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SUBUNIT STRUCTURE AND KINETIC PROPERTIES OF L-β-HYDROXYACID DEHYDROGENASE OF *DROSOPHILA*

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Summary

L-β-hydroxyacid dehydrogenase (L-gulonate:NAD^+ 3-oxidoreductase, EC 1.1.1.45) of *Drosophila* is made up of two non-identical subunits with molecular weights of 40 000 and 23 500. Michaelis constants calculated at saturating concentrations of the other substrate were 0.13 mM for NAD^+, 0.85 mM for L-gulonate, 14.8 mM for L-β-hydroxybutyrate; dissociation constants (K_{ia}) were 2.8 mM for L-gulonate, 22 mM for L-β-hydroxybutyrate. The maximum velocity with L-gulonate as substrate was ten-fold greater than with β-hydroxybutyrate. As product inhibitors, both NADH and acetoacetate are competitive vs. both substrates, suggesting a rapid equilibrium random mechanism.

Introduction

L-β-Hydroxyacid dehydrogenase (L-gulonate:NAD^+ 3-oxidoreductase, EC 1.1.1.45) is an enzyme reported from kidney and liver of a wide range of vertebrates [1] and has been partially purified and characterized from hog kidney [2]. It has also been purified from *Drosophila melanogaster* [3].

In mammals, L-β-hydroxyacid dehydrogenase is believed to participate in the glucuronate-xylulose cycle [4], the apparent primary reaction being the oxidation of L-gulonate to 3-keto-L-gulonate [2], although it uses many other L-β-hydroxyacids as substrates, notably oxidizing L-β-hydroxybutyrate to

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acetoacetate [5]. It has not been established whether the complete glucuronate-xylulose pathway exists in *Drosophila* or other insects, or what role this enzyme may play in insects. In seeking to establish such a role, we report here a kinetic study concerning the mechanism of *Drosophila* L-β-hydroxyacid dehydrogenase, as well as the subunit structure of the enzyme as indicated by SDS-polyacrylamide gel electrophoresis.

Materials and Methods

Sodium DL-β-hydroxybutyrate, lithium acetoacetate, NAD^+ and NADH were products of Sigma Chemical Co. L-1,4-Gulonolactone was purchased from Nutritional Biochemical Co. and converted to sodium L-gulonate by heating with a stoichiometric quantity of NaOH. Urea was purchased from J.T. Baker Co., and Sephadex G-150 and QAE-Sephadex A-50 from Pharmacia Fine Chemicals. Standards for gel electrophoresis (ovalbumin, rabbit γ-globulin) were purchased from Mann. Other chemicals were as previously described [3].

The enzyme was assayed, using DL-β-hydroxybutyrate as substrate, as previously described [3], in a Beckman Acta III double-beam recording spectrophotometer with cuvette chambers and assay solutions maintained at 30° C. Substrate concentrations were varied as described in the text. The buffer was 0.1 M Tris-HCl (pH 8.2) and the reaction was initiated by addition of enzyme.

Drosophila melanogaster of the Daekwanryeong strain was cultured in half-pint milk bottles. Adults were collected, immediately frozen and stored at −20° C for up to one month before use. The enzyme was purified as previously described [3], except that the Sephadex G-150 column was equilibrated with 0.01 M Tris-HCl (pH 8.3), 0.1 mM EDTA, 5 mM NaCl; the subsequent ion-exchange chromatography was carried out on QAE-Sephadex A-50 equilibrated with the same buffer, eluting the enzyme with a 600 ml linear gradient of 5—200 mM NaCl in this buffer. The specific activity was 12—13 μmol/min per mg protein.

Polyacrylamide gel electrophoresis was carried out in the system described by Davis [6], using 7.5% acrylamide gels, except that the spacer gel was omitted, the sample being overlaid on the running gels in 25 mM Tris/glycine buffer (pH 8.6), 5% sucrose. Gels were stained for protein with Coomassie brilliant blue, and for activity with the histochemical stain procedure [3]. SDS-gel electrophoresis was carried out by the method of Weber and Osborn [7].

All kinetic studies were performed with a single enzyme preparation. 12 μg protein was used when DL-β-hydroxybutyrate was the substrate, 2.1 μg protein when L-gulonate was the substrate. Two or three rate determinations were carried out at each combination of substrate conditions. In product inhibition studies, [NAD^+] was 6 mM when [DL-β-hydroxybutyrate] was varied, and [DL-β-hydroxybutyrate] was 0.1 M when [NAD^+] was varied.

The terminology of Cleland [8] is used in the description of kinetic results. Values for K_m and V of individual plots were calculated by the non-linear least-squares method of Wilkinson [9]; slopes and intercepts of replots (of slopes and intercepts of primary plots vs. 1/second substrate or vs. inhibitor) were calculated by least-squares analyses, weighted in inverse proportion to variances of the values from primary plots.

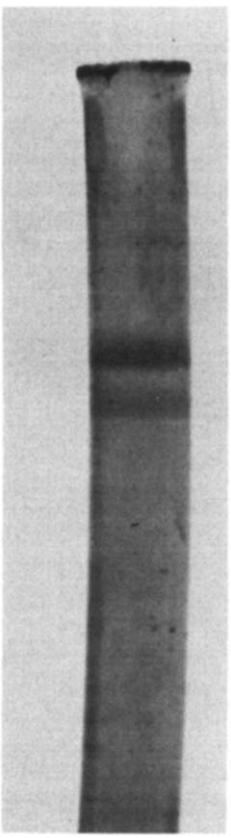
Results

Electrophoresis

Polyacrylamide gel electrophoresis of several preparations of the native enzyme at pH 8.9 reveals a single band, whether stained for protein or for activity [3]. After heating with SDS and 2-mercaptoethanol, SDS-gel electrophoresis of each preparation showed two protein bands, of molecular weight 23 500 and 40 000 (Fig. 1). The molecular weight previously reported [3] for the enzyme is 63 000.

Initial velocity studies

Reciprocal plots of velocity vs. [DL-β-hydroxybutyrate] (5—25 mM) or vs. [L-gulonate] (0.2—1.0 mM) at several NAD^+ (0.125—1 mM) levels show intersecting lines, indicating a ternary complex binding mechanism (i.e a ternary complex of enzyme, coenzyme and substrate is formed prior to release of any product). This is the normal mechanism for pyridine nucleotide-dependent



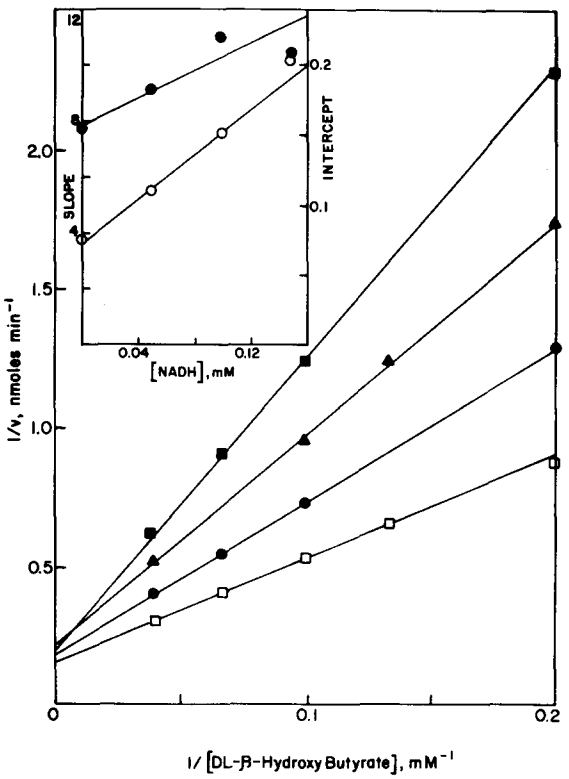


Fig. 1. SDS-polyacrylamide gel electrophoresis of *Drosophila* β-hydroxyacid dehydrogenase.

Fig. 2. Reciprocal plot of velocity vs. [DL-β-hydroxybutyrate] in presence of NADH as inhibitor. [NADH]: □, 0; ●, 0.05 mM; ▲, 0.10 mM; ■, 0.15 mM. Inset: replot of slopes (○) and intercepts (●) vs. [NADH].

dehydrogenases, in contrast to a 'ping-pong' mechanism which would show a series of parallel reciprocal plots. Replots of slopes and intercepts of β-hydroxybutyrate plots vs. $1/[NAD^+]$ indicate K_m for NAD^+ = 0.13 ± 0.013 mM, K_m for DL-β-hydroxybutyrate = 29.6 ± 3.3 mM (i.e. 14.8 mM for L-β-hydroxybutyrate) and K_{ia} for DL-β-hydroxybutyrate = 44.6 ± 7.4 mM. Replots of slopes and intercepts of L-gulonate plots indicate K_m for NAD^+ = 0.085 ± 0.005 mM, K_m for L-gulonate = 0.85 ± 0.17 mM and K_{ia} for L-gulonate = 2.79 ± 0.34 mM. These results are in reasonably good agreement with results previously obtained using only a single concentration of the other substrate [3]: K_m for NAD^+ = 0.25 mM, for DL-β-hydroxybutyrate 45 mM. The overall turnover numbers, correcting for amount of enzyme used, are different for the two substrates (444 min^{-1} with L-gulonate, 44 min^{-1} with DL-β-hydroxybutyrate), indicating that the velocity is not limited solely by the rate of NADH release, as in the Theorell-Chance [10] mechanism.

Activity with $NADP^+$ vs. NAD^+

With 40 mM L-gulonate as substrate, 0.8 mM $NADP^+$ gave activity 10% of that found with 10 mM NAD^+; higher concentrations of either coenzyme did not result in further increase in activity.

Product inhibition

The products NADH and acetoacetate were studied as inhibitors, while varying each of the substrates (NAD^+, DL-β-hydroxybutyrate) individually. Acetoacetate (12.5—80 mM) is unequivocally competitive vs. both substrates; the inhibition vs. NAD^+ is very weak (K_i = 119 mM), as expected at high [β-hydroxybutyrate]. The K_i vs. DL-β-hydroxybutyrate is 23.95 mM. NADH (0.025, 0.05, 0.10 mM) is competitive vs. NAD^+ while showing a very small effect on the intercepts of $1/v$ vs. 1/[DL-β-hydroxybutyrate] plots (Fig. 2). All replots, with the possible exception of that of slopes of $1/v$ vs. $1/[NAD^+]$ plots with acetoacetate as inhibitor, are linear. K_i values for NADH are 0.092 mM vs. β-hydroxybutyrate, 0.195 mM vs. NAD^+ (from replot of slopes).

Protection against urea denaturation

Incubation of the enzyme in 3 M urea (0.1 M NaP_i, pH 7.5) results in 83% loss of activity in 7 min; inclusion of 0.3 M DL-β-hydroxybutyrate, 0.1 M L-gulonate or 3 mM NAD^+ in the solution (separately) reduced the loss of activity to 50%, 54% and 40%, respectively, indicating that L-β-hydroxybutyrate and L-gulonate do bind to the enzyme in absence of coenzyme.

Discussion

It appears that L-β-hydroxyacid dehydrogenase of *Drosophila* contains two unlike subunits. Non-identical subunits are extremely unusual in dehydrogenases, but not unprecedented (e.g. 2-ketogluconate reductase of *Gluconobacter* [11]. NAD^+-dependent isocitrate dehydrogenase from mammalian heart muscle has recently been shown to contain non-identical, though very similar, subunits [12,13].

The kinetic results are not consistent with either of the common mechanisms

of pyridine nucleotide-dependent dehydrogenases: an ordered sequential mechanism, with coenzyme first to bind and last to leave [14], in which NADH should be a competitive inhibitor vs. NAD^+, but the other inhibitions should be non-competitive [8]; and the Theorell-Chance mechanism [10], in which alcohol binding and carbonyl product dissociation are very rapid compared to coenzyme binding and dissociation, and consequently each is a competitive inhibitor vs. the corresponding substrate. Mitochondrial D-β-hydroxybutyrate dehydrogenase has an ordered mechanism, with coenzyme binding first [15]. If the intercept effect with NADH as inhibitor vs. DL-β-hydroxybutyrate is neglected, the competitive inhibitions are consistent with a rapid equilibrium random mechanism, which is rare but not unprecedented in pyridine nucleotide-dependent dehydrogenases (e.g. yeast glucose-6-phosphate dehydrogenase [16], probably glutamate dehydrogenase [17,18]). A significant intercept effect of NADH vs. β-hydroxybutyrate is not consistent with the simplest form of the mechanism, but might be explained by addition 'dead-end' inhibition, i.e. NADH binds to E · β-hydroxybutyrate as well as to free enzyme, so that saturation with β-hydroxybutyrate cannot fully reverse the inhibition. The observation of different maximal velocities with different substrates also favors a rapid equilibrium random mechanism, and the protection against urea denaturation by substrates as well as coenzyme indicates that they can bind in absence of coenzyme.

In a subsequent study we have presented evidence that the activity of hog kidney L-β-hydroxyacid dehydrogenase is allosterically controlled by acetoacetate or a similar compound. As with the *Drosophila* enzyme, acetoacetate is a competitive inhibitor vs. both NAD^+ and L-gulonate. The possibility exists that the competitive inhibition of the *Drosophila* enzyme is similarly effected by acetoacetate binding at an allosteric site.

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