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Formation of naphthalene hydrates in the enzymatic conversion of 1,2-dihydronaphthalene by two fungal peroxygenases and subsequent naphthalene formation

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ABSTRACT

The formation of naphthalene hydrates (i.e. 1- and 2-hydroxy-1,2-dihydronaphthalene) displays a new activity (besides epoxidation) in the enzymatic transformation of 1,2-dihydronaphthalene by two fungal unspecific peroxygenases (UPOs) accounting for 16–19% of the overall turnover. These arene hydrates decayed into naphthalene that in turn was converted by UPOs into naphthols. The oxygen transferred during hydroxylation was shown to derive from hydrogen peroxide proving a true peroxygenation reaction.

1. Introduction

Enzymatic aromatizations play an important role in human and microbial metabolism. Biological aromatization occurs mainly with specifically oxyfunctionalized precursors and α-unsaturated rings (cyclic enones) like androgens [1] but also with smaller rings such as 2-cyclohexenone [2]. An example is the vertebral unspecific monooxygenase (aromatase, EC 1.14.14.1) catalyzing the oxidative deformylation at the 10-position by excessive hydroxylation of the C-19 in androgens [3]. NADPH dehydrogenase of *Saccharomyces carlsbergensis* ("Old Yellow Enzyme", EC 1.6.99.1) oxidizes 2-cyclohexenone via dehydrogenation, which yields hydroxybenzene (phenol) and subsequent reduction of a second substrate molecule cyclohexanone (considered as dismutation) [2]. Formation of 4-hydroxybenzoic acid from 4-oxocyclohexanecarboxylic acid by stepwise desaturation was reported for *Corynebacterium cyclohexanicum*, in which the final aromatization is accomplished by a spontaneous rearrangement [4].

Main source of aromatic metabolites in plants and microorganisms is the shikimic acid pathway, in the course of which the intermediate chorismate (cyclohexa-1,3-diene derivative) can be converted by a synthase into (aromatic) anthranilate or, after isomerization to prephenate (cyclohexa-1,4-diene derivative), by a specific dehydrogenase to 4-hydroxyphenylpyruvate. Beyond that, chorismate serves as the precursor for diverse aromatic substances, in particular for secondary metabolites with manifold functions [5]. However, products formed in this manner do not include unsubstituted aromatic hydrocarbons such as naphthalene.

The agaric fungi *Agrocybe aegerita* and *Coprinellus radians* secrete unspecific peroxygenases (*Aae*UPO and *Cra*UPO) belonging to the relatively new, second sub-subclass of peroxide-consuming enzymes, the peroxygenases (EC 1.11.2.1). These enzymes share spectral, structural and catalytic properties both with peroxidases and cytochrome P450-monooxygenases (P450), and catalyze various peroxygenation reactions, some of which are highly regio- and stereoselective [6–8]. Among others, it has been shown that *Aae*UPO, besides catalyzing benzylic hydroxylation [9], epoxidizes double bonds like those in styrene and its derivatives [8,10]. Thus, the conversion of 1,2-dihydronaphthalene by *Aae*UPO resulted in the preferential formation of the respective 1,2-epoxide in high yields but only with an ee of 32% that is far less than the ee-values observed for other benzylic substrates [8,10]. Surprisingly, naphthalene and its characteristic oxidation products (naphthols and naphthoquinone) were also found as products. This finding prompted the authors to investigate this aromatization more in detail by searching for an eligible decrescent intermediate.

The aromatization of 1,2-dihydronaphthalene to form naphthalene was described for the aromatic metabolism of *Pseudomonas putida* and attributed to a dioxygenase activity [11–13], while in rat liver, microsomal P450 monooxygenases were proposed to be responsible for this reaction [14]. In both cases, naphthalene hydrates emerged but naphthalene formation could not alone be attributed to their decay and rather a radical mediated dehydrogenation mechanism was assumed.

We describe here the first aromatization reaction catalyzed by a peroxygenase, starting from an unsaturated cyclic hydrocarbon,

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which is enzymatically hydroxylated adjacent to the double bond resulting in arene hydrates that spontaneously decay under water elimination.

2. Experimental

2.1. Commercially available chemicals

All chemicals were purchased at the highest purity available. 1,2-Dihydronaphthalene, 1,4-dihydronaphthalene, solvents and reagents were obtained from Sigma–Aldrich (Taufkirchen, Germany), 1,4-epoxy-1,4-dihydronaphthalene from TCI Europe (Zwijndrecht, Belgium) and ^{18}O-labeled $H_2{}^{18}O_2$ from ICON Isotopes (NJ, USA).

2.2. 1,4-Dihydronaphthalene oxide and 1,2-dihydronaphthalene oxide

1,4-Dihydronaphthalene oxide and 1,2-dihydronaphthalene oxide were synthesized by epoxidation of 1,4-dihydronaphthalene and 1,2-dihydronaphthalene, respectively, using *m*CPBA in dichloromethane as oxidant (at 0 °C, 12 h) according to Hibbert and Burt [15]. 1,4-Dihydronaphthalene oxide GC/MS (EI, *m*/*z*) 146 (72), 128 (83), 117 (79), 115 (100), 1,4-dihydronaphthalene oxide GC/MS (EI, *m*/*z*) 146 (63), 117 (29), 115 (21), 104 (100).

2.3. 2-Hydroxy-1,2-dihydronaphthalene

2-Hydroxy-1,2-dihydronaphthalene was prepared from 1,4-dihydronaphthalene oxide by rearrangement (1 M KOH/EtOH abs., 5 °C, 4 d) [16]. GC/MS (EI, *m*/*z*) 146 (100), 128 (84), 145 (64), 115 (58).

2.4. 1-Hydroxy-1,2-dihydronaphthalene

1-Hydroxy-1,2-dihydronaphthalene was prepared from 1,4-epoxy-1,4-dihydronaphthalene by hydroboration with 9-BBN (RT, 20 h, THF anhydr. under nitrogen) and subsequent oxidation (H_2O_2/3 N NaOH, RT, 5 h) according to a method of Brown and Prasad [17] (GC/MS (EI, *m*/*z*) 146 (100), 128 (68), 131 (65), 145 (55) 115 (47)). Due to their instability, naphthalene hydrates could not be isolated and therefore not directly be quantified.

2.5. Enzymatic conversions

Enzymatic conversions were carried out using 2 mM 1,2- or 1,4-dihydronaphthalene and 2 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7) with 20% acetonitrile as co-solvent in a total volume of 0.5 mL at room temperature. 90 nM *Aae*UPO (0.5 U mL^{-1} assayed with veratryl alcohol, 120 U mg^{-1}, 46 kDa) or 440 nM *Cra*UPO (0.5 U mL^{-1} assayed with veratryl alcohol, 25.8 U mg^{-1}, 44 kDa) purified with FPLC [7,18] were used in all experiments.

Initial enzymatic conversion was followed by HPLC after 2 min reaction time; after 30 min, the reaction was assumed to be finished and the reaction mixture acidified (5 μL 6 M HCl, pH < 2) to completely convert the naphthalene hydrates to naphthalene.

2.6. High performance liquid chromatography (HPLC)

HPLC analysis (Agilent 1200 Series, DAD) was performed on a Gemini-NX(2) (3 μ, 150 mm × 2 mm) column in gradient mode (0.01% HCOOH, pH 3.5 (A)/MeCN (B)) at 40 °C and 0.3 mL min^{-1}. The gradient was raised from 15% B at 2 min to 95% B at 11 min and held for further 4 min. To obtain highly pure samples of naphthalene hydrates, semi preparative HPLC on a Merck Lichrospher (5 μm, 100 mm × 4.6 mm) was run isocratically in 30% B at 1 mL min^{-1} and fractions were collected (0.2 mL min^{-1}) right after the detector, re-chromatographed and extracted with dichloromethane for subsequent GC/MS-analysis. Quantification of individual compounds was based on the peak area at 258 nm. Spectra were recorded in the range from 210 to 500 nm (0.5 nm steps) at a frequency of 10 Hz. Isomeric naphthalene hydrates were estimated as the sum derived from conversion balancing. 1,2-dihydronaphthalene oxide concentration was additionally confirmed by 1,2-dihydronaphthalene oxidation with a recombinant UPO from Novozymes (rNovo) that exclusively catalyzes epoxidation.

2.7. Gas chromatography/mass spectroscopy (GC/MS)

GC/MS analysis (Agilent 6890 GC/5790 MSD) of the extracts was accomplished using a ZB-5MS (Zebron, 30 m × 0.25 mm ID, 0.25 μm FT) capillary column at a helium flow of 1.5 mL min^{-1}. Injection was done at 190 °C in the split mode. The initial oven temperature of 65 °C was held for one minute and raised with 45 °C min^{-1} to

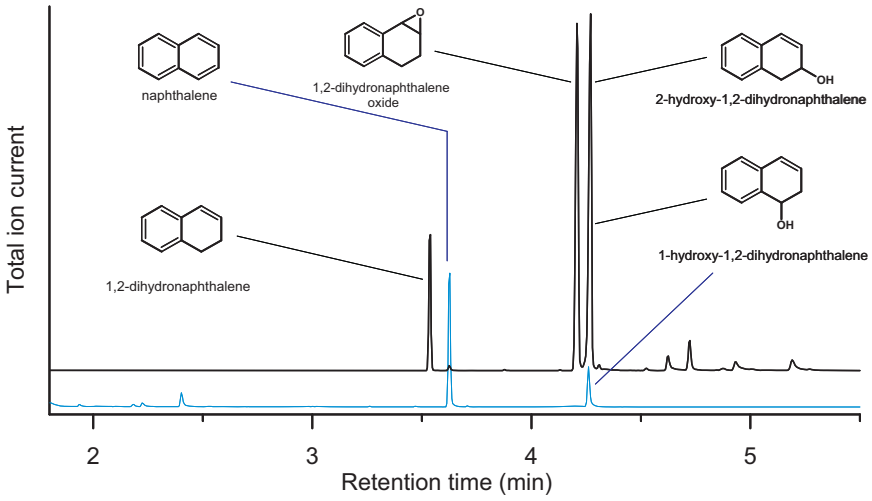

Fig. 1. GC-TIC analysis of dichloromethane extracts of the enzymatic conversion of 1,2-dihydronaphthalene by *Aae*UPO (black) and the decay of chemically synthesized 1-hydroxy-1,2-dihydronaphthalene (4.27 min) containing naphthalene (3.63 min) formed by interim spontaneous water elimination (blue). Note: 2-hydroxy-1,2-dihydronaphthalene co-elutes at 4.27 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)