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Interaction of D-β-hydroxybutyrate dehydrogenase with lecithin vesicles

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SUMMARY

Rat liver mitochondrial D-β-hydroxybutyrate dehydrogenase has an absolute requirement for lecithin. The nature of the interaction between the enzyme and phospholipid has been investigated. Single bilayer lecithin liposomes of shell-like structure bring about maximal enzyme activation, whereas the interaction with larger vesicles leads to enzyme inactivation. The strong binding of the enzyme to lecithin confers great stability to the enzyme activity as compared with the nonlipid-activated enzyme, and permits the isolation of a lipoprotein complex by chromatography on Sephadex G-200. Only 20% of the proteins solubilized with D-β-hydroxybutyrate dehydrogenase from mitochondrial membranes bind to lecithin liposomes, thus a 5-fold purification of the enzyme is achieved. The liposome-bound proteins had a significantly lower polarity than the remaining 80% of solubilized mitochondrial membrane proteins.

The mitochondrial enzyme D-β-hydroxybutyrate dehydrogenase requires lecithin for enzymic activity[1–3]. Indeed, the strong interaction between the enzyme and lecithin is the basis of a method of purification of beef heart D-β-hydroxybutyrate dehydrogenase.

In the present study we have explored the interaction of the rat liver mitochondrial enzyme with lecithin. We find that single bilayer lecithin liposomes of shell-like structure provide maximal enzymic activation. Our results suggest that the enzyme is bound within the nonpolar region of the lecithin bilayer. A method for preparing a complex of highly purified D-β-hydroxybutyrate dehydrogenase with single bilayer lecithin liposomes is described.

D-β-Hydroxybutyrate dehydrogenase was solubilized and partially purified from rat liver mitochondrial membranes as described by Gotterer[2]. The membrane fragments were extracted twice with pH 10.5 buffer and the solubilized proteins precipitated with 50% $(NH_4)_2SO_4$ and stored at −20 °C. Prior to use, the $(NH_4)_2SO_4$ precipitate was

dissolved in 75 mM Tris–HCl (pH 8.1) and insoluble material was removed by centrifugation. For the expression of maximal enzyme activity, a preincubation in the presence of thiol, NAD and lecithin is required. The standard activation procedure consisted of preincubating the enzyme (10–50 μg of protein) in 0.1–0.2 ml of 60 mM Tris–HCl (pH 8.1), 4 mM NAD, 200 mM β-mercaptoethanol, containing 0.29 μmole lipic phosphorus of egg lecithin liposomes. The enzyme was preincubated at room temperatur (20–23 °C) for 30 min, and then stored in ice until assayed (within 1 h). Enzymic activit was measured spectrophotometrically by following the change in absorbance of NAD at 340 nm with a Gilford 2400-S recording spectrophotometer. The standard assay conditio were 60 mM Tris–HCl (pH 8.1), 1 mM NAD, 11 mM β-hydroxybutyrate in a total volum of 2 ml at 25 °C. Egg lecithin liposomes were used throughout since they are almost as effective as rat liver mitochondrial lecithin vesicles[3]. Lecithin was prepared from fresh eg yolks by the method of Singleton *et al.*[5]. The liposome preparation consisted of a 3% lecithin suspension in 75 mM Tris–HCl (pH 8.1), ultrasonically irradiated for 15 min witl Branson sonifier Model LS-75, and then centrifuged at 100 000 × *g* for 60 min to remove undispersed phospholipid.

Sonicated lecithin dispersions are not homogeneous micellar preparations; they can be fractionated on Sepharose 4B into one fraction of homogeneous vesicles of shell-li structure (Fraction II) and another of higher molecular weight aggregates (Fraction I)[6]. Fig. 1 shows the fractionation of the sonicated phospholipid and the saturation curves of enzyme activation as a function of lecithin concentration for each fraction. Approx. 1 μn

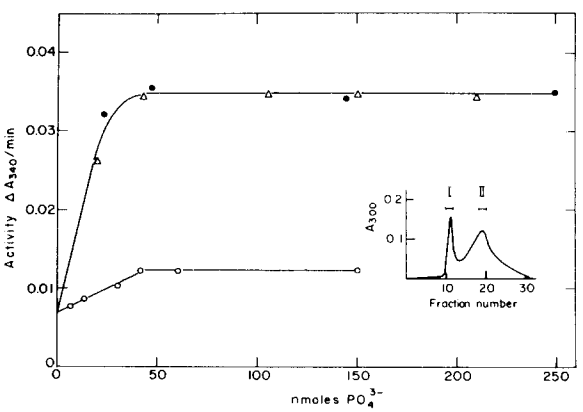

Fig. 1. Enzyme activity as a function of lecithin concentration in preincubation mixture. The standard preincubation procedure was followed, using 30 μg of protein and varying lecithin concentrations as indicated. Unfractionated sonicated lecithin (●——●); Fraction II vesicles (△——△); Fraction I vesicles (see insert) (○——○). Insert shows the fractionation of sonicated lecithin suspension on Sepharose 4B. 1 ml of a 29 mM sonicated and centrifuged suspension of lecithin in 75 mM Tris–HCl (pH 8.1) was applied to a 26 cm × 1.7 cm column at 5 °C, and eluted with the same buffer. Fractions of 1.1 ml wer collected. The indicated peaks were pooled and denoted as Fraction I and Fraction II. Phosphate determinations[7] showed that Fraction I was 0.7 mM and Fraction II 2.6 mM in lipid phosphate.

of egg lecithin phosphorus per mg of protein was required for saturation. The maximal activation obtained with the Fraction I vesicles was 35% of that obtained with vesicles of Fraction II. After incubation with a saturating amount of Fraction I, the enzyme cannot be further activated with Fraction II, although enzyme activated with Fraction II liposomes will exhibit full activity even after the addition of Fraction I liposomes. There appears to be an irreversible inactivation of the enzyme on Fraction I vesicles. Enzyme activation by lecithin is thus dependent on the physical state of the lecithin liposomes. The large multilamellar structure of an unsonicated lecithin dispersion does not activate the dehydrogenase at all. The results indicate that the lecithin is most effective in activating D-β-hydroxybutyrate dehydrogenase when presented to the enzyme as single bilayer spheres and that larger micellar structures appear to lead to surface denaturation.

Since enzyme activity is absolutely dependent upon lecithin activation, the possibility of a strong binding of the dehydrogenase to lecithin liposomes was investigated. The lecithin-activated D-β-hydroxybutyrate dehydrogenase can be isolated as a lipoprotein complex by gel chromatography. When chromatographed on a Sephadex G-200 column, 95% of the activity applied is eluted at the column void volume together with the liposomes (Fig. 2). Chromatography of the enzyme alone on Sephadex G-200, even in the presence of NAD and thiol, leads to the loss of 90% of the activity. The residual activity, however, elutes within the included volume of the column. The lipoprotein complex, once formed and isolated, shows extreme stability. In the presence of 4 mM NAD and 200 mM β-mercaptoethanol, the complex retains 94% of its activity for at least 4 days when stored at 5 °C. After 4 weeks more than 50% of the activity is still retained. Under comparable

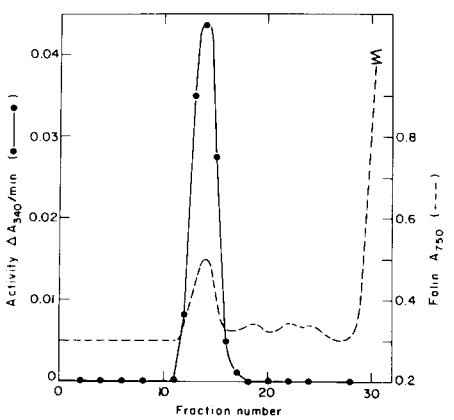

Fig. 2. Isolation of D-β-hydroxybutyrate dehydrogenase–lecithin complex on Sephadex G-200. 6.2 mg of enzyme were preincubated under standard conditions in a total volume of 1.2 ml. It was applied to a 46 cm x 0.9 cm column of Sephadex G-200 and eluted at 5 °C with 60 mM Tris–HCl (pH 8.1), 4 mM NAD. Fractions of 1.1 ml were collected. 0.02 ml of each fraction was added to 0.1 ml of 60 mM Tris–HCl (pH 8.1), 200 mM β-mercaptoethanol, 4 mM NAD, incubated at room temperature for 30 min and assayed. Protein was determined[8] on aliquots of 0.1 ml of each fraction. The large increase in absorbance at 750 nm beginning at Fraction 29 was due primarily to the β-mercaptoethanol in the sample applied to the column.

conditions, but in the absence of lecithin, the solubilized dehydrogenase preparation loses 95% of its activity within 24 h.

Chromatography of a sonicated lecithin dispersion on a Sephadex G-200 column does not separate Fractions I and II liposomes (see Fig. 1). When lecithin-activated dehydrogenase is chromatographed on Sepharose 4B, enzyme activity is associated with both fractions, the activity peaks coinciding with the liposome peaks.

Complex formation is remarkably insensitive to changes in ionic strength. The enzyme is bound to the liposomes and can be isolated as the complex, also in the presence of 0.8 M NaCl. Thiol is absolutely necessary for complex formation, and if it is not present during the enzyme–lecithin incubation, the enzymic activity is lost during the chromatography. Nevertheless, once the complex has been formed in the presence of thiol it is not necessary to have thiol in the elution buffer of the column. Attempts to determine whether NAD is required for formation of active enzyme–lecithin complex failed because the removal of NAD led to the rapid loss of enzymic activity. $1 \cdot 10^{-4}$ M NAD was sufficient for retention of activity and formation of the complex.

The specific interaction between lecithin liposomes and D-β-hydroxybutyrate dehydrogenase permits a significant purification of the solubilized enzyme. Only 20% of the protein originally solubilized from the mitochondrial membrane fragments are bound to the liposomes. The remaining 80% elute later in the chromatography. This 5-fold increase in specific activity of the enzyme is indicated in Table I. Fig. 3 compares the results of analytical gel electrophoreses of the originally solubilized enzyme preparation and that of the lecithin–enzyme complex isolated from a Sephadex G-200 column. Although the bound protein is not homogeneous, it is clear that many of the contaminating proteins have been removed. The molecular weight of the major band was determined to be 35 000 using standards of chymotrypsinogen (25 000) and ovalbumin (45 000).

Marked differences in the amino acid compositions of the solubilized enzyme preparation and the liposome-bound protein have been observed. Table II shows the ratios of corresponding amino acids in the two preparations. The ratios differ significantly only in the cases of histidine, glycine, valine, tyrosine and phenylalanine. These differences are consistently in the direction of the lipid-bound protein having a lower polarity than the

TABLE I

STAGES IN THE PURIFICATION OF D-β-HYDROXYBUTYRATE DEHYDROGENASE FROM RAT LIVER MITOCHONDRIA

Dehydrogenase activity was extracted from mitochondrial membrane fragments with pH 10.5 buffer by the method of Gotterer[2]. The enzyme–lecithin complex was then formed and isolated on a Sephadex G-200 column (see Fig. 2).

	Spec. act. (μmoles/min per mg protein)
Sonicated mitochondria	0.1
pH 10.5 extract	2.1
Enzyme–lecithin complex	10.5

originally solubilized enzyme. Indeed, calculations of polarities using the mole fractions of polar amino acids as proposed by Capaldi and Vanderkooi[9] show that the lecithin-bound protein has a "polarity index" of 41% and the originally solubilized enzyme preparation an index of 48%.

D-β-Hydroxybutyrate dehydrogenase–lecithin complex formation appears to be rather specific. Although all the protein present in the solubilized preparation is presumably of membrane origin, since it is not released on sonication of the membrane, only 20% shows any affinity for lecithin liposomes. No interaction of mitochondrial malate dehydrogenase, α-chymotrypsin, subtilisin or alkaline phosphatase with lecithin liposomes could be demonstrated. The enzymes were readily separated from the liposomes chromatographically. Negatively and positively charged liposomes, consisting of mixed micelles of lecithin–cholesterol–dicetyl phosphate (7:2:1, v/v/v) and of lecithin–cholesterol–stearylamine (7:2:1, v/v/v), were found to be effective activators of D-β-hydroxybutyrate dehydrogenase. The intensitivity of enzyme activation to the net surface charge of the micelle, as well as the ionic strength of the preincubation medium, excludes an electrostatic interaction as a major determinant in the enzyme liposome association.

The interaction appears to be predominantly a hydrophobic one. The "polarity index" of D-β-hydroxybutyrate dehydrogenase is considerably lower than the rest of the

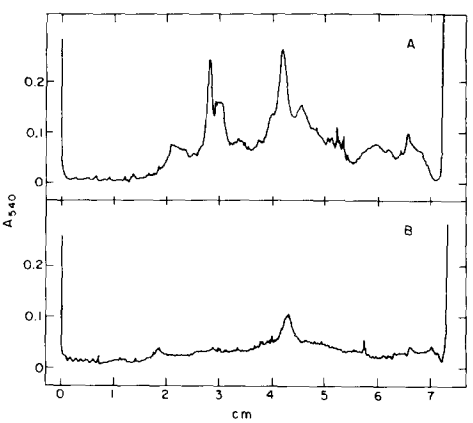

Fig. 3. Analytical sodium dodecyl sulfate gel electrophoresis of β-D-hydroxybutyrate dehydrogenase. Electrophoresis was carried out in gels of 7.5% acrylamide and 0.2% *N,N'*-methylenediacrylamide polymerized with ammonium persulfate (0.05%). 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate was used throughout. Samples were dialyzed against 0.01 M sodium phosphate (pH 7.0), 0.14 M β-mercaptoethanol, 0.1% sodium dodecyl sulfate. Aliquots of 0.1 ml, containing 5–50 μg of protein and 10% in glycerol, were applied to the gels. Gels were run at 4 mA/tube for 6–8 h and then removed and stained with 0.25% Coomassie brilliant blue in 50% methanol, 5% acetic acid for 2 h at 45 °C. They were destained by washing with the same solvent at 45 °C, and then with 5% methanol, 7.5% acetic acid in the presence of a piece of knitting wool. The destained gels were scanned at 540 nm using a Gilford 2410-S linear transport. (A) 25 mg lecithin-activated enzyme before fractionation. (B) 5 mg lecithin–enzyme complex isolated from Sephadex G-200 column as in Fig. 2. The enzymatic activities in the two samples were equal.

TABLE II

AMINO ACID RATIOS OF THE ENZYME–LECITHIN COMPLEX TO THE pH 10.5 SOLUBILIZED ENZYME PREPARATION

Amino acid	*Ratio*
Lysine	0.91
Histidine	0.40
Arginine	0.95
Aspartate	1.03
Threonine	1.00
Serine	1.08
Glutamate	1.08
Proline	0.85
Glycine	1.82
Alanine	1.18
Valine	1.82
Methionine	0.77
Isoleucine	1.01
Leucine	1.80
Tyrosine	1.86
Phenylalanine	1.72

proteins that had been solubilized together with the enzyme at high pH. This low polarity is consistent with the implication that in a hydrophobic interaction the polypeptide chain penetrates into the nonpolar regions of the bilayer. Further indication of this penetration was provided by the observation that mixed lecithin–cholesterol micelles reduce the activation by lecithin[3]. Physical studies on lecithin and lecithin–cholesterol vesicles have shown that cholesterol remarkably reduces the fluidity of the nonpolar region of the vesicles[10,11]. The reduced effectiveness of mixed micelles might thus be attributed to interference with the deep penetration of the enzyme into the vesicles.

Structural studies of the enzyme–liposome complex aimed at determining the actual relationship of the protein to the bilayer may contribute to the understanding of the organization of phospholipids and proteins within biological membranes.

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