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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Hydroxylation of naphthalene by aromatic peroxygenase from *Agrocybe aegerita* proceeds via oxygen transfer from H_2O_2 and intermediary epoxidation

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Abstract *Agrocybe aegerita* peroxidase/peroxygenase (AaP) is an extracellular fungal biocatalyst that selectively hydroxylates the aromatic ring of naphthalene. Under alkaline conditions, the reaction proceeds via the formation of an intermediary product with a molecular mass of 144 and a characteristic UV absorption spectrum (A_{max} 210, 267, and 303 nm). The compound was semistable at pH 9 but spontaneously hydrolyzed under acidic conditions (pH <7) into 1-naphthol as major product and traces of 2-naphthol. Based on these findings and literature data, we propose naphthalene 1,2-oxide as the primary product of AaP-catalyzed oxygenation of naphthalene. Using ^{18}O-labeled hydrogen peroxide, the origin of the oxygen atom transferred to naphthalene was proved to be the peroxide that acts both as oxidant (primary electron acceptor) and oxygen source.

Keywords Peroxidase · Oxygenation · Hydroxylation · P450 · Naphthol

Introduction

Aromatic peroxygenases are extracellular peroxide-consuming enzymes secreted by agaric mushrooms, which are unique in their ability to hydroxylate substrates such as naphthalene or toluene (Dau et al. 2007; Ullrich and Hofrichter 2005). The best-studied biocatalyst of this type is produced by the Black poplar mushroom (*Agrocybe aegerita*) that preferably colonizes stumps, coarse woody debris, and bark of deciduous trees (Uhart et al. 2008; Ullrich and Hofrichter 2007). In earlier publications, the enzyme has also been referred to as *A. aegerita* peroxidase or peroxygenase (AaP) or heme-thiolate haloperoxidase due to its particular catalytic and spectroscopic properties (Hofrichter and Ullrich 2006; Ullrich et al. 2004). Thus, it oxidizes phenolic substrates in the classical way of peroxidases, brominates activated aromatics in the presence of bromide and in the visible spectrum, it shows a characteristic shift of the Soret band of the reduced carbon monoxide complex towards 450 nm. In this way, aromatic peroxygenase resembles both fungal chloroperoxidase (CPO) and cytochrome P450 monooxygenases and it can be regarded as a functional hybrid of both enzyme types (Brown et al. 2008; Omura 2005; Ullrich and Hofrichter 2007). Due to their extracellular nature, the high degree of glycosylation (20–40%) and the hence high stability, AaP and related enzymes could become new model biocatalysts for oxygen transfer reactions (Hofrichter and Ullrich 2006). Here, we report on the mechanism of peroxygenase-catalyzed oxygen transfer by the example of naphthalene that is regioselectively hydroxylated into 1-naphthol (Kluge et al. 2007).

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Materials and methods

Enzyme preparation

A. aegerita peroxygenase was produced in a 10-L stirred-tank bioreactor using a 2% soybean suspension as growth

medium. The major isoform (AaP II) was concentrated by ultrafiltration and purified by fast protein liquid chromatography using different ion exchangers and an ÄKTA explorer system as described previously (Ullrich et al. 2004; Ullrich and Hofrichter 2005). The final enzyme preparation had specific activities of 74.8 and 217 U mg^{-1} for veratryl alcohol and naphthalene, respectively, and an $Rz_{(418/280)}$ value of 1.78 (comparatively low Rz values are characteristic for heme-thiolate peroxidases such as CPO or AaP; Blanke et al. 1996; Ullrich et al. 2004).

Enzyme assay

Volume activity of AaP was routinely measured by following the oxidation of veratryl alcohol into veratraldehyde photometrically (ε_{310}=9,300 M^{-1} cm^{-1}) in sodium phosphate buffer at pH 7 (Ullrich et al. 2004). In addition, the oxygenating activity of AaP was detected by the formation of 1-naphthol from naphthalene in a neutral phosphate buffer according to Kluge et al. (2007). The reaction mixture (total 1 ml) consisted of 500 µl potassium phosphate buffer (100 mM, pH 7.0), 1 mM naphthalene (added dissolved in 200 µl acetonitrile to improve solubility), 1 mM hydrogen peroxide (H_2O_2), AaP (15–300 nM corresponding to 0.05–1.0 veratryl alcohol units), and distilled water (to 1 ml). The reaction was performed at 20°C, initiated by the addition of H_2O_2, and followed as the increase in absorbance at 303 nm (calculated apparent $\varepsilon_{303\ nm}$ for 1-naphthol—2,030 M^{-1} cm^{-1}) for at least 15 s (Kluge et al. 2007). All photometric measurements were performed using a Cary Bio 50 spectrophotometer (Varian, Inc., Walnut Creek, CA, USA).

Enzymatic reaction

To study the formation of earlier metabolites (e.g., naphthalene epoxides), the reaction was performed in 1.5-ml high performance liquid chromatography (HPLC) vials (total reaction volume 1 ml) in alkaline phosphate buffer (pH 9, 50 mM) under the conditions described above except that 2 mM peroxide and naphthalene were added. After one-time addition of hydrogen peroxide, 20 µl of the reaction solution was immediately injected into the HPLC system. Acidic hydrolysis of naphthalene 1,2-oxide was achieved by the addition of 20 µl hydrochloric acid (36%) to the reaction mixture (pH <1), and the naphthols formed were subsequently identified by HPLC using authentic standards.

The origin of the oxygen incorporated by AaP into naphthalene was proved using ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$) as cosubstrate. The reaction conditions were the same as described above; mass spectral analysis of the reaction products was done by liquid chromatography (LC)/mass spectrometry (MS) and gas chromatography (GC)/MS (after acidification).

High performance liquid chromatography (LC/MS)

HPLC analyses were performed using a 1200 Series Agilent system (Waldbronn, Germany) equipped with a diode-array detector SL, a mass selective detector VL mass spectrometer, and an atmospheric pressure ionization–electrospray ionization (ESI) ion source. For chromatographic separation, a Gemini C6-Phenyl column (150× 4.6 mm, 5 µm; Phenomenex Ltd., Aschaffenburg, Germany) was used along with a mobile phase consisting of an (a) aqueous ammonium formiate buffer (5 mM, pH 9.5) and (b) acetonitrile. Separations were run at constant 40°C by using a stepwise gradient starting with (a) 45% held for 4 min, then increasing to (b) 85% within 3 min and being maintained at (b) 85% until all analytes had eluted from the column with a constant flow rate of 1 ml min^{-1}. Eluted substances were detected in the wavelength range from 225 to 275 nm (diode array range 190–600 nm). Ionization was achieved by atmospheric pressure electrospray ionization in the positive (4 kV) and/or negative (3.5 kV) mode. Mass spectra were recorded at a rate of 0.93 s^{-1} in the range from 50 to 300 m/z.

Gas chromatography–mass spectrometry

The reaction mixture (1 ml) was acidified with hydrochloric acid and intensively mixed with 300 µl of chloroform. After phase separation, the chloroform extract was used for GC/MS analysis. The latter one was performed using an Agilent 6890 model gas chromatograph equipped with a 5790 mass selective detector and a Supelco beta-DEX 120 analytical capillary column (30 m×0.25 mm i.d.×0.25 µm film thickness) that showed a high resolution for naphthol isomers. Helium was used as carrier gas at a constant flow rate of 1 ml min^{-1}. Injection was accomplished at 250°C in the split mode with a split ratio of 20:1. A temperature program was run starting with an initial temperature of 165 °C that was increased at a rate of 3 °C min^{-1} to 190°C and maintained there for 2 min. Mass spectra were recorded in the range from 35 to 300 m/z.

Chemicals

Naphthalene (1), 1-naphthol (4), 2-naphthol (3), and 1,4-naphthoquinone as well as all organic solvents used and hydrogen peroxide (H_2O_2; 30%, for medical purposes, extra pure and stabilized) were purchased from Merck (Darmstadt, Germany) with the highest purity available. ^{18}O-labeled H_2O_2 was obtained from Icon Isotopes (New York, NY, USA).

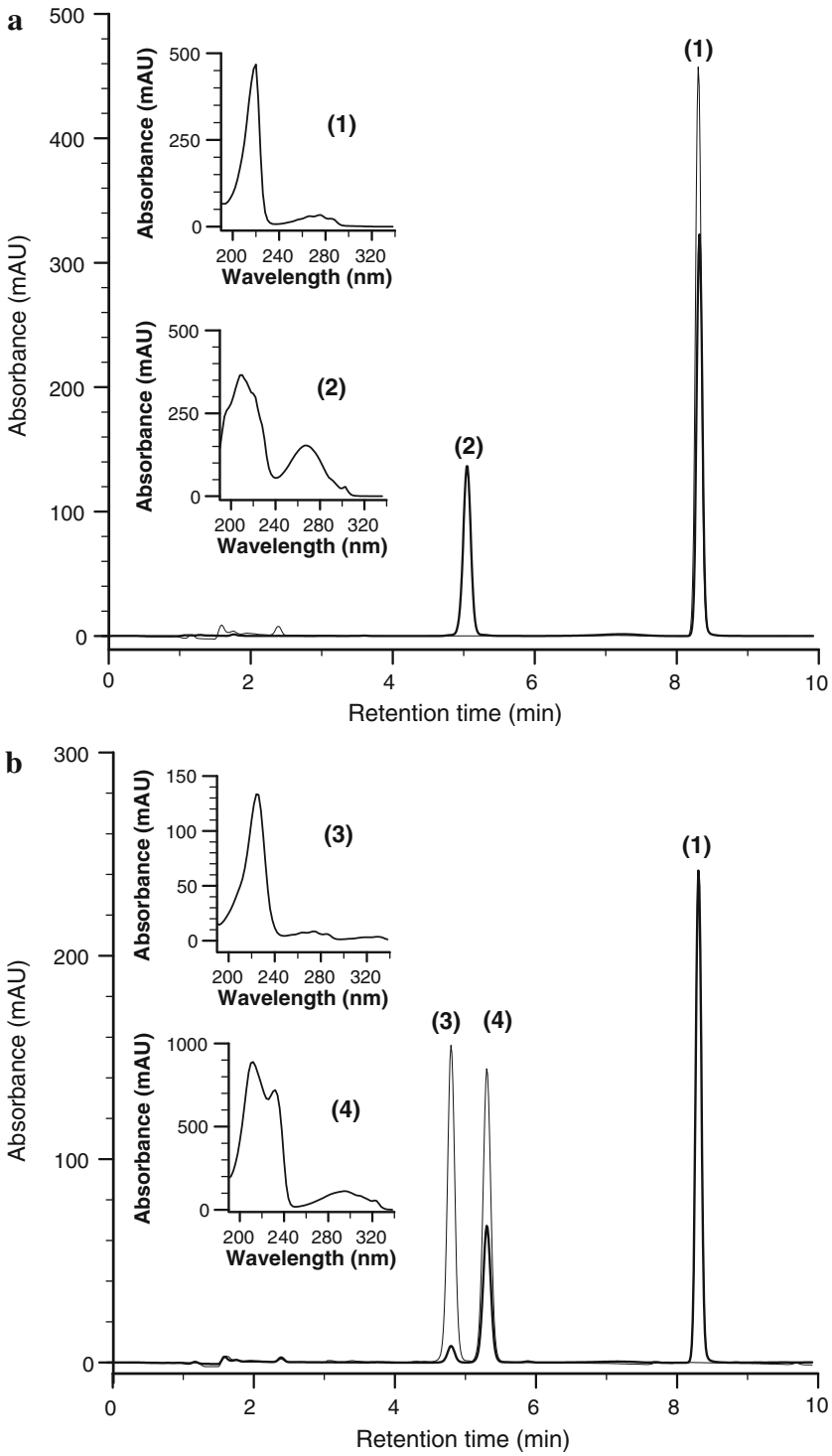

Fig. 1 **a** HPLC elution profile (275 nm) of the conversion of naphthalene by AaP under alkaline conditions (*bold line*). The reaction was performed in potassium phosphate buffer (pH 9) using 0.2 U AaP (60 nM) and 2 mM naphthalene. The *insets* show the UV spectra of naphthalene (1) on the *top* and its primary oxygenation product (2), putative naphthalene 1,2-oxide, *below*; *thin line*—naphthalene standard. **b** Chromatogram (275 nm) of the same reaction mixture after acidification with HCl to pH <1 (*bold line*). The peak of (2) disappeared while two new peaks, (3) and (4), emerged. Comparing their data with authentic standards (*thin line*) proved their naphtholic nature; *insets* show the respective UV spectra of 1-naphthol (4) and 2-naphthol (3)

Results

Naphthalene epoxidation (LC/MS analysis)

Naphthalene was rapidly enzymatically converted to one intermediate product putative naphthalene 1,2-oxide (2) that was relatively stable in the alkaline reaction buffer (pH 9) and could be analyzed by means of HPLC using an alkaline mobile phase (ammonium formiate, pH 9.5). Figure 1a shows the respective HPLC elution profile of the conversion of naphthalene (2 mM) by AaP (60 nM) in the presence of hydrogen peroxide (2 mM); (2) eluted at 5.05 min and showed a consistent UV spectrum with three maxima: the most intensive maximum at 210 nm, a second

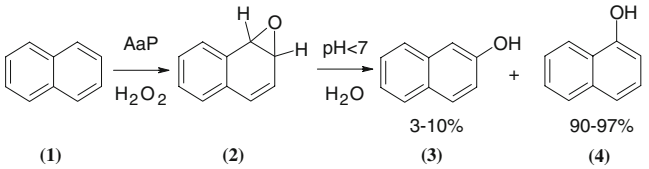

Fig. 2 Proposed reaction scheme for the AaP-catalyzed oxidation of naphthalene

one at 267 nm, and a third, characteristic maximum, at 303 nm (inset Fig. 1). In the ESI positive mode, (2) formed an $[M+H]^+$ ion with 145 m/z whereas in the negative mode, no ionization was achieved. After acidification with HCl, the peak of (2) completely disappeared while two new peaks emerged. The latter peaks were identified as the minor product 2-naphthol (retention times (RT)=4.80 min) and the major product 1-naphthol (RT=5.30 min) by comparing their retention times and UV spectra with authentic standards (Fig. 1b). Total naphthalene conversion amounted to about 50% and the contribution of (3) to the total amount of naphthols was 2.5%. Unlike (2), naphthols could not be ionized in the ESI positive mode but showed a single $[M-H]^-$ signal corresponding to 143 m/z in the negative mode (in which (2) gave no MS signal). Based on these findings, we propose that naphthalene 1,2-oxide (naphthalene 1,2-oxirane) is formed as the initial product of AaP-catalyzed naphthalene oxidation (Fig. 2).

Performing the same experiments in the presence of $H_2{}^{18}O_2$, led to the equimolar incorporation of ^{18}O into (2), (3), and (4) corresponding to an m/z shift of +2 and a $[M+H]^+$ signal of 147 m/z for (2) and $[M-H]^-$ signals of 145 m/z for (3) and (4), respectively (data not shown).

Gas chromatography–mass spectrometry

GC/MS experiments corroborated the formation of (3) and (4) as the final reaction products (after acidic hydrolysis) by comparison of their retention times and MS spectra with authentic naphthol standards as well as by the results of a NIST97 spectrum library search. A respective total ion current chromatogram is given in Fig. 3 showing the elution of residual (1) (3.59 min) and of the products (4) (6.38 min) and (3) (6.51 min). Oxidation of (1) in the presence of ^{18}O-labeled hydrogen peroxide revealed the formation of naphthols (3), (4) with a +2 amu shift in the mass spectra from 144 to 146 m/z for the molecular ion (in comparison to their native mass spectra), which corresponds to an almost complete (>99%) incorporation of one ^{18}O-atom

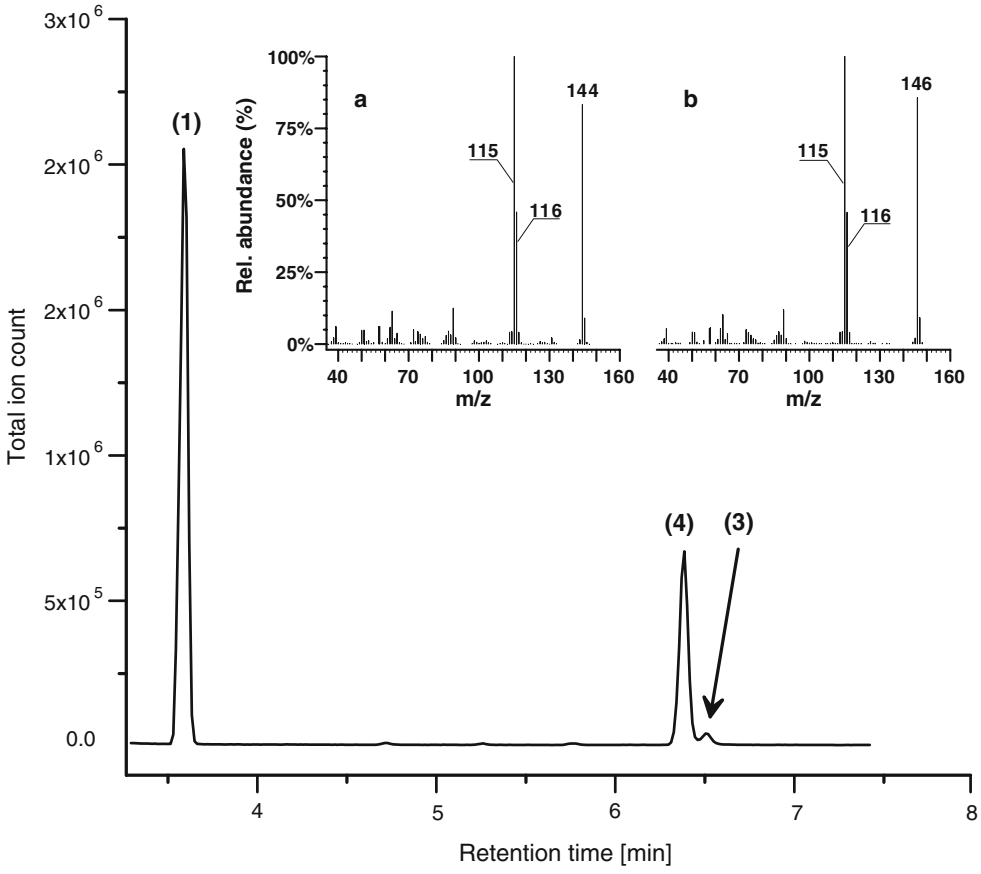

Fig. 3 GC/MS analysis of reaction products formed in the course of AaP-catalyzed naphthalene oxidation and subsequent acidification. The reaction mixtures contained either $H_2{}^{16}O_2$ (**a**) or $H_2{}^{18}O_2$ (**b**) and were extracted with chloroform after acidification; (1) naphthalene, (4) 1-naphthol, (3) 2-naphthol. The corresponding mass spectra of 1-naphthol molecules (*insets*) reveal molecular masses of 144 (^{16}O-1-naphthol) and 146 (^{18}O-1-naphthol) for **a** and **b**, respectively

from the labeled peroxide into the substrate. Insets of Fig. 3 show the respective mass spectra of (4), both ^{16}O-labeled and ^{18}O-labeled. The spectra of (3) (^{16}O- or ^{18}O-labeled) are analogous to those of (4) (data not shown). The typical fragments of (4), 115 and 116 m/z, were very probably formed by dissociation of a formyl radical and carbon monoxide, respectively, since for those fragments and all smaller ones, no +2 amu shift was observed. In contrast, (2) (the putative epoxide) was not accessible to gas chromatographic analysis due to its low thermal stability (Vogel and Klärner 1968).

Discussion

There are clear indications that *A. aegerita* peroxygenase oxygenates naphthalene to form naphthalene 1,2-oxide as primary product that in turn hydrolyzes into 1-naphthol and 2-naphthol as major and minor products, respectively. LC/MS analysis showed the formation of isobaric nominal masses for (2) and for the naphthols, (3) and (4), of 144 as well as the same elemental composition (plus one additional oxygen incorporated into (1)), which limits the number of possible regioisomers. The product formed by AaP at pH 9 in the presence of H_2O_2 exhibits a characteristic UV spectrum with three maxima (A_{max}) at 210, 267, and 303 nm and a ratio of absorbances (ε_λ) of 13.5:5.7:1, respectively. These UV spectral data sufficiently match those previously reported by Vogel and Klärner (1968) for authentic naphthalene 1,2-oxide (A_{max} at 220, 268, and 304 nm; ratio of ε_λ—11.2:5.6:1 in dioxane) and strongly indicate the formation of this oxirane (but not that of an isomeric oxepine intermediate that we can exclude because of the particular reaction conditions; Boyd and Sharma 1996; Maier 1967; Vogel and Klärner 1968). The orientation of the oxirane at the 1,2-position is additionally supported by the fact that the epoxidation of non-K regions of PAHs is generally favored (Boyd and Sharma 1996). Configurational stability is provided for individual enantiomers which do not undergo spontaneous or photo-induced racemization via an oxygen walk pathway to the respective oxepine (Boyd et al. 1987). Our study, however, could not prove the formation of a particular enantiomer of naphthalene 1,2-oxide due to the lacking of respective reference compounds (Berti 1973).

Naphthalene 1,2-oxide further undergoes isomerization within a nonenzymatic reaction as previously described by (Kasparek and Bruice 1972). Based on the fact that ^{18}O from peroxide was stoichiometrically incorporated into the naphthols and no oxygen exchange with water was observed, we can exclude a decay of (2) via a diol intermediate that would be stable in aqueous solution and hence detectable (Jerina et al. 1971).

Kinetics of the epoxide rearrangement show a pH independent first order rate constant representing a spontaneous rearrangement at alkaline conditions and an additional pH depending rate constant in acidic solution (pH <7) indicating a direct acid catalysis (Kasparek and Bruice 1972). This fact is confirmed by our present and recently published results (Kluge et al. 2007). Nevertheless, it is not entirely understood yet how a decrease in pH can lead to an increasing contribution of (3) (18% at pH 3 compared to 2.5% at pH 8) to the sum of both naphthols (Kluge et al. 2007). There has only little been reported on the dependence of the isomeric composition as a result of the decay of (2) in the literature, where (4) is always the predominating product (Vogel and Klärner 1968). Jerina et al. (1970) reported up to 12% of (3) after incubation of (2) in methylene chloride saturated with acetamide. We think that the loss of selectivity is simply due to the higher reactivity of (2) in an acidic environment.

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