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Pesticide Biochemistry and Physiology 87 (2007) 9–13
PESTICIDE
Biochemistry & Physiology
www.elsevier.com/locate/ypest

Activation kinetics of cetylpyridinium chloride on the prophenol oxidase from pupae of blowfly (*Sarcophaga bullata*)

Jin-Jin Xie [a,b], Qing-Xi Chen [a,*], Qin Wang [a], Kang-Kang Song [a], Ling Qiu [a]

[a] *Department of Biochemistry and Biotechnology, Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen 361005, China*
[b] *Biology Department of Quanzhou Normal University, Quanzhou 362000, China*

Received 25 October 2005; accepted 3 March 2006
Available online 20 October 2006

Abstract

Prophenol oxidase (PPO) (EC 1.14.18.1) is isolated from pupae of blowfly (*Sarcophaga bullata*) and purified by employing ammonium sulfate fractionation, ion-exchange chromatography on DEAE-cellulose, and gel filtration through Sephadex G-100 column chromatography. The enzyme exists in a latent or inactive state. Cetylpyridinium chloride (CPC), a cationic detergent, is found to activate the PPO activity. The activation of the enzyme by CPC has first been studied by using the kinetic method of the substrate reaction described by Tsou. The results show that the enzyme is activated by a complexing scheme that has not been previously identified. The enzyme first reversibly and quickly binds CPC and then undergoes a slow reversible active course. The activation reaction is a single molecule reaction and the apparent activation rate constant is dependent on the CPC concentration with the function relationship fit with a hyperbola. The micro rate constants of activation and the association constant are determined from the measurements. Substrate binding does not affect the micro rate constants of activation by CPC.

Keywords: Prophenol oxidase; Blowfly pupae; Kinetic; Activation; Cetylpyridinium chloride

1. Introduction

Prophenol oxidase (EC 1.14.18.1, PPO)[1] is a key enzyme associated with both melanin biosynthesis in melanocytes and sclerotization in insects [1]. It catalyzes the o-hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (hydroxylase activity) as well as the oxidation of L-dihydroxyphenylalanine to L-dopaquinone (oxidase activity). The L-dopaquinone will be oxidized to form melanin, which has a protective effect against injury by ultraviolet radiation [2]. It has been suggested that PPO also plays an important role in melanoma cells [3]. In insects, PPO is considered to be involved not only in melanin formation, but also in sclerotization of cuticles, wound healing, and defense reactions [4].

The unique roles played by PPO in insect physiology and biochemistry certainly demand a serious study on this enzyme. By taking advantage of the fact that PPO is present in the inactive proenzyme form, some scientists have successfully purified and characterized the prophenol oxidase. The PPOs from housefly larvae [5] and pupae [6] have been purified and studied on the basis of their physicochemical characteristics. Chase et al. [7] have reported the purification, characterization, and molecular cloning of prophenol oxidase from the larval hemolymph of *Sarcophaga bullata* that could be activated by cetylpyridinium chloride (CPC) specifically and the activated enzyme exhibited marked thermal instability. It has been reported that plant prophenol oxidases can be activated by different treatments such as acid shock [8], alcohols [9], fatty acids [10,11], and detergents such as sodium dodecyl sulfate (SDS) [12,13] and CPC [14]. This activation can also result from the

* Corresponding author. Fax: +86 592 2185487.
E-mail address: chenqx@jingxian.xmu.edu.cn (Q.-X. Chen).
[1] *Abbreviations used:* PPO, prophenol oxidase; L-DOPA, L-3,4-dihydroxyphenylalanine; CPC, cetylpyridinium chloride.

0048-3575/$ - see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.pestbp.2006.03.004

attack of pathogens [15]. We have also found that CPC is necessary to the enzyme from insect *S. bullata* (blowfly). In this paper, the activation mechanism is investigated. The results show that CPC first quickly binds with the pro-enzyme reversibly and then causes a slow activation course. The aim of the present experiment is, therefore, to carry out a kinetic study of the activation of prophenol oxidase by CPC and to evaluate the kinetic parameters and constants characterizing the system.

2. Materials and methods

2.1. Reagents

Blowfly pupae were purchased from Carolina Biological Supply Co. (USA); L-DOPA was a product of Sigma (USA); Cetylpyridinium chloride (CPC) was purchased from Aldrich (USA). DEAE-cellulose was from Whatman; Sephadex G-100 was a Pharmacia product. All other reagents were local products of analytical grade. The water used was re-distillated and ion-free.

2.2. Purification of the enzyme

Preparation and purification of PPO from blowfly pupae was as described previously [16]. The final preparation was homogeneous on polyacrylamide gel electrophoresis and FPLC. The specific activity of the enzyme was 770 U/mg. One unit (U) of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol dopachrome per minute from L-3,4-dihydroxyphenylalanine (L-DOPA) at 30 °C. Absorption was recorded using a Beckman UV-650 spectrophotometer.

2.3. Assay of PPO activity

The activity assay of PPO from blowfly pupae was performed with L-DOPA as substrate by the methods described by Chen [17] with slight modifications. The reaction media (3.0 ml) that contained 1.0 mM L-DOPA, 50 mM sodium phosphate buffer (pH 6.8) were first incubated at 30 °C for 5 min. Then, a portion of 20 μl of appropriately diluted enzyme solutions incubated with 50.0 μM CPC was added to the reaction mixture and the optical density was continuously monitored at 475 nm for 3 min. The enzyme activity was calculated from the linear increase in optical density at 475 nm with the molar absorption coefficient of 3700 $M^{-1}\,cm^{-1}$ [18]. Protein concentration was determined by the Bradford method with bovine serum albumin as standard.

2.4. Activation kinetics of PPO from blowfly pupae at different concentrations of CPC

The activity for oxidation of L-DOPA by PPO from blowfly pupae needs CPC for activation. The progress-of-substrate-reaction method previously described by Tsou [19] was used for the study of the activation kinetics of the enzyme. In this method, 20 μl of the purified latent enzyme was added to 3.0 ml of reaction mixture containing 0.05 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8), 0.5 mM L-DOPA and different concentrations of CPC. The substrate reaction progress curve was analyzed to obtain the rate constants as detailed below. The time course of the oxidation of the substrate in the presence of different CPC concentrations showed that at lower than 70 μM of CPC the rate increased with increasing time until a straight line was approached. The kinetic scheme of the enzyme activated by CPC can be written as Scheme 1:

$$\mathrm{E} + \mathrm{X} \underset{}{\overset{K_d}{\rightleftharpoons}} \mathrm{EX} \underset{k_{-0}}{\overset{k_{+0}}{\rightleftharpoons}} \mathrm{E}^*,$$

where E represents the enzyme and EX represents the complex formed by the fast and reversible association of the enzyme and CPC. It then undergoes a reversible slow course to form the activated form of the enzyme, E^*. K_d is the dissociation equilibrium constant of the enzyme and CPC, while k_{+0} and k_{-0} are the microscopic rate constants of the activation step. The activation rate of the enzyme at time t is given by

$$\frac{d[E^*]}{dt} = k_{+0}[EX] - k_{-0}[E^*] = B[E_0] - A[E^*] \tag{1}$$

and

$$A = \frac{k_{+0}[X]}{[X] + K_d} + k_{-0} \tag{2}$$

$$B = \frac{k_{+0}[X]}{[X] + K_d}, \tag{3}$$

where $[E_0]$, $[E^*]$, and [EX] are the concentrations of the total enzyme, activated enzyme, and the complex of enzyme and CPC, respectively. [X] is the concentration of CPC. A is the apparent activation rate constant. Because the initial activity is equal to zero, the enzyme activity at time t is calculated according to Tsou's method [20] to be

$$v = v^*(1 - e^{-At}) \tag{4}$$

and the product formation can be written as

$$[P]_t = v^* \cdot t - \frac{v^*}{A}(1 - e^{-A \cdot t}), \tag{5}$$

where $[P]_t$ is the concentration of the product formed at time t, and v^* is the enzyme activity of enzyme activated by CPC in different concentrations. When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P^*]$:

$$[P^*] = v^* \cdot t - \frac{v^*}{A}. \tag{6}$$

Combining Eqs. (5) and (6) yields

$$[P]_t - [P^*] = \frac{v^*}{A} \cdot e^{-A \cdot t}, \tag{7}$$