Synthesis and Use of a Lysolecithin Analog for the Purification of UDP-Glucuronosyltransferase

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Because of their high cost, lysolecithins are generally not considered useful detergents for the purification of membrane-bound enzymes. Therefore, we have synthesized a structural analog of lysolecithin with similar physical properties for which synthesis is straightforward. This analog is 1-palmitoylpropanediol-3-phosphocholine. To compare the efficacy of the two detergents for the purification of a membrane-bound enzyme, we have purified UDP-glucuronosyltransferase from pig liver microsomes using lysophosphatidylcholine or the synthetic analog. The catalytic properties of UDP-glucuronosyltransferase purified with 1-palmitoylpropanediol-3-phosphocholine or lysolecithin were identical. Sodium dodecyl sulfate-gel electrophoresis indicated that the purity of the UDP-glucuronosyltransferase preparation was the same whether lysophosphatidylcholine or its synthetic analog was used. The advantage of using 1-palmitoylpropanediol-3-phosphocholine in preference to lysophosphatidylcholine is that the former can be synthesized for about 1% the cost of the latter. In addition, the method for synthesis of 1-palmitoylpropanediol-3-phosphocholine is general in that the structural features of the polymethylene chain can be varied, allowing for the inexpensive synthesis of a series of detergents.

MATERIALS AND METHODS

Materials

UDP-glucuronic acid (ammonium salt), p-nitrophenol, and lysophosphatidylcholine were purchased from Sigma (St. Louis, MO). Monopalmitoyl-1,3-propanediol was prepared as described in Ref. (4). Phosphorus oxychloride was purchased from Merck (Darmstadt, FRG) and purified by distillation (bp 106–107°C). N-Methylethanolamine, triethylamine, and dimethyl sulfate were obtained from Fluka (Buchs, Switzerland). Reagent grade solvents were purchased from Baker (Deventer, The Netherlands).

Purification of integral membrane proteins is a necessary first step for detailed studies of their function and especially for investigating the consequences of interactions between these proteins and the lipids that comprise the environment in membranes. Purification depends, in turn, on removing the protein of interest from its membrane, which often can be accomplished by converting the membrane bilayer to a mixed micelle with components of the original membrane plus an added detergent. The choice of detergent for this process is important since it must be a suitable matrix for the enzyme as it is purified selectively from other membrane components. The detergent should not destabilize the enzyme, which could lead to an irreversible loss of activity during purification. We routinely have purified an isoform (GT39) of the liver microsomal enzyme UDP-glucuronosyltransferase using lysophosphatidylcholine as the detergent (1,2). Lysophosphatidylcholine provides the enzyme with an environment in which it is both active and stable (1–3). However, lysophosphatidylcholine is not a popular detergent for this purpose because of its high cost. We report in this paper a method for the synthesis of a 2-deoxy analog of lysophosphatidylcholine, 1-palmitoylpropanediol-3-phosphocholine (Fig. 1), which can be prepared easily in 200-g amounts as can other analogs differing in the chain length of the fatty acid residue. The use of 1-palmitoylpropanediol-3-phosphocholine in the purification of UDP-glucuronosyltransferase is compared with the published method using lysophosphatidylcholine.

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Methods

Preparation of microsomes. Microsomes were prepared from frozen pig liver (5). The protein concentration was determined by the biuret method (6).

Purification and delipidation of UDP-glucuronosyltransferase. Microsomes suspended in 0.25 M sucrose were diluted with 0.1 M Tris (pH 8.0 at 4°C) to 15–20 mg protein/ml. Triton X-100 was added to the homogenate to 0.2% (w/v) using 3 ml of a 10% (w/v) detergent solution. The mixture was sonicated as described above, on ice. The mixture was loaded onto a 500-ml bed of Whatman DE-52 gel, previously equilibrated with 10 mM Tris (pH 8.0, 4°C), in a 16-cm Büchner funnel. The gel was washed with 10 mM Tris (pH 8.0) and 100-ml fractions were collected. The fractions with the highest enzyme activity were pooled (approximately 700 ml). The pooled fractions were poured through a 150-ml sintered glass funnel. The CM-25 gel was washed with 100 ml of 40 mM NaCl in 20 mM Pipes, pH 7.0, at 4°C, and the enzyme was eluted with 150 ml of 250 mM NaCl in 20 mM Pipes, pH 7.0, at 4°C. Fractions of 25 ml were collected. The fractions with high activity (60–80 ml) were pooled and dialyzed against 2 liters of 20 mM Tris, pH 8.0, at 4°C overnight. Spectra/Por 2 dialysis tubing was used as above. The enzyme solution was stored at −70°C until needed.

The dialysate was concentrated to 0.5–1.0 mg/ml using an Amicon stirred cell with an XM-50 membrane. Tris (pH 8.0), diethioerythritol, EDTA (sodium salt, pH 8.0), sodium cholate, and glycerol were added to the concentrated CM-25 dialysate and adjusted to concentrations of 5 mM Tris, 0.1 mM dithioerythritol, 2 mM EDTA (sodium salt), 0.5% (w/v) sodium cholate, and 10% (v/v) glycerol. The mixture was then applied to a 50-ml column of hydroxyapatite (Bio-Rad), equilibrated with the same buffer. This step exchanges cholesterol for other detergents and delipidates the enzyme. The hydroxyapatite column was washed with 100 ml of 90 mM potassium phosphate (pH 8.0, 4°C) in the same buffer. Fractions of 180 drops were collected and the first protein peak was eluted. A second protein peak, containing the GTzp type of enzyme, was eluted with a 90–350 mM potassium phosphate gradient (100 × 100 ml, pH 8.0, 4°C) in the same buffer. The fractions with enzyme contained no phospholipid. Fractions with enzyme activity were pooled and dialyzed for 4 hours against 1 liter of the above buffer containing 125 mM NaCl. Fractions with the highest enzyme activity were pooled (600–800 ml) and dialyzed overnight against 12 liters of 10 mM Tris (pH 8.0, 4°C). Spectra/Por 2 dialysis tubing was used as above.

Egg lysophosphatidylcholine (1.2 g) or Compound II (1.2 g) was added to 800 ml of DEAE-CL-6B dialysate. The mixture was sonicated as described above, on ice. The mixture was loaded onto a 500-ml bed of Whatman DE-52 gel, previously equilibrated with 10 mM Tris (pH 8.0, 4°C), in a 16-cm Büchner funnel. The gel was washed with 10 mM Tris (pH 8.0) and 100-ml fractions were collected. The fractions with the highest enzyme activity were pooled (approximately 700 ml). The pooled fractions were poured through a 100-ml bed of CM-25 gel (prewashed with 20 mM Pipes [1,4-piperazinediethanesulfonic acid], pH 7.0, at 4°C), purchased from Sigma, in a 150-ml sintered glass funnel. The CM-25 gel was washed with 100 ml of 40 mM NaCl in 20 mM Pipes, pH 7.0, at 4°C, and the enzyme was eluted with 150 ml of 250 mM NaCl in 20 mM Pipes, pH 7.0, at 4°C. Fractions of 25 ml were collected. The fractions with high activity (60–80 ml) were pooled and dialyzed against 2 liters of 20 mM Tris, pH 8.0, at 4°C overnight. Spectra/Por 2 dialysis tubing was used as above. The enzyme solution was stored at −70°C until needed.

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Enzyme assays. UDP-glucuronosyltransferase activity was measured at 30°C with p-nitrophenol as aglycon.
by monitoring the change in absorbance at 400 nm (2). Assay mixtures contained 0.05 mM p-nitrophenol, 1 mM MgCl₂, 50 mM Tris (pH 7.5) in addition to UDP-glucuronic acid. In experiments using lysophosphatidylcholine, the enzyme was mixed with lipid in a ratio of 1:5 (w/w) and then assayed immediately. This ratio of protein to lipid has been shown previously to yield maximum activity.

Polyacrylamide gel electrophoresis. Following treatment with SDS, the enzyme was subjected to electrophoresis in a 10–15% continuous gradient of polyacrylamide gel (Phast System, Pharmacia). The protein was stained with Coomassie blue.

RESULTS AND DISCUSSION

Synthesis of 1-Palmitoylpropanediol-3-phosphocholine

Monopalmitoyl-1,3-propanediol (0.45 mol; 141.5 g) and triethylamine (0.79 mol; 80 g) were dissolved in 800 ml tetrahydrofuran. The solution was added dropwise with vigorous stirring to phosphorus oxychloride (0.52 mol; 79.4 g), precooled in an ice–water bath. The temperature never exceeded 15°C. The reaction was complete within minutes, as shown by thin-layer chromatography. The 1-palmitoylpropanediol-3-phosphoric acid dichloride intermediate was not isolated but was converted directly to 1-palmitoyl-3-propanediol 2 oxo-1,2,3-oxazaphospholane by the dropwise addition of a solution of N-methylethanolamine (0.6 mol; 45 g) and triethylamine (0.83 mol; 84 g) in 800 ml of tetrahydrofuran. The temperature of the reaction mixture should not exceed 50°C. After completion of the reaction, Rₚ values in diethyl ether are 0.9 and 0.1 for monopalmitoyl-1,3-propanediol and the oxazaphospholane respectively. Triethylamine hydrochloride was removed by filtration and the filtrate was poured into a vigorously stirred solution of 400 ml of 1 N HCl. The oxazaphospholane ring opens specifically at the P–N bond under strongly acidic conditions, leaving the P–O bond intact (7). The product formed by opening the phospholane ring, 1-palmitoylpropanediol-3-phospho-N-methylethanolamine, was extracted into chloroform by the addition of 1 liter chloroform and 1 liter water. The chloroform layer was evaporated to dryness and the intermediate N-methylethanolamine was converted to 1-palmitoylpropanediol-3-phosphocholine by methylolation using slightly alkaline conditions, as follows.

The residue after evaporation of chloroform consists primarily of 1-palmitoylpropanediol-3-phospho (N methyl)ethanolamine (Rₚ 0.7 in CHCl₃:CH₃OH:acetic acid:water, 100:60:20:5). This is dissolved in a mixture of 300 ml CH₂Cl₂ and 900 ml 2-propanol. Dimethyl sulfate (0.9 mol; 110 g) was added and methylation started by dropwise addition of K₂CO₃ (0.8 mol; 110 g) and KHCO₃ (0.4 mol; 40 g) in 200 ml of water. The precipitate was removed by filtration; 900 ml water, 900 ml CH₃OH, and 900 ml CHCl₃ were added to the filtrate and the mixture was shaken. After phase separation, the lower chloroform phase contains the product (Rₚ 0.2 in the above system). The solvent was removed by evaporation. Since the Rₚ value of the N-methyl intermediate in the above solvent system is 0.7, it is easily resolved from the...
end product. No N-methylphosphoethanolamine was detected in the chloroform layer. Hence it was converted quantitatively to the final product. The crude product was dissolved in 300 ml chloroform. Acetone (1.5 liter) was added to the solution. The precipitate was collected and washed with pentane. Elemental analysis for C_{24}H_{50}NO_{6}P \cdot 1H_{2}O, M, 497.65 (expected/found): C, 57.93/57.89; H, 10.53/10.58; N, 2.82/2.81; P, 6.22/6.24. The yield of chromatographically pure product was 140 g or 63% based on the starting monopalmitoyl-1,3-propanediol. A comparison of the physical properties of 1-palmitoylpropanediol-3-phosphocholine and palmitoyl lysophosphatidylcholine shows similar CMCs (1 \times 10^{-6} and 5.6 \times 10^{-6} mM, respectively) (8), surface areas (38 and 40 Å²/molecule, respectively) (9), and concentrations needed for the lysis of red cells (10).

Comparison of the Properties of GT_{2p}-Purified from Microsomes with Lysophosphatidylcholine or 1-Palmitoylpropanediol-3-phosphocholine

In order to determine whether the catalytic function of GT_{2p} was affected by purification with Compound II (1-palmitoylpropanediol-3-phosphocholine), enzyme activity was determined for the enzyme purified using Compound I (lysophosphatidylcholine) or II as detergent to disperse the enzyme. These data are plotted in Fig. 2 in double reciprocal form. The kinetics are Michaelis-Menten for the delipidated enzyme, which is the expected result (1,2). The specific activity of enzyme obtained with Compound II was identical with the enzyme purified with lysolecithin. In addition, the enzyme purified with Compound II responded normally to activation with egg lysophosphatidylcholine. These results demonstrate that GT_{2p} purified with Compound II was functionally identical with the enzyme purified with lysolecithin.

To confirm that Compounds I and II yielded enzymes with equal purity, gel electrophoresis of the SDS-treated enzyme was performed. As shown in Fig. 3, the enzyme purified with Compound II displayed the same pattern as the enzyme purified with Compound I.

In summary, 1-palmitoylpropanediol-3-phosphocholine, a 2-deoxy analog of lysophosphatidylcholine, was synthesized and used to purify UDP-glucuronosyltransferase from pig microsomes. This compound or an analog differing in the chain length of the fatty acid residue is likely to be a useful alternative to lysophosphatidylcholine for the purification of other integral membrane proteins. Thus, the 1-acylpropanediol-3-phosphocholines could provide a new family of detergents for the purification of membrane-bound enzymes.

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