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KINETICS OF THE COURSE OF INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY 4-(2-PYRIDYLAZO)-RESORCINOL

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The kinetic theory of the substrate reaction during modification of enzyme activity previously described by Wang and Tsou[1] has been applied to a study on the kinetics of the course of inactivation of alcohol dehydrogenase by 4-(2-pyridylazo)-resorcinol (PAR). The results showed that the inhibition of this enzyme by PAR was irreversible. A plot of $\ln([P]_\infty - [P])$ versus t give a straight line, suggesting that the inactivation kinetic course is monophasic. The kinetic analysis of the substrate reaction with different concentrations of the substrate and the inactivator has shown that the inactivation of yeast alcohol dehydrogenase by PAR involves complex formation, and that the substrate ethanol competes with PAR at the active site of the enzyme. The dissociation constants between the substrates and the enzyme as well as the microscopic rate constants for the inactivation of the enzyme have been determined.

KEY WORDS: Alcohol dehydrogenase, zinc enzyme, inactivation, kinetics, 4-(2-pyridylazo)-resorcinol

INTRODUCTION

Zinc is one of the peculiar metals having multiple coordination possibilities and various geometries, making it easily adaptable for various ligands.[2–4] Zinc enzymes are particularly interesting in that they encompass all the six main classes of enzymes.[5,6] It has been suggested[7] that the presence of Zn^{2+} helps to keep the conformation of the active site in a strained state. Although the importance of Zn^{2+} in the catalytic processes is well established by inhibition with a metal ion chelator, inactivation by the removal of Zn^{2+} from the enzyme and reactivation by its restoration,[7,8] the kinetics of the course of inactivation during metal chelation or removal have been but little explored.

A systematic study on the kinetics of the substrate reaction during irreversible modification of enzyme activity in the presence of a modifier was presented by Wang and Tsou.[1,9] It has been shown not only that the apparent rate constant for the irreversible modification of

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Abbreviation: PAR, 4-(2-pyridylazo)-resorcinol; NAD^+, β-nicotinamide adenine dinucleotide; YADH, yeast alcohol dehydrogenase.

enzyme activity can be obtained, but that the effect of substrate complexing and competition with the inactivator can also be ascertained.[10] This kinetic approach has been applied to the study on inactivation of zinc enzyme with a single substrate by removal of the Zn^{2+} from the enzyme with chelator 1,10-phenanthroline.[11] The present paper reports a study on the course of inactivation of a zinc enzyme, yeast alcohol dehydrogenase with two substrates, forming a ternary complex by a compulsory sequence, by removal of the Zn^{2+} from the enzyme with a chelator, 4-(2-pyridylazo)-resorcinol (PAR).

Yeast alcohol dehydrogenase (YADH) (alcohol: NAD^+ oxidoreductase; EC 1.1.1.1) is a tetrameric enzyme of molecular mass 150 kDa.[12,13] It is well known[13] that there are two zinc atoms per YADH subunit. In this enzyme, one zinc atom is found in the catalytic site of the enzyme. It can be removed by general chelators, which led to inactivation of this enzyme. The second zinc atom which is protected by disulfide bridges was only selectively removed by DTT.[14] The present paper reports a study on the complete kinetic course of PAR inactivation in which the oxidation of ethanol was monitored in the presence of PAR. Kinetic analysis of the results suggests a diphasic reaction involving a rapid formation of reversible enzyme-PAR, enzyme-substrate-PAR or enzyme-product-PAR complex, then a relatively slow removal of Zn^{2+} at the active site, leading to the inactivation of the enzyme.

MATERIALS AND METHODS

Yeast alcohol dehydrogenase was from Sigma. It was homogeneous on polyacrylamide gel electrophoresis in the presence and the absence of SDS. NAD^+ was also from Sigma, PAR was from Fluka. Other chemicals were local products of analytical grade.

Determination of enzyme concentration

Enzyme concentration was determined by measuring the absorbance at 280 nm and using the absorption coefficient[15] $E^{1\%}_{1cm} = 12.6$. Enzyme activity was determined in 0.1 M potassium phosphate buffer, pH 7.5, at 25°C by measuring the change of absorbance at 340 nm accompanying the formation of NADH.[15]

Determination of inactivation kinetics

Both the conventional method and the progress-of-substrate-reaction method[9] were used for the study of the inactivation kinetics of yeast alcohol dehydrogenase. In the conventional method the enzyme (1 μg/ml) was incubated with PAR (10 mM) in 0.1 M potassium phosphate buffer, pH 7.5, at 25°C, and at different time intervals 100 μl of the incubation mixture was taken and each was diluted into 1 ml of the reaction mixture containing 3 M ethanol and 15 mM NAD^+ in the same buffer for assay at the same temperature. In the progress-of-substrate-reaction method, 50 μl of 10 μg/ml YADH was added to 1 ml of a reaction mixture containing 0.3 M ethanol and 1.5 mM NAD^+ in 0.1 M phosphate buffer, pH 7.5, containing different concentrations of PAR and the progress curve for the substrate reaction was analyzed[9] to obtain the rate constants as detailed below.

Kinetic analysis

Detailed kinetic analysis of the substrate reaction during irreversible inhibition of an enzyme involving two substrates has been presented[1] and reviewed.[9] Only the relevant points will be summarized below. The concentration of product formed at time t is given by

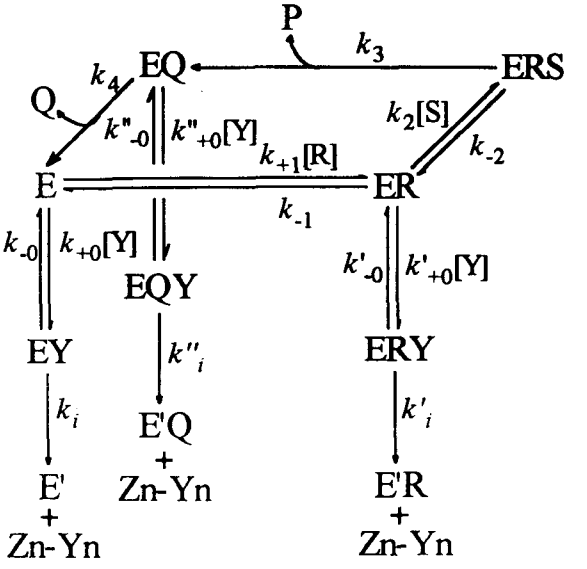
$$[P] = [P]_\infty(1 - e^{-A[Y]t}) \tag{1}$$

where $[P]$ is the concentration of the product formed at time t, $[P]_\infty$ is the product concentration at time infinity, t is reaction time, $[Y]$ is the concentration of inactivator, and A is the apparent rate constant of inactivation.

From Equation (1) the following equation is obtained:

$$\ln([P]_\infty - [P]) = \ln[P]_\infty - A[Y]t \tag{2}$$

It is known that for some enzymes, including YADH, the ternary complex can only be formed by a compulsory order of sequence in which one of the binary complex does not form. In Scheme 1 the ternary complex ESR can only be formed via the ER complex. Inhibitors of the complexing type, as Wang and Tsou[1] stated, usually sufficiently resemble one of the substrates to be able to complex with the enzyme and it is assumed that Y resembles S and complexes with E and ER only. But for PAR, we suppose that it inactivates the enzyme by removal of the active site zinc as suggested in Scheme 1.



SCHEME 1 YADH kinetics in presence of PAR. R and S are substrates NAD^+ and ethanol; P and Q are products acetaldehyde and NADH, respectively. E and Y are enzyme and inactivator PAR respectively. ER, ERS, EQ, EY, ERY and EQY are respective complexes.

For the reaction shown in Scheme 1, $1/A$, $1/v^*$ and $1/[Y][P]_\infty$ are given respectively by:

$$1/A = \frac{(1+[Y]/K_I)(K_{iR}K_{MS}+K_{MR}[S])+(1+[Y]/K'_I)K_{MS}[R]}{(k_i/K_I)(K_{iR}K_{MS}+K_{MR}[S])+(k'_i/K'_I)K_{MS}[R]}$$

$$\frac{+\{1+K_{MR}([Y]/K''_I)(k_1/k_4)\}[S][R]}{+(k''_i k_1/K''_I k_4)K_{MR}[R][S]} \tag{3}$$

where

$$K_{iR}=k_{-1}/k_{+1}, \qquad K_{MS}=k_4(k_{-2}+k_3)/k_2(k_3+k_4),$$
$$K_{MR}=k_3k_4/k_1(k_3+k_4), \qquad K_I=k_{-0}/k_{+0},$$
$$K'_I=k'_{-0}/k'_{+0}, \qquad K''_I=k''_{-0}/k''_{+0}$$

$$1/v^* = \frac{(1+[Y]/K_I)(K_{iR}K_{MS}+K_{MR}[S])+(1+[Y]/K'_I)K_{MS}[R]}{+\{1+K_{MR}([Y]/K''_I)(k_1/k_4)\}[S][R]} \tag{4}$$
$$V_{\max}[R][S]$$

$$\frac{1}{[Y][P]_\infty} = \frac{(k_i/K_I)(K_{iR}K_{MS}+K_{MR}[S])+(k'_i/K'_I)K_{MS}[R]}{+(k''_i k_1/K''_I k_4)K_{MR}[R][S]} \tag{5}$$
$$V_{\max}[R][S]$$

where v^* is the velocity of substrate reaction in the presence of inactivator, $V_{\max}$ is the maximum velocity of the substrate reaction without inactivator.

Differentiation of noncomplexing and complexing inhibition

It is necessary to make a distinction between noncomplexing and complexing types of irreversible inhibition. For the complexing type of inhibition, the rapid formation of an enzyme-inhibitor complex precedes the slow irreversible inactivation step. As has been shown previously,[1] the expression for the apparent rate constant A contains the term $[Y]$ for complexing inhibitions whereas it is independent of $[Y]$ for noncomplexing inhibitions. A plot of $1/A$ against $[Y]$ gives a straight line with positive slope cutting at the ordinate for complexing inhibition.

Determination of the microscopic rate constants

Equation (4) can be written as,

$$1/v^* = \{(1+[Y]/K_I)K_{iR}K_{MS}+(1+[Y]/K'_I)K_{MS}[R]\}/V_{\max}[R][S]+$$
$$\{(1+[Y]/K_I)K_{MR}+(1+K_{MR}[Y]k_1/K''_I k_4)[R]\}/V_{\max}[R] \tag{6}$$

and it can be seen from the above that while keeping $[R]$ constant, a plot of $1/v^*$ against $1/[S]$, should give a straight line (Figure 2a) with a slope s and an ordinate intercept i of

$$s = \{(1 + [Y]/K_I)K_{iR}K_{MS} + (1 + [Y]/K'_I)K_{MS}[R]\}/V_{\max}[R]$$

$$i = \{(1 + [Y]/K_I)K_{MR}/[R] + (1 + [Y]K_{MR}k_1/K''_I k_4)\}/V_{\max}$$

Both the slope and intercept are linear functions of $1/[R]$. A secondary plot of s against $1/[R]$ gives respectively the values of the intercept on the ordinate and the slope as,

$$\text{intercept} = \left(1 + \frac{[Y]}{K'_I}\right) K_{MS} \frac{1}{V_{\max}} \tag{7}$$

$$\text{slope} = \left(1 + \frac{[Y]}{K_I}\right) K_{iR}K_{MS} \frac{1}{V_{\max}} \tag{8}$$

and for the i against $1/[R]$ plot,

$$\text{intercept} = \left(1 + \frac{[Y]}{K''_I} K_{MR} \frac{k_1}{k_4}\right) \frac{1}{V_{\max}} \tag{9}$$

$$\text{slope} = \left(1 + \frac{[Y]}{K_I}\right) K_{MR} \frac{1}{V_{\max}} \tag{10}$$

According to Equation (5), while keeping $[R]$ constant, a plot of $1/[P]_\infty$ against $1/[S]$ gives a straight line too, and the slope s and ordinate intercept i are, respectively.

$$s = \left(\frac{k_i}{K_I} K_{iR}K_{MS} + \frac{k'_i}{K'_I} K_{MS}[R]\right) \frac{[Y]}{V_{\max}[R]} \tag{11}$$

$$i = \frac{k_i}{K_I} K_{MR} \frac{[Y]}{V_{\max}[R]} + \frac{k''_i k_1}{K''_I k_4} K_{MR} \frac{[Y]}{V_{\max}} \tag{12}$$

A secondary plot of i against $1/[R]$ gives the slope

$$\text{slope} = \frac{k_i}{K_I} K_{MR} \frac{[Y]}{V_{\max}} \tag{13}$$

From these values it can be seen that since $V_{\max}$, K_{iR}, K_{MR} and K_{MS} can be obtained in experiments without the inactivator (King-Altman Method), the dissociation and microscopic rate constants K_I, K'_I, k_i, k'_i can be calculated from Equations (7), (8), (10), (11) and (13).

RESULTS

Inactivation kinetics of YADH with PAR

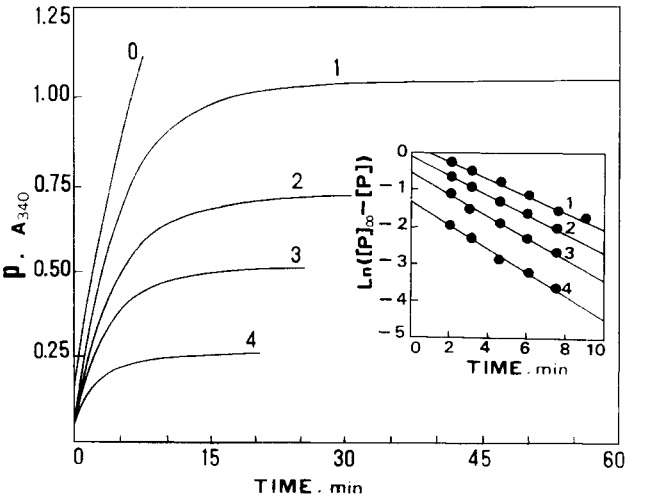
The course of substrate reaction in the presence of different concentrations of PAR are shown in Figure 1a. It can be seen that when the reaction time is sufficiently long, $[P]$ approaches a constant final value $[P]_\infty$, which decreases with the increasing concentration of PAR. Semilogarithmic plots according to Equation (2) are given in the inset of Figure 1a, and the apparent rate constant can then be calculated from the slopes of the straight lines obtained. Results presented in Figure 1a inset also show that the inactivation is a monophasic pseudo-first-order reaction. The plot of $1/A$ against PAR concentration (Figure 1b) shows that the apparent rate constant A is affected by inhibitor concentration, indicating that there is a prior complexing with the enzyme before the irreversible inhibition step[1,10] as shown in Scheme 1.

Effect of substrate on the inactivation by PAR

The initial reaction rates v^* in the presence of PAR at different ethanol concentrations were determined. According to Equation (4), a plot of $1/v^*$ against 1/[EtOH] gives a series of straight lines (Figure 2a), the slopes of which decrease with increasing concentration of NAD^+. A secondary plot of the slopes and the ordinate intercepts against $1/[NAD^+]$ gives two straight lines as shown in Figure 2b. At the different concentrations of NAD^+, the plots of $1/[P]_\infty$ against 1/[EtOH] also give a series of straight lines (Figure 3a), and the secondary plot of the ordinate intercepts against $1/[NAD^+]$ is shown in Figure 3b. The K_I, K'_I and K_{iR} can all be obtained from the above plots according to Equations (7–11). In order to obtain these constants for the inactivation reactions, it is necessary to have the respective K_m and V_{max} values for the uninhibited reaction under otherwise identical conditions. These are obtained from Lineweaver and Burk plots and suitable secondary plots of initial velocity measurements in the presence of different concentrations of NAD^+ and EtOH but in the absence of added PAR (Figures 4a and 4b). The values thus obtained are shown in Table 1. From these data, the secondary plot was given in Figures 2b and 3b. The kinetic constants for the reaction of PAR with the free enzyme and the substrate-enzyme complexes can then be obtained (Equations (7–11)), and the results are listed in Table 2. For comparison, the inactivation rate was also determined by the conventional method, giving a rate constant of 0.7×10^{-3} $mM^{-1}s^{-1}$ as compared to a value of 1.1×10^{-3} $mM^{-1}s^{-1}$ obtained from the plot in Figure 3b. Both values are listed in Table 2. It can be seen that PAR is only competitive with one substrate EtOH. The formation of the ternary complex gives protection against PAR inhibition.

TABLE 1
Kinetics parameters of YADH obtained by initial velocity measurement.

Michaelis constant	
V_{max} (min^{-1})	0.25
K_{mNAD} (mM)	0.11
K_{mEtOH} (mM)	16.00
K_{iNAD} (mM)	0.62



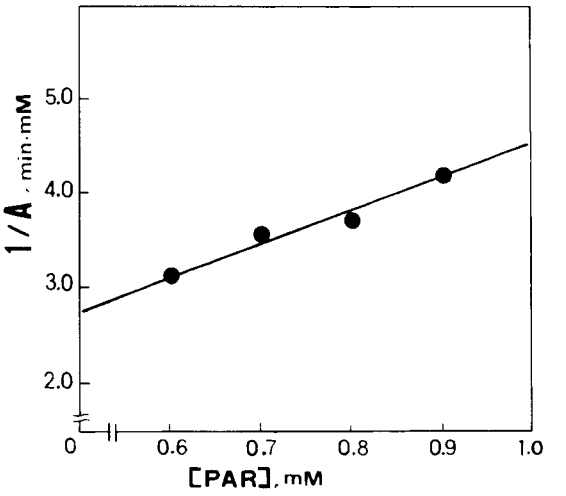


FIGURE 1 Substrate reaction of YADH in the presence of PAR. (top) Reaction course of YADH in the presence of PAR. The reaction-mixture contained 0.1 M phosphate buffer, pH 7.5, 0.01 M sodium pyrophosphate, 0.3 M EtOH and 1.5 mM NAD^+. Concentrations of PAR were 0.6, 0.7, 0.8, 0.9 mM, respectively. For curves 1–4, 50 μl YADH (10 μg/ml) was added to the reaction mixture to start the reaction. Proton generation was monitored by the absorbance at 340 nm, 25°C. The inset shows semilogarithmic plot of the lines 1–4 according to Equation (2). (bottom) Plot of $1/A$ against concentration of PAR. The data for A were calculated from Figure 1a.

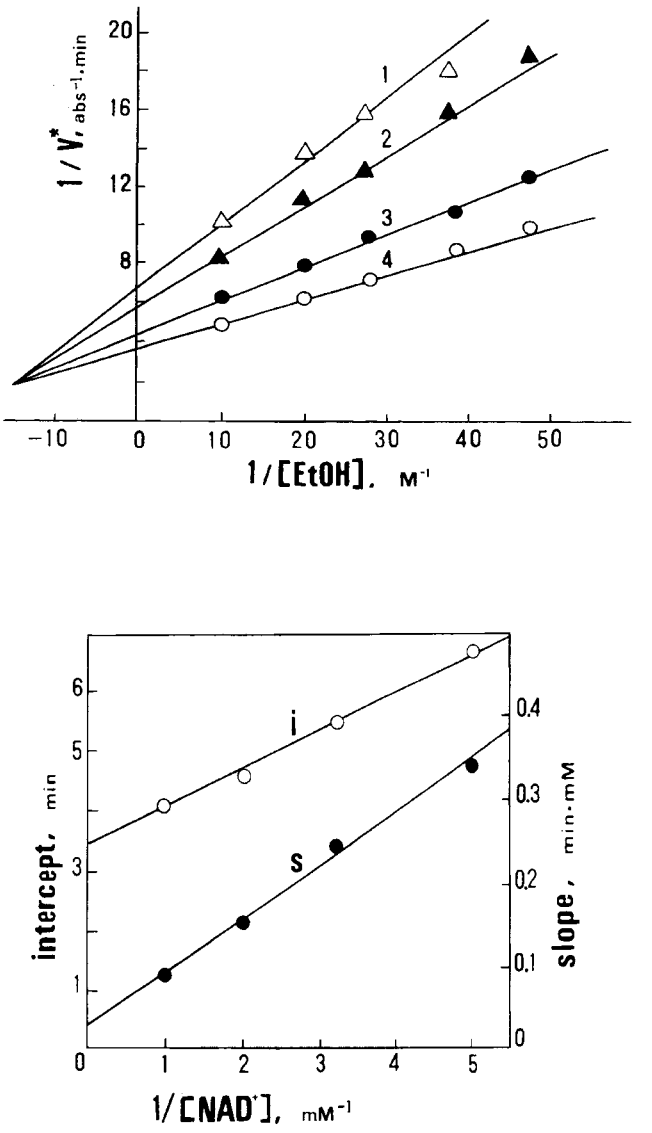


FIGURE 2 Effect of EtOH concentration on v^* during inactivation by PAR. (top) Double-reciprocal plots of $1/v^*$ against 1/[EtOH]. Experimental conditions as for Figure 1 except that the concentration for PAR 0.6 mM and those for NAD^+ and EtOH were as indicated. The concentrations of NAD^+ were 0.2, 0.31, 0.5 and 1.0 mM for lines 1–4, respectively. (bottom) The secondary plots of the ordinate intercepts (open circles and left hand scale) and the slopes (full circles and right-hand scale) against $1/[NAD^+]$.

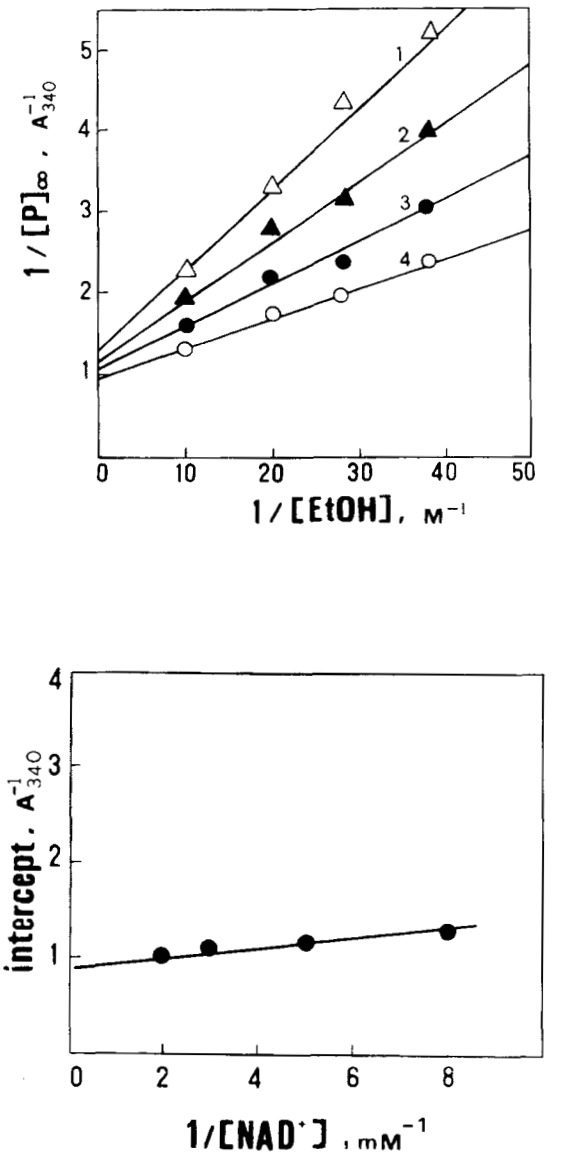


FIGURE 3 Effect of EtOH concentration on $[P]_\infty$ during inactivation by PAR. (top) Double-reciprocal plots of $1/[P]_\infty$ against $1/[S]$. Experimental conditions as for Figure 1 except that the concentrations of EtOH and NAD^+ were as indicated. The concentrations of NAD^+ were 0.12, 0.2, 0.31 and 0.5 mM for lines 1–4, respectively. (bottom) The secondary plots of the ordinate intercept against $1/[NAD^+]$.

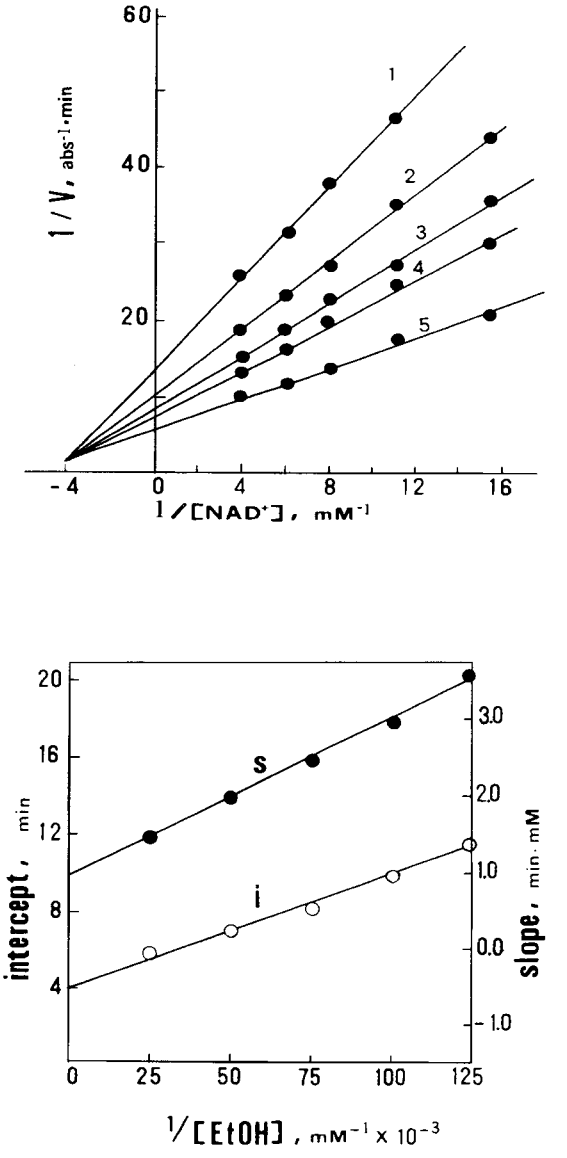


FIGURE 4 Effect of NAD^+ concentration on initial reaction rate. (top) Double-reciprocal plots of $1/v$ against $1/[NAD^+]$. Experimental conditions as for Figure 1 except not containing PAR, and concentrations for NAD^+ and EtOH were as indicated. The concentrations of EtOH were 8, 13, 20, 30 and 40 mM for lines 1–5, respectively. (bottom) The secondary plots of the ordinate intercepts (open circles and left-hand scale) and the slope (full circles and right-hand scale) against 1/[EtOH].

TABLE 2
Rate constants of the reaction of YADH with PAR.[a]

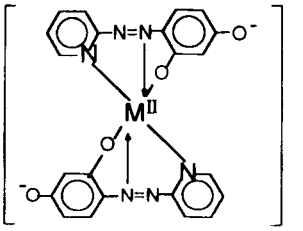
Form of enzyme reacting		*Constant*
E	$K_I(k_{-0}/k_{+0})$	0.6 (mM)
E-NAD	$K'_I(k'_{-0}/k'_{+0})$	0.1 (mM)
E-Y	k_i	1.1×10^{-3} ($mM^{-1}s^{-1}$)
		$<0.7\times10^{-3}$($mM^{-1}S^{-1}$)[b]
E-R-Y	k'_i	0.9×10^{-3} ($mM^{-1}s^{-1}$)

[a]All the rate constants were obtained by following the substrate reaction in the presence of the inactivator as described in the text except where noted. For details, see text.

[b]Obtained by the conventional method of taking aliquots of the reaction mixture at different time intervals and assaying for enzyme activity.

DISCUSSION

4-(2-Pyridylazo)-resorcinol is a metal chelator, which needs the simultaneous chelation of the two ligands to remove the zinc ion. The Zn^{2+}-PAR_2 complex, binding two PAR molecules to Zn^{2+}, is shown in Scheme 2.



M = Zn

SCHEME 2 PAR liganding to Zn^{2+}.

The presence of two zinc atoms per yeast alcohol dehydrogenase subunit has been confirmed in many publications. One zinc atom is found in the active site of the enzyme, and the second zinc atom plays a prominent conformational role. The action of the metal ion chelator PAR on YADH has been studied. Although the high-resolution X-ray structure of YADH has not yet been presented, the X-ray crystallographic structure of horse liver alcohol dehydrogenase has shown that the active site zinc ion is ligated by the sulfur atoms of cysteine residues 46 and 174, the imidazole group of His-67, and the substrate ethanol.[16] The enzyme can be inactivated by removal of Zn^{2+} at the active site with PAR. In addition, it is well known that the second zinc atom which is protected by disulfide bonds is only selectively removed by DTT. In the absence of DTT, PAR cannot remove the conformational

zinc (second zinc atom) of YADH. The results of kinetic studies in the present study show that the dissociation is preceded by a relatively rapid step of reversible binding at the active site, presumably by complexing with Zn^{2+}, followed by a relatively slow inactivation step, since the semilogarithmic plots for the inactivation reaction (Figure 1a) are monophasic. If the two steps shown in Scheme 1 had comparable rates, diphasic semilogarithmic plots should have been obtained. In plots of $1/[P]_\infty$ against $1/[S]$ for complexing inhibitors of ordered sequences of bisubstrate reaction (Figure 3a), the decreasing slopes of the lines with increasing concentrations of the substrate NAD^+, R, indicates, as described by Wang and Tsou[1] that the inhibitor Y is competitive with the substrate ethanol. These results supported the assumption shown in Scheme 1 that the quarternary E-R-S-Y complex cannot be formed.

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