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Cyclic Voltammetric Simulation for Electrochemically Mediated Enzyme Reaction and Determination of Enzyme Kinetic Constants

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A cyclic voltammetric simulation that can be applied to an electrochemically mediated enzyme reaction involving any substrate and mediator concentrations was developed. Concentration polarization of the substrate in the vicinity of an electrode was considered as well as mediator concentration. Reversible and quasi-reversible electrochemical reactions with one electron followed by an enzyme reaction with two electrons were modeled. The differential equations for the mediator and substrate were solved using digital simulation techniques. The calculated cyclic voltammograms showed prepeaks when there was a low substrate concentration, high mediator concentration, and high enzyme activity. Digital simulation was applied to the determination of the kinetic constants of glucose oxidase (GOx). Cyclic voltammetry was carried out experimentally in a phosphate buffer solution containing GOx, ferrocene derivatives, and glucose. The ratio of the catalytic to the diffusion-controlled current, i_k/i_d, was evaluated. The k_{cat}, K_{MM}, and K_{MS} values were determined from the current values obtained by simulation and by experimentation at various enzyme, mediator, and substrate concentrations. The k_{cat}, K_{MM}, and K_{MS} values for GOx, ferrocenedimethanol, and glucose were 340 s^{-1}, 110 μM, and 30 mM, respectively.

During the past two decades, cyclic voltammetry has often been used for an electrochemical reaction coupled with a mediated enzyme reaction. Since Nicholson and Shain[1] reported their theoretical treatment of cyclic voltammetry, it has commonly been used as an analytical method to explore reaction kinetics. Nicholson and Shain described an electrochemical reaction coupled with a first-order homogeneous chemical reaction as well as a simple electrochemical reaction. Their theoretical treatment has also been applied to an electrochemical reaction followed by a mediated enzyme reaction. However, enzyme reaction kinetics are quite different from a simple first-order reaction model, and, therefore, their method can be applied only to the special case in which there is a high substrate concentration and an extremely diluted mediator concentration, which defines a pseudo-first-order reaction. In addition, the kinetic parameter to be obtained from this analysis is a pseudo-first-order rate constant that corresponds to $k_{cat}C_E/K_{MM}$, where k_{cat}, C_E, and K_{MM} represent the turnover number of the enzyme reaction, the enzyme concentration, and the Michaelis constant for the mediator, respectively. These kinetic constants cannot be determined individually. Therefore, a novel analytical method for the mediated enzyme reaction is required.

Several researchers have reported simulations of mediated enzyme electrochemistry.[2−15] Bartlett and Pratt have reviewed previous works on the simulation of enzyme electrodes.[16] Simulations are classified as either approximate analytical or numerical methods. To obtain an approximate analytical solution, approximation and classification of each different condition are needed. On the other hand, digital simulation to obtain a numerical solution can be applied to any case, and neither simplification nor classification is necessary. Many digital simulations for steady-state and transient responses of enzyme electrochemistry have been reported.[2−4,7,8,10−12] However, concentration polarization of the substrate in the vicinity of an electrode surface has never been considered for cyclic voltammetric simulation of a mediated enzyme reaction. A cyclic voltammetric simulation of the homogeneous case has been described in a preliminary way by Bartlett and Pratt.[16] They compared the simulated cyclic voltammetric system with the simple EC′ system. Unfortunately, no further reports have appeared.

A digital simulation program for cyclic voltammetry, DigiSim, is commercially available from Bioanalytical Systems (West Lafayette, IN). This software, developed by Rudolph and Feldberg, was based on the fast implicit finite difference (IFD) method, and it takes a few seconds to produce results. An electrochemical reaction coupled with a first- and/or second-order chemical reaction can be simulated. However, since coupling with an enzyme reaction is not considered, it would be very inconvenient

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(1) Nicholson, R. S.; Shain, I. *Anal. Chem.* **1964**, *36*, 706−723.
(2) Mell, L. D.; Maloy, J. T. *Anal. Chem.* **1975**, *47*, 299−307.
(3) Leypoldt, J. K.; Gough, D. A. *Anal. Chem.* **1984**, *56*, 2896−2904.
(4) Bergel, A.; Comtat, M. *Anal. Chem.* **1984**, *56*, 2904−2909.
(5) Bartlett, P. N.; Whitaker, R. G. *J. Electroanal. Chem.* **1987**, *224*, 27−35.
(6) Bartlett, P. N.; Whitaker, R. G. *J. Electroanal. Chem.* **1987**, *224*, 37−48.
(7) Lucisano, J. Y.; Gough, D. A. *Anal. Chem.* **1988**, *60*, 1272−1281.
(8) Rusling, J. F.; Ito, K. *Anal. Chim. Acta* **1991**, *252*, 23−27.
(9) Albery, W. J.; Bartlett, P. N.; Driscoll, B. J.; Lennox, R. B. *J. Electroanal. Chem.* **1992**, *323*, 77−102.
(10) Battaglini, F.; Calvo, E. J. *Anal. Chim. Acta* **1992**, *258*, 151−160.
(11) Randriamahazaka, H.; Nigretto, J.-M. *Electroanalysis* **1993**, *5*, 221−230.
(12) Martens, N.; Hall, E. A. H. *Anal. Chem.* **1994**, *66*, 2763−2770.
(13) Kong, J.; Liu, H.; Deng, J. *Anal. Lett.* **1995**, *28*, 1339−1357.
(14) Bartlett, P. N.; Pratt, K. F. E. *J. Electroanal. Chem.* **1995**, *397*, 53−60.
(15) Bartlett, P. N.; Pratt, K. F. E. *J. Electroanal. Chem.* **1995**, *397*, 61−78.
(16) Bartlett, P. N.; Pratt, K. F. E. *Biosens. Bioelectron.* **1993**, *8*, 451−462.

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to simulate the cyclic voltammetry for mediated enzyme reactions. To calculate the cyclic voltammetry for enzyme reactions, the enzyme reaction should be separated into several steps of first- and second-order chemical reactions. The rate constant at each reaction step cannot be determined using electrochemical measurement. If steady-state kinetics for enzyme reaction are assumed, then determination of the first- and/or second-order kinetic constant at each step is unnecessary in order to simulate electrochemistry coupled with enzyme reaction. The enzyme kinetic constants that can easily be determined, such as turnover number and Michaelis constants, can be applied.

In this paper, digital simulation of the cyclic voltammetry of reversible and quasi-reversible electrochemical reactions coupled with mediated enzyme reactions was carried out using the explicit finite difference (EFD) method developed by Britz.[17] The EFD method has a slower processing speed than the IFD method, but it is easier to program. The EFD-based simulator is satisfactory to use because the processing speeds of modern computers are high enough to ensure its use is practical. In this work, concentration polarization of a substrate in the vicinity of an electrode surface was also considered, and, hence, a precise cyclic voltammetric simulation was achieved, even in situations of high enzyme activity and high mediator concentration. In addition, the kinetic constants of the enzyme reaction were determined using this simulation. The values of K_{MM}, k_{cat}, and the Michaelis constant for the substrate, K_{MS}, were successively determined by varying enzyme, mediator, and substrate concentrations.

DIGITAL SIMULATION

Reversible Electrochemistry Coupled with a Homogeneous Enzyme Reaction. The model of an electrochemical reaction coupled with a homogeneous enzyme reaction was considered in this study. One-electron- and two-electron-transfer reactions for the electrochemical and enzyme reactions were assumed, respectively, according to the following scheme:

$$\text{electrochemical reaction} \qquad R \rightleftharpoons O + e^- \tag{1}$$

$$\text{enzyme reaction} \qquad 2O + S \rightarrow 2R + P \tag{2}$$

where R, O, S, and P are the reduced and oxidized forms of the mediator, substrate, and product, respectively. Electrochemical reaction 1 takes place at the electrode surface with full reversible kinetics, and enzyme reaction 2 occurs in the solution. Reaction 2 represents the enzyme catalysis characterized by Michaelis–Menten kinetics. Although two molecules of the oxidant O are involved in reaction 2, in this study first-order kinetics are assumed in O, as reported previously.[10] If the diffusion of the enzyme molecule is neglected and steady-state conditions are assumed for the enzyme reaction, the rate of the homogeneous enzyme reaction is given by

$$v = \frac{k_{cat}C_E}{K_{MS}/C_S + K_{MM}/C_O + 1} \tag{3}$$

which is characteristic of the two-substrate enzyme reaction. This reaction process is valid for the ping-pong mechanism of the oxidoreductases, such as, glucose oxidase. Here, k_{cat}, C_E, K_{MS}, and K_{MM} are the turnover number, enzyme concentration, and Michaelis constants for the substrate and mediator, respectively, and C_S and C_O are the substrate and oxidized mediator concentrations, respectively.

Considering one-dimensional diffusion, coupling of reactions 1 and 2 with the diffusion described by Fick's law leads to the following equations:

$$\frac{\partial C_R}{\partial t} = D_R\frac{\partial^2 C_R}{\partial x^2} + \frac{2k_{cat}C_E}{K_{MS}/C_S + K_{MM}/C_O + 1} \tag{4}$$

$$\frac{\partial C_S}{\partial t} = D_S\frac{\partial^2 C_S}{\partial x} - \frac{k_{cat}C_E}{K_{MS}/C_S + K_{MM}/C_O + 1} \tag{5}$$

where D_R and D_S are diffusion coefficients for the reduced form of the mediator and substrate, respectively, and the diffusion coefficient for the oxidized form of the mediator was assumed to be as great as D_R.

The initial and boundary conditions were assumed to follow the following equations:

$t = 0$, $x \geq 0$, and $t > 0$, $x \rightarrow \infty$

$$C_R = C_R^{\infty} \tag{6}$$

$$C_O = 0 \tag{7}$$

$$C_S = C_S^{\infty} \tag{8}$$

$t > 0$, $x = 0$

$$C_R = C_R^{\infty}/\left[1 + \exp\left\{\frac{nF}{RT}(E - E^{\circ\prime})\right\}\right] \tag{9}$$

$$(dC_S/dx)_{x=0} = 0 \tag{10}$$

at all t and x

$$C_R + C_O = C_R^{\infty} \tag{11}$$

where C_R^{∞} and C_S^{∞} are the bulk concentrations of R and S, respectively.

Differential equations 4 and 5 were solved using the EFD method. The dimensionless model diffusion coefficients, λ_R and λ_S, for R and S, respectively, are given by

$$\lambda_R = D_R\Delta t/\Delta x^2 \tag{12}$$

$$\lambda_S = D_S\Delta t/\Delta x^2 \tag{13}$$

Since λ_R and λ_S values should be less than 0.5 for computational stability, the λ value for the greater diffusion coefficient was taken as 0.45. Time and space segments were taken as Δt (ms) $= 10/v$ and $\Delta x = (D_R\Delta t/\lambda_R)^{1/2}$ or $(D_S\Delta t/\lambda_S)^{1/2}$, respectively, where v is the scan rate value (mV/s).

(17) Britz, D. *Digital Simulation in Electrochemistry*, 2nd ed.; Springer-Verlag: Berlin, 1988.

The values of C_R and C_S at each space and each time were calculated, and the gradient of C_R at an electrode was evaluated as the current value.

Quasi-Reversible Electrochemistry Coupled with a Homogeneous Enzyme Reaction. A cyclic voltammetric simulation was also carried out on quasi-reversible electrochemistry with an enzyme reaction. The boundary condition at the electrode surface is given by

$$t > 0,\ x = 0$$

$$D_R\frac{\partial C_R}{\partial x} = k^0\left[C_R \exp\left\{\frac{\alpha nF}{RT}(E - E^{\circ\prime})\right\} - C_O \exp\left\{-\frac{(1-\alpha)nF}{RT}(E - E^{\circ\prime})\right\}\right] \quad (14)$$

Current values were also calculated for this case.

Computation. The computer program for the cyclic voltammetric simulation of the electrochemistry coupled with an enzyme reaction was written in the C language. The source code for the program can be seen at our World Wide Web site.[18] The boundary condition for C_S at $x = 0$, $t > 0$, shown as eq 10, is used as suggested by Britz.[17] Since C_S is electroinactive, the flux of C_S to the electrode surface should be zero. The boundary condition in the program was referred to in a previous report.[19]

A cyclic voltammetric curve including a reverse sweep was simulated at various scan rate values. Normally, 100 001 $i-E$ data per cyclic voltammogram were calculated and 1001 data were saved. The C_R and C_S values were calculated from the electrode surface to the point of $3(D_R t)^{1/2}$ or $3(D_S t)^{1/2}$.

The program was run on the Sparc 20 (Sun), Indigo, Onyx (Silicon Graphics), and Convex C3440 computers in the Center for Information Science, Japan Advanced Institute of Science and Technology.

EXPERIMENTAL SECTION

Chemicals. Glucose oxidase (GOx) (EC 1.1.3.4 from *Aspergillus niger*, type XS, Sigma) was used. GOx content was determined to be 8.13×10^{-9} mol/mg solid by measuring FAD content. The FAD concentration was determined from the absorbance at 450 nm using $\epsilon_{450} = 14.1\ \mathrm{mM^{-1}\ cm^{-1}}$.[20] The ratio of GOx bound to total FAD molecules was measured using gel filtration chromatography (TOSOH HPLC, TSK-gel G3000SWXL, Japan) and was found to be 89%. D-Glucose was purchased from Wako Pure Chemical, Japan, and the glucose solution was equilibrated overnight prior to measurements. Ferrocenemethanol and 1,1′-ferrocenedimethanol were purchased from Aldrich. (Ferrocenylmethyl)trimethylammonium (FMTMA) perchlorate was obtained by anion exchange from FMTMA iodide (Tokyo Chemical, Japan). All chemicals except for FMTMA perchlorate were used as received.

Cyclic Voltammetry. Glassy carbon disk electrodes with a 3 mm diameter (GC20, Tokai Carbon, Tokyo) were used throughout. The electrode surface was polished with waterproof emery paper (no. 400, 1000, and 2400, Marumoto, Tokyo) and with an alumina suspension (particle size, 20 nm, PRESI, Grenoble, France) on a wool cloth (Marumoto). The electrode was sonically cleaned and rinsed with water between the polishing steps. A platinum auxiliary electrode and a potassium chloride-saturated silver−silver chloride reference electrode were employed. All potentials herein are quoted with respect to the reference electrode. Cyclic voltammetry was carried out in a deoxygenated phosphate buffer solution (0.1 M, pH 7.0) at 30 °C with a computer-controlled potentiostat (EG&G 263A).

RESULTS AND DISCUSSION

Digital Simulation of Reversible Electrochemistry Coupled with a Homogeneous Enzyme Reaction. Figure 1 shows some examples of the calculated cyclic voltammograms of reversible electrochemistry coupled with a homogeneous enzyme reaction. These cyclic voltammograms were calculated using the same parameters except for substrate concentration. In this figure, two anodic current peaks are observed at low substrate concentrations. The value of the first peak increases with the increase in substrate concentration, and the second peak, due to the direct mediator oxidation at the electrode, gradually disappears. At extremely high substrate concentrations, no peaks are observed, and a plateau cyclic voltammogram is obtained.

These electrochemical behaviors can be elucidated by calculating concentration profiles of the mediator and substrate, as illustrated in Figure 2. Calculation was carried out at a 5 mM substrate concentration using several potential values. Most substrate in the vicinity of the electrode is consumed at −36, 20, and 200 mV versus $E^{\circ\prime}$, where the lowest level, main peak, and gradual decrease are observed in the cyclic voltammetric curve. Therefore, the recycling rate of the mediator is reduced at −36 mV. Comparing the concentration profile of the mediator with that of the substrate at the same diffusion coefficient values, the diffusion layer of the mediator is found to be shorter than that of the substrate due to recycling of the mediator.

Figure 3 shows the calculated cyclic voltammograms at four different mediator concentrations and three different enzyme activities. These curves demonstrate that the prepeak is observed at both high mediator concentration and high enzyme activity. This is due to depletion of the substrate concentration in the vicinity of the electrode when there is a high mediator concentration, high enzyme activity, and low substrate concentration.

Figure 4 shows experimental cyclic voltammograms of a mediated electrochemical reaction of GOx at high mediator and high enzyme concentrations. Prepeaks or shoulders were observed at low substrate concentrations. We confirmed the observation of the prepeak experimentally.

Observation of a prepeak was previously reported by Barker et al. in both the simulation and the experiment involving electrochemistry followed by fast second-order reactions.[19] They compared the digitally simulated cyclic voltammetry with experiments on the second-order reaction of electroactive cytochrome *c* and electroinactive plastocyanin; the concentration profiles of these two species were also calculated. Coury et al. have shown experimentally the prepeaks of the mediated electrochemical reaction of sulfite oxidase with $[Co(bpy)3]^{2+/3+}$.[21] The prepeaks

(18) http://www.jaist.ac.jp/~yokoyama/pdf/ccve2.pdf.
(19) Barker, P. D.; Hill, H. A. O.; Walton, N. J. *J. Electroanal. Chem*. **1989**, *260*, 303−326.
(20) Bright, H. J.; Gibson, Q. H. *J. Biol. Chem*. **1967**, *242*, 994−1003.
(21) Coury, L. A., Jr.; Oliver, B. N.; Egekeze, J. O.; Sosnoff, C. S.; Brumfield, J. C.; Buck, R. P.; Murray, R. W. *Anal. Chem*. **1990**, *62*, 452−458.

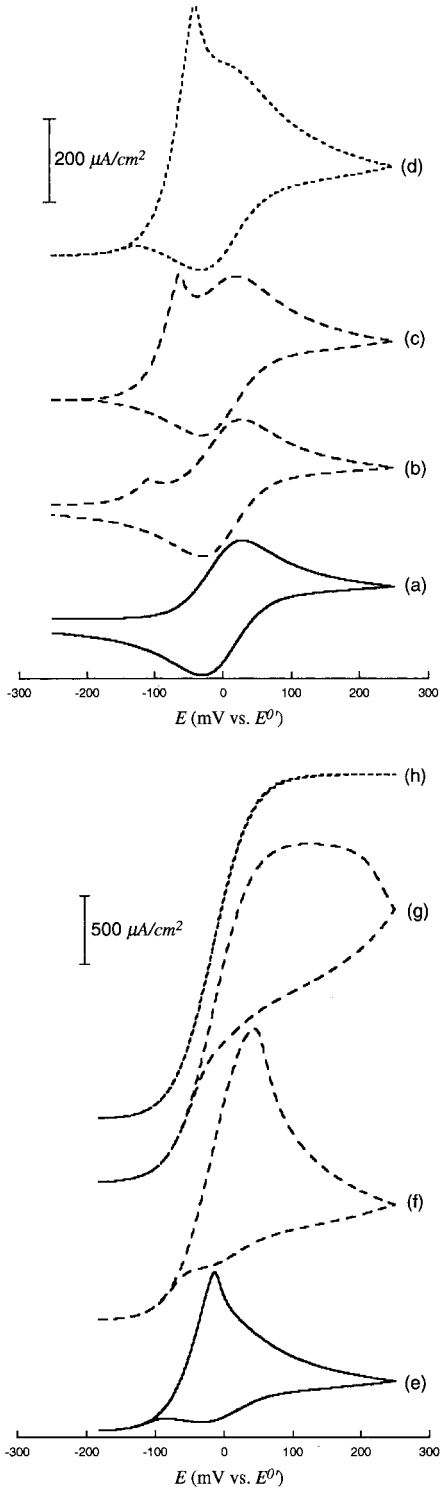

Figure 1. Calculated cyclic voltammograms of reversible electrochemistry coupled with a homogeneous enzyme reaction. $v = 1$ mV/s, $C_R^\infty = 10$ mM, $D_R = D_O = D_S = 5 \times 10^{-6}$ cm²/s, $K_{MM} = 1$ mM, $K_{MS} = 1$ mM, $k_{cat}C_E = 5$ mM/s, $C_S^\infty =$ (a) 0, (b) 1, (c) 5, (d) 10, (e) 20, (f) 50, (g) 100, and (h) 1000 mM.

were observed at low concentrations of sulfite. They also concluded that this was due to a rapid depletion of sulfate in the catalytic reaction layer by the enzymatic reaction.

Digital Simulation of Quasi-Reversible Electrochemistry Coupled with a Homogeneous Enzyme Reaction. Cyclic voltammograms of the quasi-reversible electrochemistry with an enzyme reaction were simulated and are shown in Figure 5. Catalytic current is also obtained at a low k^0 value (see Figure 5c); however, a high applied potential is necessary to obtain a high catalytic current.

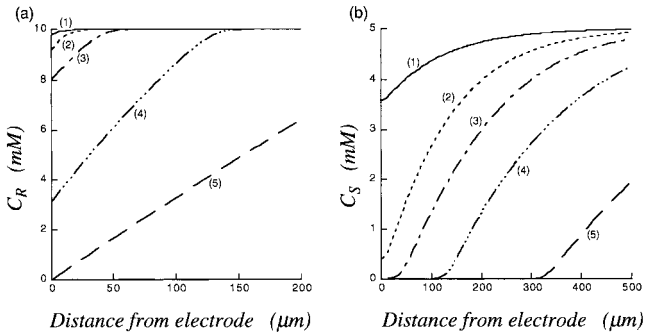

Figure 2. Calculated concentration profiles of (a) mediator and (b) substrate. The calculation conditions were the same as those in Figure 1. $C_S^\infty = 5$ mM, $E - E^{\circ\prime} =$ (1) −100, (2) −63, (3) −36, (4) 20, and (5) 200 mV at forward scan.

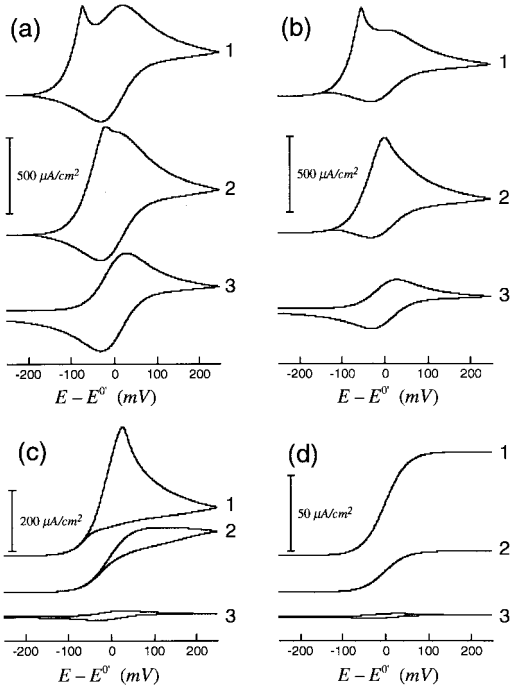

Figure 3. Calculated cyclic voltammograms of reversible electrochemistry coupled with a homogeneous enzyme reaction. $v = 1$ mV/s, $C_S^\infty = 10$ mM, $D_R = D_O = D_S = 5 \times 10^{-6}$ cm²/s, $K_{MM} = 1$ mM, $K_{MS} = 1$ mM, $C_R^\infty =$ (a) 20, (b) 10, (c) 1, and (d) 0.1 mM, $k_{cat}C_E =$ (1) 10, (2) 1, and (3) 0 mM/s.

Determination of Kinetic Constants of the Enzyme Reaction. The cyclic voltammetric simulation technique was applied to the analysis of an electrochemically mediated enzyme reaction. In this work, the values of the kinetic constants such as k_{cat}, K_{MM}, and K_{MS} for GOx were determined.

The first step is determination of the k_{cat}/K_{MM} value. At an extremely high substrate concentration and an extremely low mediator concentration, the differential eq 4 can be simplified to

$$\frac{\partial C_R}{\partial t} = D_R \frac{\partial^2 C_R}{\partial x^2} + \frac{2k_{cat}C_E}{K_{MM}} C_O \qquad (15)$$

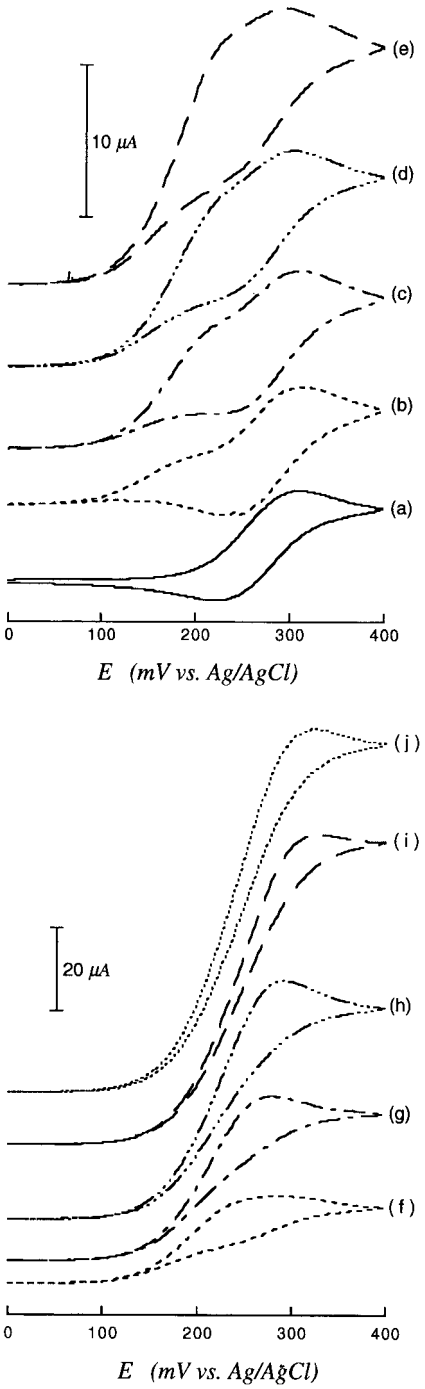

Figure 4. Cyclic voltammograms of 1,1′-ferrocenedimethanol coupled with a homogeneous enzyme reaction of GOx. $v = 1$ mV/s, $C_R^\infty = 4.0$ mM, $C_E = 32.5$ μM, $C_S^\infty =$ (a) 0, (b) 2, (c) 4, (d) 6, (e) 8, (f) 10, (g) 20, (h) 30, (i) 40, and (j) 50 mM.

This differential equation was numerically solved at various $k_{cat}C_E/K_{MM}$ values and at several scan rate values. Figure 6 shows the correlation between i_k/i_d and $k_{cat}C_E/K_{MM}$, where i_k and i_d are the current densities at 29 mV vs $E^{o\prime}$ with and without an enzyme reaction, respectively. This figure indicates that the i_k/i_d value increases with the increase in $k_{cat}C_E/K_{MM}$ and that the $k_{cat}C_E/K_{MM}$ value can be determined from the experimental i_k/i_d value using these curves.

Figure 7 shows the experimental cyclic voltammograms of the GOx reaction mediated by 1,1′-ferrocenedimethanol at various enzyme concentrations. The measurements were carried out at a glucose concentration of 100 mM, and both mediator concentration and potential scan rate were varied. This figure shows that a high catalytic current was observed as the enzyme concentration increased. The i_k/i_d value at the peak potential in the absence of glucose, E_{pd}, was evaluated to derive the $k_{cat}C_E/K_{MM}$ value. The background current was subtracted to obtain the i_k and i_d values. The experimental i_k/i_d value decreased with increases in mediator concentration, and, hence, the i_k/i_d value at extremely low mediator concentrations, $C_R^\infty \rightarrow 0$, was obtained by extrapolation of the plots of i_k/i_d and mediator concentration. The $k_{cat}C_E/K_{MM}$ values were determined from Figure 6 and the i_k/i_d values at various GOx concentrations.

Subsequently, the $k_{cat}C_E/K_{MM}$ values were plotted against C_E as shown in Figure 8. This figure demonstrates that the $k_{cat}C_E/K_{MM}$ value depended on the scan rate value. The enzyme reaction kinetics must be independent of the scan rate. This is probably due to several factors such as mutarotation from the α-glucose anomer to the β anomer, three-dimensional diffusion, and natural convection. The effect of these factors increases with increases in measurement time. The mutarotational half-life of α-glucose in 20 mM phosphate at 20 °C (37 °C) was reported to be 26.1 min (6.0 min) at pH 6.0 and 10.5 min (2.5 min) at pH 7.4.[22] Therefore, in this time scale, the catalytic current obtained at the slow scan rate is expected to be affected by mutarotation. Indeed, the correlation between $k_{cat}C_E/K_{MM}$ and C_E at the slow scan rate is less linear than that at the fast scan rate. The slope of this plot increases with the increase in C_E. This is observed especially clearly at the slow scan rate. The rate of substrate consumption is fast when there is high enzyme activity, and, therefore, the mutarotation is also fast. Three-dimensional diffusion and natural convection are also highly effective at the slow scan rate. This is probably why the $k_{cat}C_E/K_{MM}$ values derived at the slow scan rate were higher than those derived at the fast scan rate. In addition, GOx adsorption onto the electrode and the error in estimation of the $k_{cat}C_E/K_{MM}$ value should be raised as the reason for the deviation in Figure 8. In this work, the $k_{cat}C_E/K_{MM}$ value for ferrocenedimethanol was determined to be 3.12 μM^{-1} s^{-1} from the slope of the graph in Figure 8 using the i_k/i_d values at 50 mV/s.

The second step is determination of the K_{MM} value. At extremely high substrate concentrations, the differential equation 4 is partially simplified to

$$\frac{\partial C_R}{\partial t} = D_R\frac{\partial^2 C_R}{\partial x^2} + \frac{2k_{cat}C_E}{K_{MM}/C_O + 1} \qquad (16)$$

This equation was numerically solved at various C_R^∞/K_{MM} values. The calculated i_k/i_d value was plotted against C_R^∞/K_{MM} as shown in Figure 9. This figure shows that the i_k/i_d value decreases with increases in C_R^∞/K_{MM}. The C_R^∞/K_{MM} value can be determined from Figure 9 on the basis of the $k_{cat}C_E/K_{MM}$ values obtained in the previous step.

Figure 10 shows the cyclic voltammograms of the mediated enzyme reaction of GOx at various mediator concentrations. The

(22) Okuda, J.; Taguchi, T.; Tomimura, A. *Chem. Pharm. Bull.* **1987**, *35*, 4332−4337.

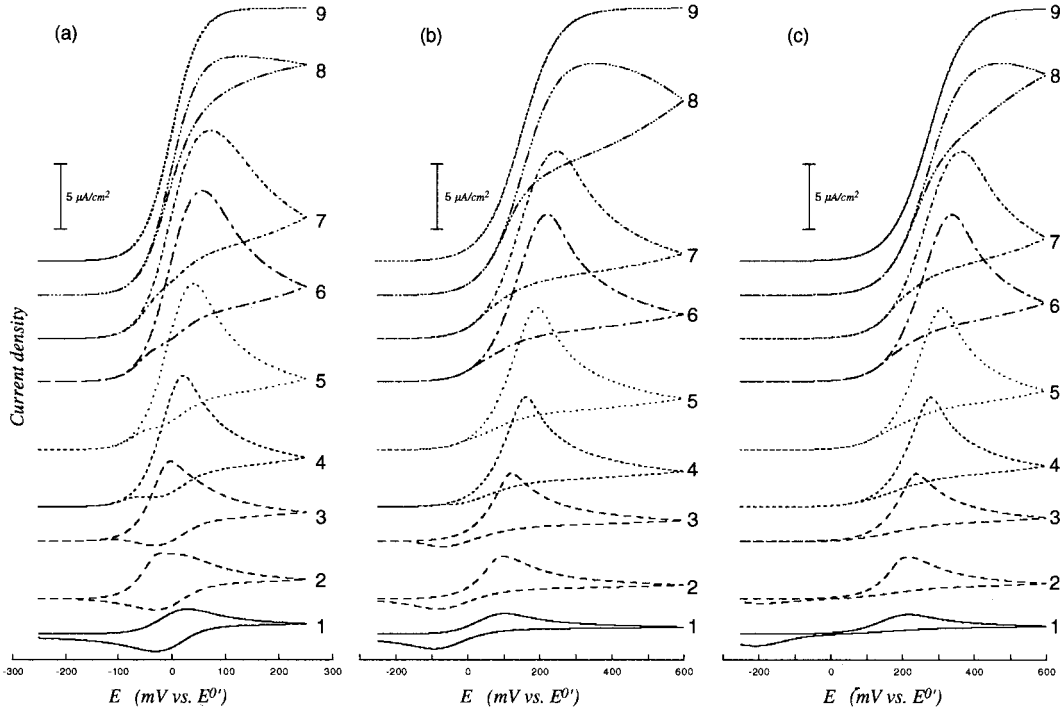

Figure 5. Calculated cyclic voltammograms of reversible and quasi-reversible electrochemistry coupled with a homogeneous enzyme reaction. (a) Reversible, (b) quasi-reversible, $k^0 = 1 \times 10^{-4}$ cm/s, $\alpha = 0.5$, (c) quasi-reversible, $k^0 = 1 \times 10^{-5}$ cm/s, $\alpha = 0.5$. $v = 1$ mV/s, $C_R^\infty = 0.1$ mM, $D_R = D_O = D_S = 5 \times 10^{-6}$ cm²/s, $K_{MM} = 1$ mM, $K_{MS} = 1$ mM, $k_{cat}C_E = 0.5$ mM/s, $C_S^\infty =$ (1) 0, (2) 0.05, (3) 0.1, (4) 0.2, (5) 0.3, (6) 0.4, (7) 0.5, (8) 1, and (9) 1000 mM.

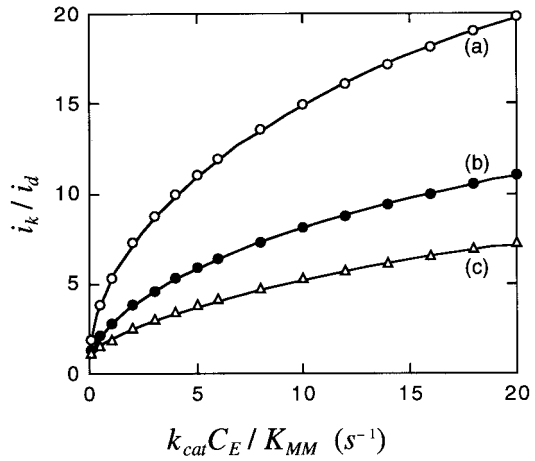

Figure 6. Correlation between i_k/i_d at 29 mV vs $E^{\circ\prime}$ and $k_{cat}C_E/K_{MM}$. Cyclic voltammograms were calculated under reversible electrochemistry at $v =$ (a) 5, (b) 20, and (c) 50 mV/s.

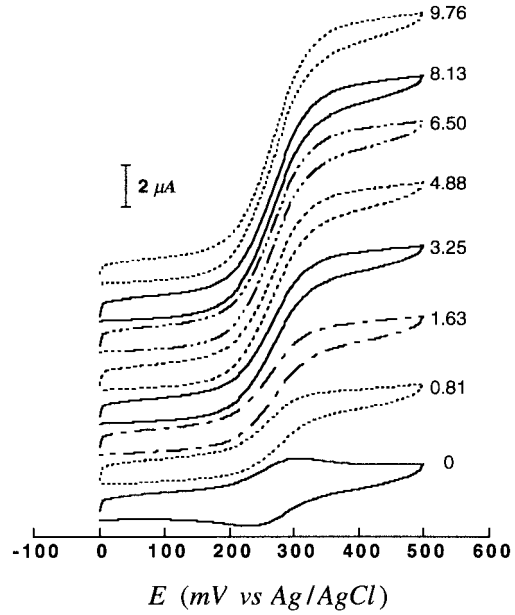

Figure 7. Cyclic voltammograms of an enzyme reaction mediated by 1,1′-ferrocenedimethanol at various GOx concentrations. The numbers in this figure show the enzyme concentration C_E (μM). $v =$ 50 mV/s, $C_R^\infty = 0.1$ mM, $C_S^\infty = 100$ mM.

current values were normalized by the i_d value at E_{pd}. These cyclic voltammograms demonstrate that the i_k/i_d value decreased with the increase in mediator concentration. The i_k/i_d values at E_{pd} were evaluated to obtain the K_{MM} value. The C_R^∞/K_{MM} values were determined by using Figure 9.

Figure 11 shows the correlation between C_R^∞/K_{MM} and C_R^∞ at $C_E = 6.50$ μM. From the slope of this graph, the K_{MM} value for ferrocenedimethanol can be derived (111 μM), and, therefore, k_{cat} can also be determined (347 s^{-1}). The k_{cat} and K_{MM} values were determined at different enzyme concentrations and are summarized in Table 1. Ferrocenedimethanol and FMTMA were also examined. These results demonstrate that the k_{cat} and K_{MM} values were essentially independent of C_E.

The K_{MS} value was determined in the last step. The differential equations 4 and 5 were solved numerically. Calculations were carried out at various enzyme and mediator concentrations at $k_{cat} = 339$ s^{-1} and $K_{MM} = 109$ μM for ferrocenedimethanol. The D_R value determined by cyclic voltammetry to be 5.14×10^{-6} cm²/s was applied to the simulation. The D_S value was assumed to be as great as D_R. The anomer ratio should be considered to determine the overall K_{MS} value, since only the β anomer is

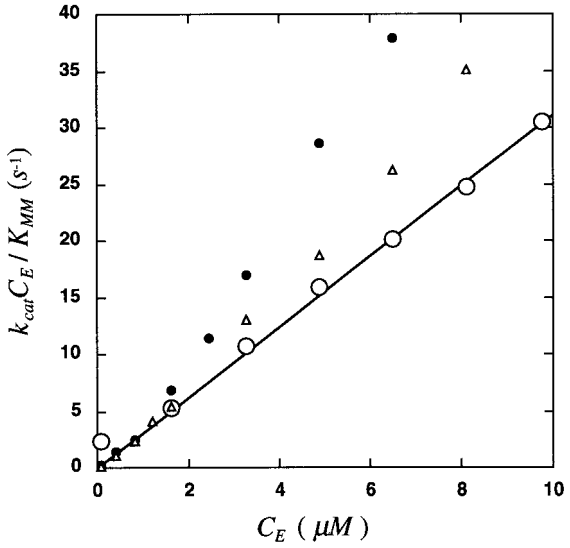

Figure 8. Correlation between $k_{cat}C_E/K_{MM}$ and C_E. The $k_{cat}C_E/K_{MM}$ values were determined using Figure 6. v = (●) 5, (△) 20, and (○) 50 mV/s. C_S^∞ = 100 mM.

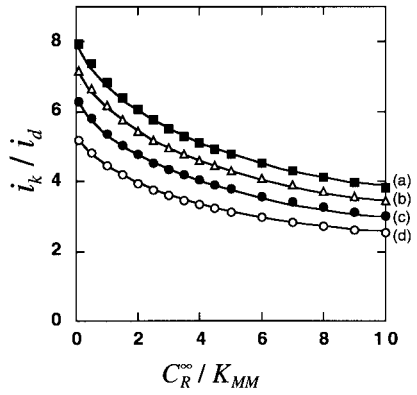

Figure 9. Correlation between i_k/i_d at 29 mV vs $E^{\circ\prime}$ and C_R^∞/K_{MM}. Cyclic voltammograms were calculated under reversible electrochemistry at v = 50 mV/s and $k_{cat}C_E/K_{MM}$ = (a) 25.3, (b) 20.3, (c) 15.2, and (d) 10.1 s^{-1}.

consumed and anomer equilibrium is not maintained at the electrode surface in the time scale of the cyclic voltammetry. If the β anomer ratio, β, is assumed to be 0.65, the bulk concentration of the β anomer is $0.65C_S^\infty$. The K_{MS} value for the β anomer, βK_{MS}, was determined by curve-fitting for the correlation between i_k/i_d and βC_S^∞, since there is no simple correlation between i_k/i_d and βC_S^∞ in the calculated results.

Cyclic voltammetry of the mediated enzyme reaction of GOx was carried out experimentally at various substrate concentrations. The resulting cyclic voltammograms showed that the i_k value increased with increases in glucose concentration. The i_k/i_d value at E_{pd} was evaluated to obtain the βK_{MS} value.

Figure 12 shows the correlation between i_k/i_d and βC_S^∞. The experimental plots demonstrate good agreement with the simulated results at βK_{MS} = 19.5 mM. Therefore, the overall K_{MS} value of GOx was determined to be 30 mM for using ferrocenedimethanol. The experimental and simulated cyclic voltammograms are compared in Figure 13. This figure also demonstrates good agreement between the simulated and experimental cyclic voltammograms after baseline compensation at several substrate concentrations.

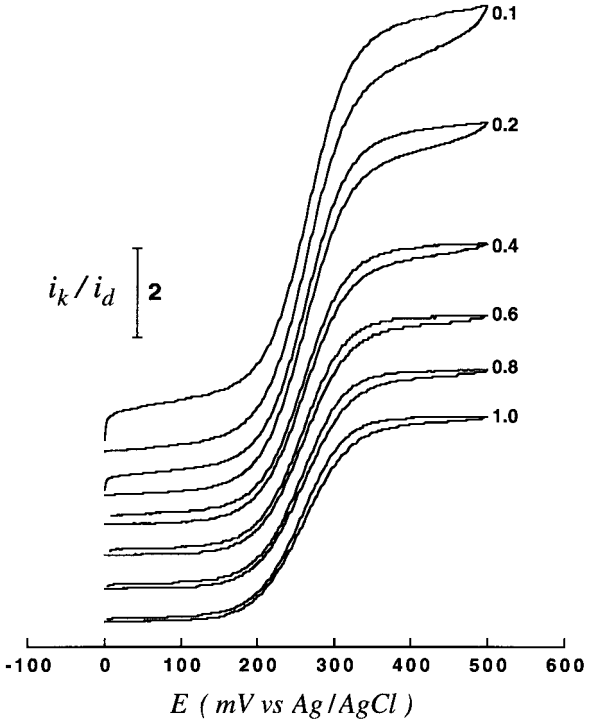

Figure 10. Cyclic voltammograms of a mediated enzyme reaction at various concentrations of 1,1′-ferrocenedimethanol. The current values were normalized by the i_d value. The numbers in this figure show C_R^∞ (mM). v = 50 mV/s, C_S^∞ = 100 mM, C_E = 6.50 μM.

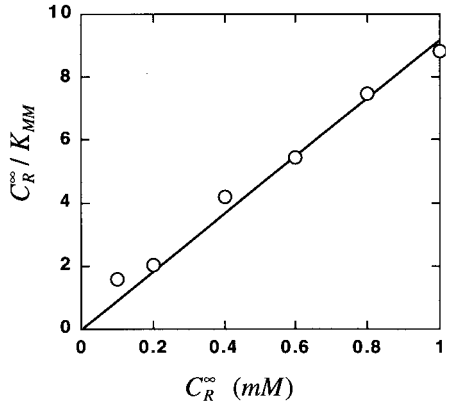

Figure 11. Correlation between C_R^∞/K_{MM} and C_R^∞. The C_R^∞/K_{MM} values were determined by using Figure 9 from the i_k/i_d values of Figure 10.

The overall K_{MS} values for ferrocenedimethanol, ferrocenemethanol, and FMTMA were determined at various enzyme and mediator concentrations, and these results are summarized in Table 2. This table demonstrates that the K_{MS} value is essentially independent of enzyme and mediator concentrations. This is likely because our work considered the concentration polarization of the substrate as well as the concentration of the mediator. If polarization of the glucose concentration in the vicinity of the electrode is neglected, the K_{MS} value that is determined is predicted to increase as the enzyme concentration increases. This has been reported elsewhere.[14]

The dependence of the k_{cat}, K_{MM}, and K_{MS} values on the mediator is discussed. If the reaction between a reduced form of GOx and two mediator molecules is the consecutive second-order chemical reactions, the reaction scheme is represented by

Table 1. K_{MM} and k_{cat} Values for Various Ferrocene Derivatives[a]

	ferrocene-dimethanol		ferrocene-methanol		FMTMA	
C_E (μM)	K_{MM} (μM)	k_{cat} (s^{-1})	K_{MM} (μM)	k_{cat} (s^{-1})	K_{MM} (μM)	k_{cat} (s^{-1})
3.25	109	339	50.5	409	1.47	221
4.88	113	352	52.3	424	1.59	240
6.50	111	347	49.8	403	1.54	233
8.13	110	344	48.7	395	1.58	238

[a] Glucose concentration, 100 mM; scan rate, 50 mV/s.

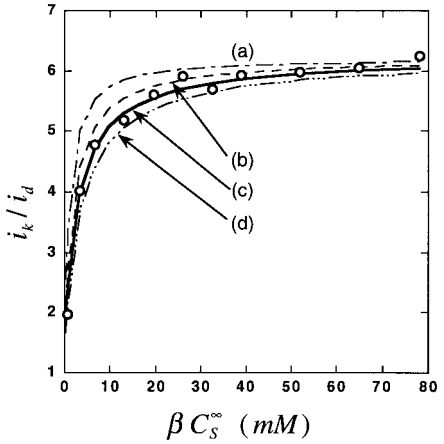

Figure 12. Correlation between i_k/i_d and C_S^{∞}. Calculation was carried out under the following conditions. K_{MS} values for the β anomer: (a) 6.5, (b) 13, (c) 19.5, and (d) 26 mM; $v = 50$ mV/s, $C_R^{\infty} = 0.1$ mM, $D_R = D_O = D_S = 5.14 \times 10^{-6}$ cm^2/s, $k_{cat} = 339$ s^{-1}, $C_E = 6.50$ μM, $K_{MM} = 109$ μM. The open circles show the experimental data. 1,1′-Ferrocenedimethanol was employed.

$$S + E_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \xrightarrow{k_2} E_{red} + P \quad (17)$$

$$O + E_{red} \xrightarrow{k_3} E_{red/ox} + R \quad (18)$$

$$O + E_{red/ox} \xrightarrow{k_4} E_{ox} + R \quad (19)$$

where E_{ox}, $E_{ox}S$, E_{red}, and $E_{red/ox}$ are the oxidized forms of GOx (GOx-FAD), the complex of GOx-FAD and substrate, the reduced form of GOx (GOx-$FADH_2$), and the semiquinoid form of GOx (GOx-$FADH^{\bullet}$), respectively. Then, k_{cat}, K_{MS}, and K_{MM} are given by

$$k_{cat} = k_2 \quad (20)$$

$$K_{MS} = \frac{k_{-1} + k_2}{k_1} \quad (21)$$

$$K_{MM} = \frac{k_2(k_3 + k_4)}{k_3 k_4} \quad (22)$$

Since neither eq 20 nor eq 21 includes a rate constant for the reaction between the enzyme and the mediator, k_3 or k_4, both k_{cat} and K_{MS} values could be independent of the mediator. The experimental results in Table 2 show that the K_{MS} values are almost independent of the mediator. Therefore, the reaction

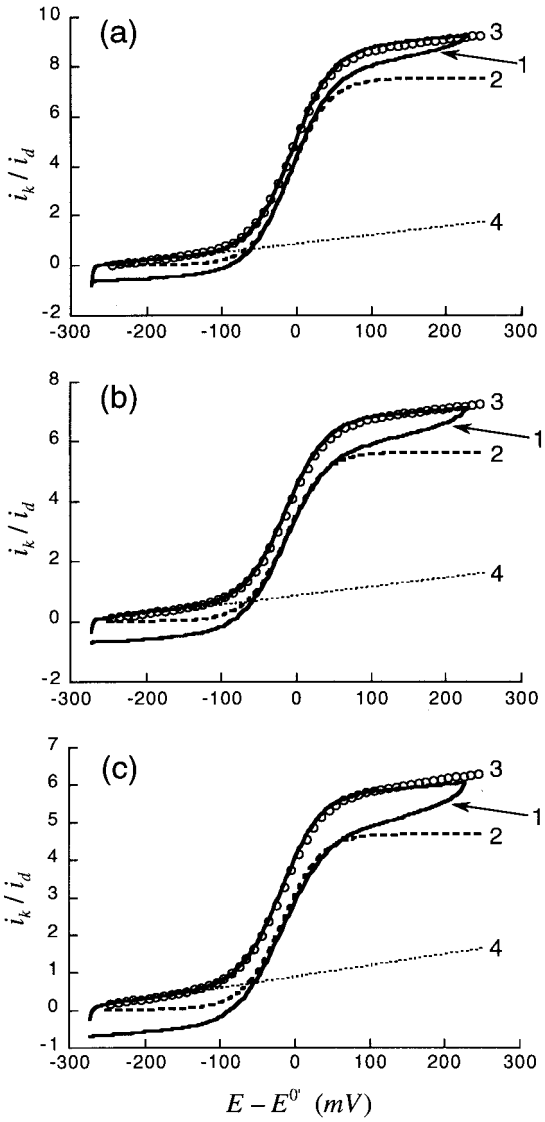

Figure 13. Comparison of simulated and experimental cyclic voltammograms of an enzyme reaction mediated by 1,1′-ferrocenedimethanol. (1) Experimental cyclic voltammogram, (2) simulated cyclic voltammogram, (3) simulated cyclic voltammogram after baseline compensation, and (4) baseline of experimental cyclic voltammogram. The current values were normalized by the i_d value. The simulation and the experiment were carried out under the following conditions: glucose concentration, (a) 100, (b) 10, and (c) 5 (mM), $v = 50$ mV/s, $C_R^{\infty} = 0.1$ mM, $D_R = D_O = D_S = 5.14 \times 10^{-6}$ cm^2/s, $K_{MM} = 109$ μM, $K_{MS} = 30$ mM, $k_{cat} = 339$ s^{-1}, $C_E = 6.50$ μM, $\beta = 0.65$.

between GOx and ferrocene derivatives may proceed through schemes 18 and 19. However, Table 1 clearly shows that the k_{cat} values depended on the mediator. In particular, the k_{cat} value derived from FMTMA is quite different from those derived from ferrocenedimethanol and ferrocenemethanol. The fact that FMTMA is positively charged may lead to association between FMTMA and the negatively charged GOx, and this could have some relevance.

Bourdillon et al. have investigated the kinetics of the electrochemically mediated enzyme reaction of GOx without considering the diffusion layer of the substrate.[23] They have treated the reaction of GOx with a ferrocene derivative as a simple second-order reaction:

Table 2. K_{MS} Values Determined at Various Mediator and Enzyme Concentrations[a]

		ferrocenedimethanol		ferrocenemethanol		FMTMA	
C_E (μM)	C_R^∞ (mM)	K_{MS}[b] (mM)	k_{cat}/K_{MS} (mM^{-1} s^{-1})	K_{MS}[c] (mM)	k_{cat}/K_{MS} (mM^{-1} s^{-1})	K_{MS}[d] (mM)	k_{cat}/K_{MS} (mM^{-1} s^{-1})
3.25	0.1	40	8.5	30	13	30	7.4
3.25	0.2	25	14	40	9.9	nd[e]	nd[e]
3.25	0.4	20	17	50	7.9	nd[e]	nd[e]
6.50	0.1	30	11	30	13	30	7.4
6.50	0.2	40	8.5	40	9.9	nd[e]	nd[e]
6.50	0.4	30	11	30	13	nd[e]	nd[e]
8.13	0.1	30	11	30	13	20	11
8.13	0.2	30	11	30	13	nd[e]	nd[e]
8.13	0.4	20	17	40	9.9	nd[e]	nd[e]

[a] Scan rate, 50 mV/s. [b] Calculated at $k_{cat} = 339$ s^{-1} and $K_{MM} = 109$ μM. [c] Calculated at $k_{cat} = 395$ s^{-1} and $K_{MM} = 48.7$ μM. [d] Calculated at $k_{cat} = 221$ s^{-1} and $K_{MM} = 1.47$ μM. [e] Not determined.

$$2\,O + E_{red} \xrightarrow{k_3} 2\,R + E_{ox} \qquad (27)$$

The k_2, k_3, and k_{red} values determined by their work were 780 s^{-1}, 6.0 μM^{-1} s^{-1}, and 12 mM^{-1} s^{-1}, respectively, for ferrocenemethanol at pH 7.0 and 25 °C, where k_{red} represents $k_1k_2/(k_{-1} + k_2)$ ($=k_{cat}/K_{MS}$). From these values, the k_{cat}, K_{MM}, and K_{MS} values can be calculated to be 780 s^{-1}, 130 μM, and 65 mM, respectively. These values are different from those obtained in our work, whereas the k_{cat}/K_{MS} values are very close to each other.

CONCLUSION

A cyclic voltammetric simulation that can be applied to the calculation at any substrate concentration as well as any mediator concentration was carried out. Concentration polarization of the substrate in the vicinity of an electrode was considered as well as mediator concentration. Reversible and quasi-reversible electrochemical reactions with one electron followed by an enzyme reaction with two electrons were modeled. The differential equations for mediator and substrate were solved using a digital simulation technique. The calculated cyclic voltammograms showed that prepeaks were observed when there was low substrate concentration, high mediator concentration, and high enzyme activity. The shape of the voltammogram changes dramatically as these values change.

Enzyme kinetic constants such as k_{cat}, K_{MM}, and K_{MS} were determined from the current values obtained by simulation and experimentation. The ratio of catalytic to diffusion-controlled current, i_k/i_d, was evaluated. The k_{cat}, K_{MM}, and K_{MS} values for GOx, 1,1′-ferrocenedimethanol, and glucose were 340 s^{-1}, 110 μM, and 30 mM, respectively.

We proved in this study that a cyclic voltammetric simulator is a powerful tool for determining enzyme kinetic constants as well as for simulating cyclic voltammograms. This technique can be applied to reactions between any redox enzymes and mediators and can contribute to the understanding of an enzyme reaction mechanism.

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(23) Bourdillon, C.; Demaille, C.; Moiroux, J.; Savéant, J.-M. *J. Am. Chem. Soc.* **1993**, *115*, 2−10.