{18}$O-incorporation (cf. Figure 19 and Figure 20; section 3.3). These results clearly prove $\text{H}_2\text{O}_2$ as the source of oxygen incorporated during substrate conversion.

4.7.6 Substrate epoxidation by *AaeUPO* – mechanism

To understand the mechanism of *AaeUPO*-catalyzed epoxidation, it is useful to look at information provided for other heme-thiolate enzymes, e.g. the well-studied P450s (Ortiz de Montellano and De Voss 2002). Two possible reaction pathways for substrate epoxidation have been proposed for P450s (Figure 36) and evidence was found for both alternatives. The concerted or synchronous transfer of the ferryl oxygen to the alkene double bond is strengthened by the fact that the alkene configuration is retained in the epoxide product (Kunze et al. 1983, Ortiz de Montellano 1995). On the other hand, two observations challenge the concerted pathway and suggest a non-concerted epoxidation mechanism. The first is the known fact that alkenes $N$-alkylate the heme and therefore inactivate the enzyme (Correia and Montellano 2005). This reaction is proof for a reactive species in the epoxidation mechanism, as a reaction of the epoxide product with the heme nitrogen was ruled out. A second indication for a radical intermediate is given by the fact that certain alkenes are not epoxidized but directly converted to the
corresponding carbonyl products, as reported for trichloroethylene and 1,1-dichloroethylene (Henschler et al. 1979, Liebler and Guengerich 1983, Miller and Guengerich 1982). An isomerization of the epoxide products to the carbonyl products was excluded in both cases. This contradictory problem with proof for both mechanisms, concerted and non-concerted, was finally clarified by density functional theoretical calculations published by de Visser, who proved that a concerted or synchronous epoxidation pathway does not exist (de Visser et al. 2001b).

Figure 36: Proposed epoxidation mechanism of cytochrome P450s with concerted (a) and non-concerted (radical) pathway (b). In (a) compound I first reacts with the alkene substrate (ethene) to form a iron-alkoxy radical complex, which, in a second step, undergoes ring closure to the epoxide product. In (b) oxygen is synchronous inserted into the π-bond of the substrate to form the epoxide. Modified according to de Visser (de Visser et al. 2001b). A similar mechanism can be assumed for AaeUPO.

They found that P450 compound I can exist in doublet and quartet spin state and both species preferentially react with ethene to produce a species with one carbon bound to the oxygen and the other carbon bearing a free radical (de Visser et al. 2001a). This species also exists in doublet and quartet spin state and while there
is no significant energetical barrier for the ring closure to the epoxide in doublet state, such a barrier exists for the quartet spin state (2.3 kcal mol$^{-1}$ or 7.2 kcal mol$^{-1}$, respectively, depending on the electron distribution), which results in much longer radical lifetimes and therefore gives a sufficient explanation for alternative reactions (e.g. heme $N$-alkylation) during P450-catalyzed epoxidation.

As AaeUPO and P450s are both heme-thiolate enzymes which share similarities in their reactive intermediate (compound I; c.f. section 4.7.1) and the heme $N$-alkylation reported for P450s was also observed for AaeUPO (c.f. section 4.6), a similar epoxidation mechanism with a non-concerted pathway is very likely. However, the possibility of an involvement of the ferric hydroperoxy complex (compound 0), as discussed for some P450-catalyzed epoxidation reactions (Kells et al. 2010, Vaz et al. 1998), cannot be completely ruled out.

### 4.7.7 AaeUPO catalytic cycle

With regard to all known and new facts of the catalytic cycle of AaeUPO obtained in mechanistic studies, an updated hypothetical model is shown in Figure 37 with cyclohexane as model substrate.

The cycle begins with AaeUPO low-spin six-coordinate iron in the ferric state [1], heme(Fe$^{III}$-H$_2$O)] with water as the sixth ligand. It remains unclear, whether a substrate molecule or hydrogen peroxide binds first. With regard to the observed ping-pong mechanism, Kinne suggested that H$_2$O$_2$ binds first to form an extremely short-lived iron-(III)-peroxide complex [2], heme(Fe$^{III}$-O-OH), known as compound 0 (Kinne 2010). In contrast, obvious and diagnostic changes in the UV/Vis spectrum of ferric AaeAPO upon substrate binding (Kinne 2010, Wang et al. 2012) indicated a very fast low-spin to high-spin inter-conversion fast (>2 x 10$^6$ M$^{-1}$ s$^{-1}$), which is also typical for cytochrome P450 enzymes (Isin and Guengerich 2008), where the higher reactivity of the high-spin enzyme supports the activation of dioxygen. A similar mechanism could also apply for AaeUPO. In a second step, the transient intermediate (compound 0) is heterolytically cleaved between the oxygen atoms by a two-electron transfer from the heme and a highly reactive iron-(IV)-porphyrin cation radical complex is formed, known as compound I [3], heme(Fe$^{IV}$=O)$^{++}$). The existence of this intermediate was proved by stopped-flow
studies and its involvement in substrate oxidation was shown in substrate kinetics (section 4.7.1). As confirmed by radical clock experiments (section 4.7.4) and the observed deuterium isotope effects (section 4.7.3), compound I then abstracts a hydrogen from the substrate to form a substrate radical intermediate and compound-II-like intermediate [4], heme(Fe^{IV}-OH)] (protonated compound II). Although several attempts failed to accumulate and capture the transient AaeUPO-II in stopped-flow experiments, promising conditions have been found and new experiments will be carried out in the near future.

Figure 37: Hypothetical catalytic cycle of AaeUPO. (1) Resting ferric AaeUPO, (2) iron-(III)-peroxide complex (compound 0), (3) iron-(IV)-porphyrin cation radical complex (compound I) and protonated compound II (heme(Fe^{IV}-OH). Modified according to Hofrichter and Kinne (Hofrichter and Ullrich 2006, Kinne 2010).

The oxygen transfer from compound II to the substrate proceeds instantaneously via oxygen rebound mechanism (section 4.7.3) and a hydroxylated substrate is released from the active site to recover resting AaeUPO (Hofrichter and Ullrich
The catalytic cycle of the AaeUPO reflects the peroxide-shunt pathway of P450s (c.f. section 1.4.3.1)

Moreover, the AaeAPO also exhibits peroxidase activity, which yields via two one-electron oxidations radicals, coupling and polymerization products from phenolic substrates (e.g. from 2,6-dimethoxyphenol) (Ullrich et al. 2004). In this kind of reaction mechanism, an acidic hydrogen atom (e.g. from a phenolic OH-group) is abstracted as described above but the ferryl oxygen is not rebounded to the substrate. Instead, one radical (in case of phenolic, a phenoxy radical) is released from (4) with formation of an oxy-ferryl heme [heme(FeIV=O)] (intermediate (4), Figure 8), which then reacts with the second substrate molecule resulting in the formation of a second radical and the native ferric enzyme. The H$_2$O$_2$-derived oxygen at the active site is then released in the form of water (i.e., two one-electron oxidation steps occur) (Kinne 2010).

4.8 Main findings and outlook

1. The unspecific peroxygenase from A.aegerita is able to catalyze the hydroxylation of various linear, branched and cyclic alkanes. Alkane substrates reach from gaseous propane (C3) to liquid $n$-hexadecane (C16).

2. Alkanes are mono-hydroxylated by AaeUPO mainly at the C2 and C3 position, rather than at the terminal carbon. In addition, the corresponding ketones are formed according to overoxidation.

3. A number of alkenes are epoxidized by AaeUPO, including linear terminal, branched and cyclic alkenes as well as linear and cyclic dienes.

4. While no reaction products could be observed for non-terminal alkynes, the conversion of terminal alkynes gave the corresponding 1-alkyn-3-ols in low yield.

5. The conversion of alkanes, alkenes, and alkynes by AaeUPO proceeds highly regio- and in some cases even stereoselectively with the ee reaching up to 99% ($n$-octane, $n$-heptane)
6. For the hydroxylation of alkanes and the epoxidation of alkenes, kinetic data were obtained from the conversion of the model substrates cyclohexane and 2-methyl-2-butene. The catalytic efficiency, $k_{cat}/K_m$ was $2.0 \times 10^3 \, \text{M}^{-1} \, \text{s}^{-1}$ and $2.5 \times 10^5 \, \text{M}^{-1} \, \text{s}^{-1}$, respectively.

7. A compound-I type species was observed in stopped-flow experiments and described kinetically.

8. The results obtained in the deuterium isotope effect experiment and the radical clock experiment clearly demonstrate that the hydroxylation of alkanes proceeds via substrate radical formation and a hydrogen abstraction mechanism.

As AaeUPO was shown to effectively catalyze certain alkane and alkene oxidation reactions, the question arises whether these reactions are part of the physiological role of the enzyme, which is still unknown. As suggested by Kinne (Kinne 2010) for the cleavage of ether bonds, AaeUPO could play a role in the biodegradation of low molecular lignin fragments or in the detoxification of fungicidal compounds derived from plants or microorganisms that contain alkane, alkene or alkyne structures. Further investigations on the AaeUPO-inducing component in the soybean medium still seems to be the most promising way to reveal AaeUPO’s physiological function.

In addition, stopped-flow studies on AaeUPO-II should be carried out, as promising conditions have recently been found to accumulate this transient intermediate.

Furthermore, a broader study on the stereoselectivity of alkane hydroxylation and alkene epoxidation seems to be promising, as it could reveal further reactions of industrial interest.
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6. Appendix
Appx. 1: GC/MS elution profiles of the *AaeUPO*-catalyzed *n*-hexane conversion with the alcohol and ketone products 2-hexanol (I), 3-hexanol (II), 2-hexanone (III) and 3-hexanone (IV), respectively. S = sample; C = control without enzyme

Appx. 2: GC/MS elution profiles of the *AaeUPO*-catalyzed 2,3-dimethylbutane conversion with the alcohol product 2,3-dimethyl-2-butanol. S = sample; C = control without enzyme
Appx. 3: GC/MS elution profiles of the *AaeUPO*-catalyzed 2-methyl-2-butene conversion with the epoxide product trimethyloxirane. S = sample; C = control without enzyme, but with H$_2$O$_2$; P = pure substrate, without enzyme and H$_2$O$_2$. Although the substrate was ordered in the highest purity available (>99%) auto-oxidized substrate was detected. This background concentration of the product was subtracted from the sample.
Appx. 4: GC/MS elution profiles of the *AaeUPO*-catalyzed cyclohexene conversion with the epoxid and alcohol products 1,2-epoxycyclohexane (7-oxabicyclo[4.1.0]heptane) and cyclohex-2-en-1-ol, respectively. S = sample; C = control without enzyme.
List of patents and publications


Acknowledgement

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

First of all I would like to express my deep gratitude to professor Hofrichter, my research supervisor, for his patient guidance, enthusiastic encouragement and useful critiques of this research work.

I would like to thank professor Katrin Scheibner for reviewing my thesis and have to offer my special thanks to professor John T. Groves for being my reviewer and for giving me the opportunity to work in his research group.

I am particularly grateful for the assistance given by Dr. Matthias Kinne especially his useful advices and critiques, as well as Martin Kluge for introducing me into the world of GC-MS analytics.

Special thanks to Dr. Gernot Kayser for useful discussions and his efforts for financial support in particular.

Thanks to all my colleagues and the laboratory teams of the IHI Zittau and the Princeton University for technical assistance and many fruitful discussions. In particular I want to thank Xiaoshi Wang for teaching me NMR-analysis and stopped-flow techniques and more importantly for being a wonderful team partner.

Finally I would like to thank my parents and my wife Ola for keeping me grounded.
Versicherung an Eidesstatt

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten:

Frau Dipl.-Übs. (FH) Gabi Ullmann


Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Zittau, den 05. Oktober 2012