Investigation of fungal peroxidase inactivation kinetics during oxidation of naphthols

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The kinetics of *Coprinus cinereus* peroxidase-catalyzed 1-naphthol and 2-naphthol oxidation was investigated at pH 5.5. The initial rate of naphthol oxidation was linearly dependent on enzyme concentration. The rate depended on naphthol concentrations and saturated at a concentration over 25–50 μM at 100 μM of hydrogen peroxide. At this peroxide concentration, the calculated EC_{50} (the concentration of naphthol at which 50% of the maximal reaction rate was reached) and the maximal rate was 74.7 μM and 0.53 μM/s, or 175 μM and 2.0 μM/s for 1- and 2-naphthol, respectively. Kinetic measurements of exhaustive naphthol oxidation showed that peroxidase was inactivated during naphthol oxidation. The catalytically inactive protein albumin retarded the enzyme inactivation. The inactivation mechanism involving intermediate formation and irreversible peroxidase inactivation by the intermediate was suggested. The kinetic parameters were calculated analyzing the integral naphthol oxidation curves.

Key words: peroxidase, *Coprinus cinereus*, naphthol, albumin

INTRODUCTION

Investigation of the oxidation of peroxidase-catalysed phenols is actual from the environmental and polymer synthesis points of view [1, 2]. Preliminary experiments showed that fungal peroxidase was inactivated during 1- and 2- naphthol oxidation [3]. The task of the current study was to explore the kinetics of this reaction, with a special emphasis on modelling the kinetics of naphthol oxidation and the action of albumins that prevented peroxidase inactivation. As peroxidase, the recombinant fungal peroxidase from *Coprinus cinereus* (rCiP) was used.

MATERIALS AND METHODS

Recombinant peroxidase from *Coprinus cinereus* (rCiP) was received from Novozymes A/S (Copenhagen, Denmark). The concentration of peroxidase was determined spectrophotometrically. The extinction of rCiP was 108 mM^{-1} cm^{-1} at 405 nm [4]. Hydrogen peroxide concentration was determined spectrophotometrically using the extinction coefficient 39.4 M^{-1} cm^{-1} at 240 nm [5].

1-naphthol and 2-naphthol were the products of Aldrich. Sodium acetate and acetic acid were received from Reachim (Russia). Human serum albumin (HSA) (purity 98%), and bovine serum albumin (BSA) (purity 99%, fatty acids 0.1%) were from Serva. The concentration of BSA and HSA was determined spectrophotometrically at 280 nm by using the extinction coefficients 43.6 mM^{-1} cm^{-1} [6] and 36.6 mM^{-1} cm^{-1} [7], respectively. All measurements were performed in 50 mM sodium acetate buffer solution, pH 5.5. All solutions were prepared in thrice-distilled water.

Spectrophotometric measurements were performed using a computer-assisted Gilford Instrument 2600 spectrophotometer. A computerized Hitachi MPF-4 spectrofluorimeter was used for fluorimetric measurements. The concentration of naphthols was less than 30 μM when the fluorescence intensity was linearly proportional to the concentration. 1-Naphthol and 2-naphthol fluorescence intensity exchange was measured at λ emission 460 nm, but λ excitation was 320 nm and 328 nm, respectively. Kinetic measurements were performed at 25 °C in 50 mM acetate buffer, pH 5.5, in the presence of 100 μM of hydrogen peroxide and rCiP. The reaction was started by enzyme addition.

CALCULATIONS

The initial reaction rate was calculated by fitting kinetic curves of naphthol fluorescence decrease with

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the second order polynomial. The kinetics of peroxidase-catalyzed naphthol oxidation was simulated according to the scheme presented below:

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

$$\text{Compound I} + \text{Naphthol} \rightarrow E + \text{Intermediate} \quad (k_2) \quad (2)$$

$$E + \text{Intermediate} \rightarrow E_{in} \quad (k_{in}) \quad (3)$$

$$\text{Compound I} + \text{Intermediate} \rightarrow E_{in} \quad (k_{in}) \quad (4)$$

$$\text{Intermediate} + \text{Intermediate} \rightarrow \text{Polymer} \quad (k_p) \quad (5)$$

where E_{in} corresponded to inactivated peroxidase.

Following this scheme, the initial rate of the reaction was expressed [8]:

$$V = k_1k_2 [H_2O_2] [N] [E] / (k_1[H_2O_2] + k_2 [N]), \quad (6)$$

where [N], $[H_2O_2]$ and [E] are the initial concentrations of naphthol, hydrogen peroxide and active enzyme.

The kinetic parameters of peroxidase reaction (k_1, k_2) during oxidation of exhausted naphthols as well as the highest constant of the enzyme inactivation (k_{in}) were calculated using the adaptive Runge–Kutta method solving a system of differential equations of the presented kinetic scheme.

RESULTS AND DISCUSSION

The initial rate of naphthol oxidation determined with a spectrofluorometer linearly depended on enzyme concentration (Fig. 1). The 1-naphthol oxidation rate increased from 0.016 to 0.48 μM/s with increasing rCiP concentration from 0.041 to 2.6 nM. The 2-naphthol oxidation rate increased more significantly (about 3 times) with enzyme concentration increasing from 0.014 to 0.43 nM.

1- and 2-naphthol oxidation rate increased with increasing hydrogen peroxide concentration, but at high concentrations the curves saturated. The initial oxidation rate was dependent on 1- and 2-naphthol concentrations (Fig. 2). The calculated EC_{50} (the concentration of naphthol at which 50% of the maximal reaction rate was reached) was 74.7 μM and 175 μM, and maximal reaction rate was 0.53 μM/s and 2.0 μM/s for 1- and 2-naphthol, respectively, at a hydrogen peroxide concentration 100 μM.

The exhausted rCiP-catalyzed naphthol oxidation curve is depicted in Fig. 3. It shows that the naphthol oxidation rate was saturated, though naphthol had not been used completely, implying that peroxidase had been inactivated during this reaction. The addition of albumin little changed the initial reaction rate, but increased the total naphthol oxidation (Fig. 3).

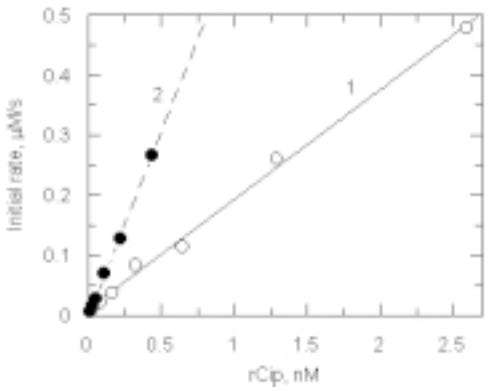

Fig. 1. Dependence of initial oxidation rate of 1-naphthol (1) and 2-naphthol (2) on rCiP concentration. 50 mM acetate buffer, pH 5.5, 25 °C; naphthols (25 μM), H_2O_2 (100 μM), λ_{em} = 460 nm, λ_{ex} = 320 nm (1-naphthol) and λ_{ex} = 328 nm (2-naphthol)

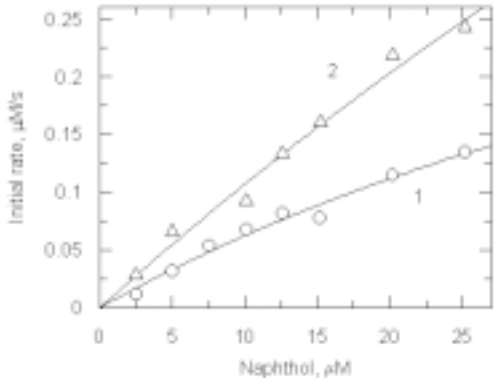

Fig. 2. Dependence of initial oxidation rate on 1-naphthol (1) and 2-naphthol (2) concentration. rCiP (0.51 nM), H_2O_2 (100 μM). Other conditions as in Fig. 1

The minimal kinetic scheme that could explain experimental facts includes the bimolecular reaction of peroxidase oxidation with hydrogen peroxide, naphthol oxidation by oxidized peroxidase, the peroxidase reaction with intermediate and the reaction of intermediates with polymer formation (eq. 1–5). The parameters of the kinetic scheme were calculated digitally for the albumin concentration 0.3–137 nM. The results of the calculations show that the bimolecular constant k_1 is similar for both naphthols; the mean value of the constant k_1 is 5.5 ± ± 0.5 $\mu M^{-1}s^{-1}$. In contrast, k_2 for 1-naphthol is 4.1 times less than for 2-naphthol. The average values of the constants were 13.2 ± 1.6 $\mu M^{-1}s^{-1}$ for 1-naphthol and 54.0 ± 1.7 $\mu M^{-1}s^{-1}$ for 2-naphthol. The values of k_{in} for 1-naphthol and 2-naphthol were $(1.1 \pm 0.3)\cdot 10^{-3}$ $\mu M^{-1}s^{-1}$ and $(0.86 \pm 0.24) \cdot 10^{-3}$ $\mu M^{-1}s^{-1}$, respectively.

The low variation of the constants k_1 indicates that the suggested scheme reflects the real process,

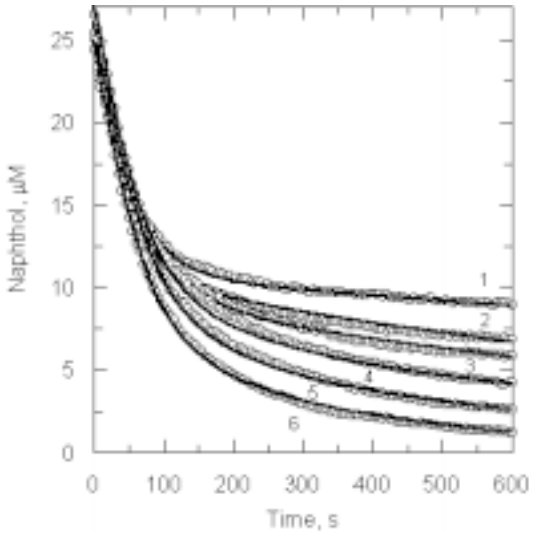

Fig. 3. Decrease of 1-naphthol fluorescence during rCiP-catalyzed substrate oxidation in the presence of different concentrations of HSA, at pH 5.5 and 25 °C. 1.0 nM rCiP (1–6); 25 μM 1-naphthol (1–6); HSA: 0 nM (1); 4 nM (2); 8 nM (3); 12 nM (4); 16 nM (5); 32 nM (6). Approximations of the data according to the kinetic scheme presented by solid curves. Other conditions as in Fig. 1

since the constant belongs to compound I formation. The k_2 values are different, since they are related to reactivity of different naphthols. The similar values of k_{in} for both naphthols and albumins implied an unspecific reaction of the intermediate with peroxidase. The mechanism of albumins action, however, is not clear. Since albumins are active at submicromolar concentrations, the effect cannot be related to the chemical interaction with intermediates. Most likely the albumin acts as an accelerator of the recombination of intermediates. The process can be described as follows:

$$\text{Intermediate} + \text{Albumin} \rightarrow [\text{Intermediate} / \text{Albumin}] \quad (7)$$

$$[\text{Intermediate} / \text{Albumin}] + \text{Intermediate} \rightarrow \text{Albumin} + \text{Polymer}. \quad (8)$$

Under steady state conditions the recombination rate will be dependent on the complex intermediate and albumin formation (eq. 6), and albumin will not be used. The complex is probably formed by hydrophobic interaction.

Inactivation of peroxidase has also been indicated during the oxidation of other phenolic substances [1, 2]. The mechanism-based enzyme inactivation involving covalent binding of the oxidized substrate to the enzyme has been postulated in many publications [1, 9]. The kinetic analysis performed in this work permitted to characterize quantitatively the process following which the quenching of reactive intermediates (possibly naphthoyl radicals [9]) by albumin was the main reason for peroxidase inactivation retarding.

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GRYBELINĖS PEROKSIDAZĖS INAKTYVACIJOS KINETIKOS TYRIMAS OKSIDUOJANT NAFTOLUS

S a n t r a u k a

Grybelinės peroksidazės katalizuojama 1-naftolo ir 2-naftolo oksidacija buvo tirta esant pH 5,5. Pradinis oksidacijos greitis priklauso nuo naftolų koncentracijos. Priklausomybė įsisotina, kai naftolų koncentracija didesnė nei 25–50 μM, esant 100 μM vandenilio peroksido. Esant šiai peroksido koncentracijai apskaičiuotas EC_{50} (t. y. naftolo koncentracija, kai stebima pusė maksimalaus greičio) ir maksimalus greitis buvo 74,7 μM ir 0,53 μM/s arba 175 μM ir 2,0 μM/s atitinkamai 1-naftolui ir 2-naftolui. Naftolo sunaudojimo kinetiniai matavimai parodė, kad naftolo oksidacijos metu peroksidazė inaktyvuojasi. Katalitiškai neaktyvus baltymas – albuminas – sumažina fermento inaktyvaciją. Pasiūlytas inaktyvacijos mechanizmas susijęs su tarpinio produkto susidarymu ir negrįžtama peroksidazės inaktyvacija tarpiniu produktu. Kinetiniai parametrai gauti analizuojant integralines naftolų oksidacijos kreives.