ON THE ISOLATION AND PROPERTIES OF THE D(-)β-HYDROXYBUTYRIC DEHYDROGENASE OF BEEF HEART MITOCHONDRIA

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The D(-)β-hydroxybutyric dehydrogenase, discovered by Green et al. in 1937 and shown to be a pyridinoprotein enzyme, has hitherto resisted all efforts aimed at purification. The root of the difficulty has been the tight association of this enzyme with mitochondria and mitochondrial particles. The application of procedures designed to detach the enzyme from particles invariably has led to loss of activity [cf., e.g., Lehninger et al. (1960)]. The β-hydroxybutyric dehydrogenase is unique among the pyridinoproteins of the mitochondrion in the tenacity with which it remains associated with the particulate electron transport chain. It was, therefore, of considerable theoretical importance to ascertain the basis of this association and to determine what sets apart the β-hydroxybutyric dehydrogenase from the classical pyridinoprotein enzymes

When a suspension of beef heart mitochondria is clarified with cholate (0.4 mg/mg protein) and then fractionated with ammonium sulfate, soluble extracts can be obtained which catalyze the oxidation of D(-)β-hydroxybutyrate by DPN (but not by TPN). In crude extracts, the enzyme is associated with cytochrome-containing complexes which are at the borderline of solubility. To separate the enzyme from these contaminants, a cycle of solubilization at pH 8.5 and then precipitation at pH 5.7 is carried through twice (all other fractions being discarded). A summary of the yields and purity at various stages in the purification is given in Table I. At the highest purity level, the enzyme has a pale yellow color and the specific activity is 135 times greater than that of the starting mitochondrial

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suspension. The overall yield at this purity level is about 8%. Tests for other mitochondrial enzymes such as lipoyl, DPNH and α-ketoglutaric dehydrogenases and for bound flavin were negative when applied to high purity material.

TABLE I

Purification of the D(-)β-hydroxybutyric dehydrogenase of beef heart mitochondria.

Stage		Total Protein (mg)	Specific Activity*	Units**	Recovery %
A	Mitochondria	172,000	0.1	17,200	100
B	Green residue	70,000	0.2	14,000	81
C	Extract of B ppt. with $(NH_4)_2SO_4$ (0-45% sat.)	8,300	1.4	11,600	67
D	C clarified by centrifugation 30,000 rpm for 40 min.	4,900	2.1	10,300	60
E	D ppt. at pH 5.6,*** after discarding ppt. at pH 6.3	2,000	3.5	7,000	41
F	E adjusted to pH 8.5 (discarding insoluble fraction), ppt. with $(NH_4)_2SO_4$ (40% sat.), and dissolved in 0.25 M sucrose-0.01 M Tris, pH 7.7	594	6.8	4,000	24
G	F ppt. at pH 5.7,*** after discarding ppt. at pH 6.7	198	11.6	2,300	13
H	G adjusted to pH 8.0 (discarding insoluble fraction), ppt. with $(NH_4)_2SO_4$ (40% sat.), and dissolved in 0.25 M sucrose-0.01 M Tris, pH 7.7	102	13.5	1,400	8

* Specific Activity: μmoles DPN^+ reduced per min. per mg. protein at 35°.
** Units = Specific Activity X protein (mg.).
*** For assay purposes all ppt. were suspended routinely in 0.25 M sucrose-0.01 M Tris, pH 7.7, and assayed directly.

At all stages subsequent to the extraction of mitochondria with cholate the enzyme shows an absolute requirement for lecithin (cf. Figure 1). Triglycerides, fat soluble vitamins and various coenzymes were all inactive in this regard and no phospholipid yet tested other than lecithin showed any activity. When a suboptimal level of lecithin is used in the assay and in-

creasing amounts of other phospholipids are added, the rate of reaction either remains unchanged or declines. Thus no substitution for lecithin by other phospholipids can be made either in part or whole. Other aspects of the relationship between lecithin and enzyme protein will be considered in the companion communication by Jurtshuk et al. (1961).

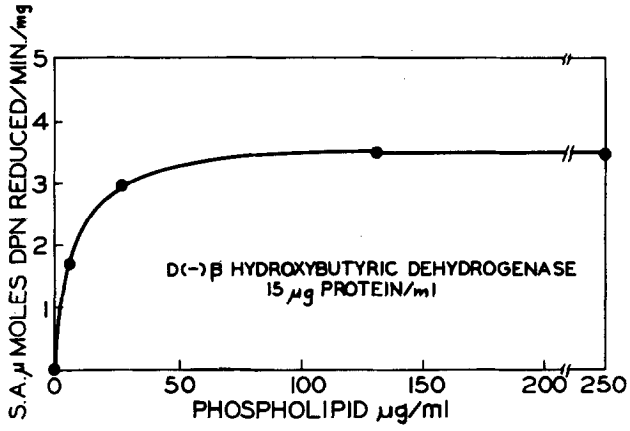

Figure 1 - Activity of the β-hydroxybutyric dehydrogenase system as a function of the concentration of lecithin.

The assay system (1 ml. final volume) contained 50 μmoles of Tris chloride, 0.4 mg. of bovine serum albumin, 50 μmoles of cysteine, 0.5 μmoles of EDTA, 3% ethanol, 4 μmoles of DPN, β-hydroxybutyric dehydrogenase in the amount indicated and variable amounts of lecithin. The above components were incubated for 15 min. at 35° and then the reaction was initiated by addition of 40 μmoles of DL-β-hydroxybutyrate (potassium salt). The change in absorbancy at 340 mμ was measured over a period of 90 sec. - readings being taken each 15 sec. The pH of the reaction mixture and of the Tris chloride was 8.1.

In general, the β-hydroxybutyric enzyme is completely inactive unless some sulfhydryl reducing agent such as cysteine, glutathione or dimercaptopropanol is present in the assay mixture (cf. Figure 2). Whether the requirement is complete or partial depends upon the stage of purity and the length of time of storage of the enzyme. The addition of cysteine to preparations of the enzyme which are being stored is useful for preservation of activity.

When the enzyme is incubated for 15 min. at 38° with the components shown in the legend for Figure 1 and then substrate is introduced to initiate the reaction, a linear relation is observed between the rate of reduction of DPN and time. However, if the enzyme is left out of the incubation mixture (now includ-

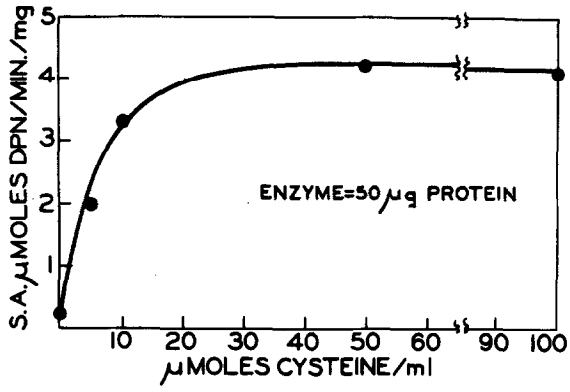

Figure 2 - Activity of the β-hydroxybutyric dehydrogenase system as a function of cysteine concentration.

Assay details as in legend for Figure 1 except that lecithin in the form of mitochondrial lipid (200 μg), and variable amounts of cysteine as indicated were used. The concentration of enzyme used in the assay was 50 μg per ml.

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ing substrate) and the reaction is initiated by addition of enzyme, the relation between rate and time is entirely altered (cf. Figure 3). A considerable lag

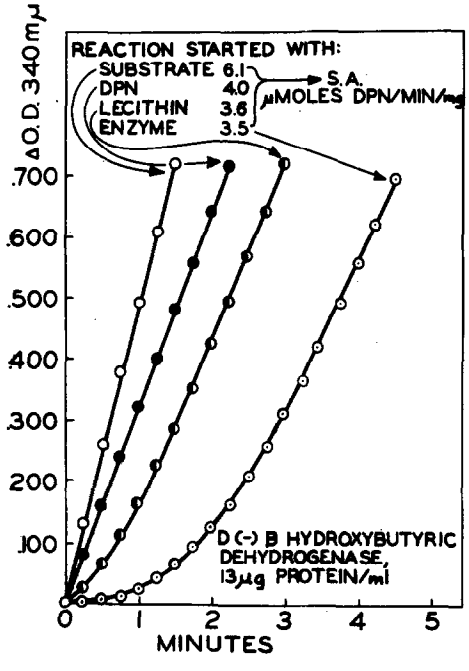

Figure 3 - The kinetic consequences of omitting in turn each of four components from the assay mixture during the preincubation period.

The component omitted from the preincubation mixture was added to initiate the reaction. Details as in legend for Figure 1 except for the order of addition and the nature of the component used for initiating the reaction. Lecithin was added in the form of mitochondrial lipid (200 μg). The values for the specific activities have been calculated from the linear portions of the respective curves

period intervenes and the limiting rate approaches but never equals the rate which obtains when the enzyme is incubated with the other components. From studies of this kind we have come to recognize that the interaction of enzyme protein with both cysteine and lecithin under the conditions of the assay (to some degree this is also true of DPN) is not instantaneous and a definite incubation period is required to convert the enzyme to the fully active form in presence of excess lecithin.

As demonstrated previously by Green et al. (1), the β-hydroxybutyric dehydrogenase system is reversible. The optimum pH for the forward reaction (reduction of DPN^+) is 8.1 and for the back reaction (oxidation of DPNH), 7.0. The lecithin and cysteine requirements also apply to the reduction of acetoacetate by DPNH.

The enzyme at the highest purity level shows only a single sedimenting component but there is insufficient data now available for assessing homogeneity and the molecular weight of the protein.

Both the crude and purified enzyme preparations are stable at -20° for considerable periods providing the enzyme is kept in precipitated form in 40% saturated ammonium sulfate containing 0.01 M cysteine (pH 7.0).

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