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PIGEON LIVER DIACETYL REDUCTASE KINETIC AND THERMODYNAMIC STUDIES WITH NADH AS COENZYME

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SUMMARY

1. The dependence of the initial rate of the reaction catalyzed by pigeon liver diacetyl reductase on the concentration of substrates (diacetyl and NADH) and the product (acetoin and NAD^+) inhibition pattern are consistent with both a rapid equilibrium random Bi–Bi mechanism with two dead-end ternary complexes and a Theorell–Chance one.

2. The inhibition patterns with acetone and pentane-3-one (competitive for diacetyl and uncompetitive for NADH) discard the random Bi–Bi mechanism and prove that the reaction follows that of Theorell–Chance with NADH as the leading substrate.

3. $K_m^{diacetyl}$ (3.1 mM), K_m^{NADH} (100 μM), $K_s^{diacetyl}$ (5 mM), K_s^{NADH} (162 μM), activation energy (13.8 kcal/mole), ΔH^* (13.3 kcal/mole), ΔF^* (14.5 kcal/mole) and ΔS^* ($= -4$ cal/mole per degree) were estimated.

INTRODUCTION

Diacetyl reductase, formerly thought to be exclusively present in microorganisms, is now known to have a much wider distribution in nature [1–4]. The various diacetyl reductases isolated differ largely in affinity for diacetyl [2, 4–7] and NADH [5–7] and in substrate and coenzyme specificity [2–5, 8–11]. There is one single entry in the Enzymes Nomenclature for all these enzymes (acetoin:NAD^+ oxidoreductase, EC 1.1.1.5) but it is obvious that some of them are not adequately described by it [4, 10–11].

Most of the data until now reported for the diacetyl reductases have been obtained with beef liver and *Aerobacter aerogenes* preparations, two enzymes which show very different properties and whose catalyzed reactions could, according to the published reports [6, 7], follow different kinetic mechanisms. Pigeon liver diacetyl reductase catalyzes the irreversible reduction of diacetyl to acetoin coupled to the oxidation of either NADH or NADPH (it is, therefore, one of the diacetyl reductases that do not conform to entry EC 1.1.1.5). This enzyme is similar to that from *A. aerogenes* in molecular weight and affinity for diacetyl and to the beef liver one in substrate specificity, differing from both of them in pH profile and other properties [4].

In the present paper the kinetics of the catalyzed reaction with NADH as coenzyme is studied, showing that it follows a Theorell–Chance mechanism; values of the kinetic constants and thermodynamic parameters are also given.

MATERIALS AND METHODS

NAD^+ and NADH were obtained from Boehringer; acetoin, diacetyl, hexane-2,5-dione, acetone and pentane-3-one from BDH; α-ketoglutaric acid from Merck. Acetoin was purified by washing it with peroxide-free diethyl ether until the diacetyl content was less that 0.05%, as already described [6].

Enzyme preparations were obtained as reported in a previous paper [4]. These preparations were entirely free of butyleneglycol and alcohol dehydrogenases, as well as nonspecific NADH-dehydrogenase.

The enzyme activities were spectrophotometrically determined at pH 6.1 (optimum) in 0.05 M sodium potassium phosphate buffer and 25 °C, except when otherwise stated.

RESULTS

The initial rates in the diacetyl–acetoin direction were measured at several concentrations of each substrate in the range 0.3–60 mM diacetyl and 0.025–0.4 mM NADH. The primary double-reciprocal plots were linear (within experimental error) except at diacetyl concentrations higher than 20 mM at which substrate inhibition was observed; up to this concentration of diacetyl, two families of straight lines (one for each variable substrate) which intercept in the upper left quadrant (Fig. 1) are obtained. *K*s (dissociation constants) values were calculated from these plots.

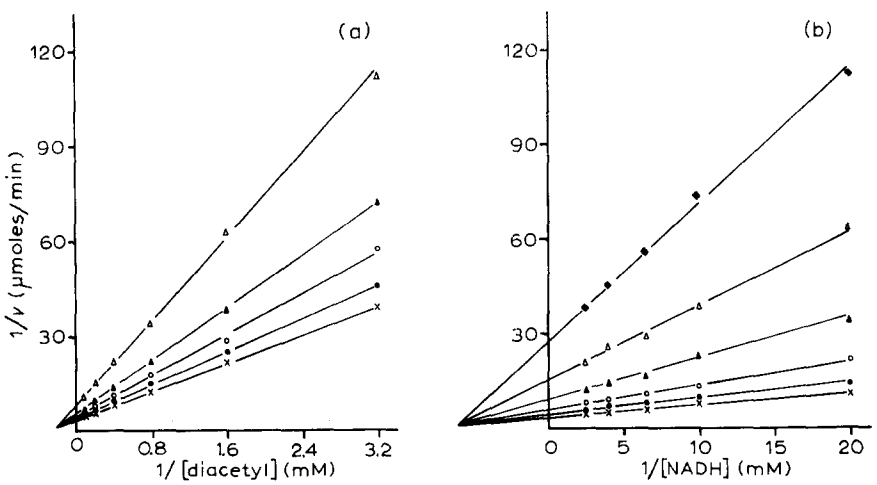

Fig. 1. Primary plots: (a) Double-reciprocal plots of $1/v$ versus 1/[diacetyl] at the fixed NADH concentrations (mM) indicated: ×, 0.4; ●, 0.25; ○, 0.15; ▲, 0.1; △, 0.05. (b) Double-reciprocal plots of $1/v$ versus 1/[NADH] at the fixed concentrations of diacetyl indicated (mM): ×, 10; ●, 5; ○, 2.5; ▲, 1.25; △, 0.625; ◆, 0.312.

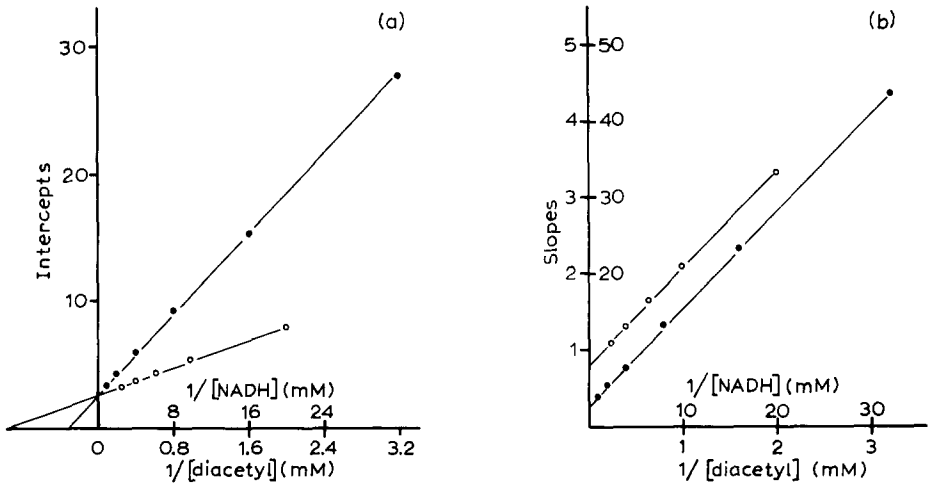

Fig. 2. Secondary plots of intercepts (a) and slopes (b) from Fig. 1 against 1/[fixed-variable substrate]: (●), diacetyl; (○), NADH.

Secondary plots of the slopes versus 1/[substrate] are parallel straight lines (Fig. 2b). Those of intercepts cut the vertical axis at the same point (Fig. 2a)· K_m (Michaelis constant) values were obtained from this figure.

The K_s and K_m values found were: $K_m^{diacetyl} = 3.1$ mM; $K_m^{NADH} = 100\,\mu$M; $K_s^{diacetyl} = 5$ mM; $K_s^{NADH} = 162\,\mu$M.

Product inhibition pattern

With NADH as the variable substrate and diacetyl at a fixed unsaturating concentration, inhibition by NAD^+ is competitive and acetoin inhibition is noncompeti-

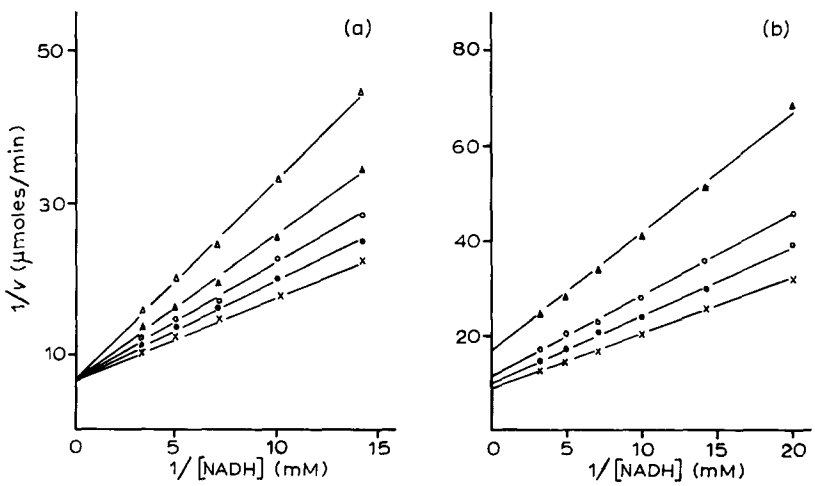

Fig. 3. NAD^+ (a) and acetoin (b) as product inhibitors versus NADH. (a) NAD^+ concentrations (mM): ×, none; ●, 0.33; ○, 0.9; ▲, 1.5; △, 3. Diacetyl concentration, 10 mM. (b) Acetoin concentrations (mM): ×, none; ●, 40; ○, 100; ▲, 300. Diacetyl concentration, 2.5 mM.

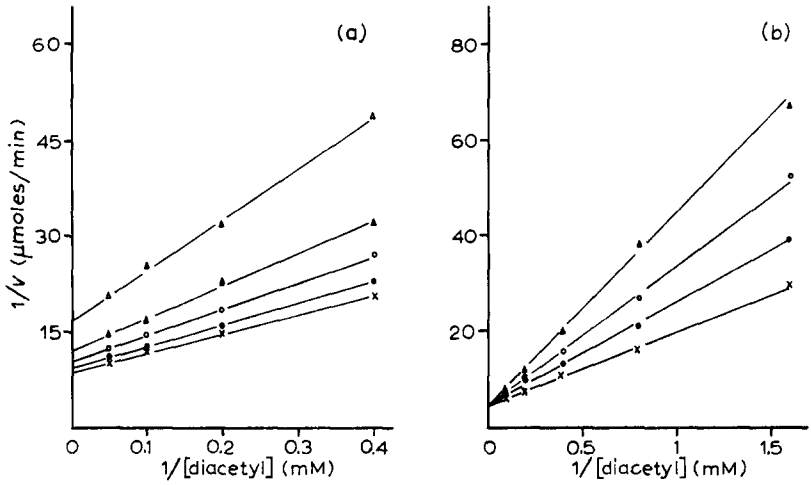

Fig. 4. NAD^+ (a) and acetoin (b) as product inhibitors versus diacetyl. (a) NAD^+ concentrations (mM): ×, none; ●, 0.4; ○, 0.8; ▲, 1.6; △, 3.2. NADH concentration, 0.2 mM. (b) Acetoin concentrations (mM): ×, none; ●, 50; ○, 150; ▲, 300. NADH concentration, 0.2 mM.

tive (Fig. 3). With an unsaturating fixed concentration of NADH and a variable diacetyl, NAD^+ inhibits non-competitively and acetoin competitively (Fig. 4).

Inhibition by diacetyl analogues

Several diacetyl analogues were studied. α-Ketoglutarate and hexane-2,5-dione showed noncompetitive inhibition for both substrates. Acetone and pentane-3-one inhibit competitively for diacetyl (Fig. 5) and uncompetitively for NADH (Fig. 6).

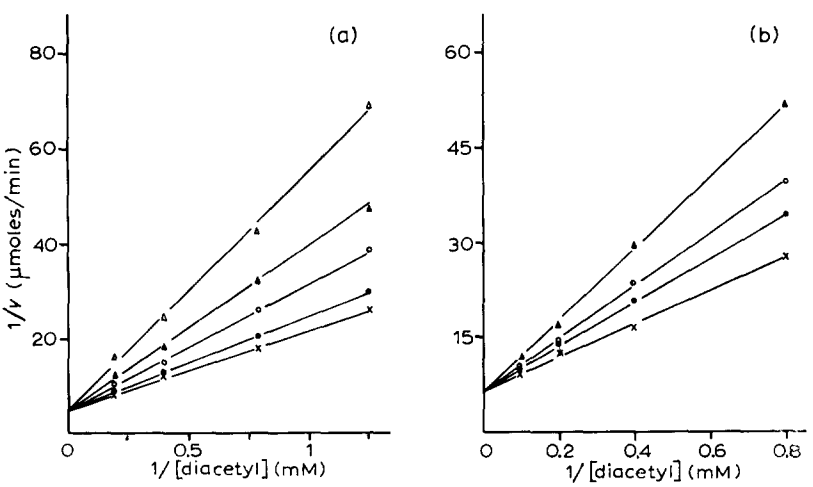

Fig. 5. Inhibition by acetone (a) and pentane-3-one (b) versus diacetyl. (a) Acetone concentrations (mM): ×, none; ●, 28; ○, 85; ▲, 170; △, 340. NADH concentration, 0.2 mM. (b) Pentane-3-one concentrations (mM): ×, none; ●, 5; ○, 10; ▲, 20. NADH concentration, 0.2 mM.

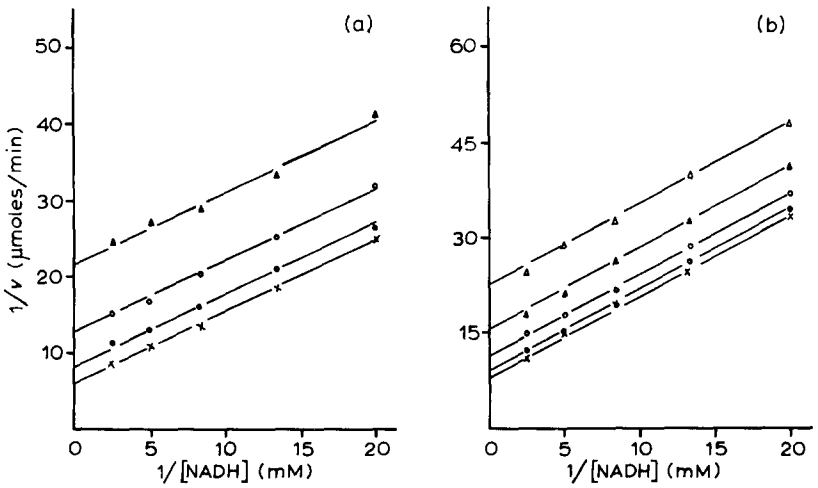

Fig. 6. Inhibition by acetone (a) and pentane-3-one (b) versus NADH. (a) Acetone concentrations (mM): ×, none; ●, 85; ○, 170; ▲, 340. Diacetyl concentration, 2.5 mM. (b) Pentane-3-one concentrations (mM): ×, none; ●, 5; ○, 10; ▲, 20; △, 40. Diacetyl concentration, 2.5 mM.

Effects of temperature on activity and thermodynamic parameters of the reaction

Fig. 7 shows the effects of temperature on the enzyme activity with standard assay conditions (pH 6.1 in 0.05 M sodium potassium phosphate buffer, 0.2 mM NADH and 10 mM diacetyl). From this figure a Q_{10} between 15 and 25 °C = 2 was calculated.

The activation energy (13.8 kcal/mole, ± 0.35) was obtained from Fig. 8 and ΔH^* (13.3 kcal/mole, ± 0.35) was obtained from similar plots of ln (V/T) versus $1/T$ (°K). V values were calculated from the secondary plots (intercepts) of the initial rates obtained at each temperature with several concentrations of diacetyl and NADH.

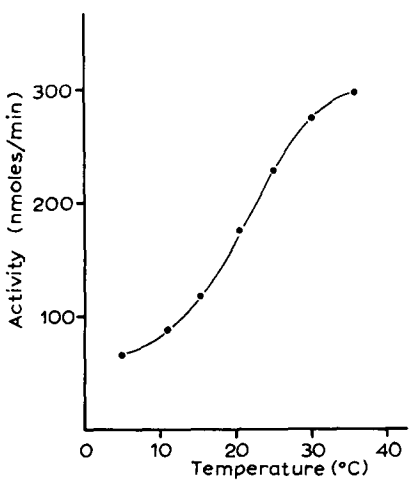

Fig. 7. Effects of temperature on enzyme activity at the standard assay conditions.

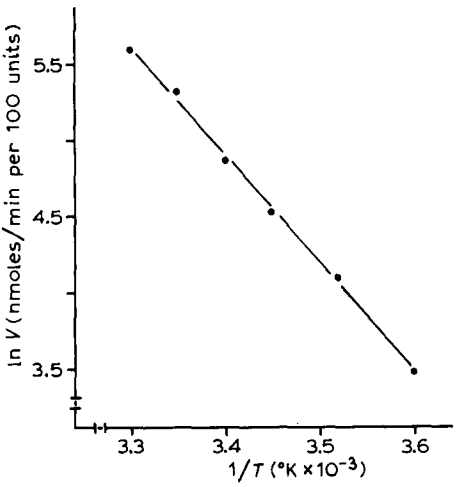

Fig. 8. Arrhenius plots of the changes of V with temperature.

From an estimation of the purity of 40% for the preparations of specific activity 12 000 units/mg of protein and assuming one single active center per unit of molecular weight 110 000 (see [4]) a catalytic constant at 25 °C of 142.1 s^{-1} was calculated. By using the equation

$$\Delta F^{\star} = - R \cdot T \cdot \ln \frac{k \cdot h}{\boldsymbol{k} \cdot T} \tag{I}$$

where R, h, and $\boldsymbol{k}$ are the gas, Plank and Boltzmann constants, respectively, and k the catalytic constant, the free energy change was found to be 14.5 kcal/mole.

From the equation

$$\Delta S^{\star} = \frac{\Delta H^{\star} - \Delta F^{\star}}{T} \tag{II}$$

an activation entropy of -4 ($\pm$ 1.2) cal/mole per degree was calculated.

DISCUSSION

The convergency of the primary double-reciprocal plots demonstrate that the reaction obeys the following initial rate law:

$$v = \frac{V}{1 + (K_m^a/[a]) + (K_m^b/[b]) + (K_s^a \cdot K_m^b/[a] \cdot [b])} \tag{III}$$

According to Cleland rules [12], the product inhibition pattern found for diacetyl reductase from pigeon liver in our experimental conditions (neither of the fixed substrates at saturating concentrations) is only consistent with two of the mechanisms obeying Eqn III: a Theorell–Chance and a rapid equilibrium random Bi–Bi with two dead-end ternary complexes (for details of these mechanisms, see [13]).

By applying the Cleland rules for dead-end inhibition [12] to those of the dia-

cetyl analogues tested, it can be deduced that: (a) The inhibition patterns for α-ketoglutarate and hexanedione lack specificity and are compatible with either of the two mechanisms in discussion assuming that these inhibitors react with more than one enzyme form. (b) The patterns of inhibition with acetone and pentane-3-one show that they can only react with the E–NADH complex whatsoever the mechanism (random Bi–Bi or Theorell–Chance) considered. This discards the random mechanism here discussed unless a higher selectivity for the inhibitors than for the substrate binding were admitted, and leaves as the only possible alternative a Theorell–Chance scheme with NADH as the "leading" substrate. This mechanism has also been reported as the most probable one for the enzyme from beef liver [6].

In *A. aerogenes* the diacetyl reductase reaction could follow a different mechanism, an ordered Bi–Bi [7]. This statement is based on the dependence of the reaction rate on substrate concentration and on the product inhibition pattern, however, since the *A. aerogenes* enzyme catalyzes not only the reduction of diacetyl to acetoin but also that of acetoin to butyleneglycol, the inhibition by acetoin in the diacetyl reductase reaction could not be studied and butyleneglycol was substituted for it to obtain the inhibition pattern (NAD^+: competitive for NADH, none for diacetyl. Butyleneglycol: uncompetitive for NADH, noncompetitive for diacetyl. The reaction rates are measured from the consumption of NADH). In our opinion this pattern proves that the reaction follows a compulsory order with NADH as the leading substrate, but does not discard a Theorell–Chance mechanism.

Since the reduction of acetoin by the *A. aerogenes* enzyme follows an ordered Bi–Bi mechanism with the coenzyme as the first substrate to be added [14], a stable E–NAD^+–butyleneglycol complex must be present in this reaction. It is to be expected that while using butyleneglycol as inhibitor for the diacetyl reduction an alternate pathway will be operating in which kinetically significant amounts of the same complex will also participate. In such a case, the aplication of Cleland's rules [12] predicts that the reported inhibition pattern could be equally consistent with both an ordered Bi–Bi and a Theorell–Chance mechanism.

Affinity for diacetyl and NADH

The value of K_m^{NADH} calculated for pigeon liver diacetyl reductase is very similar to those of the beef liver [6] and *Lactobacillus casei* [5] enzymes, but differs by two orders of magnitude from that reported for the *A. aerogenes* one [7]. The K_m for diacetyl is similar to those reported for the diacetyl reductase from other sources [5, 7] except for beef liver diacetyl reductase which is two orders of magnitude lower [6], and for the enzyme from rat liver which is 10-fold higher [2]. It is not easy to understand the reasons for such a wide variation in substrate and coenzyme affinity. They may partially reflect the need for the enzyme to operate under very different diacetyl and NADH concentrations in the various biological systems but, on the other hand, some of these values have been obtained with preparations of low purity and it must be remembered that diacetyl is accepted, although poorly, as substrate by alcohol dehydrogenase [9].

Thermodynamic data

The activation energy calculated agrees well with that reported for the reaction catalyzed by the enzyme from beef liver [3].

Since the reaction follows a Theorell–Chance mechanism with NADH as the first substrate to add to the enzyme, the rate-limiting step in the forward direction is the release of NAD^+ from the E–NAD^+ complex. The negative value of the activation entropy found, shows that from a thermodynamic point of view this step is reversible and does not forbid the backward reaction. The assumption, that there is one single active center per unit of molecular weight 110 000 which was made to calculate ΔF^*, may not be correct, since it has been shown that other diacetyl reductases are polymers of subunits of mol. wt about 25 000 ([15]; see also [3]). If such were the case here and four active centers were present per unit of mol. wt 110 000, the free energy change would be 15.3 kcal/mole and the entropy change -6.7 ($\pm$ 1.2) cal/mole per degree.

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