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KINETICS OF THE REACTION CATALYSED BY RAPE ALCOHOL DEHYDROGENASE

MARIE STIBOROVÁ and SYLVA LEBLOVÁ
Department of Biochemistry, Charles University, Faculty of Natural Sciences, Prague, Czechoslovakia

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Key Word Index—*Brassica napus*; Cruciferae; alcohol dehydrogenase; coenzyme; enzyme kinetics.

Abstract—The kinetics of the enzyme reaction of ethanol oxidation and acetaldehyde reduction catalysed by alcohol dehydrogenase (ADH) (EC 1.1.1.1) isolated from germinating rape seeds obeys the bi-bi ordered mechanism of Theorell and Chance. The enzyme reaction depends on the pH and temperature. The K_m values for the basic substrates have the lowest values around the pH optimum of the reaction. The enzyme is most stable at pH 6.5–7. The K_m values for ethanol and NAD increase with increasing temperature. The maximum rate of the ethanol oxidation satisfies the Arrhenius equation. The activation energy for the given temperature range is 40.11 kJ/mol. The rape ADH is denatured by heating above 60° but the enzyme–NAD complex is thermally more stable than the enzyme alone.

INTRODUCTION

The present paper attempts to verify whether the enzyme reaction catalysed by plant alcohol dehydrogenase (ADH) isolated from germinating rape seeds obeys the kinetic model of Theorell and Chance [1]. The effect of pH and temperature on these kinetics was also studied, as well as the temperature and pH stability of the isolated enzyme.

RESULTS AND DISCUSSION

The kinetics of two substrate reactions can be generally described by the equation

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{1/2}}{[S_1][S_2]} \qquad (1)$$

where the symbols are described in refs. [2, 3]. The values of kinetic coefficients ϕ were obtained by methods used in a previous paper [4]. The values of kinetic coefficients for oxidation of ethanol and for reduction of acetaldehyde obtained for the rape ADH are in good agreement with the conditions for the Theorell and Chance [1] mechanism. The Michaelis constants and dissociation contants can be calculated from these kinetic coefficients [4] (Table 1).

Table 1. Michaelis constants for the basic substrates and the dissociation constants for the binary complexes of rape ADH with NAD and NADH, calculated from the kinetic coefficients

	mM	
	pH 7.5	pH 8.5
$K_{m(NAD)}$	0.2	0.133
$K_{m(NADH)}$	0.15	0.2
$K_{m(ethanol)}$	30	20
$K_{m(acetaldehyde)}$	1	1.42
$K_{ADH-NAD}$	0.4	0.266
$K_{ADH-NADH}$	0.075	0.1

Assay conditions: 0.1 M sodium phosphate buffer; 10–100 mM ethanol; 1–10 mM acetaldehyde; 0.1–0.8 mM NAD, NADH; reaction medium vol., 1 ml.

The calculated equilibrium constant of the overall reaction [4]

$$\text{Ethanol} + \text{NAD}^+ \overset{k}{\rightleftharpoons} \text{acetaldehyde} + \text{NADH} + \text{H}^+ \qquad (2)$$

equals 3.7×10^{-11} M at pH 8.5. The thermodynamic equilibrium constant value is 0.98×10^{-11} M at 25° [5].

In view of the kinetics (mechanism of Theorell and Chance), the rape ADH is similar to liver ADH [6] and pea seeds ADH. [4].

Effect of pH

The pH optimum for ethanol oxidation differs from that for acetaldehyde reduction [7]. The optimum pH for the reduction of acetaldehyde is close to 7, which is connected with the role of the enzyme in the seeds. Higher plants in the period of natural anaerobiosis, i.e. during seed germination, first produce lactate and then, if the cell pH is reduced by the lactate formed, ethanol [8]. Ethanol is formed by the reduction of acetaldehyde, produced by decarboxylation of pyruvate at lower pH values when pyruvate decarboxylase is active [9] (pH optimum 5.6). On changing the conditions from anaerobic to aerobic, the ethanol is again oxidized by ADH to acetaldehyde. The pH optimum of this reaction lies in the alkaline region (pH 8.5). We have found that a change in the pH also causes a change in the kinetic coefficients in Equation (1) and thus also the Michaelis constants for the basic substrates. The difference in the K_m was found for two pH values, 7.5 and 8.5. The K_m values are lowest at the pH close to the pH optimum of the given reaction (Table 1).

Rape ADH is very unstable in acidic media. The denaturation rate was monitored at pH 4–6. First order

Table 2. Dependence of the Michaelis constants on temperature

Temp.	mM $K_{m(NAD)}$	$K_{m(ethanol)}$
23°	0.136	20.0
27°	0.155	25.0
33°	0.185	30.0
42.8°	0.224	41.3

Assay conditions: 0.1 M sodium phosphate buffer pH 8.5; 10–100 mM ethanol; 0.1–0.8 mM NAD; reaction medium vol. 1 ml.

kinetics were used to express the denaturation rate quantitatively, as this is a pseudomonomolecular reaction [10]. The rate constant of such a reaction is a linear function of the hydrogen ion concentration and therefore the rate of denaturation is suitably expressed by the function, $\log k = f(pH)$. At pH 4, 5 or 6 the values of $-\log k$ were respectively 3.8, 4.5 and 5.2 and these experimental rate constants therefore obey this function.

The rape ADH activity decreases in alkaline media, but the denaturation rate is not as high as that in acids. A decrease in the rape ADH activity in the alkaline region occurs only after prolonged incubation of the enzyme. The enzyme is most stable in a pH range of 6.5–7.

Effect of temperature

The rate of the enzyme reaction (oxidation of ethanol) increased from 10 to 45° and then sharply decreased. Between 20 and 30° the reaction rate increases 1.8 times, which is in good agreement with the temperature coefficient (Q_{10}) for enzyme reactions. With varying temperature, kinetic coefficients in Equation (1) also vary and thus the Michaelis constants for ethanol and NAD also change. The K_m values for 4 different temperatures are given in Table 2. The K_m values for NAD and ethanol increase with increasing temperature.

Coefficient ϕ_0 in Equation (1) is essentially the reciprocal maximum rate of the ethanol oxidation. The plot of the log of the maximum rate of the ethanol oxidation against the reciprocal temperature (296–316 K) is linear; consequently coefficient ϕ_0 obeys the Arrhenius equation. From this dependence, the activation energy for the given temperature range was calculated to be 40.11 kJ/mol.

The enzyme is denatured at temperatures above 60°. The denaturation rate is affected by the presence of the coenzyme (NAD). NAD substantially protects rape ADH against thermal denaturation; with increasing coenzyme concentration, the thermal denaturation depends on the equilibrium reaction between the enzyme and NAD. As can be seen from Fig. 1 the denaturation of the enzyme alone and of the enzyme-NAD complex obeys first order kinetics.

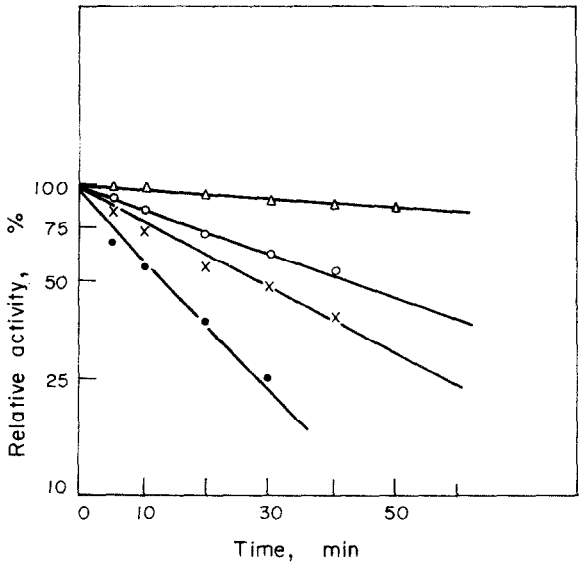

Fig. 1. Thermal denaturation of rape ADH and its protection by the coenzyme. Assay conditions: 0.025 M Tris–acetate buffer in 0.01 M mercaptoethanol (pH 7) with different concns of NAD; —●——● without NAD; —×——×— 0.393 mM NAD; –○–○– 0.786 mM NAD; –△——△– 1.57 mM NAD. Abscissa: time in min; Ordinate: relative activity in log % activity.

EXPERIMENTAL

Plant material and chemicals. The enzyme was isolated from rape seeds (*Brassica napus* L., cv Třebíčská) by the procedure described in ref. [7].

Enzyme activity was measured as the increase (with EtOH) or decrease (with CH_3CHO) of the A at 366 nm [7]. Statistical treatment of the values obtained yielded $\bar{x} = 413.3$, $s = 5.6$, $v = 1.4\%$.

Thermal denaturation of rape ADH was followed in 25 mM Tris–acetate buffer of pH 7 in 10 mM mercaptoethanol. A suitable amount of the enzyme was added to 2 ml of the buffer and heated to the required temp. When protecting the enzyme against thermal denaturation by means of NAD, amounts of NAD were added so that its resultant concns were 0.393, 0.786 and 1.57 mM. After a certain incubation time, an aliquot was taken from the incubation mixture, pipetted immediately into ice-cold Tris–acetate buffer of pH 7 in 10 mM mercaptoethanol and the enzyme activity was immediately measured by adding 0.1 ml of the dilute enzyme into the reaction medium containing 0.1 M NaPi buffer (pH 8.5), 100 mM EtOH and 0.5 mM NAD, with total vol. of the reaction medium of 1 ml at 20°.

All measurements were repeated at least 5 × and the results given are the mean values of these measurements. If not stated otherwise, the measurements were performed at 20°.

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