EFFECT OF pH AND TEMPERATURE ON ALCOHOL DEHYDROGENASE

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Received October 5th, 1977

Alcohol dehydrogenase isolated from germinating pea seeds catalyzes ethanol oxidation at pH 8·7 and acetaldehyde reduction at pH 7·0. The values of the Michaelis constants are the lowest in the range of pH-optimums. The effect of temperature on the reaction rate was investigated over the range 15—55°C. The initial and maximal rates increase with the increasing temperature and attain a maximum at 40°C. The values of the Michaelis constants are the lowest at 21°C. Pea alcohol dehydrogenase looses its activity at 70°C, the binary enzyme–NAD complex is more thermostable.

In a number of mono- and dicotyledone plants with lipids, carbohydrates, or proteins as reserve products in the seed, lactate is the first product formed during natural anaerobiosis (*i.e.* during the germination of seeds before the rupture of the testa), followed approximately after 20 h of swelling by ethanol[1,2]. The formation of lactate and subsequently of ethanol has been observed using *in vitro* cell free extracts prepared from pea seeds and parsnip root by Davies and coworkers[3] who ascribed their origin to an alteration of the activity of enzymes involved in the anaerobic metabolism caused by a change in the concentration of hydrogen ions. In this study the effect of pH and temperature on isolated pea alcohol dehydrogenase was investigated.

EXPERIMENTAL

The vegetal material used to start with was pea (*Pisum arvense* L. c. Raman Elita) grown as described in our preceding paper[4].

Pea alcohol dehydrogenase was prepared from seeds, germinated for 24 h, by extraction of the plant tissue with a phosphate buffer, precipitation of the enzyme in the extract by ammonium sulfate, and chromatography of the desalted sulfate fraction of the enzyme on DEAE-cellulose and Sephadex G-200. The enzyme preparation had a specific activity of 80 000 units/mg.

The enzymic activity was measured in terms of absorbance increase (ethanol oxidation) or decrease (acetaldehyde reduction) at 366 nm as described in our preceding paper[5]. When it was necessary to express the reaction rate in absolute units ($\mu M\ s^{-1}$), a molar absorption coefficient $\varepsilon_{\frac{NADH}{366}} = 5{\cdot}7 \cdot 10^3 M^{-1}\ cm^{-1}$ was used. K_m and K_i were determined from the Lineweaver–Burke plot[6] or according to Dixon[7].

A two-substrate reaction can generally be described by

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]},$$

where v_0 is the initial reaction rate, $[S_1]$, $[S_2]$ the concentrations of both substrates (S_1 = NAD; S_2 = ethanol), ϕ the kinetic coefficients and e the concentration of active sites.

The values of the kinetic coefficients were obtained as described in This journal[8]. The relation between kinetic coefficients and rate constants can be simply expressed on the assumption[9-14] that the coenzyme is the first substance bound to the enzyme and the last substance to dissociate off, and that the rate limiting step is the dissociation of the coenzyme from the enzyme–coenzyme complex. If we involve the formation of the ternary complex and the redox reaction itself in one equation we arrive at the following mechanism:

$$E + S_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_1$$

$$ES_1 + S_2 \underset{k'_{+5}}{\overset{k_{+5}}{\rightleftharpoons}} ES'_1 S'_2$$

$$ES'_1 \underset{k'_{+1}}{\overset{k'_{-1}}{\rightleftharpoons}} E + S'_1$$

(NAD = S_1, NADH = S'_1, ethanol = S_2, acetaldehyde = S'_2). The values of the kinetic coefficients and of the corresponding rate constants involved in this mechanism are then governed by the following simple relations:

$$\phi_0 = \frac{1}{k'_{-1}}, \quad \phi_1 = \frac{1}{k_{+1}}, \quad \phi_2 = \frac{1}{k_{+5}}$$

$$\phi'_0 = \frac{1}{k_{-1}}, \quad \phi'_1 = \frac{1}{k'_{+1}}, \quad \phi'_2 = \frac{1}{k'_{+5}}.$$

RESULTS AND DISCUSSION

Effect of pH

We found that pea alcohol dehydrogenase catalyzes ethanol oxidation with a pH-optimum at 8·7 and acetaldehyde reduction with an optimum at pH 7·0. This finding can be reconciled with processes which take place during the germination of seeds by pyruvate conversion. Pyruvate is reduced by the arising NADH to lactate; because of the latter the pH in the cell drops to 6·6. This pH drop activates pyruvate decarboxylase[3] with a pH-optimum in the weakly acidic region; pyruvate decarboxylase subsequently gives rise to acetaldehyde. The latter is next reduced to ethanol, the reduction being catalyzed by alcohol dehydrogenase whose pH-optimum lies in the neutral range.

Experiments designed to investigate the effect of the pH of the medium on the reaction rate have shown that both the maximal reaction rate and the value of the Michaelis constant for both substrates vary with the varying concentration of hydrogen ions, as shown in Table I. Ionizing groups obviously play a role both in the free enzyme and in the enzyme–substrate complex. The K_m-values were determined for two fundamental substrates; the measurements were made in the range of optimal pH for both reversible processes. The Michaelis constants are the lowest in the range around the pH-optimum of pea alcohol dehydrogenase. Fig. 1 shows the stability of the enzyme as a function of hydrogen ion concentration over the pH-range 4·5 to 9·0. It follows from the diagram that the enzyme is most stable in the neutral range,

TABLE

Values of Michaelis Constants as Function of pH

Experimental conditions: [ethanol] = 5–40 mM; [acetaldehyde] = 1–8 mM; [NAD) = 50 to 500 μM; 20°C.

Value	pH 8·5	pH 7·4
$K_{m(NAD)}$, μM	150	200
$K_{m(NADH)}$, μM	200	160
$K_{m(ethanol)}$, mM	30	40
$K_{m(acetaldehyde)}$, mM	6	4

TABLE II

Values of Michaelis Constants as Function of Temperature

Experimental conditions: 0·1M Na-phosphate buffer, pH 8·5; see Table I for substrate concentrations.

°C	$K_{m(NAD)}$, mM	$K_{m(ethanol)}$, mM
21	0·15	30
32·4	0·21	42
41·3	0·29	51

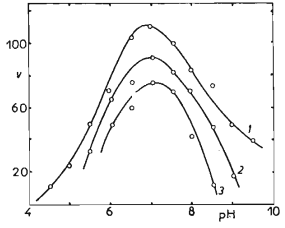

FIG. 1

pH-Stability of Pea Alcohol Dehydrogenase

1 Incubation time at 20°C 2 h, *2* 6 h, *3* 48 h.

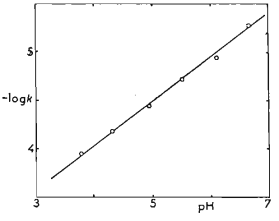

FIG. 2

Dependence of log of Rate Constant k (s^{-1}) on pH

i.e. at physiological pH in the cell. The inactivation both in the acidic and alkaline medium is marked. Fig. 2 illustrates an effort to quantitate the denaturation rate in the acidic medium. If the kinetics of the order (pseudomonomolecular) is applied, the rate constant is a linear function of $[H^+]$ and the function expressing the denaturation rate is given by $-\log k = f(pH)$. The denaturation rate was examined over the pH-range 4·0–6·5 after 4-h incubation at 20°C by determination of the rate of ethanol oxidation. The denaturation rate constants thus determined $k\,(s^{-1})$ are high.

Effect of Temperature

The effect of temperature on the reaction rate catalyzed by pea alcohol dehydrogenase was examined over the range 15–55°C at intervals of 5°C. The measurements were made in a thermostated water bath with an accuracy of ±0·5°C. Both the initial rate

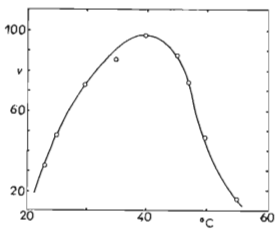

FIG. 3
Temperature Optimum of Alcohol Dehydrogenase for Ethanol Oxidation

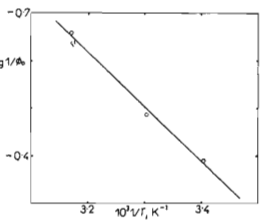

FIG. 4
Dependence of log of Reciprocal Coefficient ϕ_0 for Ethanol Oxidation on Reciprocal Temperature Value

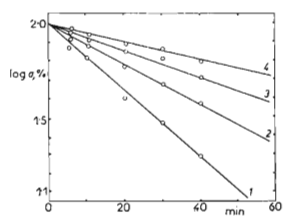

FIG. 5
Thermal Denaturation of Pea Alcohol Dehydrogenase and Enzyme–Coenzyme Complex
1 Incubation at 70°C in absence of NAD, *2* with 0·393 mM NAD, *3* with 0·786 mM, *4* with 1·57 mM NAD.

and the maximal rate increase with the increasing temperature and attain a maximum at 40°C with respect to both substrates, *i.e.* ethanol and NAD (Fig. 3). The values of the Michaelis constants decrease with the increasing temperature and are the lowest at 21°C (Table II). Pea alcohol dehydrogenase is thermolabile, loses its activity at 70°C; the stability of the enzyme–NAD complex depends on the concentration of the coenzyme (Fig. 5). The thermal denaturation of both the enzyme and the binary enzyme–NAD complex follows the kinetics of the first order. The coefficient ϕ_0 complies with the Arrhenius equation and since $1/\phi_0$ actually expresses the maximal oxidation rate V_0/e under the given conditions, Fig. 4 permits us to estimate how the reaction rate depends on temperature.

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Translated by V. Kostka.