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Oxidation of 1-pyrene butyric acid by fungal peroxidase in acidic media

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The oxidation of 1-pyrene butyric acid (PBA) as a model of polycyclic aromatic hydrocarbon catalyzed with recombinant *Coprinus cinereus* peroxidase was studied at pH 4.0–7.5. The apparent bimolecular constant of PBA oxidation varied from $5.4 \cdot 10^3$ to $7.3 \cdot 10^2$ $M^{-1}s^{-1}$ at pH 4.0–7.0. The apparent pK_a of reactivity change was 4.9 ± 0.3.

PBA oxidation at pH 4.0 proceeded in absence of extra-added hydrogen peroxide. The scheme of PBA oxidation at acid pH was suggested.

Key words: 1-pyrene butyric acid, peroxidase, oxidation

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), compounds composed of two or more fused aromatic rings, are widely distributed in the natural environment. Coal and petroleum are two major natural sources of PAHs [1]. These compounds are generated during incomplete combustion of solid and liquid fuels or derived from industrial activities. PAHs are hydrophobic compounds with a low water solubility, thus they are easily adsorbed onto organic matter as soils and sediments [2]. PAHs are known to be mutagenic or carcinogenic, and their contamination in soil and aquifer is of great environmental concern [1].

Among other possibilities, an environmentally friendly approach to PAHs degradation could be based on the use of white rot fungi which are known to degrade a great variety of compounds due to their complex enzymatic system [2]. It is known that white rot fungi, among others, produce Mn and lignin peroxidases which are responsible for the oxidation of lignin, PAHs and other congeners [2].

The peroxidase from ink mushroom, *Coprinus cinereus* peroxidase (CiP; the previous name ARP), was cloned in *Aspergillus oryzae* and produced at large quantities [3]. It shows a broad pH interval of activity. The mutants of recombinant CiP (rCiP) demonstrate an elevated stability to high hydrogen peroxide concentration [3]. The redox potential of the active center of CiP is less in comparison to lignin peroxidase, but it is much higher than in laccases – other oxidoreductases which are produced by white rot fungi [4–6].

The task of our investigation was to study the kinetics of 1-pyrene butyric acid (PBA; Fig. 1) oxidation catalyzed by rCiP. PBA belongs to the PAH compound group. In comparison to pyrene it shows an increased solubility in neutral and alkaline solutions due to presence of dissociated butyric acid residue. The reaction was performed at pH 4.0–7.5.

MATERIALS AND METHODS

1-pyrene butyric acid (PBA; Fig. 1) was a product of Serva (Heidelberg, Germany). Solution of PBA was prepared by weight in methanol. Methanol was received from Achema (Lithuania). The spectral pure methanol was prepared by boiling with charcoal and CaO following redistillation.

Recombinant *Coprinus cinereus* peroxidase was prepared as described in [7] and used without further purification. The concentration of peroxidases was determined spectrophotometrically. The extinction coefficient used for rCiP is 109 $mM^{-1} \cdot cm^{-1}$ at 405 nm [7]. *Aspergillus niger* catalase (Cat) was received from Novozymes A/S (Copenhagen, Denmark). The extinction coefficient used for catalase was 150 $mM^{-1} \cdot cm^{-1}$ at 280 nm [8].

Perhydrol (~30%), sodium acetate, acetic acid, potassium dihydrophosphate, and potassium hydroxide were received

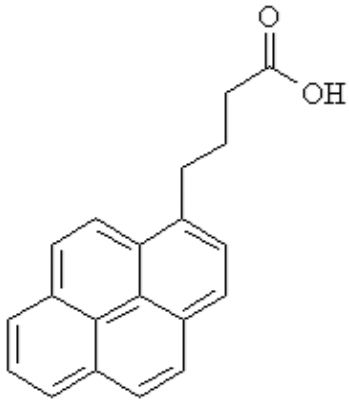

Fig. 1. Structure of 1-pyrene butyric acid

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from Reakhim (Moscow, Russia). The hydrogen peroxide solution was prepared from perhydrol. The concentration of hydrogen peroxide was determined by using the extinction coefficient at 240 nm (ε_{240} = 39.4 $M^{-1}\cdot cm^{-1}$ [9]). The solutions were prepared in three-distilled water.

Kinetic measurements were performed at 25 ± 0.1 °C in a 1-cm thermostated quartz cuvette by using a computer-controlled equipment: Ultrospec II LKB UV/Visible and Hitachi MPF–4 spectrofluorimeter. The decrease of PBA fluorescence during reaction was measured at 398 nm with an excitation 321 nm. The initial PBA fluorescence intensity was normalized to the PBA concentration. The initial reaction rate was calculated as a slope of the PBA concentration change during 100 s. Measurements were performed in 50 mM sodium acetate buffer (4–5.5 pH) and in 50 mM phosphate buffer (6–7.5 pH). The reaction mixture contained 75 and 150 nM PBA, 100 nM rCiP and 0 or 50 μM of H_2O_2. The final concentration of methanol in the PBA solution was 2% (v/v). The reaction started with addition of the enzyme solution.

Cyclic voltammetry of PBA was performed using an electroanalytical system (Cypress Systems, USA) equipped with a glassy carbon electrode (model CS-1087, Cypress Systems, USA). A saturated calomel electrode (SCE, saturated with KCl, model K-401, Radiometer, Denmark) was used as a reference electrode, and as an auxiliary electrode a Pt wire (diameter 0.2 mm, length 4 cm) was mounted on the end of the reference electrode. The measurements were performed in 50% ethanol/buffer (50 mM phosphate, pH 7.0) solution at scan rate 6, 13, 25, 50, 100, 200 mV/s and at room temperature. In electrochemical measurements, the potential values were expressed vs. SCE. To calculate the potential values vs. a normal hydrogen electrode (NHE), 241 mV should be added.

CALCULATIONS

Kinetic and spectral data were transferred to GraFit 3.01 and MathCAD PLUS 5.0, which were used for calculations and plots. The pK_a and logP of PBA were calculated with ACD/ChemSkech version 4.56.

The kinetics of peroxidase-catalyzed PBA oxidation was simulated according to the ordered bi-bi ping-pong scheme [7]:

$$E + H_2O_2 \rightarrow \text{cpd I} + H_2O \quad (k_1) \quad (1)$$

$$\text{cpd I} + AH_2 \rightarrow \text{cpd II} + AH^{\cdot} \quad (k_2) \quad (2)$$

$$\text{cpd II} + AH_2 \rightarrow E + AH^{\cdot} + H_2O \quad (k_3) \quad (3)$$

where E, cpd I and cpd II are ferryperoxidase, compound I and compound II, respectively. AH_2 and $AH^{\cdot}$ are substrate and single oxidized compound (in our case PBA and radical cation, respectively).

Following the scheme, the initial rate of the reaction can be expressed:

$$V = 2\cdot k_1\cdot k_2\cdot k_3\cdot [E]_t\cdot [PBA]\cdot [H_2O_2] / k_2\cdot k_3\cdot [PBA] + k_1\cdot k_3\cdot [H_2O_2] + k_1\cdot k_2\cdot [H_2O_2], \quad (4)$$

where $[E]_t$, [PBA] and $[H_2O_2]$ are the total concentration of peroxidase, 1-pyrene butyric acid and hydrogen peroxide, respectively.

To simplify the expression (eq. 4), the rate limiting constant (k_{lim}) corresponding to a slower cpd I or cpd II reaction rate can be used (if $k_3 > k_2$ $k_{lim} = k_2$; if $k_2 > k_3$ $k_{lim} = k_3$):

$$V = 2\cdot k_1\cdot k_{lim}\cdot [E]_t\cdot [PBA]\cdot [H_2O_2] / (k_{lim}\cdot [PBA] + k_1\cdot [H_2O_2]). \quad (5)$$

To obtain the dependence of the initial PBA oxidation rate on pH, the dependency of bimolecular constants on pH should be added to the mathematical expression of the initial rate (eq. 5):

$$k_1 = k_1^{*}\cdot 10_1^{-pH}\cdot (10_1^{-pk} + 10^{-pH}), \quad (6)$$

$$k_{lim} = k_{lim}^{*}\cdot 10^{-pH} / (10_2^{-pK} + 10^{-pH}), \quad (7)$$

where pK_1 and pK_2 is pK_a of ferryperoxidase and cpd I (or cpd II), respectively.

RESULTS AND DISCUSSION

Kinetics of PBA oxidation

The PBA contains a pyrene ring (Fig. 1). Therefore it is a hydrophobic compound. The solubility of PBA increases due to dissociation of butyric acid residue. However, at acid pH when butyric acid residue is in undissociated form (pH < pK_a = 4.76), the solubility of PBA in water solution decreases, since the calculated logP of this compound is 5.37 ± 0.20. It was determined that 150 nM of PBA did not precipitate in the buffer solutions containing 2% (v/v) of methanol. For this reason, the experiments were performed using 150 or 75 nM of PBA.

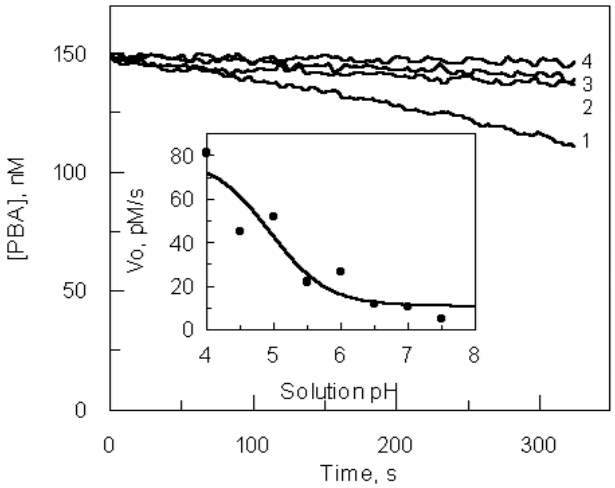

Fig. 2. Kinetics of PBA concentration change at different pH. The reaction mixture contained 150 nM PBA, 100 nM rCiP and 50 μM H_2O_2 in 50 μM acetate buffer pH 4.0 (1), pH 5.0 (2), in 50 mM phosphate buffer pH 6.0 (3), pH 7.0 (4), 25 °C. The insert graph shows dependence of initial reaction rate on solution pH. The curve is data fit by the single proton transfer

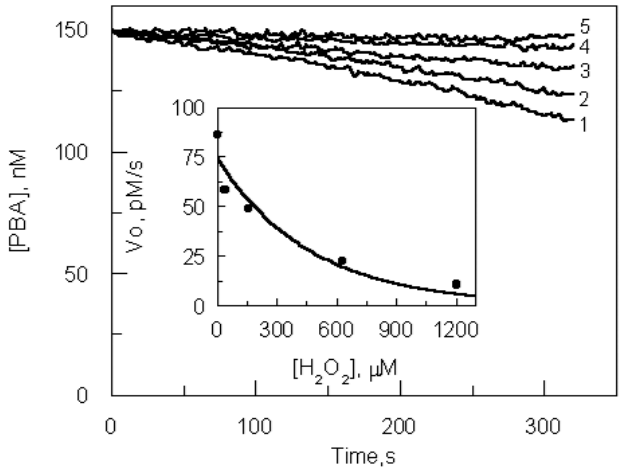

Fig. 3. Dependence of peroxidase-catalyzed PBA oxidation rate on hydrogen peroxide concentration in 50 mM acetate buffer pH 4.0, 25 °C. The reaction mixture contained 150 nM PBA, 100 nM rCiP. Hydrogen peroxide: 0 μM (1), 39 μM (2), 157 μM (3), 627 μM (4) and 1.2 mM (5) The inserted graph shows the dependence of initial reaction rate on hydrogen peroxide concentration; the curve is an approximation with exponential function

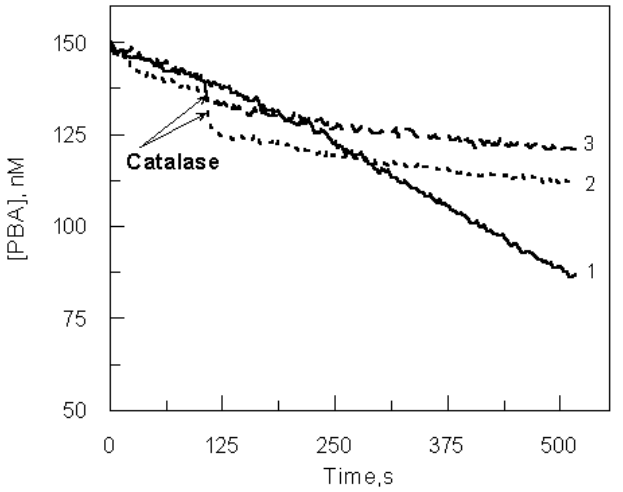

Fig. 4. Kinetics of peroxidase-catalyzed PBA oxidation in absence (1, 3) and in presence of hydrogen peroxide (2). The addition of catalase (61 nM) is shown by arrows. Hydrogen peroxide concentration was 50 μM (2). The reaction mixture contained 150 nM PBA, 100 nM rCiP in 50 mM acetate buffer pH 4.0, 25 °C

The PBA fluorescence decreased in presence of rCiP and hydrogen peroxide. The decrease of fluorescence was attributed to PBA oxidation. It was ascertained that products of PBA oxidation did not fluoresce. The oxidation of PBA proceeded at a rather high rCiP concentration (Fig. 2). The reaction rate increased at acid pH.

At pH 4.0, the initial reaction rate was equal to 81 ± 3 pM/s. At pH 7.0, the rate decreased down to 11 ± 3 pM/s. The calculated apparent bimolecular constant (k_{lim}) was $5.4 \cdot 10^3$ $M^{-1}s^{-1}$ and $7.3 \cdot 10^2$ $M^{-1}s^{-1}$ at pH 4.0 and 7.0, respectively. The dependence of the PBA oxidation rate on pH fitted a single proton transfer scheme with an apparent $pK_a = 4.9 \pm 0.3$ (Fig. 2). The decrease of PBA concentration down to 75 nM changed the pK_a value 5.0 ± 0.4 (data not shown in Fig. 2).

The increase of PBA oxidation rate at low pH was investigated in more detail at pH 4.0. It was indicated that the initial rate was directly proportional to the enzyme and PBA concentration lower than 150 nM. The most unexpected result was that the oxidation decreased with increasing the hydrogen peroxide concentration (Fig. 3). The largest reaction rate was in the absence of added hydrogen peroxide.

To estimate the role of hydrogen peroxide, the oxidation of PBA at pH 4.0 was performed with catalase. The results (Fig. 4) show that addition of catalase retarded PBA oxidation in absence and in presence of hydrogen peroxide. This indicates that PBA oxidation in the absence of hydrogen peroxide is still a peroxidase reaction.

The redox potential of PBA, which was essential for the analysis of PBA oxidation mechanism, was determined using electrochemical oxidation. Cyclic voltammetry of PBA solution in a mixture of phosphate buffer and

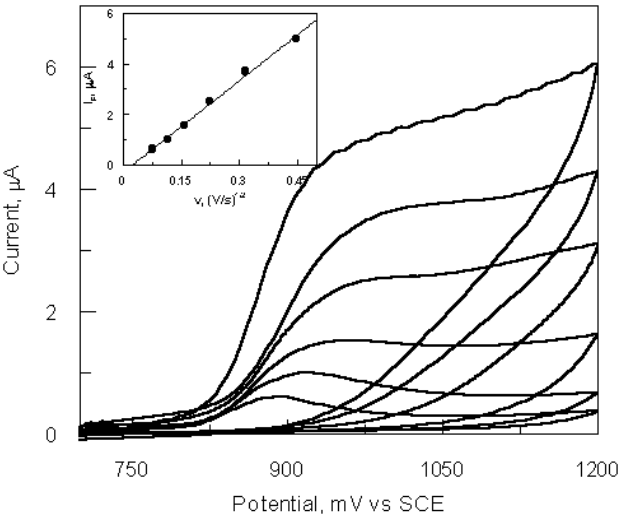

Fig. 5. Cyclic voltammetry of 0.87 mM PBA in 50% ethanol/buffer (50 mM phosphate pH 7.0) mixture at scan rate 6, 13, 25, 50, 100, 200 mV/s and at room temperature. The insert shows dependence of peak current on square root from the potential scan rate

ethanol shows that PBA is a redox-active compound. It can be oxidized electrochemically at a high potential, i.e. about 1.1 V vs. NHE (Fig. 5). The electrochemical conversion of PBA proceeds at a diffusion limiting condition as follows from the results of the insert. However, the oxidation is irreversible, implying that PBA does not form relatively stabile intermediate products.

Mechanism of PBA oxidation

Electrochemical measurements show that PBA is a redox-active compound. However, its oxidation proceeds at a high potential which similar to the redox potential of rCiP [5].

For this reason, the rate of rCiP-catalyzed reaction is low. It is known that the redox potential of rCiP increases at acid pH [5]. This was an incentive for us to perform PBA oxidation in acid solutions. As expected, the oxidation rate of PBA increased. The apparent pK_a of transition was 4.9 ± 0.3.

The analysis of the kinetic scheme of peroxidase action reveals that this transition is not associated with compound I formation, since the calculated (eq. 6) rate of the process should be 11.9 nM/s at pH 4 (k_1=7.1 ·10^6 $M^{-1}s^{-1}$, pK_a = 4.9 [7]). Therefore, the pK_a of PBA oxidation transition may be referred to the reaction of cpd I (cpd II) with PBA.

It is tempting to speculate that this pK_a is related to pK_a of butyric acid residue dissociation (calculated pK_a = 4.76). Molecular docking calculations, however, indicate that the undissociated and dissociated state of PBA forms very similar complexes, with rCiP. In these complexes the pyrene core is in close proximity to the ferryl oxygen of compound I, and butyric acid residue lies outside the active center of peroxidase (Fig. 6). It seems that the small difference in docking energies cannot explain the activity change with pK_a = 4.9.

The observations that PBA oxidation proceeds in absence of added hydrogen peroxide show that the mechanism of PBA oxidation is much more complicated in comparison to the scheme (eqs. 1–3), and that the determined pK_a may be attributed to other processes.

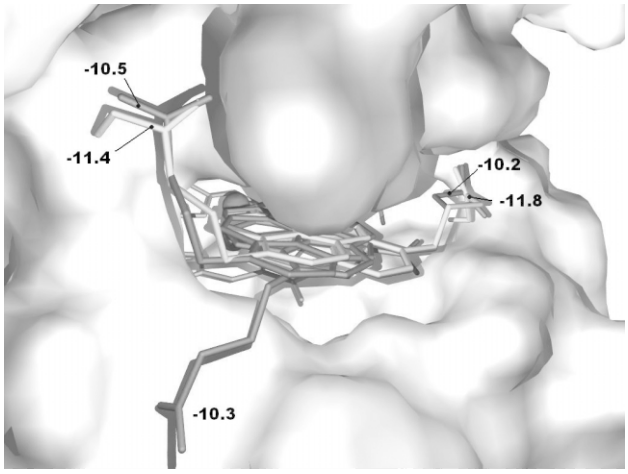

Fig. 6. The docking of PBA in the active center cpd I of rCiP. Numbers show docking energy in kcal/mol

The inhibition of reaction with hydrogen peroxide can be associated with compound III formation [10]. The rate of this reaction increases in an acid solution. Therefore, at a low pH the oxidation of PBA may proceed only at a very low hydrogen peroxide concentration. The inhibition of PBA oxidation with catalase shows that at a low pH the reaction is peroxidase-like even in absence of added hydrogen peroxide. The question arises how hydrogen peroxide is generated. The scheme below may explain the process:

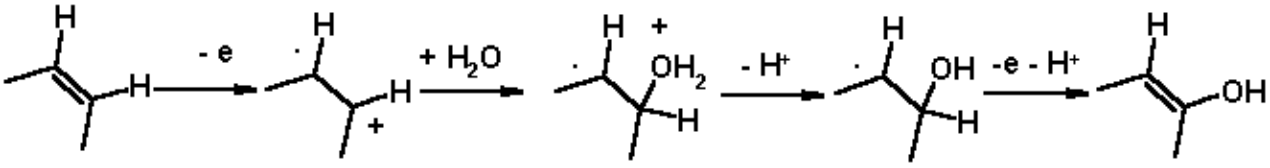

Fig. 7. Scheme of a fragment of PBA oxidation in acid solution

The reaction starts with a single electron oxidation of PBA by cpd I. The addition of water molecule followed by proton transfer produces a low potential radical which can be oxidized with oxygen (eq. 7). The redox potential of this reaction is –0.29 V vs. NHE [11]:

$$O_2 + e^- + H^+ \rightarrow HO_2^{\cdot}. \quad (7)$$

The disproportionation of protonated superoxide generates hydrogen peroxide:

$$2\,HO_2^{\cdot} \rightarrow H_2O_2 + O_2. \quad (8)$$

Formally, in an acid solution the peroxidase acts as an oxidase:

$$PBA + 1/2\,O_2 + H^+ \rightarrow PBA\text{-}OH. \quad (9)$$

Due to a complex reaction of PBA oxidation at a low pH when electron transfer is associated with proton transfer, each stage may determine the apparent pK_a. The reaction (eq. 7) is most probable, since the pK_a of superoxide protonation is 4.7 [11].

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R. Ivanec, J. Kulys

1-PIRENO SVIESTO RŪGŠTIES OKSIDACIJA GRYBINE PEROKSIDAZE RŪGŠTINĖJE TERPĖJE

Santrauka

Tirta vieno iš policiklinių aromatinių junginių grupės atstovo – 1-pireno sviesto (butano) rūgšties (PBA) – oksidacija, katalizuojama rekombinantine *Coprinus cinereus* peroksidaze. pH intervale 4,0–7,5 PBA oksidacijos tariamoji bimolekulinė konstanta kito nuo $5{,}4 \cdot 10^3\ M^{-1}s^{-1}$ iki $7{,}3 \cdot 10^2\ M^{-1}s^{-1}$. Tariamoji reaktyvumo pK_a 4,9 ± 0,3.

Nustatyta, kad PBA oksidacija, esant pH 4,0, vyksta be peroksido. Pasiūlyta PBA oksidacijos rūgštinėje terpėje schema.