*Biochimica et Biophysica Acta*, 484 (1977) 9—23


BBA 68226

# KINETIC STUDIES OF THE OLD YELLOW ENZYME

## I. THE REACTION MECHANISM OF THE ENZYME WITH REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

TAKESHI HONMA * and YASUYUKI OGURA **

*Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo (Japan)*

(Received February 12th, 1977)

## Summary

The reaction mechanism of old yellow enzyme (NADPH:(acceptor) oxidoreductase, EC 1.6.99.1) was kinetically investigated using NADH as substrate. The enzyme was reduced by NADH via a reaction intermediate which has a specific absorption spectrum. This intermediate decomposed to yield a reduced enzyme and $NAD^+$ through an irreversible first-order reaction step. The reduced enzyme was reoxidized by oxygen through a second-order reaction process. Individual values of elementary rate constants were measured and a computer simulation of the reaction process was carried out. No involvement of free radical of flavin semiquinone in the reaction process could be shown.

## Introduction

The reaction mechanism of the old yellow enzyme (NADPH:(acceptor) oxidoreductase, EC 1.6.99.1) was previously studied by Nakamura, Yoshimura and Ogura [1]. In their reaction scheme using NADH as substrate, the oxidized form of the enzyme formed a complex with the substrate and this complex reacted with oxygen. Additionally, the complex was considered to be identical with Haas's red-colored complex [2], which was produced by titration of the reduced enzyme with $NAD^+$ under anaerobic conditions.

In the present experiment, a more detailed investigation was carried out into the reaction mechanism of old yellow enzyme using NADH as substrate.

* To whom reprint requests should be addressed. Present address: Research and Development Center, Pharmaceuticals Division, Sumitomo Chemical Co., Ltd., 4-2-1, Takatsukasa, Takarazuka, Hyogo, 665, Japan.

** Present address: School of Pharmaceutical Science, Toho University, Narashino, Chiba, Japan.

## Materials and Methods

*NADH.* NADH was prepared from NAD (Sigma Chemical Co.) by reduction with ethanol in the presence of alcohol dehydrogenase from baker's yeast.

*The preparation of the old yellow enzyme.* The old yellow enzyme was prepared and purified using a modified method of Theorell and Åkeson [3]. Brewer's bottom yeast (*Saccharomyces carlsbergensis*) was kindly supplied from Ebios Yakuhin Kogyo Co. The enzyme was finally purified by gel filtration with a Biogel P-150 column. The solution of the purified enzyme had a absorption ratio, $A_{383nm}/A_{464nm}$, of 0.98, which is equal to the ratio of crystalline enzyme [3]. The concentration of the enzyme was expressed as that of the enzyme-bound FMN and it was estimated spectrophotometrically by measuring the absorbance of the enzyme solution at 464 nm, using the millimolar extinction coefficient of 10.6 $cm^{-1}$ [3].

*Experimental conditions.* All the experiments were performed at pH 7.0 and 25°C in 0.1 M phosphate buffer, except for the ESR measurement, which was carried out at liquid nitrogen temperature.

*Measurement of oxygen uptake.* When oxygen was used as an electron acceptor, the overall rate of reaction was estimated by measuring the consumption of molecular oxygen dissolved in the reaction medium by means of a Clark oxygen electrode made by the Yellow Spring Instrument Co. The reaction was initiated by the addition of 0.1 ml of enzyme solution into 2.9 ml of the reaction mixture which had been previously saturated with air and placed in the reaction vessel. The concentration of oxygen in the reaction mixture was increased or decreased by bubbling with oxygen gas or nitrogen gas, respectively, before the reaction.

*Measurement of the reduction of ferricyanide.* When ferricyanide was used as an electron acceptor, the overall rate of reaction was estimated by measuring the reduction of ferricyanide. The concentration of ferricyanide was determined by using the millimolar extinction coefficient of 1.04 $cm^{-1}$ at 420 nm. The reaction was initiated by the addition of 0.1 ml of enzyme solution into the cuvette containing 2.9 ml of reaction mixture. The decrease of the optical density at 420 nm was recorded with a Hitachi Perkin-Elmer spectrophotometer, model 139.

*Spectrophotomeric measurements by the Flow Method.* For analysis of each reaction step, a flow apparatus was used. The apparatus used comprised a sensitive spectrophotometer combined with a flow system which had been designed by Chance and Legallais [4]. The changes in absorbance were displayed on a San-ei oscillograph, model ER 101.

*Computer simulation.* A digital computer, Hitachi 5020E, was used for the simulation of the enzyme reaction. A set of first-order simultaneous differential equations was numerically solved using a Runge-Kutta-Gill method [5]. The programme was written in FORTRAN IV.

*Electron spin resonance spectrometry.* ESR measurement of the reaction intermediate, which appeared immediately after the mixing of the enzyme with the substrate, was made according to Bray's rapid freezing technique [6]. The ESR measurement of the red-colored complex produced by the anaerobic titration was performed at room temperature according to the method of

Ogura, Nakamura and Nakamura [7]. For ESR measurements performed at liquid nitrogen temperature, the sample was anaerobically introduced into a glass tube of 3 mm internal diameter. The tube containing the sample was frozen in liquid nitrogen, and the signal was measured at 77 K. The ESR spectrometer used was a model P-10 X-band instrument of Japan Electron Optics Laboratory Co.

*Titration of the reduced enzyme with $NAD^+$.* A stock solution of old yellow enzyme was anaerobically reduced with an equimolar amount of NADH. This reduced enzyme was titrated by adding various amounts of $NAD^+$ solution in an anaerobic cuvette and the mixtures were made up to equal volume by the addition of buffer solution. The solutions of $NAD^+$ and buffer had been kept under anaerobic conditions before the experiments. The absorption spectrum was recorded using a Shimazu recording spectrophotometer, model MPS-50.

## Results

*The kinetics of overall reaction*

The rate of overall reaction was followed by measuring the oxygen uptake in the presence of various concentrations of substrate (NADH) and electron acceptor (oxygen). The Lineweaver-Burk plot of initial velocity is shown in Figs. 1a and 1b. As Fig. 1 shows, the plots were linear and a set of parallel lines was obtained in which the ordinate intercept varied. From Figs. 1a and 1b, the following equation is deduced:

$$\frac{e_0}{v} = \frac{e_0}{V} + \frac{A}{[NADH]} + \frac{B}{[O_2]} \quad (1)$$

where $e_0$ is the concentration of the enzyme, $v$ is the initial velocity of the oxygen uptake, $V$ is the maximum velocity of the reaction and A and B are constants determined from the gradients of the parallel lines in Figs. 1a and 1b, respectively. The rate of the monomolecular reaction step was obtained by extrapolation of the lowest line to the ordinate in Fig. 1a, and the value of $V/e_0$ was 0.71 $s^{-1}$. The values $A = 3.1 \cdot 10^{-4}$ M · s and $B = 9.1 \cdot 10^{-4}$ M · s were obtained.

In the presence of ferricyanide as an electron acceptor, no consumption of oxygen was observed and the rate of the reduction of ferricyanide was independent of the concentration of ferricyanide. After the ferricyanide was used up, the residual substrate was oxidized by oxygen. The Lineweaver-Burk plot in the presence of ferricyanide, with NADH as substrate, was the same as the lowest line in Fig. 1a when an infinite concentration of oxygen was available. Values for the kinetic constants relating to conditions of infinite oxygen concentration and ferricyanide addition are shown in Table I. These results would indicate identical enzyme reaction pathways with oxygen or ferricyanide used as electron acceptor. However, the reoxidation by ferricyanide was far more rapid than that by oxygen.

*The absorption spectrum change during the anaerobic reduction of the oxidized form of the enzyme*

Using the flow apparatus, the solution of the oxidized enzyme was mixed

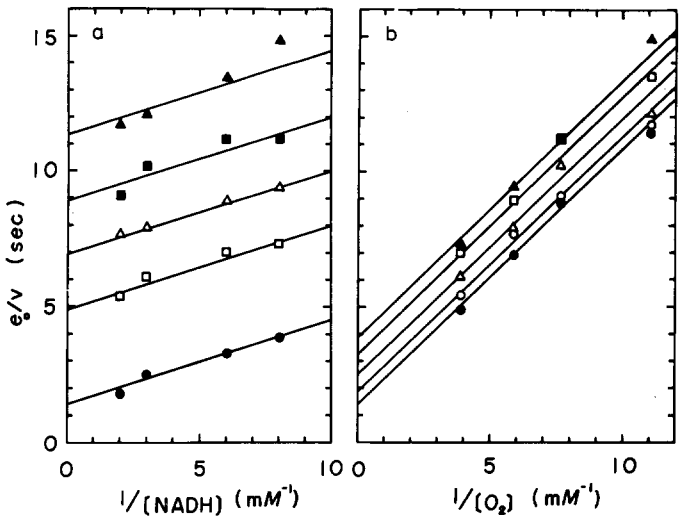


Fig. 1. Lineweaver-Burk plots of the overall reaction for NADH concentration (a) and oxygen concentration (b). Enzyme concentration (based on FMN): 2.88 μM. Molecular oxygen concentration in a: 90 μM (▲), 130 μM (■), 170 μM (△), 255 μM (□) and ∞ (●). NADH concentration in b: 127 μM (▲), 168 μM (□), 338 μM (△), 506 μM (○) and ∞ (●). In b, each line was extrapolated to the ordinate i.e. $1/[O_2] = 0$. Each ordinate intercept was replotted in a (●). In a, each line was extrapolated to the ordinate ($1/[NADH] = 0$) and each ordinate intercept was replotted in b (●). Buffer, 0.1 M phosphate (pH 7.0); temperature, 25°C.

with 750 μM NADH under anaerobic conditions, and the absorption spectrum change was observed over the wavelength range 420—700 nm. The absorption spectrum of the enzyme at 100 and 300 ms after mixing with NADH are shown in Fig. 2. Both oxidized and reduced enzyme have no absorption over the range 560—700 nm. However, a rapid increase and a subsequent slow decrease in absorbance were observed in this wavelength region immediately after mixing. This absorbance increase was thought to be result of the formation of a reaction intermediate. By measuring the rate of the decrease in the absorbance at 560 nm, the rate of decay of the intermediate (0.69 $s^{-1}$) was obtained as a first-order rate constant. The formation of the intermediate (increase in absorbance) was so rapid that the rate of formation was measured by using the continuous flow technique at different NADH concentrations. The wavelength used was 560 nm. An apparent rate constant of formation ($k_{app}$) was estimated

TABLE I

KINETIC CONSTANTS OBTAINED FROM OVERALL REACTIONS

The values of $V/e_0$, A and B were estimated from the Lineweaver-Burk plot of the initial velocity of oxidation of NADH by molecular oxygen (Figs. 1a and b) or ferricyanide in the presence of the enzyme.

| | Electron acceptor | |
|---|---|---|
| | $O_2$ | $Fe(CN)_6^{3-}$ |
| $V/e_0$ ($s^{-1}$) | 0.71 | 0.67 |
| A (M · s) | $3.1 \cdot 10^{-4}$ | $3.0 \cdot 10^{-4}$ |
| B (M · s) | $9.1 \cdot 10^{-4}$ | — |

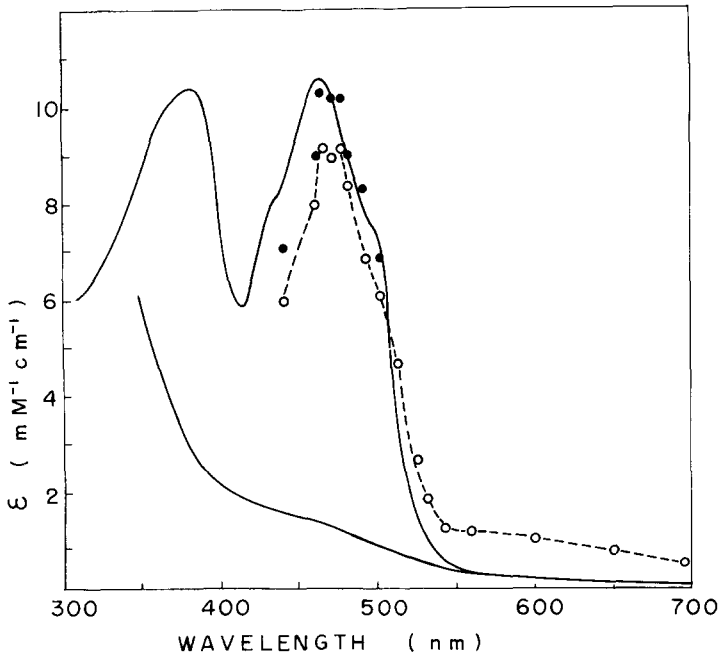


Fig. 2. Absorbance change on reacting the oxidized enzyme with NADH in the absence of oxygen. Absorption spectra recorded at 100 (•) and 300 (○) ms after mixing in flow apparatus. $\epsilon$ ($mM^{-1} \cdot cm^{-1}$) represents millimolar extinction coefficient. In the wavelength range 520—700 nm, the level of absorption at 100 ms was almost the same as at 300 ms. The continuous lines represent the oxidized (upper line) and reduced (lower line) enzyme. Enzyme concentration, 5 $\mu$M; NADH concentration, 750 $\mu$M.

according to the following equation:

$$k_{app} = 2.303 \log\left[\frac{\Delta A_t}{\Delta A_m - \Delta A_t}\right] \cdot \frac{1}{t} \qquad (2)$$

Where $\Delta A_m$ represents the maximum increase in absorbance (the height of the peak of absorbance increase) after the mixing of the enzyme and substrate solutions, and $\Delta A_t$ represents the absorbance increase at $t$ s after mixing, observed before absorbance increase reached maximum. In Fig. 3, values of $k_{app}$ are plotted against NADH concentrations. A plot of increase of $k_{app}$ did not give a straight line but levelled off at high substrate concentration. This result indicates that a simple, one step, reversible reaction is not suitable for explaining the mechanism of formation of intermediate from the oxidized enzyme and NADH. It may most simply be ascribed to a two step, reversible reaction in which the first step is in rapid equilibrium and the second step is a slower isomerization process [8]:

$$E_{ox} + NADH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}NADH \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} X \qquad \text{(Scheme 1)}$$

$$k_{+1}[NADH] + k_{-1} \gg k_{+2} + k_{-2},$$

where X has been written for the intermediate that has a particular absorbance at 560 nm, and $E_{ox}$ + NADH and $E_{ox}$NADH exhibit the same spectrum. Then

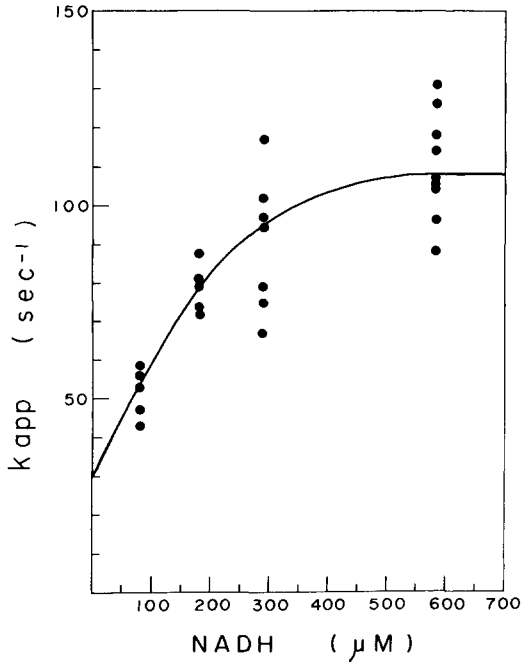


Fig. 3. Plot of the apparent rate constant of formation of reaction intermediate ($k_{app}$) against the NADH concentration. $k_{app}$ was calculated according to Eqn. 2 in the text. Solid line represents the theoretical curve obtained from Eqn. 3 and numerical values in the text.

$k_{app}$ is given by

$$k_{app} = k_{-2} + \frac{k_{+2}[\text{NADH}]}{K_1 + [\text{NADH}]} \tag{3}$$

where,

$K_1 = k_{-1}/k_{+1}$,

since [NADH] >> [E], and [NADH] remains constant in the reaction. [NADH] and [E] represent the initial concentrations of NADH and enzyme, respectively. Assuming the two step reaction, the values of $k_{+2}$, $k_{-2}$ and $K_1$ were estimated to be 80 $s^{-1}$, 30 $s^{-1}$ and $1.7 \cdot 10^{-4}$ M, respectively, as the best fit for the experimental values.

It appears from the absorbance change that the following sequential reactions occurred when the solution of the enzyme previously deaerated with nitrogen was anaerobically mixed with the solution of NADH in flow apparatus:

$$E_{ox} + \text{NADH} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}\text{NADH} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} X \xrightarrow{k_{+3}} E_{red} + \text{NAD}^+ \qquad \text{(Scheme 2)}$$

According to this scheme, the following equation can be derived relating to

$\Delta A_m$, $\Delta\epsilon_{560}$, $e_0$ and the rate constants.

$$\frac{1}{\Delta A_m} = \frac{1}{\Delta\epsilon_{560} \cdot e_0}\left(1 + \frac{k_{-2} + k_{+3}}{k_{+2}} + \frac{K_m}{[NADH]}\right) \tag{4}$$

$$K_m = \frac{k_{-1}k_{-2} + k_{-1}k_{+3} + k_{+2}k_{+3}}{k_{+1}k_{+2}},$$

where $\Delta\epsilon_{560}$ represents the differential millimolar extinction coefficient (intermediate minus oxidized or reduced form) of the enzyme at 560 nm and $e_0$ represents the total concentration of the enzyme (see Appendix I). A linear relationship was obtained when plotting reciprocals of $\Delta A_m$ against those of NADH. Using the values of $k_{+2}$, $k_{-2}$ and $k_{+3}$ obtained previously, a millimolar extinction coefficient of 1.03 $cm^{-1}$ and $K_m$ value of $1.6 \cdot 10^{-4}$ M were obtained. Thus, the extinction coefficient of the intermediate in the wavelength range greater than 560 nm could be estimated. However, in the range 420—540 nm, $E_{ox}$ and $E_{ox}$NADH in addition to X contribute to the absorbance. Using the values of $k_{+2}$ and $k_{-2}$, the amounts of $E_{ox}$NADH and X which exist at infinite concentration of NADH were estimated as 27 and 73% of the total concentration of enzyme, respectively. Since $E_{ox}$ and $E_{ox}$NADH exhibit the same spectrum, the extinction coefficient of X could be calculated from the linear relationship between $1/\Delta A_m$ and 1/[NADH] at different wavelengths by eliminating the contribution of $E_{ox}$ through the extrapolation of the plot to the ordinate. The absorption spectra of X thus estimated are shown in Fig. 4.

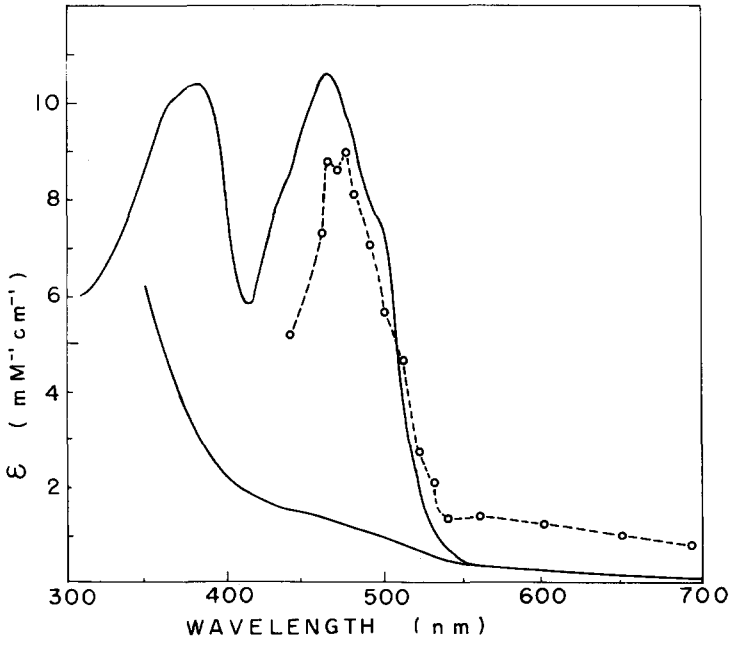


**Fig. 4. Absorption spectrum of the reaction intermediate. From the anaerobic reduction of the oxidized enzyme with NADH, the millimolar extinction coefficient ($\epsilon$) of the intermediate was calculated according to Eqn. 4 and numerical values in the text.**

*The measurement of the rate of reoxidation of $E_{red}$ using the flow method*

The solutuons of the oxidized enzyme and NADH were kept separately and saturated with air before experiments. The enzyme solution was rapidly mixed with substrate by means of the stopped flow method under aerobic conditions. The absorbance change of the mixed enzyme solution at 464 nm was recorded. The recorder trace is shown in Fig. 5. The absorbance rapidly decreased due to the formation of the intermediate and the reduced enzyme, and the enzyme returned to the oxidized form on exhaustion of the added NADH. Since the absorbance of the reaction intermediate is similar to that of the oxidized enzyme at 464 nm, the contribution of the intermediate to the decreased absorbance could be neglected. The rate constant $k_{ox}$ of the oxidation of the enzyme by oxygen was estimated according to the following equation [9] [10] (see Appendix II):

$$k_{ox} = \frac{[NADH]}{[O_2] \cdot P_{max} \cdot t_{1/2}}$$

where $P_{max}$ has been written as the maximum concentration of the reduced enzyme formed during the course of reaction and $t_{1/2}$ represents the time required for the return to the level of half the decreased absorbance. The values obtained are given in Table II. The values of $k_{ox}$ were constant within the limits of experimental error. The values in Table II show that the plot of $1/k_{ox}[O_2]$ against $1/[O_2]$ is linear and crosses the ordinate at $1/[O_2] = 0$. This fact suggests that the reoxidation process in the sequential reactions does not involve a first-order rate step and follows second-order kinetics with respect to the concentration of oxygen.

*Computer simulation of the enzyme reaction*

From the experiment of anaerobic reduction of the oxidized enzyme with NADH, scheme 2 was proposed for the mechanism of oxidation of NADH by old yellow enzyme and each value for the elementary rate constant was estimated. To confirm these values, computer simulation was attempted and the change in the amount of the reaction intermediate was calculated using the given rate constants. On representing the amounts of $E_{ox}$, $E_{ox}NADH$, X and $E_{red}$ by functions which vary with time, the simulation of this reaction scheme

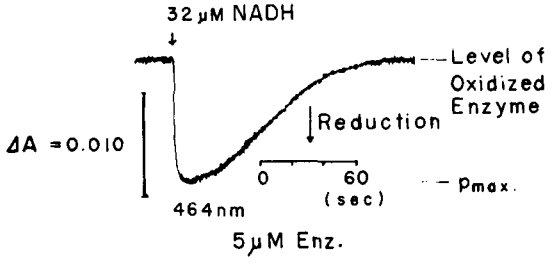


Fig. 5. Oscillographic trace of absorbance change at 464 nm obtained in an aerobic flow experiment. The solution of oxidized enzyme was rapidly mixed with NADH solution in the flow apparatus, and the change in absorbance was recorded in the presence of oxygen. Enzyme concentration, 5 μM; NADH concentration, 32 μM; oxygen concentration, 260 μM.

TABLE II

$k_{ox}$ VALUES OBTAINED IN THE AEROBIC FLOW EXPERIMENT

The rate constants of oxidation of NADH by molecular oxygen ($k_{ox}$) were estimated from the oscillographic trace in Fig. 5 at different NADH concentrations. $k_{ox}$ was estimated according to the equation in Appendix II.

| NADH (μM) | $k_{ox}$ ($M^{-1} \cdot s^{-1}$) |
|---|---|
| 32 | $1.3 \cdot 10^3$ |
| 44 | $1.1 \cdot 10^3$ |
| 67 | $1.2 \cdot 10^3$ |
| 126 | $1.0 \cdot 10^3$ |

may be reduced to a mathematical problem solving simultaneous ordinary differential equations with four unknowns. Unfortunately the complete solution of this problem leads to equations of great complexity. For that reason, the Runge-Kutta-Gill method was adopted as a numerical solution for these equations [5]. An initial condition, that is $[E_{ox}] = 1$ and $[E_{ox}NADH] = [X] = [E_{red}] = 0$ at $t = 0$, was given. Five elementary rate constants were given as the coefficients of these equations. Among these rate constants, $k_{+2}$, $k_{-2}$, $k_{+3}$ and the ratio $k_{-1} : k_{+1}$ were already obtained. However, there are no data available regarding the magnitudes of the individual values of $k_{-1}$ and $k_{+1}$, so appropriate values were chosen such as to be in accordance with the assumption $k_{+1}[NADH] + k_{-1} >> k_{+2} + k_{-2}$, used when the equation for $k_{app}$ had been derived. As step size was $10^{-2}$, the accumulated truncation error of the approximate solution was less than $10^{-7}$. The program was written so as to plot the calculated values of [X] vs. time. Figs. 6a and 6b show the calculated and experimental curves, respectively. An actual value of $k_{+3}$ seems to be a little greater than the estimated one of $V/e_0$, so that the value of 1.0 $s^{-1}$ was used for $k_{+3}$.

*ESR measurement*

Using the rapid freezing technique [6], ESR measurement on the reaction intermediate was undertaken. In the flow apparatus, the oxidized form of the enzyme was anaerobically mixed with NADH and the solution was rapidly introduced into a solution of isopentane previously cooled in liquid nitrogen so that the solution was frozen. The reaction was stopped by rapid freezing 110 ms after mixing. Measurement was performed at 77 K. No difference in signal between the reaction mixture with NADH and the enzyme solution without NADH was observed.

*Titration of the reduced enzyme with $NAD^+$*

On reaction with $NAD^+$, the enzyme which was reduced with equimolar NADH formed a red-colored complex which was similar to that formed with $NAD^+$ and the enzyme reduced by hydrosulfite as performed by Haas [2]. $NAD^+$ solution was added to the solution of the reduced enzyme in the absence of oxygen. By plotting the reciprocals of the increase in the absorbance ($1/\Delta A$) against those of the concentration of $NAD^+$ (final concentration: 2.2—14.8 mM) at different wavelengths, straight lines were obtained. This finding indi-

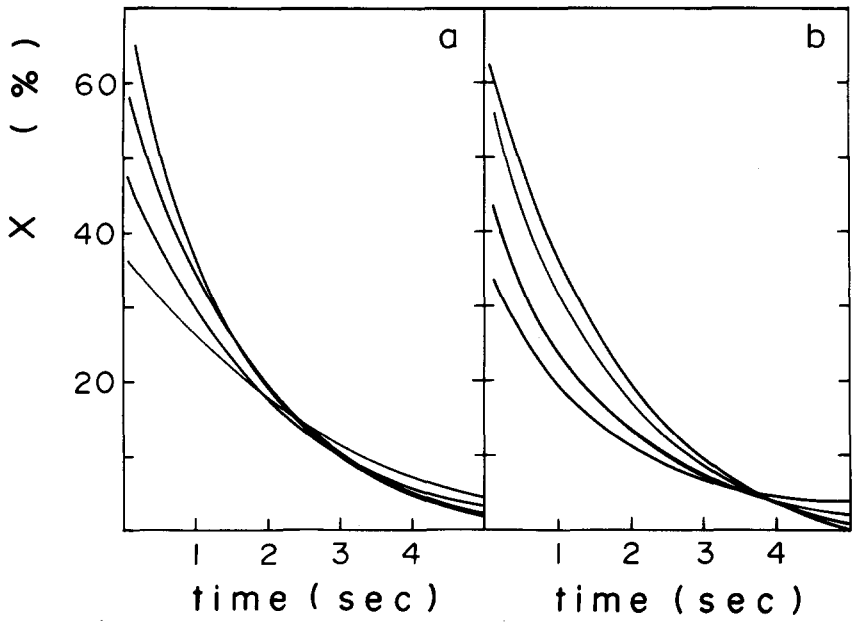


**Fig. 6. The change in the amount of intermediate, X, against reaction time. The amount of X is expressed as a percentage of total concentration of enzyme. The experimental curves are shown in b. The NADH concentrations are 590, 292, 183 and 81 $\mu$M from top to bottom. The simulation curves are shown in a. The values $k_{+1} = 1.7 \cdot 10^6\ M^{-1} \cdot s^{-1}$, $k_{-1} = 500\ s^{-1}$, $k_{+2} = 80\ s^{-1}$, $k_{-2} = 30\ s^{-1}$ and $k_{+3} = 1.0\ s^{-1}$ were used for calculation. The NADH concentrations correspond to those in b, respectively, from top to bottom.**

cates the following equilibrium exists among the reduced enzyme, $NAD^+$ and the complex:

$$E_{red} + NAD^+ \underset{k'}{\overset{k}{\rightleftharpoons}} \text{complex}$$

According to this scheme, the following equation can be derived:

$$\frac{1}{\Delta A} = \frac{1}{\Delta\epsilon \cdot e_0}\left(1 + \frac{K}{[NAD^+]}\right)$$

where

$$K = k'/k,$$

where $\Delta\epsilon$ represents the differential millimolar extinction coefficient (complex minus reduced form) of the enzyme and $e_0$ represents the total concentration of the enzyme. Thus, the absorption spectrum of the complex was estimated and shown in Fig. 7. The dissociation constant calculated ($K = 1.5 \cdot 10^{-2}$ M) was almost constant at 440—700 nm and 25°C, and the value at 5°C was not different.

ESR measurement of the complex formed in this way was performed at 77 K and at room temperature. No significant signal caused by flavin free radicals formation was observed.

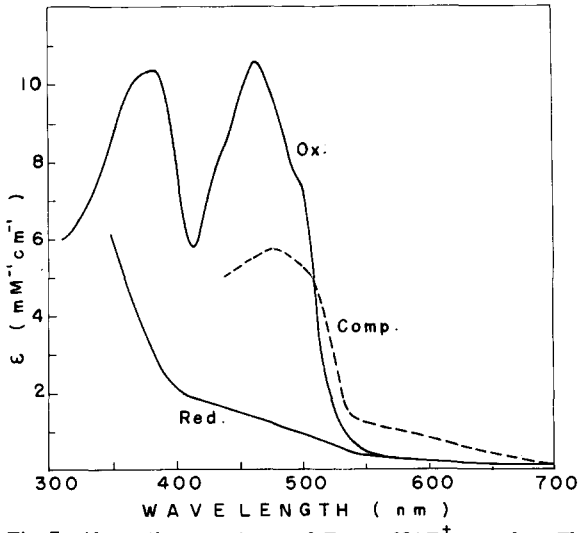


Fig. 7. Absorption spectrum of $E_{red} \cdot NAD^+$ complex. The reduced enzyme was titrated with $NAD^+$ in the absence of oxygen. The millimolar extinction coefficient ($\epsilon$) of the complex formed by titration was calculated according to the method described in the text.

## Discussion

The rate of decomposition of the intermediate (0.69 $s^{-1}$) to products $E_{red}$ and $NAD^+$ is in fair agreement with that of the first-order reaction step in overall reaction (0.71 $s^{-1}$). This finding indicates that the reduced form of the enzyme is included in the turnover process, and that a molecular species of the enzyme which is reoxidized by oxygen or ferricyanide is the reduced form of the enzyme. The kinetic data suggested that the intermediate was formed with $E_{ox}$ and NADH via an ES complex.

The results obtained can be summarized by the following reaction scheme when NADH and oxygen were used as substrate and electron acceptor, respectively:

$$E_{ox} + NADH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox}NADH \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{intermediate} \xrightarrow{k_{+3}} E_{red} + NAD^+$$

$$E_{red} + O_2 \xrightarrow{k_{0x}} E_{ox} + H_2O_2 \qquad \text{(Scheme 3)}$$

The following equation is given for the rate of overall reaction in scheme 3:

$$\frac{e_0}{v} = \frac{1}{k_{+3}} + \frac{K_m}{k_{+3}} \frac{1}{[NADH]} + \frac{1}{k_{ox}} \frac{1}{[O_2]} \qquad (5)$$

$$K_m = \frac{k_{-1}k_{-2} + k_{-1}k_{+3} + k_{+2}k_{+3}}{k_{+1}k_{+2}}$$

It is thought that $e_0/V$, A and B in Eqn. 1 correspond to $1/k_{+3}$, $K_m/k_{+3}$ and $1/k_{ox}$ in Eqn. 5, respectively. From the value of $V/e_0$ (0.71 $s^{-1}$), A ($3.1 \cdot 10^{-4}$ M · s) and B ($9.1 \cdot 10^{-4}$ M · s), the values of $k_{+3}$, $K_m$ and $k_{ox}$ were calculat-

TABLE III

KINETIC CONSTANTS OBTAINED BY VARIOUS METHODS

| | | Overall * | Anaerobic reduction ** | Stopped flow *** |
|---|---|---|---|---|
| $k_{+3}$ | ($s^{-1}$) | 0.71 | 0.69 | |
| $K_m$† | (M) | $2.2 \cdot 10^{-4}$ | $1.6 \cdot 10^{-4}$ | |
| $k_{ox}$ | ($M^{-1} \cdot s^{-1}$) | $1.1 \cdot 10^{3}$ | | $1.2 \cdot 10^{3}$ |
| $k_{-1}/k_{+1}$ | (M) | | $1.7 \cdot 10^{-4}$ | |
| $k_{+2}$ | ($s^{-1}$) | | 80 | |
| $k_{-2}$ | ($s^{-1}$) | | 30 | |

* Obtained from the overall reaction when oxygen was used as electron acceptor.

** Obtained from the absorbance change at 560 nm when the enzyme was anaerobically mixed with NADH.

*** Obtained from the absorbance change at 464 nm when the oxidized enzyme was mixed with NADH in the presence of oxygen.

† $K_m$ is defined in the text.

ed. The values of elementary rate constants measured by various methods are shown in Table III. The values of $K_m$, $k_{+3}$ and $k_{ox}$ so obtained were constant.

From the results of the ESR measurement by the rapid freezing technique, there is little possibility of a flavin semiquinone acting as an actual reaction intermediate. Therefore, the FMN of the intermediate may not take a semiquinone form in the reaction. The red-colored complex formed by titration of the reduced enzyme with $NAD^+$ had a specific absorption spectrum but showed no ESR signal. Its absorption spectrum differs to a small extent from that of the intermediate. The $E_{red} \cdot NAD^+$ complex has no absorption in the wavelength region greater than 700 nm. On the other hand, the intermediate has considerable absorption even at 700 nm or greater wavelengths. When old yellow enzyme was titrated with hydrosulfite, a flavin semiquinone appeared and showed an ESR signal [1]. The absorption spectrum of this semiquinone apparently differs from that of $E_{red} \cdot NAD^+$ complex and from that of the reaction intermediate. It appeared that old yellow enzyme takes different forms with NADH substrate.

Further experiments are being carried out on the reaction mechanisms of this enzyme when compounds other than NADH are used as substrate.

## Appendix I

In the text, scheme 2 was proposed for the anaerobic reduction of the enzyme. The concentrations of various forms of the enzyme are expressed as follows:

$$[E_{ox}] = y_1, \quad [E_{ox}NADH] = y_2, \quad [X] = y_3, \quad [E_{red}] = y_4,$$

The concentration of the substrate is represented as $s$. From the sequential reactions in scheme 2:

$$\frac{dy_2}{dt} = k_{+1} y_1 s - k_{-1} y_2 - k_{+2} y_2 + k_{-2} y_3$$

$$\frac{dy_3}{dt} = k_{+2} y_2 - k_{-2} y_3 - k_{+3} y_3 .$$

At maximum increase of absorbance,

$$\frac{dy_2}{dt} = 0\,, \tag{1}$$

$$\frac{dy_3}{dt} = 0\,. \tag{2}$$

The formation of the intermediate is extremely rapid but its breakdown is markedly slow, so that the amount of the reduced enzyme which is formed and accumulated up to the time of the maximum increase in absorbance can be neglected, i.e. $y_4 \approx 0$. The total concentration of the enzyme is given as:

Since $y_4 \approx 0$,

$$e_0 = y_1 + y_2 + y_3\,. \tag{3}$$

From a combination of Eqns. 1, 2 and 3,

$$e_0 = \frac{k_{-1}k_{-2} + k_{-1}k_{+3} + k_{+2}k_{+3}}{k_{+1}k_{+2}} \cdot \frac{y_3}{s} + \frac{k_{-2} + k_{+3}}{k_{+2}} \cdot y_3 + y_3\,, \tag{4}$$

From the definition of the differential millimolar extinction coefficient of the intermediate, $\Delta A_m$ is related to $\Delta\epsilon_{560}$ and $y_3$ by

$$\Delta A_m = \Delta\epsilon_{560} \cdot y_3\,.$$

On applying the above relationship in Eqn. 4,

$$\frac{1}{\Delta A_m} = \frac{1}{\Delta\epsilon_{560} \cdot e_0}\left(1 + \frac{k_{-2} + k_{+3}}{k_{+2}} + \frac{K_m}{s}\right).$$

Here $K_m$ is defined by

$$K_m = \frac{k_{-1}k_{-2} + k_{-1}k_{+3} + k_{+2}k_{+3}}{k_{+1}k_{+2}}\,.$$

## Appendix II

The same notation is used as in Appendix I. The concentration of electron acceptor is represented as $a$.

From the reaction scheme 3 proposed in Discussion, the differentiated forms of rate equations are:

$$\frac{ds}{dt} = k_{-1}y_2 - k_{+1}y_1 s$$

$$\frac{dy_2}{dt} = k_{+1}y_1 s - k_{-1}y_2 - k_{+2}y_2 + k_{-2}y_3$$

$$\frac{dy_3}{dt} = k_{+2}y_2 - k_{-2}y_3 - k_{+3}y_3$$

$$\frac{dy_4}{dt} = k_{+3}y_3 - k_{ox}y_4 a.$$

By summation of these equations, we obtain

$$\frac{ds}{dt} + \frac{dy_2}{dt} + \frac{dy_3}{dt} + \frac{dy_4}{dt} = -k_{ox}y_4 a.$$

If $a$ is greatly in excess and does not vary appreciably during the course of reaction, it may be regarded as constant. On integrating between $t = 0$ and $t = t$,

$$s + y_2 + y_3 + y_4 = -k_{ox}a \int_0^t y_4 \, dt + C \, , \tag{1}$$

where C is the integration constant.

At $t = 0$, $s = s_0$ and $y_2 = y_3 = y_4 = 0$. Therefore $C = s_0$, where $s_0$ is written as the initial concentration of substrate.

At $t = \infty$, $s = y_2 = y_3 = y_4 = 0$. So that Eqn. 1 reduces to

$$-k_{ox}a \int_0^\infty y_4 \, dt + s_0 = 0,$$

and therefore, $k_{ox}$ is represented as follows:

$$k_{ox} = \frac{s_0}{a \int_0^\infty (E_{red}) dt}$$

The value of the above integration can be approximately estimated by the following equation:

$$\int_0^\infty (E_{red}) dt = P_{max} \cdot t_{1/2}.$$

Therefore,

$$k_{ox} = \frac{s_0}{a \cdot P_{max} \cdot t_{1/2}} \quad .$$

## Acknowledgement

The authors express their gratitude to Dr. T. Nakamura for helpful discussion in the course of preparing this manuscript.

## References

1 Nakamura, T., Yoshimura, J. and Ogura, Y. (1965) J. Biochem. 57, 554—564
2 Haas, E. (1937) Biochem. Z. 290, 291—292

3 Theorell, H. and Åkeson, Å. (1956) Arch. Biochem. Biophys. 65, 439—448
4 Chance, B. and Legallais, V. (1951) Rev. Sci. Instr. 22, 627—634
5 Scarborough, J.B. (1958) Numerical Mathematical Analysis, IVth. edn., pp. 309—323, Johns Hopkins Press, Baltimore, Md.
6 Bray, R.C. (1964) in Rapid Mixing and Sampling Techniques in Biochemistry (Chance, B., ed.), pp. 195—203, Academic Press, New York
7 Ogura, Y., Nakamura, S. and Nakamura, T. (1963) Acta Chem. Scand. 17, s184—s189
8 Eigen, M. and de Maeyer, L. (1963) in Techniques of Organic Chemistry (Weissberger, A., ed.) vol. VIII, Part II, pp. 895—1054, Interscience, New York
9 Chance, B. (1943) J. Biol. Chem. 151, 553—577
10 Chance, B. (1957) Arch. Biochem. Biophys. 71, 130—136