European Journal of Medicinal Chemistry 122 (2016) 138–148

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Non-specific binding sites help to explain mixed inhibition in mushroom tyrosinase activities

Sorour Hassani [a], Kamahldin Haghbeen [b, *], Mostafa Fazli [a]

[a] Department of Chemistry, Faculty of Science, Semnan University, Semnan, Iran
[b] Department of Plant Bioproducts, National Institute for Genetic Engineering and Biotechnology, Tehran, Iran

ARTICLE INFO

Article history:
Received 16 April 2016
Received in revised form
30 May 2016
Accepted 10 June 2016
Available online 15 June 2016

Keywords:
Phthalic acid
Cinnamic acid
Mixed-cooperativity
Molecular dynamics
Channel

ABSTRACT

Inhibition and activation studies of tyrosinase could prove beneficial to agricultural, food, cosmetic, and pharmaceutical industries. Although non-competitive and mixed-inhibition are frequent modes observed in kinetics studies on mushroom tyrosinase (MT) activities, the phenomena are left unexplained. In this study, dual effects of phthalic acid (PA) and cinnamic acid (CA) on MT during mono-phenolase activity were demonstrated. PA activated and inhibited MT at concentrations lower and higher than 150 μM, respectively. In contrast, CA inhibited and activated MT at concentrations lower and higher than 5 μM. The mode of inhibition for both effectors was mixed-type. Complex kinetics of MT in the presence of a modulator could partly be ascribed to its mixed-cooperativity. However, to explain mixed-inhibition mode, it is necessary to demonstrate how the ternary complex of substrate/enzyme/effector is formed. Therefore, we looked for possible non-specific binding sites using MT tropolone-bound PDB (2Y9X) in the computational studies. When tropolone was in MTPa (active site), PA and CA occupied different pockets (named MTPb and MTPc, respectively). The close Moldock scores of PA binding posed in MTPb and MTPa suggested that MTPb could be a secondary binding site for PA. Similar results were obtained for CA. Ensuing results from 10 ns molecular dynamics simulations for 2Y9X-effector complexes indicated that the structures were gradually stabilized during simulation. Tunnel analysis by using CAVER Analyst and CHEXVIS resulted in identifying two distinct channels that assumingly participate in exchanging the effectors when the direct channel to MTPa is not accessible.

1. Introduction

Studies on inhibition and activation of tyrosinase could prove beneficial to agricultural, food, cosmetic, and pharmaceutical industries [1,2]. Studies on synthetic and natural substances with inhibitory potential have resulted in discovery of some strong inhibitors [3,4], as well as an understanding of some structural requirements for tyrosinase inhibition [3–6]. In contrast, fewer studies examine the activation of tyrosinase. Tyrosinase activities are modified in the presence of some compounds, such as sodium dodecyl sulfate, because of the known effects of a surfactant on the tertiary structure of a protein [7]. However, in some cases, the complex kinetics of tyrosinase cannot be easily explained.

Homotropic effects were observed for tyrosinase substrates in rather early works [8]. Increasing concentration of L-tyrosine inhibited its oxidation by tyrosinase, while the addition of a small amount of dihydroxyphenols, such as catechol or L-dopa, increased the rate of oxidation [9]. Although the former phenomenon has remained unexplained, the activation effect of catecholic substances on the mono-phenolase activity (known also as cresolase activity) was later explained by proposing a mechanism in which the copper ions in the active site of tyrosinase were considered in three intermediate states: deoxy (Cu^{I}–Cu^{I}), oxy (Cu^{II}–O_2–Cu^{II}), and met (Cu^{II}–Cu^{II}) [10]. Phenolic substrates react only with oxy-tyrosinase, but catecholic substrates can react with both oxy- and met-tyrosinase. Therefore, the activation effect of catecholic compounds on cresolase reaction has been ascribed to their ability to change met-tyrosinase into deoxy-tyrosinase [11].

Activation of tyrosinase was also observed in early works [12,13]. Sanjust et al. reported that 3-hydroxykynurenine works as an activator on the mono-phenolase activity of mushroom tyrosinase (MT) [14]. Using a direct method for assay of tyrosinase based on monitoring the rate of the substrate consumption [15], we showed the homotropic activation effect of L-tyrosine, phenol, and

* Corresponding author.
E-mail address: kamahl@nigeb.ac.ir (K. Haghbeen).

http://dx.doi.org/10.1016/j.ejmech.2016.06.013
0223-5234/

p-cresol on mono-phenolase activity of MT [16]. Considering some key structural requirements for competitive inhibition, we extended our studies on some molecules that could not be considered MT substrates. Consequently, we could demonstrate heterotropic activation of both activities of MT [17,18].

Dual response of MT to a modulator made kinetics studies of MT more interesting. For instance, ethyl and propyl xanthate activated cresolase activity at low concentrations (1–12 μM) but worked as inhibitors at higher concentrations [17]. In a different study, it was shown that some selected α-keto acids activated catecholase activity but worked as inhibitors in cresolase reaction [18]. Structural features and concentration of the effector were two determinant factors affecting the modulator impact on MT activities. For example, increasing the length of the alkyl residue in the structure of the dithiocarbamate modulator had no effect on the mode of inhibition of MT activities [19], but change in the length of the alkyl residue of xanthate inhibitor changed the mode of inhibition from uncompetitive to mixed and then to competitive [17]. These observations were in parallel with the reports of other researchers on the effect of para alkyl and alkoxy-substituted benzoic acid [20,21].

Dual response of an enzyme to an effector is a known phenomenon in enzymology. Nonylphenol inhibits cytochrome P450 3A at higher concentrations but works as its activator at lower concentrations [22]. To explain the dual response of MT, we carried out a comprehensive kinetic study on both activities of MT in the presence of both natural and synthetic substrates [23]. Although the double reciprocal analysis of the kinetic data produced convex graphs with negative curvature, indicating the dominancy of negative cooperativity, Hill analysis of the data suggested that negative cooperativity was dominant at low concentrations and positive cooperativity at higher concentrations of the substrates in both cresolase and catecholase reactions.

Although the assumption of mixed-type cooperativity shed light on the complex kinetics of MT activities, changes in the mode of inhibition and the advent of non-competitive and mixed-type modes of inhibition in various studies have led scientists to assume the existence of a regulatory site on MT [9,10,24–26]. However, a literature review reveals that, despite the reports of MT crystal structures (with and without an inhibitor) [27] and some valuable molecular docking and simulation studies [28], no allosteric/regulatory site for MT has been reported. To explain non-competitive and mixed-type modes of inhibition in MT kinetics studies, it is necessary to explain how the ternary complex of substrate/MT/effector is formed during the enzymatic reactions. Considering the results of previous works, as well as recent advances in MT studies, we have taken a different approach by looking for possible non-specific binding sites that could help explain the complex kinetics of MT.

Phthalic acid (PA) and cinnamic acid (CA) (Fig. 1) were selected as the effectors for this study. There were several reasons for selecting these molecules as the modulators. First, both molecules have benzene ring in their structures. Benzene ring is one of the structural requirements for a molecule to be a competitive inhibitor for tyrosinase activities [6,20,26]. Second, both molecules have also carboxylic acid functional group (CAFG) in their structures. There are some reports on the effects of molecules carrying CAFG on tyrosinase activities [10,13,29,30]. Although the role of CAFG has not been clearly explained, the ability to chelate the copper ions has been considered important [30].

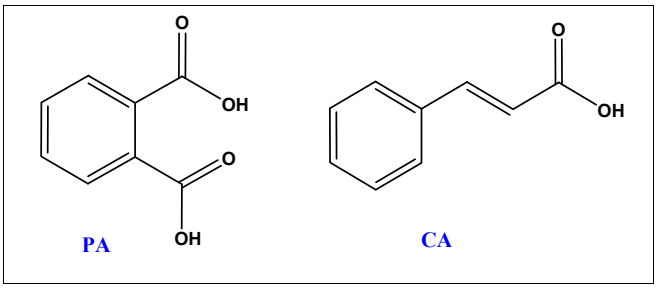

Fig. 1. Chemical structure PA and CA.

Although both molecules have planar structures, in contrast to CA, CAFG is directly attached to the benzene ring in PA. These structural differences lead to a better understanding of the way MT responds to the effectors. PA has an extra CAFG located at the ortho position of the first CAFG (Fig. 1). Previous studies showed that molecules carrying substituents at ortho position to the binding terminal were poor inhibitors [10,20]. We also showed that guaiacols are not oxidized by MT, assumingly due to the spatial hindrance created by the methoxy group [31]. PA and CA have both benzene ring and CAFG in their structures, but neither serves as a substrate for MT. Nonetheless, computational simulation suggested that PA could be a potent inhibitor. Yin et al. demonstrated that PA inhibits oxygenase activity of MT on L-dopa ($K_i = 65.84 \pm 1.10$ mM) in a mixed-type inhibition mode [32]. Studies on the structural requirements for a potent tyrosinase inhibitor led scientists to molecules such as benzoic acid and CA. A benzene ring carrying an unsaturated open chain with a carboxylic terminal showed inhibitory potential as important as that observed for benzoic acid [13,29]. Shi et al. reported that CA reversibly inhibits diphenolase activity of MT on L-dopa. The mode of inhibition was non-competitive with a K_i value of 1.99 mM [33]. Structural features, the mode of inhibition during the diphenolase activity, and the lack of information about the effects of PA and CA on the mono-phenolase activity of MT were the main reasons for selecting these modulators for this study.

2. Materials and methods

MT (EC 1.14.18.1) [34] and 4-[(4-Methylphenyl) azo]-phenol (MePAPh, $\lambda_{max} = 352$ nm, $\varepsilon = 20800\ M^{-1}\ cm^{-1}$) [35] were both prepared as previously described. CA and PA were purchased from Merck (Darmstadt, Germany). The other chemicals were taken from the authentic samples. Double distilled water was used for making a phosphate buffer solution (PBS). Spectrophotometric measurements were carried out in conventional quartz cells (4 mL) using a Analytik, SPECORD 210 (Jena, Germany) spectrophotometer.

2.1. Assay method and kinetics studies

Freshly prepared stock solution of MT was used for each set of measurements. Enzymatic reactions were run in PBS (0.05 M, pH 6.8) at 293 K in a total volume of 3 mL. Cresolase activity was measured through the depletion of the substrate, MePAPh, in the presence of a constant amount of MT for 15 min. Details of the assay method have already been explained [15,19].

To study the effect of PA on cresolase activity of MT, enzymatic oxidation of fixed amounts of MePAPh (5–50 μM) was measured in the presence of various concentrations of PA in PBS. Similar experiments were carried out for CA. Substrate addition followed after the incubation of MT with the effector. Possible interactions between the substrate and the effector were examined prior to the kinetics studies. In all cases, no changes were observed in the overall spectra of the substrate-inhibitor mixtures for at least 2 h under the applied conditions. Each result reported here is the average of three measurements for that point. The reproducibility of the results was tested in three series of measurements. The applied concentrations of the substrate and effectors are