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Interactions of the mitochondrial membrane rat liver D-3-hydroxybutyrate dehydrogenase with glass beads during adsorption chromatography

Relationships with the activation of the enzyme by phospholipids

P. ADAMI and B. NASSER

Laboratoire de Biochimie et Biologie Moléculaire (UA CNRS 531), Université de Franche Comté, 25030 Besançon Cedex (France)

and

N. LATRUFFE[*,a]

Laboratoire de Biologie Moléculaire et Cellulaire, Université de Bourgogne, B.P. 138, 21004 Dijon (France)

ABSTRACT

D-3-Hydroxybutyrate dehydrogenase (BDH) is an NAD^+-dependent dehydrogenase of the mitochondrial inner membrane involved in the energetic balance between the liver and peripheral organs in mammals. It allows the conversion of ketone bodies (acetoacetate and D-3-hydroxybutyrate) and it is one of the best documented lipid-requiring enzymes with a dependence on lecithins. After release of proteins from the membrane by phospholipase A2 treatment of salt-treated mitochondria, the rat liver enzyme is absorbed on controlled-pore glass beads. After batch washing, the enzyme, devoid of lipids (apoBDH), is specifically eluted at pH 8.05–8.15 with a 0.1 M Tris 1 M LiBr buffer under reducing conditions (5 mM dithiothreitol). It appears that during BDH absorption, the glass beads mimic the phospholipid surface of biomembranes.

INTRODUCTION

D-3-Hydroxybutyrate dehydrogenase (BDH), EC 1.1.1.30, is a ketone body converting enzyme (for a review, see ref. 1). This mammalian NAD(H)-dependent enzyme plays a central role in the energetic balance between hepatic and peripheral tissues under physiological and pathological conditions. Indeed, the concentrations of the ketone bodies (acetoacetate and D-3-hydroxybutyrate) in the plasma and in the cells depend on the interrelationships between the metabolic pathways of lipids, of

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[a] Present address: Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences de la Vie–Mirande, Université de Bourgogne, B.P. 138, 21004 Dijon, France.

carbohydrates and of some ketogenic amino acids [2]. Under normal conditions, D-3-hydroxybutyrate dehydrogenase is essential in the oxidization of D-3-hydroxy-butyrate in the brain of newborns, providing energy and biosynthetic precursors (acetyl-CoA) of cholesterol and myelin. In addition, extrahepatic tissues such as the kidney, heart and skeletal muscle use ketone bodies preferentially as energy fuels. It is also well known that an overproduction of ketone bodies appears in diseases or non-physiological conditions, *e.g.*, diabetes mellitus, starvation or hyperlipidic diet [3].

Lehninger *et al.* [4] showed that D-3-hydroxybutyrate dehydrogenase is located in the inner mitochondrial from mammalian tissue. Later, it was found that the orientation of the catalytic site is on the matrix side of the inner membrane [5].

BDH is one of the few enzymes so far known which show a specific lipid dependence (absolute requirement for phosphatidylcholine for enzymatic activity).

The enzyme cannot be released from the membrane by sonication, osmotic shock or high ionic strength [4]. Three methods have been used to solubilize the BDH from mitochondrial membrane: cholate treatment [6], phospholipid hydrolysis by snake venom phospholipase A_2 [7] which released BDH almost completely, although only 30–40% of phospholipids were hydrolysed whatever the nature of the hydrolysed phospholipid, and incubation of mitochondria at high pH which partially released the enzyme [8].

In order to understand the phospholipid–apoBDH interaction at the molecular level, it was necessary to obtain a purified homogeneous and lipid-free apoenzyme. With this aim, Fleischer's group [9] proposed an original technique for purifying beef heart apoBDH based on the release of the enzyme by treatment of mitochondria with phospholipase A_2, adsorption of the enzyme on untreated controlled-pore glass beads (CPG) and selective elution of the protein from the column by lithium bromide, a chaotropic agent, at defined pH. Purified beef heart apoBDH, devoid of phopho-lipids and water soluble under defined conditions, consists of a single polypeptide chain of 31 500 dalton as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [9]. Several purification procedures, partial or complete, were successively proposed by different groups in order to improve the purity, stability, yield, time required or simplicity, or to adapt the technique to BDH from different species [10 18].

However, some of these purification procedures were not suitable for studying lipid protein interactions as BDH was purified as a mitochondrial phospholipid complex and not as apoBDH [10 14]. To our knowledge, no-one has tried to explain the nature of specific interactions between BDH and the glass surface of CPG which are essential in the purification process. In this paper, we describe the purification procedure adapted in our laboratory for the rat liver mitochondrial membrane-bound BDH and show a relationship between phospholipid–BDH interaction and glass–BDH adsorption.

EXPERIMENTAL

Materials

Controlled-pore glass beads (CPG-10-350; mesh size 120–200; pore diameter *ca.* 350 Å) were obtained from Electro-Nucleonics (Fairfield, NJ, U.S.A.). These beads were not treated with polyethylene glycol or polyethylene oxides as indicated for

exclusion chromatography [19]. Chemicals were of the highest grade available. Solutions were prepared in ultrapure water.

Methods

Rat liver mitochondria were prepared on a large scale as described previously [20] and were stored frozen ($-70°C$) at 40 mg ml^{-1} in 0.25 M sucrose until used. Protein was determined as described by Lowry *et al.* [21] and modified by Ross and Schatz [22] for assays containing free thiols. Lipid phosphorus was determined according to Chen *et al.* [23].

Mitochondrial lipids (MPL) were extracted from rat liver mitochondria. Aqueous dispersions of MPL were prepared by the dialysis or the sonication procedures described previously [24].

SDS-PAGE was performed according to Laemmli [25].

D-3-Hydroxybutyrate dehydrogenase activity was measured spectrophotometrically at 334 nm at 20 or 37°C as described previously [26], in a "cocktail medium" containing 10 mM potassium phosphate, 0.05 mM EDTA, 0.04% bovine serum albumin, 1.27% (v/v) ethanol, 0.3 mM dithiothreitol (DTT) and 2 mM NAD$^+$ (final volume 450 μl). The reaction was started by addition of 50 μl of 0.2 M D,L-3-hydroxybutyrate.

Preparation of "crude intrinsic mitochondrial membrane proteins fraction"

Frozen–thawed mitochondria were submitted to a high ionic strength (1 volume of 3 M KCl) in order to remove soluble and membrane-associated proteins. Crude intrinsic mitochondrial membrane proteins fraction (about 20% of the original protein) was obtained after ultracentrifugation for 30 min at 100 000 g. This pellet was frozen at $-80°C$

Solubilization and purification of D-3-hydroxybutyrate apodehydrogenase

Frozen crude intrinsic mitochondrial membrane protein fraction (20 g of original mitochondrial protein) from about 150 rats was thawed by gentle swirling in a water-bath at 37°C. This suspension (*ca.* 0.5 l) was added to a beaker containing 0.1 M Tris–HCl (pH 7.4), 1 mM NAD$^+$, 3 mM CaCl$_2$, 5 mM DDT and water to give a final volume of 1 l at 30°C. The mitochondrial membrane fraction was digested by addition of 80 ml (equivalent to 80 mg of original snake venom) of phospholiphase A$_2$ [8], *i.e.*, in a ratio of 4 μl per milligram of original mitochondrial protein, at 30°C for the optimum time (usually 3–4 min) previously determined in a trial digestion [8]. Digestion was terminated by adding the mixture to a cold carboy (*ca.* 5 l capacity) containing 36.66 ml of 0.3 M EDTA, 55 ml of 2 M KCl and 125 ml of "ice cubes" ($-20°C$) consisting of 0.1 M KCl, 20 mM EDTA and 2 mM DTT, to cool the mixture rapidly to *ca.* 8°C. The pH was reduced by addition of 30.66 ml of 2 M potassium phosphate (pH 6.5).

An aliquot was removed to determine the amount of solubilized D-3-hydroxybutyrate apodehydrogenase in the supernatant after ultracentrifugation (3 min) at 140 000 g in a Beckman Airfuge. The digested suspension was stirred slowly with a paddle and 100 ml (packed volume) of untreated CPG previously degassed in water and stored under nitrogen were added with an equal volume of buffer VI (see Table I). Adsorption of the apodehydrogenase on the glass beads was nearly complete (90%) after 30 min.

The glass beads with bound enzyme were recovered in 1-l centrifuge bottles by centrifugation for 30 s at 1000 rpm in a Sorvall RC5B and GS3 rotor. The first five washed with potassium phosphate buffers (buffers I–VI; see Table I) were carried out batchwise in the centrifuge bottles so as to remove mitochondrial membrane fragments trapped in the sedimented glass beads.

The glass beads were then packed into a column (ca. 20 cm × 8 cm I.D.) already containing 50 ml of packed CPG as scavenger, under a nitrogen atmosphere, and washing was continued with buffer VII at a flow-rate of ca. 3 ml cm^{-2} min^{-1}.

The enzyme was eluted (see Table I) using 1.0 M LiBr–0.1 M Tris–HCl–2.5 mM DTT elution buffer with an initial pH of 8.00 and a flow-rate of ca. 3 ml cm^{-2} min^{-1}, then with pH 8.05 buffer until enzymatic activity was detected in the effluent (usually after 5–7 column volumes). Elution was continued with buffer in the pH range 8.10–8.15 until the activity began to decrease, at which point the pH of the effluent was increased to a maximum of 8.15. Elution was terminated when the enzymatic activity per litre fell below 1% of the initial activity solubilized by phospholipase A$_2$. A small amount of additional enzymatic activity could be eluted at higher pH (8.20–8.30), but elution of contaminants began to predominate, attended by a yellow band which migrated slowly down the second column during the course of elution of the enzyme.

When the activity was first detected in the eluate from the purification column, the eluate was passed directly into a 1-l stirred vessel and titrated with 1 M HCl to pH 7.2. This titrated effluent was applied directly to a second column [concentration column, consisting of 50 ml of CPG-10 packed in buffer A (see Table I), column dimensions ca. 18 cm × 3.5 cm I.D.] at a flow-rate to match the elution rate of the

TABLE I

BUFFERS USED FOR PURIFICATION OF D-3-HYDROXYBUTYRATE APODEHYDROGE-
NASE ON CONTROLLED-PORE GLASS BEADS

Buffer description	Buffer composition (containing 2.5 mM DTT)	pH	Elution volume (ml)	
			Purification column	Concentration column
Washing buffers				
I = A	1 M potassium phosphate	6.5	1500	200
II	1.5 M potassium phosphate	8.15	1500	Omitted
B	1 M potassium phosphate	7.5	Omitted	200
III = C	1 M potassium phosphate	8.15	3000	600
IV = D	0.75 M potassium phosphate	6.5	1500	600
V = E	0.2 M potassium phosphate	6.5	1500	200
VI = F	0.02 M potassium phosphate	6.5	750	200
VII = G	0.01 M Tris	7.75	750	200
Elution buffers				
8.00	0.1 M Tris–1 M LiBr	8.00 at 4°C	750	200
8.05	0.1 M Tris–1 M LiBr	8.05 at 4°C	750	400
8.10	0.1 M Tris–1 M LiBr	8.10 at 4°C	750	400
8.15	0.1 M Tris–1 M LiBr	8.15 at 4°C	3000	1400
8.20	0.1 M Tris–1 M LiBr	8.20 at 4°C	750	600
8.30	0.1 M Tris–1 M LiBr	8.30 at 4°C	500	600

purification column. After all the eluate had been applied, the column was washed with buffers A–G.

The second (concentration) column was then eluted with 1 M LiBr–0.1 M Tris–HCl–5 mM DTT (pH 8.00–8.15) buffers. Fractions containing enzymatic activity were pooled, adjusted to 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES), titrated to pH 7.0 with 1 M HCl and concentrated to a volume of $ca.$ 50 ml using 400-ml Amicon stirred cells with a PM-10 membrane. The enzyme solution was adjusted to 0.4 M LiBr, 5 mM HEPES (pH 7.0) and 5 mM DTT as essential reducing agent to prevent BDH disulphide bond formation, and concentrated to a final volume of 4–5 ml ($ca.$ 1–2 mg protein/ml) in a 60-ml Amicon stirred cell using a PM10 membrane.

The concentrated enzyme was ultracentrifuged (Type 40 rotor for 30 min at 105 000 g). A small opalescent glass bead pellet was obtained which had a low protein content. The supernatant, containing the purified apodehydrogenase, was quick-frozen in small aliquots and stored in a liquid nitrogen refrigerator.

RESULTS AND DISCUSSION

Purification scheme and properties of purified apoBDH
The purification of D-3-hydroxybutyrate dehydrogenase from rat liver mito-chondria has been adapted from methods described 15 years ago for beef heart mitochondria BDH [9,19] and for other proteins [27]. Although the basic elements of the method have remained unchanged, the procedure has been improved and simplified: a previous treatment of mitochondria with a high ionic strength of KCl already removes 80% of contaminating proteins. The enzyme is then released from the mitochondrial inner membrane with the use of phospholipase A_2. The ammonium sulphate precipitation step [8] has been omitted. BDH solubilized in the supernatant is now directly adsorbed in batch to controlled pore glass beads (CPG-10), which are then washed to remove the mitochondrial membranes and any other non-adsorbed proteins. The first five elution buffers are applied batchwise prior to packing the CPG-10 with adsorbed proteins into a column for the chromatographic elution. From 20 g of rat liver mitochondrial protein, we obtain more than 10 mg of the purified enzyme with a specific activity at 37°C of about 80 μmol min^{-1} mg protein^{-1} when reactivated with mitochondrial phospholipids. The procedure has been appreciably shortened; the entire preparation now takes $ca.$ 18–20 h with an additional 8 h for concentration and centrifugation.

Fig. 1 shows the elution profile of apoBDH from the CPG concentration column. The purification procedure results in the elution of two separate fractions of enzyme (apoBDH) from the second column. The first fraction elutes in the pH 8.15, 1 M phosphate buffer (contaminated phosphate fraction) and the second (highest activity) in the 1.0 M LiBr–0.1 M Tris–HCl (pH 8.05–8.15) buffer (called pure "LiBr–BDH fraction").

After diafiltration and concentration, the pooled "LiBr–BDH" active fractions are checked for purity and for lipid dependence. Fig. 2 shows that after two CPG adsorption–elution cycles (purification column and concentration column), BDH appears to be at least 95% pure. Fig. 3 indicates that the purified lipid-free apoBDH is catalytically inactive and requires reassociation with either total mitochondrial
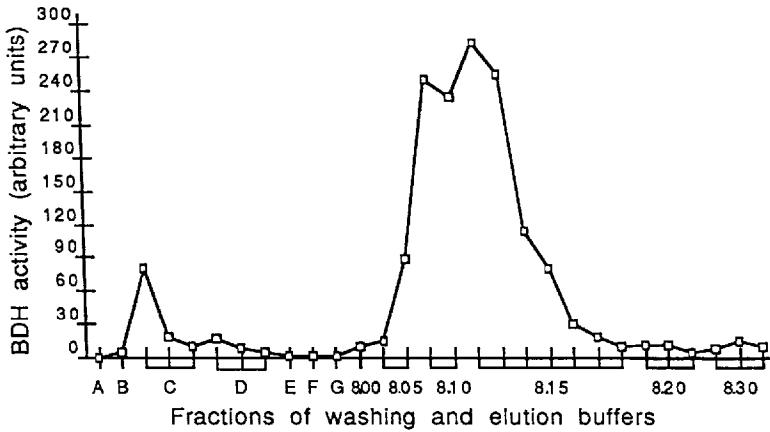
Fig. 1. Elution profile of apoBDH from a controlled-pore glass bead concentration column. Buffers A–G are washing buffers and buffers 8.00–8.30 correspond to elution buffers (see Table I). Each bar on the abscissa corresponds to a fraction of 200 ml. BDH activity is expressed in arbitrary units and corresponds to 40 μl of fraction reactivated with 1.8 μg of lipid phosphorus MPL and measured as described under Experimental.
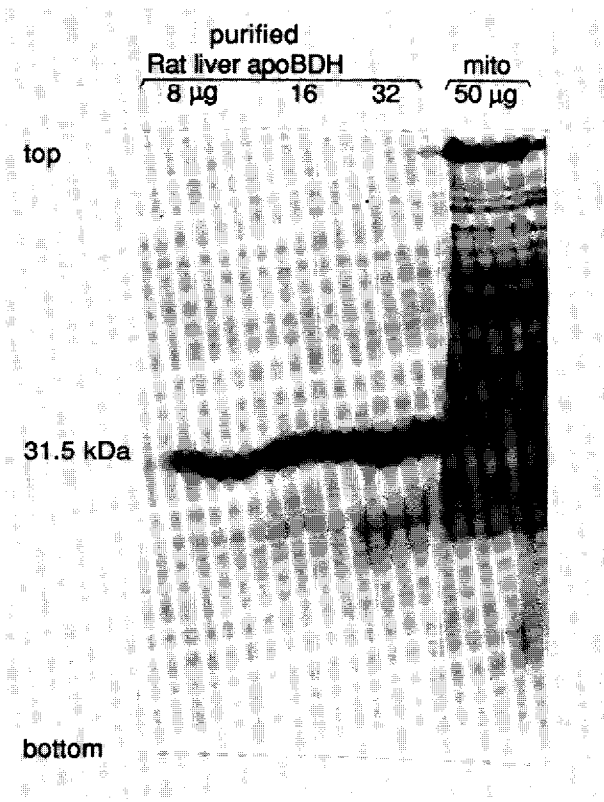

Fig. 2. Purified apoBDH separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. For conditions, see Experimental. kDa = Kilodalton.
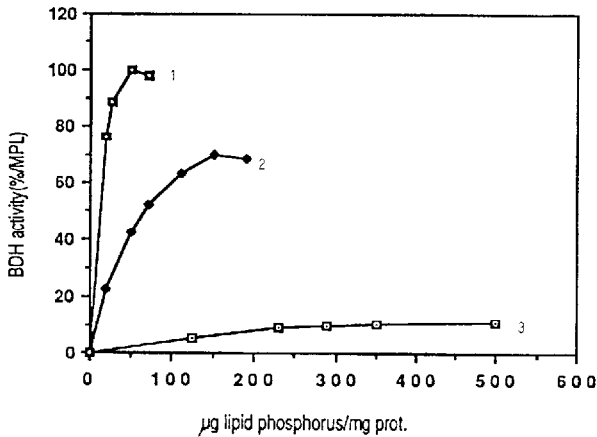
Fig. 3. BDH reactivation by differents amounts of natural [MPL (1), mitoPC (2)] or synthetic DMPC (3) phospholipids. The apoBDH (10 μg) was incubated for 10 min at room temperature in 100 μl (final volume) containing 20 mM Tris HCl–1 mM EDTA–5 mM DTT (pH 8.1) and the appropriate phospholipid concentrations. The 100-μl aliquot was added at 37 C to a cuvette containing 450 μl of "cocktail" medium as described under Experimental. Then the enzyme reaction was started by addition of 50 μl of 0.2 M DL-β-hydroxybutyrate at 37 C and the activity was followed by measuring the increase in absorbance at 334 nm.

phospholipid containing lecithin (MPL), or pure mitochondrial lecithin (mitoPC), or synthetic lecithin (dimyristoylphosphatidylcholine, DMPC). The different extents of reactivation according to the nature of the lipid have been explained previously [28 32].

Several other attempts to purify rat liver apoBDH by different methods have been tried in our laboratory. All of them failed, including Burnett and Khorana's method involving DEAE-cellulose chromatography [17] or an immunoaffinity procedure based on the use of rabbit monospecific anti-rat liver BDH antibody [33].

Relationships between glass beads–BDH adsorption and phospholipid–BDH reactivation

Taking into account the specificity of BDH for either CPG or phospholipids it was important to establish whether there was any relationship between the two interacting mechanisms.

Fig. 4 shows that the addition of CPG prevents the reactivation of purified apoBDH by phospholipids while not modifying the enzymatic activity of BDH previously reconstituted with MPL. These results indicate that the interaction between CPG and the BDH polypeptide chain changes the conformation of the enzyme or, more probably, that the glass surface interacts with phospholipid-binding sites of BDH. However, there is an important difference between glass beads and membrane phospholipid surfaces, as only the latter is able to reactivate BDH.

On the other hand, Fig. 5 shows that LiBr is needed to reactivate BDH by lecithin (PC) or phospholipid containing lecithin (MPL), previously associated with phospholipid containing no lecithin [phosphatidylethanolamine–diphosphatidylglycerol–phosphatidylinositol (PE–DPG–PI)]. This displacement of BDH from a non-reactivating phospholipid surface to a reactivating phospholipid surface is due to the
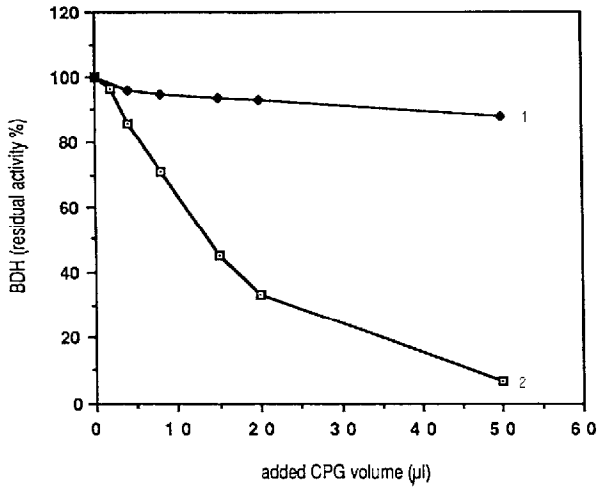
Fig. 4. Effect of controlled-pore glass beads (CPG) on BDH enzyme activity. The untreated glass beads (CPG) were added at room temperature to a cuvette containing 20 mM Tris–HCl–1 mM EDTA–5 mM DTT (pH 8.1) and 20.4 µg of the enzyme (apoBDH or BDH–MPL complex), final volume 450 µl. After 15 min of gentle shaking, the enzyme activity was measured at 334 nm either immediately for BDH–MPL complex assay (1), or after previous incubation (10 min) of MPL (80 µg of lipid phosphorus per mg protein) in the assay containing CPG–apoBDH (2).

ionic strength and to the chaotropic effect of LiBr in addition to the alkaline pH. An evident similarity is found with BDH elution from the glass bead column (Fig. 1). To our knowledge, such a relationship between BDH reactivation by phospholipid and BDH purification by glass beads adsorption has not been reported before.
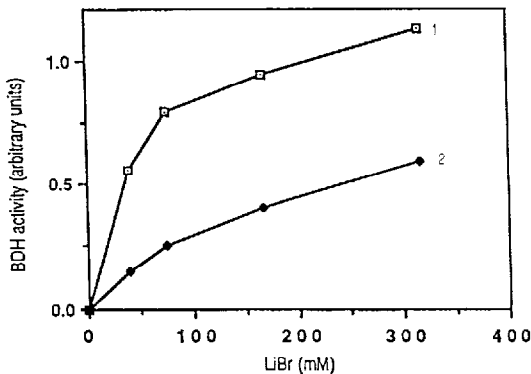

Fig. 5. Displacement of the non-active complex BDH–PE–DPG–PI by reactivating phospholipid [PC (2) or MPL (1)] in the presence of LiBr. ApoBDH (35 µg) was incubated for 15 min at room temperature in a medium (650 µl) containing a microdispersed PE–DPG–PI preparation (200 µg lipid phosphorus per mg protein) and 20 mM Tris–HCl–1 mM EDTA–5 mM DTT (pH 8.1). At the end of incubation, 50 µl of the mixture were removed and added to a medium (650 µl final volume) containing the same buffer and a fixed amount (180 µg lipid phosphorus per mg protein) of reactivating phospholipid (MPL or PC) and an increasing concentration of LiBr. After incubation for 15 min at room temperature, an aliquot of 70 µl was removed and measured as described under Experimental.

It appears that glass beads mimic phospholipids in their interaction with BDH. Indeed, it is well known that a phospholipid membrane is amphipathic and shows a polar surface mostly negatively charged and a hydrophobic domain. On the other hand, untreated glass beads show a negatively polarized surface of the silicate network in addition to a hydrophobic character.

The combination of a high pH (8.05–8.15), high ionic strength and the chaotropic effect of LiBr is able to break the bonds between surface negative charges and positive amino groups of the BDH amino acid side-chain.

This work extends our knowledge of both the basic principles of the adsorption chromatography of membrane proteins and the mechanism of lipid–protein interactions.

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