A Kinetic Study on the Diffusion-coupled Reaction of a Basic Horseradish Peroxidase Adsorbed on the Carboxymethylcellulose Membrane*

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The rate of reactions of a basic horseradish peroxidase adsorbed on the carboxymethylcellulose membrane was measured spectrophotometrically by monitoring the state of the enzyme. For the reactions with hydrogen peroxide and cyanide dissolved in media the diffusion process was rate limiting both in the static and flowing media, and kinetic features of the enzyme were masked.

The rate of CO recombination of the ferrous enzyme was measured by flash photolysis in the static media containing varying amounts of CO. Apparently, the initial rate of CO recombination was no more affected by the diffusion factor, and the second order rate constant could be measured for the reaction of the enzyme adsorbed on the membrane. The value was about 70% of that obtained from the enzyme in solution and exhibited the same pH dependence as did the value for the enzyme in solution. The difference of the reaction pattern between the enzymes on the membrane and in solution became distinct when flash photolysis was applied to the reaction system without free CO in the medium.

The kinetic data were analyzed by computer simulation.

In biology most enzymes function in the heterogeneous milieu, where the diffusion of substances might be a fundamental problem. The kinetics of enzyme action, however, has been treated principally in homogeneous systems. Recently much attention has been paid to the subject of immobilized enzymes mostly for practical purposes. With the aid of immobilized enzyme systems it may be possible to evaluate the diffusion limitations governing the activities of enzymes in the intracellular milieu.

Theoretical works on diffusion-controlled enzyme reactions have been reported (1-3), but the direct experimental support is lacking. Reactions of immobilized enzymes have been studied kinetically by monitoring the product formation in stationary states (4-11). It can be said that more direct kinetic information is obtained by monitoring spectrophotometrically the state of immobilized enzymes. Peroxidase is fit for the purpose, and in this paper we describe the diffusional behavior of substrate and ligand molecules in the reactions of a basic isoenzyme of horseradish peroxidase tightly adsorbed on the carboxymethyl cellulose membrane.

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MATERIALS AND METHODS

A basic isoenzyme of horseradish peroxidase was purified from the precipitate of the horseradish root extract at 50% saturation with ammonium sulfate by CO-50 column chromatography and by crystallization from ammonium sulfate solutions. The RZ value (ε200/cm/ε200 cm2/mol) of the enzyme used in this experiment was about 3.0. This enzyme was usually isolated from plant tissues as the form of cyanide complex, and the cyanide-free enzyme was prepared by the treatment with mercuric chloride.

Carboxymethylated cellophane membranes (1 x 2 cm) were prepared from cellulose tubing (type 8/32, Visking Company) by a slight modification of the method of Peterson and Sober (13) and stored in 5 mM sodium acetate, pH 5.0. The basic peroxidase of horseradish was tightly adsorbed on the carboxymethyl cellophane membrane in 10 mM sodium acetate, pH 5.5. The amount of adsorbed enzyme could be controlled by the time of immersion and the enzyme concentration in the adsorption solution. The membrane adsorbing the enzyme was then washed and immersed in appropriate buffer solutions to be used for reactions. The immersion was conducted for 30 min at room temperature. No desorption of the enzyme from the membrane was observed in buffer solutions used in the present experiments.

For CO recombination experiments in CO-free solutions, the enzyme was adsorbed on the membrane in the form of CO complex from the enzyme solution, which was freed from unbound CO by Sephadex G-25 filtration in dark at 0°C. The fixation of the membrane to the frame was performed under dim light.

Spectra of the peroxidase membrane in static media were measured with a quartz cell (1 x 1 x 4 cm), where the frame fixing the membrane was inclined at an angle of 45° with the axis of measuring light. The reaction was started by immersion of the membrane.

A block diagram for flash photolysis measurements is shown in Fig. 1A. The Xe flash had a pulse width of 10 μs, and the power was supplied by a Union Giken RA 410 instrument. By this flash light most of the CO complex of ferrous peroxidases in solution was photodissociated, but about 50% of that on the membrane remained undissociated. Two monochrometers were equipped to avoid the photodissociation by measuring light and to reduce the effect of flash light on the detector. The change in absorbance occurring after photodissociation was recorded in a digital memory. The concentration of CO in the bulk solution was controlled by bubbling a CO/N2 gas mixture into the solution for 10 min before flash photolysis.

The measurement of the peroxidase membrane in flowing media was performed in an apparatus shown in Fig. 1B. The membrane was fixed on a quartz plate inside the flow cell (Fig. 1C). Measuring light passing through the membrane was led to a photomultiplier via a light guide with a diameter of 8 mm. In order to maintain constant flow velocity during the measurement, an air chamber (15 liters) was placed between the cavity and the aspirator. The flow velocity was measured with a Nikon Koden multichannel square wave electromagnetic flow meter, RPM-6004. The changes in absorbance and flow rate were both recorded on a two-pen recorder, directly or via a digital memory. The memory was started by a trigger signal from the flow meter.

The smoothness of the membrane surface was measured with a Hitachi scanning electron microscope, HSM-2B. Gold was evaporated onto the membrane surface in thickness of 5 nm after the membrane was treated with ethanol and dried. The surface was found to be smooth within the resolution of 1 μm.

All reactions were carried out at 20°C unless otherwise noted.
Reactions of Peroxidase Membrane

Fig. 1. Schematic illustration of systems designed to measure the rate of reactions of peroxidase membrane. A, block diagram for flash photolysis measurements; B, block diagram for flow experiments; and C, flow apparatus with the membrane sticking to the inside surface of one quartz plate. The numerals in the figure denote lengths in millimeters. The inlet tube in C was inclined at a small angle with the membrane so as to reduce the unstirred layer.

Fig. 2. Spectra of the peroxidase and its derivatives adsorbed on the carboxymethylcellulose membrane. The membrane was suspended in 20 mM potassium phosphate, pH 6.0. Enzyme forms are ferric (---), ferrous (-----), ferrous-CO complex (— — —), ferric-cyanide complex (— — —), and Compound I, a product of reaction of the ferric enzyme with H$_2$O$_2$ (— — —). The ordinate denotes absorbance normalized by setting that of the ferric enzyme at 403 nm = 100.

RESULTS

Absorption Spectra—Absorption spectra of various forms of basic horseradish peroxidase adsorbed on the membrane are shown in Fig. 2. Absorbance was plotted by normalizing the absorbance of the ferric enzyme as 100 at 403 nm. No significant difference was seen in the spectra between the enzymes on the membrane and in solution. Since the absorption spectra of peroxidases are very sensitive to the heme environment, the result may indicate that the enzyme was kept intact on the membrane. It was found that the Soret band of the ferric enzyme became lower and broader irreversibly when the membrane was suspended in a salt-free water. This denaturation could be avoided in the presence of more than 1 mM of acetate, phosphate, or glycyI-glycine buffer.

Reactions with H$_2$O$_2$ and Cyanide in Static Milieus (Case

Fig. 3. The reactions of hydrogen peroxide and cyanide with peroxidase adsorbed on the membrane suspended in the static medium of 20 mM sodium acetate, pH 6.0. A, effect of the amount of adsorbed enzyme on the rate of Compound I formation. The initial absorbance of the membranes was 0.23 (a), 0.40 (b), 0.56 (c), and 0.73 (d) at 403 nm. The medium contained 150 µM H$_2$O$_2$. ----- reaction of 7 µM enzyme in solution. B, shows the semilogarithmic plots. C, effect of H$_2$O$_2$ concentration in the medium on the rate of Compound I formation. The initial absorbance of the membrane was 0.40 at 403 nm. The concentration of H$_2$O$_2$ was 30 (a), 60 (b), 90 (c), 120 (d), and 150 µM (e). D, effect of cyanide concentration on the rate of formation of the cyanide complex. The initial absorbance of the membrane was 0.40 at 403 nm. The concentration of cyanide was 30 (a), 60 (b), and 150 µM (c).
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**Fig. 4.** Effect of viscosity of the medium on the rate of Compound I formation. The initial absorbance of the membrane was about 0.60 at 403 nm. The medium contained 150 µM H₂O₂, varying amounts of glycerol, and 20 mM sodium acetate, pH 6.0. The weight per cent of glycerol was 0 (a, 1.01), 10 (b, 1.30), 20 (c, 1.77), 30 (d, 2.48), 40 (e, 3.74), and 50 (f, 5.94). The numerals in parentheses denote the coefficient of viscosity. A, reaction curves; B, semilogarithmic plots; and C, the slope of the middle portion of the curves in B was plotted against reciprocal of the viscosity of medium.

**Fig. 5.** Reactions of hydrogen peroxide with peroxidase on the membrane exposed to the flowing medium of 20 mM sodium acetate, pH 6.0. A, reactions at varying concentrations of H₂O₂ in the medium, 50 (a), 100 (b), 200 (c), and 300 µM (d). The flow rate was 80 cm/s. The inset shows a plot of ΔA occurring during the fast phase against the concentration of H₂O₂. B, effect of repetition of the flow and stop of the medium. The H₂O₂ concentration in the medium was 50 µM. C, reactions at varying flow rates, 50 (a), 123 (b), and 223 (c) cm/s. The H₂O₂ concentration in the medium was 100 µM. Absorbance of the membrane at 403 nm was 0.6 for A and C and 0.4 for B.

**Fig. 6.** The reactions of hydrogen peroxide and cyanide with peroxidase on the membrane exposed to the flowing medium. Absorbance of the membrane was 0.6 at 403 nm. The medium contained 150 µM H₂O₂ (a) or 150 µM potassium cyanide (b), and 20 mM potassium phosphate pH 6.0. The flow velocity was 50 cm/s. Even in the presence of excess H₂O₂ in the bulk solution (Fig. 3B), the initial rate depended on the concentration of H₂O₂ in the bulk solution (Fig. 3C). Similar results were obtained in the reaction with cyanide (Fig. 3D). When the concentrations of H₂O₂ and cyanide were the same the rates of the two reactions were about the same, though in solution the rate constant for H₂O₂ was about 100 times higher than that for cyanide. These results indicate that a diffusion process was rate limiting in the reaction of the enzyme adsorbed on the membrane with ligands present in the bulk solution. The effect of viscosity of the bulk solution on the Compound I formation is shown in Fig. 4. The viscosity was changed by the addition of glycerol and measured with a Ubbelohde's viscometer. The rate of Compound I formation decreased with the increase in viscosity (Fig. 4A). The slope at the middle portion (near the inflection point of the semilogarithmic plots (Fig. 4B) was found to be proportional to the reciprocal of the viscosity (Fig. 4C). Computer analysis of a simple model having a diffusion layer at the surface of membrane showed that the slope was proportional to the diffusion constant.

**Reactions in Flowing Milieus (Case 2)—In order to gain some insight into properties of the initial reaction and of the diffusion layer, the solution containing H₂O₂ was flowed along
the membrane surface. The apparatus used is shown in Fig. 1 (B and C). It was possible to observe the initial rapid reaction followed by the slow stationary reaction. The transition between the two phases occurred about 0.5 s after the flow was started, and ΔA occurring during the initial phase was approximately proportional to the concentration of H₂O₂ in the flowing solution (Fig. 5A). The acceleration in the initial phase might be ascribable to the turbulent flow transiently appearing at the proximity of the membrane surface. A laminar flow might then be attained, forming a steady unstirred layer over the membrane surface. The formation of Compound I slowed down immediately after the flow was stopped. By the repetition of pulsed flow it was shown that the effect of the flow was distinct in the earlier stage of reaction (Fig. 5B). As the flow rate was increased the rate of reaction in the second phase increased (Fig. 5C), but it reached a maximum at a velocity of about 200 cm s⁻¹.

Fig. 6 shows comparison between the reactions of H₂O₂ and cyanide under the same flow conditions. The initial rate of the two reactions was nearly the same. The fact that the rate of Compound I formation slightly decreased as the reaction proceeded might be explained in terms of a small increase in the thickness of diffusion layer, although the unstirred layer remained unchanged at a constant flow rate. The decrease in the rate was more distinct in the reaction of cyanide. The comparison led us to conclude that the chemical reaction began to affect the rate of formation of cyanide complex.

**CO Recombination after Flash Photolysis (Case 3)**—It was found that the kinetic study for CO recombination after photodissociation was well suited for the comparison of reactivity between the enzymes in solution and on the membrane. Fig. 7A shows kinetic traces for CO recombination at pH values of 5.0 and 8.0 of the ferrous enzyme adsorbed on the membrane. The difference spectrum obtained by flash photolysis of the CO complex of the ferrous enzyme on the membrane was the same as that obtained with the enzyme in solution (Fig. 7B). In the presence of 1 mM CO, CO recombination of the enzyme in solution obeyed first order kinetics. For the enzyme adsorbed on the membrane suspended in 1 mM CO solution, the reaction was approximately of first order (the insets of Fig. 7A).

The semilogarithmic plot became nonlinear as the concentration of CO in the bulk solution was decreased, particularly at pH 8.0 (Fig. 8, A and B). Therefore, the slopes in the initial and the stationary phases were respectively plotted versus the concentration of CO in the bulk solution (Fig. 8, C and D). The results show that the initial slope increased linearly with the concentration of CO in the bulk solution. By extrapolation of the linear line in Fig. 8C it was found that flash photolysis increased the effective concentration of CO near the membrane by about 100 μM. From the initial rate of CO recombination and the effective concentration of CO, the second order rate constant for the reaction of the enzyme could be tentatively calculated. The rate constant thus obtained is plotted versus pH in Fig. 9, in comparison with data obtained with the enzyme in solution. The close similarity between the two data indicates that the kinetic property of the enzyme remained essentially unchanged after adsorption on the membrane and also that the diffusion was no more rate limiting under such conditions. Fig. 9 also shows that the rate constant for reaction of CO with ferrous horseradish peroxidase is much different between basic and neutral isoenzymes. The rate constant for the basic enzyme varied with pH and was much higher than that for the neutral one. The latter one was reported to be about 2 × 10⁴ M⁻¹ s⁻¹, almost independent of pH (Fig. 9 and Ref. 14).

Fig. 10 shows the effects of temperature and buffer concentration on the apparent rate constant for CO recombination of the enzymes both in solution and on the membrane. The activation energy was calculated to be about 6.0 kcal for all the reactions shown in the figure. It also shows that the rate decreased with the decrease of the buffer concentration in the bulk solution. It should be noted here that the enzyme adsorbed on the membrane became gradually denatured when it was suspended in salt-free water.

The difference in the reactions between the enzymes in
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The enzyme was free in the medium for sodium acetate for pH 8.0; the medium was saturated with potassium phosphate, pH 8.0, and no CO before flash photolysis. Transmittance changes during CO recombination of peroxidase on the membrane (A) and in solution (B). The other conditions were as described in Fig. 7. The enzyme was free in the medium (C) or adsorbed on the membrane (D). The rate constant for horseradish peroxidase C in solution is shown (Δ).

The medium contained 20 mM potassium phosphate, pH 8.0, and no CO before flash photolysis. Transmittance changes during CO recombination of peroxidase on the membrane (A) and in solution (B). The other conditions were as described in Fig. 7. The enzyme was free in the medium (C) or adsorbed on the membrane (D). The rate constant for horseradish peroxidase C in solution is shown (Δ).

The adsorbed peroxidase amounts to about 2 nmol/cm² of the membrane in the experiment of Fig. 2, for instance. If one assumes that the Stokes radius of the peroxidase (M₀ = 30,000) is 3.0 nm and that the enzyme is closely packed on the flat membrane surface, the number of the enzyme layers will be 20 to 50 on each side of the membrane. The binding force between the peroxidase and the carboxymethylcellulose appears to be mostly electrostatic. It is, therefore, difficult to explain the tight binding of excess enzyme on the flat membrane. Although the membrane surface was found to be smooth within the resolution of 1 μm, it is still possible that the membrane surface has fine structures, being covered by the single layer of the enzyme.

The kinetic characteristics of the present enzyme reactions are similar to those of reaction of O₂ with hemoglobin in red blood cells. The reaction of hemoglobin involving diffusion processes has been investigated by many workers (15-18). It is shown that the O₂ uptake by erythrocytes is slowed down by the presence of unstirred layers of solvent adjacent to the red cell surface (17, 18). The thickness of the layer is estimated at 1.0 to 5.0 μm when the stopped flow experiments are carried out. The present reaction systems can be grouped into three cases, as shown in Fig. 12, where the concentration of substrate or ligand is plotted against the distance perpendicular to the membrane surface at times indicated. In static milieus (Case 1), the initial rate of reactions is independent of the amount of peroxidase adsorbed on the membrane (Fig. 3A) and of the kind of chemical reaction (Fig. 3, C and D), but it is dependent on the viscosity of the bulk solution. The result can be explained by assuming that every molecule reaching the membrane surface is trapped by the enzyme. As the reaction proceeds, the diffusion layer increases in thickness and the reaction rate decreases. The reaction profile can be simulated on the basis of Fick's law for the diffusion of substrate or ligand, the results being shown in the appendix. ¹ In Case 1, the thickness of diffusion layer reaches 200 to 500 μm, and the kinetic feature of the enzyme itself is not seen in the results.

When the bulk solution flows (Case 2), the reaction rate

¹ Portions of this paper (including an appendix and Figs. 1 to 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document 80M-779, cite authors, and include a check or money order for $1.00 per set of photocopies. Full sized photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
**Reactions of Peroxidase Membrane**

during the laminar flow is increased as much as 2- to 5-fold, as compared with the rate of Case 1 reaction. In Case 2, the diffusion layer approaches the unstrained layer, the thickness of which is about 20 to 50 μm, depending on the flow velocity (Fig. 12B). Fig. 6 suggests that, in Case 2, the kinetic feature of enzyme reactions is still masked by the diffusion process of substrate when the second order rate constant is $10^2 \text{m}^{-1}\text{s}^{-1}$, but does appear when the rate constant decreases to about $10^5 \text{m}^{-1}\text{s}^{-1}$.

The spectrophotometry is a powerful method to analyze rapid reactions, but the diffusion process is mainly measured in Case 1 and Case 2 reactions. The flash photolysis method has great advantages to initiate a reaction in heterogeneous systems, although the kind of reactions applicable is limited. The conclusion obtained from data of CO recombination is as follows. First, the second order rate constant can be measured in the reaction of membrane-bound peroxidase, and its value is found to be about 70% of that obtained from the reaction in solution. The pH dependence of the rate constant (Fig. 9) indicates that the pH of reaction milieu of the adsorbed peroxidase is the same as that of bulk solution and that the adsorbed enzyme behaves nearly as it is free in solution. Goldstein et al. (19) reported displacement of the pH activity profile of an immobilized enzyme at low ionic strength, as compared to the enzyme in solution. Changes in the pH activity profile have also been reported in other reactions of immobilized enzymes (6, 8). The small effect of the buffer concentration (Fig. 10) can be interpreted in terms of the increase in mobility of peroxidase molecules at higher ionic strength of the bulk solution. Second, the increase in the effective concentration of CO after photolysis enables us to estimate the average thickness of CO dispersion layer. The increase of 100 μm in the effective concentration means that about 1 nmol of CO released has diffused into the medium of thickness of about 100 μm. However, approximate calculation described in the appendix gives a value of 10 μm for the dispersion layer (Fig. 12C). The contradiction remains to be solved.

Although the heterogeneous model system discussed here is far different from that in vivo, the present kinetic study will serve to analyze diffusion-coupled reactions occurring in biology. Particularly, the flash photolysis method can be applied for the analysis of rapid reactions in tissues, which will be reported elsewhere.

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Reactions of Peroxidase Membrane

Appendix
to
A kinetic Study on the Diffusion-Coupled Reaction of a Basic Horseradish Peroxidase Adsorbed on the Carboxyphosphoryl Cellulose Membrane

by
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Method of calculation. In order to analyze the experimental results, a one-dimensional model was assumed and the following second order differential equation was calculated numerically with a computer.

$$ \frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} - v \frac{\partial c}{\partial x} - k \cdot c \cdot g(t) $$

where \( c \) refers to substrate or ligand concentration; \( t \), time after reaction starts; \( D \), diffusion constant; and \( v \), distance from the enzyme locus. The term \( g(t) \) denotes the rate of chemical reaction. In order to calculate the equation, one-dimensional space is divided into a number of unit cells. The length of unit cells corresponds to a small deviation, \( dx \). At the end of a series of unit cells enzyme is filled. The unit cell with enzyme is numbered \( 0 \) and from this cell a series of cells are numbered \( 1, 2, 3 \ldots \) and so on, in order. In this way \( 200 \) unit cells are assumed.

$$ \frac{d^2 c}{dx^2} \bigg|_{x=0} = \frac{c(2, t) - 2c(1, t) + c(0, t)}{dx^2} $$

Substrate is distributed in the cells with number from \( 1 \) to \( 200 \) and cell \( 200 \) is connected with the reservoir where the substrate concentration is kept constant. It is assumed that enzyme in cell \( 0 \) reacts only with substrate in cell \( 1 \) according to mass action law within second order rate constant \( k \). Substrate in each cell from \( 1 \) to \( 200 \) is diffused to the nearest cells and the overall flux of substrate occurs according to diffusion equation.

The amount of enzyme in cell \( 0 \) and the concentration of substrate in each cell are determined as initial conditions. At time \( t \), the amount of free substrate and the concentration of substrate in the \( j \)-th cell is denoted by \( c(j, t) \) and \( c(j, 0) \), respectively. The changes in their concentration occurred from \( t \) to \( t+dt \) are expressed as follows:

$$ a(t+dt) - a(t) = -k \cdot a(t) \cdot c(j, t) \cdot dt $$

where \( a = k \cdot (c(j-1, t) - 2c(j, t) + c(j+1, t)) \) and \( a = k \cdot (c(j-1, t) - 2c(j, t) + c(j+1, t)) \). The calculation was performed with a FACOM 230-75 computer and the results were displayed with an X-Y plotter.

Case 1 reaction. By setting \( a(t) = a(t) \) at a given value and \( c(j, t) = c(j) \) at appropriate parameters \( a \), \( b \), and \( c \), the calculation yields a value of \( a(t) \) and \( c(j) \). The data in Fig. 1A were calculated result. Rectangular plus sign (+) denotes the calculated results. Rectangular minus sign (−) denotes the experimental results.

Method of calculation. In order to understand the space-time correlation, the temporal change in the distribution of substrate is plotted in a three-dimensional graph of concentration, time, and distance. The characteristic features of Case 1 reactions can be summarized as follows. (1) Substrate near the enzyme locus takes \( 200 \) immediately after the reaction starts and there appears a sharp gradient of substrate concentration near the enzyme locus. Then, substrate is supplied to the enzyme locus along the concentration gradient. (2) At one end of the reaction process the diffusing layer increases in thickness and the apparent rate of reaction decreases. In Case 2 reactions the amount of free enzyme decreases and the substrate concentration near the enzyme locus increases again.

Case 2 reactions. The above principle can be applied to the simulation for the distribution of substrate at flash photolysis. Fig. 6A shows an experimental result when CO is absent in the black solution. In this case, the reaction starts immediately after the flash photolysis is induced. A special pulse of CO is successively added at \( t = 0 \), as shown in the three-dimensional graph (Fig. 6B). It clearly shows that a part of CO present near the enzyme locus combines with the enzyme while the other disperses into the bulk solution.

Fig. 6. A, time course for the decrease of free enzyme (a) and three-dimensional display of concentration (c)-time-distance for reaction in Fig. 5. b, The ordinates denote a(t) and c(j, t).

Fig. 5. A, three-dimensional display of the result shown in Fig. 4b. Conditions were the same as described in Fig. 4a.