A Bioelectrocatalysis Method for the Kinetic Measurement of Thermal Inactivation of a Redox Enzyme, Bilirubin Oxidase

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The thermal stability of a redox enzyme, bilirubin oxidase (BOD), has been quantitatively evaluated by measuring the inactivation kinetics of BOD at several temperatures. The enzyme activity is directly related to the mediated bioelectrocatalytic current for the BOD-catalyzed reduction of O_2 . Thus, the inactivation process is measured by the time-dependent decrease in the bioelectrocatalytic current. The results reveal that the inactivation obeys first-order kinetics, whose rate constants (k) are determined at pH 7.0 and at 50–70°C. The half life of BOD activity, calculated from the k value at 50°C is 114 min, which is in harmony with the thermal-stability data given in a catalog by Amano Enzyme Inc. The bioelectrocatalysis method allows *in situ* measurements of the inactivation kinetics in the period of a few minutes at relatively high temperatures. The rate constants show a large temperature dependence, leading to a large Arrhenius activation energy (E_A) of 221 kJ mol⁻¹. The activation Gibbs energy ($\Delta G^\ddagger$), activation enthalpy ($\Delta H^\ddagger$), and activation entropy ($\Delta S^\ddagger$) are also determined.

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Introduction

Enzymes have found a variety of industrial applications. They have been used, for example, in fiber processing, environmental purification,¹ and clinical analysis,¹⁻³ and as ingredients of detergents,¹ and electrocatalysts of biofuel cells.³⁻⁵ In such industrial applications, enzymes are required to be stable for an appropriate period of time, and the technology of stability improvement has been intensively developed by the method of trial and error. Recently, protein engineering based on structural biology has also been introduced for an improvement.⁶ In combination with structural information, kinetic studies providing macroscopic information on the inactivation mechanism are helpful for designing stable enzymes. The kinetic study is also useful for quantitative evaluations of the thermal stability of enzymes, which is in many cases expressed as the residual activity of an enzyme after a treatment for a given period of time, say, 30 min at a given temperature of, say, 60°C.⁷

Mostly, enzyme activity is measured by a spectrophotometric method, in which the rate of the enzymatic reaction is measured by recording the absorbance-time curve reflecting the time-dependent increase (decrease) of the product (substrate). The slope of the curve is proportional to the enzyme activity. When inactivation proceeds during the measurement, the curve will bend downward. It is a tedious and difficult task to extract the inactivation effect from the curve. In the bioelectrocatalysis

method,⁸ on the contrary, enzyme activity is measured as a steady state bioelectrocatalytic current, which is time independent. A time dependent decrease, if occurs, it is attributed to inactivation of the enzyme accordingly, and the data of the time-dependent current decrease are directly utilized for a kinetic analysis of the inactivation.

In this work, we attempted to apply the bioelectrocatalysis method for measurements of the inactivation kinetics of a redox enzyme. We have taken bilirubin oxidase (BOD) as the redox enzyme, which has been proved to be a promising enzyme as a cathode catalyst in biofuel cells.^{3-5,9-11} BOD is a multicopper (one Type 1, one Type 2, and one set of Type 3 coppers)¹² oxidase catalyzing the oxidation of bilirubin to biliverdin and other substrates. We have shown¹³ that $Fe(CN)_6^{4-}$ functions as an excellent electron donor of BOD and as an electron-transfer mediator to produce a large catalytic current for the reduction of O_2 to water. Here, we tried to apply the $Fe(CN)_6^{4-}$ mediated bioelectrocatalytic current for *in situ* measurements of the inactivation process of BOD at relatively high temperatures.

Experimental

Reagents and chemicals

Bilirubin oxidase (BOD; EC 1.3.3.5) from *Myrothecium verrucaria* was donated by Amano Enzyme Inc. (2.83 U/mg, Lot No. BOBO551301), which was stored at -80°C. One milliliter of a BOD solution was prepared with 50 mM phosphate buffer (pH 7.0), and the BOD concentration was determined spectrophotometrically using $\epsilon_{600} = 4800 \text{ M}^{-1} \text{ cm}^{-1}$.¹⁴ The BOD solution was divided into 10 portions of 0.1 mL volume each and stored at -80°C until use. A 0.1-mL portion of the BOD solution was brought to 0°C in ice and used up in a

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series of experiments in the same day. Stock solutions of 10 mM $\text{Fe}(\text{CN})_6^{3-}$ and 0.1 M $\text{Fe}(\text{CN})_6^{4-}$ were freshly prepared every two weeks from $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$, respectively. All chemicals used were of analytical reagent grade, and all solutions were prepared with distilled water.

Apparatus and procedure

Electrochemical measurements were carried out with a home-made electrochemical analyzer. A glassy carbon electrode ($\phi = 3$ mm; BAS Co.), a platinum coil, and an $\text{Ag}|\text{AgCl}$ (0.1 M KCl) electrode were used as the working electrode, counter electrode, and reference electrode, respectively. The potential is referred to as the $\text{Ag}|\text{AgCl}$ (0.1 M KCl) electrode in the following. A home-made electrolysis cell of 10 mL volume with a water jacket was used, whose temperature was controlled by circulating water kept at a constant temperature with a water bath (Thermominder 50; Taitec Co.). The temperature of a test solution (5 mL) in the electrolysis cell was monitored with a digital thermometer (SK-250WP II-N, 15849; Sato Keiryoku Co.). All electrochemical measurements were performed in air-saturated solutions. Spectrophotometric measurements were performed with a JASCO V-530 UV VIS spectrophotometer at room temperature.

Results and Discussion

BOD catalyzes the following reaction very effectively:

The rate of the catalytic reaction (v) is written by the Michaelis-Menten type equation,

$$v = \frac{k_{\text{cat}}[\text{BOD}]}{1 + (K_s/[\text{O}_2]) + (K_M/[\text{Fe}(\text{CN})_6^{4-}])}, \quad (2)$$

where k_{cat} is the catalytic constant, $[\text{BOD}]$ is the concentration of BOD, K_s and K_M are the Michaelis constant for O_2 and $\text{Fe}(\text{CN})_6^{4-}$, respectively. Reaction (1) can be coupled with the electrode reaction,

to produce a mediated bioelectrocatalytic current for the reduction of O_2 .¹⁵

The catalytic current is given by Eq. (4) in a steady state in a limiting current region under the condition $K_s/[\text{O}_2] \ll 1$,¹⁶ which is fulfilled in an air-saturated solution.

$$I_{\text{L,cat}} = n_{\text{M}}FA \sqrt{(n_s/n_{\text{M}})D_{\text{M}}k_{\text{cat}}K_{\text{M}}[\text{BOD}]} \sqrt{2 \left[\frac{[\text{M}]^*}{K_{\text{M}}} - \ln \left(1 + \frac{[\text{M}]^*}{K_{\text{M}}} \right) \right]} \quad (4)$$

$$= f([\text{M}]^*)[\text{BOD}]^{1/2}, \quad (4')$$

where $I_{\text{L,cat}}$ denotes the limiting catalytic current in a steady state; n_s and n_{M} are the number of electrons involved in Eqs. (1) and (3), respectively; F and A are the Faraday constant and the electrode surface area; D_{M} and $[\text{M}]^*$ are the diffusion coefficient and the concentration of $\text{Fe}(\text{CN})_6^{3-}$, respectively.

Equation (4) (Eq. (4')) shows that $I_{\text{L,cat}}$ is proportional to $[\text{BOD}]^{1/2}$. To confirm this, we measured cyclic voltammograms (CVs) of $\text{Fe}(\text{CN})_6^{3-}$ in an air-saturated solution containing BOD at several concentrations at 30°C. The results shown in Fig. 1A

Fig. 1 A: Cyclic voltammograms of: a, air saturated 50 mM phosphate buffer (pH 7.0); b, 50 μM $\text{Fe}(\text{CN})_6^{3-}$ in a; c – g, 89 nM, 0.27 μM , 0.62 μM , 0.98 μM , and 1.69 μM BOD in b, 30°C; scan rate, 5 mV s⁻¹. B: Dependence of $-I_L$ at -0.3 V on $[\text{BOD}]^{1/2}$.

demonstrate that the CVs in the presence of BOD (c – g in Fig. 1A) exhibit an S-shaped pattern with steady state limiting currents, which are typical of mediated bioelectrocatalytic reactions. A plot of the limiting current ($-I_L$) against $[\text{BOD}]^{1/2}$ (Fig. 1B) shows that $-I_L$ is proportional to $[\text{BOD}]^{1/2}$ in satisfying Eq. (4), at least up to 1 μM of BOD. It should be noted that since the cathodic current is defined to have a minus sign, the positive quantity $-I_L$ is used in the analysis.

We have next recorded CVs at a higher temperature 50°C in a phosphate buffer solution containing 0.1 mM $\text{Fe}(\text{CN})_6^{3-}$ and 0.36 μM BOD. When the CVs were recorded several times at appropriate intervals for a few hours, the limiting current (I_L) gradually decreased as illustrated in Fig. 2A. The time-dependent decrease in I_L may be attributed to a decrease in the BOD concentration (Eq. (4)) to form an inactive form, or a decrease in the catalytic constant, k_{cat} , of BOD (Eqs. (2) and (4)) due to a conformational change of the BOD. When we plot $\ln(-I_L)$ against time (t), it gives a straight line (B in Fig. 2), suggesting a first-order kinetics of the inactivation. Thus, we assume the following simple mechanism of inactivation:

where iBOD denotes an inactive form of BOD. The rate of inactivation (v_i) is written as

$$v_i = -\frac{d[\text{BOD}]}{dt} = k[\text{BOD}] \quad (6)$$

and

$$\ln[\text{BOD}] = -kt + \ln[\text{BOD}]_{t=0}, \quad (7)$$

where k is defined as the inactivation rate constant. Taking

Fig. 2 A: Dependence of $-I_1$ (-0.3 V) on the incubation time t at 50°C . B: Plot of $\ln(-I_1)$ against t .

Eq. (4) into account, we can write

$$\ln(-I_1) = -(1/2)kt + \ln(-I_1)_{t=0}. \quad (8)$$

The k value in Eq. (8) is determined to be $1.12 \times 10^{-4} \text{ s}^{-1}$ from the slope of the plot in Fig. 2B.

We have also measured the inactivation kinetics by the conventional method of spectrophotometry. To start the inactivation process, $4 \mu\text{L}$ of BOD solution ($86.3 \mu\text{M}$) was injected into 1 mL of a phosphate buffer (50 mM , $\text{pH } 7.0$) solution kept at 50°C in a water bath. Immediately after injection, the solution was gently stirred; then, $25 \mu\text{L}$ of the solution was taken and injected into a phosphate buffer containing 2 mM $\text{Fe}(\text{CN})_6^{4-}$ (2.8 nM in BOD concentration) for the absorbance measurement at room temperature. The absorbance at 420 nm increased linearly for the first 100 s due to the production of $\text{Fe}(\text{CN})_6^{3-}$. The slope gives the initial rate of the BOD catalyzed reaction (Eq. (2)), which reflects the BOD activity. In the same way, $25 \mu\text{L}$ portions of the BOD solution in the water bath were taken at appropriate intervals during incubation; then, the enzyme activities were measured. The activity is expressed as $\Delta(\text{absorbance})/25 \text{ s}$ and plotted against the incubation time t in Fig. 3A. When plotted as $\ln[\Delta(\text{absorbance})/25 \text{ s}]$ vs. t (Fig. 3B), it gives a straight line, from which the k value is determined by Eq. (7) to be $9.17 \times 10^{-5} \text{ s}^{-1}$. This agrees well with that obtained as mentioned above by the electrochemical method. The half life (τ) of the BOD activity is calculated from the k values as $\tau = 114 \text{ min}$ at 50°C , and the residual activity after 30 min is 83.3% of the original activity. These are in harmony with the thermal-stability data given in the Catalog of Amano Enzyme Inc.,⁷ where the data are given as the residual activities after a treatment for 30 min at several temperatures.

An advantage of the electrochemical method is that it allows an *in situ* measurement at a given temperature. Figure 4 shows a current time curve recorded at -0.3 V and at 30°C of the $\text{pH } 7.0$ phosphate buffer (50 mM) containing $50 \mu\text{M}$ $\text{Fe}(\text{CN})_6^{3-}$ and $0.36 \mu\text{M}$ BOD. When the circuit was closed, the current for the mediated bioelectrocatalytic reaction appeared, and attained a

Fig. 3 A: Dependence of $\Delta(\text{absorbance})/\Delta t$ (initial rate of reaction (2)) on the incubation time t at 50°C . B: Plot of $\ln[\Delta(\text{absorbance})/\Delta t]$ against t .

Fig. 4 Limiting current ($-I_1$ at -0.3 V) vs. time t curve of the $50 \mu\text{M}$ $\text{Fe}(\text{CN})_6^{3-}$, $0.36 \mu\text{M}$ BOD phosphate buffer ($\text{pH } 7.0$) solution kept at 30°C . The electrochemical circuit was closed at the point indicated by the arrow.

steady state within 1 min . The steady state current was kept practically constant for, at least for 25 min , indicating that the BOD activity remained unchanged during the measurement. In contrast, when we measured the current time curve at 70°C , the current decreased rapidly with time, as illustrated in Fig. 5. The current was first measured at -0.3 V in a 50 mM phosphate buffer ($\text{pH } 7.0$) containing 0.1 mM $\text{Fe}(\text{CN})_6^{3-}$. When the solution was stirred with a magnetic stirring bar (arrow a), a spike appeared and returned after about 20 s to the original magnitude of the diffusion current of $\text{Fe}(\text{CN})_6^{3-}$ in a quiet solution. BOD was then added at arrow b to make $0.35 \mu\text{M}$ in BOD with gentle stirring of the solution. Immediately after BOD addition, a large current for the mediated bioelectrocatalytic reaction appeared, but the current decreased rapidly to the original current in the absence of BOD. The result indicates that the BOD activity disappears within several minutes.

It is noted that the diffusion current for the reduction of $\text{Fe}(\text{CN})_6^{3-}$ seems to remain constant during the course of recording. This is because the edge effect at the GC electrode becomes significant at longer times of recording¹⁷ (e.g. 69% at

Fig. 5 Current ($-I$ at -0.3 V) vs. time t curve of a 0.1 mM $\text{Fe}(\text{CN})_6^{3-}$ phosphate buffer (pH 7.0) solution kept at 70°C . At the point indicated by arrow a, the solution was stirred with a magnetic stirring bar for a few seconds, and at arrow b, BOD was added with gentle stirring to make $\text{BOD} = 0.35$ μM .

Fig. 6 A: Plot of $\ln(-I)$ against t in the region from $t = 175$ to 295 s of the curve in Fig. 5. B: Experimental $-I$ vs. t curve (solid curve) and calculated one (broken curve); the calculation is detailed in the text.

300 s). Anyway, we must take into account the contribution of the diffusion current to the total current when the bioelectrocatalytic current is small. The theory of a simple catalytic current (I_{cat}) for the reaction $\text{O} + \text{e}^- \rightarrow \text{R}$, $\text{R} + \text{Z} \rightarrow \text{O} + \text{Y}$ predicts that the equation for the current in a steady state is applicable only when $I_{\text{cat}} \gg 2.3I_d$, I_d is the diffusion current of O in the absence of Z.¹⁸ The same consideration is valid for I_{bcat} in Eq. (4).¹⁹ Taking into account this effect and the stirring effect mentioned above, we took the data in Fig. 5 in the region corresponding a pure bioelectrocatalytic current (from 20 s after the BOD addition to 140 s) to plot as $\ln(-I)$ vs. t in Fig. 6A. The plot gives a straight line, from which the k value is determined as $1.19 \times 10^{-2} \text{ s}^{-1}$. The broken curve in Fig. 6B illustrates the $-I$ vs. t curve by Eq. (8) using $k = 1.19 \times 10^{-2} \text{ s}^{-1}$ and the $-I$ data at $t = 0$ ($3.76 \mu\text{A}$), which well reproduces the measured $-I$ vs. t curve (solid curve) at $-I > 2 \mu\text{A}$. In conclusion, the electrochemical method based on mediated

Fig. 7 Arrhenius plot of the rate constant k for thermal inactivation of BOD.

bioelectrocatalysis allows *in situ* measurements of the inactivation kinetics of BOD within a few minutes. The k value at 70°C ($1.19 \times 10^{-2} \text{ s}^{-1}$) corresponds to a half life of 1 min. It seems not to be easy to measure such fast inactivation kinetics by the conventional spectrophotometric method.²⁰ The fact that the decay kinetics of I_1 depends on half of the rate constant ($k/2$) (Eq. (8)) favors measurements of faster inactivation kinetics.

The k value at 70°C ($1.19 \times 10^{-2} \text{ s}^{-1}$) is 100-times larger than the k value at 50°C (1.12×10^{-4} , $0.917 \times 10^{-5} \text{ s}^{-1}$), suggesting a large activation energy. We measured I - t curves by the method in Fig. 2 or Fig. 5 at various temperatures between 50 and 70°C , and plotted the results as $\ln k$ vs. $1/T$ in Fig. 7. Since the plot satisfies a linear relationship, we may assume the same reaction mechanism in the temperature range studied. The Arrhenius activation energy (E_A) was calculated to be 221 kJ mol^{-1} from the slope of the plot. The large activation energy indicates that BOD is stable at lower temperatures. For example, the half life at 60°C was calculated to be 7.5 min, while the half life at 25°C is 39 days. We calculated the activation enthalpy ($\Delta H^\ddagger$) from the Arrhenius activation energy and activation Gibbs energy ($\Delta G^\ddagger$) from the k values by $k = (k_B T/h) \exp(-\Delta G^\ddagger/RT)$ and then the activation entropy ($\Delta S^\ddagger$) by $\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger)/T$, where k_B , h , and R are the Boltzmann constant, the Planck constant and the gas constant, respectively. The results are summarized in Table 1. The activation Gibbs energy is less than half of the activation enthalpy, because of a large positive value of the activation entropy. A large Arrhenius activation energy and positive activation entropy are relatively common in the inactivation kinetics of oxidoreductases; for example, $E_A = 699 \text{ kJ mol}^{-1}$, $\Delta S^\ddagger = 696 \text{ J mol}^{-1} \text{ K}^{-1}$ for methanol dehydrogenase,²¹ $E_A = 330 \text{ kJ mol}^{-1}$, $\Delta S^\ddagger = 676 \text{ J mol}^{-1} \text{ K}^{-1}$ for glucose oxidase (*Penicillium adametzi*),²² $E_A = 252 \text{ kJ mol}^{-1}$, $\Delta S^\ddagger = 434 \text{ J mol}^{-1} \text{ K}^{-1}$ for glucose oxidase (*Aspergillus niger*),²³ and $E_A = 195$ and 145 kJ mol^{-1} , $\Delta S^\ddagger = 94$ and $237 \text{ J mol}^{-1} \text{ K}^{-1}$ for peroxidase isozymes (*Eupatorium odoratum*).²⁴

BOD exists in solution as a monomer of molecular weight 59950, composed of 534 amino acid residues containing one cysteine.²⁵ The cysteine residue is supposed to play a role in the intramolecular electron transfer between a type 1 copper site and the trinuclear center composed of the type 2 and type 3 copper sites.²⁶ Thus, the possibility of a monomer-dimer change, intra- and intermolecular disulfide bond formation (or cleavage) is precluded during the course of inactivation. The activation enthalpy would be a result of the cleavage of many numbers of weak non-covalent bonds within a BOD molecule, and the positive activation entropy would reflect an increasing local disorder in the inactivation process. Further studies are needed to understand the structural change behind the $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values of BOD. Our studies are underway in such a direction.

Table 1 Rate constant (k) and kinetic parameters for thermal inactivation of BOD

T/K	k/s	$\Delta G^\circ/kJ\ mol^{-1}$	$\Delta H^\circ/kJ\ mol^{-1}$	$\Delta S^\circ/J\ mol^{-1}\ K^{-1}$
323	$(1.02 \pm 0.10) \times 10^{-4}$ (2) ^a	104.1 ± 0.2	218.7	355 ± 1
328	$(3.08 \pm 0.20) \times 10^{-4}$ (2) ^a	102.7 ± 0.2	218.7	354 ± 1
330	4.80×10^{-4}	102.1	218.7	353
333	$(1.56 \pm 0.12) \times 10^{-3}$ (3) ^a	99.8 ± 0.2	218.6	357 ± 1
336	3.09×10^{-3}	98.8	218.6	357
338	$(4.20 \pm 0.27) \times 10^{-3}$ (3) ^a	98.5 ± 0.2	218.6	355 ± 1
341	7.58×10^{-3}	97.7	218.6	354
343	$(1.16 \pm 0.08) \times 10^{-2}$ (4) ^a	97.1 ± 0.2	218.6	354 ± 1

a. (n): number of run.

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