Evaluation of an Electrochemical Method for the Analysis of Enzymatic Inhibition Reactions

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An approximate equation for bioelectrocatalytic current was applied to an inhibition reaction analysis of bilirubin oxidase by anion (Cl^- , SCN^- , and F^-) in order to assess the possibility of the electrochemical method for the analysis of enzymatic inhibition reactions. The approximate equation can be transformed into the Michaelis-Menten form, so that the bioelectrocatalytic current can be analyzed by the usual graphical manner, that is, Lineweaver-Burk, Hanes-Woolf, Dixon and Cornish-Bowden plots, if the rate of inhibition reaction is described as a simple Michaelis-Menten form. From the electrochemical assay, it was found that the inhibitor of Cl^- and SCN^- anions exhibit non-competitive inhibition while that of F^- exhibits competitive inhibition, and their inhibition constants were 220, 45, and 22 mM, respectively. The results were essentially similar to those obtained from the conventional spectrophotometric assay.

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Introduction

Several oxidoreductase reactions can be connected to the electrode reaction through electron transfer mediators. The process is called mediated bioelectrocatalysis, and the process in an homogeneous system where the enzyme, mediator and substrate are dissolved in a test solution has been utilized for the evaluation of enzyme kinetics.¹⁻⁴ Some advantages of the electrochemical method over the usual spectrophotometrical method have been presented for several cases, such as a capability of evaluating a considerably small value of Michaelis constant *etc.*^{1,2,4} Kano *et al.* presented an approximate equation for bioelectrocatalytic current that can be transformed into the Michaelis-Menten form.^{1,2} The approximate equation may provide an electrochemical method for analyzing an inhibition reaction by using conventional graphical methods, that is, Lineweaver-Burk (L-B), Hanes-Woolf (H-W), Dixon (D) and Cornish-Bowden (C-B) plots, if the rate of the inhibition reaction can be described as a simple Michaelis-Menten form.

Electrochemical evaluations of inhibition reactions have been widely conducted using an immobilized enzyme electrode for the application of the electrode as a biosensor for detecting and quantifying toxic materials.⁵ The enzyme immobilized electrode is very useful because it can be used repeatedly and allows for a reduction in the required amount of enzyme. However, the immobilization of the enzyme on the electrode surface sometimes changes the properties of the enzyme, such as inhibition type and its constants.⁵⁻⁷ Therefore, in certain cases, the electrochemical method based on the enzyme immobilized electrode is not suited for the evaluation of the inhibition reaction itself.

In contrast with electrochemical assay using an enzyme immobilized electrode, there has been little reported on

electrochemical assay for inhibition reaction under homogeneous conditions, as far as we know. In this study, the approximate equation for the bioelectrocatalytic currents were applied to an inhibition reaction analysis of bilirubin oxidase (BOD) by anions, including Cl^- , SCN^- , and F^- , and the results were compared with the results obtained from the spectrophotometric assay in order to assess the validity and utility of the electrochemical assay for the inhibition reaction.

Bilirubin oxidase is a multicopper oxidase and reduces oxygen to water conjugated with the oxidation of bilirubin.^{8,9} BOD has proven to be a promising enzyme for use as a cathode catalyst in biofuel cells,^{10,11} and its stability in solutions has been also studied electrochemically.¹²⁻¹⁴ Like other multicopper enzymes, BOD from *Trachyderma tsunodae* (*T.t.* BOD) is inhibited by some anions such as N_3^- , SCN^- , F^- , Cl^- , and Br^- ions. The inhibition, *i.e.*, anion binding properties, in *T.t.* BOD has been investigated in detail by inhibition kinetics of enzyme activity, spectrophotometric, EPR and stopped-flow methods.¹⁵ The inhibition type of these anions are reported to be non-competitive with respect to bilirubin. Because bilirubin does not exhibit a well-defined redox reaction at the electrode surface,¹⁶ we chose hexacyanoferrate(II) ion ($[\text{Fe}(\text{CN})_6]^{4-}$) as the reductant for the BOD enzymatic reaction instead of bilirubin,^{13,17} allowing for a comparison between the electrochemical and the spectrophotometrical assays.

Experimental

Reagents

Bilirubin oxidase (BOD; EC 1.3.3.5) from *Myrothecium verrucaria* was donated by Amano Enzyme Inc. (2.83 U/mg, Lot No. BOBO551301). The concentration of BOD in stock solution was determined spectrophotometrically using $\epsilon_{600} = 4800 \text{ M}^{-1} \text{ cm}^{-1}$.¹⁸ Reagent grade potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6]$), potassium hexacyanoferrate (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$), potassium chloride (KCl),

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sodium thiocyanate (NaSCN) and sodium fluoride (NaF) were obtained from Wako Pure Chemical Industries. All other chemicals were of reagent grade.

Measurements

The electrochemical and spectrophotometric measurements were carried out in the following mediator and inhibitor concentration ranges: $[K_3[Fe(CN)_6]] = 20 - 196 \mu M$ and $[K_4[Fe(CN)_6]] = 30 - 200 \mu M$, and $[KCl] = 0 - 400 mM$, $[NaSCN] = 0 - 160 mM$, and $[NaF] = 0 - 60 mM$. The concentrations of BOD in the spectroscopic and electrochemical measurements were $0.35 nM$ and $0.35 - 0.76 \mu M$, respectively. Although the concentrations of BOD for the two measurements were different, we assumed that the difference had no effect on the inhibition reaction mechanism of BOD. Throughout the study, the test solution was buffered at pH 7.0 with $50 mM NaH_2PO_4-Na_2HPO_4$, and its ionic strength was adjusted to 0.5 with KNO_3 . The solution was saturated with O_2 and its temperature was kept at $25^\circ C$.

The electrochemical measurements, cyclic voltammetry (CV) and amperometry were carried out with a three electrode system. A glassy carbon electrode ($\phi = 3 mm$; BAS Co.), a platinum coil and an Ag|AgCl (0.1 M KCl) electrode were used as the working electrode, counter electrode, and reference electrode, respectively. Spectrophotometric measurements were performed with a JASCO V-630 UV VIS spectrophotometer. The enzymatic reaction was followed by a time-dependent increase of the absorbance at 420 nm (A_{420}), which corresponds to the absorption maxima of $[Fe(CN)_6]^{3-}$ ion.¹³ The enzymatic reaction rate, v , was determined from the initial slope of the $A_{420}-t$ curve.

Results and Discussion

Prior to the electrochemical assay, we confirmed from the spectrophotometrical assay whether the inhibition reaction by anion obeys the simple Michaelis-Menten form with respect to the mediator. The equation can be expressed as:¹⁹

$$v = \frac{V_{max,i}}{K_{M,i}/[M] + 1} \quad (1)$$

where $V_{max,i}$ and $K_{M,i}$ are the apparent maximum rate and apparent Michaelis constant in the presence of an inhibitor, respectively, and $[M]$ is the concentration of reductant, *i.e.* $[Fe(CN)_6]^{4-}$ (or the concentration of mediator in the electrochemical method). The $V_{max,i}$ and $K_{M,i}$ may be a function of inhibitor concentration, $[i]$, and the forms of the equations are dependent on the inhibition type.²⁰ Figure 1 shows L-B (a), H-W (b), D (c) and C-B (d) plots obtained from the v values through spectrophotometrical measurements at different concentrations of $[Fe(CN)_6]^{4-}$ and Cl^- . All the plots showed good linearity and gave regression lines that intersect at a point on the x-axis. These results indicate that the inhibition type of Cl^- is non-competitive with respect to $[Fe(CN)_6]^{4-}$, and that the v value can be described by Eq. (1). Then, $K_{M,i}$ and $V_{max,i}$ are given by:¹⁹

$$K_{M,i} = K_M \quad (2)$$

$$V_{max,i} = V_{max}/(1 + [i]/K_i) \quad (3)$$

where, V_{max} , K_M and K_i are the maximum rate, Michaelis constant of mediator and inhibition constant, respectively. In the non-competitive inhibition, the x-coordinate of the

Fig. 1 Dependence of the enzymatic reaction rate (v) on the concentrations of the inhibitor ($[i]$, here Cl^-) and the mediator ($[M]$, $[Fe(CN)_6]^{4-}$) in the form of the (a) L-B and (b) H-W plots when $[i] = 0$ ($\bullet$), 100 ($\circ$), 200 ($\blacktriangle$), 300 ($\triangle$) and 400 ($\blacksquare$) mM, and the (c) D and (d) C-B plots when $[M] = 30$ ($\bullet$), 60 ($\circ$), 90 ($\blacktriangle$), 150 ($\triangle$) and 200 ($\blacksquare$) μM at $[BOD] = 0.35 nM$.

Fig. 2 Cyclic voltammograms (corrected for the base current) recorded in the absence (dashed line) and presence (solid line) of $0.4 M Cl^-$ in a O_2 -saturated phosphate buffer solution (pH 7, the ionic strength = 0.5 adjusted with KNO_3) containing $0.66 \mu M BOD$ and $50 \mu M K_3[Fe(CN)_6]$. Scan rate, $2 mV s^{-1}$.

intersection point in the D and C-B plots is equal to $-K_i$, and the K_i value of Cl^- was determined to be $220 mM$ from both plots c and d. The inhibition type and the K_i value of Cl^- correspond with those of *T.t.* BOD.¹⁵

To determine an appropriate experimental condition for the electrochemical assay, CVs of a test solution containing $K_3[Fe(CN)_6]$ and BOD in the absence and presence of an inhibitor were recorded. The dashed line in Fig. 2 shows CV (corrected for the base current) of the O_2 -saturated test solution (pH 7.0) containing $0.66 \mu M BOD$ and $50 \mu M K_3[Fe(CN)_6]$. The CV exhibited the S-shaped current vs. potential ($I-E$) curve with a well-defined limiting current (I_L), and the I_L substantially decreased when the test solution was deaerated by argon gas (data not shown). The results indicate that a $[Fe(CN)_6]^{4-/3-}$ -mediated bioelectrocatalytic reaction conjugated

Fig. 3 Amperometric response to the successive addition of the mediator ($K_3[Fe(CN)_6]$) to the test solution containing 0.76 μM BOD in the absence (dashed line) and presence (solid line) of 0.4 M Cl⁻. The concentration of mediator was increased from 0 to 196 μM by successive additions (arrows).

with the reduction of O₂ by BOD takes place.⁸ When the Cl⁻ was added to the test solution up to 0.4 M, the bioelectrocatalytic reduction current decreased as shown by the solid line in Fig. 2, indicating the inhibition of the BOD enzymatic reaction by Cl⁻. The half wave potential was essentially unchanged by the addition of Cl⁻, and I_t was obtained below -0.05 V in both the absence and presence of Cl⁻. Thus, the current at a constant potential of $E = -0.10$ V was determined as the I_t in the following inhibition assays. Figure 3 shows examples of the amperometric response at -0.10 V upon successive additions of the mediator into O₂-saturated test solution (pH 7.0) containing 0.76 μM BOD in the absence (dashed line) and presence (solid line) of 0.4 M KCl. The arrows in this figure indicate the addition of the mediator to the test solution with the solution stirred. After the stirring was stopped, a steady-state current was recorded as the I_t . As seen in the experimental results, in the electrochemical assay, the concentration dependence of the enzymatic activity can be obtained readily by successive addition of the mediator or the inhibitor.

The analytical and approximate equations for I_t have been derived as a function of mediator concentration under conditions of $K_s \gg [S]$, where K_s and $[S]$ are Michaelis constant and concentration of substrate.¹⁻³ In that case, v can be expressed as $V_{max}/(K_M/[M] + 1)$. Then, the approximate equation for I_t can be expressed in the Michaelis-Menten form, as given by:^{1,2}

$$\frac{I_t^2}{[M]} = \frac{2(n_M F A)^2 D_M (n_S/n_M) V_{max}}{2K_{M,i}/[M] + 1} \quad (4)$$

where n_M and n_S are the number of electron in the electrode reaction and enzymatic redox reaction, respectively, A the surface area of the electrode, D_M the diffusion coefficient of M, and F the Faraday constant. The V_{max} in Eq. (4) corresponds to $k_{cat}[E]$ in Ref. 2. In the presence of an inhibitor, if v is described by the simple Michaelis-Menten form, that is, Eq. (1), the equation of I_t also can be described by the same form as Eq. (4) because $V_{max,i}$ and $K_{M,i}$ are not functions of $[M]$. Then, the

Fig. 4 Dependence of the limiting current (I_t) on the concentrations of Cl⁻ ([i]) and $[Fe(CN)_6]^{3-}$ ([M]) in the form of the (a) $[M]I_t^{-2}$ vs. $[M]^{-1}$ (L-B type) and (b) $[M]^2I_t^{-2}$ vs. [M] (H-W type) plots when [i] = 0 (●), 100 (○), 200 (▲), 300 (△) and 400 (■) μM, and the (c) $[M]I_t^{-2}$ vs. [i] (D type) and (d) $[M]^2I_t^{-2}$ vs. [i] (C-B type) plots when [M] = 30 (●), 50 (○), 99 (▲), 148 (△) and 196 (■) μM at [BOD] = 0.64 μM.

equation of I_t in the presence of an inhibitor is given by:

$$\frac{I_t^2}{[M]} = \frac{2(n_M F A)^2 D_M (n_S/n_M) V_{max,i}}{2K_{M,i}/[M] + 1} \quad (5)$$

The reciprocal of the Eqs. (4) and (5) indicates that $[M]I_t^{-2}$ and $[M]^2I_t^{-2}$ are proportional to $[M]^{-1}$ and $[M]$, respectively.

Figures 4(a) and 4(b) show $[M]I_t^{-2}$ vs. $[M]^{-1}$ and $[M]^2I_t^{-2}$ vs. [M] plots at different concentrations of Cl⁻. These plots correspond to the L-B and H-W plots, that is, Figs. 1(a) and 1(b). The other type plots, D and C-B plots, are also obtained by the plots of $[M]I_t^{-2}$ vs. [i] and of $[M]^2I_t^{-2}$ vs. [i] at different [M]s, respectively, as shown by in Figs. 4(c) and 4(d). All plots gave good linearity and gave regression lines that intersect at a point on the x-axis. These results indicate that the inhibition type of Cl⁻ is non-competitive with respect to $[Fe(CN)_6]^{4-}$ and that the inhibition constant was determined to be 220 mM. These results of electrochemical assay agree well with spectrophotometrical assay results.

For SCN⁻ ion, the inhibition type was also deduced to be non-competitive from both the electrochemical and the spectrophotometrical assays. However, the values of K_i , which were determined to be 45 and 120 mM from the electrochemical and spectrophotometrical assays, respectively, were somewhat different. One of the possible reasons is a difference in the affinity of the BOD for SCN⁻ according to the redox state of the BOD. It has been reported that some multicopper oxidases exhibit a strong dependency of the affinity of anions on the redox state of the enzyme.^{21,22} In the case of spectrophotometrical assay, the enzymatic reaction occurs in the bulk solution, meaning that the BOD is surrounded by substantial $[Fe(CN)_6]^{4-}$. On the other hand, in the electrochemical assay, the enzymatic reaction takes place in the proximity of the electrode surface, meaning that substantial $[Fe(CN)_6]^{4-}$ exists only in the vicinity of the electrode surface. The different conditions for the enzymatic reaction may result in different ratios of the oxidized and reduced BOD, leading to different values of K_i .

Fig. 5 Dependence of v on the concentrations of F^- ($[i]$) and $[Fe(CN)_6]^{4-}$ ($[M]$) in the form of the (a) L-B and (b) H-W plots when $[i] = 0$ (●), 10 (○), 25 (▲), 40 (△) and 60 (■) mM, and the (c) D and (d) C-B plots when $[M] = 30$ (●), 60 (○), 90 (▲), 150 (△) and 200 (■) μM at $[BOD] = 0.35$ nM.

The inhibition type of SCN^- for the BOD is consistent with the type for *T.t.* BOD.¹⁵ However, the K_i value is considerably larger than that of *T.t.* BOD (94 μM). This implies that the inhibition property of anions against BOD is dependent on the origin of BOD.

For F^- ion, the inhibition type was not determined to be non-competitive. Figure 5 shows L-B, H-W, D and C-B plots obtained from the spectrophotometrical assay in the presence of F^- . The regression lines in the L-B and D plots intersect at a point on the y-axis and in the second quadrant, and those of H-W and C-B plots are parallel. The results indicate that the inhibition is competitive. In competitive inhibition, $K_{M,i}$ and $V_{max,i}$ of Eq. (1) can be given as:¹⁹

$$K_{M,i} = K_M / (1 + [i]/K_i) \quad (6)$$

$$V_{max,i} = V_{max} \quad (7)$$

Then, the x-coordinate of the intersection point for the regression lines in the D plot is equal to $-K_i$ and the K_i value of F^- was determined to be 17 mM. The difference of the inhibition type from other anions (Cl^- and SCN^-) might be related to the size of anion. The K_i value is in agreement with the value for *T.t.* BOD.¹⁵ However the inhibition type is different from that of *T.t.* BOD (non-competitive inhibition).

In the electrochemical assay for inhibition by F^- , atypical results were obtained. In the lower concentration region of F^- (< 5 mM), the catalytic current steeply decreased with the concentration of F^- and the decline of I_1 led to a deviation from a regression line derived from the higher concentration region in the graphical analysis. An example of the deviation is shown in Fig. 6, where $[M]I_1^{-2}$ is plotted against $[i]$ when $[M] = 50$ μM . The $[M]I_1^{-2}$ values below 5 mM of $[i]$ were smaller than those calculated by the linear function. The unexpected behavior also may be connected with the difference in the affinity of the BOD for F^- depending on the redox state of the BOD.

Using I_1 value when $[i] > 10$ mM, $[M]I_1^{-2}$ vs. $[M]^{-1}$, $[M]^2I_1^{-2}$ vs. $[M]$, and $[M]I_1^{-2}$ vs. $[i]$, and $[M]^2I_1^{-2}$ vs. $[i]$ are plotted in Fig. 7. The results indicate also that the inhibition of F^- ion is

Fig. 6 Dependence of $[M]I_1^{-2}$ on the concentration of F^- at $[BOD] = 0.35$ μM and $[M] = 50$ μM .

Fig. 7 Dependence of the I_1 on the concentrations of F^- ($[i]$) and $[Fe(CN)_6]^{3-}$ ($[M]$) in the form of the (a) $[M]I_1^{-2}$ vs. $[M]^{-1}$ (L-B type) and (b) $[M]^2I_1^{-2}$ vs. $[M]$ (H-W type) plots when $[i] = 10$ (○), 25 (▲), 40 (△) and 60 (■) mM, and the (c) $[M]I_1^{-2}$ vs. $[i]$ (D type) and (d) $[M]^2I_1^{-2}$ vs. $[i]$ (C-B type) plots when $[M] = 30$ (●), 50 (○), 99 (▲), 148 (△) and 196 (■) μM at $[BOD] = 0.35$ μM .

competitive. The K_i value was determined to be 22 mM from plot (c), which is in harmony with that obtained by spectrophotometric assay.

Consequently, the above results suggest that the inhibition reaction of BOD can be studied also by the electrochemical method. The electrochemical assay essentially gave similar results to those from spectrophotometric assay. To evaluate the utility of the electrochemical assay for the inhibition reaction, further research using other enzymatic inhibition reactions are required.

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