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A rapid and efficient procedure for the purification of mitochondrial β-hydroxybutyrate dehydrogenase

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A new, rapid and efficient procedure for the purification of the mitochondrial enzyme β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) to homogeneity is described. It involves the following steps. The mitochondria are solubilized with potassium cholate and the 100 000 × *g* supernate is fractionated with ammonium sulfate. This is followed by precipitation of the enzyme at pH 5.2 and then selective solubilization at pH 8.8. This key step removes eighty percent of the contaminating proteins and allows subsequent DEAE-Sepharose and glass bead column chromatography to be performed in the absence of detergents. The overall yield is consistently around 35% and the purified protein is homogeneous on polyacrylamide gel electrophoresis. The purified enzyme is absolutely dependent upon phosphatidylcholine for activity.

Introduction

A wide variety of important biological processes are membrane-associated. The specificity and dynamics of these processes are governed primarily by the proteins in the membranes whose activity is often modulated by the phospholipids present in the membrane. While many enzymes merely require a hydrophobic environment in which to attain their correct conformation, several enzymes have been shown to have a preferential or absolute specificity for the polar headgroup of phospholipids [1]. One mitochondrial enzyme, β-hydroxybutyrate dehydrogenase, discovered and recognized early as a phospholipid-dependent enzyme [2], has been unequivocally shown to possess an absolute requirement for the polar choline headgroup of phosphatidylcholine for activity [3]. Because of its stringent specificity, this enzyme has been an ideal candidate for studies of protein-phospholipid interactions. Previous studies of this specificity have been concerned with the effects of structural changes in phosphatidylcholine on enzyme activity [4–6]. However, it still remains unknown how phosphatidylcholine interacts with the protein and brings about a stimulation of activity.

Recently new approaches using photoactivable groups have been introduced for the study of phospholipid-protein interactions [7]. These involve the use of photoactivable groups as integral parts of the fatty acyl chains in phospholipids. Photolysis of the proteins reconstituted with such phospholipids leads to reactive intermediates such as carbenes that result in crosslinking between specific sites on the proteins and the phospholipids. Amino acid sequence analysis of the sites of crosslinking in the proteins have provided structural information concerning the domains undergoing interactions with the lipids [8,9].

In an accompanying paper, we are reporting on a method complementary to that described above

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for the study of interactions between the membrane enzymes and their polar headgroups [10]. During application of this approach to the mitochondrial β-hydroxybutyrate dehydrogenase, relatively large amounts of the pure enzymes were required. While procedures have previously been described for the purification of the enzyme [11,12], we did not find them amenable to the facile purification of sufficient quantities. We have therefore reinvestigated the procedures for the purification and now report a simplified and efficient procedure. A brief report of this procedure has been made previously [13].

Experimental Procedures

Materials. DL-β-Hydroxybutyrate, NAD, NADH, dithiothreitol, and cholic acid were obtained from Sigma. The cholic acid was recrystalized from aqueous acetone. The reagents for electrophoresis were obtained from the indicated commercial sources [14]. DEAE-Sepharose and Sepharose 4B were obtained from Pharmacia Fine Chemicals. Synthetic phospholipids were obtained from Avanti Polar Lipids, Birmingham, AL. Soybean lecithin (phosphatidylcholine) was obtained from Associated Concentrates, Woodside, Long Island, New York, and partially purified as described [15]. Enzyme grade ammonium sulfate and sucrose were obtained from Schwarz, Mann. All other chemicals were of reagent grade. Controlled Pore Glass Beads 10-240 were obtained from Electro-Nucleonics, Fairfield, NJ. Bovine serum albumin was obtained from Pentex; bovine trypsin inhibitor, DNAase and lysozyme were from Worthington.

General methods. Mitochondria from bovine heart were prepared as described [16]. We found the mitochondrial preparations to be primarily composed of the light (leaky mitochondria) fraction as opposed to the dark (intact mitochondria) fraction. The β-hydroxybutyrate dehydrogenase activity was assayed essentially as described in Ref. 12. Phospholipid vesicles were prepared by drying a phospholipid solution in chloroform with a stream of nitrogen in a test tube, twice dissolving the lipid in ether and drying under nitrogen, and then placing the dried lipid film under a high vacuum for at least two hours. The vesicles were formed by sonication in a bath sonicator, (Laboratory Supplies Company, Hicksville, NY), at a concentration of 4 mg/ml in 50 mM Tris-HCl (pH 7.8)/0.1 M KCl, until the solution was optically clear.

Protein concentration was determined by the method of Lowry et al. [18]. When the samples contained DTT, the modified procedure of Ross and Schatz [19] or the method of Bradford [20] was used. Phosphorus was determined by the method of Ames [21]. Amino acid analyses were carried out on the purified apoprotein, after extensive dialysis and lyophylization, by hydrolysis in constant boiling 6 M HCl at 110°C for 24 h. Analyses were performed on a Beckman 120-C amino acid analyzer with an automatic integrator.

Polyacrylamide gel electrophoresis was performed as reported [14]. Glass beads for column chromatography were washed as reported [12]. All solutions were prepared with distilled deionized water. The pH of all buffers was adjusted at 4°C. All operations were carried out at 4°C unless otherwise noted.

Results

Purification procedure

Solubilization. Eight grams of mitochondria (as determined by Lowry assay) were suspended in 360 ml of 50 mM Tris-HCl (pH 8.0)/0.1 M KCl/0.25 M sucrose/5 mM dithiothreitol (Buffer 1, pH 8.0) and then solubilized with the slow addition of 40 ml of 20% potassium cholate (w/v) (pH 8.0). The optimal conditions for the solubilization of the enzyme were determined as follows. When mitochondria were resuspended in Buffer 1 (pH 8.0) at a final concentration of 60 mg/ml, with various amounts of potassium cholate, the amount of enzymatic activity solublized is shown in Fig. 1A. The activity is termed solubilized if it is in the supernate after centrifugation for 1 h at $100\,000 \times g$. The cholate concentration which resulted in the highest amount of solubilized activity was 20 mg/ml. At higher cholate concentrations, irreversible denaturation of the enzyme was observed. When the cholate concentration was maintained at 20 mg/ml, and the protein concentration varied, the result was quite different and is shown in Fig. 1B. The highest amount of enzymatic activ-

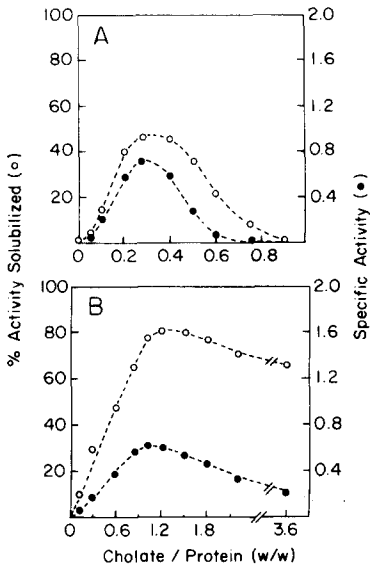

Fig. 1. (A) Solubilization of β-hydroxybutyrate dehydrogenase activity (O- - - - - -O) with increasing concentrations of potassium cholate. To centrifuge tubes containing mitochondrial protein, the appropriate amount of 20% potassium cholate was added to bring the final concentration of protein in each tube to 60 mg/ml and increasing ratios of cholate to protein. The protein solutions were mixed, allowed to stand on ice for 15 min, and then centrifuged for 1 h at 100000 × *g*. The supernatant solutions were then assayed as stated in Methods. For the determination of the specific activity (●- - - - -●), the protein concentration was determined by the method of Lowry et al. (B) Solubilization of β-hydroxybutyrate dehydrogenase activity (O- - - - - -O) with a fixed concentration of potassium cholate and varying concentrations of mitochondrial protein. To centrifuge tubes containing various amounts of mitochondrial protein, the same amount of 20% potassium cholate was added to bring the final concentration of cholate in each tube to 20 mg/ml and increasing ratios of cholate to protein. The protein solutions were mixed, allowed to stand on ice for 15 min, and then centrifuged for 1 h at 100000 × *g*. The supernatant solutions were then assayed as stated in Methods. For determination of the specific activity (●- - - - -●), the protein concentration was determined by the method of Lowry et al.

ity solubilized was over 80% of the total activity present in the mitochondria. The optimum protein concentration was 20 mg/ml.

Other detergents were tested for their ability to solubilize β-hydroxybutyrate dehydrogenase activity from mitochondria. These included sodium deoxycholate, Triton X-100, and octyl glucoside. However, none of these detergents led to improved solubilization of the enzyme. The cholate-solubilized fraction (100000 × *g* for 60 min at 4°C) described above is designated Fraction I.

Ammonium sulfate fractionation. Fraction I (340 ml) was immediately adjusted to 50% ammonium sulfate by adding 340 ml of a saturated solution of ammonium sulfate (pH 7.0). The rate of addition was approx. 20 ml/min and the solution was stirred constantly. The solution was allowed to stand for at least two hours at 4°C to insure the complete precipitation of the β-hydroxybutyrate dehydrogenase activity. The ammonium sulfate precipitate was sedimented by centrifugation at 8000 × *g* for 20 min. The reddish supernate was discarded and the pellet dissolved in 150 ml of Buffer 1 (pH 7.5) at approx. 10 mg/ml. The enzyme can be stored at this stage at −20° for several months with little or no loss in activity. The solution is slightly cloudy due to some insoluble material, and is clarified by centrifugation at 15000 × *g* for 30 min resulting in a clear yellowish-brown solution (Fraction II).

Acid precipitation and selective solubilization at alkaline pH. The pH of Fraction II was lowered to pH 5.2 by the slow and careful addition of 1 M potassium acetate (pH 5.2). The cloudy solution was centrifuged at 10000 × *g* for 30 min and the supernate discarded. A titration curve for the activity remaining in the supernate as the pH is lowered is shown in Fig. 2A. The precipitate was resuspended in 50 ml of 50 mM potassium phosphate (pH 7.0)/5 mM dithiothreitol. The precipitate does not dissolve in this buffer and the solution is very cloudy. The pH of the solution was raised to pH 8.8 by the slow addition of 1 M Tris-HCl (pH 9.6) and then centrifuged at 10000 × *g* for 30 min. This resulted in 55 ml of a clear, lightly colored solution and is labeled Fraction III. A curve showing the amount of β-hydroxybutyrate dehydrogenase activity solubilized as a function of pH is shown in Fig. 2B. The acidification and the alkalinization steps routinely gave a 85–100% recovery of activity.

DEAE-Sepharose chromatography. The material of Fraction III was applied to a column 3.5 × 22 cm containing DEAE-Sepharose which had been equilibrated with 50 mM Tris-HCl (pH 8.8)/5 mM dithiothreitol/10 mM KCl. The column was washed with 100 ml of equilibration buffer and then a gradient from 10 to 600 mM KCl in 50 mM

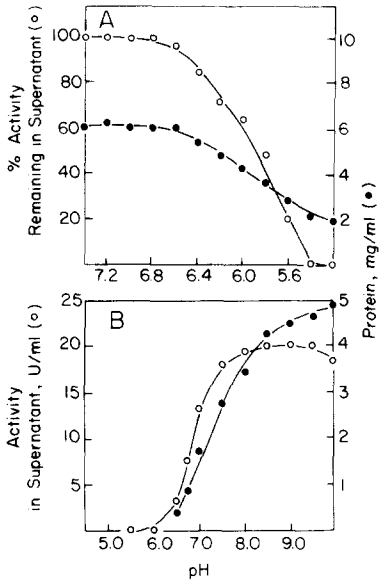

Fig. 2. (A) Precipitation of β-hydroxybutyrate dehydrogenase activity as a function of the buffer pH. To a flask containing a solution of solubilized protein (Fraction II) was added 1 M potassium acetate (pH 5.2). Aliquots of the protein solution were taken at various values of pH, and then subjected to centrifugation for 10 min at 10000 × *g*. The activity remaining in the supernate was then determined as before. At all times the solution was maintained at 4°C. (B) Resolubilization of β-hydroxybutyrate dehydrogenase activity as a function of the buffer pH. A solution of protein (Fraction II) was adjusted to pH 5.2, and then centrifuged at 10000 × *g* for 10 min. The supernate was discarded and the pellet resuspended in 50 mM potassium phosphate (pH 7.0)/5 mM dithiothreitol. To this suspension was added 1 M Tris-HCl (pH 9.6). Aliquots of the protein solution were taken at various values of pH, and then subjected to centrifugation at 10000 × *g* for 30 min. The activity remaining in the supernate after centrifugation was then determined as before.

Tris-HCl (pH 8.8)/5 mM dithiothreitol (total volume of the gradient, 1000 ml) was started. The activity eluted between 0.1 M and 0.15 M KCl, and the elution profile is shown in Fig. 3. The fractions containing β-hydroxybutyrate dehydrogenase activity were pooled (108 ml; Fraction IV).

Fractionation on glass beads. The pH of the pooled Fraction IV was lowered to pH 7.5 with 1 M potassium acetate (pH 5.2) and loaded onto a 15 ml glass beads column. The column was washed with 30 ml of 50 mM potassium phosphate (pH 6.5)/5 mM dithiothreitol, 30 ml of 1.0 M potassium phosphate (pH 8.15)/5 mM dithiothreitol, and 45 ml of 50 mM Tris-HCl (pH 7.9)/5 mM dithiothreitol. A 100 ml gradient of 0–1 M LiBr in 100 mM Tris-HCl (pH 8.1)/5 mM dithiothreitol was then used to elute β-hydroxybutyrate dehydrogenase activity. The activity eluted at approx. 0.6 M LiBr. The activity was pooled and dialyzed twice against 200 ml of 50 mM Tris-HCl (pH 7.8)/0.1 M KCl/5 mM dithiothreitol/50% glycerol. The activity can be stored at −20°C in this buffer for several months with little loss of activity. For long term storage, the enzyme solution was stored at −80°C.

The recovery of β-hydroxybutyrate dehydrogenase at various stages of its purification is shown in Table I. The purity of β-hydroxybutyrate dehydrogenase at various stages of the purification as monitored by SDS-polyacrylamide gel electrophoresis is shown in Fig. 4. The molecular weight of β-hydroxybutyrate dehydrogenase was estimated to be 31500. This was calculated plotting the mobility of the purified protein relative to the

TABLE I

RECOVERY OF ACTIVITY IN THE PURIFICATION OF β-HYDROXYBUTYRATE DEHYDROGENASE

	Total vol. (ml)	Protein concn. (mg/ml)	Total protein (mg)	Activity		Yield (%)	Purification (fold)
				Total units	Spec. act.		
Mitochondria	360	22.6	8150	2000	0.25	100	1
Fraction I	340	6.8	2312	1418	0.61	71	2.4
Fraction II	150	9.3	1390	1403	1.0	70	4
Fraction III	55	4.6	253	1280	5.1	64	20
Fraction IV	108	0.24	26	1115	43	56	172
Fraction V	8.5	0.92	7.8	782	100	39	400

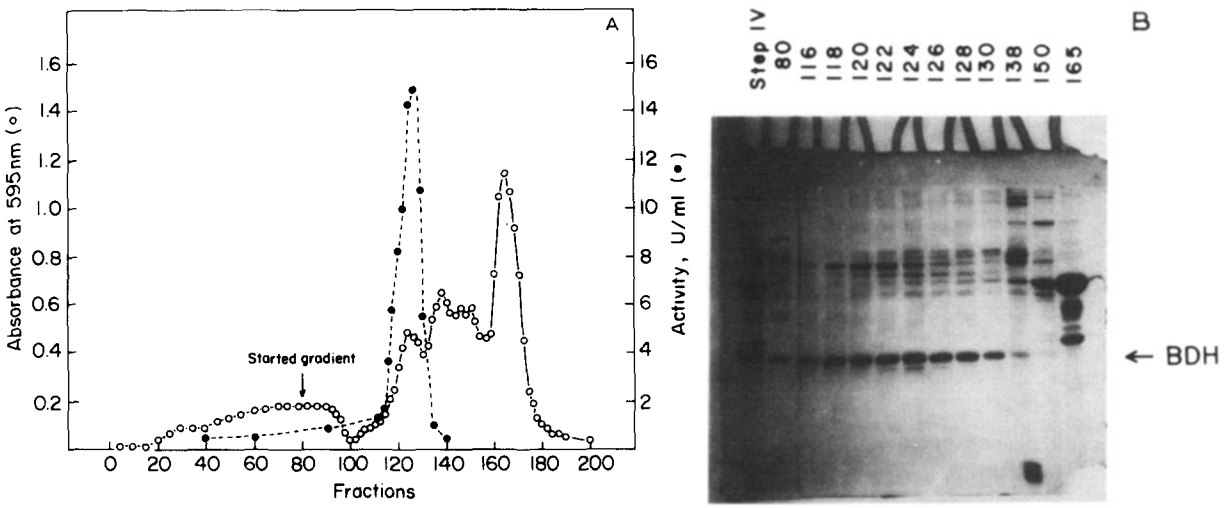

Fig. 3. (A) Elution profile of a solution of Fraction III after chromatography over a column of DEAE-Sepharose. The column dimensions were 3.5×22 cm, and fractions of 7.5 ml were collected. A 1000 ml gradient of 50–600 mM KCl was used to elute the protein from the column. The protein concentrations were determined by the protein-staining method of Bradford. (B) Different fractions from the column as analyzed by SDS-polyacrylamide gel electrophoresis.

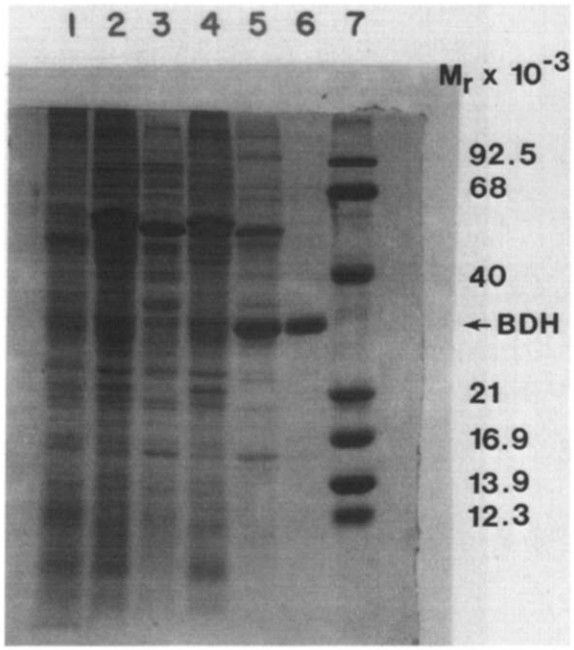

Fig. 4. A 12% SDS-polyacrylamide gel of protein samples taken from each step in the purification. Lane 1 = total mitochondria, Lane 2 = Fraction II, Lane 3 = Fraction III, Lane 4 = acid-base pellet, Lane 5 = Fraction IV, Lane 6 = Fraction V, Lane 7 = protein standards: phosphorylase *a* (92500), bovine serum albumin (68000), alcohol dehydrogenase (40000), trypsin inhibitor (21000), myoglobin (16900), lysozyme (13900), cytochrome *c* (12300).

protein standards as shown on the SDS-polyacrylamide gel electrophoresis of Fig. 4. (Semi-log plot not shown.)

Discussion

Purification of integral membrane enzymes usually presents greater difficulties than that of soluble enzymes. The main reason is the strong affinity of the phospholipids for the proteins and the tendency of the enzymes to aggregate and/or denature once the lipids have been removed. The two procedures described previously for the purification of β-hydroxybutyrate dehydrogenase have involved the use of phospholipase A_2 [12] or potassium cholate [11] for the solubilization of the enzyme from mitochondria. However, in both procedures, the solubilized activity represented only 25–35% of the starting activity. It has been possible in the present procedure to solubilize at least 70% of the original activity by determining the optimal conditions for potassium cholate solubilization.

In the purification method of Menzel and Hammes [11], the enzyme was purified to homogeneity by formation of an enzyme-phospholipid complex and subsequent column chromatography over Agarose Bio-Gel A-1.5 m. Since the authors

were unable to delipidate the enzyme without loss of activity, the procedure is of little utility for the study of phospholipid-protein interaction under well-defined conditions. It has the additional drawback of not being amenable to large scale purification of the protein. In the method of Bock et al. [12], the enzyme is purified to homogeneity by adsorption and elution from controlled pore glass beads. While the major drawback of this procedure is its low final yield of only 5–6%, the method is extremely laborious, requiring seven buffer changes and almost constant attention for 20–30 h.

The present procedure is fast, efficient and amenable to large scale purification. The enzyme is also isolated in a lipid-free and detergent-free state, allowing subsequent studies involving protein-phospholipid interaction.

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