KINETICS OF REDOX REACTION CATALYZED BY PEA ALCOHOL DEHYDROGENASE

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The oxidation of ethanol by NAD and the reduction of acetaldehyde by NADH, catalyzed by alcohol dehydrogenase isolated from pea, were studied. The reaction obviously follows the mechanism proposed by Theorell and Chance: the coenzyme is bound as the first one to the enzyme and in its reversed form the coenzyme dissociates off as the last one. The dissociation of the coenzyme from the complex with alcohol dehydrogenase is a step which determines the rate of the whole reaction at the same time.

We have reported in this Journal on the preparation and characterization of alcohol dehydrogenase isolated from pea during the so-called natural anaerobiosis. This dehydrogenase resembles in certain features (susceptibility to the action of some inhibitors) the enzyme from animal liver and in certain features the enzyme from yeast.

The kinetics of the redox reaction of horse liver alcohol dehydrogenase and of yeast alcohol dehydrogenase (EC 1.1.1.1) with various alcohols and aldehydes has been studied in detail[1,2]. It has been shown that the oxidation of ethanol by NAD and the reduction of acetaldehyde by NADH follow the Theorell and Chance mechanism[3], *i.e.* NAD* or NADH, is bound first to the enzyme and NADH or NAD, dissociates last from the enzyme after completion of the redox reaction.

$$E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ES_1S_2 \underset{k'}{\overset{k}{\rightleftharpoons}} ES'_1S'_2 \underset{k'_{+2}}{\overset{k'_{-2}}{\rightleftharpoons}} ES'_1 \underset{k'_{+1}}{\overset{k'_{-1}}{\rightleftharpoons}} E$$

The validity of these equations, referred to as the Theorell and Chance model, for animal liver ADH has been demonstrated experimentally.

Another feature of the Theorell and Chance mechanism is that the formation of a ternary ADH–NAD–ethanol complex and a ternary ADH–NADH–acetaldehyde complex, and their reciprocal conversions proceed faster than the remaining

* Abbreviations used: alcohol dehydrogenase-ADH, enzyme-E, NAD-S_1, NADH-S'_1, ethanol -S_2, acetaldehyde -S'_2.

processes; therefore none of these reactions represents a limiting step. If the initial concentration of the coenzyme and of the substrate is sufficiently high, the dissociation of the product, *i.e.* of ADH-coenzyme[4] is the limiting step of the whole reaction.

In accordance with such a simple mechanism obviously proceed the reduction of aldehydes and the oxidation of ethanol whereas a partial dissociation of NAD from the ternary ADH–NAD–ethanol complex is supposed to be necessary for the oxidation of higher alcohols[2].

In this study we endeavored to determine whether the Theorell and Chance model holds true also for pea alcohol dehydrogenase. As far as we know similar measurements have not been made with the plant enzyme.

EXPERIMENTAL

Isolation of alcohol dehydrogenase. Pea (*Pisum arvense* L. cv. *Raman*) was the vegetal material used to start with. ADH was prepared from pea seeds which had been allowed to germinate 24 h. Plant tissues were pulverized and the material was extracted with 0·1M phosphate buffer, pH 8·5. The part of the material precipitated at 40–60% saturation with ammonium sulfate was used in the subsequent isolation process. This material was desalted on Sephadex G-25 and proteins were fractionated on a column of DEAE-cellulose. A linear gradient from 0·025 to 0·5M Tris-acetate buffer, pH 6·4, containing 10 mM L-cysteine was used[5].

The measurement of the initial rates of the reaction catalyzed by pea ADH was carried out according to Racker[6] at 20°C; the total volume of the reaction mixture was 1 ml.

The concentration of the active sites of pea ADH was determined by titration with 8-anilino-1-naphthalene sulfonate[7].

The kinetics of a two-substrate reaction can be expressed by the following general formula

$$\frac{e}{v_0} = \Phi_0 + \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \frac{\Phi_{12}}{[S_1][S_2]}, \qquad (1)$$

where e is the concentration of active sites, $[S_1]$ and $[S_2]$ the concentration of the coenzyme and the substrate, respectively, and v_0 the initial reaction rate[8,9]. The values of the kinetic coefficients Φ (for oxidation of alcohols) and Φ' (for reduction of aldehydes) are obtained as follows. When the reciprocal reaction rate is plotted *versus* reciprocal substrate concentration, we obtain a series of lines. The slopes and intercepts of the latter are plotted *versus* reciprocal coenzyme concentration and we obtain two lines whose intercepts and slopes directly indicate the set of four kinetic coefficients[10]. The necessary and sufficient conditions for the reaction to follow the Theorell and Chance mechanism are

$$\Phi'_0 = \frac{\Phi_1\Phi_2}{\Phi_{12}}, \quad \Phi_0 = \frac{\Phi'_1\Phi'_2}{\Phi'_{12}}. \qquad (2)$$

These equations guarantee that the rate-determining step of the entire reaction is the dissociation of the enzyme from the ADH-coenzyme complex[8].

RESULTS AND DISCUSSION

The kinetic coefficients, given in Table I and determined at two pH-values, comply with the conditions of equations (*2*), valid for the Theorell and Chance mechanism. We can calculate from the values of these kinetic coefficients not only the Michaelis constants for all the participating substrates (Table II) but we can also determine the values of the dissociation constants of binary complexes of the enzyme with the two coenzymes (Table III).

The equilibrium constant K for the reaction ethanol + NAD $\overset{K}{\rightleftharpoons}$ acetaldehyde + + NADH + H^+ can be calculated from the kinetic coefficients as

$$K = \frac{\Phi'_{12}}{\Phi_{12}} [H^+]$$

The calculated value of K for pH 8·5 is $1{\cdot}7 \cdot 10^{-11}$M. The thermodynamic value of the constant at 25°C is $0{\cdot}98 \cdot 10^{-11}$M (ref.[11]).

TABLE I

Values of Kinetic Coefficients from Equation (*1*) for Oxidation of Ethanol by NAD and Reduction of Acetaldehyde by NADH, Catalyzed by Pea ADH at pH 8·5 and 7·4

Experimental conditions: [ethanol] = 5—40 mM, NAD = 50—500 μM, [acetaldehyde] = = 1—8 mM, [NADH] = 50—500 μM.

ϕ_0, s	5·5	7	ϕ'_0, s	1·5	1
ϕ_1, mM s	0·8	0·35	ϕ'_1, mM s	0·3	0·15
ϕ_2, mM s	170	280	ϕ'_2, mM s	9	4
ϕ_{12}, mM^2 s	90	90	ϕ'_{12}, mM^2 s	0·5	0·1

Measured at pH: [a] 8·5; [b] 7·4; [c] 8·5; [d] 7·5.

TABLE II

Michaelis Constants of Basic Substrates of Pea ADH Calculated from Values of Kinetic Coefficients

K_m(NAD) = ϕ_1/ϕ_0, μM	150	200
K_m(NADH) = ϕ'_1/ϕ'_0, μM	200	160
K_m(ethanol) = ϕ_2/ϕ_0, mM	30	40
K_m(acetaldehyd) = ϕ'_2/ϕ'_0, mM	6	4

Measured at pH: [a] 8·5; [b] 7·4.

TABLE III

Dissociation Constants of Binary Complexes of Pea ADH with NAD and NADH, Calculated from Values of Kinetic Coefficients

$K_{ADH-NAD} = \phi_1/\phi'_0$, μM	500	300
$K_{ADH-NADH} = \phi'_1/\phi_0$, μM	60	20

Measured at pH: [a] 8·5; [b] 7·4.

The results obtained show that the oxidation of ethanol by NAD and the reduction of acetaldehyde by NADH, catalyzed by pea ADH, follow the Theorell and Chance mechanism. *i.e.* a mechanism supposed to be also operative in the reactions catalyzed by horse liver and yeast alcohol dehydrogenase[12]. The Theorell and Chance mechanism points to the decisive role of binary ADH–coenzyme complexes whose dissociation constants can be determined. The interpretation of the formation, reciprocal conversion, and dissociation of ternary ADH–NAD–ethanol and ADH–NADH–acetaldehyde complexes rests on somewhat uncertain grounds since these reactions are faster than the dissociation of binary ADH–coenzyme complexes and hence are not reflected in the kinetic schemes.

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