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PIGEON LIVER DIACETYL REDUCTASE

EFFECTS OF pH ON THE KINETIC PARAMETERS OF THE REACTION

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Summary

(1) The pH dependence of the kinetic parameters of the reaction catalyzed by pigeon liver diacetyl reductase (EC 1.1.1.5) was investigated in the pH range 5.1—8.6.

(2) From the results obtained it is postulated that: (a), a group of p*K* around 7, active in the protonated form, participates in the interaction of the enzyme with NADH and NAD. (b), a second group with a p*K* of 8.4, active in the protonated form too, takes part in the binding of diacetyl to E-NADH. (c) A third group of p*K* about 4.7—5, active in the unprotonated form, is involved at least in the dissociation of the complex E-NAD and in the attachment of diacetyl to E-NADH.

Introduction

Pigeon liver diacetyl reductase catalyzes, for all practical purposes, the irreversible reduction of diacetyl to acetoin coupled to the oxidation of either NADH or NADPH [1]; it is, therefore, one of the diacetyl reductases which are not adequately described by the entry EC 1.1.1.5 (Acetoin: NAD^+ oxidoreductase) in the Enzymes Nomenclature (1972). In earlier papers [1,2] the authors have reported a method which allows the purification of 400-fold of this enzyme and have described some of its kinetic properties: with NADH as hydrogen donor and at standard assay conditions [1] the maximal activity is attained in the pH range 5.6—6.2 and, at optimum pH, the reaction follows a Theorell-Chance mechanism the rate-limiting step being the dissociation of the E-NAD complex.

In the present paper the effects of pH on the kinetic parameters of the reaction are studied in order to obtain information about the groups involved in the interaction of the enzyme with its substrates and products.

Materials and Methods

NADH was obtained from Boehringer and diacetyl from BDH.

Enzyme preparations were acetone (1.2—2.5 vol.) precipitates free of non-specific NADH dehydrogenase, obtained as previously reported [1]; they were dissolved in 1.5 M sucrose to stabilize the diacetyl reductase activity during the experiments [1]. One unit is taken as the amount of enzyme that oxidizes 1 nmol of NADH per min at 25° C under the following conditions: pH 6.1 phosphate buffer, 0.15 mmol; diacetyl, 30 μmol; NADH, 0.6 μmol; total volume, 3 ml [1].

Initial reaction rates have been determined at 25° C from measurements of the changes in absorbance at 340 nm. Assays were performed in 0.05 M potassium phosphate buffer; pHs were adjusted within ± 0.01 units. The reaction was started by addition of the enzyme.

Results

Enzyme stability versus pH

Aliquots of an enzyme preparation were incubated for 5 min in 0.05 M phosphate buffers, at the assay temperature and several pH values. Under these conditions diacetyl reductase activity is fully stable in the pH range 5.4—7.6 but quickly decays at higher hydrogenions concentrations, with loss of about 20, 30 and 60% at pH 5.1, 5.0 and 4.8, respectively; in the alkaline side the loss of activity is slower: 15, 30 and 50% at pH 8.0, 8.5 and 8.8, respectively. Similar experiments with an incubation period of 2 min gave the following inactivation data: pH 4.8, 34%; pH 5.1, 10%; pH 8.5, 14%; pH 8.8, 26%.

Effects of pH on the kinetic parameters

Table I shows the variations of K_s^{NADH}, $K_s^{diacetyl}$, K_m^{NADH}, $K_m^{diacetyl}$ and V with pH in the range 5.1—8.6, in which the loss of activity was nil or estimated

TABLE I

pH-DEPENDENCE OF THE KINETIC PARAMETERS

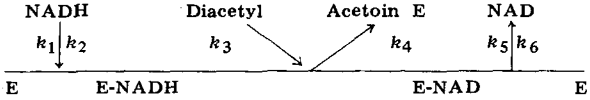
V is expressed as nmol of substrate transformed in one min by 100 enzyme units.

pH of the reaction mixture	K_s^{NADH} (μM)	$K_s^{diacetyl}$ (mM)	K_m^{NADH} (μM)	$K_m^{diacetyl}$ (mM)	V
5.1	147	5.26	65	2.36	133
5.4	149	5.13	79	2.7	171
5.6	150	5.28	77	2.71	195.5
5.9	154	5	87	2.81	206
6.3	164	4.3	116	3	210.7
6.7	190	3.65	135	2.64	195.3
7.1	198	3	142	2.12	142.2
7.4	242	2.67	145	1.62	109.6
7.7	286	2.15	167	1.26	75.6
8	330	1.88	147	0.84	43.4
8.3	380	1.8	151	0.72	24.8
8.5	500	1.6	155	0.49	14.9
8.6	520	1.55	155	0.44	12

to be negligible along the few seconds that the conditions approach those of initial rate. K_s values were calculated from primary plots of $1/v$ versus $1/[S]$ at each possible combination of five NADH concentrations (in the range 0.05—0.4 mM) and five other of diacetyl (in the range 1.25—20 mM). K_m and V values were obtained by replotting the intercepts from the primary plots versus the reciprocal of the fixed-variable substrate concentration; in this paper V is always expressed as nmol of substrate transformed in one min per 100 enzyme units.

Discussion

It has been previously proved by the authors [2] that the NADH-linked diacetyl reductase reaction catalyzed by the enzyme from pigeon liver follows at optimum pH (6.1) a Theorell-Chance mechanism, the coenzyme being the leading substrate:



(I)

Our results will be discussed on the assumption that the same mechanism holds along the whole pH range under study.

Only three forms of the enzyme are kinetically significant in this scheme: the free enzyme (E) and the two binary complexes, E-NADH and E-NAD. From the rate equation for Theorell-Chance mechanisms as calculated by the method of King and Altman [3,4] and since in this type of mechanism $K_s^a = k_2/k_1$, $K_m^a = k_5/k_1$ and $K_m^b = k_5/k_3$, it is deduced that:

$$V/K_m^a = k_1 \cdot E_t \qquad \text{(II)}$$

$$V' = V \cdot (K_s^a/K_m^a) = k_2 \cdot E_t \qquad \text{(III)}$$

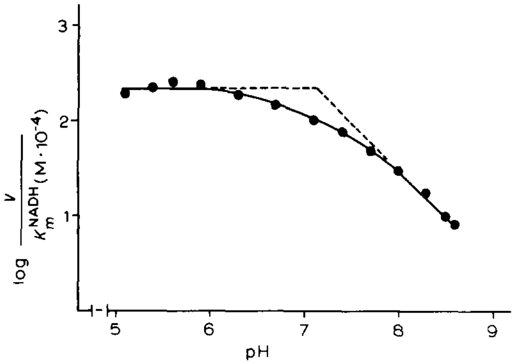
$$V/K_m^b = k_3 \cdot E_t \qquad \text{(IV)}$$

$$V = k_5 \cdot E_t \qquad \text{(V)}$$

where V and V' are the maximum velocity of the reaction in the forward (V) and backward (V') direction, E_t the total enzyme concentration, K_m^a and K_s^a the Michaelis and the dissociation constants (respectively) for the first substrate to add to the enzyme and K_m^b the Michaelis constant for the second one; k_1, k_2, k_3 and k_5 are rate constants of individual steps of the reaction, as indicated in Eqn. I (for details about this equation, see [4]).

Ionizing groups on the free enzyme

By applying Eqn. II to the diacetyl reductase reaction under study it is infered that, at constant E_t concentration, a plot of log V/K_m^{NADH} versus pH must evidence the changes of log k_1 with pH and will show the pK of any group on either the free enzyme or NADH whose ionization affects to their ability to combine with each other. Lines of slope zero and -1 intercept in this plot (Fig. 1) at pH 7.0—7.2, displaying the pK of a group whose active form is the pro-



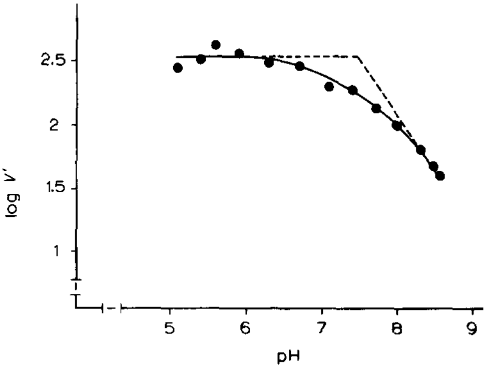


Fig. 1. Ionizing groups on the free enzyme; plot of log (V/K_m^{NADH}) versus pH.

Fig. 2. Ionizing groups on E-NADH; plot of log V' versus pH.

tonated one; since NADH has none in the pH range investigated, this pK value must be due to a group on the free enzyme at which NADH combines.

Ionizing groups on E-NADH

Plots of log V' versus pH will reveal (Eqn. III) the changes of log k_2 with pH and, therefore, the pK values of the groups on E-NADH whose ionization state regulates the dissociation of NADH from the binary complex it forms with the enzyme. This plot (Fig. 2) shows a pK around pH 7.3—7.5 for a group active in the protonated form.

As the reaction under study is not mensurable in the reverse direction, V' had to be calculated as the ratio $V \cdot K_s^{NADH}/K_m^{NADH}$ (Eqn. III). This procedure, when used to determine V' in other cases to which the same relationship applies, has sometimes yielded values widely different from the true maximal velocity of the backward reaction, resulting in anomalous plots of log V' versus pH [5,6]. However, the results of our estimates for V' in the diacetyl reductase reaction seem reliable since the plot of Fig. 2 is a normal two branched curve with the lines of slope zero and -1 intercepting at a vertical distance of about 0.3 units from the bend of the graph. In spite of the fact that the reduction of diacetyl catalyzed by the enzyme from pigeon liver is practically irreversible, V' so calculated was higher than V in the whole pH range used, which could look awkward; nevertheless, this paradoxical situation is probably due to the very low affinity of the enzyme for acetoin and NAD which has to hinder strongly the backward reaction:

Let us consider the following Haldane relationship, valid for Theorell-Chance mechanisms (4)

$$K_{eq} = \frac{V \cdot K_s^p \cdot K_m^q}{V' \cdot K_m^a \cdot K_s^b} \qquad \text{(VI)}$$

where K_{eq} is the thermodynamic equilibrium constant of the reaction, "p" the first product released from the enzyme and "q" the second one. From the data of product inhibition at pH 6.1 (optimum) already published by the authors

[2] it can be calculated that $K_{\mathrm{s}}^{\mathrm{acetoin}} = 180$ mM and $K_{\mathrm{s}}^{\mathrm{NAD}} = 1.1$ mM. At this pH $K_{\mathrm{m}}^{\mathrm{NADH}} = 0.1$ mM, $K_{\mathrm{s}}^{\mathrm{NADH}} = 0.162$ mM and $K_{\mathrm{s}}^{\mathrm{diacetyl}} = 5$ mM [2]. From Eqn. III it is derived that $V/V' = K_{\mathrm{m}}^{\mathrm{a}}/K_{\mathrm{s}}^{\mathrm{a}} = 0.1/0.162 = 0.62$; on the other hand (since for Theorell-Chance mechanisms $K_{\mathrm{m}}^{\mathrm{q}} = k_2/k_6$, $K_{\mathrm{s}}^{\mathrm{q}} = k_5/k_6$, $K_{\mathrm{s}}^{\mathrm{a}} = k_2/k_1$ and $K_{\mathrm{m}}^{\mathrm{a}} = k_5/k_1$) $K_{\mathrm{m}}^{\mathrm{q}} = K_{\mathrm{s}}^{\mathrm{q}} \cdot K_{\mathrm{s}}^{\mathrm{a}}/K_{\mathrm{m}}^{\mathrm{a}} = 1.1 \,.\, 0.162/0.1 = 1.78$ mM. By introducing these values in Eqn. VI

$$K_{\mathrm{eq}} = \frac{0.62 \cdot 180 \cdot 1.78}{0.1 \cdot 5} = 397.3 \qquad \text{(VII)}$$

Therefore, at pH 6.1 the equilibrium of the reaction must be displaced about 400-fold in favor of the forward (diacetyl-acetoin) direction. This would explain the difficulties in detecting the backward reaction.

Eqn. IV indicates that the plots of log $V/K_{\mathrm{m}}^{\mathrm{diacetyl}}$ versus pH must show the changes of log k_3 with hydrogen ion concentration and will, therefore, allow the estimation of the pK values of the groups on the binary complex E-NADH whose ionization state affects its interaction with diacetyl. By plotting our results in this way (Fig. 3) a curve is obtained with a wide flat portion and two descending branches, one dropping toward higher pH values and the other toward lower pH values. The branch dropping toward higher pH values reveals the existence of a group active in the protonated form, but the graph does not become steep enough to reach a slope of −1. Therefore, the pK of this group had to be estimated from plots of the values of $K_{\mathrm{m}}^{\mathrm{diacetyl}}/V$, in the pH range 7.4—8.6, versus $1/[\mathrm{H}^+]$ (Fig. 4a) according to the equation

$$\frac{K_{\mathrm{m}}^{\mathrm{diacetyl}}}{V} = \frac{\bar{K}_{\mathrm{m}}^{\mathrm{diacetyl}}}{\bar{V}} + \frac{\bar{K}_{\mathrm{m}}^{\mathrm{diacetyl}} \cdot K_{\mathrm{h}}}{\bar{V} \cdot (\mathrm{H}^+)} \qquad \text{(VIII)}$$

where $\bar{V}$ and $\bar{K}_{\mathrm{m}}^{\mathrm{diacetyl}}$ are kinetic parameters independent of pH, K_{h} the ionization constant of the group under study and (H^+) the hydrogenions concentration. Eqn. VIII has been obtained by rearranging

$$\frac{V}{K_{\mathrm{m}}^{\mathrm{diacetyl}}} = \frac{\bar{V}}{\bar{K}_{\mathrm{m}}^{\mathrm{diacetyl}} \cdot (1 + K_{\mathrm{h}}/(\mathrm{H}^+))} \qquad \text{(IX)}$$

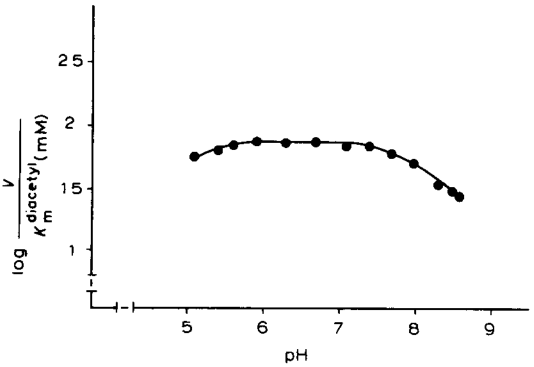


Fig. 3. Ionizing groups on E-NADH; plot of log $(V/K_{\mathrm{m}}^{\mathrm{diacetyl}})$ versus pH.

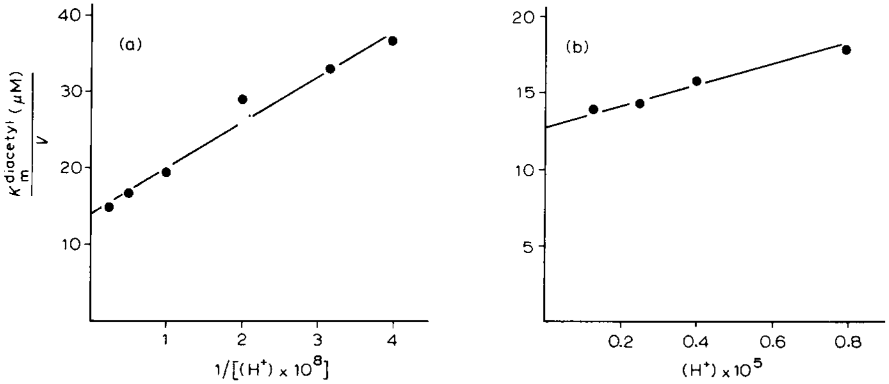


Fig. 4. Ionizing groups on E-NADH: (a) plot of $[K_m^{diacetyl}/V]$ versus $1/[H^+]$; (b) plot of $[K_m^{diacetyl}/V]$ versus $[H^+]$.

valid in the pH region around pK_h for groups whose active form is the protonated one (for details, see [7]). The pK was determined as the negative logarithm of the ratio slope/vertical intercept of Fig. 4a, giving a value of 8.4. Since diacetyl is not ionizable, this pK ought to be due to a group on the E-NADH complex.

The branch in Fig. 3 dropping toward the acid side indicates the existence of another pK, corresponding to a group which is active in the unprotonated form. The value of this pK was calculated by plotting the data obtained in the pH range 5.1—5.9 as $K_m^{diacetyl}/V$ versus $[H^+]$ (Fig. 4b) in accordance with the equation:

$$\frac{K_m^{diacetyl}}{V} = \frac{\bar{K}_m^{diacetyl}}{\bar{V}} + \frac{\bar{K}_m^{diacetyl} \cdot [H^+]}{\bar{V} \cdot K_h} \tag{X}$$

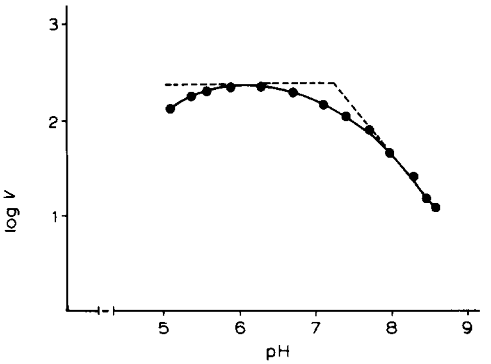
derived by rearangement of

$$\frac{V}{K_m^{diacetyl}} = \frac{\bar{V}}{\bar{K}_m^{diacetyl} \cdot (1 + [H^+]/K_h)} \tag{XI}$$

which applies to groups active in the unprotonated form [7]. The pK was calculated by taking the negative log of the ratio vertical intercept/slope in the plot of Fig. 4b. A value of 4.71 was obtained; since the pK values falling out of the pH range investigated can be very inaccurate, the standard error was calculated and found to be ± 0.23.

Ionizing groups on E-NAD

From Eqn. V it can be deduced that plots of log V against pH must show up any change of log k_5, revealing the pK values of the groups on the E-NAD complex whose ionization state affects the dissociation of NAD from the complex. This graph (Fig. 5) gives evidence for a well defined pK with a value around 7.2, corresponding to a group active in the protonated form. As in Fig. 3, a branch descending toward the acidic side is observed, indicating a second pK for a group whose active form is the unprotonated one; the value of this pK



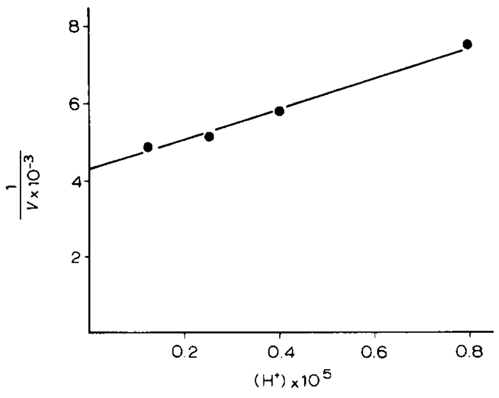


Fig. 5. Ionizing groups on E-NAD; plot of log V versus pH.

Fig. 6. Ionizing groups on E-NAD; plot of $1/V$ versus $[H^+]$.

was calculated in 4.99 ± 0.08 by taking the negative log of the ratio intercept/slope in Fig. 6, where it has been plotted $1/V$, in the range 5.1—5.9, versus $[H^+]$ according to the equation [7]

$$\frac{1}{V} = \frac{1}{\overline{V}} + \frac{[H^+]}{\overline{V} \cdot K_h} \qquad \text{(XII)}$$

Ionizing groups participating in the overall reaction

Summarizing, the following pK values corresponding to groups whose ionization degree someshow affects the overall reaction, have been detected: (a) one showing a value of 7.0—7.2 pH units for a group, active in the undissociated state, implied in the binding of NADH to the free enzyme; (b) another around pH 7.2 for a group, active in the protonated form too, involved in the dissociation of the E-NAD complex; (c) a third at pH 7.3—7.5 corresponding to a group, whose active form is also the protonated one, implied in the dissociation of E-NADH; (d) another about pH 8.4 belonging to a group, active in the protonated form, involved in the binding of diacetyl to E-NADH; (e) a fifth toward pH 4.7 which is due to a group active in the unprotonated form whose ionization state affects to the interaction between diacetyl and E-NADH, and, finally, (f) one more of a similar value (4.99) affecting the E-NAD dissociation.

It is postulated that the first three pK values (involved in the interaction of the enzyme with NADH and NAD) correspond to one single group, the third probably shifted toward a higher pH by the binding of NADH; the pK values calculated for this group are in good agreement with that of an imidazolium one from an histidyl residue. The group of pK 8.4 appears to play a role only in the binding of diacetyl to E-NADH, since it has not been detected in the plots of log V/K_m^{NADH}, log V' or log V versus pH; it seems most likely that it is either a sulphydryl or an ammonium. All these groups have been frequently considered essential for the catalysis of a large number of NAD(P)-linked dehydrogenases.

The two pK values detected with a value of 4.71—4.99 must correspond also to one single group, probably a γ or δ-carboxyl, implied at least in the binding of diacetyl to the binary complex E-NADH and in the dissociation of E-NAD.

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