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AFFINITY CHROMATOGRAPHY OF THE PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN FROM BOVINE LIVER

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Summary

Affinity chromatography has been used to purify the phosphatidylcholine exchange protein from bovine liver. The affinity resin consisted of 1-acyl-2-(9-carboxy)nonyl-glycero-3-phosphocholine linked to AH-Sepharose 4 B via the carboxyl group. Application of a crude exchange protein fraction to the affinity column resulted in a complete adsorption of the phosphatidylcholine exchange protein. The exchange protein eluted with a buffer containing 0.15% sodium deoxycholate. The most active fraction was 130-fold purified and accounted for 62% of the activity.

Introduction

Affinity chromatography has been successfully applied in the purification of enzymes that are involved in the metabolism of lipids, e.g. *sn*-glycero-3-phosphate:CMP phosphatidyltransferase [1], phospholipase A_2 [2] and lecithin-cholesterol acyltransferase [3]. Specific ligands used were cytidinediphospho-1,2-diacylglycerol [1], *rac*-1-(9-carboxy)nonyl-2-hexadecylglycero-3-phosphocholine [2] and high density lipoprotein [3].

The phosphatidylcholine exchange protein from bovine liver specifically transfers phosphatidylcholine between membrane interfaces [4,5]. It appears that the specificity resides in a binding site on the protein that recognizes the phosphorylcholine head group [6,7]. This specificity makes affinity chromatography a potentially attractive method to purify the exchange protein.

In the present study it will be shown that the exchange protein binds to phosphatidylcholine covalently coupled to 1,6-diamino-hexane agarose. In addition we report that phosphatidylcholine-agarose can be used to achieve a partial purification of the exchange protein.

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Materials and Methods

Materials

Pure phosphatidylcholine exchange protein from bovine liver was prepared according to a slight modification of the procedure described previously [8]. In step 2, the pH of the solution was raised from 3 to approx. 6.2 by the slow addition of 1 M Tris-HCl (pH 8.0). The purified protein (110 μg/ml) was stored at −10°C in 50% glycerol (v/v). Before use the protein was diluted with an equal volume of 50 mM Tris-HCl (pH 7.8). As a crude exchange protein solution we used the protein that precipitated with ammonium sulfate (step 3 of procedure). After sedimentation the protein pellet was stored at −20°C; before use the protein was dissolved in 0.01 M sodium phosphate/0.001 M EDTA (pH 7.0) and dialysed overnight at 4°C against the same buffer. Egg yolk phosphatidylcholine was obtained from Makor Chemicals (Jerusalem, Israel); the purity of the lipid was established by thin layer chromatography. The *Me*-^{14}C-labeled derivative of egg yolk phosphatidylcholine (55–60 μCi · μmol^{-1}) was synthesized as described previously [6]. AH-Sepharose 4 B (1,6-diaminohexane agarose) and Sepharose 4 B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); sodium deoxycholate and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride from E. Merck (Darmstadt, G.F.R.) and 2,4,6-trinitrobenzene sulfonic acid from Sigma (St. Louis, U.S.A.). Additional chemicals were of reagent grade.

Preparation of phosphatidylcholine-AH-Sepharose 4 B

Oxidative cleavage of egg yolk phosphatidylcholine. The double bonds of the unsaturated fatty acids in egg yolk phosphatidylcholine were cleaved by oxidation as described by Shimojo et al. [9]. In this way, phosphatidylcholine with a free carboxyl group at the ω-terminal end of the 2-acyl chain was obtained. Phosphatidylcholine (1 g) was dissolved in 100 ml acetic acid (90%, v/v); [^{14}C]-phosphatidylcholine (2 μCi) was added as an internal standard. A solution of 0.024 M potassium permanganate and 0.02 M sodium periodate was added dropwise under constant stirring at room temperature until the color of the solution remained purple; approximately 200 ml of the oxidant solution were required. Stirring of the reaction mixture was continued for 30 min. After the solution had been decolorized with 40 ml of a 20% sodium bisulfite solution, the pH was adjusted to 2 with 3 M HCl [2]. The reaction mixture was extracted with 750 ml chloroform/methanol (2 : 1, v/v). The chloroform phase which contained both the carboxy- and non-oxidized phosphatidylcholine, was washed three times with 250 ml water. After evaporation of the chloroform the lipid residue was dissolved in 50 ml of chloroform/methanol (2 : 1, v/v) and stored at −20°C. Recovery of total phosphatidylcholine was 94%.

The reaction product was applied to a thin layer plate of silicagel H (E. Merck, Darmstadt, G.F.R.). The plate was developed with chloroform/methanol/ammonia (65 : 35 : 8, v/v) and the lipid spots visualized by iodine vapor (Fig. 1). The areas corresponding to carboxy- phosphatidylcholine (R_F 0.17), lysophosphatidylcholine (R_F 0.10) and non-oxidized phosphatidylcholine (R_F 0.35) were scraped off and the ^{14}C radioactivity was determined with liquid scintillation counting. Carboxy-phosphatidylcholine comprised 68 ± 4%

(standard deviation of three preparations), lysophosphatidylcholine 9 ± 2% and non-oxidized phosphatidylcholine 23 ± 2% of the total ^{14}C radioactivity. The total phosphatidylcholine reaction mixture was used without further purification to prepare the affinity resin.

Coupling of carboxy-phosphatidylcholine to AH-Sepharose 4 B. The affinity resin was prepared by a modification of the method described by Rock and Snijder [2]. AH-Sepharose 4 B (3 g) was prepared and washed according to the procedure described by Pharmacia Fine Chemicals. The gel was resuspended in 25 ml dioxane/water (1 : 1, v/v), and sedimented by centrifugation. Phospholipid (480 μmol) was dissolved in 24 ml dioxane/water (1 : 1, v/v) and added to the packed gel. The pH of the suspension was adjusted to 5.3 with sodium hydroxide (0.5 M). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (680 mg) was added gradually and the reaction proceded for 24 h at room temperature under constant stirring. The pH was maintained at approximately 5.0. At the end of the reaction, the gel (12 ml of packed volume) was washed with dioxane (100 ml), methanol (100 ml), 1 M sodium chloride (200 ml), 0.02% sodium azide (200 ml, twice), resuspended in 0.02% sodium azide up to a total volume of 30 ml and stored at +4°C. After the washing with 1 M sodium chloride some preparations of phosphatidylcholine-AH-Sepharose 4 B and AH-Sepharose 4 B were resuspended in 0.1 M sodium chloride and the unreacted amino-groups were acetylated with acetic anhydride as described by Inman and Bintzis [10]. Completion of the acetylation was established by addition of 2,4,6-trinitrobenzene sulfonic acid [11]. The amount of carboxy-phosphatidylcholine bound to the agarose, was determined directly on the gel by measuring the ^{14}C radioactivity and the phospholipid phosphorus as described previously [6]. Both methods of analysis indicated that 14 to 30 μmol of phosphatidylcholine (four preparations) were bound to 12 ml of gel. According to the manufacturer, 3 g of AH-Sepharose 4 B contains 70—120 μmol of free amino-groups.

Additional methods

Phosphatidylcholine transfer activity was measured for 20 min at 25°C with the assay described by Kamp et al. [8]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the procedure of O'Farrell [12]. Protein was determined by the method of Lowry et al. [13] with bovine serum albumin as standard.

Results

Binding of phosphatidylcholine exchange protein

The major fatty acids of egg yolk phosphatidylcholine are palmitic acid (33%), stearic acid (14%), oleic acid (30%) and linoleic acid (15%). Because the unsaturated fatty acids are located at the 2-acyl position, the oxidation product mainly consists of 1-acyl-2(9-carboxy)nonyl-glycero-3-phosphocholine. Under the coupling conditions used only phosphatidylcholine with a carboxyl group at the ω-terminal end of the 2-acyl chain will form a covalent bond with the 1,6-diaminohexane agarose. The major constituent of phosphatidylcholine-agarose is depicted in Fig. 1.

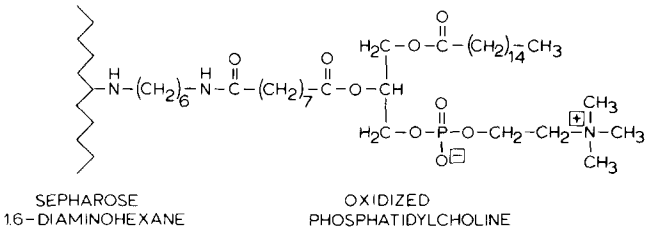

Fig. 1. Structure of phosphatidylcholine-AH-Sepharose 4 B.

An affinity column was prepared of a phosphatidylcholine-agarose slurry (0.25 ml) containing 400 nmol of phosphatidylcholine to determine the binding capacity for phosphatidylcholine exchange protein. Application of 55 μg protein (2.5 nmol) resulted in a complete retention of transfer activity. Activity, i.e., 25% of control value appeared in the effluent upon application of 5 nmol of protein. From this one can deduce that approximately 1 nmol protein binds per 100—150 nmol ligand.

In order to ascertain that binding of the exchange protein is caused by the phosphatidylcholine ligand, columns were prepared from AH-Sepharose 4 B containing free and acetylated amino-groups. It was found that the free amino-groups caused considerable binding of the exchange protein; binding was virtually eliminated after acetylation of the AH-Sepharose 4 B. Acetylation of the unreacted amino-groups of phosphatidylcholine-AH-Sepharose 4 B, however, had no adverse effect on the binding capacity of the affinity resin. This indicates that phosphatidylcholine functions as a ligand for the exchange protein.

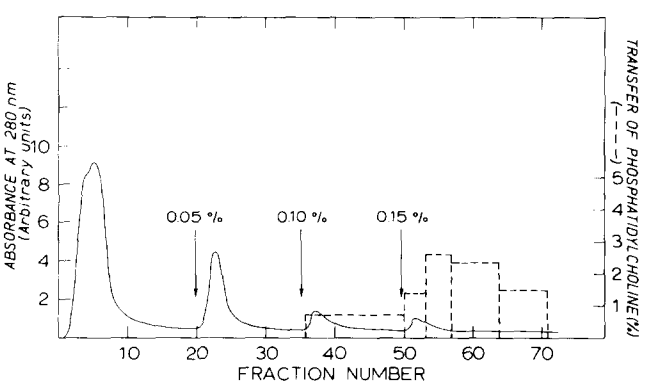

Fig. 2. Affinity column chromatography of phosphatidylcholine exchange protein. Column (0.65 X 4 cm) contained 1.6 ml of packed acetylated phosphatidylcholine-AH-Sepharose 4 B (1.9 μmol phosphatidylcholine) in 0.01 M sodium phosphate/0.001 M EDTA (pH 7.0). Crude exchange protein (43 mg) was applied and the column eluted stepwise with 0.01 M sodium phosphate/0.001 M EDTA (pH 7.0), 0.05% (w/v), 0.10% and 0.15% sodium deoxycholate in the phosphate/EDTA buffer. The arrows indicate where the composition of the elution buffer was changed. Flow rate, 30 ml/h. Fractions of 2 ml were collected at 4°C. Protein was measured by absorbance at 280 nm (———) and 0.1 ml aliquots of pooled fractions were assayed for phosphatidylcholine transfer activity (- - - - - -).

TABLE I

PURIFICATION OF PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN FROM BOVINE LIVER

Fraction numbers relate to fractions in Fig. 2. Specific activity is expressed as nmol phosphatidylcholine transferred/min per mg protein at 25°C.

Fraction	Volume (ml)	Protein (mg)	Specific activity	Recovery (%)	Purification factor
Crude protein	5	43.0	6	100	1
37—50	28	1.2	49	22	8
51—53	6	0.4	63	10	10
54—67	8	0.4	156	24	26
58—71	28	0.2	780	62	130

Purification of phosphatidylcholine exchange protein

A crude exchange protein fraction (43 mg protein) was applied to a column of acetylated phosphatidylcholine-AH-Sepharose 4 B (1.6 ml packed volume). It is seen from Fig. 2 that the phosphatidylcholine transfer activity was retained by the affinity resin under conditions where approximately 85% of the contaminating protein did not bind. The exchange protein was released by addition of sodium deoxycholate to the elution buffer; the bulk of the activity eluted with 0.15% sodium deoxycholate. Determination of activity on pooled fractions indicated that the recovery exceeded 100% with approximately 60% of the activity in pool 58—71 (Table I). This pool contained 0.2 mg of protein and was purified 130-fold relative to the crude protein fraction; this would correspond to 780-fold relative to the pH 5.1 supernatant [8]. It is of interest to note that after elution with 0.15% sodium deoxycholate the transfer activity did not coincide with the protein peak.

Starting from a pH 5.1 supernatant a 2800-fold purification is required to obtain a homogeneously pure exchange protein [8]. This implies that affinity chromatography under the conditions employed, gives rise to a partial purification only. This was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Electrophoresis of pool 58—71 indicated that still a considerable number of contaminating proteins were present.

In some experiments the exchange protein was released from the affinity resin by use of a linear gradient of sodium deoxycholate (0—0.3%). It was found that the bulk of the transfer activity eluted between 0.15 and 0.2% sodium deoxycholate. Although the peak of the contaminating protein eluted at 0.075% sodium deoxycholate, purification of the exchange protein was comparable to that observed by stepwise elution.

Discussion

The phosphatidylcholine exchange protein from bovine liver contains one molecule of non-covalently bound phosphatidylcholine [5]. Upon interaction with a membrane the protein exchanges its endogenous lipid molecule for a phosphatidylcholine molecule from the interface [5]. The present study demonstrates that the protein interacts also with phosphatidylcholine covalently bound to a solid support. This agrees with previous evidence that the protein

has a site that recognizes and accomodates the phosphorylcholine head group [6]. As yet, we do not know whether the endogenous phosphatidylcholine remains on the protein upon binding to the affinity resin or is released into the hydrophobic interface of the resin.

Phosphatidylcholine can be used as ligand to isolate a highly active, 780-fold purified phosphatidylcholine exchange protein fraction by affinity chromatography. In a previous study [14] it was observed that sodium deoxycholate above the critical micelle concentration (>0.1%) delipidated the exchange protein without affecting the transfer activity. It is under similar conditions that sodium deoxycholate releases the exchange protein from the affinity resin. This suggests that micelles of sodium deoxycholate need to be present to disrupt the exchange protein-ligand complex.

It is of interest to note that the bulk of the contaminating proteins that are bound to the affinity resin, are eluted by sodium deoxycholate ahead of the exchange protein. Most of these proteins will be aspecifically adsorbed onto the hydrophobic interface of the affinity resin [15,16]. The fact that only a partial purification of the exchange protein is obtained may, in part, be explained by the aspecific hydrophobic binding of these proteins, the elution of which coincides with the elution of the exchange protein. This aspecific adsorption affects the binding capacity of the phosphatidylcholine-AH-Sepharose 4 B and, therefore, limits its use to purify the exchange protein at a large scale. Preliminary experiments, however, have indicated that this affinity resin discriminates between the exchange protein and a contaminating protein which is sometimes present after the last step of the standard purification procedure [8]. In addition, to its potential use in a modified purification procedure, the affinity resin offers the possibility of establishing how chemical modifications will affect the binding site of the exchange protein.

Eukaryotic cells contain a variety of phospholipid exchange proteins with different affinities for the polar head groups of phospholipids [17—19]. It is currently investigated whether phosphatidylcholine-AH-Sepharose 4 B discriminates between these various proteins.

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