Communication

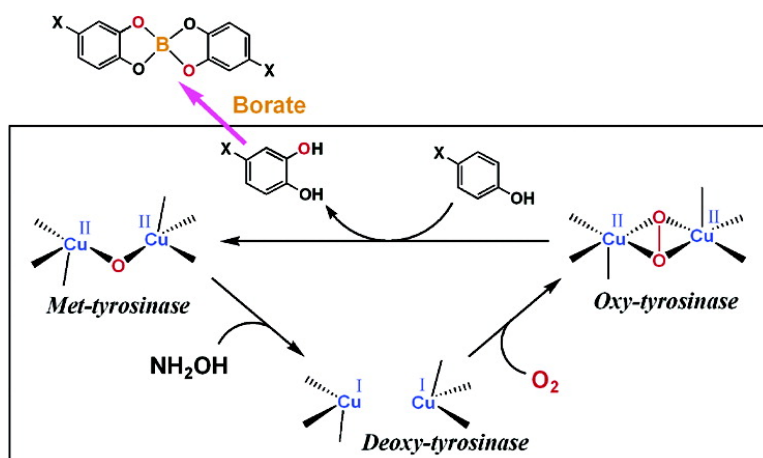# Kinetic Evaluation of Phenolase Activity of Tyrosinase Using Simplified Catalytic Reaction System

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Kinetic Evaluation of Phenolase Activity of Tyrosinase Using Simplified Catalytic Reaction System

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Tyrosinase is a copper monooxygenase that catalyzes oxygenation of phenols to catechols (phenolase activity) and oxidation of catechols to the corresponding *o*-quinones (catecholase activity).[1,2] The enzyme has a dinuclear copper active site, where a (μ-η^2:η^2-peroxo)dicopper(II) species (oxy-form) has been suggested to perform the above oxidation reactions, producing the corresponding oxidation products and the oxo-bridged dicopper-(II) form of the enzyme (met-form).[1-3] Met-tyrosinase also reacts with catechols to generate deoxy-tyrosinase (dicopper(I) form), from which oxy-tyrosinase is regenerated by the reaction with O_2.[1,4-7]

Among the catalytic processes of tyrosinase, the phenol oxygenation step (phenolase reaction) has attracted much interest not only in bioinorganic chemistry but also in the fields of coordination chemistry and synthetic organic chemistry. To clarify the mechanism of the phenol oxygenation reaction, substituent effects of phenols in the enzymatic catalytic reaction have been examined in detail.[5] However, the reported data contain the substituent effects not only on the phenolase reaction but also on the catecholase reaction. Thus, the discussion based on such data might be problematic.

Model studies on the phenolase activity of tyrosinase have provided profound insights into the aromatic hydroxylation mechanism by the (μ-η^2:η^2-peroxo)dicopper(II) complex.[8-11] Since the pioneering work by Karlin and co-workers was reported in the early 1980s,[8] several examples of intramolecular aromatic ligand hydroxylation reaction in a dinuclear copper(I) complex by O_2 have been reported.[9,12] Recently, our group and others have achieved the intermolecular oxygenation of phenolates by the peroxo complex, providing more insights into the phenolase activity of tyrosinase.[10,11] Detailed kinetic studies of the model reaction have suggested that *electrophilic aromatic substitution* is the most plausible reaction mechanism.[10]

In this study, we present a very simple enzymatic reaction system using borate anion as a trapping agent of catechols and hydroxylamine as an external reductant (Scheme 1). With this simplified enzymatic reaction system, we succeeded to examine the phenolase activity without the interference of catecholase reactions and perform the first direct comparison of the reactivities of variously para-substituted phenols between in the enzymatic reaction and in the model reaction.

Met-tyrosinase was purified from mushroom by following the reported procedures with some modifications as described in Supporting Information. The catalytic reaction of tyrosinase was monitored by following O_2-consumption using a Clark-type oxygen electrode.[13] As mentioned above, catechols affect the phenol oxygenation process in a complicated manner. Therefore, we first intend to minimize the influence of catechols by using a borate buffer as the reaction media, since the borate anion is well-known to form a stable complex with catechols.[14] Such a complex formation may exclude the catechols from the catalytic cycle by preventing the reaction with oxy-tyrosinase as well as with met-

Scheme 1
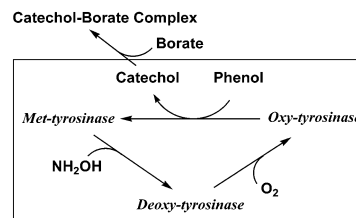
tyrosinase as indicated in Scheme 1. In fact, O_2-consumption by the catechol oxidation was successfully suppressed when tyrosinase and exogenous catechols were treated in 0.5 M borate buffer (pH = 9.0) (data not shown). More importantly, the oxygenation of phenols was also completely prohibited in the borate buffer [line (a) in Figure 1A]. Thus, the catalytic cycle may stop at the stage of met-tyrosinase as we expected, since the reductant of met-tyrosinase (free catechol) is excluded from the catalytic cycle by the complex formation with borate anion (Scheme 1).

Addition of hydroxylamine (NH_2OH) into the borate buffer solution containing tyrosinase and *p*-fluorophenol drastically enhanced the O_2-consumption rate [line (b) in Figure 1A], but no O_2-consumption was observed when NH_2OH and tyrosinase were treated without the substrate. Thus, NH_2OH functions as an external reductant for met-tyrosinase but not as a substrate for oxy-tyrosinase (Scheme 1). Product analysis of the reaction mixture by HPLC indicated a formation of the corresponding catechol (42% based on oxygen consumption).[15] The observed O_2-consumption profile exhibits little lag phase at the high NH_2OH and low phenol concentrations (Figure 1A). Thus, the modified enzymatic reaction system is suitable for the kinetic studies of the phenolase activity.

The initial rates of O_2-consumption were then plotted against the concentrations of *p*-fluorophenol to afford a Michaelis–Menten type saturation curve as shown in Figure 1B. No dependence on the concentrations of NH_2OH, O_2, and borate was observed under the present experimental conditions, indicating that these three components are kinetically saturated. Thus, the rate equation can be simply drawn as $v = V_{max}/(1 + K_M^P/[P])$, where [P] denotes the concentrations of the phenol.[16]

The kinetic parameters (K_M^P and V_{max}) for several para-substituted phenols were determined similarly by the Hanes–Woolf plot, and are listed in Table S2. The parameter V_{max} might reflect not only the phenol oxygenation process but also the NH_2OH-assisted reduction process of met-tyrosinase (also see Supporting Information). However, the reduction process is relatively fast and common in each reaction. Thus, we can use V_{max} as an indicator of the phenolase activity of tyrosinase. As seen in Table S2, the V_{max} value increases as the electron donor ability of the para-substituent increases. This clearly indicates that the peroxo intermediate of tyrosinase exhibits an electrophilic nature as suggested by the model studies.[12,17] Then, the para-substituent effect of the enzymatic
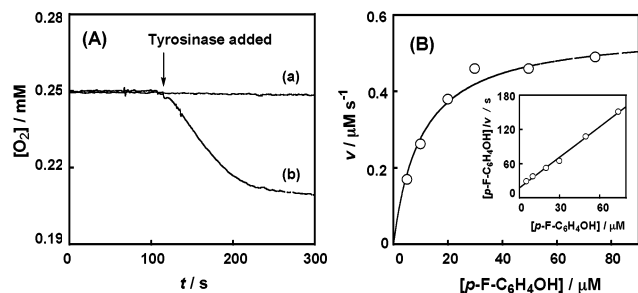
Figure 1. (A) Time course of O_2-consumption in the tyrosinase-catalyzed reaction in 0.5 M borate buffer (pH 9.0) (a) in the absence of NH_2OH and (b) in the presence of NH_2OH (6.8 mM); [tyrosinase] = 0.014 μM, [p-F$-$C$_6$H$_4$OH] = 30 μM, under air-saturated conditions at 25 °C. (B) Plot of v vs [p-F$-$C$_6$H$_4$OH]. Inset: Hanes$-$Woolf plot ([p-F$-$C$_6$H$_4$OH]/v vs [p-F$-$C$_6$H$_4$OH]).
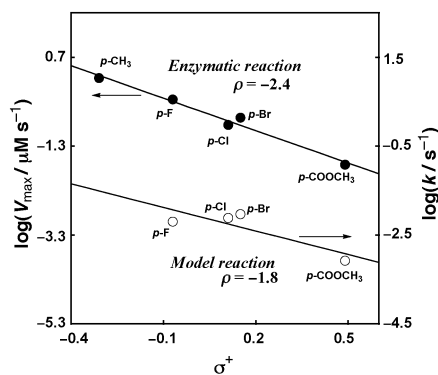

Figure 2. Plots of [log V_{max}] (enzymatic reaction, closed circle) and [log k] (model reaction, open circle) against σ^+ values of the p-substituents.

reaction has been evaluated by comparing the Hammett plot ([log k] vs σ^+) with that of the model reaction (Figure 2). The ρ value (the slope of the Hammett plot) of the enzymatic reaction (-2.4, $R^2 = 0.98$) is fairly close to that of our model reaction (-1.8)[10] and that of the aromatic ligand hydroxylation reaction reported by Karlin and co-workers (-2.1).[17] The negatively small ρ values of both the enzymatic and model reactions clearly demonstrated that the phenolase reaction of tyrosinase proceeds via the same mechanism as that of the model reaction, that is, an *electrophilic aromatic substitution mechanism*.[5,18]

In summary, we have successfully constructed a very simple enzymatic reaction system that allows us to perform a quantitative evaluation of the phenolase activity of tyrosinase for the first time. One of the key features of the present system is the exclusion of catechols from the catalytic cycle by using borate anion as the trapping agent (Scheme 1). In the absence of borate anion (in a nonborate buffer), catechols, the primary oxidation product of phenols, may be further oxidized to the corresponding o-quinones at the active site of met-tyrosinase as reported previously.[1b] In the presence of high concentration of borate anion (0.5 M), however, the primary oxidation product catechol may be withdrawn from the active site before being oxidized to the o-quinone. This means that the catechol release by the complex formation with borate anion is fast enough to prevent such the over oxidation of catechols.

Supporting Information Available: Experimental details including tyrosinase purification, the spectrum data of oxy-tyrosinase (S1), kinetic data of the phenolase reaction (S2), and the kinetic equations based on the detailed reaction scheme (S3) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) The low yield of catechol product may be due to nonenzymatic side reactions such as a quinone-induced polymerization of catechols and a reaction of the product (and/or the substrate) with oxidized NH_2OH. It is, however, apparent that the enzymatic over oxidation of catechols to the quinones is not involved, since the catechol oxidation does not proceed at all in the present borate buffer system.

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