Kinetic Basis for Artificially Designing Enzymes

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Kinetic Basis for Artificially Designing Enzymes

（人工酵素設計のための速度論的基礎）

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Contents

Introduction

Enzymes are naturally designed catalysts with high efficiency and specificity. To know how to design such enzymes is a major goal for enzymologists, and they have tried to elucidate the structural basis of enzyme catalysis. The information on structure-function relationship has made it possible to design or redesign enzymes by protein engineering and also to design artificial enzymes (Wiseman, 1984; Breslow, 1987; Pluckthun et al., 1987), although such artificially designed or redesigned enzymes are still primitive.

MDH-PEG-NAD^+ (Eguchi et al., 1986) and GlcDH-PEG-NAD^+ (Nakamura et al., 1987) were prepared by covalently linking NAD^+ to malate and glucose dehydrogenases, respectively, via a long spacer of poly(ethylene glycol) (M_r 3000). These two enzyme-NAD^+ conjugates show quite different effects of the covalent linking of NAD^+ on the rate of the reaction between the enzyme and the NAD^+ moieties: the rate is much enhanced for GlcDH-PEG-NAD^+, but not for MDH-PEG-NAD^+. Recently, I have prepared EP^+-PEG-NAD^+ by covalently linking NAD^+ to 5-ethylphenazine via the poly(ethylene glycol) spacer(Yomo et al., 1989b); this conjugate behaves as an enzyme-like catalyst having an intramolecular reaction step in its catalytic cycle, and provides us with valuable kinetic information on enzymatic catalysis.

These findings on the kinetic properties of these conjugates have shown us the importance of kinetic information for designing enzymes and enzyme-like catalysts. Thus, I have prepared several types of enzyme-cofactor conjugates by covalently linking 5-ethylphenazine (and also NAD^+) to various dehydrogenases. These conjugates show new catalytic activities, and the ethylphenazine moiety works as an artificial catalytic group for the oxidation of NADH (or the NADH moiety) with

oxygen or MTT (Yomo et al., 1989a,b; see Fig. i) in the new catalytic reactions; thus, I have converted dehydrogenases into new and unique semisynthetic enzymes by these covalent modifications. The investigation of the kinetic properties of these semisynthetic enzymes provides us with a kinetic basis for artificially designing enzymes. The results on the semisynthetic enzymes are reported in this thesis in the following order.

This first chapter describes EP^+-PEG-GltDH, an NADH oxidase; the coenzyme-binding site of the glutamate-dehydrogenase moiety is used as a substrate-binding site of this semisynthetic enzyme. Kinetic analysis of the NADH oxidase activity of EP^+-PEG-GltDH quantitatively shows the effect of the presence of the substrate-binding site near the catalytic group, the ethylphenazine moiety. The second chapter describes about EP^+-GlcDH-NAD^+, a glucose oxidase. The glucose oxidase activity of this semisynthetic enzyme is due to the coupling of the two catalytic reactions of the glucose dehydrogenase moiety and the ethylphenazine moiety, and the kinetic analysis of this activity have shown the following three rate-acceleration mechanisms in enzymatic catalysis: high effective concentration, intramolecular coupling of successive catalytic reactions, and multiple connection between the two kinds of the catalytic sites. The third chapter describes EP^+-LDH-NAD^+, a lactate oxidase. The kinetic analysis of the lactate oxidase activity shows the effects of hiding the NADH moiety by the coenzyme-binding site of the lactate dehydrogenase moiety. On the basis of the results obtained from the kinetic analysis of these semisynthetic enzymes, the fourth chapter describes the kinetic principle for designing enzyme-like catalysts.

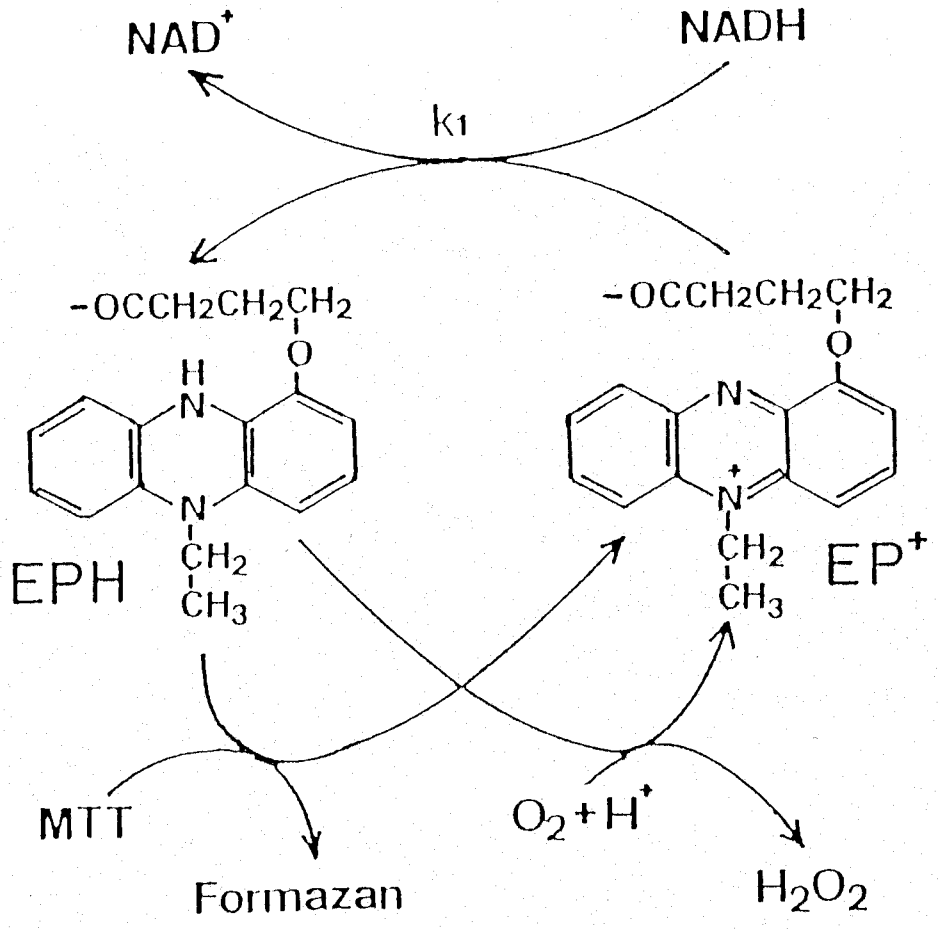


Fig. i. Schematic representation of the catalytic cycles of NADH oxidation by a 5-ethylphenazine moiety.

Chapter I

Presence of Substrate-Binding Site

Introductory remarks

In catalytic reactions, their substrates contact with catalytic groups and are converted into the products. Thus, the efficiency of catalysts is greatly due to how frequently their catalytic groups make the collisions with the substrates in the proper manner.

Enzymes increase the probability of such a collision by binding their substrates to enhance the rate of the reaction. A molecule of the substrate scattered in the solution is fixed into its binding site near the catalytic group, and held to the binding site until it is scattered again. This holding keep the substrate longer in the vicinity of the catalytic group. The trap let the catalytic group have more opportunity to collide with the substrate. This major characteristic of enzymes has been studied analyzing the natural enzymes. This chapter describes EP^+-PEG-GltDH, an semisynthetic NADH oxidase, where the substrate binding is reproduced; the coenzyme binding site of the glutamate dehydrogenase moiety and the ethylphenazine moiety function as a substrate binding site of this semisynthetic enzyme and a catalytic group, respectively. Kinetic analysis of the NADH oxidase activity of EP^+-PEG-GltDH quantitatively shows the effect of the presence of the substrate binding site near the catalytic group.

Experimental Procedures

Materials

Glutamate dehydrogenase (EC 1.4.1.3) from beef liver, lactate

dehydrogenase (EC 1.1.1.27) from rabbit muscle, and NADH were purchased from Oriental Yeast Co. Ltd. (Tokyo); 5-ethylphenazine ethylsulfate was from Nakarai Chemicals (Kyoto); MTT was from Dojindo Laboratories (Kumamoto). NAD^+ was a generous gift from Kohjin Co. Ltd. (Tokyo). 1-(3-Carboxypropyloxy)-5-ethylphenazine (Yomo et al., 1989a), EP^+-PEG (Yomo et al., 1989b) and DHBT (Eguchi et al., 1986) were prepared as described previously.

Concentration of Protein, Nucleotides, and 5-Ethylphenazine Derivatives

The concentrations of glutamate and lactate dehydrogenases were measured spectrophotometrically at 280 nm using molar absorption coefficients for the subunits of 54400 $M^{-1}cm^{-1}$ (Eisenberg & Tomkins, 1968; M_r 55900) and 40500 $M^{-1}cm^{-1}$ (Fromm, 1963), respectively. The concentrations of NAD^+, NADH, and 5-ethylphenazine derivatives were measured using the following molar absorption coefficients: NAD^+, 18000 $M^{-1}cm^{-1}$ at 260 nm; NADH, 6300 $M^{-1}cm^{-1}$ at 340 nm; 5-ethylphenazine, 26300 $M^{-1}cm^{-1}$ at 386 nm (Zaugg, 1964); 1-(3-carboxypropyloxy)-5-ethylphenazine and EP^+-PEG, 17000 $M^{-1}cm^{-1}$ at 386 nm (Yomo et al., 1989b). The concentrations of the ethylphenazine moiety and the enzyme moiety (as a subunit) in EP^+-PEG-GltDH were calculated from the ultraviolet absorption spectrum of the conjugate using the following molar absorption coefficients: 17000 $M^{-1}cm^{-1}$ at 386 nm and 43000 $M^{-1}cm^{-1}$ at 280 nm for the ethylphenazine moiety; 0 $M^{-1}cm^{-1}$ at 386 nm and 40500 $M^{-1}cm^{-1}$ at 280 nm for the enzyme moiety.

Preparation of EP^+-PEG-GltDH

EP^+-PEG-GltDH was prepared by the method for the preparation of GlcDH-PEG-NAD^+ (Nakamura et al., 1986). EP^+-PEG (1 μmol) and DHBT (86 μmol) were dissolved in CH_2Cl_2 (1 ml), and the mixture was kept at 4°C

for 2 h. Then, 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.5, was added to the mixture, and CH_2Cl_2 was removed by evaporation. One ml of 0.13 mM glutamate dehydrogenase solution in the same buffer was added to the residue, the reaction mixture was kept at 15°C for 18 h, and the mixture was diluted to 107 ml with water to reduce the conductivity to 0.8 mS. The solution was applied to a DEAE-Sephadex CL-6B column (2 x 25 cm) equilibrated with 1 mM sodium phosphate buffer, pH 7.5. The column was washed with this buffer (300 ml), and EP^+-PEG and its derivatives that had not reacted with the enzyme were eluted out; then, EP^+-PEG-GltDH was eluted with an NaCl gradient of 0 - 1.0 M (total 400 ml). The main fractions (No. 29 - 33, 4 ml each; Fig. I-3) having the two activities due to glutamate dehydrogenase and the ethylphenazine moiety were combined and used as EP^+-PEG-GltDH. There was no other peak with the dehydrogenase activity.

Assay of Reaction Rates

Initial rates of reactions were measured at 30°C in 0.1 M sodium phosphate buffer, pH 7.5, with a Hitachi 220 spectrophotometer with a thermostated cell compartment. Glutamate dehydrogenase activity was measured in an assay mixture (3.2 ml) containing 50 mM L-glutamate, 0.93 mM NAD^+, 1.0 mM 5-ethylphenazine, 1.5 mM MTT, and 0.1 M sodium phosphate (pH 7.5). The activity of the ethylphenazine moiety was measured in an assay mixture (3.15 ml) containing 0.76 mM NADH, 1.5 mM MTT, and 0.1 M sodium phosphate (pH 7.5). For the other assays, the components of the reaction mixtures are given in the legends to Figs. I-2 and I-3. The reactions were recorded as the increase in absorbance at 570 nm (formazan concentration), and the reaction rates were calculated from the initial linear parts. The assays were made at least in duplicate

with a reproducibility of less than 5% error. The concentration of the formazan produced from the reduction of MTT was calculated using a molar absorption coefficient of 13000 $M^{-1}cm^{-1}$ at 570 nm (Eguchi et al., 1985).

Results

Reactivity of NADH Bound in a Substrate Binding Site

Enzymes have substrate-binding sites and catalytic groups in their active sites. To investigate the kinetic effects of the presence of a substrate-binding site near a catalytic group, I planned to make a semisynthetic NADH oxidase by covalently linking EP^+-PEG to a dehydrogenase. In this oxidase, the ethylphenazine moiety and the dehydrogenase moiety are expected to work as a catalytic group and a substrate-binding site, respectively, and the catalytic cycle is expressed as shown in Fig. I-1, where MTT, instead of oxygen, is used as a terminal electron acceptor. To select an appropriate dehydrogenase for the NADH-binding site of the semisynthetic oxidase, it is important to know the reactivity of NADH bound in the coenzyme site of the dehydrogenase toward the ethylphenazine moiety. To know this reactivity is also essential for analyzing the kinetic data of the NADH oxidase.

The reactivity of NADH bound in the site of a dehydrogenase was estimated by measuring the reaction rate of NADH with 5-ethylphenazine derivatives in the presence of various concentrations of the dehydrogenase and an excess amount of MTT (see the legend to Fig. I-2). In this system, almost all the ethylphenazine moiety is in the oxidized form (Yomo et al., 1989a), and the reaction rate (v) at the initial steady state is expressed as:

$$v = k_{1f}[EP^+]_0[NADH] + k_{1b}[EP^+]_0[\text{site NADH}] \qquad (I\text{-}1)$$

where $[EP^+]_0$ is the initial concentration of the ethylphenazine moiety,

[NADH] is the concentration of free NADH, [site NADH] is the concentration of NADH bound in the site (or that of the site having NADH), and k_{1f} and k_{1b} are the second-order rate constants of the reactions of the ethylphenazine moiety with free and bound NADH, respectively.

The material balances for the reaction system at the initial steady state, and the dissociation constant (K_d) of the site NADH complex are expressed as:

$$[NADH]_0 = [NADH] + [site\ NADH] \tag{I-2}$$

$$[site]_0 = [site] + [site\ NADH] \tag{I-3}$$

$$K_d = [site][NADH]/[site\ NADH] \tag{I-4}$$

where $[NADH]_0$ and $[site]_0$ are the initial concentrations of NADH and the binding sites, respectively, and [site] is the concentration of the free sites.

The fraction (α) of NADH bound in the site is defined as:

$$\alpha = [site\ NADH]/[NADH]_0 \tag{I-5}$$

From Eqns (I-2)-(I-5), α is expressed as:

$$\alpha = ([site]_0 + [NADH]_0 + K_d)/(2[NADH]_0) - \left\{([site]_0 + [NADH]_0 + K_d)^2 - 4[NADH]_0[site]_0\right\}^{\frac{1}{2}}/(2[NADH]_0) \tag{I-6}$$

From Eqns I-1,I-2, and I-5,

$$v = [EP^+]_0[NADH]_0\{k_{1f} + (k_{1b} - k_{1f})\alpha\} \tag{I-7}$$

Equation I-6 shows that α changes depending on $[site]_0$, and Eqn 7 shows that v is linearly related to α.

I examined the reactivity of NADH bound in the coenzyme site of glutamate dehydrogenase and also that in the site of lactate dehydrogenase; the values of v were measured as the rate of formazan formation (Yomo et al., 1989a) at different concentrations of one of the enzymes as described in the legend to Fig. I-2. Assuming an arbitrary value of

K_d, the value of α was calculated from Eqn I-6 for each $[site]_0$. The plot of v vs. α thus obtained generally shows a curved line, and the shape of the line varies depending on the value of K_d assumed. As Eqn I-7 requires a linear relationship between v and α, the K_d values that satisfy the linear relationship were calculated for glutamate and lactate dehydrogenases to be 1.0 μM and 23 μM, respectively, so as to make the correlation coefficient maximum (0.9969 for glutamate dehydrogenase and 0.9990 for lactate dehydrogenase) in the range of $K_d \geqq 0$ and $k_{1b} \geqq 0$ by the least-squares method. Figure I-2 shows the straight lines obtained for the two enzymes.

Equation I-7 shows that the slope of the line thus obtained corresponds to the difference between k_{1b} and k_{1f}. This means that the slope is positive when the bound NADH has higher reactivity, the slope is zero when the site has no effect on the reactivity of NADH (or $K_d = \infty$), and the slope is negative when the bound NADH has lower reactivity. Equation I-7 also shows that the value of k_{1b} can be obtained from the value of v at $\alpha = 1$ or from the α-intercept (α_0) of the straight line using the following equation:

$$k_{1b} = k_{1f}(1 - 1/\alpha_0) \qquad \text{(I-8)}$$

For lactate dehydrogenase, $\alpha_0 = 1.0$ from Fig. I-2, and so $k_{1b} = 0$ $mM^{-1}s^{-1}$ from Eqn I-8. For glutamate dehydrogenase, k_{1b} is calculated to be 1.20 $mM^{-1}s^{-1}$ using a k_{1f} value of 2.78 $mM^{-1}s^{-1}$ for the reaction of NADH with EP^+-PEG (Yomo et al., 1989b) and an α_0 value of 1.76 (Fig. I-2). These k_{1b} values indicate that the NADH molecule bound in the site of glutamate dehydrogenase reacts with the ethylphenazine moiety of EP^+-PEG at a rate of 43% of that of free NADH, but the NADH molecule bound in the site of lactate dehydrogenase does not react with the ethylphenazine moiety of 1-(3-carboxypropyloxy)-5-ethylphenazine, a smaller derivative

than EP^+-PEG. Therefore, I used glutamate dehydrogenase as a substrate-binding site for preparing a semisynthetic NADH oxidase.

Characterization of EP^+-PEG-GltDH

EP^+-PEG was covalently linked to glutamate dehydrogenase by the procedure described for the preparation of MDH-PEG-NAD^+ (Eguchi et al., 1986) and GlcDH-PEG-NAD^+ (Nakamura et al., 1986). Figure I-3 shows the DEAE-Sephadex CL-6B column chromatography of EP^+-PEG-GltDH. The conjugate thus prepared has the following characteristics. The average number of the ethylphenazine moieties bound per molecule of enzyme subunit (EP^+ content) is 0.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (Laemmli, 1970) shows at least four bands with apparent M_r of 54000, 60000, 640000, and 67000; these bands seem to correspond to the subunits with 0, 1, 2, and 3 molecules of EP^+-PEG, respectively. The glutamate dehydrogenase activity of EP^+-PEG-GltDH measured as described under Experimental Procedures is 0.6 kat per mole of subunit; this value is 39% of that of the native enzyme.

NADH Oxidase Activity of EP^+-PEG-GltDH

Both 5-ethylphenazine and EP^+-PEG-GltDH catalyze the following reaction when MTT, instead of oxygen, is used as an electron acceptor:

$$NADH + MTT + H^+ = NAD^+ + formazan \qquad (I\text{-}9)$$

but the mechanism of the catalysis is different. 5-Ethylphenazine catalyzes this reaction by the cycle shown in Fig. i, and EP^+-PEG-GltDH catalyzes it either by the cycle shown in Fig. I-1 or Fig. i. In the reaction cycle shown in Fig. I-1, EP^+-PEG-GltDH works as an enzyme, NADH oxidase, having a catalytic group of the ethylphenazine moiety and the NADH-binding site of the glutamate dehydrogenase moiety.

Now let us consider the active site of this NADH oxidase. The

active site is composed of one NADH-binding site and some ethylphenazine moieties as catalytic groups. The number of the catalytic groups may be different for each active site, the distances between the binding site and the catalytic groups may be different for each catalytic group, and the affinity of the binding site for NADH may be different for each active site because of the effects of the chemical modification. Therefore, these active sites are grouped by the affinity of the substrate-binding site and the turnover number: the i'th active site has a dissociation constant of $K_{d,i}$ and a turnover number of $k_{cat,i}$. In this case, the initial steady-state rate of the reaction (Eqn I-9) catalyzed by EP^+-PEG-GltDH is expressed by the following equation, if the NADH concentration is much larger than the active-site concentration, the MTT concentration is in excess, and the pH is kept constant:

$$d[\text{formazan}]/dt = 0.7 \cdot k'_{lf}[EP^+\text{-PEG-GltDH}]_0[\text{NADH}]_0 + \sum_i \{k_{cat,i}\, \gamma_i [EP^+\text{-PEG-GltDH}]_0[\text{NADH}]_0/(K_{d,i} + [\text{NADH}]_0)\} \qquad \text{(I-10)}$$

where k'_{lf} is the second-order rate constant of the reaction between the ethylphenazine moiety and free NADH (the value is assumed to be the same for all the ethylphenazine moieties), $[EP^+\text{-PEG-GltDH}]_0$ is the initial concentration of the conjugate expressed as the subunit (i.e., active site) concentration, γ_i is the fraction of the i'th active site. The first term of Eqn I-10 is the rate of the reaction between free NADH and the ethylphenazine moiety by the catalytic cycle shown in Fig. i, and the second term is the activity of the active site by the catalytic cycle shown in Fig. I-1; that is, the effects of the presence of the NADH-binding site are expressed by the second term. It should be noted that for a dehydrogenase with an oligomeric structure, one ethylphenazine moiety may be used commonly by two or more active sites on the enzyme, but the form of Eqn I-10 does not change. It should also be

noted that almost all the ethylphenazine moieties are in the oxidized form and are always ready to react with NADH in the presence of excess MTT (Yomo et al., 1989a).

The activity of EP^+-PEG-GltDH was measured at different initial concentrations of NADH, and the results are shown in Fig. I-4. The reaction rate increases with the increase in the NADH concentration, and reaches a straight line with a slope of 1.94×10^{-3} s^{-1}. When $[NADH]_0 >> K_{d,i}$, Eqn I-10 is simplified to:

$$d[\text{formazan}]/dt = 0.7 \cdot k'_{1f}[EP^+\text{-PEG-GltDH}]_0[NADH]_0 + k'_{cat}[EP^+\text{-PEG-GltDH}]_0 \qquad (I\text{-}11)$$

where k'_{cat} is the apparent k_{cat} of this preparation of the semisynthetic oxidase, and is corresponds to $\sum_i k_{cat,i}\,\delta_i$ in Eqn I-10. The values of k'_{1f} and k'_{cat} are thus obtained to be 1.60 $mM^{-1}s^{-1}$ and 0.38 s^{-1}, respectively, from the straight line shown in Fig. I-4 and by using Eqn I-11. This k'_{1f} value is 58% of the second-order rate constant (k_{1f}) of the reaction between EP^+-PEG and NADH; this may be due to some effect of the enzyme molecule on the reactivity of the 5-ethylphenazine moiety. The k'_{cat} value of 0.38 s^{-1} is much smaller than the turnover number of 56 s^{-1} for the reductive amination of 2-oxoglutarate by glutamate dehydrogenase (Engel & Dalziel, 1970). Therefore, the dissociation rate constant of NAD^+ must be much larger than the k'_{cat} value, and the k'_{cat} value corresponds to the apparent intramolecular rate constant of the oxidation of NADH bound in the active site.

If the NADH-binding site of EP^+-PEG-GltDH does not work (i.e., for all i, $k_{cat,i} = 0$ or $K_{d,i} >> [NADH]_0$), the reaction rate is expressed by the first term of Eqn I-10 and by the broken line in Fig. I-4 with a slope of $0.7\ k'_{1f}[EP^+\text{-PEG-GltDH}]_0$ (= 1.94×10^{-3} s^{-1}). Thus the rate-acceleration effect of the NADH-binding site (i.e., the second term of

Eqn I-10) is shown in Fig. I-4 as the difference between the data points and the broken line. As expected from Eqns I-10 and I-11, the difference increases with the increase in $[NADH]_0$ and reaches a constant value of $k'_{cat}[EP^+\text{-PEG-GltDH}]_0$ (= 0.65 $\mu M\ s^{-1}$) when $[NADH]_0 \geqq 1.1$ mM. In this constant region, almost all the binding sites are occupied with NADH.

Discussion

When NADH is bound in the coenzyme sites of glutamate and lactate dehydrogenases, the reactivity of NADH with the ethylphenazine moiety decreases to 43% and 0%, respectively, of that of free NADH. This decrease seems to be due to the steric effect that limits the accessibility of the ethylphenazine moiety to the reduced nicotinamide ring of bound NADH. If so, the difference in the steric effect of dehydrogenases on bound NAD^+ reflects the difference in the structures of the coenzyme-binding sites of these enzymes; the coenzyme site of glutamate dehydrogenase may be more open than that of lactate dehydrogenase. This is also supported by the fact that glutamate dehydrogenase has a higher activity than lactate dehydrogenase toward NAD^+ derivatives modified at the nicotinamide ring (Inoue et al., 1985).

I obtained K_d values from the experiment shown in Fig. I-2 based on the fact that the reactivity of bound NADH is different from that of free NADH. These K_d values are a little different from the values of 8.6 μM for glutamate dehydrogenase (Engel & Dalziel, 1970) and 5.1 μM for lactate dehydrogenase (Katayama et al., 1983) obtained from enzyme kinetics; this may be due to the difference in the principle of the methods. It should be pointed out that our method is applicable not only for enzymatic systems but also for nonenzymatic systems.

5-Ethylphenazine catalyzes the oxidation of NADH by the cycle shown

in Fig. i. When 5-ethylphenazine is covalently linked to an NADH-binding site, the oxidation rate is enhanced by the additional enzyme-like catalytic cycle shown in Fig. I-1. Kinetic constants obtained in this work for EP^+-PEG-GltDH show the effects of the presence of the substrate-binding site near the catalytic group of the ethylphenazine moiety. One of the effects is that the intermolecular reaction between the ethylphenazine moiety and NADH is changed to the intramolecular reaction between the ethylphenazine moiety and bound NADH. The ratio of the rate constants (based on the ethylphenazine moiety) of the intramolecular and intermolecular reactions, i.e., $k'_{cat}/(0.7 \cdot k'_{lf})$, is the apparent maximum effective concentration of bound NADH for the catalytic group, and the value is 0.33 mM. This value is also obtained from Fig. I-4 as the intercept at d[formazan]/dt = 0 of the straight line. This means that when all the binding sites are occupied by NADH, the ethylphenazine moiety feel as if it were in a solution with an NADH concentration of $([NADH]_0 + 0.33\ mM)$. This increase in the local NADH concentration is the effect of the presence of the substrate-binding site near the catalytic group, and this is one of the rate-acceleration mechanisms used by native enzymes. It should be noted that this rate-acceleration mechanism is effective even when the binding sites are not saturated with NADH. In this case, the apparent effective concentration varies depending on the NADH concentration, in contrast to the constant effective concentration obtained for EP^+-PEG-NAD^+ (Yomo et al., 1989b); this difference is due to the difference between the absence and the presence of the covalent linkage between the two reactants.

The apparent effective concentration of the NADH-binding site for the ethylphenazine moiety is calculated to be 0.77 mM from the apparent maximum effective concentration (0.33 mM) and the relative reactivity

(0.43) of bound NADH to free NADH. The effective concentration of 0.77 mM is about twice the effective concentration (0.4 mM) of the NAD^+ moiety for the ethylphenazine moiety of EP^+-PEG-NAD^+, and this corresponds to the situation that the ethylphenazine moiety is linked with two NADH-binding sites by the spacer of poly(ethylene glycol) (M_r 3000, about 25 nm). In reality, the ethylphenazine moiety is linked by the spacer at a point on the surface of glutamate dehydrogenase, and the point is generally not at the binding site. In this case, the maximum distance between the binding site and the ethylphenazine moiety is longer than the length of the spacer of poly(ethylene glycol), and the effective concentration of one NADH-binding site must be less than 0.4 mM. Therefore, the ethylphenazine moiety can interact with more than three binding sites of a hexameric glutamate dehydrogenase molecule. The apparent effective concentration of the ethylphenazine moiety for the NADH-binding site, on the other hand, is 0.54 mM. The effective concentration of 0.54 mM means that one NAD-binding site can interact with more than two ethylphenazine moieties. It should be noted that these effective concentrations obtained in this work are apparent values for this sample, because the attachment point and the number of EP^+-PEG on the enzyme molecule are not constant for each subunit.

Kaiser et al. have reported many kinds of semisynthetic enzymes made by covalently linking flavin analogs to various enzymes (Kaiser & Lawrence, 1984; Hilvelt & Kaiser, 1985). From their results on flavopapains (Kaiser & Lawrence, 1984), the apparent maximum effective concentrations of various substrates for the flavin moiety can be calculated, and the values are in the range of 0.05 - 3.5 mM. The length of the spacer used in their work is only 4 carbon units including the cysteine residue, and is much shorter than ours. Therefore, if all the

flavin groups can react with the substrate molecules bound in the substrate-binding sites neighboring each group, the effective concentration must be much larger. Probably, due to the shortness of the spacer, the most of the flavin groups cannot react with the substrate bound in the specific site. The importance of a spacer in these semisynthetic enzymes will be discussed in the chapter IV.

Summary

5-Ethylphenazine-poly(ethylene glycol)-glutamate dehydrogenase conjugate (EP^+-PEG-GltDH) was prepared by linking poly(ethylene glycol)-bound 5-ethylphenazine (EP^+-PEG) to glutamate dehydrogenase. The average number of the ethylphenazine moieties bound per enzyme subunit was 0.7. This conjugate is a semisynthetic enzyme having NADH oxidase activity; the ethylphenazine moiety works as a catalytic group, and the coenzyme-binding site of glutamate dehydrogenase works as a substrate-binding site. The effects of the presence of the substrate-binding site near the catalytic group were studied by using EP^+-PEG-GltDH. Before the preparation of the conjugate, the reactivity of NADH bound in the coenzyme-binding site toward the ethylphenazine moiety was estimated for glutamate and lactate dehydrogenases. The results show that the NADH molecule bound in the site of glutamate dehydrogenase reacts with EP^+-PEG at a rate of 43% of that of free NADH, but the NADH molecule bound in lactate dehydrogenase does not react with 1-(3-carboxypropyloxy)-5-ethylphenazine. Therefore, glutamate dehydrogenase was used as the substrate-binding site of the semisynthetic NADH oxidase. The results of the kinetic analysis of the activity of EP^+-PEG-GltDH show that the apparent turnover number of the active site is 0.38 s^{-1}, which corresponds to the apparent intramolecular rate constant of the

oxidation of NADH bound in the active site. The apparent maximum effective concentration of bound NADH for the catalytic group of the ethylphenazine moiety is 0.33 mM. This means that the presence of the substrate-binding site near the catalytic group increases the local NADH concentration by at most 0.33 mM, and this is the rate-acceleration effect of the binding site.

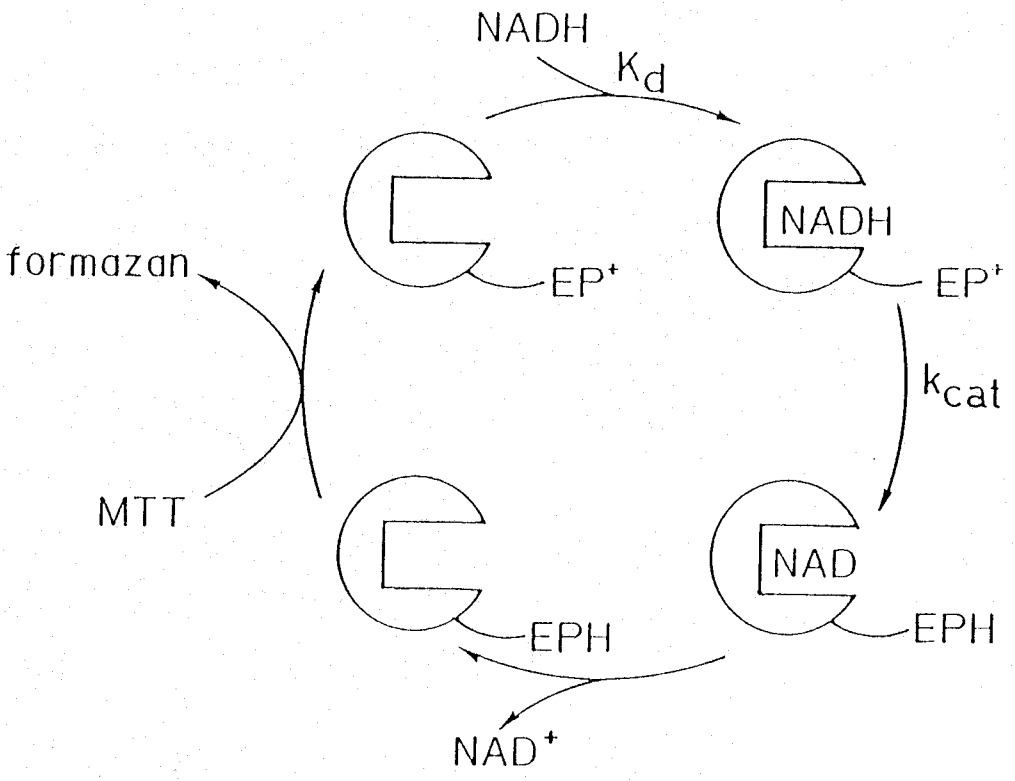


Fig. I-1. Schematic representation of the catalytic cycles of NADH oxidation by semisynthetic NADH oxidase. K_d, the dissociation constant of the binding-site NADH complex; k_{cat}, the turnover number of the active site.

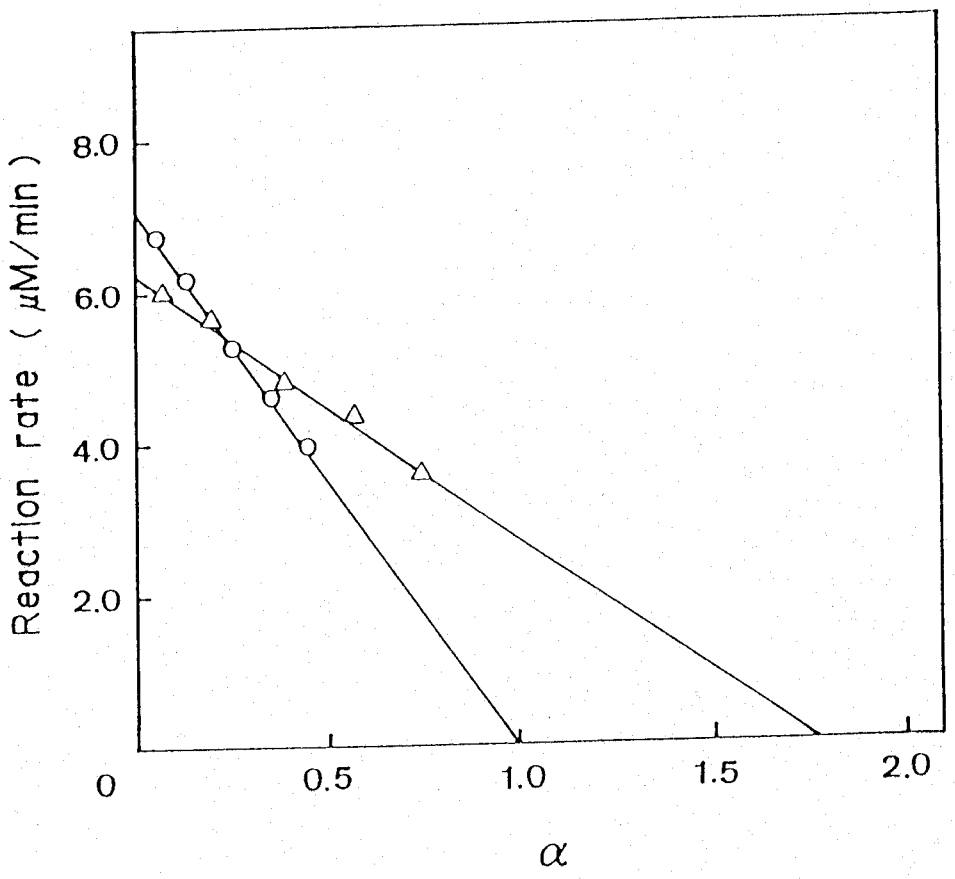


Fig. I-2. Plot of Eqn I-7. For glutamate dehydrogenase (Δ), the reaction mixture contained 90 μM NADH, 0.60 μM EP^+-PEG, 1.5 mM MTT, 0.1 M sodium phosphate (pH 7.5), and various subunit concentrations (7.02, 17.6, 35.1, 52.7, and 70.3 μM) of glutamate dehydrogenase; reactions were started by adding 50 μl of EP^+-PEG solution to the solution (3.05 ml) containing the other components described above. For lactate dehydrogenase (O), the reaction mixture contained 83.1 μM NADH, 0.99 μM 1-(3-carboxypropyloxy)-5-ethylphenazine, 1.5 mM MTT, 0.1 M sodium phosphate (pH 7.5), and various subunit concentrations (5.55, 13.9, 27.7, 41.6, 55.5 μM) of lactate dehydrogenase; reactions were started by adding 50 μl of the solution of the 5-ethylphenazine derivative to the solution (3.05 ml) containing the other components described above. The α values were calculated from Eqn I-6 using the K_d values of 1.0 μM for glutamate dehydrogenase and 23 μM for lactate dehydrogenase.

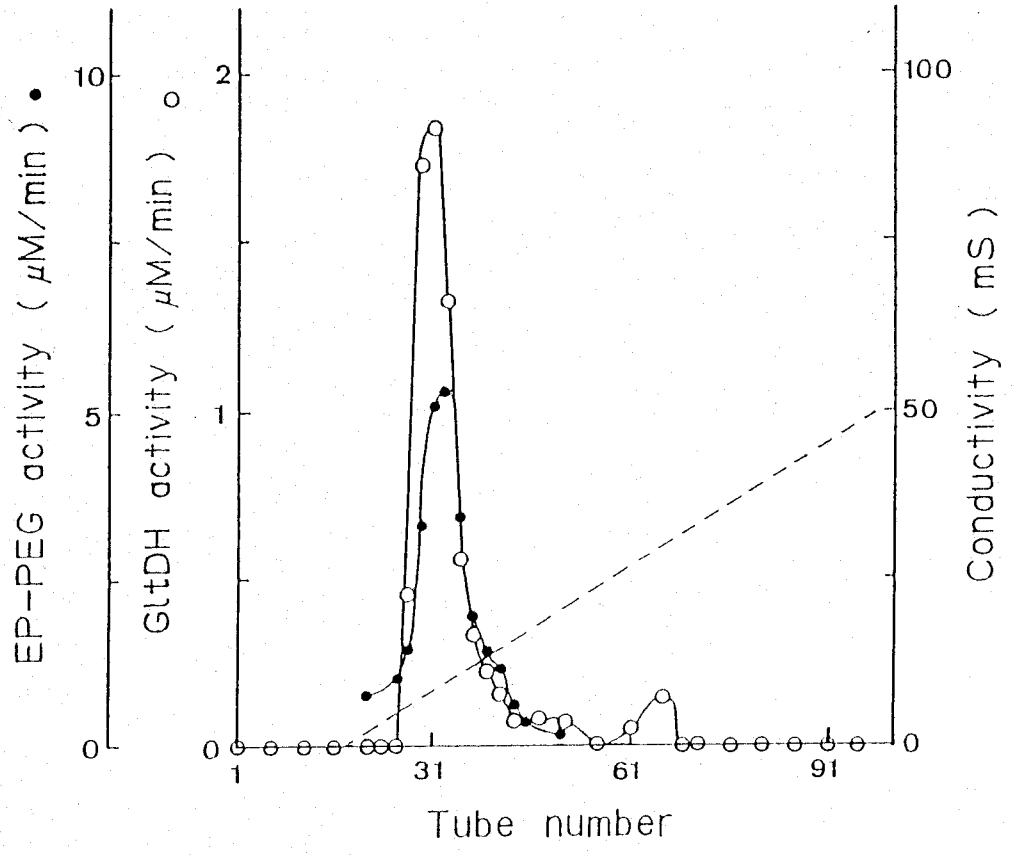


Fig. I-3. DEAE-Sephadex CL-6B column chromatography of EP^+-PEG-GltDH. O , Glutamate dehydrogenase (GltDH) activity; ●, activity of the ethylphenazine (EP^+) moiety; - - -, conductivity. The activities of GltDH and EP^+ moiety were measured as described under "Experimental Procedures".

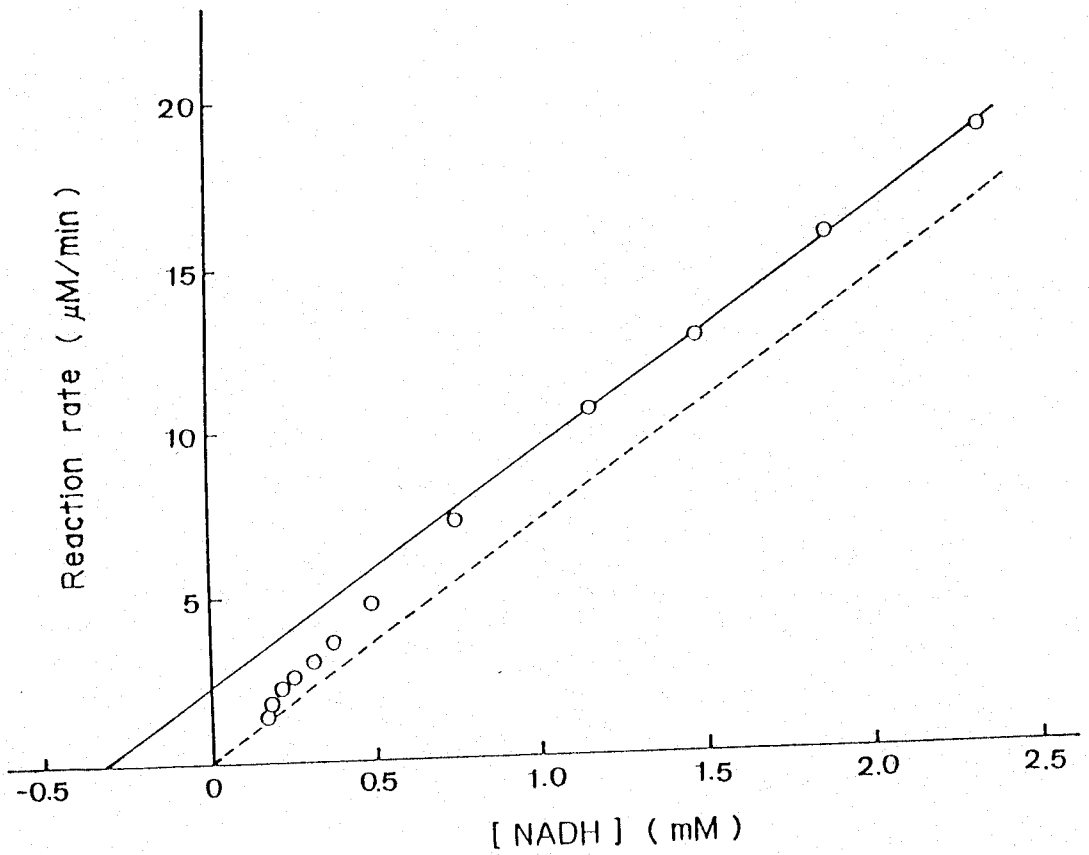


Fig. I-4. Effect of NADH concentration on the activity of EP^+-PEG-GltDH. The reaction mixture (3.2 ml) contained 1.73 μM (as subunit concentration) EP^+-PEG-GltDH, 1.5 mM MTT, 0.1 M sodium phosphate (pH 7.5), and the indicated concentrations of NADH. - - -, theoretical line for the first term of Eqn I-10 with a k'_{if} value of 1.60 $mM^{-1}s^{-1}$.

Chapter II

Intramolecular Coupling of Two Catalytic Reaction

Introductory remarks

In the chapter I, EP^+-PEG-GltDH, a semisynthetic NADH oxidase, was used to investigate the effects of the presence of a substrate-binding site near a catalytic group. In this chapter, EP^+-GlcDH-NAD^+, a semisynthetic glucose oxidase, is used to investigate rate-acceleration effects by linking NAD^+, 5-ethylphenazine, and glucose dehydrogenase into one molecule. The catalytic cycle of this oxidase has three reactions and is schematically shown in Fig. II-1. First, the NAD^+ moiety is reduced with glucose at the active site of the glucose-dehydrogenase moiety, then the NADH moiety is reoxidized by the reaction with the ethylphenazine moiety, and finally, the reduced ethylphenazine moiety is reoxidized with oxygen or MTT. In this cycle, the ethylphenazine moiety works as a catalytic group, and so this cycle has two catalytic steps. The characteristic point of the cycle is that the product (NADH) of the first step does not diffuse out, but reacts with the second catalytic group of the ethylphenazine moiety on the same EP^+-GlcDH-NAD^+ molecule. The rate-acceleration effect by this intramolecular coupling of the two successive catalytic steps are quantitatively shown together with the other mechanisms of rate acceleration from the kinetic point of view. These rate-acceleration mechanisms are generally seen in the catalytic cycles of natural enzymes.

Experimental Procedures

Materials

Glucose dehydrogenase (EC 1.1.1.47) from Bacillus megaterium IWG3 was a generous gift from Amano Pharmaceutical Co. Ltd. (Nagoya) and was used after purification by DEAE-Sephadex A-50 column chromatography. Horseradish peroxidase (EC 1.11.1.7; Grade III) was purchased from Toyobo Co. Ltd. (Osaka); 5-ethylphenazine ethylsulfate and MBTH were from Nakarai Chemicals (Kyoto); MTT was from Dojindo Laboratories (Kumamoto); primaquine diphosphate was from Aldrich (Milwaukee). EP^+-PEG was prepared by linking 1-(3-carboxypropyloxy)-5-ethylphenazine to α,ω-diaminopoly(ethylene glycol) with an M_r of 3000 (Yomo et al., 1989b). NAD^+ was a generous gift from Kohjin Co. Ltd. (Tokyo). PEG-NAD^+ was prepared by linking N^6-(2-carboxyethyl)-NAD^+ (Sakamoto et al., 1986) to α,ω-diaminopoly(ethylene glycol) with an M_r of 4000 by the method described previously (Katayama et al., 1983) with further purification. DHBT was prepared as described previously (Eguchi et al., 1986).

Concentration of Protein, Nucleotides, and 5-Ethylphenazine Derivatives

The concentrations of glucose dehydrogenase, NAD^+, NADH, PEG-NAD^+, PEG-NADH, 5-ethylphenazine, and EP^+-PEG were measured spectrophotometrically using the following molar absorption coefficients: the subunit of glucose dehydrogenase, 33000 $M^{-1}cm^{-1}$ at 280 nm (Nakamura et al., 1986; M_r 28100); NAD^+, 18,000 $M^{-1}cm^{-1}$ at 260 nm; PEG-NAD^+, 21500 $M^{-1}cm^{-1}$ at 266 nm (Yomo et al., 1989b); NADH and PEG-NADH, 6300 $M^{-1}cm^{-1}$ at 340 nm; 5-ethylphenazine, 26300 $M^{-1}cm^{-1}$ at 386 nm (Zaugg, 1964); and EP^+-PEG, 17000 $M^{-1}cm^{-1}$ at 386 nm (Yomo et al., 1989b). The concentrations of the ethylphenazine moiety, the enzyme moiety (as a subunit), and the NAD^+ moiety in EP^+-GlcDH-NAD^+ were calculated from the absorption spectrum of the conjugate using the following molar absorption coefficients:

for the ethylphenazine moiety, 17000 $M^{-1}cm^{-1}$ at 386 nm, 43000 $M^{-1}cm^{-1}$ at 280 nm, and 28000 $M^{-1}cm^{-1}$ at 266 nm; for the enzyme moiety, 0 $M^{-1}cm^{-1}$ at 386 nm, 33000 $M^{-1}cm^{-1}$ at 280 nm, and 23000 $M^{-1}cm^{-1}$ at 266 nm; for the NAD^+ moiety, 0 $M^{-1}cm^{-1}$ at 386 nm, 12000 $M^{-1}cm^{-1}$ at 280 nm, and 21500 $M^{-1}cm^{-1}$ at 266 nm. Unless otherwise stated, the concentration of EP^+-GlcDH-NAD^+ was expressed as the concentration of the subunit of the glucose dehydrogenase moiety.

Preparation of EP^+-GlcDH-NAD^+

EP^+-GlcDH-NAD^+ was prepared by the method for the preparation of EP^+-PEG-GltDH (see the chapter I). EP^+-PEG (4 μmol), PEG-NAD^+ (4 μmol), and DHBT (350 mg) were dissolved in CH_2Cl_2 (4 ml), and the mixture was kept at 4°C for 2 h. Then, 0.5 ml of 50 mM sodium phosphate buffer, pH 6.5, was added to the mixture, and CH_2Cl_2 was removed by evaporation. One ml of 0.94 mM glucose dehydrogenase solution in the same buffer was added to the residue, the reaction mixture was kept at 15°C for 40 h, and the mixture was diluted to 50 ml with 25 mM sodium phosphate buffer, pH 6.5, to reduce the conductivity to 4.5 mS. The solution was applied to a DEAE-Sephadex CL-6B column (2 x 25 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.5. The column was washed with this buffer (50 ml), and EP^+-PEG, PEG-NAD^+, and their derivatives that had not reacted with the enzyme were eluted out (the first peak in Fig. II-2); then, EP^+-GlcDH-NAD^+ was eluted with an NaCl gradient of 0 - 0.5 M (total 600 ml). The main fractions (No. 85 - 94, 5 ml each; Fig. II-2) having three kinds of activities due to the presence of the conjugate of 5-ethylphenazine, glucose dehydrogenase, and NAD^+ were combined and used as EP^+-GlcDH-NAD^+. There was no other peak having the dehydrogenase activity.

Assay of Reaction Rates

Initial rates of reactions were measured at 30°C in 0.1 M sodium phosphate buffer, pH 7.5, with a Hitachi 220 spectrophotometer with a thermostated cell compartment. MTT or oxygen was used as a terminal electron acceptor. For the MTT method, the reactions were recorded as the increase in absorbance at 570 nm (the concentration of formazan produced from the reduction of MTT), and the reaction rates were calculated from the initial linear parts using a molar absorption coefficient of 13000 $M^{-1}cm^{-1}$ (Eguchi et al., 1985). For the O_2 method, the reactions were recorded as the increase in absorbance at 510 nm (the concentration of the red dye produced from the reaction of H_2O_2, MBTH, and primaquine catalyzed by peroxidase), and the reaction rates, which correspond to the production rates of H_2O_2, were calculated from the initial linear parts using a molar absorption coefficient of 53000 $M^{-1}cm^{-1}$. The assays were made at least in duplicate with a reproducibility of less than 5% error.

The following four reactions were measured by the MTT method. Glucose dehydrogenase activity in the presence of exogenous NAD^+ and 5-ethylphenazine was measured by adding 50 μl of enzyme solution (154 nM for native enzyme and 23.7 nM for EP^+-GlcDH-NAD^+) to the substrate solution (3.2 ml) containing 94 mM D-glucose, 0.90 mM NAD^+, 1.34 mM 5-ethylphenazine, and 1.4 mM MTT. Glucose dehydrogenase activity in the presence of exogenous PEG-NAD^+ and 5-ethylphenazine was measured by adding 0.1 ml of 52.5 μM native enzyme solution to the substrate solution (3.1 ml) containing 97 mM D-glucose, 5.84 mM 5-ethylphenazine, 1.5 mM MTT, and PEG-NAD^+; the concentration of PEG-NAD^+ was varied from 8.03 μM to 151.5 μM for obtaining the second-order rate constant of the reaction between glucose dehydrogenase and PEG-NAD^+. The intramolecular

reaction rates of the reduction of the NAD^+ moiety by the active site of the glucose-dehydrogenase moiety on the same EP^+-GlcDH-NAD^+ molecule were measured in the presence of excess 5-ethylphenazine by adding 0.2 ml of 6.84 μM EP^+-GlcDH-NAD^+ to the substrate solution (3.0 ml) containing 0.1 M D-glucose, 5.84 mM 5-ethylphenazine, and 1.5 mM MTT. The reaction rates of the 5-ethylphenazine moiety with exogenous PEG-NADH was measured by adding 0.1 ml of 6.84 μM EP^+-GlcDH-NAD^+ to the substrate solution (3.1 ml) containing 1.5 mM MTT and PEG-NADH; the concentration of PEG-NADH was varied from 34.7 μM to 249 μM for obtaining the second-order rate constant.

Glucose oxidase activity of EP^+-GlcDH-NAD^+ was measured by the MTT method and also by the O_2 method. For the MTT method, reactions were started by adding 0.2 ml of 6.09 μM EP^+-GlcDH-NAD^+ to the substrate solution (3.0 ml) containing 0.1 mM D-glucose and MTT; the concentration of MTT was varied from 0.015 mM to 1.5 mM. The procedure for the O_2 method is as follows. The enzyme solution containing 5.54 μM EP^+-GlcDH-NAD^+ and 18 μg/ml peroxidase was deaerated by bubbling argon gas at 30°C. The substrate solution containing 0.1 M sodium phosphate buffer, pH 7.5, 0.1 M D-glucose, 0.1 mM MBTH, 0.1 mM primaquine, and 1 mM EDTA was bubbled with argon gas or O_2 gas at 30°C. A volume of the argon-bubbled substrate solution and a volume of the O_2-bubbled substrate solution (total 3.0 ml) were taken into a syringe, and the mixture was transferred into a cuvette filled with argon gas; the concentration of dissolved oxygen of the substrate solution in the cuvette was varied by taking different volumes of the argon-bubbled and the O_2-bubbled solutions into the syringe. The oxygen concentration of the O_2-bubbled solution is assumed to be 1.2 mM. The reactions were started by adding the enzyme solution (0.2 ml) with a syringe to the cuvette containing the

substrate solution; the cuvette was shielded with Parafilm during the reaction. It was confirmed that the amount of peroxidase is in excess. Blank tests were done without EP^+-GlcDH-NAD^+.

Results and Discussion

Characterization of EP^+-GlcDH-NAD^+

EP^+-PEG and PEG-NAD^+ were covalently linked to glucose dehydrogenase as described under "Experimental Procedures". Figure II-2 shows the DEAE-Sephadex CL-6B column chromatography of EP^+-GlcDH-NAD^+. The conjugate thus prepared has the following characteristics. The average number of the ethylphenazine moieties bound per molecule of enzyme subunit (EP^+ content) is 0.8, and that of the NAD^+ moieties (NAD^+ content) is 1.2. The glucose dehydrogenase activity of EP^+-GlcDH-NAD^+ in the presence of exogenous NAD^+ and 5-ethylphenazine (see "Experimental Procedures") is 55 kat per mole of subunit; this value is 53% of that of the native enzyme.

Glucose Oxidase Activity of EP^+-GlcDH-NAD^+

EP^+-GlcDH-NAD^+ catalyzes the oxidation of glucose by oxygen or MTT, and H_2O_2 or formazan is produced, respectively. This reaction strictly depends on the presence of the conjugate, glucose, and one of the electron acceptors (data not shown); and therefore, EP^+-GlcDH-NAD^+ works as glucose oxidase. This means that natural glucose dehydrogenase is artificially converted to glucose oxidase.

Figure II-3 shows the effects of oxygen concentration on the activity of the semisynthetic oxidase. From these results, the values of the parameters for the Michaelis-Menten equation are calculated by the Marquardt method (Marquardt, 1963): V_{max} = 8.23 μM/min and K_m = 1.57 mM

with a standard deviation of 0.036. The values of the parameters for the reaction system with MTT are also obtained from the results shown in Fig. II-4: V_{max} = 2.50 uM/min and K_m = 0.072 mM with a standard deviation of 0.066. From these V_{max} values, the turnover numbers (k_{cat}) per subunit are calculated to be 0.40 s^{-1} and 0.11 s^{-1} for the systems with oxygen and with MTT, respectively. It is interesting that the k_{cat} value varies depending on the kind of the electron acceptor. The reactivity of the ethylphenazine moiety may be affected by the kind of the acceptor. In our previous work, I found that the reactivity of the reduced form of the ethylphenazine moiety toward MTT is decreased to about one third by covalently linking the moiety to poly(ethylene glycol), but that the reactivity toward oxygen is not affected by the linking (Yomo et al., 1989b).

It is interesting that EP^+-GlcDH-NAD^+ has no binding site for oxygen or MTT but has K_m values for them. This is because the ethylphenazine moiety takes two forms, EP^+ and EPH, in the catalytic cycle of the semisynthetic oxidase (see Fig. II-1). That is, this oxidase reaction follows a ping-pong mechanism. In this case, the reaction kinetics fit the Michaelis-Menten equation, even if the catalyst has no binding site for the substrates. Generally, K_m is thought to be a dissociation constant of an enzyme-substrate complex, but these findings clearly show it is not always true. Kovar et al. reported that their alcohol dehydrogenase-NAD^+ conjugate obeys a ping-pong mechanism (Kovar et al., 1984); this can also be explained by the fact that the conjugate takes two forms, the oxidized and the reduced forms, in its catalytic cycle.

The catalytic cycle in Fig. II-1 is shown as a simplified form. In fact, there is the other cycle in which the oxidized forms are EP^+-

GlcDH-NADH and EPH-GlcDH-NAD^+ and the reduced form is EPH-GlcDH-NADH. The ratio of the fluxes of these two cycles changes depending on the ratio of the rate constants for the reduction of the NAD^+ moiety and for the oxidation of the reduced form of the ethylphenazine moiety as shown in our previous paper on EP^+-PEG-NAD^+ (Yomo et al., 1989b).

Heterogeneity in the Reactivity of EP^+-GlcDH-NAD^+

The preparation of EP^+-GlcDH-NAD^+ is not homogeneous in the numbers of the ethylphenazine moiety and the NAD^+ moiety and also in the reactivities between the NAD^+ moiety and the active site of the glucose dehydrogenase moiety and between the NADH moiety and the ethylphenazine moiety. Therefore, the tetrameric molecules of EP^+-GlcDH-NAD^+ are grouped as follows: the tetramers of EP^+-GlcDH-NAD^+ in a group (for example, the i'th group) have the same number of the ethylphenazine moieties and the same number of the NAD^+ moieties; each tetramer in the i'th group has the same first-order rate constant, k_{ij}, for the reduction of the j'th NAD^+ moiety; and each tetramer in the i'th group has the same intramolecular rate constant, $k_{hj,i}$, for the reoxidation of the j'th NADH moiety with the h'th ethylphenazine moiety.

The molecular species of tetrameric EP^+-GlcDH-NAD^+ in the i'th group can be divided into two groups with respect to the oxidation/reduction state of the ethylphenazine or NAD^+ moiety, and the material balances for these molecular species are expressed as follows:

$$[EP(H)\text{-}GlcDH_i\text{-}NAD(H)]$$
$$= [EP^+_h\text{-}GlcDH_i\text{-}NAD(H)] + [EPH_h\text{-}GlcDH_i\text{-}NAD(H)] \quad \text{(II-1)}$$
$$= [EP(H)\text{-}GlcDH_i\text{-}NAD^+_j] + [EP(H)\text{-}GlcDH_i\text{-}NADH_j] \quad \text{(II-2)}$$
$$[EP^+_h\text{-}GlcDH_i\text{-}NAD(H)]$$
$$= [EP^+_h\text{-}GlcDH_i\text{-}NAD^+_j] + [EP^+_h\text{-}GlcDH_i\text{-}NADH_j] \quad \text{(II-3)}$$

$$[EP(H)\text{-}GlcDH_i\text{-}NADH_j]$$

$$= [EP^+{}_h\text{-}GlcDH_i\text{-}NADH_j] + [EPH_h\text{-}GlcDH_i\text{-}NADH_j] \quad \text{(II-4)}$$

$$d[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j]/dt$$

$$= \sum_h (k_{hj,i}[EP^+{}_h\text{-}GlcDH_i\text{-}NADH_j]) - k_{ij}[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j] \quad \text{(II-5)}$$

$$= \sum_h (k_{hj,i}\beta_{EP^+{}_h\text{-}i\text{-}NADH_j})[EP(H)\text{-}GlcDH_i\text{-}NADH_j] - k_{ij}[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j] \quad \text{(II-6)}$$

$$d[EP^+{}_h\text{-}GlcDH_i\text{-}NAD(H)]/dt$$

$$= (k_{ox}[O_2] + k_{MT}[MTT])[EPH_h\text{-}GlcDH_i\text{-}NAD(H)] - \sum_j (k_{hj,i}[EP^+{}_h\text{-}GlcDH_i\text{-}NADH_j]) \quad \text{(II-7)}$$

$$= (k_{ox}[O_2] + k_{MT}[MTT])[EPH_h\text{-}GlcDH_i\text{-}NAD(H)] - \sum_j (k_{hj,i}\gamma_{EP^+{}_h\text{-}i\text{-}NADH_j})[EP^+{}_h\text{-}GlcDH_i\text{-}NAD(H)] \quad \text{(II-8)}$$

where [ ] shows the concentration, and for the conjugate, the concentration is expressed as the concentration of the tetramer; k_{ox} and k_{MT} are the second-order rate constants of the reactions of the reduced ethylphenazine moiety with oxygen and with MTT, respectively. The molecular species of the conjugate are expressed as follows: $EP(H)\text{-}GlcDH_i\text{-}NAD(H)$, every form of the conjugate in the i'th group; $EPH_h\text{-}GlcDH_i\text{-}NAD(H)$, every form of the i'th group conjugate whose h'th ethylphenazine moiety is in the reduced form; $EP^+{}_h\text{-}GlcDH_i\text{-}NAD^+{}_j$, every form of the i'th group conjugate whose h'th ethylphenazine moiety and j'th NAD^+ moiety are in the oxidized forms; and so on. The fractions, $\beta_{EP^+{}_h\text{-}i\text{-}NADH_j}$, $\beta_{EPH_h\text{-}i}$, $\gamma_{EP^+{}_h\text{-}i\text{-}NADH_j}$, and $\gamma_{i\text{-}NADH_j}$ are defined as:

$$\beta_{EP^+{}_h\text{-}i\text{-}NADH_j} = [EP^+{}_h\text{-}GlcDH_i\text{-}NADH_j]/[EP(H)\text{-}GlcDH_i\text{-}NADH_j] \quad \text{(II-9)}$$

$$\beta_{EPH_h\text{-}i} = [EPH_h\text{-}GlcDH_i\text{-}NAD(H)]/[EP(H)\text{-}GlcDH_i\text{-}NAD(H)] \quad \text{(II-10)}$$

$$\gamma_{EP^+{}_h\text{-}i\text{-}NADH_j} = [EP^+{}_h\text{-}GlcDH_i\text{-}NADH_j]/[EP^+{}_h\text{-}GlcDH_i\text{-}NAD(H)] \quad \text{(II-11)}$$

$$\gamma_{i\text{-}NADH_j} = [EP(H)\text{-}GlcDH_i\text{-}NADH_j]/[EP(H)\text{-}GlcDH_i\text{-}NAD(H)] \quad \text{(II-12)}$$

Here, I neglected the concentration of the enzyme forms having an

NAD(H) moiety in the coenzyme-binding site of the conjugate, because the glucose dehydrogenase moiety seems to have a large K_m value for the NAD^+ moiety (Nakamura et al., 1986), and almost all the NAD(H) moieties are considered to be in the free state. In Eqns II-5 and II-7, it is assumed that the rates of intermolecular reactions between the conjugates are negligible compared to those of the corresponding intramolecular reactions. The evidence to justify this assumption is the fact shown later that the effective concentrations of the NAD^+, the ethylphenazine, and the dehydrogenase moieties are much higher than their actual concentrations.

At steady state,

$$d[EP(H)\text{-}GlcDH_i\text{-}NAD^+_j]/dt = d[EP^+_h\text{-}GlcDH_i\text{-}NAD(H)]/dt = 0 \qquad (II\text{-}13)$$

From Eqns II-2, II-6, II-12, and II-13, $\gamma_{i\text{-}NADH_j}$ is expressed as:

$$\gamma_{i\text{-}NADH_j} = k_{ij}/\{k_{ij} + \sum_h (k_{hj,i}\beta_{EP^+_h\text{-}i\text{-}NADH_j})\} \qquad (II\text{-}14)$$

From Eqns II-1, II-8, II-10, and II-13, $\beta_{EPH_h\text{-}i}$ is expressed as:

$$\beta_{EPH_h\text{-}i} = \sum_j (k_{hj,i}\gamma_{EP^+_h\text{-}i\text{-}NADH_j})/\{\sum_j (k_{hj,i}\gamma_{EP^+_h\text{-}i\text{-}NADH_j}) + k_{ox}[O_2] + k_{MT}[MTT]\} \qquad (II\text{-}15)$$

The reaction rate measured by the O_2 method is expressed as:

$$d[H_2O_2]/dt = k_{ox}[O_2]\sum_i\sum_h [EPH_h\text{-}GlcDH_i\text{-}NAD(H)] \qquad (II\text{-}16)$$

Using Eqns II-10 and II-15 and the conditions of [MTT] = 0, Eqn II-16 is converted to:

$$d[H_2O_2]/dt = k_{ox}[O_2]\sum_i\sum_h \{[EP(H)\text{-}GlcDH_i\text{-}NAD(H)] \times \sum_j (k_{hj,i}\gamma_{EP^+_h\text{-}i\text{-}NADH_j})/(\sum_j (k_{hj,i}\gamma_{EP^+_h\text{-}i\text{-}NADH_j}) + k_{ox}[O_2])\} \qquad (II\text{-}17)$$

On the other hand, the reaction rate measured by the MTT method is expressed as:

$$d[formazan]/dt = k_{MT}[MTT]\sum_i\sum_h [EPH_h\text{-}GlcDH_i\text{-}NAD(H)] \qquad (II\text{-}18)$$

Using Eqns II-10 and II-15, Eqn II-18 is converted to:

$$d[\text{formazan}]/dt = k_{MT}[MTT] \sum_i \sum_h \{ \sum_j (k_{hj,i} \gamma_{EP^+_h} \text{-i-NADH}_j) \times [EP(H)\text{-GlcDH}_i\text{-NAD(H)}] / \{ \sum_j (k_{hj,i} \gamma_{EP^+_h} \text{-i-NADH}_j) + k_{ox}[O_2] + k_{MT}[MTT] \} \} \quad \text{(II-19)}$$

Figures II-3 and II-4 show that the experimental results fit the Michaelis-Menten kinetics well. This means that the reactivities of the NAD^+ and the ethylphenazine moieties are homogeneous at least for the values of K_m and V_{max}, though it is difficult to express these parameters in a simple form from Eqn II-17 or II-19 and Eqns II-14 and II-15. To confirm this homogeneity, I assumed that the preparation of EP^+-GlcDH-NAD^+ is composed of four groups with the same V_{max} value but with different K_m values ($K_{m,1}$, $K_{m,2}$, $K_{m,3}$, and $K_{m,4}$), and obtained these values by fitting the following equation (Eqn II-20) to the data points for the MTT system by the Marquardt method (Marquardt, 1963) with a standard deviation of 0.021; the values are: V_{max} = 0.65 μM/min, $K_{m,1}$ = $K_{m,2}$ = 0.115 mM, $K_{m,3}$ = 0.116 mM, and $K_{m,4}$ = 0.024 mM.

$$d[\text{formazan}]/dt = V_{max}[MTT]\{1/(K_{m,1} + [MTT]) + 1/(K_{m,2} + [MTT]) + 1/(K_{m,3} + [MTT]) + 1/(K_{m,4} + [MTT])\} \quad \text{(II-20)}$$

The resulting curve for Eqn II-20 is shown in Fig. II-4A as a broken line. The broken line is almost the same as the solid line obtained for the simple Michaelis-Menten kinetics, and the value of the standard deviation is also similar to that for the solid line (0.036). Figure II-4B shows the Eadie plot for the same results. This plot shows the difference between the curves for the homogeneous and the heterogeneous systems most clearly, but the difference is so small that the experimental results can well be explained by either system. These results indicate that the the the values of $\sum_j (k_{hj,i} \gamma_{EP^+_h} \text{-i-NADH}_j)$ (h, i = 1, 2, ...) are almost constant or only a few of them are much larger than the others, and the preparation of EP^+-GlcDH-NAD^+ behaves just like

a kinetically homogeneous system.

Kinetic Parameters for Intramolecular Reactions

To understand the kinetic mechanism of the semisynthetic glucose oxidase, I estimated the values of the kinetic parameters for the intramolecular steps of the oxidase reaction (Fig. II-1) using MTT as the electron acceptor. The value of the apparent intramolecular rate constant (k_{en}) for the reduction of the NAD^+ moiety by the active site of the glucose dehydrogenase moiety is estimated by the following procedure. In the presence of exogenous 5-ethylphenazine, Eqn II-6 is converted to:

$$\begin{aligned} &d[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j]/dt \\ &= \sum_h (k_{hj,i}\beta_{EP^+_h\text{-}i\text{-}NADH_j})[EP(H)\text{-}GlcDH_i\text{-}NADH_j] \\ &\quad + k_{PS}[EP(H)\text{-}GlcDH_i\text{-}NADH_j][PES] \\ &\quad - k_{ij}[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j] \end{aligned} \qquad \text{(II-21)}$$

where k_{PS} is the second-order rate constant of the reaction between the NADH moiety and exogenous 5-ethylphenazine, and [PES] is the concentration of the 5-ethylphenazine.

In the presence of high concentration of MTT, Eqn II-15 becomes

$$\beta_{EPH_h\text{-}i} \cong 0 \qquad \text{(II-22)}$$

This means that almost all the ethylphenazine moieties are in the oxidized form. Therefore,

$$\beta_{EP^+_h\text{-}i\text{-}NADH_j} \cong 1 \qquad \text{(II-23)}$$

Under these conditions, Eqn II-21 becomes

$$\begin{aligned} &d[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j]/dt \\ &= (\sum_h k_{hj,i} + k_{PS}[PES])[EP(H)\text{-}GlcDH_i\text{-}NADH_j] \\ &\quad - k_{ij}[EP(H)\text{-}GlcDH_i\text{-}NAD^+{}_j] \end{aligned} \qquad \text{(II-24)}$$

At steady state, from Eqns II-2, II-12, II-13, and II-24,

$$\gamma_{i\text{-NADH}_j} = k_{ij}/(k_{ij} + \sum_h k_{hj,i} + k_{PS}[PES]) \qquad \text{(II-25)}$$

In this case, the reaction rate is expressed as:

$$d[\text{formazan}]/dt = \sum_i \sum_j \{(\sum_h k_{hj,i} + k_{PS}[PES])[EP(H)\text{-}GlcDH_i\text{-}NADH_j]\} \qquad \text{(II-26)}$$

Using Eqns II-12 and II-25, Eqn II-26 is converted to:

$$d[\text{formazan}]/dt = \sum_i \sum_j \{k_{ij}(\sum_h k_{hj,i} + k_{PS}[PES])[EP(H)\text{-}GlcDH_i\text{-}NAD(H)]/(k_{ij} + \sum_h k_{hj,i} + k_{PS}[PES])\} \qquad \text{(II-27)}$$

In the presence of excess 5-ethylphenazine, Eqn II-27 is simplified to:

$$d[\text{formazan}]/dt = \sum_i \sum_j (k_{ij}[EP(H)\text{-}GlcDH_i\text{-}NAD(H)]) = k_{en}[NAD(H)]_t \qquad \text{(II-28)}$$

where $[NAD(H)]_t$ is the total concentration of the NAD^+ plus NADH moieties, and k_{en} is the average value of k_{ij}. The k_{en} value was found to be 0.39 s^{-1} by measuring the activity of EP^+-GlcDH-NAD^+ in the presence of an excess of 5-ethylphenazine (5.84 mM) and MTT (1.5 mM); the reaction conditions are described under "Experimental Procedures".

The value of the apparent intramolecular rate constant ($k_{in,ND}$) for the oxidation of the NADH moiety was obtained by the following procedure. From Eqns II-1 and (II-9)-(II-12),

$$\gamma_{i\text{-NADH}_j} = \gamma_{EP^+_h\text{-}i\text{-NADH}_j}(1 - \beta_{EPH_h\text{-}i})/\beta_{EP^+_h\text{-}i\text{-NADH}_j} \qquad \text{(II-29)}$$

In the presence of excess MTT, $\gamma_{EP^+_h\text{-}i\text{-NADH}_j}$ is expressed from Eqns II-14, II-22, II-23, and II-29 as:

$$\gamma_{EP^+_h\text{-}i\text{-NADH}_j} = \gamma_{i\text{-NADH}_j} = k_{ij}/(k_{ij} + \sum_h k_{hj,i}) \qquad \text{(II-30)}$$

When MTT concentration is in excess, the rate of formazan formation expressed by Eqn II-19 becomes V_{max} as shown in Fig. II-4. In this case, Eqn II-19 is simplified by using Eqn II-30 to:

$$V_{max} = \sum_i \sum_j \{k_{ij}(\sum_h k_{hj,i})[EP(H)\text{-}GlcDH_i\text{-}NAD(H)]/(k_{ij} + \sum_h k_{hj,i})\} \qquad \text{(II-31)}$$

This equation is also obtained from Eqn II-27 and [PES] = 0.

For a simplified system with a constant ratio of $(\sum_h k_{hj,i})/k_{ij}$, Eqn II-31 is further simplified to:

$$V_{max} = k_{en}k_{in,ND}[NAD(H)]_t/(k_{en} + k_{in,ND}) \quad \text{(II-32)}$$

where $k_{in,ND}$, the apparent intramolecular rate constant for the oxidation of the NADH moiety, is written as follows and corresponds to an average value of $\sum_h k_{hj,i}$:

$$k_{in,ND} = (\sum_h k_{hj,i})k_{en}/k_{ij} \quad (i, j = 1, 2, \ldots.) \quad \text{(II-33)}$$

Thus, the $k_{in,ND}$ value can be calculated to be 0.12 s^{-1} using a V_{max} value of 2.50 μM/min and a k_{en} value of 0.39 s^{-1}.

The apparent intramolecular rate constant ($k_{in,EP}$) for the reduction of the ethylphenazine moiety is related to $k_{in,ND}$ by the following relationship:

$$k_{in,EP}[EP(H)]_t = k_{in,ND}[NAD(H)]_t \quad \text{(II-34)}$$

where $[EP(H)]_t$ is the total concentration of the ethylphenazine moiety. Thus the $k_{in,EP}$ value is calculated to be 0.18 s^{-1}.

When oxygen is used as the electron acceptor, V_{max} is expressed by the same equation as Eqn II-34, and the values of $k_{in,ND}$ and $k_{in,EP}$ are calculated to be 2.2 s^{-1} and 3.2 s^{-1}, respectively, using a V_{max} of 8.23 μM/min and a k_{en} value of 0.39 s^{-1}. These calculated values of the intramolecular rate constants are much larger than those obtained in the system with MTT, and this difference corresponds to the difference in the k_{cat} values obtained from Figs. II-3 and II-4. The values of the kinetic constants obtained so far are listed in Table II-1.

Effective Concentration and Rate-acceleretion Mechanisms

To know the rate-acceleration effect by the covalent linking of PEG-NAD^+

to glucose dehydrogenase as reported previously (Nakamura et al., 1986), the glucose dehydrogenase activity of EP^+-GlcDH-NAD^+ in the presence of exogenous PEG-NAD^+ and 5-ethylphenazine was measured as described under "Experimental Procedures", and a second-order rate constant ($k_{2,en}$) of 0.30 $mM^{-1}s^{-1}$ was obtained for the reaction between the glucose dehydrogenase moiety and PEG-NAD^+; this value is almost the same as that obtained previously (Nakamura et al., 1986). The apparent effective concentration (Page, 1973; Nakamura et al., 1986; Yomo et al., 1989b) of the active site of the dehydrogenase moiety for the NAD^+ moiety is then calculated to be 1.3 mM using the k_{en} value of 0.39 s^{-1} and the $k_{2,en}$ value of 0.30 $mM^{-1}s^{-1}$. The apparent effective concentration of the NAD^+ moiety for the active site is also calculated to be 1.6 mM using the NAD^+ content of 1.2. These values mean, for example, that the NAD^+ moiety feels as if it were in a solution containing 1.3 mM glucose dehydrogenase irrespective of the actual concentration of the conjugate. As the concentration of EP^+-Glcdh-NAD^+ used for the assay of the glucose oxidase activity was about 0.4 μM, the activity for PEG-NAD^+ is increased more than 3000 times by this covalent linking.

The apparent effective concentration of the NAD^+ moiety for the ethylphenazine moiety is estimated from a $k_{in,EP}$ value of 3.2 s^{-1} or 0.18 s^{-1} (Table II-1) and the second-order rate constant ($k_{2,EP}$) of 1.49 $mM^{-1}s^{-1}$ for the reaction of PEG-NADH with the ethylphenazine moiety of EP^+-LDH-NAD^+ (see the chapter III); the estimated values are 2.1 mM and 0.12 mM for the reaction systems with oxygen and with MTT, respectively. The apparent effective concentration of the ethylphenazine moiety for the NAD^+ moiety is also estimated to be 1.5 mM and 0.08 mM for the reaction systems with oxygen and with MTT, respectively, from the values of $k_{in,ND}$ and $k_{2,EP}$. The effective concentrations obtained for the

system with oxygen are similar to the effective concentration (1.6 mM) of the NAD^+ moiety for the active site of the dehydrogenase moiety, but those obtained for the system with MTT are much smaller. As the NAD^+ and EP^+ contents are similar and about one moiety per subunit, the values of the kinetic constants obtained for the system with oxygen seems to be normal. The low values for the system with MTT are related to the low k_{cat} value, and this point will be discussed in the chapter III.

These effective concentrations are much higher than the actual concentration of EP^+-GlcDH-NAD^+ used for the assay. Therefore, the rates of the two catalytic steps in Fig. II-1 are much increased by the covalent linking of the reactants. This kind of rate acceleration is one of the important catalytic mechanisms of enzymes (Jencks, 1975). It should be pointed out that the rates of the intermolecular reactions between different molecules of EP^+-GlcDH-NAD^+ are negligible under our reaction conditions; this point has been discussed in the chapter I.

The rate of each catalytic step in Fig. II-1 is increased just by linking glucose dehydrogenase with NAD^+ or linking NAD^+ with 5-ethylphenazine. It is evident, however, from the large difference between the effective and the actual concentrations, that the rate of the overall oxidase reaction is increased not by linking two of the three reactants but by linking all the three into one conjugate. In this conjugate, the two successive reactions catalyzed by the glucose dehydrogenase moiety and the ethylphenazine moiety are coupled intramolecularly, and this intramolecular coupling is the most characteristic point of the catalytic mechanism of the semisynthetic glucose oxidase. This effect of the intramolecular coupling can be estimated by the comparison of the V_{max} values for the two catalytic systems, EP^+-GlcDH-NAD^+ and EP^+-PEG-NAD^+ plus GlcDH-PEG-NAD^+. When the EP^+ content and the NAD^+ content are the

same and one molecule per subunit, and when the two systems have the same concentration of the ethylphenazine moiety, V_{max} for the separate system is expressed as:

$$V_{max,sp} = k_{en}k_{2,EP}[EP(H)]_t^2/(k_{en} + k_{2,EP}[EP(H)]_t) + k_{in,ND}k_{2,en}[EP(H)]_t^2/(k_{in,ND} + k_{2,en}[EP(H)]_t) \quad \text{(II-35)}$$

Using the following values of the rate constants: k_{en} = 0.39 s^{-1}, $k_{in,ND}$ = $k_{in,EP}$ = 2.7 s^{-1}, $k_{2,en}$ = 0.30 $mM^{-1}s^{-1}$ and $k_{2,EP}$ = 1.49 $mM^{-1}s^{-1}$, and when $[EP(H)]_t$ = 0.4 μM, the values of V_{max} (Eqn II-32) and $V_{max,sp}$ (Eqn II-35) are calculated to be 0.14 μM/s and 0.29 nM/s, respectively. That is, the the reaction rate is accelerated about 500 times by this intramolecular coupling. This rate acceleration by the intramolecular coupling of the two successive catalytic steps is also another important catalytic mechanism used in natural enzymes.

As reported previously, the effective concentration of the NAD^+ moiety for the ethylphenazine moiety (and also that of the ethylphenazine moiety for the NAD^+ moiety) of EP^+-PEG-NAD^+ is 0.4 mM (Yomo et al., 1989b). This means that two ligands linked with the spacer of poly(ethylene glycol) (M_r 3000) show an effective concentration of 0.4 mM for each other. A tetrameric EP^+-GlcDH-NAD^+ molecule prepared in this work has five NAD^+ moieties and three ethylphenazine moieties in average. Therefore, the effective concentration of 1.3 mM of the active site of the glucose dehydrogenase moiety for the NAD^+ moiety means that the NAD^+ moiety can interact with all the active sites on the tetramer, as the effective concentration of each site for the NAD^+ moiety must be lower than 0.4 mM. This and the other values of the effective concentrations show that all the active sites, the NAD^+ moieties, and the catalytic groups of the ethylphenazine moieties on a tetrameric EP^+-GlcDH-NAD^+ can interact with each other. That is, the two kinds of the cata-

lytic sites are coupled with each other in multiple ways on the tetrameric molecule. This multiple connection is due to the long and flexible chain of poly(ethylene glycol), and is the third rate-acceleration mechanism found in the reaction of the semisynthetic oxidase. Similar multiple connection between catalytic sites has been suggested in the catalytic mechanism of the multienzyme complexes of α-ketoglutarate dehydrogenase (Hackert et al., 1983a) and pyruvate dehydrogenase (Hackert et al., 1983b). The importance of the flexibility of a spacer in designing enzymes will be discussed in the chapter IV.

Summary

5-Ethylphenazine-glucose dehydrogenase-NAD^+ conjugate (EP^+-GlcDH-NAD^+) was prepared by linking both poly(ethylene glycol)-bound 5-ethylphenazine and poly(ethylene glycol)-bound NAD^+ to glucose dehydrogenase. The average number of the ethylphenazine moieties bound per molecule of enzyme subunit was 0.8, and that of the NAD^+ moieties was 1.2. This conjugate is a semisynthetic enzyme having glucose oxidase activity using oxygen or MTT as an electron acceptor. When the concentration of oxygen or MTT is varied, the oxidase activity fits the Michaelis-Menten equation with the following apparent values of the kinetic constants: for the system with oxygen, the turnover number per subunit is 0.40 s^{-1} and K_m for oxygen is 1.57 mM; and for the system with MTT, the turnover number is 0.11 s^{-1} and K_m for MTT is 0.072 mM. The catalytic cycle of the semisynthetic oxidase has two catalytic steps: reduction of the NAD^+ moiety by the active site of the glucose dehydrogenase moiety and oxidation of the NADH moiety by another catalytic site of the ethylphenazine moiety. The apparent intramolecular rate constants of these steps were estimated, and the values are as follows: 0.39 s^{-1} for the reductions of

the NAD^+ moiety, 2.2 s^{-1} and 0.12 s^{-1} for the oxidation of the NADH moiety in the systems with oxygen and with MTT, respectively, and 3.2 s^{-1} and 0.18 s^{-1} for the reduction of the ethylphenazine moiety in the systems with oxygen and with MTT, respectively. On the bases of these results, the following three rate-acceleration mechanisms of the semi-synthetic glucose oxidase are discussed: high effective concentration, intramolecular coupling of successive catalytic reactions, and multiple connection between the two kinds of the catalytic sites.

Table II-1. Kinetic Parameters for the Reaction of EP^+-GlcDH-NAD^+

Electron acceptor	Kinetic constants				
	k_{cat} (s^{-1})	K_m (mM)	k_{en} (s^{-1})	$k_{in,ND}$ (s^{-1})	$k_{in,EP}$ (s^{-1})
O_2	0.40	1.57	0.39[a]	2.2	3.2
MTT	0.11	0.072	0.39	0.12	0.18

[a]The k_{en} value for the reaction system with oxygen is assumed to be the same as that for the system with MTT.

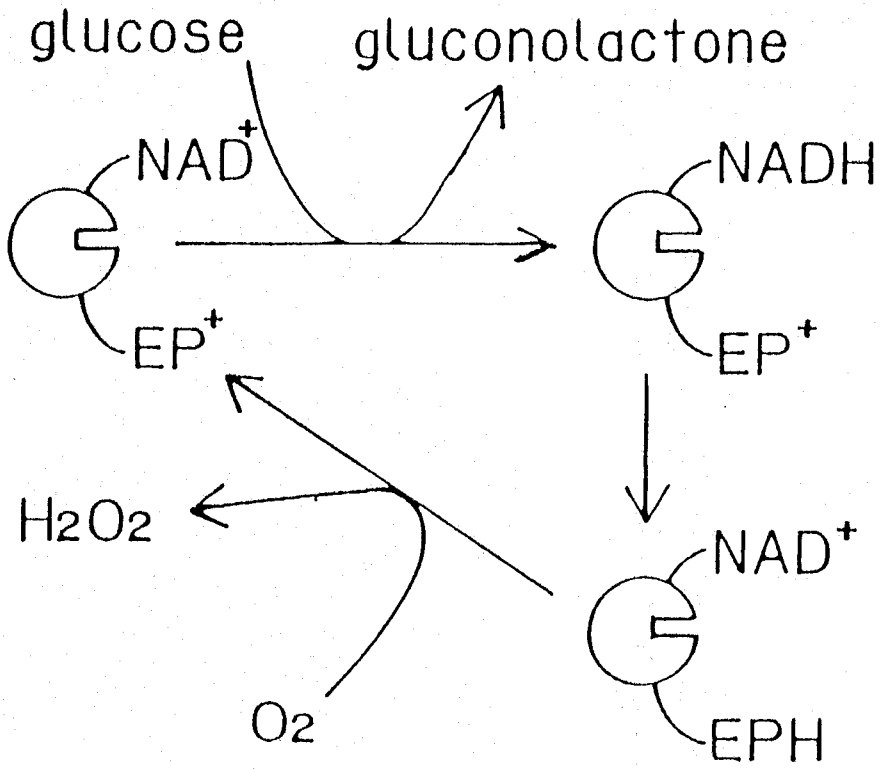


Fig. II-1. Schematic representation of the catalytic cycle of semisynthetic glucose oxidase. Only the cycle with the more oxidized forms of EP^+-GlcDH-NAD^+ is shown. EP^+, the oxidized form of the ethylphenazine moiety; EPH, the reduced form of the ethylphenazine moiety.

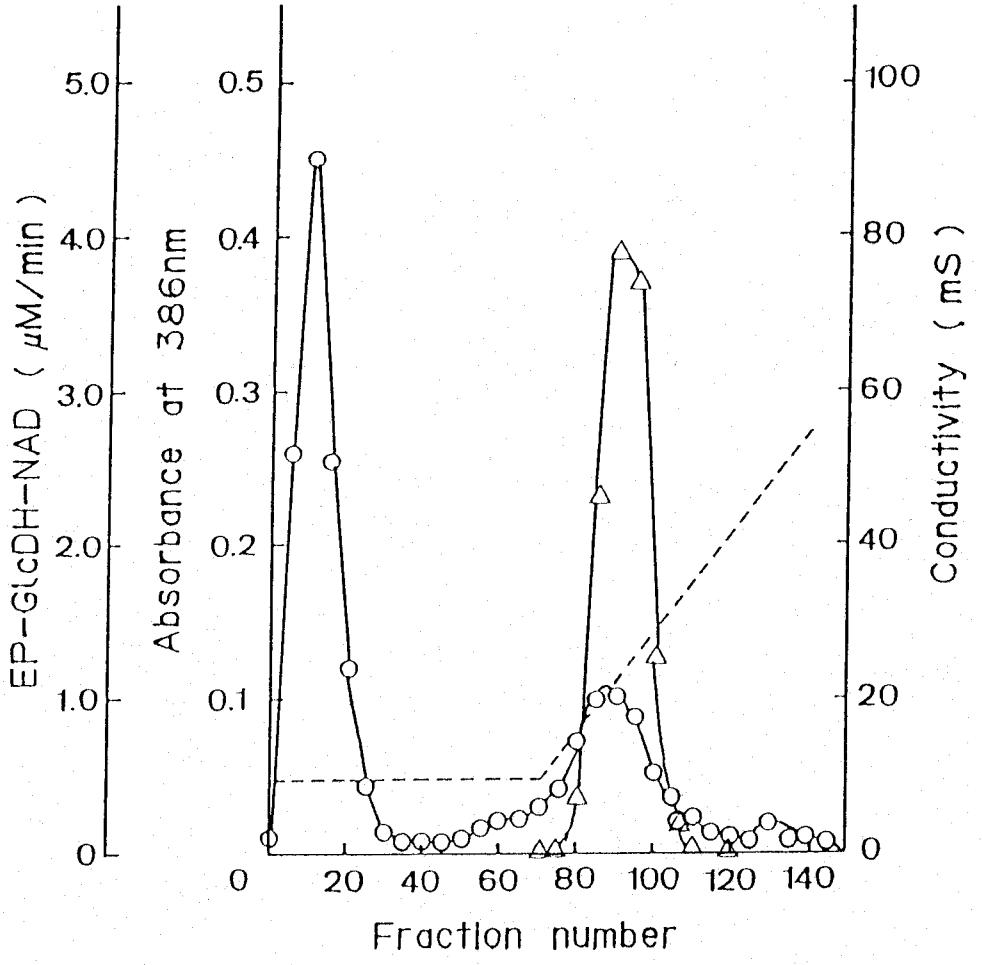


Fig. II-2. DEAE-Sephadex CL-6B column chromatography of EP^+-GlcDH-NAD^+. O , Absorbance at 386 nm; Δ, EP^+-GlcDH-NAD^+ activity; - - -, conductivity. The EP^+-GlcDH-NAD^+ activity is expressed as glucose oxidase activity measured by the MTT method as described under "Experimental Procedures".

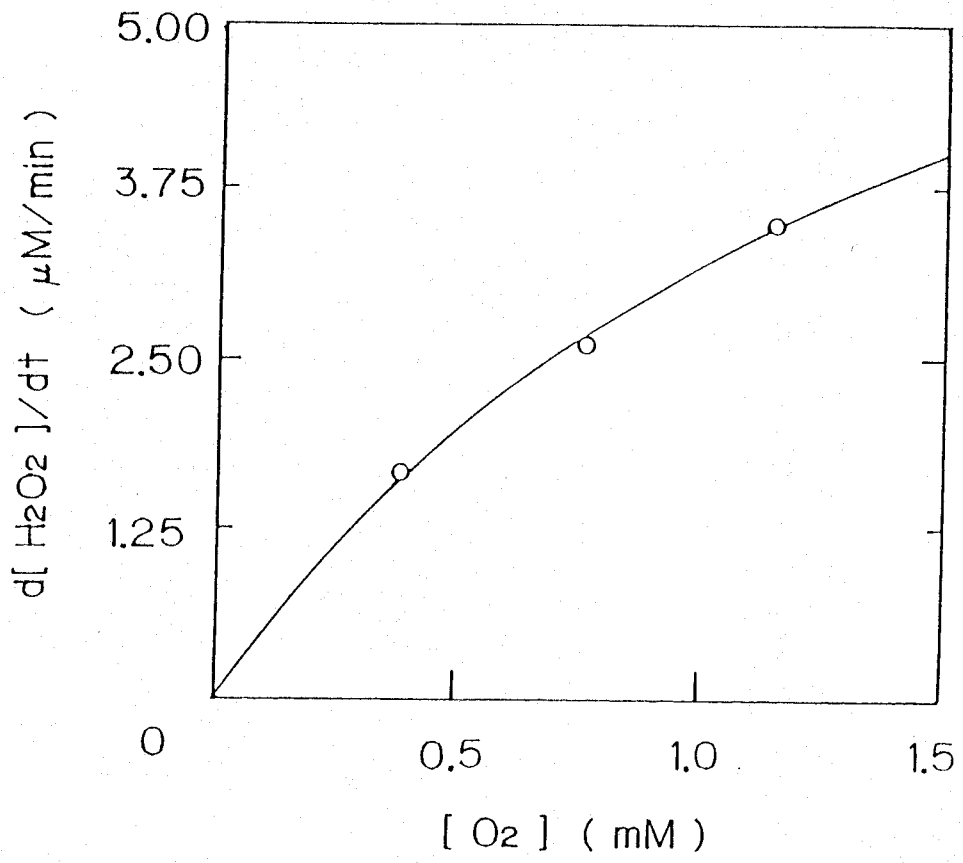


Fig. II-3. Effects of oxygen concentration on the activity of semisynthetic glucose oxidase. ——, the activity calculated from the Michaelis-Menten equation with V_{max} = 8.23 μM/min and K_m = 1.57 mM.

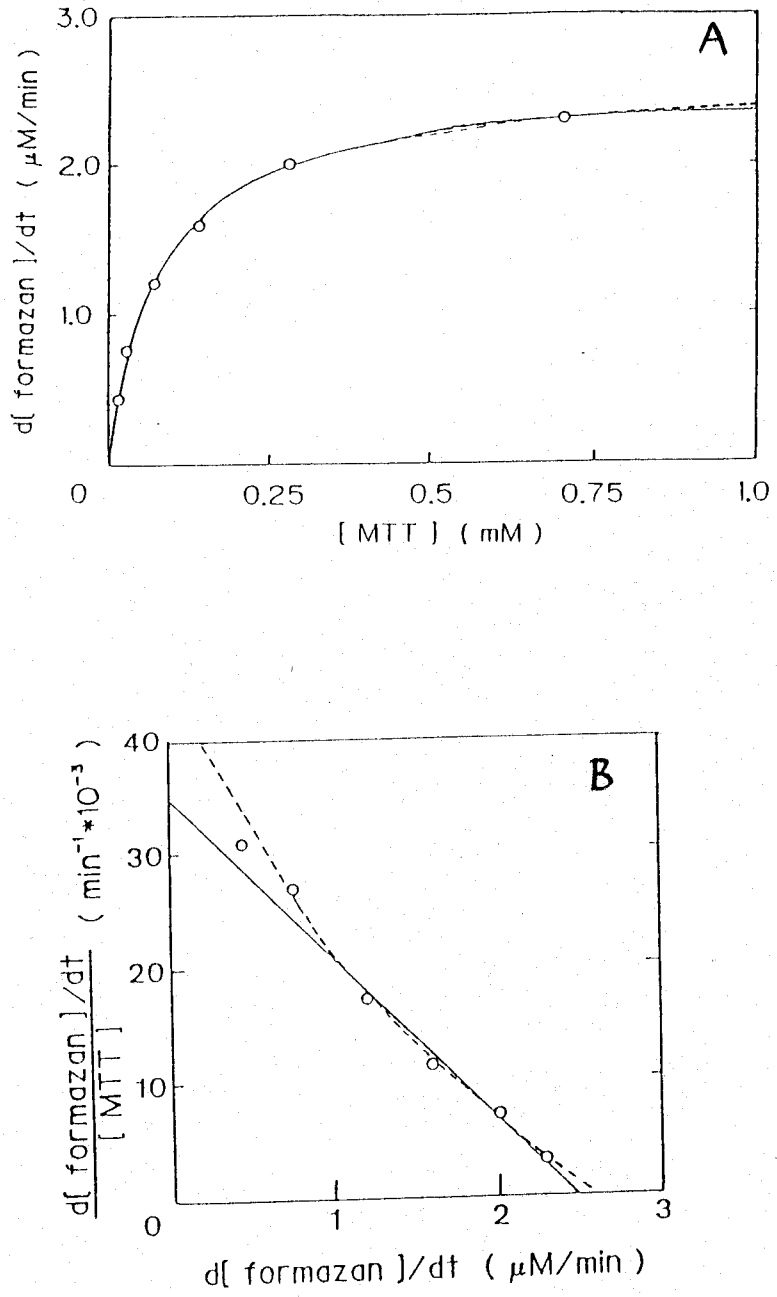


Fig. II-4. Effects of MTT concentration on the activity of semisynthetic glucose oxidase shown by a saturation curve (A) and by Eadie plot (B). ——, the activity calculated from the Michaelis-Menten equation with V_{max} = 2.50 μM/min and K_m = 0.072 mM; – – –, the activity calculated from Eqn II-19 with V_{max} = 0.65 μM/min, $K_{m,1} = K_{m,2}$ = 0.115 mM, $K_{m,3}$ = 0.116 mM, and $K_{m,4}$ = 0.024 mM.

Chapter III

Hide-and-Seek Effect

Introductory remarks

In our preceding chapter, EP^+-GlcDH-NAD, a semisynthetic glucose oxidase, was used to investigate the rate-acceleration effects by intramolecular coupling of two successive catalytic steps. In this chapter, EP^+-LDH-NAD^+, a semisynthetic lactate oxidase, is used to investigate the effects of hiding the NADH moiety in the binding site of the lactate dehydrogenase moiety. The catalytic cycle of this semisynthetic oxidase is schematically shown in Fig. III-1. This cycle is different from that of EP^+-GlcDH-NAD^+ in the point that there are additional two enzyme forms having NAD^+ or NADH moiety in the coenzyme-binding site of the conjugate, because lactate dehydrogenase has a much higher activity and affinity for PEG-NAD^+ than glucose dehydrogenase (Katayama et al., 1983; Okuda et al., 1985; Nakamura et al., 1986). In the chapter I, I reported that the NADH molecule bound in the coenzyme-binding site of lactate dehydrogenase does not react with a 1-substituted 5-ethylphenazine derivative (see the chapter I). Therefore, the ethylphenazine moiety of EP^+-LDH-NAD^+ cannot react with the hidden NADH moiety and must seek the free NADH moiety. This hide-and-seek effect quantitatively shown in this chapter is important for designing artificial enzymes.

Experimental Procedures

Materials

Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle was pur-

chased from Oriental Yeast Co. Ltd. (Tokyo); nucleotide pyrophosphatase (EC 3.6.1.9) was from Sigma (St. Louis); horseradish peroxidase (Grade III) was from Toyobo Co. Ltd. (Osaka); Sephacryl S-200 was purchased from Pharmacia (Uppsala). The other chemicals are the same as those described in our preceding chapter.

Concentration of Protein, Nucleotides, and 5-Ethylphenazine derivatives

The concentration of lactate dehydrogenase was measured spectrophotometrically at 280 nm using a molar absorption coefficient for the subunit of 40500 $M^{-1}cm^{-1}$ (Fromm, 1963). The concentrations of the ethylphenazine moiety, the enzyme moiety (as a subunit), and the NAD^+ moiety in EP^+-LDH-NAD^+ were calculated from the absorption spectrum of the conjugate using the following molar absorption coefficients: for the ethylphenazine moiety, 17000 $M^{-1}cm^{-1}$ at 386 nm, 43000 $M^{-1}cm^{-1}$ at 280 nm, and 28000 $M^{-1}cm^{-1}$ at 266 nm; for the enzyme moiety, 0 $M^{-1}cm^{-1}$ at 386 nm, 40500 $M^{-1}cm^{-1}$ at 280 nm, and 30600 $M^{-1}cm^{-1}$ at 266 nm; for the NAD^+ moiety, 0 $M^{-1}cm^{-1}$ at 386 nm, 12000 $M^{-1}cm^{-1}$ at 280 nm, and 21500 $M^{-1}cm^{-1}$ at 266 nm. Unless otherwise stated, the concentration of EP^+-LDH-NAD^+ was expressed as the concentration of the subunit of the lactate-dehydrogenase moiety. The concentrations of NAD^+, NADH, PEG-NAD^+, PEG-NADH, 5-ethylphenazine, and EP^+-PEG were measured spectrophotometrically using the molar absorption coefficients described in our preceding chapter.

Preparation of EP^+-LDH-NAD^+

EP^+-LDH-NAD^+ was prepared by the method for the preparation of EP^+-GlcDH-NAD^+ (see the chapter II). EP^+-PEG (10 μmol), PEG-NAD^+ (10 μmol), and DHBT (0.3 mmol) was dissolved in CH_2Cl_2 (4 ml), and the mixture was kept at 4°C for 3 h. Then 0.5 ml of 0.1 M sodium phosphate buffer, pH

7.5, was added to the mixture, and CH_2Cl_2 was removed by evaporation. The mixture was transferred to a centrifuge tube with 1.5 ml of the same buffer, and a precipitate was removed by centrifugation. The supernatant was concentrated to 1 ml, and 0.5 ml of 2.3 mM lactate dehydrogenase solution in the same buffer was added to the supernatant. The reaction mixture was kept at 15°C for 15 h, and EP^+-LDH-NAD^+ thus prepared in the mixture was purified by gel filtration through a Sephacryl S-200 (Pharmacia) column (5 x 70 cm) equilibrated with the same buffer at a flow rate of 20 ml/h. Fractions (3.4 ml) were collected, and absorbances at 386, 280, and 266 nm and lactate oxidase activity were monitored (Fig. III-2). The fractions 32-38 were combined and used as EP^+-LDH-NAD^+.

Assay of Reaction Rates

Initial rates of reactions were measured at 30°C in 0.1 M sodium phosphate buffer, pH 7.5, with a Hitachi 220 spectrophotometer with a thermostated cell compartment. MTT or oxygen was used as an electron acceptor. The reactions were recorded as the increase in absorbance at 570 nm (formazan concentration) or at 510 nm (corresponds to H_2O_2 concentration) for the MTT or O_2 method, respectively, as described in our preceding chapter. The assays were made at least in duplicate with a reproducibility of less that 5% error.

The reaction rates of 5-ethylphenazine with PEG-NADH were measured by the MTT method in the reaction mixture (3.07 ml) containing 1.5 mM MTT, 2.3 mM 5-ethylphenazine, and PEG-NADH; the concentration of PEG-NADH was varied from 0 to 47.4 μM for obtaining the second-order rate constant. Lactate dehydrogenase activity in the presence of exogenous NAD^+ and 5-ethylphenazine was measured by the MTT method, and the components of assay mixtures are given in the legend to Table III-1. The

lactate oxidase activity of EP^+-LDH-NAD^+ was measured by the MTT method and also by the O_2 method as described in our preceding chapter; the components of the assay mixtures are given in the legends to Figs. III-2 - III-6.

Results and Discussion

Characterization of EP^+-LDH-NAD^+

EP^+-PEG and PEG-NAD^+ were covalently linked to lactate dehydrogenase. Figure III-2 shows the Sephacryl S-200 column chromatography for purification of EP^+-LDH-NAD^+. The first small peak of absorbance at 386 nm, which corresponds to the concentration of the ethylphenazine moiety, coincides with the first peak of lactate oxidase activity. This first peak of absorbance at 386 nm is well separated from the other higher peaks (fractions 43-70) corresponding to EP^+-PEG activated with DHBT and its hydrolyzed product. Although Fig. III-2 shows, for simplicity, only the profiles of absorbance at 386 nm and lactate oxidase activity, activated PEG-NAD^+ and its hydrolyzed products are also in these higher peaks (fractions 42-67) eluted later than the first peak, and the peak (fractions 29-43) of the subunit concentration of lactate dehydrogenase overlaps the two peaks (fractions 29-44) of lactate oxidase activity; the approximate concentrations of the NAD^+ moiety and the lactate dehydrogenase moiety can be estimated from absorbances at 386, 280, and 266 nm as described under Experimental Procedures. Therefore, fractions 32-38 were combined and used as EP^+-LDH-NAD^+.

The average number of the ethylphenazine moieties bound per molecule of enzyme subunit (EP^+ content) is 0.46, and that of the NAD^+ moieties (NAD^+ content) is 0.32. Almost all the NAD^+ moieties are

reduced by the lactate-dehydrogenase moieties in the presence of L-lactate. In the presence of 0.1 mM NAD^+ and 0.82 mM 5-ethylphenazine exogenously, the lactate dehydrogenase activity of EP^+-LDH-NAD^+ was very low. Then I measured the activity in the presence of much higher concentrations of exogenous NAD^+ (1.6 mM and 68.8 mM), and the results are shown in Table III-1. The activity of the conjugate is still lower than that of the native enzyme, and increases with the increase in the NAD^+ concentration, while the activity of the native enzyme at 68.8 mM NaD^+ is the same as that at 1.6 mM NAD^+. This means that the conjugate has much larger apparent K_m value for exogenous NAD^+ than the native enzyme. MDH-PEG-NAD^+ also has a larger K_m value (Eguchi et al., 1986). These larger K_m values for exogenous NAD^+ of the conjugates are considered to be due to competitive inhibition by the bound NADH moiety. To confirm this point, the pyrophosphate bond of the NAD^+ moiety of EP^+-LDH-NAD^+ was hydrolyzed by nucleotide pyrophosphatase, and then the activity was measured (Table III-1). These results show that the inhibition by the bound NADH moiety is relieved by the pyrophosphatase treatment because of much lower affinity of the AMP moiety than that of the NADH moiety. These results also show that all the lactate-dehydrogenase moiety is active, though the chemical modification of lysine residues may cause some alterations in the kinetic parameters.

Lactate Oxidase Activity of EP^+-LDH-NAD^+

EP^+-LDH-NAD^+ catalyzes the oxidation of L-lactate by oxygen or MTT, and H_2O_2 or formazan is produced, respectively, and therefore EP^+-LDH-NAD^+ works as lactate oxidase. At this point, EP^+-LDH-NAD^+ is very similar to EP^+-GlcDH-NAD^+ (see the chapter II), and these successful results show that this is a general method for artificial conversion of a dehydrogenase into an oxidase. By this method, new oxidases can be

prepared from the corresponding dehydrogenases.

Figure III-3 shows the effects of oxygen concentrations on the activity of the semisynthetic lactate oxidase. The kinetic parameters for the Michaelis-Menten equation were calculated from these results by the Marquardt method (Marquardt, 1963), and the values are: V_{max} = 3.51 μM/min and K_m = 1.91 mM with a standard deviation of 0.028. Figure III-4 shows the effects of MTT concentrations on the oxidase activity. The kinetic parameters were similarly calculated (omitting the point at [MTT] = 0.176 mM) and the values are: V_{max} = 0.42 μM/min and K_m = 0.076 mM with a standard deviation of 0.022. From these V_{max} values, the turnover numbers (k_{cat}) per subunit are calculated to be 2.3 min^{-1} and 0.25 min^{-1} for the assay systems with oxygen and with MTT, respectively. The dependence of the k_{cat} value on the kind of the electron acceptor was also observed for EP^+-GlcDH-NAD^+ (see the chapter II).

Figures III-3 and III-4 show that the experimental results fit the Michaelis-Menten kinetics well; this means that each EP^+-LDH-NAD^+ molecule has similar apparent V_{max} and K_m values, though the preparation of EP^+-LDH-NAD^+ is not homogeneous in the reactivities of the the NAD^+ and the ethylphenazine moieties. To confirm this homogeneity, I divided EP^+-LDH-NAD^+ into four groups with the same V_{max} value but with different K_m values ($K_{m,1}$, $K_{m,2}$, $K_{m,3}$, and $K_{m,4}$), and obtained these values by fitting the following equation to the data points (omitting the point at [MTT] = 0.176 mM) for the MTT system by the Marquardt method (Marquardt, 1963) with a standard deviation of 0.0028: V_{max} = 0.107 μM/min, $K_{m,1}$ = 0.058 mM, $K_{m,2}$ = $K_{m,3}$ = 0.060 mM, and $K_{m,4}$ = 0.214 mM.

$$d[\text{formazan}]/dt = V_{max}[MTT]\{1/(K_{m,1} + [MTT]) + 1/(K_{m,2} + [MTT]) + 1/(K_{m,3} + [MTT]) + 1/(K_{m,4} + [MTT])\} \quad \text{(III-1)}$$

The resulted curve for Eqn III-1 is shown in Fig. III-4A as a broken line, and is almost the same as the solid line obtained for the simple Michaelis-Menten equation. Figure III-4B shows the Eadie plot for the same results. These analyses indicate that the experimental results can well be explained by both equations, and for simplicity, I assume the homogeneity of EP^+-LDH-NAD^+ in its apparent V_{max} and K_m.

Kinetic Equation for the Semisynthetic Lactate Oxidase

As discussed above and in our preceding chapter, the preparation of EP^+-LDH-NAD^+ is not homogeneous in the numbers of the ethylphenazine moieties and the NAD^+ moieties and also in the reactivities between the NAD^+ moiety and the active site of the lactate dehydrogenase moiety and between the NADH moiety and the ethylphenazine moiety. Therefore, the tetrameric molecules of EP^+-LDH-NAD^+ are grouped as follows: the tetramers of the conjugate in a group (for example, the i'th group) have the same number of the ethylphenazine moieties and the same number of the NAD(H) moieties; each tetramer in the i'th group has the same apparent first-order rate constant, k_{ij}, for the reduction of the j'th NAD^+ moiety; and each tetramer in the i'th group has the same intramolecular rate constant, $k_{hj,i}$, for the reoxidation of the j'th NADH moiety in the free state (i.e., not bound to the coenzyme site of the lactate dehydrogenase moiety) with the h'th ethylphenazine moiety.

The molecular species of tetrameric EP^+-LDH-NAD^+ in the i'th group can be divided into two groups with respect to the oxidation/reduction state of the ethylphenazine or NAD^+ moiety or to the free/bound state of the NAD^+ moiety, and the material balances for these molecular species are expressed as follows:

[EP(H)-LDH_i-NAD(H)]

$$= [EP^{+}_{h}\text{-}LDH_{i}\text{-}NAD(H)] + [EPH_{h}\text{-}LDH_{i}\text{-}NAD(H)] \quad \text{(III-2)}$$

$$= [EP(H)\text{-}LDH_{i}\text{-}NAD(H)_{j,f}] + [EP(H)\text{-}LDH_{i}\text{-}NAD(H)_{j,b}] \quad \text{(III-3)}$$

$[EP^{+}_{h}\text{-}LDH_{i}\text{-}NAD(H)]$

$$= [EP^{+}_{h}\text{-}LDH_{i}\text{-}NAD(H)_{j,f}] + [EP^{+}_{h}\text{-}LDH_{i}\text{-}NAD(H)_{j,b}] \quad \text{(III-4)}$$

$[EP(H)\text{-}LDH_{i}\text{-}NAD(H)_{j,f}]$

$$= [EP(H)\text{-}LDH_{i}\text{-}NAD^{+}_{j,f}] + [EP(H)\text{-}LDH_{i}\text{-}NADH_{j,f}] \quad \text{(III-5)}$$

$[EP(H)\text{-}LDH_{i}\text{-}NADH_{j,f}]$

$$= [EP^{+}_{h}\text{-}LDH_{i}\text{-}NADH_{j,f}] + [EPH_{h}\text{-}LDH_{i}\text{-}NADH_{j,f}] \quad \text{(III-6)}$$

$d[EP(H)\text{-}LDH_{i}\text{-}NAD^{+}_{j,f}]/dt$

$$= \sum_{h}(k_{hj,i}[EP^{+}_{h}\text{-}LDH_{i}\text{-}NADH_{j,f}]) - k_{ij}[EP(H)\text{-}LDH_{i}\text{-}NAD^{+}_{j,f}] \quad \text{(III-7)}$$

$$= \sum_{h}(k_{hj,i}\beta_{EP^{+}_{h}\text{-}i\text{-}NADH_{jf}})[EP(H)\text{-}LDH_{i}\text{-}NADH_{j,f}] - k_{ij}[EP(H)\text{-}LDH_{i}\text{-}NAD^{+}_{j,f}] \quad \text{(III-8)}$$

$d[EP^{+}_{h}\text{-}LDH_{i}\text{-}NAD(H)]/dt$

$$= (k_{ox}[O_2] + k_{MT}[MTT])[EPH_{h}\text{-}LDH_{i}\text{-}NAD(H)] - \sum_{j}(k_{hj,i}[EP^{+}_{h}\text{-}LDH_{i}\text{-}NADH_{j,f}]) \quad \text{(III-9)}$$

$$= (k_{ox}[O_2] + k_{MT}[MTT])[EPH_{h}\text{-}LDH_{i}\text{-}NAD(H)] - \sum_{j}(k_{hj,i}\gamma_{EP^{+}_{h}\text{-}i\text{-}NADH_{jf}})[EP^{+}_{h}\text{-}LDH_{i}\text{-}NAD(H)_{j,f}] \quad \text{(III-10)}$$

where [ ] shows the concentration, and for the conjugate, the concentration is expressed as the concentration of the tetramer; k_{ox} and k_{MT} are the second-order rate constants of the reactions of the reduced ethylphenazine moiety with oxygen and with MTT, respectively. The molecular species of the conjugate are expressed as follows: $EP(H)\text{-}LDH_{i}\text{-}NAD(H)$, every form of the conjugate in the i'th group; $EPH_{h}\text{-}LDH_{i}\text{-}NAD(H)$, every form of the i'th group conjugate whose h'th ethylphenazine moiety is in the reduced form; $EP(H)\text{-}LDH_{i}\text{-}NAD(H)_{j,b}$, every form of the i'th group conjugate whose j'th NAD(H) moiety is in the bound state (i.e., bound in the coenzyme site of the lactate dehydrogenase moiety); $EP^{+}_{h}\text{-}LDH_{i}\text{-}NADH_{j,f}$, every form of the i'th group conjugate whose h'th ethyl-

phenazine moiety is in the oxidized form and j'th NAD(H) moiety is in the free state and in the reduced form; and so on. The fractions, $\beta_{EP^+_h\text{-}i\text{-}NADH_{jf}}$, $\beta_{EPH_h\text{-}i}$, $\gamma_{EP^+_h\text{-}i\text{-}NADH_{jf}}$, $\gamma_{i\text{-}NADH_{jf}}$, and $\delta_{i\text{-}j}$ are defined as:

$$\beta_{EP^+_h\text{-}i\text{-}NADH_{jf}} = [EP^+{}_h\text{-}LDH_i\text{-}NADH_{j,f}]/[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}] \quad \text{(III-11)}$$

$$\beta_{EPH_h\text{-}i} = [EPH_h\text{-}LDH_i\text{-}NAD(H)]/[EP(H)\text{-}LDH_i\text{-}NAD(H)] \quad \text{(III-12)}$$

$$\gamma_{EP^+_h\text{-}i\text{-}NADH_{jf}} = [EP^+{}_h\text{-}LDH_i\text{-}NADH_{j,f}]/[EP^+{}_h\text{-}LDH_i\text{-}NAD(H)_{j,f}] \quad \text{(III-13)}$$

$$\gamma_{i\text{-}NADH_{jf}} = [EP(H)\text{-}LDH_i\text{-}NADH_{j,f}]/[EP(H)\text{-}LDH_i\text{-}NAD(H)_{j,f}] \quad \text{(III-14)}$$

$$\delta_{i\text{-}j} = [EP(H)\text{-}LDH_i\text{-}NAD(H)_{j,f}]/[EP(H)\text{-}LDH_i\text{-}NAD(H)] \quad \text{(III-15)}$$

In Eqns III-7 and III-9, I assumed that the NADH moiety bound in the coenzyme-binding site does not react with the ethylphenazine moiety (see the chapter I), and that the rates of intermolecular reactions between the conjugates are negligible compared to the corresponding intramolecular-reaction rates; the latter simplification is justified from the fact shown later that the effective concentrations of the NAD^+, ethylphenazine, and dehydrogenase moieties are much higher than their actual concentrations. I also neglected the reverse reaction of the lactate dehydrogenase moiety, because the rates were assayed at the initial steady state of the reaction.

At steady state,

$$d[EP(H)\text{-}LDH_i\text{-}NAD^+]/dt = d[EP^+{}_h\text{-}LDH_i\text{-}NAD(H)]/dt = 0 \quad \text{(III-16)}$$

From Eqns III-5, III-8, III-14, and III-16, $\gamma_{i\text{-}NADH_{jf}}$ is expressed as:

$$\gamma_{i\text{-}NADH_{jf}} = k_{ij}/\{k_{ij} + \sum_h (k_{hj,i}\beta_{EP^+_h\text{-}i\text{-}NADH_{jf}})\} \quad \text{(III-17)}$$

From Eqns III-2, III-10, III-12, III-15 and III-16, $\beta_{EPH_h\text{-}i}$ is expressed as:

$$\beta_{EPH_h\text{-}i} = \sum_j (k_{hj,i}\delta_{i\text{-}j}\gamma_{EP^+_h\text{-}i\text{-}NADH_{jf}})/\{k_{ox}[O_2] + k_{MTT}[MTT] + \sum_j (k_{hj,i}\delta_{i\text{-}j}\gamma_{EP^+_h\text{-}i\text{-}NADH_{jf}})\} \quad \text{(III-18)}$$

The reaction rate measured by the O_2 method is expressed as:

$$d[H_2O_2]/dt = k_{ox}[O_2]\sum_i\sum_h[EPH_h\text{-}LDH_i\text{-}NAD(H)] \quad \text{(III-19)}$$

Using Eqns III-12 and III-18 and the conditions of [MTT] = 0, Eqn III-19 is converted to:

$$d[H_2O_2]/dt = k_{ox}[O_2]\sum_i\sum_h[\sum_j(k_{hj,i}\delta_{i-j}\gamma_{EP^+_h}\text{-i-}\mathbf{NADH}_{jf}) \times [EP(H)\text{-}LDH_i\text{-}NAD(H)]/\{k_{ox}[O_2] + \sum_j(k_{hj,i}\delta_{i-j}\gamma_{EP^+_h}\text{-i-}\mathbf{NADH}_{jf})\}] \quad \text{(III-20)}$$

On the other hand, the reaction rate measured by the MTT method is expressed as:

$$d[\text{formazan}]/dt = k_{MT}[MTT]\sum_i\sum_h[EPH_h\text{-}LDH_i\text{-}NAD(H)] \quad \text{(III-21)}$$

Using Eqns III-12 and III-18, Eqn III-21 is converted to:

$$d[\text{formazan}]/dt = k_{MT}[MTT]\sum_i\sum_h[\sum_j(k_{hj,i}\delta_{i-j}\gamma_{EP^+_h}\text{-i-}\mathbf{NADH}_{jf}) \times [EP(H)\text{-}LDH_i\text{-}NAD(H)]/\{k_{ox}[O_2] + k_{MT}[MTT] + \sum_j(k_{hj,i}\delta_{i-j}\gamma_{EP^+_h}\text{-i-}\mathbf{NADH}_{jf})\}] \quad \text{(III-22)}$$

State of the NAD(H) Moiety

During the catalytic cycle of EP^+-LDH-NAD^+ (Fig. III-1), the NAD(H) moiety takes four kinds of states by the combination of the free and bound states and the oxidized and reduced states. To understand the kinetic mechanism of this lactate oxidase reaction, it is important to know what kind of state the NAD(H) moiety mainly takes at steady state. For this purpose, I investigated a reaction system containing EP^+-LDH-NAD^+, L-lactate, MTT, and exogenously added 5-ethylphenazine.

In the presence of exogenous 5-ethylphenazine, Eqns III-17 and III-21 are converted respectively to:

$$\gamma_{i\text{-}\mathbf{NADH}_{jf}} = k_{ij}/\{k_{ij} + \sum_h(k_{hj,i}\beta_{EP^+_h}\text{-i-}NADH_{jf}) + k_{PS}[PES_{ox}]\} \quad \text{(III-23)}$$

$$d[\text{formazan}]/dt = k_{MT}[MTT](\sum_i\sum_h[EPH_h\text{-}LDH_i\text{-}NAD(H)] + [PES_{rd}]) \quad \text{(III-24)}$$

where k_{PS} is the second-order rate constant of the reaction between the NADH moiety and exogenous 5-ethylphenazine, and $[PES_{ox}]$ and $[PES_{rd}]$ are the concentrations of the oxidized and reduced forms of 5-ethylphenazine, respectively. The material balances for 5-ethylphenazine are:

$$[PES]_t = [PES_{ox}] + [PES_{rd}] \quad \text{(III-25)}$$

$$d[PES_{ox}]/dt = (k_{ox}[O_2] + k_{MT}[MTT])[PES_{rd}] - k_{PS}\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}][PES_{ox}] \quad \text{(III-26)}$$

where $[PES]_t$ is the total concentration of 5-ethylphenazine. At steady state, the following equations are obtained from Eqns III-25 and III-26:

$$[PES_{rd}] = k_{PS}\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}][PES]_t/(k_{ox}[O_2] + k_{MT}[MTT] + k_{PS}\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}]) \quad \text{(III-27)}$$

$$[PES_{ox}] = (k_{ox}[O_2] + k_{MT}[MTT])[PES]_t/(k_{ox}[O_2] + k_{MT}[MTT] + k_{PS}\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}]) \quad \text{(III-28)}$$

In the presence of excess MTT,

$$k_{MT}[MTT] >> k_{ox}[O_2] + k_{PS}\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}] \quad \text{(III-29)}$$

In this case, Eqns III-27 and III-28 are simplified respectively to:

$$[PES_{rd}] = k_{PS}\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}]/(k_{MT}[MTT]) \quad \text{(III-30)}$$

$$[PES_{ox}] = [PES]_t \quad \text{(III-31)}$$

Using Eqns III-10, III-11, III-13, III-16, and III-30, Eqn III-24 is converted to:

$$d[formazan]/dt = \sum_i\sum_h\sum_j(k_{hj,i}\beta_{EP^+_h\text{-}i\text{-}NADH_{jf}}[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}]) + k_{PS}[PES]_t\sum_i\sum_j[EP(H)\text{-}LDH_i\text{-}NADH_{j,f}] \quad \text{(III-32)}$$

In the presence of high concentrations of MTT, Eqn III-18 becomes $\beta_{EPH\text{-}i} \cong 0$. This means that almost all the ethylphenazine groups are in the oxidized form. Therefore,

$$\beta_{EP^+_h\text{-}i\text{-}NADH_{jf}} \cong 1 \quad \text{(III-33)}$$

Using Eqns III-31 and III-33, Eqn III-23 is simplified to:

$$\gamma_{i\text{-}NADH_{jf}} = k_{ij}/(k_{ij} + \sum_h k_{hj,i} + k_{PS}[PES]_t) \quad \text{(III-34)}$$

Using Eqns III-14, III-15, III-33 and III-34, Eqn III-32 is converted to:

$$d[\text{formazan}]/dt = \sum_i \sum_j \{k_{ij}\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)](\sum_h k_{hj,i} + k_{PS}[PES]_t)/(k_{ij} + \sum_h k_{hj,i} + k_{PS}[PES]_t)\} \quad (III\text{-}35)$$

The lactate oxidase activity of EP^+-LDH-NAD^+ was measured by the MTT method in the presence of excess MTT and various concentrations of exogenous 5-ethylphenazine, and the results are shown in Fig. III-5. This figure indicates that the reaction rate is proportional to $[PES]_t$. Therefore, the following relationship is satisfied in Eqn III-35 under these experimental conditions:

$$k_{ij} + \sum_h k_{hj,i} >> k_{PS}[PES]_t \quad (III\text{-}36)$$

Then, Eqn III-35 is simplified to:

$$d[\text{formazan}]/dt = \sum_i \sum_j \{k_{ij}\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)](\sum_h k_{hj,i} + k_{PS}[PES]_t)/(k_{ij} + \sum_h k_{hj,i})\} \quad (III\text{-}37)$$

Using the values of the slope and the y-intercept of the straight line shown in Fig. III-5, the following equations are obtained, respectively:

$$k_{PS}\sum_i \sum_j \{k_{ij}\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)]/(k_{ij} + \sum_h k_{hj,i})\} = 1.98 \times 10^{-5}\ s^{-1} \quad (III\text{-}38)$$

$$\sum_i \sum_j \{k_{ij}\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)](\sum_h k_{hj,i})/(k_{ij} + \sum_h k_{hj,i})\} = 6.68 \times 10^{-6}\ mM\ s^{-1} \quad (III\text{-}39)$$

On the other hand, the k_{PS} value was obtained to be 1.74 $mM^{-1}s^{-1}$ from the reaction of 5-ethylphenazine and PEG-NAD^+; the reaction conditions are described under "Experimental Procedures". If every NADH moiety has the same value of $\sum_h k_{hj,i}$, the value is estimated to be 0.59 s^{-1} from Eqns III-38 and III-39 and the k_{PS} value. Therefore, even if the $\sum_h k_{hj,i}$ values are heterogeneous, most of the values are considered to be less than 6 s^{-1}. In addition, under the experimental

conditions for Fig. III-5, the value of $k_{PS}[PES]_t$ is less than 1 s^{-1}. Thus, the $k_{PS}[PES]_t$ value is the same order as the $\sum_h k_{hj,i}$ values, and the following relationship is derived from Eqn III-36:

$$k_{ij} >> \sum_h k_{hj,i} \qquad \text{(III-40)}$$

Using this relationship, Eqns III-38 and III-39 are simplified to:

$$k_{PS}\sum_i\sum_j(\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)]) = 1.98 \times 10^{-5}\ s^{-1} \qquad \text{(III-41)}$$

$$\sum_i\sum_j\sum_h(k_{hj,i}\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)]) = 6.68 \times 10^{-6}\ mM\ s^{-1} \qquad \text{(III-42)}$$

For a homogeneous system with a constant $\sum_h k_{hj,i}$, these equations are simplified to:

$$k_{PS}\delta[NAD(H)]_t = 1.98 \times 10^{-5}\ s^{-1} \qquad \text{(III-43)}$$

$$k_{in,ND}\delta[NAD(H)]_t = 6.68 \times 10^{-6}\ mM\ s^{-1} \qquad \text{(III-44)}$$

where $[NAD(H)]_t$ is the total concentration of the NAD^+ plus NADH moieties, $k_{in,ND}$ is the apparent intramolecular rate constant for the oxidation of the NADH moiety defined as:

$$k_{in,ND} = \sum_h k_{hj,i} \quad (i, j = 1, 2, \ldots.) \qquad \text{(III-45)}$$

and δ is the fraction of the free NAD(H) moiety defined as:

$$\delta = \sum_i\sum_j(\delta_{i-j}[EP(H)\text{-}LDH_i\text{-}NAD(H)])/[NAD(H)]_t \qquad \text{(III-46)}$$

From Eqns III-43 and III-44, the values of δ and $k_{in,ND}$ are calculated to be 0.021 and 0.59 s^{-1}, respectively. Using Eqns III-36 and III-40, Eqn III-34 is simplified to:

$$\gamma_{i\text{-}NADH;jf} = 1 \qquad \text{(III-47)}$$

This equation and the δ value mean that only 2.1 % of the NAD(H) moieties are in the free state under our experimental conditions, and that almost all the free NAD(H) moieties are in the reduced form irrespective of the concentration of the electron acceptors. This state of the NAD(H) moiety reflects the high affinity of the coenzyme-binding site of the lactate-dehydrogenase moiety and a much larger catalytic

rate constant of the dehydrogenase moiety than that of the ethylphenazine moiety. As the reaction rates are measured at the initial steady state, the reverse reaction by the dehydrogenase moiety must be negligible; therefore, almost all the bound NAD(H) moieties must also be in the reduced form. It should be noted that the state and form of each NAD(H) moiety are not constant, but change depending on time.

Kinetic Parameters for Intramolecular Reactions

As described above, almost all the NAD(H) moieties of EP^+-LDH-NAD^+ are in the reduced form, and therefore Eqns III-20 and III-22 are simplified, respectively, using $\gamma_{EP^+_h\text{-}i\text{-}NADH_j f} = 1$ from Eqn III-47 to:

$$d[H_2O_2]/dt = k_{ox}[O_2]\sum_i\sum_h[\sum_j(k_{hj,i}\delta_{i-j})[EP(H)\text{-}LDH_i\text{-}NAD(H)]/\{\sum_j(k_{hj,i}\delta_{i-j}) + k_{ox}[O_2]\}] \quad \text{(III-48)}$$

$$d[\text{formazan}]/dt = k_{MT}[MTT]\sum_i\sum_h\{\sum_j(k_{hj,i}\delta_{i-j})[EP(H)\text{-}LDH_i\text{-}NAD(H)]/(\sum_j(k_{hj,i}\delta_{i-j}) + k_{ox}[O_2] + k_{MT}[MTT])\} \quad \text{(III-49)}$$

These equations indicate that the value of $\sum_j(k_{hj,i}\delta_{i-j})$ must be constant or zero, when they are simplified to the Michaelis-Menten equation as suggested from the results shown in Figs. III-3 and 4. As most of the NADH moieties are hidden in the coenzyme-binding sites at steady state, and as the NAD^+ content per tetramer of EP^+-LDH-NAD^+ is 1.3, most of the tetramers do not have a free NADH moiety, that is, the number of the free NADH moieties on each tetramer is zero or at most one. In this case, the fraction of the tetramers having one free NADH moiety can be expressed as $\delta \cdot n$ assuming that almost all the NAD(H) moieties are in the reduced form, where n is the NAD^+ content per tetramer, and the fraction of the ethylphenazine moieties on the tetramers having one free NADH moiety is also expressed as $\delta \cdot n$. For such ethylphenazine moieties, $\sum_j(k_{hj,i}\delta_{i-j}) = k_{hj,i}$, and for the other ethylphenazine moieties, $\sum_j(k_{hj,i}\delta_{i-j}) = 0$. Therefore, Eqns III-48 and

III-49 are converted, respectively, to:

$$d[H_2O_2]/dt = \delta n k_{ox}[O_2] \sum_i \sum_h \{k_{hj,i}[EP(H)\text{-}LDH_i\text{-}NAD(H)]/ (k_{hj,i} + k_{ox}[O_2])\} \quad \text{(III-50)}$$

$$d[\text{formazan}]/dt = \delta n k_{MT}[MTT] \sum_i \sum_h \{k_{hj,i}[EP(H)\text{-}LDH_i\text{-}NAD(H)]/ (k_{hj,i} + k_{ox}[O_2] + k_{MT}[MTT])\} \quad \text{(III-51)}$$

For a homogeneous system with a constant $k_{hj,i}$, these equations are simplified to:

$$d[H_2O_2]/dt = \delta n k_{in} k_{ox}[EP(H)]_t[O_2]/(k_{in} + k_{ox}[O_2]) \quad \text{(III-52)}$$

$$d[\text{formazan}]/dt = \delta n k_{in} k_{MT}[EP(H)]_t[MTT]/(k_{in} + k_{ox}[O_2] + k_{MT}[MTT]) \quad \text{(III-53)}$$

where $[EP(H)]_t$ is the total concentration of the ethylphenazine moiety, and k_{in} is the intramolecular rate constant for the reaction between a free NADH moiety and an oxidized ethylphenazine moiety, and is defined as:

$$k_{in} = k_{hj,i} \ (h, i, j = 1, 2, \ldots.) \quad \text{(III-54)}$$

On the other hand, K_m for oxygen ($K_{m,ox}$) and that for MTT ($K_{m,MT}$) are expressed from Eqns III-52 and III-53, respectively as:

$$K_{m,ox} = k_{in}/k_{ox} \quad \text{(III-55)}$$

$$K_{m,MT} = (k_{in} + k_{ox}[O_2])/k_{MT} \quad \text{(III-56)}$$

Therefore, the k_{in} value is calculated from Eqn III-55 to be 2.3 s^{-1}, using the $K_{m,ox}$ and k_{ox} values of 1.91 mM (Fig. III-3) and 1.22 $mM^{-1}s^{-1}$ (Yomo et al., 1989b), respectively. The k_{in} value is also calculated from Eqn III-56 to be 2.1 s^{-1}, using the $K_{m,MT}$, k_{MT}, and $[O_2]$ values of 0.076 mM (Fig. III-4), 32 $mM^{-1}s^{-1}$ (Yomo et al., 1989b), and 0.24 mM, respectively; these k_{in} values agree well.

The apparent intramolecular rate constant ($k_{in,EP}$) for the reduction of the ethylphenazine moiety is expressed as:

$$k_{in,EP} = \delta n k_{in} \quad \text{(III-57)}$$

The $k_{in,EP}$ values for the reaction systems with oxygen and with MTT are thus calculated to be 0.062 s^{-1} and 0.056 s^{-1}, respectively, assuming the same δ value for the two systems. These $k_{in,EP}$ values are much smaller than those obtained for EP^+-GlcDH-NAD^+ (see the chapter II) due to the effect of hiding the NADH moieties by the coenzyme sites of the dehydrogenase moieties. If $\delta = 1$, the value of $k_{in,EP}$ is calculated to be about 3 s^{-1}; this value is the same order as that of EP^+-GlcDH-NAD^+ obtained in the assay system with oxygen.

The apparent intramolecular rate constant, $k_{in,ND}$, defined by Eqn III-45 is expressed, similarly to Eqn III-58, by the following equation:

$$k_{in,ND} = mk_{in} \quad \text{(III-58)}$$

where m is the EP^+ content per tetramer. The $k_{in,ND}$ values for the reaction systems with oxygen and with MTT are calculated to be 4.2 s^{-1} and 3.9 s^{-1}, respectively. The value for the MTT system is much larger than that obtained experimentally from Fig. III-5. This discrepancy is related to the lower k_{cat} value for the MTT system, as will be discussed in the next section. The values of the kinetic parameters obtained so far are listed in Table III-2.

State of the Ethylphenazine Moiety

EP^+-LDH-NAD^+, as well as EP^+-GlcDH-NAD^+, has K_m values for oxygen and MTT without binding sites for them. This is explained in our preceding chapter by the fact that these conjugates take two forms in their catalytic cycle, oxidized and reduced forms. In the case of EP^+-LDH-NAD^+, almost all the free NAD^+ moieties are in the reduced form (see Eqn III-47), and therefore the oxidized and the reduced forms can be considered to be EP^+-LDH-NADH and EPH-LDH-NADH, respectively. The

fraction of the oxidized ethylphenazine moiety is expressed from Eqns III-18, III-47, and III-54 as:

$$1 - \beta_{EPH_h\text{-}i} = (k_{ox}[O_2] + k_{MT}[MTT])/(k_{in} + k_{ox}[O_2] + k_{MT}[MTT]) \quad \text{(III-59)}$$

For the system with oxygen, [MTT] = 0, and Eqn III-59 becomes

$$1 - \beta_{EPH_h\text{-}i} = k_{ox}[O_2]/(k_{in} + k_{ox}[O_2]) = [O_2]/(K_{m,ox} + [O_2]) \quad \text{(III-60)}$$

Eqns III-52 and III-60 show the same dependence on the oxygen concentration. This means that the oxidase activity is proportional to the fraction of the oxidized form of the conjugate, and that the value of the fraction becomes 0.5 when the oxygen concentration becomes the $K_{m,ox}$ value.

As described above (Figs. III-3 and III-4), the value of $K_{m,ox}$ is 25 times larger than that of $K_{m,MT}$. Equations III-55 and III-56 clearly show that this difference is not due to the difference in the affinity to these substrate, but mainly due to the difference in the values of the rate constants, k_{ox} and k_{MT}.

On the other hand, V_{max} is expressed from Eqn III-52 or III-53 by the following same equation:

$$V_{max} = \delta n k_{in}[EP(H)]_t \quad \text{(III-61)}$$

At present, it is impossible to explain by Eqn III-61 the difference in V_{max} (or k_{cat}) values obtained from Figs. III-3 and III-4 for the assay systems with oxygen and with MTT, respectively. The V_{max} value calculated from Eqn III-61 is 2.6 μM/min for both assay systems; this is similar to that obtained from Fig. III-3, but is about 6 times as high as that obtained from Fig. III-4. A similar difference in k_{cat} value was also observed in the glucose oxidase activity of EP^+-GlcDH-NAD^+ (see the chapter II). There must be some factors concerned with the low k_{cat}

value when MTT is used as the electron acceptor. Such factors seems to be related to the number of the ethylphenazine moieties that are actively recycled at steady state, though the actual mechanism is still not clear.

Effective Concentration

The x-intercept of the straight line shown in Fig. III-5 gives the value of the apparent effective concentration of the ethylphenazine moiety for the free NADH moiety, and the value is 0.34 mM; this value corresponds to the ratio of $k_{in,ND}/k_{PS}$. This means that, in the absence of exogenous 5-ethylphenazine, the free NADH moiety feels as if it were in a solution containing 0.34 mM 5-ethylphenazine irrespective of the actual concentration of the conjugate.

The lactate oxidase activity of EP^+-LDH-NAD^+ was also measured by the MTT method in the presence of excess MTT and various concentrations of exogenous PEG-NADH, and the results are shown in Fig. III-6. This figure indicates that the reaction rate is proportional to the initial concentration of PEG-NADH, and the x-intercept of the straight line gives the value of the apparent effective concentration of the free NADH moiety for the ethylphenazine moiety; the value is 5.5 μM. This means that, in the absence of exogenous PEG-NADH, the ethylphenazine moiety feels as if it were in a solution containing 5.5 μM PEG-NADH. This value is much lower than the effective concentration (0.34 mM) of the ethylphenazine moiety, and the difference is mainly due to the effect of hiding the NADH moiety in the coenzyme-binding site. In fact, if $\delta = 1$, the effective concentration becomes 0.26 mM.

In the presence of exogenous PEG-NADH, Eqn III-18 is converted to:

$$\beta_{EPH_h\text{-}i} = \left\{\sum_j (k_{hj,i}\delta_{i-j}\gamma_{EP^+_h\text{-}i\text{-}NADH_j} f)\right\} + k_{PNH}[\text{PEG-NADH}] \Big/ \left\{\sum_j (k_{hj,i}\delta_{i-j}\gamma_{EP^+_h\text{-}i\text{-}NADH_j} f)\right\} + k_{PNH}[\text{PEG-NADH}]$$

$$+ k_{ox}[O_2] + k_{MT}[MTT] \} \qquad \text{(III-62)}$$

where k_{PNH} is the second-order rate constant of the reaction between the ethylphenazine moiety and exogenous PEG-NADH. From Eqns III-12, III-21, III-47, III-54, and III-62, the reaction rate is expressed, similarly to Eqn III-53, as:

$$d[\text{formazan}]/dt = k_{MT}[MTT][EP(H)]_t(\delta n k_{in} + k_{PNH}[\text{PEG-NADH}])/$$
$$(k_{in} + k_{PNH}[\text{PEG-NADH}] + k_{ox}[O_2] + k_{MT}[MTT]) \qquad \text{(III-63)}$$

In the presence of excess MTT and using Eqn III-61, Eqn III-63 is simplified to:

$$d[\text{formazan}]/dt = V_{max} + k_{PNH}[\text{PEG-NADH}][EP(H)]_t \qquad \text{(III-64)}$$

The values of V_{max} and k_{PNH} are calculated from the straight line shown in Fig. III-6 to be 0.40 μM/min and 1.49 $mM^{-1}s^{-1}$. The V_{max} value is almost the same as that obtained from Fig. III-4. The k_{PNH} value is 61% of the second-order rate constant for the reaction of PEG-NADH with EP^+-PEG (Yomo et al., 1989); a similar reduction was observed in the reactivity of the ethylphenazine moiety of EP^+-PEG-GlcDH (see the chapter I).

Hide-and-Seek Effect

The main difference between EP^+-GlcDH-NAD^+ and EP^+-LDH-NAD^+ is that lactate dehydrogenase has a much higher activity and affinity for PEG-NAD^+ (Katayama et al., 1983; Okuda et al., 1985; Nakamura et al., 1986). While the K_m value of glucose dehydrogenase for PEG-NAD^+ is estimated to be much larger than 4.2 mM (Nakamura et al., 1986), lactate dehydrogenase has dissociation constant for PEG-NAD^+ and PEG-NADH of 0.54 mM and 2.8 μM, respectively (Katayama et al., 1983). It looks strange that in spite of this higher affinity and activity of lactate dehydrogenase,

the activity of EP^+-LDH-NAD^+ is lower than that of EP^+-GlcDH-NAD^+; the k_{cat} value of EP^+-LDH-NAD^+ is only 10% or 38% of that of EP^+-GlcDH-NAD^+ for the assay system with oxygen or with MTT, respectively. However, this strange but interesting discrepancy can be explained as follows. The high affinity and activity of lactate dehydrogenase is reflected in the results for EP^+-LDH-NAD^+ that almost all the NAD^+ moieties are in the reduced form and only 2.1% of the NADH moieties are in the free state. As the ethylphenazine moiety cannot react with the NADH moiety hidden in the coenzyme-binding site (see the chapter I), this hide-and-seek effect greatly reduces the oxidase activity of EP^+-LDH-NAD^+ by lowering the effective concentration of the free NADH moiety. Therefore, such a hide-and seek effect is very important for designing artificial enzymes, and will be analyzed further in the chapter IV.

Summary

5-Ethylphenazine-lactate dehydrogenase-NAD^+ conjugate (EP^+-LDH-NAD^+) was prepared by linking poly(ethylene glycol)-bound 5-ethylphenazine and poly(ethylene glycol)-bound NAD^+ to lactate dehydrogenase. The average number of the ethylphenazine moieties bound per molecule of enzyme subunit was 0.46, and that of the NAD^+ moieties was 0.32. This conjugate is a semisynthetic enzyme having lactate oxidase activity using oxygen or MTT as an electron acceptor; to make such conjugates seems to be a general method for artificially converting a dehydrogenase into an oxidase. When the concentration of oxygen or MTT is varied, the oxidase activity fits the Michaelis-Menten equation with the following apparent kinetic constants: for the reaction system with oxygen, the turnover number per subunit is 2.3 min^{-1} and K_m for oxygen is 1.91 mM; and for the system with MTT, the turnover number is 0.25 min^{-1} and K_m

for MTT is 0.076 mM. At the initial steady state of the oxidase reaction, only 2.1% of the NAD^+ moieties are in the free state and the rest are hidden in the coenzyme-binding site of the dehydrogenase moiety, and almost all the NAD^+ moiety are in the reduced form . The apparent intramolecular rate constant for the reaction between a free NADH moiety and an oxidized ethylphenazine moiety is 2.3 s^{-1} and 2.1 s^{-1} for the systems with oxygen and with MTT, respectively. The apparent effective concentration of the free NADH moiety for the ethylphenazine moiety is 5.5 μM and is much smaller than that (0.34 mM) of the ethylphenazine moiety for the free NADH moiety; this difference is due to the effect of hiding the NADH moiety in the binding site, as the hidden NADH moiety cannot react with the ethylphenazine moiety.

Table III-1. Effects of exogenous NAD^+ concentration on the lactate dehydrogenase activity of EP^+-LDH-NAD^+ before and after the treatment with nucleotide pyrophosphatase[a]

NAD^+ concentration (mM)	Lactate dehydrogenase activity (%)[b]	
	before pyrophosphatase treatment	after pyrophosphatase treatment
1.6	34.7	60.9
68.8	79.8	98.2

[a]Lactate dehydrogenase activity was measured by the MTT method. Reaction mixture (3.02 ml) contained 0.1 M sodium phosphate, pH 7.5, 50 mM lithium L-lactate, 0.82 mM 5-ethylphenazine, 1.5 mM MTT, the indicated concentrations of NAD^+, and 0.97 nM lactate dehydrogenase or 8.82 nM EP^+-LDH-NAD^+. Pyrophosphatase treatment was done at 15°C for 3 h by mixing 50 µl of 27 µM EP^+-LDH-NAD^+ solution containing 0.1 M sodium phosphate, pH 7.5, with 50 µl of dinucleotide pyrophosphatase solution (78 units/ml) containing 0.1 M sodium phosphate, pH 7.5, and 40 mM $MgCl_2$.

[b]The specific activity of native lactate dehydrogenase was 100 kat/mol at 1.6 and 68.8 mM NAD^+, and this value was used as 100%.

Table III-2. Kinetic parameters for the reaction of EP^+-LDH-NAD^+

Electron acceptor	Kinetic constants					
	k_{cat} (min^{-1})	K_m (mM)	k_{in} (s^{-1})	$k_{in,ND}$ (s^{-1})	$k_{in,EP}$ (s^{-1})	δ
O_2	2.3	1.91	2.3	4.2	0.062	0.021[a]
MTT	0.25	0.076	2.1	0.59	0.056	0.021

[a]The δ value for the reaction system with oxygen is assumed to be the same as that for the system with MTT.

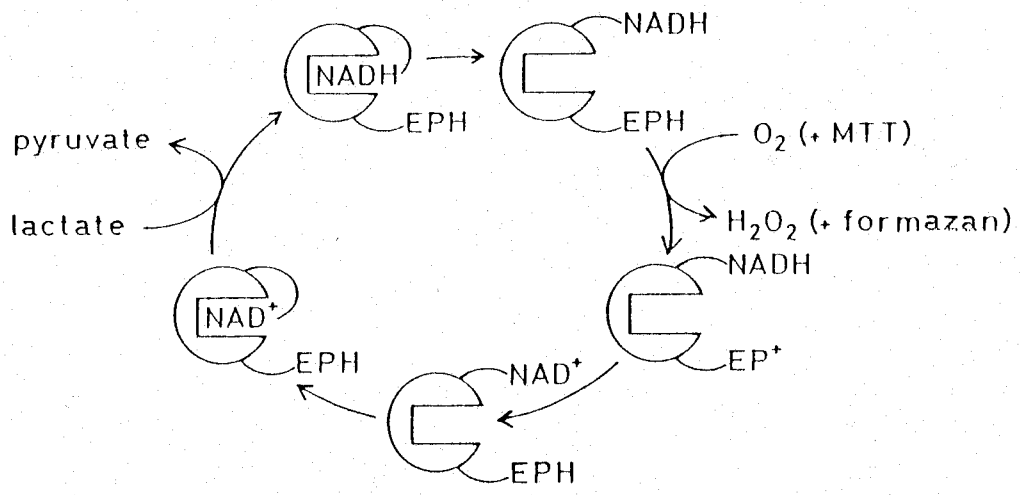


Fig. III-1. Schematic representation of the catalytic cycle of semisynthetic lactate oxidase. Only the cycle with the more reduced forms of EP^+-LDH-NAD^+ is shown.

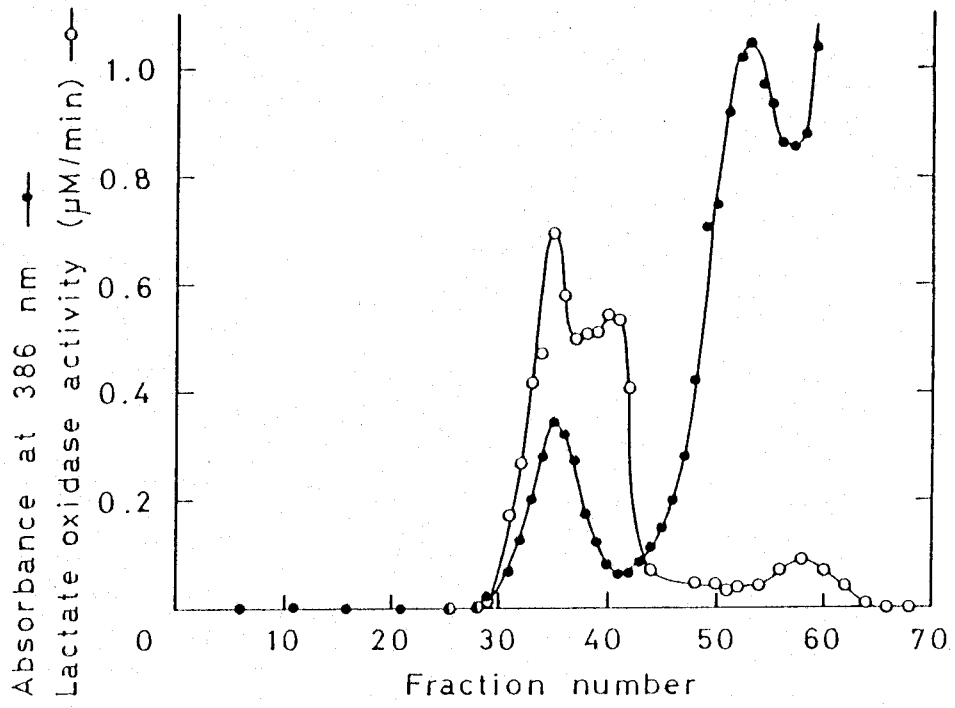


Fig. III-2. Sephacryl S-200 column chromatography of EP^{+}-LDH-NAD^{+}. ●, absorbance at 386 nm; O, lactate oxidase activity. Lactate oxidase activity was measured by the MTT method as described under "Experimental Procedures"; reactions were started by adding 0.2 ml of sample solution to a substrate solution (3.0 ml) contained 0.1 M sodium phosphate, pH 7.5, 50 mM lithium L-lactate, and 1.5 mM MTT.

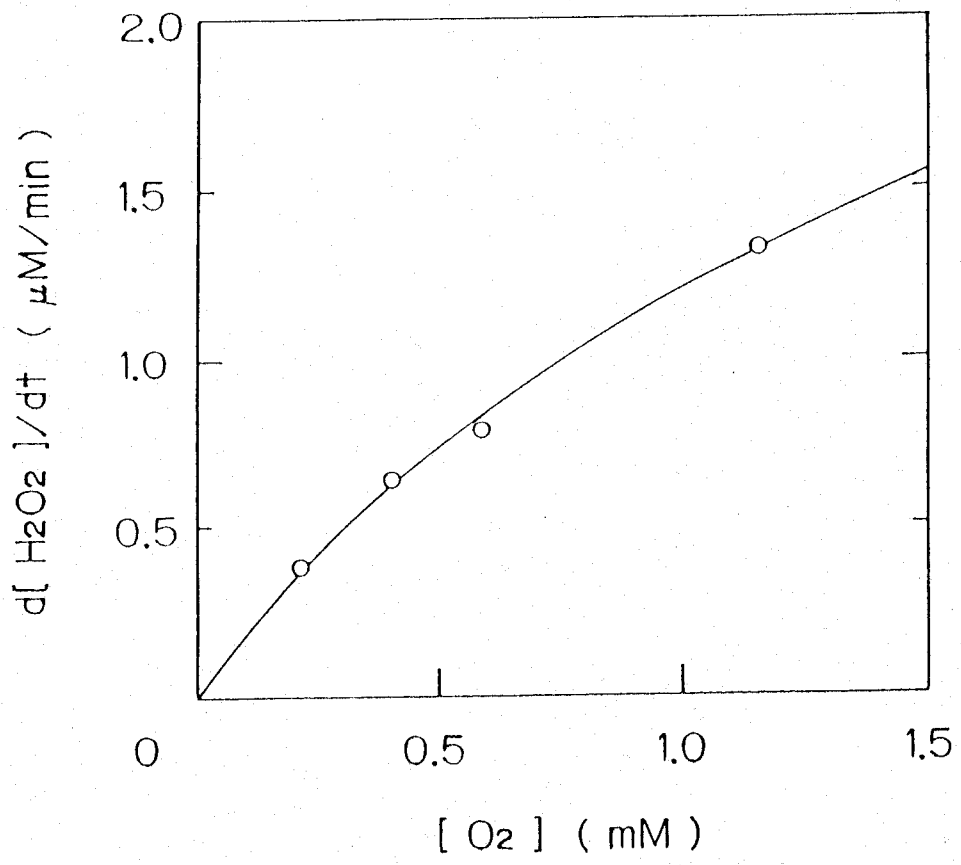


Fig. III-3. Effects of oxygen concentration on the activity of semisynthetic lactate oxidase. ——, the activity calculated from the Michaelis-Menten equation with V_{max} = 3.51 μM/min and K_m = 1.91 mM. Lactate oxidase activity was measured by the O_2 method; reactions were started by adding 0.2 ml of enzyme solution containing 0.1 M sodium phosphate, pH 7.5, 24.5 μM EP^+-LDH-NAD^+, and 18 μg/ml peroxidase to a substrate solution (3.0 ml) containing 0.1 M sodium phosphate, pH 7.5, 50 mm lithium L-lactate, 0.1 mM MBTH, 0.1 mM primaquine, 1 mM EDTA, and oxygen; the initial oxygen concentration in the reaction mixture was varied and the values are indicated on the abscissa.

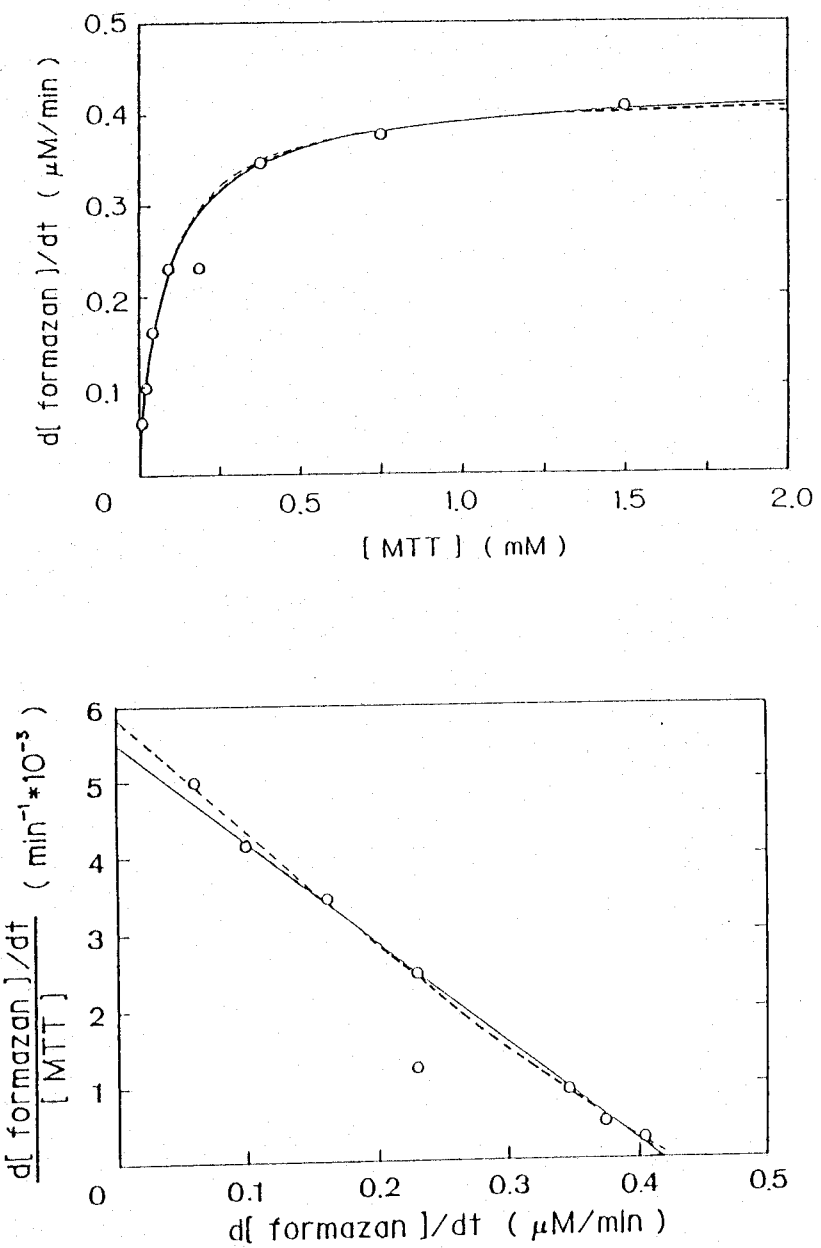


Fig. III-4. Effects of MTT concentration on the activity of Semisynthetic lactate oxidase shown by a saturation curve (A) and by an Eadie plot (B). ——, the activity calculated from the Michaelis-Menten equation with V_{max} = 0.42 μM/min and K_m = 0.076 mM; - - -, the activity calculated from Eqn III-1 with V_{max} = 0.107 μM/min, $K_{m,1}$ = 0.058 mM, $K_{m,2} = K_{m,3}$ = 0.060 mM, and $K_{m,3}$ = 0.214 mM. Lactate oxidase activity was measured by the MTT method; reactions were started by adding 0.2 ml of 27 μM EP^+-LDH-NAD^+ to a substrate solution (3.0 ml) containing 0.1 M sodium phosphate, pH 7.5, 50 mM lithium L-lactate, and MTT; the initial concentration of MTT was varied and the values are shown on the abscissa.

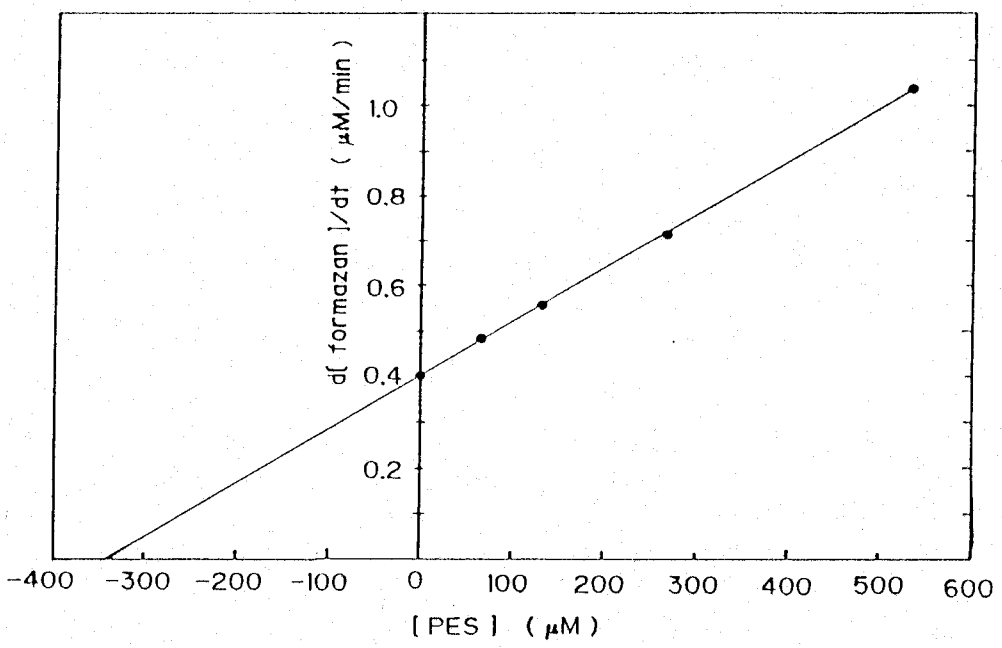


Fig. III-5. Effects of the exogenous 5-ethylphenazine on the activity of semisynthetic lactate oxidase. Lactate oxidase activity was measured by the MTT method; reactions were started by adding 0.2 ml of 27 μM EP^+-LDH-NAD^+ into a substrate solution (3.02 ml) containing 0.1 M sodium phosphate, pH 7.5, 50 mM lithium L-lactate, 1.5 mM MTT, and 5-ethylphenazine; the initial concentration of 5-ethylphenazine was varied and the values are shown on the abscissa.

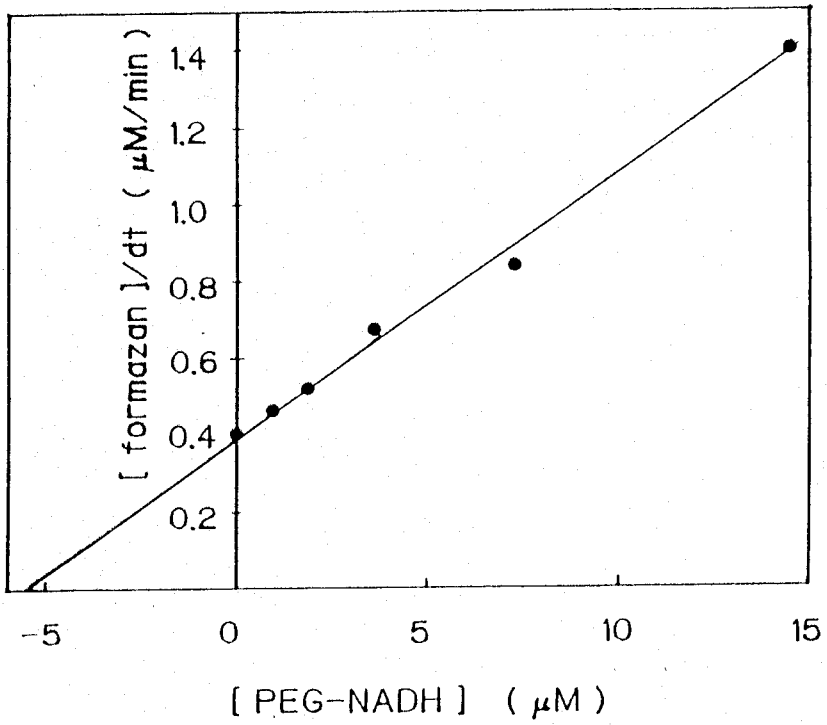


Fig. III-6. Effects of exogenous PEG-NADH on the activity of semisynthetic lactate oxidase. Lactate oxidase activity was measured by the MTT method. The reaction mixture contained the same components as described in the legend to Fig. III-5, except that PEG-NADH was added instead of 5-ethylphenazine; the initial concentration of PEG-NADH was varied and the values are shown on the abscissa.

Chapter IV

Principles for Designing Enzyme-like Catalysts

Introductory remarks

Enzymes are natural catalysts that have been created by trial and error. The structure and catalytic mechanism of enzymes have been investigated, and now we have some knowledge on how they work; however, we cannot know the purpose of nature for designing enzymes, as nature does not have any purposes. When we prepare a catalyst, on the other hand, we have a purpose to use it. We have long wanted to prepare an enzyme-like catalyst with high efficiency and specificity, but for this purpose, we must establish the principles of designing enzyme-like catalysts from our point of view.

In our preceding chapters, three kinds of semisynthetic oxidases are described: EP^+-PEG-GltDH (see the chapter I) , EP^+-GlcDH-NAD^+ (see the chapter II), and EP^+-LDH-NAD^+ (see the chapter III). They have the 5-ethylphenazine moiety as an artificial catalytic group for the oxidation of NADH (or NADH moieties) with oxygen or MTT. The results of the kinetic analysis of these semisynthetic oxidases provide us basic information about the mechanism of enzyme-like catalysts: the presence of a substrate-binding site near a catalytic group, the intramolecular coupling of two catalytic reactions, and a hide-and-seek effect. After generalization of these results, this chapter describes the principle for designing enzyme-like catalysts from the kinetic point of view.

Results and Discussion

Rate Acceleration by Substrate Binding

The presence of a substrate-binding site is one of the most characteristic points of enzymes. By the specific binding of their substrate, enzymes express their specificity and accelerate the chemical reactions that they catalyze. In the chapter I of this issue, I demonstrated the effects of the presence of a substrate-binding site near the catalytic group of the ethylphenazine moiety of EP^+-PEG-GltDH.

The reaction scheme for such artificial enzymes having one substrate-binding site and one catalytic group per enzyme molecule is generally expressed by the following equations:

$$S + E \xrightarrow{k_{ex}} P + E \qquad \text{(IV-1)}$$

$$S + E \underset{}{\overset{K_d}{\rightleftharpoons}} ES \xrightarrow{k_{in}} EP \xrightarrow{k_{of}} P + E \qquad \text{(IV-2)}$$

where E is an artificial enzyme, S is a substrate, P is a product, k_{ex} is the second-order rate constant of the intermolecular reaction between S and the catalytic group of E, k_{in} is the first-order rate constant of the intramolecular enzyme reaction, k_{of} is the first-order rate constant for the step regenerating the free form of the enzyme, and K_d is the dissociation constant of the ES complex. The rate of the intermolecular reaction of Eqn IV-1 is expressed as:

$$v_{ex} = k_{ex}[E]_t[S] \qquad \text{(IV-3)}$$

where $[E]_t$ is the total concentration of the enzyme defined by:

$$[E]_t = [E] + [ES] \qquad \text{(IV-4)}$$

The rate of the enzymatic reaction of Eqn IV-2 at the initial steady state is expressed by the following Michaelis-Menten-type equation assuming that $[E]_t \ll [S]$ and $k_{in} \ll k_{of}$:

$$v_{en} = k_{in}[E]_t[S]/(K_d + [S]) \qquad \text{(IV-5)}$$

The overall reaction rate is then expressed as:

$$v_t = v_{ex} + v_{en} \qquad \text{(IV-6)}$$

Eqn IV-6 means that the reaction rate between the substrate and the catalytic group increases from v_{ex} to v_t by the presence of the substrate-binding site near the catalytic group. This increase in the reaction rate is regarded as the increase in the effective concentration of the substrate around the catalytic group. This effective concentration, $[S]_{ef}$, is expressed as:

$$[S]_{ef} = v_t/(k_{ex}[E]_t) \qquad \text{(IV-7)}$$

and is related to the effective radius, r, by the following equation:

$$[S]_{ef} = 3/(4\pi N \cdot r^3) \qquad \text{(IV-8)}$$

where N is the Avogadro number. Equation IV-8 means that $[S]_{ef}$ corresponds to the concentration of one molecule per the volume of a sphere with a radius of r, and that the effective radius decreases with the increase in the effective concentration. From Eqns IV-3 and IV-5 - IV-8, the effective radius is expressed as:

$$r = [3(K_d + [S])/\{4\pi N[S](K_d + k_{in}/k_{ex} + [S])\}]^{1/3} \qquad \text{(IV-9)}$$

and this relationship between [S] and r is shown in Fig. IV-1.

The straight line shown in Fig. IV-1A and IV-1B corresponds to the situations of $k_{in} = 0$ and $K_d = \infty$, respectively. In these cases, Eqn IV-9 is simplified to:

$$r_0 = \{3/(4\pi N[S])\}^{1/3} \qquad \text{(IV-10)}$$

This effective radius of r_0 corresponds to the actual substrate concentration ([S]), and in this case, there is no effect of the presence of the substrate-binding site and Eqn IV-6 is simplified to:

$$v_t = v_{ex} \qquad \text{(IV-11)}$$

Therefore, the ratio of r_0 and r shows the rate-acceleration effect of the substrate-binding site quantitatively; the ratio is expressed as:

$$\ln(r_0/r) = (1/3)\ln\{(K_d + k_{in}/k_{ex} + [S])/(K_d + [S])\} \qquad \text{(IV-12)}$$

Figure IV-1 and Eqn IV-12 show that the effect of the binding site is negligible (i.e., $r \cong r_0$) in the region of $k_{in}/k_{ex} << [S]$ or under the conditions of $k_{in}/k_{ex} << K_d$. But under other conditions, the rate-acceleration effect appears and increases with the decrease in [S], and reaches a maximum in the region of $[S] << K_d$; in the latter case, Eqn IV-12 is simplified to:

$$\ln(r_0/r)_{max} = (1/3)\ln\{1 + k_{in}/(k_{ex}K_d)\} \qquad \text{(IV-13)}$$

Figure IV-1A shows that $(r_0/r)_{max}$, the maximum value of r_0/r, increases with the increase in k_{in}/k_{ex}, which corresponds to the effective concentration of the bound substrate for the catalytic group. Figure IV-1B shows that the maximum value increases with the decrease in K_d, unless k_{of} in Eqn IV-2 is smaller than k_{in}. It is interesting that when $[S] << K_d$, almost all the binding sites do not have the substrate but the rate-acceleration effect of the site is maximum.

In the case of EP^+-PEG-NAD^+ (Yomo et al., 1989b), the substrate is covalently linked to the catalytic group and the product is converted again to the substrate by a different reaction, and therefore the K_d value is zero and the corresponding line is shown in Fig. IV-1B.

Energetics of Rate-acceleration Effect

The kinetic constants, K_d, k_{in}, and k_{ex}, in Eqns IV-1 and IV-2 are related to the standard free energy change for the substrate binding (ΔG_b^o) and the activation free energies ($\Delta G_{in}^{\ddagger}$ and $\Delta G_{ex}^{\ddagger}$), respectively, illustrated in Fig. IV-2.

For a good catalyst, $k_{in}/k_{ex} >> K_d$. In this case, Eqn IV-13 is further simplified and rewritten to:

$$\ln(r_0/r)_{max} = (1/3)\ln\{k_{in}/(k_{ex}K_d)\} \qquad \text{(IV-14)}$$
$$= (1/3)\{\Delta G_{ex}^{\ddagger}/RT - (\Delta G_{in}^{\ddagger} - \Delta G_b^o)/RT\}$$

$$= (1/3)(\Delta\Delta G^{\ddagger}/RT) \qquad \text{(IV-15)}$$

where R is the gas constant and T is the absolute temperature. Eqn IV-15 means that the catalyst accelerates the reaction by lowering the activation energy by $\Delta\Delta G^{\ddagger}$, and this expression is generally used for explaining the rate acceleration by a catalyst. However, it should be noted that Eqn IV-15 must be used only for the "maximum" rate-acceleration effect and not for the actual effect.

In Eqn IV-14, the term of $k_{in}/(k_{ex}K_d)$ corresponds to the equilibrium constant of the interconversion between E and ES (the first step of Eqn IV-2) at $[S] = k_{in}/k_{ex}$, and Eqn IV-14 is converted to:

$$\ln(r_0/r)_{max} = (1/3)(\Delta G_b^{o'}/RT) \qquad \text{(IV-16)}$$

where $\Delta G_b^{o'}$ is the free energy change for the substrate-binding step at $[S] = k_{in}/k_{ex}$. This means that the binding energy is directly related to the maximum rate-acceleration effect due to the presence of the substrate-binding site. The relationship between the binding energy and enzyme catalysis has long been discussed from a number of aspects (Jenckes 1975; Jenckes 1980; Page 1984; Fersht, 1987); however, Eqn IV-16 most clearly and directly shows this relationship irrespective of the physicochemical mechanism of rate acceleration that corresponds to the value of k_{in}/k_{ex}. The important point our results show is that just the presence of a simple substrate-binding site near a catalytic group can accelerate the reaction.

Under the conditions of $K_d << [S] << k_{in}/k_{ex}$, Eqn IV-12 is simplified to:

$$\ln(r_0/r) = (1/3)\ln\{k_{in}/(k_{ex}[S])\} \qquad \text{(IV-17)}$$

$$= (1/3)\{(\Delta G_{ex}^{\ddagger} - \Delta G_{in}^{\ddagger})/RT - \ln([S]/c_0)\} \qquad \text{(IV-18)}$$

$$= (1/3)\{(\Delta\Delta G^{\ddagger} - \Delta G_b^{o})/RT - \ln([S]/c_0)\} \qquad \text{(IV-19)}$$

where c_0 is the concentration at the standard conditions. These equa-

tions mean that when [S] = c_0, the rate acceleration effect corresponds to the difference between $\Delta G_{ex}^{\ddagger}$ and $\Delta G_{in}^{\ddagger}$, and that the effect is smaller than the maximum value by the term of ΔG_b^{o}. The catalytic efficiency of enzymes has been explained by $\Delta\Delta G^{\ddagger}$ and/or ($\Delta G_{ex}^{\ddagger}$ - $\Delta G_{in}^{\ddagger}$) (Albery and Knowles, 1976; Page, 1984; Hermes et al., 1987), but it is not clear how to use these terms properly. Our Eqns IV-15, IV-18, and IV-19 clearly express the relationship between the rate-acceleration effect and these thermodynamic terms. Especially, Eqns IV-14 and IV-15 show a new concept of the maximum rate-acceleration effect, that is the upper limit of the effect, and Eqns IV-12 and IV-17 - IV-19 show the effects of substrate concentration and are applicable to actual reaction systems.

Importance of Intramolecular Coupling

Coupling of two or more catalytic reactions enables us to prepare various kinds of new artificial enzymes. In this case, one of the products of the first reaction becomes one of the substrates of the second reaction, one of the products of the second reaction becomes one of the substrates of the third reaction, and so on; thus two or more reactions are coupled by these intermediates. Let us consider an artificial bifunctional enzyme having one substrate-binding site and each one of the two kinds of catalytic groups that catalyzes the following successive reactions:

$$ES \xrightarrow{k_1} EM \xrightarrow{k_2} EP \qquad \text{(IV-20)}$$

where k_1 is the first-order rate constant for the conversion of the bound substrate (S) to the bound intermediate (M), and k_2 is that for the conversion of the bound intermediate to the bound product (P). At

the initial steady state, the overall intramolecular rate constant (k_t) is expressed as:

$$k_t = k_1k_2/(k_1 + k_2) \qquad \text{(IV-21)}$$

When the intermediate diffuses out from the binding site with a dissociation-rate constant of k_{of} as shown in the following scheme:

$$\begin{array}{ccccc} & & k_1 & & k_2 \\ & ES & \longrightarrow & EM & \longrightarrow EP \\ & & & \downarrow k_{of} & \\ & & & E + M & \end{array} \qquad \text{(IV-22)}$$

then Eqn IV-21 is converted to:

$$k_t = k_1k_2/(k_1 + k_2 + k_{of}) \qquad \text{(IV-23)}$$

As the two reactions are coupled with the intermediate, the dissociation of it from the site decreases the coupling yield (Y) that is expressed as:

$$Y = k_2/(k_2 + k_{of}) \qquad \text{(IV-24)}$$

Using Eqn IV-24, Eqn IV-23 is converted to:

$$k_t = k_1k_2/(k_1 + k_2/Y) \qquad \text{(IV-25)}$$

This equation indicates that the overall reaction rate increases with the increase in the coupling yield of the two successive reactions.

It should be noted that for an enzyme with $k_1 >> k_2$ and $k_1 >> k_{of}$, both Eqns IV-21 and IV-23 are simplified to:

$$k_t = k_2 \qquad \text{(IV-26)}$$

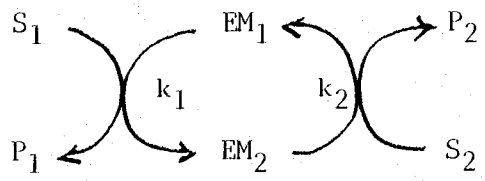
In this case, the overall reaction rate is not affected by the Y value; however, if the Y value is small, most of the substrate (S) is not converted to the desired product (P) but is accumulated or wasted as the intermediate (M).

EP^+-PEG-GltDH prepared in the chapter I may work as a glutamate oxidase. In this case, the first reaction is the oxidation of glutamate

with NAD^+ by the dehydrogenase reaction, the reduced NAD^+ (i.e., NADH) is reoxidized with oxygen by the catalysis of the ethylphenazine moiety, and NADH works as an intermediate. However, this semisynthetic oxidase will not work well considering a k_2 value of 0.38 s^{-1} (see the chapter I) and a k_{of} value of 340 s^{-1} (Malcolm, 1972); using these values, Y is calculated to be 1.1×10^{-3}.

EP^+-GlcDH-NAD^+ (see the chapter II) and EP^+-LDH-NAD^+ (see the chapter III), on the other hand, work well as semisynthetic glucose and lactate oxidases, respectively. This is because the intermediate of the NADH moiety is covalently linked to the enzyme and does not diffuse out from the reaction system. That is, even if the NADH moiety dissociates from the binding site, it stays near the second catalytic group of the ethylphenazine moiety and reacts with it; in this case, the coupling yield is 100%. Especially when the reactivity of the intermediate in the binding site with the second catalytic group is very low, the intermediate must be linked covalently to the enzyme as the case of EP^+-LDH-NAD^+. Thus, covalent linking of both NAD^+ and 5-ethylphenazine is a good general strategy for converting a dehydrogenase to a corresponding oxidase.

There are two types of coupling of two successive reactions: linear coupling and cyclic coupling. For the linear coupling, S and P in Eqn IV-20 are different, but for the cyclic coupling, S and P are the same compound (M_1) and the two reactions (S_1 to P_1 and S_2 to P_2) are coupled by the interconversion of M_1 and M_2 as shown in the following scheme:



(IV-27)

For example, the glucose oxidase activity of EP^+-GlcDH-NAD^+ (see the

chapter II) is due to the cyclic coupling of the two reactions: NAD^+ reduction by the dehydrogenase moiety and the NADH oxidation by the ethylphenazine moiety. In this case, M_1, M_2, S_1, S_2, P_1, and P_2 in Equ IV-27 are NAD^+, NADH, glucose, gluconolactone, and the oxidized and the reduced forms of the ethylphenazine moieties, respectively. For the linear coupling, the intermediate must not be linked to the enzyme, and the coupling yield can be improved only by decreasing the ratio of k_{of}/k_2. For cyclic coupling, on the other hand, the coupling yield can be 100% by covalent linking of the intermediate as described above, and in addition, the intermediate is efficiently recycled by this linking.

In the above discussion, it is assumed that once the intermediate dissociates from the binding site, it diffuses out rapidly from the reaction system and does not react with the second catalytic group, unless the intermediate is covalently linked to the enzyme; that is, there is no gradient in the concentration of the intermediate from the binding site to the bulk solution. This is definitely true for the case of the concentration of a substrate of which the binding reaction with the site is at an equilibrium. For the steady-state concentration of an intermediate (or a product), on the other hand, there is a concentration gradient. Therefore, I must estimate the concentration of the intermediate around the the second catalytic group.

For this estimation, I considered a situation that an intermediate (M) continuously springs out with a rate constant of k_{of} from point A, which corresponds to the binding site on an enzyme (Fig. IV-3). As for the motion of the enzyme, the following two situations are considered: one is that the enzyme dose not move against the bulk solution, and the other is that the enzyme rotates rapidly against the bulk solution. For the first case, considering the vicinity of point A as shown in Fig. IV-

3A, the material balance in a half shell with a radius of x from point A and a thickness of dx is:

$$2\pi x^2 dx(d[M]/dt) = k_{of}/N + 2\pi Dx^2(d[M]/dx) \qquad \text{(IV-28)}$$

where N is the Avogadro number and D is the diffusion constant. At steady state, d[M]/dt = 0, and Eqn IV-28 becomes:

$$d[M]/dx = -k_{of}/(2\pi ND \cdot x^2) \qquad \text{(IV-29)}$$

Integrating Eqn IV-29,

$$[M] = k_{of}/(2\pi ND \cdot x) + [M]_{bk} \qquad \text{(IV-30)}$$

where $[M]_{bk}$ is the concentration of the intermediate in the bulk solution, and $[M]_{bk}$ = 0 at the initial steady state.

When the enzyme rotates rapidly, the concentration gradient is the same as that when the intermediate continuously springs out from the center (point O) of the enzyme whose radius is R (Fig. IV-3B), and is expressed as:

$$[M] = k_{of}/\{4\pi ND(x + R)\} + [M]_{bk} \qquad \text{(IV-31)}$$

Using the values of N = 6.02 x 10^{23}, D = 10^{-5} cm^2s^{-1}, k_{of} = 340 s^{-1}, R = 10 nm, and $[M]_{bk}$ = 0, the value of [M] at x = 10 nm, that is the effective radius for the system of EP^+-PEG-NAD^+ (Yomo et al., 1989b), is calculated to be 9.0 nM from Eqn IV-30 or 2.3 nM from Eqn IV-31; even at x = 1 nm, the value is 90 nM (Eqn IV-30) or 4.1 nM (Eqn IV-31). Therefore, the concentration of the intermediate around the second catalytic group is very low, and the reaction rate between them is negligible comparing to the diffusion rate unless the second-order rate constant for the reaction is very large. This means that, for most artificial bifunctional enzymes without covalently linked intermediates, the intermediate coming out of the binding site diffuses out from the reaction system without reacting with the second catalytic group.

For multifunctional enzymes, much research on substrate channeling

have been reported; for example, the first three reactions of the de novo pyrimidine biosynthesis (Mally et al., 1980; Christopherson & Jones, 1980), and chorismate mutase-prephenate dehydratase (Duggleby et al., 1978; Heyde, 1979), a covalently linked alcohol dehydrogenase-lactate dehydrogenase conjugate (Mansson et al., 1983). Considering our results above, however, the actual coupling yield of these multienzyme systems having separate active sites must be very small, unless the successive enzymes have such a high affinity for their substrates that they can catch almost all the substrate coming from the neighboring site and reduce the substrate concentration below the nM order.

Effects of the Number of Covalently Linked Intermediates

As described in the above section, covalent linking of an intermediate to the catalyst is the best way to increase the coupling efficiency of two successive reactions connected by cyclic coupling. In this case, the number of intermediates linked to the catalyst is an important factor affecting the catalytic rate.

Let us consider the latter half reaction (i.e., the right-hand half of Eqn IV-27) of an artificial bifunctional enzyme; for this enzyme, the number of the intermediate-binding site is m, and the average number of the covalently linked intermediate is $\tilde{i}$. As the intermediates are linked generally by the method of chemical modification, the prepared artificial enzyme is heterogeneous in the number of the intermediates. Therefore, the enzyme molecules are grouped by the number of the linked intermediates: the number of the intermediates linked to the enzyme molecule in the i'th group is i. In addition, the enzyme molecules are at different states as for the number of the intermediates bound in the site. In the i'th group, the fraction of the enzyme molecule whose j out

of i intermediates are in the binding sites is expressed as:

$$p_{j,i} = f_{j,i}K^j / \sum_{j=0}^{\min(m,i)} (f_{j,i}K^j) \qquad \text{(IV-31)}$$

where K is the equilibrium constant for the binding of the intermediate with its binding site, and $f_{j,i}$ and min(m, i) are defined as:

$$f_{j,i} = m!\cdot i!/\{(m - j)!\cdot(i - j)!\cdot j!\} \qquad \text{(IV-32)}$$

$$\min(m, i) = m \ (m \leq i) \text{ or } i \ (m > i) \qquad \text{(IV-33)}$$

In these equations, it is assumed for simplicity that all the intermediates and the binding sites can interact with each other with the same equilibrium constant of K.

As described in the chapter I, the reactivity of the bound intermediate in the binding site is different from that of the free (i.e., unbound) intermediate. By correcting this effect, the effective number of the intermediate (calculated in terms of the free state) per molecule of the i'th enzyme is expressed as:

$$N_i = \sum_{j=0}^{\min(m,i)} [\{i - (1 - \varepsilon)j\}p_{j,i}] \qquad \text{(IV-34)}$$

where ε is the reactivity of the bound intermediate relative to the free one. On the other hand, the fraction of the i'th enzyme molecule is expressed as:

$$q_i = [n!/\{(n - i)!\cdot i!\}](\tilde{i}/n)^i(1 - \tilde{i}/n)^{n-i} \qquad \text{(IV-35)}$$

where n is the maximum number of the intermediates that can be linked to the enzyme molecule. Then the average effective number of the intermediates per molecule is expressed as:

$$\tilde{N} = \sum_{i=0}^{n}(N_i q_i) \qquad \text{(IV-36)}$$

The value of $\tilde{N}$ can be calculated using Eqns IV-31 - IV-36 when the values of K, m, n, $\tilde{i}$, and ε are given. Figure IV-4 shows the plot of $\tilde{i}/m$ vs. $\tilde{N}/m$. In this figure, the following values are used: K = 120, m = 4, n = 2, and ε = 0, 1, or 2. In the case of ε = 1 (Fig. IV-4, curve B), the reactivity of the bound intermediate is the same as that of the

free one, and $\tilde{N}/m$ is proportional to $\tilde{i}/m$ (i.e., $\tilde{N} = \tilde{i}$ from Eqn IV-34). In the case of $\mathcal{E} > 1$ (Fig. IV-4, curve A), the reactivity of the bound intermediate is higher than that of the free one, and therefore the effective number of the intermediates is larger than the actual number. The difference between the effective and the actual numbers increases with the increase in the $\tilde{i}/m$ value, and reaches at a maximum value of $m(\mathcal{E} - 1)$. In the case of $\mathcal{E} < 1$ (Fig. IV-4, curve C), on the other hand, the effective number of the intermediates is smaller than the actual number. Especially, when the reactivity of the bound intermediate is negligible, the artificial enzyme behaves as if it has few intermediates in the region of $\tilde{i}/m < 1$, and the activity of the enzyme becomes very low; this is the hide-and-seek effect observed for EP^+-LDH-NAD^+ (see the chapter III). In this case of $\mathcal{E} = 0$, the $\tilde{i}/m$ value must be higher than 1 to make an efficient catalyst (see Fig. IV-4, curve C).

The change in the m value has little effect on the shape of the plot of $\tilde{i}/m$ vs. $\tilde{N}/m$ shown in Fig. IV-4. However, the activity of the catalyst depends on m, because the activity is proportional to $\tilde{N}$ and not to $\tilde{N}/m$. The K value, on the other hand, affects the shape of this plot. With the decrease in the K value, the curvature of the lines decreases, and at $K = 0$, the same straight line as that for $\mathcal{E} = 1$ (Fig. IV-4, curve B) is obtained. EP^+-G1cDH-NAD^+ (see the chapter II) is an example for the cases of $K \cong 0$.

Design of Enzyme-like Catalysts

An enzyme-like catalyst has substrate-binding sites and catalytic groups. At first, let us consider the simplest type having one substrate-binding site and one catalytic group per molecule. The catalytic activity of a catalyst of this type can be evaluated by the effective radius, and is expressed by Eqn IV-12; that is, the larger the

ratio of r_0/r, the higher the catalytic activity. Equation IV-12 and Fig. IV-1 show that it is important to select a substrate-binding site with a lower K_d value and to make the ratio of k_{in}/k_{ex} as large as possible, when we want to design a catalyst with high activity. As for a substrate-binding site, the sites of natural enzymes are good candidates, because they have low K_d values and high specificity for their substrates, and they also have functional groups available for covalent linking of artificial catalytic groups; EP^+-PEG-GltDH (see the chapter I), EP^+-GlcDH-NAD^+ (see the chapter II), and EP^+-LDH-NAD^+ (see the chapter III) are examples of this type. In future, we will be able to prepare a variety of binding sites with desired properties artificially.

The length and flexibility of a linker connecting a catalytic group to the substrate-binding site of a catalyst is an important factor for making a catalyst with a large k_{in}/k_{ex} value. Generally, the shorter the distance between the catalytic group and the site, the larger the k_{in}/k_{ex} value. In the case of natural enzymes, catalytic groups are included in the site, and the distance between the catalytic groups and the bound substrate is so short that even solvent molecules are excluded between them; thus the k_{in}/k_{ex} values of natural enzymes are very large. To design and prepare such exquisite molecules is very attractive, but in the case of artificial enzymes prepared by chemical methods, the distance between the attachment point of the catalytic group and the reactive point of the bound substrate is much longer and not constant. Therefore, the catalytic group cannot react with the bound substrate, if the length of the linker is shorter than the distance, or if the linker is rigid and its direction is wrong. Thus, it is better to use a flexible linker, and there is an optimum value for the length of the linker.

When the catalytic group of an artificial enzyme is linked in the outside of the substrate-binding site, the reactivity of the catalytic group toward a bound substrate may be different from that toward a free substrate. For example, the reactivity of the 5-ethylphenazine group toward NADH bound in the site of glutamate dehydrogenase is 43% of that toward free NADH, and the catalytic group does not react with NADH bound in the site of lactate dehydrogenase (see the chapter I). Therefore, it is also important to select a substrate-binding site that does not reduce the reactivity of a bound substrate so much. The method described in the chapter I is useful for estimating the reactivity of bound substrates. It should be noted that the K_d value of the binding site can also be estimated at the same time by this method.

It is easy to increase the numbers of the catalytic groups and the substrate-binding sites of an enzyme-like catalyst. When the catalytic groups are connected with long and flexible linkers, the specific activity of the catalyst will be proportional to the number of the catalytic groups and also to the number of the binding sites, provided that all the catalytic groups have similar reactivity toward the bound substrate, and that all the binding sites can interact with all the catalytic groups intramolecularly. In this case, a subunit structure is effective in improving the catalytic activity. For example, when a monomer has one catalytic site and one substrate-binding site, the dimer prepared by linking two monomers has four times as high activity as the monomer; in general, the activity of the n-mer will be n^2 times as high as that of the monomer. This rate acceleration effect is due to the long and flexible linker. If the linker is short and each catalytic group can interact with only one binding site, the activity of the n-mer will be only n times as high as that of the monomer.

It is possible to prepare an enzyme-like catalyst having more than two kinds of catalytic groups. Such a catalyst can accelerate a multistep reaction more efficiently than a mixture of catalysts each having one of the catalytic groups. For a multistep reaction with linear coupling, the smaller the ratio of the dissociation rate constant and the intramolecular rate constant of each intermediate, the higher the coupling yield of the reaction (see Eqn IV-24). For a multistep reaction with cyclic coupling, covalent linking of the intermediates to the catalyst is the best way to increase both the coupling yield of the reaction and the recycling rate of the intermediate. If the intermediates are covalently linked to the catalyst, restrictions to the binding sites available for multi-functional catalysts are relaxed; for example, even a site with low affinity for the intermediates can be used as the case of EP^+-GlcDH-NAD^+ (see the chapter II), and even a site in which the intermediates cannot react with the catalytic groups can also be used as the case of EP^+-LDH-NAD^+ (see the chapter III). In addition, a catalyst having more than two kinds of substrate- (and intermediate-) binding sites can also accelerate a multistep reaction, if the intermediates are covalently linked; without the covalent linkage, the yield of the transfer of the intermediates between the sites must be very low, and the activity of the multifunctional catalyst must be similar to that of the mixture of catalysts having each one of the binding sites.

The number of the covalently linked intermediate is an important factor for designing multifunctional catalysts with linked intermediates. When the reactivity of the intermediate bound in the site of a catalyst is much lower than that of the free intermediate, the number of the linked intermediate must be more than the number of the binding site. Otherwise, the activity of the catalyst becomes very low due to

the hide-and-seek effect (Fig. IV-4).

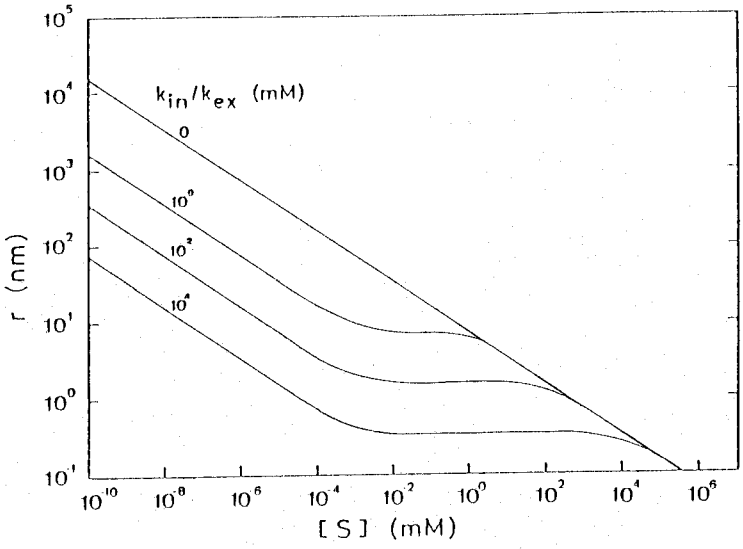
At present, materials and methods for preparing enzyme-like catalysts are still limited, but in the future, we will be able to prepare a variety of such catalysts according to the principle described here, and we will have more profound ideas of enzymes.

Summary

An enzyme-like catalyst has substrate-binding sites and catalytic groups. The catalytic activity of the catalysts depends on the affinity of the binding site for its substrate and on the ratio of the rate constants (k_{in}/k_{ex}) for intramolecular and intermolecular reactions between the substrate and the catalytic group. For a catalyst having one substrate-binding site and one catalytic group, an equation was obtained which shows the relationship among the catalytic activity, the affinity of the site, and the ratio of k_{in}/k_{ex}. This equation provides the following principle for designing catalysts of this type with high activity: the binding site is required to have a high affinity for the substrate and not to reduce the reactivity of the bound substrate, and the linker connecting the binding site and the catalytic group is required to be flexible and have an appropriate length. As for the substrate-binding sites, the sites of natural enzymes are good candidates. In addition, a subunit structure is found to be effective to improve the catalytic activity: the activity of an n-mer is at most n^2 times as high as that of the monomer.

The principle for designing a multifunctional catalyst having higher activity than a mixture of catalysts having each one of the functions was then investigated. In this case, the diffusion of the

intermediates of the multistep reaction strongly affect the activity of the multifunctional catalyst, and such a diffusion process was also analyzed. On the basis of these analyses, the following principle was obtained: for a multistep reaction by which a substrate is converted to intermediates successively and finally to a product (linear coupling), the catalyst must have one kind of binding site, and the ratio of the dissociation rate constant and the intramolecular rate constant for each intermediate must be made as small as possible; for a multistep reaction in which reaction steps are coupled by recycling of the intermediates (cyclic coupling), covalent linking of the intermediates to the catalyst is the best way, and the more the number of the linked intermediates, the higher the activity of the catalyst becomes.



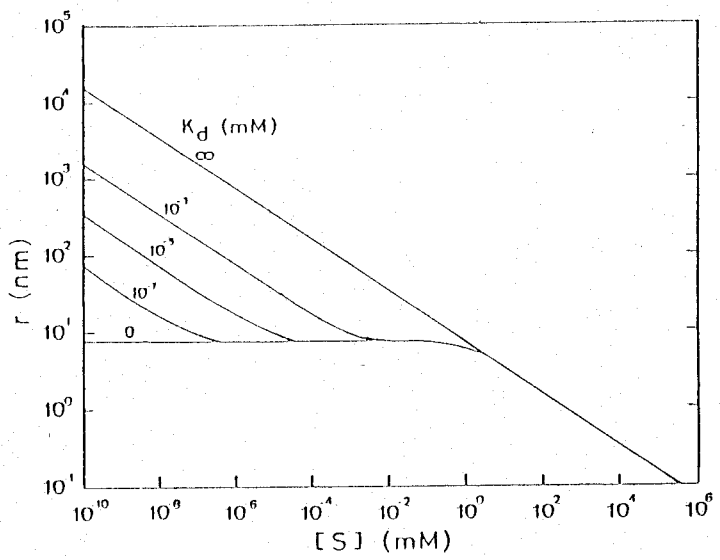


Fig. IV-1. Plot of Eqn IV-9. For A, the value of K_d is fixed at 10^{-3} mM, and the value of [S] is varied at the indicated values of k_{in}/k_{ex}; for B, the value of k_{in}/k_{ex} is fixed at 1 mM, and the value of [S] is varied at the indicated values of k_d.

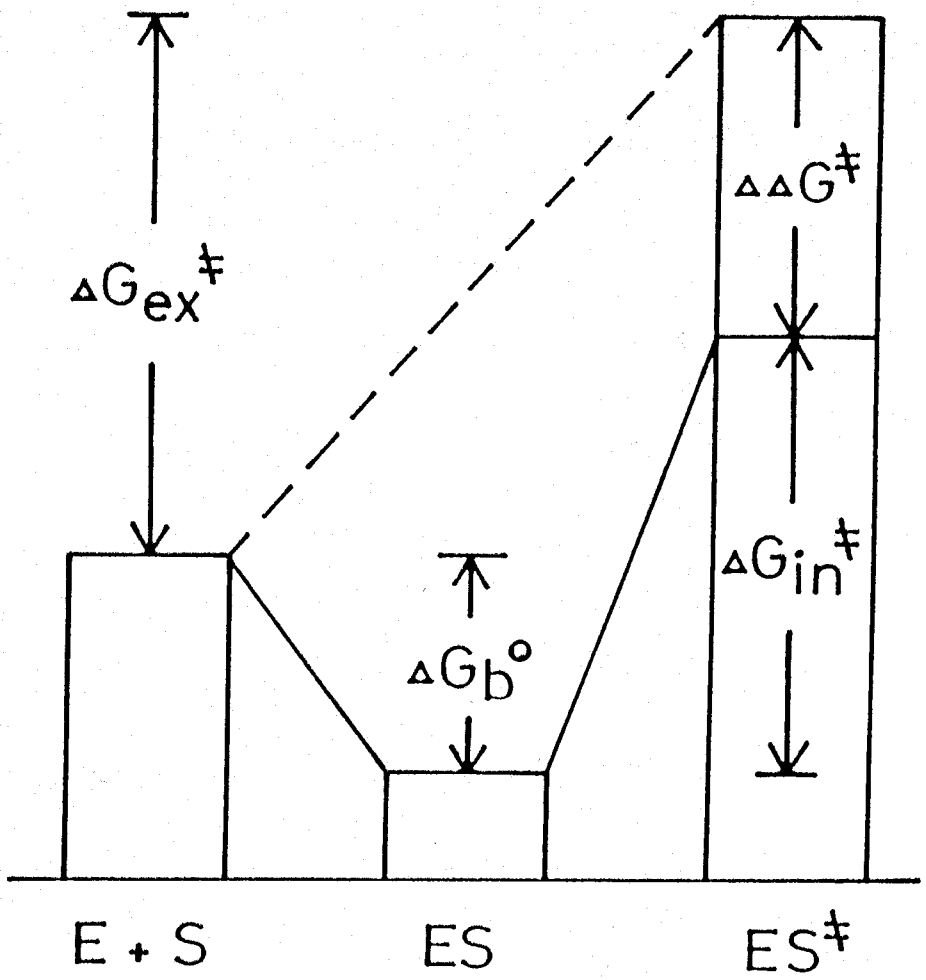


Fig. IV-2. Free-energy diagrams for enzymatic and non-enzymatic catalysis. ——, Enzymatic process; - - -, non-enzymatic process. ΔG_b°, the standard free energy change for the binding of the substrate with its binding site and defined by $\Delta G_b^{\circ} = -RT\ln K_d$; $\Delta G_{in}^{\ddagger}$ and $\Delta G_{ex}^{\ddagger}$ are the activation free energies corresponding to the intramolecular and the intermolecular rate constants, respectively, and are defined by $\Delta G_{in}^{\ddagger} = -RT\ln k_{in}$ and $\Delta G_{ex} = -RT\ln k_{ex}$; $\Delta\Delta G^{\ddagger}$ corresponds to the rate-acceleration by the enzymatic process and is defined by Eqn IV-15.

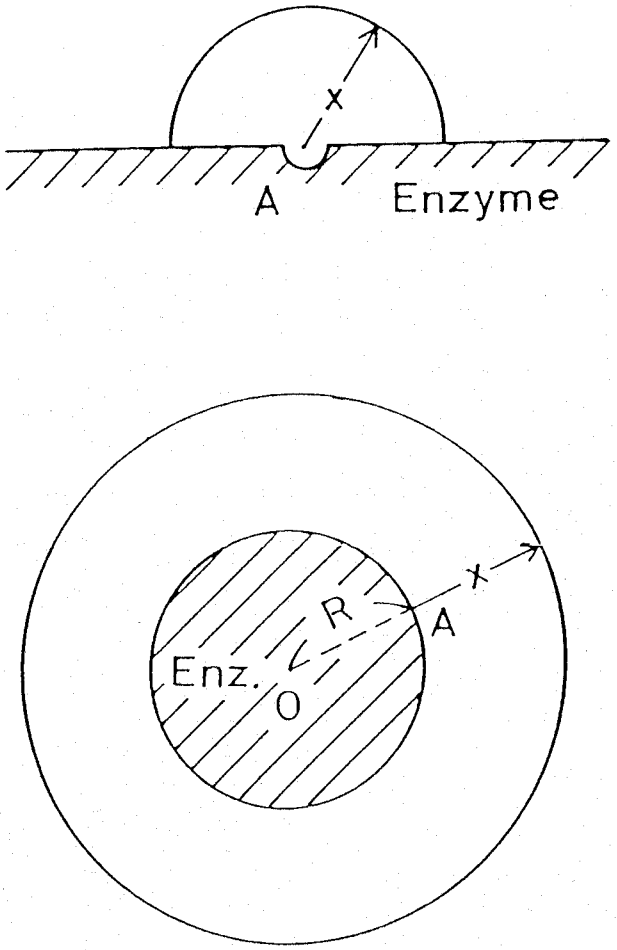


Fig. IV-3. Diffusion of an intermediate from its binding site makes a concentration gradient of the intermediate. The intermediate continuously springs out from point A (the binding site) (Fig. IV-3A) or point O (Fig. IV-3B) with a rate constant of k_{of}. The concentration of the intermediate at a half sphere with a radius of x from point A is expressed by Eqn IV-30, and that at a sphere with a radius of (x + dx) from point O is by Eqn IV-31. Here, the size of the binding site is assumed to be much smaller than the enzyme molecule.

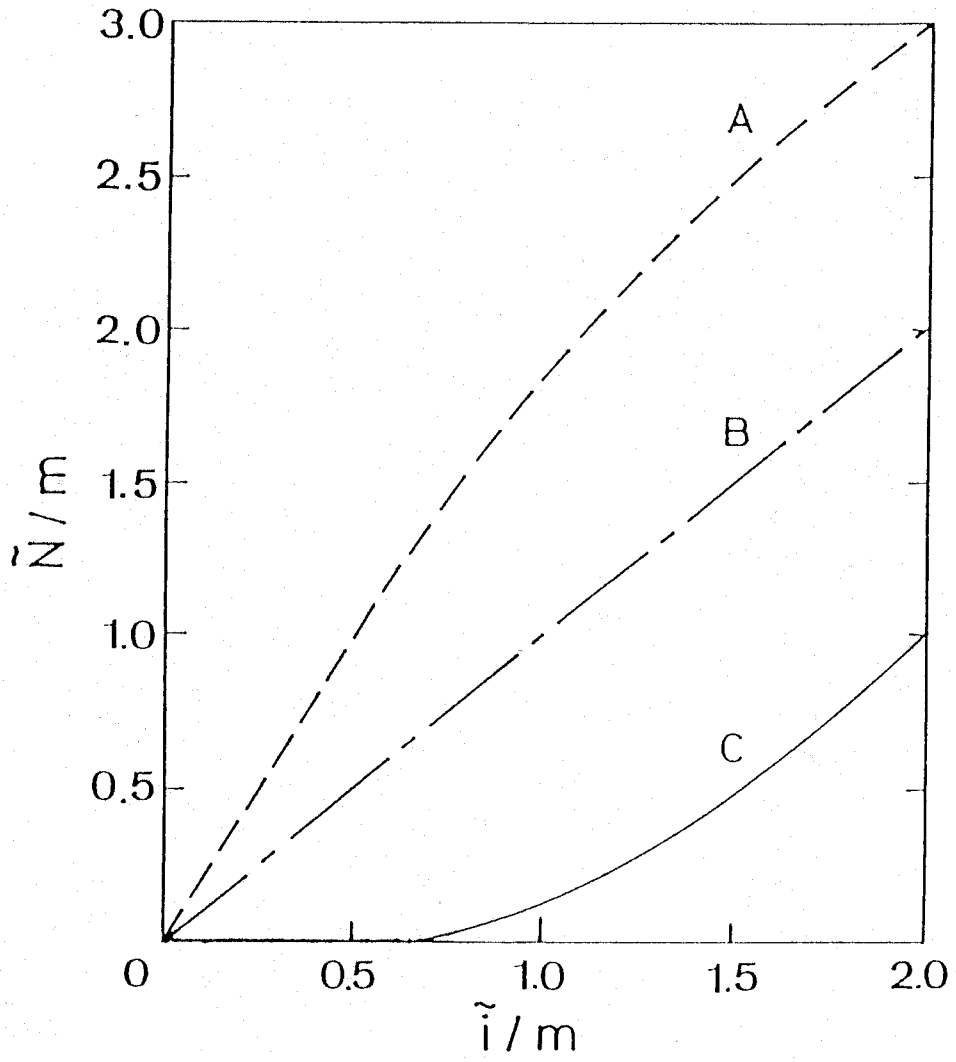


Fig. IV-4. Plot of $\tilde{i}/m$ vs. $\tilde{N}/m$. The values of $\tilde{N}/m$ were calculated using the following constants: K = 120, m = 4, n = 2, and ε = 2 (for curve A), 1 (for curve B), or 0 (for curve C).

Conclusion

There are three main characters in an enzyme reaction: a substrate-binding site, a catalytic group and substrate. I prepared these characters separately, made three type of the enzyme-like catalysts by linking two or three of them:EP-PEG-GltDH, EP-GlcDH-NAD, EP-LDH-NAD. The kinetic analysis of these conjugates provided us the better understanding of the rate-acceleration mechanism used in enzyme reactions and the basic information for designing artificial enzymes.

In chapter I, I showed EP-PEG-GltDH to work as an NADH oxidase. The coenzyme-binding site of the GltDH moiety is used as a substrate-binding site. The kinetic analysis of the NADH-oxidase activity shows the effect of the presence of the substrate-binding site near the catalytic group of the EP moiety. In chapter II, I showed EP-GlcDH-NAD to work as a glucose oxidase. This activity is due to the coupling of the two catalytic reactions of the GlcDH and the EP moieties, and the kinetic analysis shows the presence of the following rate-acceleration mechanisms: high effective concentration, intramolecular coupling of successive catalytic reactions, and multiple connection between the two kinds of the catalytic sites. In chapter III, I showed EP-LDH-NAD to work as a lactate oxidase. The kinetic analysis shows the effect of hiding the NADH moiety by the coenzyme-binding site of the LDH moiety. In the chapter IV, I showed the several equations expressing the relationship between the activity of catalysts and several effects as described above. The general knowledges concerned with to what extent those effects influence the activity were obtained analyzing the equations, and the following strategies for designing an artificial enzyme are presented.

For simple catalysts having one substrate-binding site and one

catalytic group, use a binding site with higher affinity for the substrate, and make the ratio of the intramolecular and intermolecular rate constants as large as possible. To increase the intramolecular rate constant, select a good binding site and use a flexible linker with an appropriate length. A catalyst with a subunit structure is also effective to increase the specific activity.

For a more complex catalyst having one substrate-binding site and two kinds of catalytic groups, it is important to couple the two catalytic reactions intramolecularly. For the reaction system with cyclic coupling, covalent linking of the intermediate is the best way. If the intermediates are covalently linked, the catalyst does not need to have the binding sites for the intermediates.

List of abbreviations

DHBT	3,3'-(1,6-dioxo-1,6-hexanediyl)bis-2-thiazolidinethione
EP^+-DHG-NAD^+	5-ethylphenazine-dehydrogenase-NAD^+ conjugate
EP^+-GlcDH-NAD^+	5-ethylphenazine-glucose dehydrogenase-NAD^+ conjugate
EP^+-LDH-NAD^+	5-ethylphenazine-lactate dehydrogenase-NAD^+ conjugate
EP^+-PEG	poly(ethylene glycol)-bound 5-ethylphenazine
EP^+-PEG-DHG	5-ethylphenazine-poly(ethylene glycol)-dehydrogenase conjugate
EP^+-PEG-GltDH	5-ethylphenazine-poly(ethylene glycol)-glutamate dehydrogenase conjugate
EP^+-PEG-NAD^+	5-ethylphenazine-poly(ethylene glycol)-NAD^+ conjugate
GlcDH-PEG-NAD^+	glucose dehydrogenase-poly(ethylene glycol)-NAD^+ conjugate
MBTH	3-methyl-2-benzothiazolinone-hydrazone hydrochloride
MDH-PEG-NAD^+	malate dehydrogenase-poly(ethylene glycol)-NAD^+ conjugate
MTT	3-(4',5'-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
PEG-NAD^+	poly(ethylene glycol)-bound NAD^+

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Publications related to this thesis

1. Synthesis and characterization of 1-substituted 5-alkylphenazine derivatives carrying functional groups

Yomo, T., Sawai, H., Urabe, I., Yamada, Y., & Okada, H. (1989a) Eur. J. Biochem. 179, 293-298.

2. Preparation and kinetic properties of 5-ethylphenazine-poly(ethylene glycol)-NAD^+ conjugate, a unique catalyst having an intermolecular reaction step

Yomo, T., Sawai, H., Urabe, I., & Okada, H. (1989b) Eur. J. Biochem. 179, 299-305.

3. Enzymatic method of measuring the absolute value of oxygen concentration

Yomo, T., Urabe, I., & Okada, H. (1989) Anal. Biochem. 179 124-126

4. Electrostatic and redox potential effects on the rate of electron-transfer reaction of nicotinamide adenine dinucleotides with 1-substituted 5-ethylphenazines

Yomo, T., Urabe, I., & Okada, H. (1990) Biochem. Biophys. Acta 1017 139-142

5. Preparation and kinetic properties of 5-Ethylphenazine-poly(ethylene glycol)-glutamate dehydrogenase conjugate, a semisynthetic NADH oxidase

Yomo, T., Urabe, I., & Okada, H. Eur. J. Biochem. in press.

6. Preparation and kinetic properties of 5-Ethylphenazine-glucose dehydrogenase-NAD^+ conjugate, a semisynthetic glucose oxidase

Yomo, T., Urabe, I., & Okada, H. in preparation.

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