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AFFINITY CHROMATOGRAPHY OF β-HYDROXYBUTYRATE DEHYDROGENASE ON NAD AND HYDROPHOBIC CHAIN DERIVATIVES OF SEPHAROSE

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SUMMARY

Partially purified beef heart apo-β-hydroxybutyrate dehydrogenase, which requires phospholipids for catalytic activity, binds to NAD covalently linked to Sepharose through 6-aminocaproic acid. The apodehydrogenase is denatured by free carboxyl groups covalently linked to Sepharose so that it is not possible to ascertain if the apoenzyme binds specifically to affinity columns made from Sepharose derivatized with β-hydroxybutyrate analogs. The apoenzyme, because of its hydrophobicity, is retarded on Sepharose columns to which various aminoacylaminoalkanes have been covalently attached. Affinity chromatography of the apodehydrogenase on NAD–Sepharose and a combination of NAD–Sepharose and hydrophobic chain–Sepharose have been utilized to purify the enzyme.

INTRODUCTION

The enzyme D(−)-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) from mammalian mitochondria is tightly bound to the membrane [1–3], but can be released from the membrane either by digestion of the lipids with phospholipase [4, 5] or with detergents such as cholic acid [6–9]. The lipid-free protein prepared by either of the two procedures is enzymatically inactive. However, it can be reconstituted with mitochondrial lipids, a variety of lecithins or simpler compounds such as stearylphosphorylcholine to form an active enzyme–lipid complex [6, 10] (Glover, A. K., Slotboon, A. J., De Haas, G. H. and Hammes, G. G., unpublished). Although the lipid–protein complex has been purified to homogeneity [9] only a denatured form of the apoenzyme has been obtained as a homogeneous protein. Thus it has not been possible to study the properties of the apoenzyme using classical protein chemistry methods.

In this communication the properties of the apo-β-hydroxybutyrate dehydrogenase have been studied using affinity chromatography. Interactions of the apodehydrogenase with substrates and with long chain aliphatic groups coupled to a solid matrix have been investigated. The results obtained indicate the partially purified apoenzyme binds specifically to immobilized NAD and interacts strongly with long aliphatic chains. Affinity chromatography has also been used for purification of the protein.

EXPERIMENTAL METHODS

Materials

Glycine, threonine, glycylthreonine, NAD, NADH, DL-β-hydroxybutyrate sodium salt, sodium pyruvate, dithiothreitol, Sepharose 4B, cholic acid, BRIJ-35, Triton X-100, bovine serum albumin and rabbit muscle lactate dehydrogenase were purchased from Sigma. The (carbonyl-^{14}C) NAD (59 Ci/mole) was obtained from Radiochemical Centre, Amersham. CNBr, dicyclohexylcarbodiimide, 6-aminocaproic acid, dodecylamine and octadecylamine were obtained from Aldrich Chemical Co. and *N*-hydroxysuccinimide from Pierce Chemical Co. The Bio-Gel A 1.5 M and sodium dodecylsulfate were purchased from Bio-Rad. Soybean lecithin was a gift from Dr Betzing of Natterman and Co., and beef heart mitochondria were a gift from Dr E. Racker. Cholic acid was recrystallized twice from ethanol before use. All other chemicals were reagent grade. Distilled deionized water was used for preparation of all solutions.

Preparation and assay of enzymes

Lactate dehydrogenase was dialyzed against 0.1 M sodium phosphate buffer (pH 7.5) before use, and lactate dehydrogenase assays were performed at 25 °C by the method of Kornberg [11]. The apo-β-hydroxybutyrate dehydrogenase and β-hydroxybutyrate dehydrogenase–lecithin complex were prepared as previously described [9]. Assays for the apodehydrogenase and the lecithin complex were performed at 37 °C as described by Sekuzu et al. [6]. A unit of β-hydroxybutyrate dehydrogenase or lactate dehydrogenase was defined as the amount of enzyme required to catalyze the conversion of 1 μmole of the nucleotide substrate into product per min at the assay temperature.

Protein concentrations were determined by the method of Lowry et al. [16] using bovine serum albumin as a standard. The protein samples were prepared by precipitation in 5 % trichloroacetic acid whenever compounds interfering with the colorimetric assay were present in the samples.

Sodium dodecylsulphate–gel electrophoresis was carried out as described by Weber and Osborn [12].

Synthesis of NAD–Sepharose

The NAD–resins were prepared by first linking a 6-aminocaproic acid "arm" to Sepharose 4B using CNBr activation [13] and coupling NAD to the "arm". For preparation of Sepharosyl-6-aminocaproic acid, 10 ml of the settled gel were activated with 1 g of CNBr by maintaining the pH at 11 for 6 min, washing in 200 ml of ice-cold 0.1 M $NaHCO_3$, and then placing the product in 50 ml of 0.1 M 6-aminocaproic acid solution in 0.1 M sodium carbonate (pH 9.5) for 16 h at 4 °C. The resultant gel was washed successively with 500 ml of water, 0.1 M $NaHCO_3$, 0.01 M HCl, 0.5 M NaCl, and then thoroughly with water. The coupling of NAD to the carboxyl groups of the resin using dicyclohexylcarbodiimide as a coupling agent was carried out by two different procedures. The first procedure (I) was as described by Larsson and Mosbach [14]. In this procedure the gel was washed in pyridine and suspended in 12 ml of 80 % pyridine–water (v/v) along with 80 mg NAD, 4 g of the carbodimide and 10 ml of the derivatized gel. The reaction mixture was shaken in a brown

bottle for 10 days and then filtered. The gel was washed successively with water, ethanol, *n*-butanol, ethanol, water and pyridine, and resuspended in the first filtrate along with an additional 1 g of dicyclohexylcarbodiimide. After 7 days of shaking, the gel was again filtered and washed thoroughly in water, ethanol, *n*-butanol, ethanol, water, 0.1 M $NaHCO_3$, 0.01 M HCl, 0.5 M NaCl and water. The NAD–Sepharose prepared by this procedure contained 3–4 μmoles of NAD/ml of the settled gel as determined from [^{14}C]NAD incorporation. The second procedure (II) was identical to the first except for the proportions of various reactants. The reaction mixture contained 10 ml of Sepharose derivatized with 6-aminocaproic acid, 670 mg NAD and 5 g of the carbodiimide reagent in 20 ml of the pyridine–water solvent. After 10 days the reaction mixture was washed and the reaction carried out as described in the first procedure, using another 5 g of the coupling reagent. After another 8–10 days the gel was washed with various solvents as previously described. The incorporation of ^{14}C label indicated that 10–12 μmoles of NAD were bound per ml of the settled resin.

Preparation of Sepharose derivatives of threonine

Affinity gels were also prepared from threonine, an analogue of the substrate β-hydroxybutyrate. The glycylthreonine derivative of the gel was prepared in a manner analogous to the method described above for preparation of Sepharosyl-6-aminocaproic acid. For synthesis of Sepharosyl-6-aminocaproylthreonine, the carboxyl groups of the 6-aminocaproic acid–Sepharose derivative were converted into *N*-hydroxysuccinimide active ester groups [15]. The Sepharosyl-6-aminocaproic acid was washed in dioxane, suspended in the same solvent, and *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were added to concentrations of 0.5 M; after shaking for 5 h at room temperature, the gel was washed with dioxane, dimethylformamide, methanol, and again with dioxane. The gel was then dried to a cake, placed in 50 ml of 0.75 M solution of DL-threonine (pH 6.3 ± 0.1) and shaken for 18–20 h. The gel was then washed successively with water, methanol, *n*-butanol, methanol, water, 0.1 M $NaHCO_3$, 0.01 M HCl, 1 M NaCl, and again thoroughly in water.

Synthesis of hydrophobic derivatives of Sepharose

Glycine and 6-aminocaproic acid derivatives of Sepharose were prepared by the CNBr activation procedure described above. Sepharosylglycylaminomethane was synthesized from 10 ml of the glycine–Sepharose derivative using 10 ml of a 40 % aqueous solution of methylamine and 5 g of dicyclohexylcarbodiimide in 100 ml of dimethylformamide. The reaction mixture was shaken for 48 h and the gel then was washed sequentially in dimethylformamide, water, ethanol, *n*-butanol, ethanol, water, 0.1 M $NaHCO_3$, 0.01 M HCl, 0.5 M NaCl and again in water. Glycylaminobutane and glycylaminoheptane derivatives of the gel were prepared by the same procedure using 10 ml of each amine in the reaction mixture. For synthesis of the 6-aminocaproylaminododecane derivative, 10 ml of the 6-aminocaproic acid derivative of Sepharose, 15 mmoles of *n*-dodecylamine and 6.25 g of dicyclohexylcarbodiimide were suspended in 31 ml of pyridine. The reaction mixture was shaken for 24 h. After filtration, the gel was washed serially in water, ethanol, *n*-butanol, ethanol and pyridine. The washed gel and the first filtrate were mixed with another 5 g of the carbodiimide reagent, shaken for 24 h, and then washed as described above for preparation

of other similar derivatives. An identical procedure was used for synthesis of the *n*-octadecylamine derivative.

Packing and equilibration of columns

The affinity gels were washed thoroughly in water, packed into columns (0.5 or 1 cm diameter) and equilibrated with the appropriate buffer. The used gels were regenerated by washing in 2 M urea. All column chromatography was carried out at 4 °C.

RESULTS

NAD-affinity chromatography

The biological activity of the NAD-bound to Sepharose was tested by affinity chromatography of rabbit muscle lactate dehydrogenase. The enzyme when applied on the gel in 0.1 M sodium phosphate buffer (pH 7.5), is not eluted on washing with buffer. However, it can be released quantitatively from the column on application of 3 mM NADH in the same buffer, as shown in Fig. 1A.

The partially purified apo-β-hydroxybutyrate dehydrogenase also binds to NAD–Sepharose, and can be eluted from these columns with NAD (Fig. 1B). The interaction between the NAD–Sepharose and the apodehydrogenase under a variety of conditions, is summarized in Table I. The apoenzyme binds to the NAD–Sepharose prepared by both procedures, I and II. However, only 10–15 % of the enzyme activity can be eluted with NAD from the columns prepared by procedure I, whereas the recovery is almost quantitative with columns prepared by procedure II. The specific activity of the protein eluted from the latter columns is also substantially higher (Table I). The presence of a sulfhydryl reagent such as dithiothreitol is necessary to prevent enzyme denaturation. The maximum specific activity of the apoenzyme eluted from these columns is 2–3 units/mg protein, even when apoenzyme of a comparable specific activity is applied to the gel (Table I). As shown in Table I, if the apoenzyme bound to the NAD-gels is washed with pH 5 or 4 buffers before elution with NAD, preparations of somewhat higher specific activity can be obtained. The apoenzyme elutes from the column as a broad peak when it is washed with 0.5 M NaCl. However, only 32 % of the enzyme activity can be eluted by this treatment, and a 5 ml pulse of 0.2 M NAD elutes an additional 31 % of the activity with an increased specific activity. Treatment of the enzyme–NAD–Sepharose complex with protein denaturants, such as urea, cholic acid, Triton X-100 and BRIJ-35, inactivates the protein at concentrations lower than those required to remove any extraneous proteins from the column and therefore cannot be used for purification.

The β-hydroxybutyrate dehydrogenase–soybean lecithin complex is prepared in 2 mM NAD. It was passed through two Sephadex G-25 columns to remove the coenzyme. The complex, when free of NAD, binds to the NAD–Sepharose gels, and can be eluted with NAD solutions (Fig. 1C).

β-Hydroxybutyrate affinity chromatography

Affinity chromatography employing the analogues of the second substrate of the enzyme included chromatography of the apodehydrogenase on Sepharose derivatized with 6-aminocaproic acid, glycylthreonine and 6-aminocaproylthreonine.

TABLE I

NAD-AFFINITY CHROMATOGRAPHY OF β-HYDROXYBUTYRATE DEHYDROGENASE

The enzyme was suspended in the application buffer and applied on the column. The column was then washed with the application buffer (3–5 times the column volume). All buffers except the starred (★) entries contained 5 mM dithiothreitol. After washing with the application buffer, the column was washed with 3–5 times the column volume of the washing buffer, and again with a similar volume of the application buffer. Elution was carried out using 5 ml of 200 mM NAD, 0.05 M sodium phosphate, 5 mM dithiothreitol (pH 7.5). After the NAD had entered the gel, the application buffer was applied.

Column preparation procedure	Application buffer	Washing buffer	Spec. act. (units/mg protein)		Recovery (%)	
			Initial	Peak fractions eluted with NAD	Without NAD elution	Peak fractions eluted with NAD
I	0.1 M phosphate (pH 7.5)★	—	0.9	0.4	9	3
I	0.1 M phosphate (pH 7.5)	—	0.9	1.3	8	12
II	0.05 M phosphate (pH 7.2)★	—	1.0	0.6	3	32
II	0.05 M phosphate (pH 7.2)	—	1.0	2.1	1	87
II	0.05 M phosphate (pH 7.2)	—	2.1	2.1	5	90
II	0.05 M phosphate (pH 7.2)	0.5 M NaCl, 0.05 M phosphate (pH 7.2)†	1.0	2.6	2	31
II	0.05 M phosphate (pH 7.2)	0.05 M phosphate (pH 5.0)	1.0	2.6	6	95
II	0.05 M phosphate (pH 7.2)	0.05 M sodium acetate (pH 4.0)	0.9	3.9	8	66

† 32 % of the activity was eluted by the wash-buffer; the specific activity in the fraction with maximum activity was 0.6. The specific activity and recovery indicated in the table are those obtained on subsequent elution with NAD.

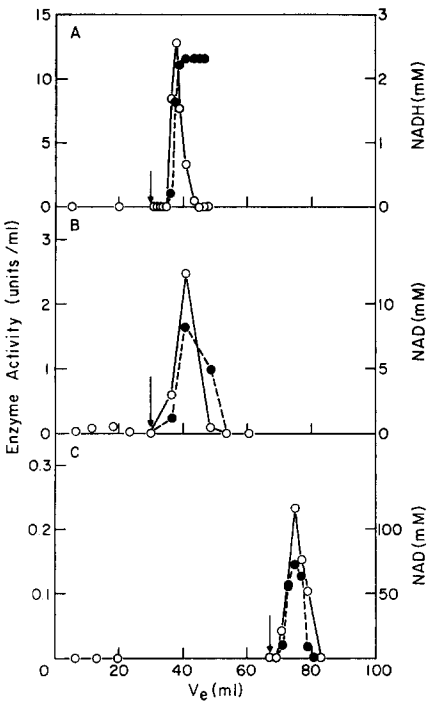

Fig. 1. Affinity chromatography of enzymes on NAD–Sepharose. Arrows indicate when the nucleotide was applied on the column; solid lines and open circles represent the enzyme activity, while dotted lines and filled circles indicate the nucleotide concentration. (A) Lactate dehydrogenase. 1 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 59 units of lactate dehydrogenase was applied on a 10 ml NAD–Sepharose column prepared by Procedure I. The column was washed with the same buffer, and the enzyme was eluted with 3 mM NAD in the same buffer. (B) Apo-β-hydroxybutyrate dehydrogenase. 2 ml of the β-hydroxybutyrate dehydrogenase containing 14 units of the enzyme, spec. act. 0.96 units/mg protein in 0.05 M sodium phosphate buffer (pH 7.1), 5 mM dithiothreitol, was applied to a 10 ml NAD–Sepharose column prepared by Procedure II. The column then was washed with the application buffer, and the enzyme was eluted with 5 ml of 200 mM NAD prepared in the same buffer, readjusted to pH 7.2, followed by the application buffer. (C) β-Hydroxybutyrate dehydrogenase–lecithin complex. β-hydroxybutyrate dehydrogenase–soybean lecithin complex was prepared as described in the Experimental Methods and NAD was removed from it by passage through two Sephadex G-25 columns equilibrated with 0.05 M sodium phosphate buffer (pH 7.8), 1 mM EDTA, 5 mM dithiothreitol. 4.5 ml of this preparation containing 4.6 units of the enzyme were applied on the gel in the same buffer. The column was washed with the application buffer and eluted as in B.

When the apoenzyme is applied on these gels in the presence or absence of 2 mM NAD in 0.05 M sodium phosphate buffer (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, 20–30 % of the activity elutes unretarded. The remaining activity cannot be recovered on elution with buffers containing high salt concentrations or with the substrate β-hydroxybutyric acid.

Hydrophobic chain–Sepharose chromatography

The apoenzyme was also chromatographed on "hydrophobic" columns of various Sepharosylaminoacylaminoalkanes. The ratio of the enzyme elution volume,

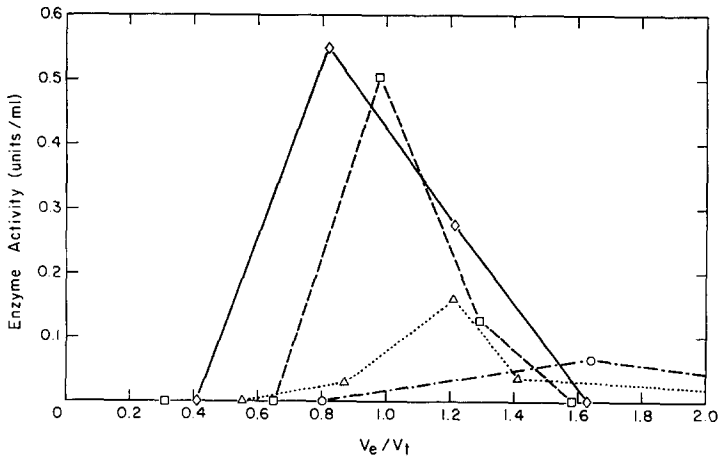

Fig. 2. Chromatography of β-hydroxybutyrate dehydrogenase on hydrophobic derivatives of Sepharose. Various hydrophobic gels were prepared, packed into 8–14 ml bed volume, 0.5 cm diameter columns and pre-equilibrated with the buffer, 0.05 M sodium phosphate, 1 mM EDTA, 2 mM NAD, 5 mM dithiothreitol (pH 8.0). 300 μl of the β-hydroxybutyrate apodehydrogenase containing 4.7 units of the apoenzyme were applied to the columns in the same buffer. The enzyme was eluted from these columns with the application buffer, with a flow-rate of 6–8 ml/h. The ratio of the elution volume to the total bed volume, V_e/V_t, has been plotted against the apodehydrogenase activity (units/ml). ◇——◇, Sepharosylglycylaminomethane; □- - -□, Sepharosylglycylaminobutane; △ · · · △, Sepharosylglycylaminoheptane; ○ - · - · -○, Sepharosyl-6-aminocaproylaminododecane. No enzyme activity was recovered from Sepharosyl-6-aminocaproylaminooctadecane columns.

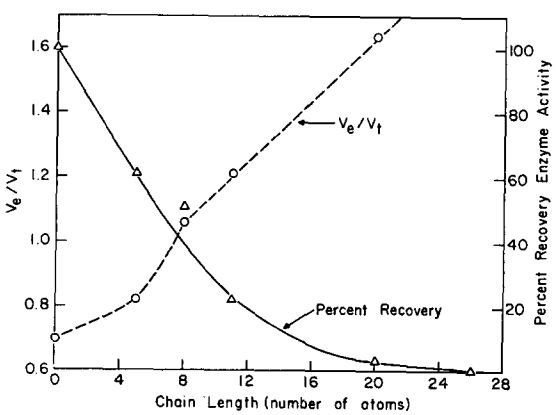

Fig. 3. Interaction of β-hydroxybutyrate apodehydrogenase with hydrophobic gels. The percent recovery of enzyme activity applied (△–△) and the ratio of the elution volume to the total bed volume (V_e/V_t, ○- - -○) plotted versus the chain length of the hydrophobic chain, that is the total number of atoms in the linear chain from the N-terminus to the methyl group. The data were taken from Fig. 2.

V_e, to the total bed volume of the hydrophobic gel, V_t, and the total amount of enzyme recovered were determined. The elution patterns of the enzyme from various gels are shown in Fig. 2. The retardation of the enzyme, as measured by V_e/V_t, increases with increasing length of the aliphatic chains. Similarly the total amount of the apode-

hydrogenase activity eluted from these columns was observed to decrease with increasing hydrophobicity of the gel (Figure 3). No enzyme activity could be recovered from chromatography on the Sepharosyl-6-aminocaproylaminooctadecane, even when washing the gels with bovine serum albumin or soybean lecithin. Hydrophobic column chromatography cannot be used for purification of the apo-β-hydroxybutyrate dehydrogenase since the apoenzyme elutes, along with the extraneous proteins, as a very broad peak.

Combination NAD–hydrophobic chain affinity chromatography

In a 25 cm × 1 cm column, 10 ml of a 1:1 mixture of the NAD–Sepharose prepared by Procedure II and Sepharosylglycylaminobutane were layered at the bottom, and covered with another 10 ml of the glycylaminobutane derivative of Sepharose. The apoenzyme was applied on this two layer column in 0.05 M sodium phosphate buffer (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, and the column washed with 85 ml of the same buffer. The apodehydrogenase was eluted from the column with NAD, as described in the legend to Fig. 1B. The apoenzyme eluted from this column corresponded to 75 % of the activity applied, and had a specific activity of 9–10 units/mg protein. This represents a 9–10-fold purification of the apodehydrogenase. Sodium dodecylsulfate–acrylamide gels show 30–50 bands in the starting material and only 6–10 in the apoenzyme purified by this method. The enzyme obtained is about 7 % pure, as estimated from the specific activity of the pure enzyme–lecithin complex [9]. However, the purified apoenzyme is quite unstable even in the presence of NAD and dithiothreitol, and has a half life of about 1 h at 4 °C.

DISCUSSION

Affinity chromatography of β-hydroxybutyrate dehydrogenase on Sepharosyl-6-aminocaproyl-NAD indicates that the partially purified apoenzyme binds to NAD. However, a sulfhydryl group protecting reagent is required to maintain the enzymatic activity (Table I). The binding of the apoenzyme to immobilized NAD is a specific interaction rather than a non-specific ionic interaction since the enzyme cannot be eluted by high salt concentrations or variation of pH but is eluted by NAD solutions. Other evidence suggesting that NAD binds to the apoenzyme is the increased stability of the apoenzyme in buffers containing NAD [7, 9] and the requirement of NAD for enzymelipid complex formation [4, 6]. The maximum specific activity of the apoenzyme obtained from NAD columns alone (about 4 units/mg protein) is considerably less than the specific activity of the purified enzyme–lipid complex (130 units/mg protein) [9] and indicates that the apoenzyme is tightly bound to other proteins. The large number of bands observed on sodium dodecylsulfate–acrylamide-gel electrophoresis with the apoenzyme having a specific activity of 9 units/mg protein obtained from the combined NAD–Sepharose, hydrophobic chain–Sepharose column confirms this interpretation. Since the apoenzyme is tightly bound to other proteins, the possibility that its binding to the NAD affinity gels is due to its being bound to another protein which in turn binds to NAD cannot be excluded.

The apoenzyme is inactivated by affinity chromatography on NAD–Sepharose prepared by Procedure I, but not with gels prepared by Procedure II. The amounts of NAD incorporated into the Sepharose by the two procedures are 3–4 μmoles/ml and

10–12 μmoles/ml, respectively. The concentration of the 6-aminocaproic acid linked to Sepharose is about 10–12 μmoles/ml of settled Sepharose [14]. Thus over half of the carboxyl groups attached to Sepharose remain free with Procedure I, while essentially no free carboxyl groups remain with Procedure II. The former gel then can act as a cation-exchange resin as well as an affinity resin. The apoenzyme is denatured in Sepharose derivatized with 6-aminocaproic acid alone indicating that free carboxyl groups denature the enzyme. This accounts for the denaturing effect of the NAD-Sepharose prepared by procedure I. Clearly procedure II is preferable for the preparation of NAD-affinity resins.

Attempts to determine if the apoenzyme binds to the substrate β-hydroxybutyrate were inconclusive. The apoenzyme is denatured on binding to Sepharose derivatives in which a free α-carboxyl group is present (6-aminocaproic acid–Sepharose) or a β-hydroxyl-group and an α-carboxyl group are present (glycylthreonine–Sepharose, 6-aminocaproylthreonine–Sepharose). Since the protein binds to these columns in the presence or absence of NAD, the interaction is probably of a non-specific nature: most dehydrogenases are believed to bind substrates in an ordered sequence with the nucleotide binding first [17–19].

Many proteins isolated from membranes display hydrophobic surface properties rendering them insoluble in water [20–21]. Solubilization and purification of such proteins often utilize detergents [22]. Detergent treatments, however, may lead to irreversible protein denaturation at the detergent concentrations required for the breakdown of hydrophobic protein–protein and protein–lipid interactions. A less drastic treatment is to immobilize hydrophobic molecules on Sepharose as described earlier. This has the effect of eliminating the formation of micelles, which probably are the major denaturing species in detergent denaturation. The elution patterns of the apoenzyme on the hydrophobic affinity columns indicate that the protein is progressively retarded as the hydrophobicity of the column increases; unfortunately the enzyme also is increasingly denatured with increasing hydrophobicity (Fig. 3). However, only 20–30 % of the apoenzyme activity is lost during chromatography on glycylaminobutane–Sepharose over a period of 2–3 h at 4 °C, whereas incubation of the apoenzyme at a *sec*-butanol concentration as low as 0.5 % for 15 min at 1 °C leads to a 50 % loss of activity. Treatment of the apoenzyme bound to NAD–Sepharose with detergent solutions leads to rapid denaturation even when the detergents are present at extremely low concentrations. The concentrations of the hydrophobic groups present in these columns, assuming that all of the 6-aminocaproic acid carboxyl groups are derivatized, is about 10–12 μmoles/ml of settled Sepharose. Despite this high concentration, the gels with short aliphatic chains can be used to dissociate the apoenzyme from other proteins without a major loss of activity.

The usefulness of the hydrophobic column is illustrated by the two layer hydrophobic chain–NAD–Sepharose column used for purification of the apodehydrogenase. The top layer, which is lipophilic, dissociates the protein–protein complexes formed due to hydrophobic interactions. The apoenzyme is then stabilized by binding to the immobilized NAD, and other inactive proteins are released. The almost quantitative recovery of activity (75 %) and the high specific activity of the apoenzyme purified with this method (9–10 units/mg protein) attest to the success of this "double barreled" affinity column. Hydrophobic affinity columns may be of general utility in purifying lipophilic proteins, particularly when combined with other affinity gels.

To summarize, affinity chromatography has been used to show that the inactive partially purified apo-β-hydroxybutyrate dehydrogenase binds to NAD and hydrophobic aliphatic chains. Furthermore, a substantial purification of the enzyme has been achieved with affinity chromatography.

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