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Stepwise oxygenations of toluene and 4-nitrotoluene by a fungal peroxygenase

Matthias Kinne [a,*], Christian Zeisig [a], René Ullrich [a], Gernot Kayser [a], Kenneth E. Hammel [b], Martin Hofrichter [a]

[a] Unit of Environmental Biotechnology, International Graduate School of Zittau, Markt 23, 02763 Zittau, Germany
[b] USDA Forest Products Laboratory, Madison, WI 53726, USA

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ABSTRACT

Fungal peroxygenases have recently been shown to catalyze remarkable oxidation reactions. The present study addresses the mechanism of benzylic oxygenations catalyzed by the extracellular peroxygenase of the agaric basidiomycete *Agrocybe aegerita*. The peroxygenase oxidized toluene and 4-nitrotoluene via the corresponding alcohols and aldehydes to give benzoic acids. The reactions proceeded stepwise with total conversions of 93% for toluene and 12% for 4-nitrotoluene. Using $H_2{}^{18}O_2$ as the co-substrate, we show here that H_2O_2 is the source of the oxygen introduced at each reaction step. *A. aegerita* peroxygenase resembles cytochromes P450 and heme chloroperoxidase in catalyzing benzylic hydroxylations.

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1. Introduction

Selective C–H oxidations occur in a wide range of biological transformations and are one of the most challenging reactions in organic chemistry. Therefore, it is of interest to understand the reaction mechanisms of enzymes that are capable of these reactions, and to apply these biocatalysts as tools for organic synthesis.

Recently a new group of extracellular fungal heme biocatalysts, the aromatic peroxygenases (APOs), was found in agaric basidiomycetes. These heme-thiolate enzymes may represent a new superfamily of heme peroxidases [1,2], and show some functional similarities with the latter enzymes in catalyzing the H_2O_2-dependent oxidation of substrates such as phenols and halide ions (e.g. Br^-), but also resemble cytochrome P450-dependent monooxygenases (P450s) in mediating selective epoxidations/hydroxylations of numerous aromatic and aliphatic substrates. ^{18}O-Labeling studies have established that H_2O_2 is the source of oxygen introduced during a variety of peroxygenase-catalyzed oxidations, including the epoxidation and hydroxylation of aromatics [3–6], the sulfoxidation of dibenzothiophene [4], the *N*-oxidation of pyridine derivatives [7] and the cleavage of diverse ethers to produce aldehydes and ketones [8].

Previous work has shown that the best-characterized fungal peroxygenase, from *Agrocybe aegerita,* can oxidize toluene to form benzyl alcohol, benzaldehyde, and benzoic acid [9]. However, the mechanism of the benzylic oxidations catalyzed by *A. aegerita* aromatic peroxygenase (*Aae*APO) remains unclear. The work we report here suggests that *Aae*APO-catalyzed reactions resemble the benzylic hydroxylations catalyzed by P450s via their H_2O_2-dependent "peroxide shunt" mechanism.

2. Materials and methods

2.1. Chemicals and enzyme preparation

All chemicals used were purchased from Sigma–Aldrich except $H_2{}^{18}O_2$ (90 atom%, 2% wt/vol), which was obtained from Icon Isotopes. *Aae*APO (isoform II, p*I* 5.6) was produced and purified as described previously [10,11]. The enzyme preparation was homogeneous by SDS polyacrylamide gel electrophoresis and exhibited an A_{418}/A_{280} ratio of 1.75. The specific activity of the peroxygenase was 117 U mg^{-1}, where 1 U represents the oxidation of 1 μmol of 3,4-dimethoxybenzyl alcohol to 3,4-dimethoxybenzaldehyde in 1 min at 23 °C [10].

Abbreviations: *Aae*APO, aromatic peroxygenase from *Agrocybe aegerita*; P450, cytochrome P450.

* Corresponding author. Fax: +49 3583 612734.
E-mail address: kinne@ihi-zittau.de (M. Kinne).

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2.2. Reaction conditions

Typical reaction mixtures (0.5 ml) contained purified peroxygenase (4 U ml^{-1}), potassium phosphate buffer (50 mM, pH 7.0), acetonitrile (5% vol/vol) and the substrate (0.5 mM). The reactions were started by the addition of H_2O_2 (1 mM).

2.3. Product identification

The reaction products benzoic acid and 4-nitrobenzoic acid were analyzed by high performance liquid chromatography (HPLC) using an Agilent Series 1100 instrument equipped with a diode array detector and an electrospray ionization mass spectrometer on a Luna 5-μm-pore-size C18 column (Phenomenex). The column was eluted at 40 °C and 0.35 ml min^{-1} with an aqueous ammonium formate solution (0.1% vol/vol, pH 3.5)/acetonitrile, 95:5 (70:30 for nitro-substituted compounds), for 5 min, followed by a 25-min linear gradient to 100% acetonitrile. Products were identified relative to authentic standards, based on their retention times, UV absorption spectra, and $[M–H]^-$ ions.

The reaction products benzyl alcohol, 4-nitrobenzyl alcohol, benzaldehyde and 4-nitrobenzaldehyde were analyzed by gas chromatography (GC) of benzene extracts, using a Hewlett Packard 6890 chromatograph equipped with a Hewlett Packard 5973 mass spectrometer. GC was performed with a temperature program starting at 40 °C for 2 min and then increasing at 15 °C min^{-1} to 220 °C, using helium as the carrier gas at a column flow rate of 1 ml min^{-1} on a 5% polysiloxane column (Zebron ZB-5, 250 μm diameter by 30 m length, 0.25 μm film thickness, Phenomenex). The products were identified relative to authentic standards by their retention times and by electron impact MS at 70 eV. For each *m/z* value, the average total ion count within the product peak was used after background correction to generate the ion count used for mass abundance calculations. Calculation of ^{18}O-incorporation was performed by dividing the sum of the natural species abundance and the isotope abundance with the isotope abundance. The 10% of natural abundance H_2O_2 in the $H_2{}^{18}O_2$ preparation was taken into account in these calculations.

2.4. Kinetics experiments

The time course of product release during *Aae*APO-catalyzed hydroxylation of toluene and 4-nitrotoluene was analyzed in stirred reactions (0.20 ml, 23 °C) that contained 1 U ml^{-1} of the peroxygenase, potassium phosphate buffer (50 mM, pH 7.0), and 0.50 mM of the substrate. The reactions were initiated with 1 mM H_2O_2 and stopped with 0.02 ml of 50% (w/vol) trichloroacetic acid after 5, 10, 30, 60 and 120 s. The reaction products were quantified by HPLC as described above.

3. Results

*Aae*APO hydroxylated toluene and 4-nitrotoluene to give the corresponding benzyl alcohols, benzaldehydes and benzoic acids. The reactions proceeded rapidly with total conversions of 93% for toluene and 12% for 4-nitrotoluene (Fig. 1). The low extent of 4-nitrotoluene oxidation is attributable to inhibition of the enzyme by the substrate, which has also been observed during P450-catalyzed oxidations of nitroaromatics [12,13]. The initial product of toluene oxidation was benzyl alcohol, which then declined with concomitant production of benzaldehyde, which in turn declined with concomitant production of benzoic acid. When benzyl alcohol was used instead of toluene as the starting substrate, the products were benzaldehyde and benzoic acid (Supplementary Fig. 1A), whereas with benzaldehyde as the starting material, only benzoic acid was formed (Supplementary Fig. 1B). In reactions with 4-nitrotoluene as the starting substrate, the reaction sequence was not as apparent (Fig. 1), but other experiments with 4-nitrobenzyl alcohol or 4-nitrobenzaldehyde as starting substrates showed the same precursor-product relationships as in the experiments with toluene (Supplementary Fig. 1C and D).

An ^{18}O-labeling study established that H_2O_2 supplied the oxygen incorporated during *Aae*APO-catalyzed oxidation of the two toluenes. (Fig. 2). When we conducted the reaction with toluene and $H_2{}^{18}O_2$, mass spectral analysis of the resulting benzyl alcohol showed that its principal ion had shifted from the natural abundance *m/z* of 108 to *m/z* 110. Similarly, the reaction with 4-nitrotoluene and $H_2{}^{18}O_2$ yielded 4-nitrobenzyl alcohol in which the principal ion had shifted from *m/z* 153 to *m/z* 155.

We also observed incorporation of ^{18}O from $H_2{}^{18}O_2$ in the benzaldehyde and benzoic acid formed from toluene in these experiments. To clarify this finding, we performed labeling experiments using each of the intermediate products as *Aae*APO substrates, and thus showed that ^{18}O was incorporated from $H_2{}^{18}O_2$ at each oxidation step. With benzyl alcohol as the substrate, some of the resulting benzaldehyde shifted from its natural abundance *m/z* of 106 (100%) to *m/z* 108 (22%), which indicates 18% incorporation of ^{18}O from $H_2{}^{18}O_2$. The *m/z* values for the benzoic acid formed in this experiment also shifted, in this case from *m/z* 121 to *m/z* 123 (100%) and *m/z* 125 (9.5%). When benzaldehyde was used as the substrate instead, the resulting benzoic acid shifted quantitatively from its natural abundance *m/z* of 121 to *m/z* 123 (Fig. 3).

The same trend was apparent with 4-nitro-substituted substrates. In reactions started from 4-nitrobenzyl alcohol, some of the resulting 4-nitrobenzaldehyde shifted from the natural abundance *m/z* of 151 (100%) to *m/z* 153 (15.5%), thus indicating 13% incorporation of ^{18}O from $H_2{}^{18}O_2$. The *m/z* values for the 4-nitrobenzoic acid formed in this experiment also shifted, in this case from *m/z* 166 to *m/z* 168 (100%) and *m/z* 170 (9.5%). The shift from the natural abundance *m/z* of 166 to *m/z* 168 was quantitative for 4-nitrobenzoic acid when 4-nitrobenzaldehyde was used instead as the starting substrate (Fig. 3).

4. Discussion

Our results show that *Aae*APO can convert toluenes to benzoic acids via sequential two-electron oxidations, and that the intermediate benzyl alcohols and benzaldehydes are released from the enzyme active site. In addition to side chain oxidation, *Aae*APO also catalyzes the oxygenation of the aromatic ring of toluene (but not of 4-nitrotoluene) leading to mixtures of *p*- and *o*-cresol and their oxidation products [9]. As reported earlier, these reactions may compete with side chain oxidation. In the present study, where the focus has been on side chain oxidations, ring oxygenation of toluene was ignored.

The ^{18}O-labeling experiments establish that the oxygens introduced during oxidations originate from H_2O_2. The results support a mechanism similar to that envisaged for the peroxygenase activity of P450s [14,15] and for ether cleavage catalyzed by *Aae*APO [8], in which the enzyme heme is oxidized by H_2O_2 to give a ferryl oxygen intermediate [16] that carries one of the peroxide oxygens and can be depicted formally as $(FeO)^{3+}$. The latter (very probably a Compound I-type intermediate) abstracts a hydrogen from the benzylic carbon to give an enzyme-bound benzylic radical, after which rebound of an •OH equivalent occurs to introduce a new hydroxyl group on the same carbon (Fig. 4).

According to this model, oxygen incorporation from H_2O_2 should be quantitative when a toluene is oxidized to a benzyl alcohol, and our data agree with this picture. When the substrate is a benzyl alcohol instead, the enzyme-bound intermediate will be

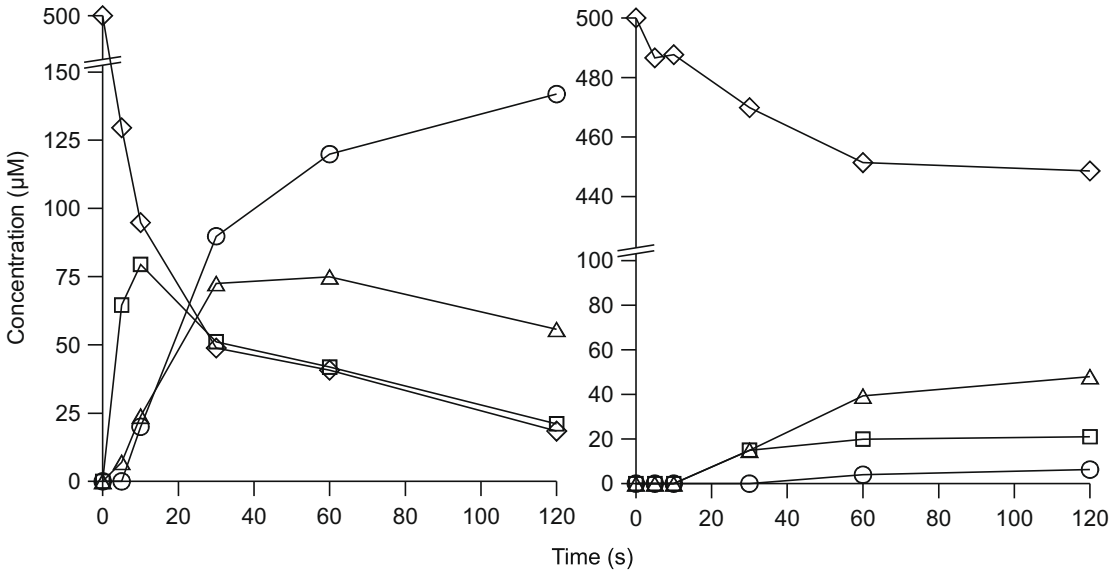

Fig. 1. Time course of *Aae*APO-catalyzed oxidation of toluene (left) and 4-nitrotoluene (right). Toluenes (◇), benzyl alcohols (□), benzaldehydes (Δ), benzoic acids (o).

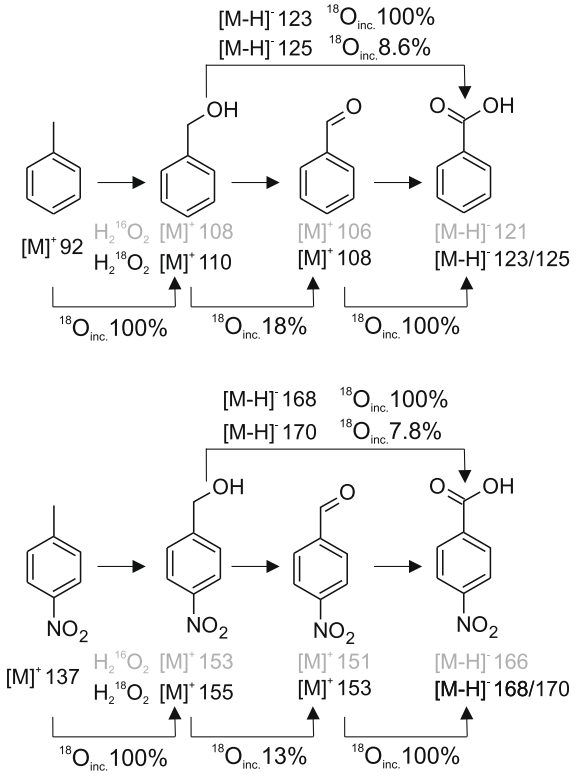

Fig. 2. Reaction scheme showing the yields of ^{18}O-incorporation into reaction products during *Aae*APO-catalyzed oxidation of toluene (top) and 4-nitrotoluene (bottom) in the presence of $H_2{}^{18}O_2$.

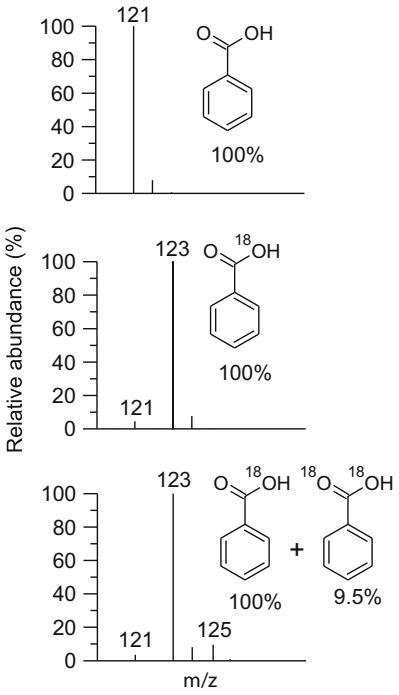

Fig. 3. Incorporation of ^{18}O from $H_2{}^{18}O_2$ into the carboxyl group of benzoic acid after oxidation of benzaldehyde (middle) and benzyl alcohol (bottom) by *Aae*APO. MS of the product obtained with natural abundance H_2O_2 (top).

an α-hydroxybenzylic radical and the resulting initial product will be a *gem*-diol in equilibrium with the benzaldehyde. Consequently, some of the oxygens introduced from H_2O_2 will be lost via non-stereospecific exchange with water, again in accord with our results. Finally, when the substrate is a benzaldehyde, the enzyme-bound intermediate will be an α-oxobenzylic radical, oxygen incorporation from H_2O_2 will be quantitative, and the resulting product will be the benzoic acid, once more in agreement with our data. In theory, this last oxidation could proceed via the *gem*-triol, but this intermediate can be ruled out because we found that no exchange of incorporated oxygen occurred during the oxidation of either benzaldehyde (Fig. 4).

The sequential *Aae*APO-catalyzed oxidations we have described here are also typical of P450s [17,18], but the latter enzymes are intracellular, whereas *Aae*APO is secreted into the surrounding environment by the fungal hyphae. Some other oxidative fungal enzymes such as lignin peroxidases and chloroperoxidases also have an extracellular location, but are more limited than *Aae*APO in the variety of compounds they can utilize as electron donors. For example, lignin peroxidase does not oxidize benzyl alcohol, and neither of these peroxidases is able to oxidize 4-nitrotoluene

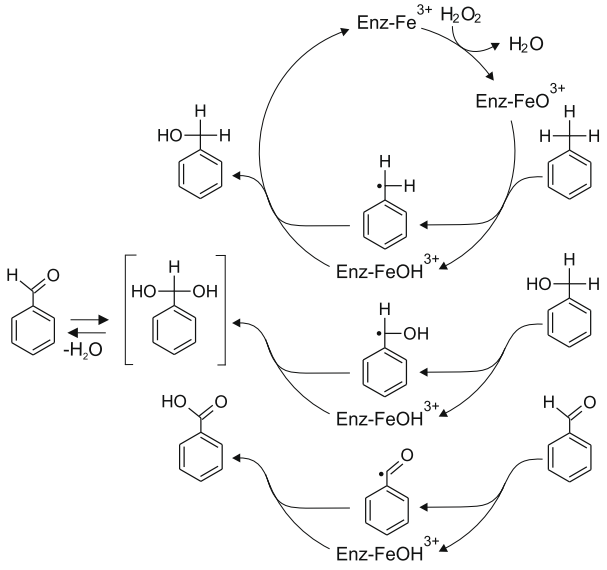

Fig. 4. Proposed reaction mechanism for benzylic oxidation by *Aae*APO.

[17,19,20]. The broad substrate range and ubiquitous distribution of extracellular APOs may indicate an important environmental role for them in the oxidation of both natural and anthropogenic aromatics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.05.036.

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