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CHARACTERIZATION OF RECONSTITUTED PARTIALLY PURIFIED GLYCEROPHOSPHATE ACYLTRANSFERASE FROM *ESCHERICHIA COLI*

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A modification of the method of Snider and Kennedy (J. Bacteriol. (1977) 130, 1072–1083) was worked out to solubilize *sn*-glycero-3-phosphate acyltransferase from whole cells by Triton X-100. The solubilized preparation was used for a systematic study of the reconstitution of enzymatic activity as observed by addition of phospholipid vesicles. Although enzymatic activity was regained by addition of vesicles and not by addition of multilayered liposomes, subsequent Sepharose 4B chromatography revealed the enzyme to be incorporated in large lipid aggregates of undefined structures. Incorporation of glycerophosphate acyltransferase in single bilayer vesicles composed of phosphatidylcholine and phosphatidylglycerol (4:1) was obtained after removal of Triton X-100 from the enzyme solution, co-dispersion of enzyme and phospholipids with cholate and Sephadex G-50 gel filtration of this mixture to remove cholate. The optimal conditions for this reconstitution procedure with respect to phospholipid/protein and phosphatidylcholine/phosphatidylglycerol ratio were established. The active site of glycero-3-phosphate acyltransferase in the reconstituted system was localized for at least 90% at the outside surface of the vesicle, as revealed by proteolysis experiments under conditions of vesicle intactness as shown by ^{13}C -NMR experiments. The reconstituted systems produced only lysophosphatidate from *sn*-[^{14}C]glycero-3-phosphate and palmitoyl-CoA and showed identical apparent K_m for *sn*-glycero-3-phosphate and identical pH- and temperature-dependencies as the enzyme in isolated *Escherichia coli* membranes.

Introduction

The first step in glycerolipid biosynthesis in *Escherichia coli* involves the transfer of an acyl group from acyl-CoA or acyl-acyl carrier protein to *sn*-glycero-3-phosphate. The *sn*-glycero-3-phosphate acyltransferase condenses two water-soluble substrates to form a lipid-soluble, membrane-associated, product. The enzyme is firmly anchored in the inner membrane of *E. coli* [1,2] with its active site facing the cytoplasm [3]. Earlier reports described various aspects of glycerophosphate

acyltransferase, using crude membrane preparations [4–13]. More recently, partially purified preparations have been obtained [14,15] following solubilization of the enzyme with the detergent, Triton X-100. Once solubilized, the enzyme is virtually inactive, but can be reactivated simply by mixing with phospholipids [14,15]. This paper describes a modification of the procedure of Snider and Kennedy [15] to obtain a partially purified glycerophosphate acyltransferase and focuses on the type of lipid structures that result from the reconstitution process. In addition, we describe a different reconstitution procedure, based on the cholate-dispersion method described by Brunner et al. [16], which yields better-defined lipid structures. A comparison of some properties of the

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol.

glycerophosphate acyltransferase in isolated *E. coli* membranes and the reconstituted system is made. During the course of this work Bell and coworkers [17,18] reported on the complete purification of the glycerophosphate acyltransferase from an *E. coli* strain bearing a hybrid plasmid which resulted in an overproduction of the enzyme. The purified enzyme was characterized in detail by Bell and colleagues [19] using a reconstitution procedure similar to that described originally by Snider and Kennedy [15].

Materials and Methods

Materials

E. coli K12 frozen cells, silica gel G and deuterium oxide (minimum 99.75% $^2\text{H}_2\text{O}$, for NMR spectroscopy) were obtained from Merck, Darmstadt, F.R.G. Biobeads SM-2 are a product of Bio-Rad Lab., Richmond, U.S.A. Sodium cholate was obtained from Fluka AG, Buchs, Switzerland. Bovine trypsin and trypsin inhibitor from soybean were obtained from Boehringer, Mannheim, F.R.G. Triton X-100 was from Rohm and Haas, U.S.A. ^3H -labeled Triton X-100 (0.22 mCi/g) was kindly donated by Dr. W. Gerritsen. [2,4(n)- ^3H]Cholic acid (spec. radioact., 14 Ci/mmol) was obtained from New England Nuclear, Boston, U.S.A., whereas L-[U- ^{14}C]glycero-3-phosphate (spec. radioact. 177 mCi/mmol) was a product of The Radiochemical Centre, Amersham, U.K. Palmitoyl-CoA was synthesized according to the method of Al-Arif and Blecher [20]. Sephadex G-50 (fine) and Sepharose 4B were products from Pharmacia, Sweden.

Lipids

Lipids from thawed *E. coli* K12 cells were extracted according to the method of Bligh and Dyer [21] and stored in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4:1, v/v) under nitrogen at -20°C . Egg phosphatidylcholine, purified by HPLC, was kindly donated by Mr. W. Geurts van Kessel. Phospholipase D-catalyzed transphosphatidylation was used to convert this to egg phosphatidylglycerol, which was purified by CM-cellulose chromatography as described by Comfurius and Zwaal [22]. Dipalmitoylphosphatidyl[*N*-methyl- ^{14}C]choline (spec. radioact. 58 mCi/mmol) was obtained from The Radiochemi-

cal Centre, Amersham, U.K. Dioleoylphosphatidyl[*N*-methyl- ^{13}C]choline was prepared as described [23] and kindly donated by Dr. B. De Kruijff. [9,10- $^3\text{H}_2$]Palmitic acid (spec. radioact., 23.3 Ci/mmol) was bought from New England Nuclear, Boston, U.S.A.

Methods

Solubilization of glycerophosphate acyltransferase. Glycerophosphate acyltransferase was solubilized and partially purified from *E. coli* K12 cells by a modification of the procedure of Snider and Kennedy [15]. Cells were thawed and washed twice in 50 mM Tris-HCl (pH 8.4) containing 5 mM β -mercaptoethanol and 5 mM MgCl_2 at 0°C (1 g cells/10 ml). After the second wash, the cells were resuspended (1 g/5 ml) in 25 mM Tris-HCl (pH 8.4) containing 5 mM β -mercaptoethanol, 5 mM MgCl_2 , 20% (v/v) glycerol and 0.05% (w/v) Triton X-100. Cells (25-ml aliquots) were broken by sonication at 15-s intervals for a total of 5 min under nitrogen with a Branson Sonifier at 70 W with cooling in an ice/water bath. Residual particulate matter was collected by centrifugation at $100\,000 \times g$ for 60 min. The pellet was resuspended in the same volume of buffer as described above but now containing 0.2% (w/v) Triton X-100. After a 15 min incubation at 0°C the mixture was centrifuged again at $100\,000 \times g$ for 60 min. Glycerophosphate acyltransferase activity was recovered almost completely in the supernatant, which contained about 1 mg protein/ml. This supernatant was stored at -40°C .

Preparation of liposomes and vesicles. Appropriate amounts of lipids in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4:1, v/v) were mixed. The solvents were evaporated under nitrogen and residual traces were removed under vacuo overnight. To the dry lipid film 0.1 M Tris-HCl (pH 8.4), 5 mM β -mercaptoethanol, 100 mM NaCl and 20% (v/v) glycerol was added (10 μmol lipid P/ml) and liposomes were formed by vigorous mixing for 2 min at 0°C . When sonicated vesicles had to be prepared these suspensions were sonicated for 30 min at 70 W under nitrogen at 0°C . Lipid aggregates and metal particles were removed by centrifugation at $160\,000 \times g$ for 30 min. The clear supernatant was stored at 4°C and used the same day.

Membrane preparation. Membranes were prepared from thawed cells as described previously [3].

Reconstitution procedures

Simple mixing. Glycerophosphate acyltransferase was reconstituted by gentle mixing of 1 vol. of solubilized glycerophosphate acyltransferase solution and 1–4 vol. of lipid suspension at 0°C as described by Snider and Kennedy [15]. From these mixtures 50- μ l aliquots were taken for assay of glycerophosphate acyltransferase activity.

Removal of Triton X-100 by Bio-Beads SM2. Reconstitution was carried out as described by Gerritsen et al. [24] for erythrocyte band 3 protein. Briefly, solubilized glycerophosphate acyltransferase was added to a dried lipid film at a lipid/Triton X-100 ratio of 10:1 (w/w). After mixing for 30 s, Bio-Beads SM2 (300 mg/ml) were added and the mixture was incubated for 18 h at 4°C. Beads were removed by centrifugation.

Cholate dispersion and gel filtration. For this reconstitution method the solubilized glycerophosphate acyltransferase was first freed of Triton X-100 by treatment with Bio-Beads SM2 (300 mg/ml) for 18 h at 4°C as described [25]. In initial experiments ^3H -labeled Triton X-100 was added to calculate residual concentrations. To 15 μ mol of dried lipid 0.9 ml of the Triton-depleted glycerophosphate acyltransferase solution and 23 mg of cholate (stock solution, 300 mg/ml water) was added. In some experiments trace amounts of highly labelled phosphatidyl[*N*-methyl- ^{14}C]choline and/or [2,4(n) ^3H]cholic acid were added to monitor phospholipid and cholate elution, respectively. After gentle mixing for 60 min at 4°C, a clear suspension was obtained, which was passed over a Sephadex G-50 (fine) column (60 \times 1 cm) equilibrated and eluted with 85 mM Tris-HCl (pH 8.4) containing 5 mM β -mercaptoethanol, 1 mM MgCl_2 , 80 mM NaCl and 20% (v/v) glycerol. 1.75-ml fractions were collected at a flow rate of 2.3 ml/h.

Characterization of lipid structures. Vesicle dispersions were sized on a Sepharose 4B column (45 \times 2 cm), equilibrated and eluted with the same buffer as used for the Sephadex G-50 column. 4.0-ml fractions were collected at a flow rate of 8 ml/h. Void and internal volumes were determined

with Blue Dextran 2000 and potassium dichromate, respectively.

Freeze-fracture electron microscopy was performed on samples frozen from 4°C, with 25% glycerol as cryoprotectant and fractured at –100°C according to established procedures [26].

Discontinuous sucrose-gradient centrifugation of reconstituted glycerophosphate acyltransferase was carried out as described by Low and Zilversmit [27].

For NMR experiments egg phosphatidylcholine was replaced by dioleoylphosphatidyl[*N*-methyl- ^{13}C]choline. Sonicated and gel-filtrated vesicles were prepared in 10 mM Tris-HCl (pH 7.0) containing 80 mM NaCl, 5 mM β -mercaptoethanol and 1 mM MgCl_2 . NMR experiments were carried out similarly as described [23]. After addition of 20% (v/v) of the $^2\text{H}_2\text{O}$ analogue of the buffer a 1.5 ml sample was transferred to a 10 mm NMR tube. ^{13}C -NMR measurements were done at 50.3 MHz using a Bruker WP 200 wide-bore spectrometer. Spectra were recorded at 30°C from 350 transients using 18 μ s 90° radiofrequency pulses, a 2.5 kHz sweepwidth, 4000 datapoints and gated decoupling. The interpulse time was 5 s and the broad band ^1H decoupling (input power 2 W) was on during the 0.8192 s acquisition time. Peak intensities were determined by computer integration with respect to a dioxane reference. Spectra were recorded first in the absence and then in the presence of 10 mM Mn^{2+} .

Trypsin treatment of reconstituted glycerophosphate acyltransferase. To 2 ml of a suspension of reconstituted glycerophosphate acyltransferase vesicles was added 100 μ l of a 2 mg/ml trypsin solution in 1 mM CaCl_2 and 10 mM KCl adjusted to pH 3.0. Controls received the same solution without trypsin. The mixtures were incubated at 25°C. At the indicated times, 100- μ l samples were withdrawn and proteolytic action was stopped by addition of soybean trypsin inhibitor (trypsin: inhibitor = 1:2, w/w). After 120 min a 1 ml sample, after addition of inhibitor, was sonicated at 0°C for 30 or 60 s, respectively. A sample to which substrates as in the standard glycerophosphate acyltransferase assay were added before sonication was treated similarly.

Glycerophosphate acyltransferase activity assay. Glycerophosphate acyltransferase activity was

determined according to the method of Snider and Kennedy [15] as described in detail previously [3]. For analysis of lipid products the reaction mixtures were extracted [21] under acidic conditions. Lipids were separated on silica gel G thin-layer plates impregnated with oxalic acid [28] using $\text{CHCl}_3/\text{CH}_3\text{OH}/10\text{ N HCl}$ (87:13:0.5, v/v) as developing solvent. Spots were detected by iodine vapour, identified by comparison with standards and scraped into Packard Emulsifier scintillation fluid for measurement of radioactivity in a Packard TriCarb liquid scintillation spectrometer.

Analytical procedures. Protein was measured by the procedure of Ross and Schatz [29] or Peterson [30] with bovine serum albumin as standard. Lipid phosphorus was determined as described previously [31].

Results and Discussion

Solubilization of glycerophosphate acyltransferase. Glycerophosphate acyltransferase of *E. coli* can be almost quantitatively solubilized from isolated membranes by Triton X-100 [15,19]. In initial studies we noticed, however, that during the preparation of membranes from whole cells by sonication large amounts (up to 70%) of the glycerophosphate acyltransferase activity were recovered in the cells debris fraction. Therefore, we modified the procedure by combining the cell disruption step and the first extraction of the membranes at low (0.05%, w/w) Triton X-100 concentration [15] as described in Materials and Methods. Table I shows the result of a typical experiment and indicates that the modified procedure solubilizes the glycerophosphate acyltransferase

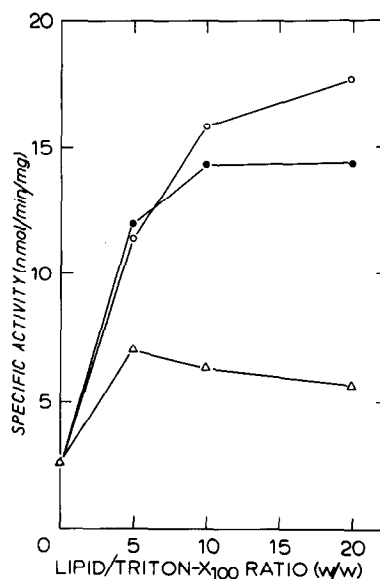


Fig. 1. Reconstitution efficiency of different lipids. Solubilized glycerophosphate acyltransferase was reconstituted by mixing with increasing amounts of vesicles prior to assay of enzymatic activity. Vesicles were prepared by sonication and consisted of total *E. coli* lipids (○), PC/PG (4:1, molar ratio, ●) or PC (Δ).

ase activity in high yield and with a 25-fold increase in specific activity. However, the final specific activity obtained by our procedure is lower than that achieved by Snider and Kennedy [15] after extraction of glycerophosphate acyltransferase from membranes, due to the lower specific activity of the starting material. The solubilized enzyme stored in detergent solution at -40°C remained stable for at least 12 months. In agreement with results obtained by Snider and Kennedy [15] the enzyme appeared extremely labile during attempts to purify it further by ion-ex-

TABLE I

SOLUBILIZATION OF GLYCEROPHOSPHATE ACYLTRANSFERASE FROM *E. COLI* K12

Glycerophosphate acyltransferase was solubilized from *E. coli* K12 cells by a combination of sonication and a two-step extraction with Triton X-100. Values, obtained after reconstitution of the fractions with phospholipid, are expressed per g wet cells.

Fraction	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)
Wet cells	130	75	0.58
0.05% Triton X-100, pellet	28	91	3.25
0.2% Triton X-100, supernatant	5.9	90	15.3

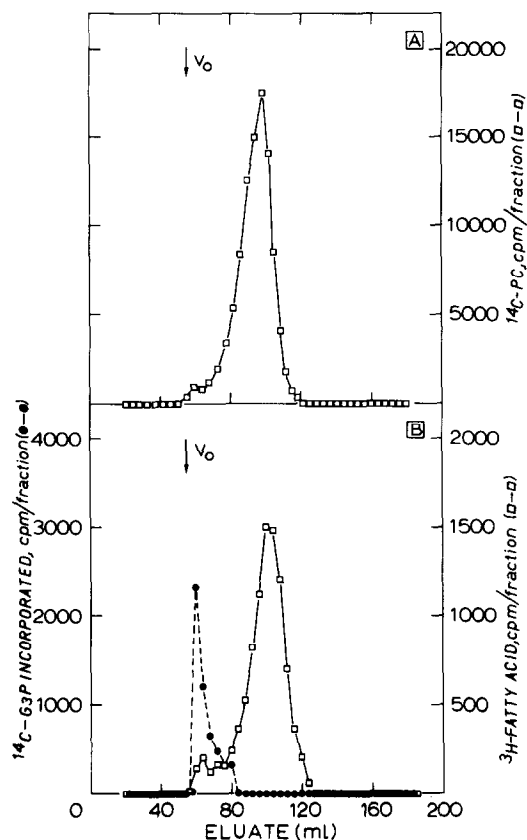


Fig. 2. Sepharose 4B column chromatography of sonicated vesicles and reconstituted glycerophosphate acyltransferase. In A, sonicated vesicles (PC/PG, 4:1) were chromatographed. Lipid elution was monitored by ^{14}C radioactivity from a trace amount of dipalmitoylphosphatidyl[*N*-methyl- ^{14}C]choline added prior to vesicle preparation. Lipid recovery was 93%. In B, a vesicle population prepared by mixing solubilized glycerophosphate acyltransferase and sonicated vesicles (PC/PG, 4:1) followed by Bio-Beads treatment was applied. Lipid was monitored by addition of a trace amount of [9,10- $^3\text{H}_2$]palmitate prior to vesicle formation. Recoveries were 86% for lipids and 33% for glycerophosphate acyltransferase activity. G3P, glycerol-3-phosphate.

change chromatography, gel filtration, hydrophobic interaction chromatography, affinity chromatography [14] and isoelectric focussing under a variety of conditions. Also the procedures worked out by Bell and coworkers [18,19] to obtain homogeneous glycerophosphate acyltransferase from constructed *E. coli* strains that overproduce the enzyme about 15-fold, when applied to wild-type *E. coli* led to unacceptable losses of enzymatic activity. Thus, glycerophosphate acyltransferase

from wild-type *E. coli* is still only present in partially purified preparations of relatively low specific activity.

Reconstitution and characterization. Upon solubilization, glycerophosphate acyltransferase loses most of its activity, which can be regained to various extents by addition of phospholipid vesicles [15,19]. In all preparations we found about 10–15% residual activity, which could be stimulated by addition of *E. coli* phospholipid vesicles (Fig. 1). Others [15,19] have found that optimal activity was obtained by addition of mixtures of phosphatidylethanolamine with phosphatidylglycerol and/or cardiolipin. However, the physical chemistry of and the lipid structures present in these dispersions are not well understood and are subject to variation in response to changes in temperature and bivalent cation concentrations [32–35]. Thus, it has been shown by ^{31}P -NMR spectroscopy and freeze-fracture electron microscopy that dispersions of total *E. coli* lipids contain lamellar, isotropic and hexagonal lipid phases [35]. Phospholipids in isolated cytoplasmic membranes were mainly organized in a bilayer state at 25°C but a sizeable fraction experienced isotropic motion at 37°C. This fraction was much smaller, however, than observed in aqueous dispersions of total *E. coli* lipids. It is quite conceivable that the polymorphic phase behaviour of these lipid dispersions is one of the factors which determines their efficiency for glycerophosphate acyltransferase reconstitution. Yet, in view of the predominant bilayer state of the phospholipids in cytoplasmic *E. coli* membranes we attempted to reconstitute glycerophosphate acyltransferase activity by addition of phospholipids that prefer to adopt a bilayer phase rather than a hexagonal phase as preferred by phosphatidylethanolamine [34]. In line with previous reports [15,19] poor reconstitution of enzymatic activity was obtained in the presence of only phosphatidylcholine, but addition of sonicates composed of phosphatidylcholine (PC) and phosphatidylglycerol (PG) (4:1, mol/mol) led to acceptable, though slightly suboptimal, reconstitution (Fig. 1). It is noteworthy that multilayered liposomes of PC and PG in this molar ratio did not stimulate glycerophosphate acyltransferase activity. Sonicates of PC and PG in a molar ratio of 4:1 are known to form unilamellar vesicles [36].

This is confirmed by chromatography of the dispersed lipids on a Sepharose 4B column (Fig. 2A). More than 95% of the phospholipids elute in the vesicle peak, with only a few percent appearing as aggregated structures in the void volume peak. Addition of such a PC/PG vesicle preparation to solubilized glycerophosphate acyltransferase in 0.2% (w/v) Triton X-100 solution and subsequent removal of Triton X-100 by Bio-Beads SM2 treatment followed by Sepharose 4B filtration indicated that almost all glycerophosphate acyltransferase activity eluted at the void volume (Fig. 2B). This had been observed before by Snider and Kennedy [15] for enzyme reconstituted with phosphatidylcholine/cardiophilin (4:1, wt/wt) dispersions. To rule out the influence of Triton X-100 on the reconstitution process this detergent was first removed by treatment of the solubilized glycerophosphate acyltransferase with Bio-Beads SM2. Surprisingly, virtually complete (over 99.5%) removal of Triton X-100 from the enzyme preparation (see Materials and Methods) had no deleterious effect on reconstitutable glycerophosphate acyltransferase activity (data not shown). Treatment of such detergent-depleted enzyme with PC/PG vesicles gave upon Sepharose 4B filtration phospholipid and glycerophosphate acyltransferase activity elution profiles very similar to those observed in Fig. 2B, where Triton X-100 was removed after addition of lipid. Glycerophosphate acyltransferase activity was again associated almost exclusively with the phospholipid present in larger structures. Addition of an extra amount of lipid vesicles to the column fractions prior to assay of glycerophosphate acyltransferase activity did not reveal any non-lipid associated glycerophosphate acyltransferase peak (data not shown). Thus, it appeared that glycerophosphate acyltransferase had more affinity for the few percent larger structures than for the single bilayer vesicles. This is in line with the observation of Green et al. [19] that reconstituted purified glycerophosphate acyltransferase could be pelleted by centrifugation, but contrasts to the finding mentioned above that PC/PG liposomes did not stimulate glycerophosphate acyltransferase activity. This discrepancy was investigated further by separating the PC/PG sonicates into a void volume and a vesicle fraction by gel-filtration as in Fig. 2A

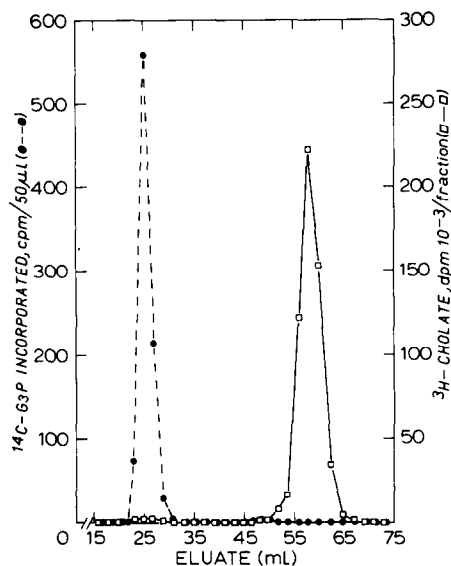


Fig. 3. Sephadex G-50 reconstitution of glycerophosphate acyltransferase. Solubilized glycerophosphate acyltransferase was reconstituted with PC/PG (4:1) via the cholate dispersion and gel filtration technique. Cholate distribution in column fractions was monitored by addition of [^3H]cholate prior to chromatography. Glycerophosphate acyltransferase activity was determined by assaying 50- μl fractions under standard assay conditions. Recoveries were 83% for lipid and 104% for protein. G3P, glycerio-3-phosphate.

and testing each fraction for its ability to reconstitute glycerophosphate acyltransferase activity. Only the small vesicles were able to do so, but subsequent sizing by the method used in Fig. 2 indicated that void volume material had been formed again and that glycerophosphate acyltransferase activity was associated at least 50% with the void volume aggregates (data not shown). Apparently, glycerophosphate acyltransferase requires small phospholipid vesicles for reconstitution, but during this reconstitution becomes associated with larger structures, perhaps as a consequence of fusion or aggregation. This property apparently persists with the purified enzyme which upon reconstitution with vesicles still formed lipid-protein structures that could be pelleted by centrifugation [19]. Since the aggregated lipids in the void volume fraction are of undefined structure, this reconstitution method of adding pre-formed vesicles to solubilized protein was abandoned.

Attempts were then made to reconstitute glycerophosphate acyltransferase by a method successfully applied to Triton X-100-solubilized band 3 protein from human erythrocytes [24] and ($\text{Na}^+ + \text{K}^+$)-ATPase from lamb kidney [37]. This method, based on dispersing a dry lipid film in the Triton X-100 protein solution, yielded single bilayer vesicles of diameters up to 200 nm with the above-mentioned enzymes. When applied to glycerophosphate acyltransferase, turbid solutions were obtained and reconstituted activity amounted to only 20% of that obtained by the method described above.

By dispersing phosphatidylcholine in cholate and removing the detergent by Sephadex G-50 gel filtration, Brunner et al. [16] developed a method to prepare single bilayer vesicles. This procedure has since also been used for the incorporation of various membrane proteins in phospholipid bilayers [38–42]. When Triton X-100-depleted glycerophosphate acyltransferase was mixed with phospholipids (PC/PG = 4:1) and cholate in a ratio of 1 mg protein:15 μmol phospholipid:53 μmol cholate a clear solution of mixed micelles was obtained. Upon gel filtration on Sephadex G-50, the void volume peak contained all glycerophosphate acyltransferase activity (Fig. 3),

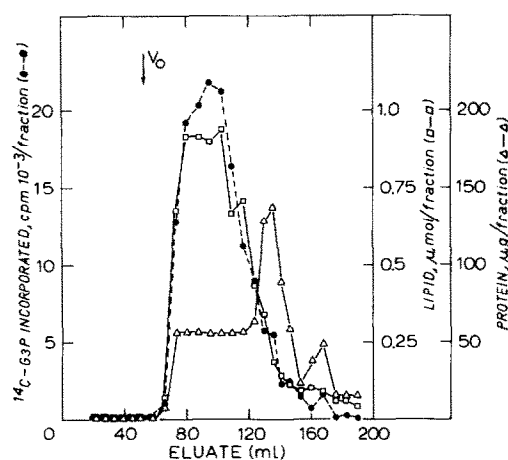


Fig. 4. Sephadex 4B chromatography of glycerophosphate acyltransferase reconstituted via the cholate dispersion and gel filtration technique. Combined Sephadex G-50 void volume fractions from an experiment similar to that of Fig. 3 were applied on a Sephadex 4B column. Recoveries were 69% for protein and 92% for lipid. G3P, glycerol-3-phosphate.

protein and phospholipid (data not shown) virtually free of cholate. The molar ratio of phospholipid to cholate in the void volume peak was 200:1. Freeze-fracture electron microscopy of the void volume material showed only small unilamellar vesicles of 25–30 nm. This and the close association of lipid and glycerophosphate acyltransferase activity in a rather homogeneous population of small vesicles was also seen upon sizing the Sephadex void volume peak on Sepharose 4B (Fig. 4). Interestingly, only part of the proteins become associated with the vesicles. The close association of lipid and glycerophosphate acyltransferase activity could be confirmed by discontinuous sucrose gradient centrifugation [27] where both entities distributed identically over high- and low-density fractions (data not shown). These results strongly suggest that the enzyme becomes incorporated in the vesicle membrane during cholate removal. Table II lists the lipid mixtures that have been

TABLE II

DEPENDENCY OF GLYCEROPHOSPHATE ACYLTRANSFERASE ACTIVITY ON LIPID MIXTURES AND LIPID/PROTEIN RATIO

Glycerophosphate acyltransferase was reconstituted with the indicated lipid mixtures at the indicated lipid/protein ratios via the cholate dispersion and gel filtration technique. The resulting Sephadex G-50 void volume peaks were assayed for glycerophosphate acyltransferase activity and protein. ^a, value represents endogenous lipid. In the course of these comparative experiments three preparations of enzyme reconstituted with PC/PG (4:1) at a lipid/protein ratio of 15.7 were used.

Lipid mixture	Lipid protein ($\mu\text{mol}/\text{mg}$)	Glycerophosphate acyltransferase activity (nmol/min per mg)
PC	15.7	5.5
Total <i>E. coli</i> lipids	15.7	15.4
PC/PG (4:1)	15.7	20.8
PC/PG/total <i>E. coli</i> lipids (7.2:1.8:1)	15.7	18.4
PC/PG (9:1)	15.7	8.5
PC/PG (4:1)	15.7	16.6
PC/PG (1:1)	15.7	10.9
None	0.3 ^a	5.4
PC/PG (4:1)	6.9	13.6
PC/PG (4:1)	15.7	21.6
PC/PG (4:1)	25.5	20.9

used to reconstitute glycerophosphate acyltransferase by the cholate dispersion method. Similarly to the direct mixing method as used by Snider and Kennedy [15] (compare Fig. 1) phosphatidylcholine alone is a poor reconstituent whereas total *E. coli* phospholipids and PC/PG (4:1) are about equally active. Incorporation of 10 mol% *E. coli* lipids in the PC/PG system did not give rise to a further stimulation of glycerophosphate acyltransferase activity, again demonstrating that phosphatidylethanolamine and cardiolipin are not absolutely required for regaining glycerophosphate acyltransferase activity. In view of the different capacities of PC and PC/PG mixtures to reconstitute glycerophosphate acyltransferase activity it is tempting to speculate that a negative surface charge is required. Due to the presence of the anionic phospholipid, differences in local pH at the membrane surface of PC and PC/PG membranes could have been introduced at identical bulk pH values. It has been shown for reconstituted D-lactate dehydrogenase from *E. coli* that this can give rise to different pH profiles and optima [43]. However, glycerophosphate acyltransferase reconstituted with either PC or PC/PG (4:1) showed identical activity-pH profiles (data not shown). The data in Table II further demonstrate that optimal reconstitution with PC/PG mixtures is achieved at lipid/protein ratios of about 15 $\mu\text{mol}/\text{mg}$ and at about 20 mol% phosphatidylglycerol. Under these conditions enzymatic activity is linear with time up to 25 min (5 μg protein) and with protein up to 8 μg (20 min incubation). Analysis of the lipid products formed indicated 99.2% of the recovered radioactivity to be present in lysophosphatidate. The remainder was present in phosphatidate (0.2%) and monoglyceride (0.6%). Therefore, the reconstituted system measures only the first enzyme of phospholipid biosynthesis.

Comparison of native and reconstituted glycerophosphate acyltransferase. Kito et al. [44] have reported that solubilization of glycerophosphate acyltransferase from *E. coli* membranes with Triton X-100 resulted in a 20-fold increase of the enzyme's K_m value for glycer-3-phosphate. It was suggested that removal of the native membrane matrix introduced a conformational change in the protein which could not be reversed by reconstitu-

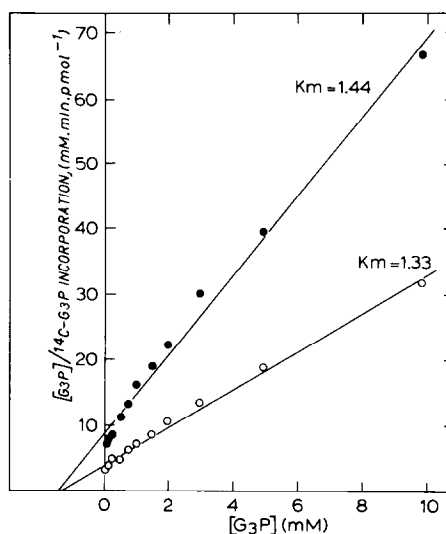


Fig. 5. Determination of apparent K_m values of glycerophosphate acyltransferase for glycer-3-phosphate (G3P) in native and reconstituted matrix. Glycer-3-phosphate concentration in otherwise standard assay mixtures was varied as indicated. Data were subjected to computer analysis to obtain best-fit curves and K_m values. Errors in the latter are within 10%. O, Isolated *E. coli* membranes, ●, enzyme reconstituted with PC/PG (4:1) by cholate dispersion and gel filtration.

tion with phospholipids. In contrast, Cronan and co-workers [13] have argued that glycerophosphate acyltransferase contains both a high- and a low-affinity site for glycer-3-phosphate and that possibly the high-affinity site might have been inactivated by solubilization. In Fig. 5 Hanes plots for the glycer-3-phosphate dependency of glycerophosphate acyltransferase in isolated membranes and reconstituted vesicles are shown. Although for unknown reasons the values found are considerably higher than those reported in the literature (see Refs 19 and 45 for reviews) it is obvious that no differences in apparent K_m were introduced by the solubilization and reconstitution procedure. Native and reconstituted glycerophosphate acyltransferase also showed similar pH and temperature dependency (Fig. 6).

Localization of the active site of reconstituted glycerophosphate acyltransferase. In line with expectation we have recently demonstrated [3] that the active site of glycerophosphate acyltransferase in *E. coli* is located at the inner aspect of the cytoplasmic membrane. We have next investigated

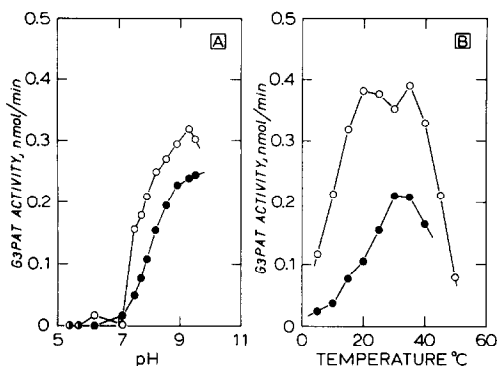


Fig. 6. Dependency of native and reconstituted glycerophosphate acyltransferase (G3PAT) on pH and temperature. In A, 20 μ l of enzyme preparation was mixed with 30 μ l 2 M Tris-maleic acid buffer to yield the indicated pH and containing 80 mM NaCl, 5 mM β -mercaptoethanol, 1 mM $MgCl_2$ and 20% glycerol. Then, a standard glycerophosphate acyltransferase assay was done on this mixture. In B, the temperature of standard assays was varied as indicated. ●, Isolated *E. coli* membranes; ○, reconstituted glycerophosphate acyltransferase.

whether glycerophosphate acyltransferase in the reconstituted system is incorporated in a symmetrical or asymmetrical way with respect to the two monolayers of the bilayer. Incubation of reconstituted glycerophosphate acyltransferase in the presence of trypsin resulted in complete inactivation of acyltransferase activity. Control incubations, either in the absence of trypsin (Fig. 7A) or with trypsin and trypsin-inhibitor showed only a 25% decrease in glycerophosphate acyltransferase activity, presumably due to thermal inactivation. These results suggested an outside surface localization of the active site of glycerophosphate acyltransferase. It remained possible, however, that part of the active sites were present at the inside surface but that these were not detected because they could not be reached by the added substrates. To investigate this possibility both trypsin-treated and control vesicles after addition of trypsin inhibitor were sonicated in the absence or presence of substrates (Fig. 7B). In the absence of substrates sonication had no effect. This indicates that glycerophosphate acyltransferase activity itself is not influenced by sonication and that no redistribution of glycerophosphate acyltransferase takes place during sonication. In the presence of substrates a small increase, equivalent to about 10% of

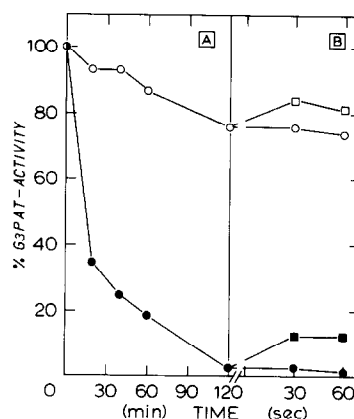


Fig. 7. Localization of the active site of glycerophosphate acyltransferase (G3PAT) in vesicle membranes. Reconstituted vesicular glycerophosphate acyltransferase was incubated in the presence (●) or absence (○) of trypsin. At the indicated times (panel A) samples were removed and after addition of trypsin-inhibitor were used for assay of glycerophosphate acyltransferase activity. At $t = 120$ min the remainder of the incubation mixture, after addition of trypsin inhibitor, was divided in two parts. These were sonicated (panel B) for 30 and 60 s in the absence (circles) or presence (squares) of substrates as under standard assay conditions. Aliquots of each sonicate were then assayed for glycerophosphate acyltransferase activity under standard assay conditions. Results are expressed as percent of initial glycerophosphate acyltransferase activity. The experiments in panel A were repeated four times with essentially the same results.

initial glycerophosphate acyltransferase activity, is seen in both control and trypsin-treated vesicles. We attribute this increase to abolition of latency of active sites originally located at the inside of the vesicles. From these results we conclude that at least 90% of the active sites in reconstituted glycerophosphate acyltransferase vesicles are present at the outside surface of the vesicle membrane. For correct interpretation of the proteolysis experiments it is necessary to demonstrate that the vesicle membrane is impermeable for the proteolytic enzyme. We have used the capacity of Mn^{2+} to broaden ^{13}C -NMR signals to discriminate between phospholipid present in the outer and inner monolayer in vesicles composed of 1,2-di-oleoylphosphatidyl[*N*-methyl- ^{13}C]choline and 20 mol% phosphatidylglycerol. It is known that PC and PG distribute in the same way over both monolayers of the vesicles such that about 65–70% of each is present in the outer monolayer [36].

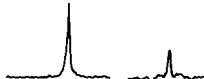


TYPE VESICLE	¹³ C-NMR SPECTRA UNTREATED 10 mM Mn ²⁺	% SIGNAL REMAINING
WITHOUT PROTEIN		30
WITH PROTEIN INCUBATED 120 min 25°C		24
WITH PROTEIN INCUBATED WITH TRYPSIN 120 min 25°C		31

Fig. 8. ¹³C-NMR spectra of reconstituted glycerophosphate acyltransferase vesicles. Dioleoylphosphatidyl[*N*-methyl-¹³C]choline/phosphatidylglycerol (4:1) vesicles were prepared in absence or presence of solubilized glycerophosphate acyltransferase. Vesicles containing protein were incubated for 2 h at 25°C with or without trypsin prior to addition of trypsin inhibitor and 20% (v/v) of the ²H₂O analogue of the buffer. ¹³C-NMR spectra were recorded first in the absence and then in the presence of 10 mM Mn²⁺. Chemical shifts of the peaks are 13 ppm upfield from external dioxane.

With control vesicles, prepared by sonication, addition of Mn²⁺ up to 10 mM resulted in a 63% decrease in signal intensity. This residual signal did not decrease further during a 2 h incubation, indicating that the vesicles are not leaky for Mn²⁺. Vesicles prepared in buffer with 10 mM Mn²⁺ showed only a very broadened spectrum with no peak signal at all. Similar observations with vesicles prepared by the cholate dispersion and gel filtration technique showed that 70% of the phospholipids were present in the outer monolayer (Fig. 8, top). Reconstituted glycerophosphate acyltransferase vesicles after a 2 h incubation with and without trypsin, under the conditions used in the experiment of Fig. 7, showed a 69 and 76% drop in signal intensity, respectively, upon addition of Mn²⁺ (Fig. 8). In these experiments with the vesicles containing proteins a further slow decrease in signal intensity accompanied by a slow formation of aggregated structures in the NMR tube was seen. We attribute these changes to induction of aggregation and/or fusion of the vesicles containing protein in the presence of Mn²⁺. However, since directly after trypsin treatment the vesicles

respond identically to Mn²⁺ addition as do control vesicles, and taking into account the difference in size of Mn²⁺ and trypsin, it can be concluded that the vesicles are impermeable for trypsin. A similar result was reported [42] for vesicles containing glycophorin which were prepared by the cholate dispersion and gel filtration technique.

Summarizing, it can be stated that at least 90% of the active site of reconstituted glycerophosphate acyltransferase in PC/PG (4:1) vesicles is located at the outside surface. This reconstituted system comprises a useful model to characterize further the properties of glycerophosphate acyltransferase in membrane-bound form, not least because lysophosphatidate is the only product formed, thus circumventing interference of side reactions such as occur in isolated *E. coli* membranes.

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