The Association of Phosphatidylserine Synthetase with Ribosomes in Extracts of Escherichia coli*

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SUMMARY

The biosynthesis of phospholipids in Escherichia coli is catalyzed by enzymes most of which are associated with the bacterial membrane. In the present work, it has been found that the enzyme CDP-diglyceride:l-serine phosphatidyltransferase (phosphatidylserine synthetase) is exceptional in that it is not bound to membrane fragments in cell-free extracts of E. coli, but is predominantly associated with ribosomes. The enzyme resists extraction from ribosomes in buffers of high ionic strength. When ribosomes were allowed to dissociate in buffers containing low concentrations of magnesium, the enzyme was associated with both 50 S and 30 S subunits.

Phosphatidylserine is found only in trace amounts in E. coli, being rapidly converted to phosphatidylethanolamine by a decarboxylase which is a membrane-bound enzyme. Despite the apparently different intracellular localization of the two enzymes, their function in vivo must be tightly coupled. When the decarboxylase in living cells of E. coli was inhibited by the addition of hydroxylamine to the medium, phosphatidyl[1-14C]serine was shown to accumulate within 1 min. In the control cells, in which the decarboxylase was not inhibited, no labeled phosphatidylserine could be detected, showing that the decarboxylation of the newly formed phosphatidylserine must take place without detectable time lag.

Previous studies in this laboratory led to the finding that enzymes responsible for the biosynthesis of phospholipids in Escherichia coli (Scheme 1) are generally recovered in the particulate, membrane-containing fraction. Patterson and Lemarz (1) have reported similar findings for Bacillus megaterium. In their study of phosphatidylserine synthetase (Scheme 1, Reaction 2) Kanfer and Kennedy (2) noted, however, a persistent tendency of this enzyme to be recovered in fractions containing nucleic acid. In the present work, aimed at purification and more complete characterization of the synthetase, we have obtained evidence that the enzyme is associated with ribosomes, and not with membrane fragments. Phosphatidylserine decarboxylase, the next enzyme in the biosynthetic pathway (Scheme 1) is recovered almost entirely in the membrane-containing fraction. With hydroxylamine to block the action of the decarboxylase in living cells of E. coli, we have found that newly synthesized phosphatidylserine is recovered almost entirely in the membrane fraction, suggesting that a rapid, intracellular translocation of the lipid may be involved in the coordinate function of the synthetase and the decarboxylase.

MATERIALS AND METHODS

**[1-14C]Serine, DL-[3-14C]serine, DL-[3-14C]serine, [2-3H]glycerol, and ATP labeled with 32P in the γ position were products of the New England Nuclear Co., Boston. L-α-[2-3H]Glycerophosphate was prepared enzymatically by the procedure of Chang and Kennedy (3). (Dipalmitoyl) phosphatidyl[1-14C]-serine was prepared as described by Kanfer and Kennedy (2) with cytidine diphosphate dipalmityl, which was synthesized by a modification of the method of Agranoff and Suomi (4). A mixture of 1,2-sn-diglycerides was prepared from egg lecithin by treatment with phospholipase C from Clostridium perfringens (Sigma Co., St. Louis). Cardiolipin was obtained from the Sylvania Co. (Milburn, N. J.). Triton X-100 (octylphenoxypolyethoxyethanol) and Brij 58 (polyoxyethylene cetyl ether) both nonionic detergents, were obtained from Rohm and Haas (Philadelphia) and Atlas Chemical Co. (Wilmington), respectively.

**Growth Conditions for E. coli B**

Cells were usually grown in a rotary shaker at 37° on a simple medium, supplemented with 1% glycerol, which contained 3 mM MgSO4, 3 mM KCl, 0.5 mM K2HPO4, 5 mM (NH4)2SO4, 0.01 mM FeSO4, and 0.1 mM Tris-HCl, pH 7. In several experiments cells were also grown on mineral medium 63 (5) with glycerol (1%) or on a minimal glucose medium 6 (6).

**Preparation of Cell Extracts**

The bacteria were harvested at the end of log phase at a concentration of 109 per ml. In a typical experiment 1 liter of bacterial suspension was centrifuged at 0°, and the cells were washed once with 100 ml of the same buffer, usually 10 mM Tris of pH 8.0, employed subsequently for sonication. After a second
centrifugation (3000 × g for 15 min) the washed bacteria were resuspended in the appropriate buffer at a protein concentration of 10 mg per ml. Portions of the bacterial suspension (5 to 10 ml) were disrupted at 0° with an MSE 100-watt sonicator at an amplitude of 7 to 8 μm and a frequency of 22 kc. Four 30-s bursts were sufficient to assure complete disruption, with half-minute intervals to allow cooling of the sample. Sonicates were subsequently stored on an ice bath.

Penicillin lysates were made by a modification of the procedure of Lederberg (7). Cells grown to a density of 10⁹ per ml on Tris-glycerol were treated with the potassium salt of penicillin G (added as a concentrated solution) at a level of 1 mg per ml. After an hour of further shaking at 37° over 99% of the cells had lysed as judged by the viability count. Freshly grown E. coli B were also lysed by the freeze-thaw-lysozyme-deoxycholate method (with 0.3% detergent) as described by Ron et al. (8).

Differential Centrifugation and Sucrose Gradient Analysis

These were performed at 0-2°C. The gradients were prepared in 12-ml polyallomer tubes (9.5 cm in length), and an International B-60 preparative ultracentrifuge equipped with an SB 283 rotor was employed for high speed sedimentation. Gradient fractions were collected by piercing the bottom of the tube with a short No. 22 needle, which was left in place to assure even drainage. Details of each centrifugation accompany the legends to the figures and tables.

Enzyme Assays

Phosphatidylserine Synthetase and CDP-diglyceride:1-α-Glycerophosphate Phosphatidylyl Transferase The conversion of DL-[3-¹⁴C]serine and 1-α-[2-³H]glycerophosphate to lipid in the presence of CDP-dipalmitin (Scheme 1, Reactions 2 and 4) were measured by similar techniques. The final reaction mixture, usually in a volume of 0.06 ml, was incubated in a 12-ml plastic tube or a conical 40-ml glass-stoppered vessel. The reaction was terminated by the addition of 3 ml of chloroform-methanol (2:1, v/v) containing 0.01 N HCl. Extraction was performed with 5 ml of 2 M KCl by agitation the tightly stoppered reaction vessel for 15 s with a mechanical vibrator until both phases were thoroughly mixed. After separation of the phases, the upper

PATHWAYS OF PHOSPHOLIPID METABOLISM IN ESCHERICHIA COLI

Scheme 1
layer was discarded and the extraction repeated. A sample (1 ml) of the CHCl₃ layer was transferred to a scintillation vial and dried in a 90° water bath. Patterson-Green (9) counting solution (10 ml) and water (1 ml) were added, and the radioactivity was determined on a Packard liquid scintillation counter.

In the case of phosphatidylether synthetase, the final incubation mixture included 0.3 mm CDP-diacylglycerol, 1 mm DL-[3-¹⁴C]-serine, 0.1 m potassium phosphate buffer, pH 7.4, 0.1% Triton X-100, and an appropriate amount of enzyme protein. The reaction, which was linear with enzyme concentration, was allowed to proceed for 10 min at 37°C. A unit of activity is defined as the amount of enzyme which forms 1 nmole of product per min.

To assay the phosphatidylglycerophosphate synthetase (Scheme 1, Reaction 4) the incubation conditions described by Chang and Kennedy (3) were employed, except that the final reaction volume was 0.06 ml. A unit of activity was defined as above, except that the incubations were carried out at 37°C.

Diglyceride Kinase This enzyme catalyzes the synthesis of phosphatidic acid from ATP and 1,2-di-α-glycerides (10, 11). A final reaction volume of 0.1 ml was employed. The incubation mixture included 2 mm Na₂P₃ATP, 2 mm diglyceride from egg lecithin, 25 mm MgCl₂, 0.7% Triton X-100, 0.08% beef heart cardiolipin, 0.1 m NaCl, 0.1 m Tris-phosphate, pH 6.5, and enzyme extract. After 10 min at 37°C, the reaction was stopped with 1.5 ml of chloroform-methanol, the lipid phase was extracted with 2 m KCl solution, and 0.1 ml of the CHCl₃ phase was counted directly in Patterson-Green fluid.

Phosphatidylserine Decarboxylase—This enzyme (Scheme 1, Reaction 3) was assayed as described by Kanfer and Kennedy (2). A unit of activity of decarboxylase or diglyceride kinase was defined as for the other enzymes.

None of these enzymes were inhibited by 2 to 10% sucrose in the incubation mixture, under conditions in which the samples from gradient centrifugations were assayed.

**TABLE I**

| Additions to medium | Percentage of synthetase activity in supernatant |
|---------------------|-----------------------------------------------|
|                     | 40,000 x g (1 hr) | 100,000 x g (3 hrs) |
| 1. MgCl₂ (1 mm)     | 107%            | 29%               |
| 2. MgCl₂ (1 mm) + 0.1 m KCl | 91%            | 27%               |
| 3. MgCl₂ (2 mm)     | 82%             | 12%               |
| 4. MgCl₂ (10 mm)    | 57%             | 4%                |
| 5. MgCl₂ (10 mm) + 0.1 m KCl | 74%            | 4%                |
| 6. 0.10 m potassium phosphate, pH 7.4; no Tris buffer | 90% | 13% |

Thin Layer Chromatography of Lipid Produced from [1-¹⁴C]Serine in Presence of Hydroxylamine

Portions of the chloroform extracts of labeled lipids produced at varying time intervals in the experiment of Fig. 4 were pooled to increase the amount of radioactivity available for analysis, washed with water to remove nonlipid impurities, and dried under a stream of nitrogen. The residue was redissolved in 0.05 ml of chloroform-methanol (2:1, v/v) and applied to an Eastman Chromagram thin layer sheet (8000 silica gel). This was developed with the solvent chloroform-methanol-water-acetic acid (25:15:4:2, v/v) at room temperature. After the position of the lipids had been determined by staining with iodine vapor, I-cm strips were cut out and counted. Nearly all of the radioactivity was recovered in a sharp band coincident with marker authentic phosphatidylserine.

Other Procedures

Protein was determined by the method of Lowry et al. (21). RNA was measured by the pentose reaction with orcinol (13) with yeast RNA as a standard. The presence of deoxycholate in the samples caused a slight turbidity during the orcinol reaction; this was removed by centrifugation without interference with the RNA determination.

**RESULTS**

Sedimentation of Phosphatidylserine Synthetase during Differential Centrifugation—When extracts prepared by sonic disruption of cells of *E. coli* B were subjected to differential centrifugation, the pattern of sedimentation of phosphatidylserine synthetase strongly suggested association of this enzyme with ribosomes (Table I). Thus, when centrifugation was carried out in a medium of low magnesium concentration (1 mm), little or no activity was sedimented during centrifugation at 40,000 x g for 1 hour. The fraction precipitated under these conditions contains membrane fragments, and is enriched in membrane-bound enzymes. When the 40,000 x g supernatant fraction was subjected to centrifugation at 100,000 x g under conditions expected to sediment ribosomes, the greater part of the enzyme activity was recovered in the pellet (Table I). Furthermore, increasing the magnesium concentration to 10 mm, which is known to cause aggregation of ribosomes, caused the sedimentation of 43% of the enzyme activity in the 40,000 x g pellet, together with a comparable percentage of the RNA of the extract.

Essentially similar results were obtained upon analysis of cells lysed by the gentler penicillin method, indicating that the result obtained is not an artifact produced by sonic disruption of the cells.

The sedimentation pattern was identical when cells were grown on minimal medium 63 (5) with glycerol as carbon source, or when minimal glucose was employed. Extracts prepared from frozen cells of *E. coli* B also presented much the same pattern.

Treatment of Ribosomal Pellets with Buffers of High Ionic Strength—When ribosomal pellets collected by centrifugation at 100,000 x g were treated with buffers containing high concentrations of salt, or 50 mm EDTA, the bulk of the enzyme activity remained associated with the ribosomal pellet upon subsequent recentrifugation (Table II). Washing with 0.5 M NaCl has been used by Stanley et al. (14) to extract from ribosomes factors
TABLE II

Binding of phosphatidylserine synthetase to ribosomes
during extraction with buffers of high ionic strength.

Ribosomal pellets, sedimented by centrifugation at 100,000 × g for 5 hours as described in Table I, were suspended in 10 mM Tris buffer of pH 8.0, to which various salts were added in the final concentrations indicated, and centrifuged again at 100,000 × g for 5 hours. The activity of phosphatidylserine synthetase in each supernatant fraction was then measured.

| Additions to extraction medium | Percentage of synthetase not sedimented at 100,000 × g |
|-------------------------------|-----------------------------------------------|
| 1. MgCl₂ (10 mM)              | 10                                           |
| 2. MgCl₂ (10 mM) + NH₄Cl (0.5 M) | 26                                           |
| 3. MgCl₂ (10 mM) + (NH₄)₂SO₄ (0.6 M) | 36                                           |
| 4. MgCl₂ (1 mM) + KCl (1.0 M)  | 20                                           |
| 5. EDTA (50 mM)               | 15                                           |

Fig. 1. Sedimentation profile of phosphatidylserine synthetase (PS synthetase) and of phosphatidylserine decarboxylase during centrifugation in a sucrose gradient. A sample (0.4 ml) of cells of E. coli B, containing 10 mg of protein per ml, sonically disrupted in a buffer containing 10 mM Tris-HCl of pH 8.0 and 2.0 mM MgCl₂, was layered above a 5 to 20% sucrose gradient (10.5 ml) with a 70% sucrose shelf (1.5 ml) at the bottom, containing the same buffer as the sample. Fractions of 0.5 ml were collected after centrifugation at 200,000 × g for 90 min at 2°C. Absorbance at 260 nm, phosphatidylserine synthetase, and phosphatidylserine decarboxylase were measured as indicated.

These conditions (2 mM MgCl₂) most of the ribosomes are undissociated; the second, smaller peak of synthetase activity is probably coincident with dissociated ribosomes.

In an effort to localize the synthetase on the subunits of the ribosomes, the experiment shown in Fig. 3 was carried out at 0.1 mM MgCl₂ and with a longer period of centrifugation. The enzyme appeared to be bound to both 50 and 30 S subunits. The presence of 0.1 M KCl added to the medium did not alter the sedimentation pattern (data not shown). It should be noted that the smaller, trailing peak of enzyme activity at about Fraction 31, sedimented distinctly faster than the bulk of the soluble, ultraviolet-absorbing fraction.

Although phosphatidylserine decarboxylase and the other membrane-bound enzymes were recovered principally at the bottom of the gradient in the experiments of Figs. 1 and 2, significant amounts of activity were spread throughout the entire gradient. This broad distribution may reflect heterogeneity...
of size and density in membrane fragments in extracts prepared by sonic disruption of cells.

**Treatment with Deoxycholate in Presence of Magnesium**—When ribosomes were prepared from frozen-thawed cells by the method of Ron et al. (8) which utilizes deoxycholate as a detergent in buffers containing magnesium ion, they were found to contain very little phosphatidylserine synthetase. However, a precipitate (presumably magnesium deoxycholate) was observed to form in these extracts, and nearly all of the synthetase was recovered with this precipitate. These observations suggested that the treatment of ribosomal pellets with deoxycholate might remove phosphatidylserine synthetase.

Frozen cells of *E. coli* B (obtained from Grinn Processing Co., Muscatine, Ia.) were suspended at a protein concentration of 10 to 60 mg per ml in 0.1 M potassium phosphate buffer, pH 6.8, containing 5 mM MgSO4 and 0.01 M β-mercaptoethanol. The cells were thawed and dispersed in a Waring Blender, and disrupted by four passages through a Mantin Gaulin press at 9000 p.s.i. All procedures were performed between 0 and 4°.

Membranes were sedimented by a 6-hour centrifugation at 45,000 × g. The supernatant was stored frozen at −20° over a period of months without detectable loss of activity.

Streptomyein precipitation of the 45,000 × g supernatant was performed at 0°. The frozen material was thawed and diluted 1:4 with distilled water, yielding a solution (Fraction 1, Table III) of 5 to 10 mg per ml in protein. A concentrated streptomyein sulfate solution (10%) was added over a period of several minutes with stirring in a volume equal to 15% of that of Fraction 1. After 2 hours at 0°, the suspension was centrifuged at 30,000 × g for 20 min. The streptomyein supernatant, which was free of enzymatic activity, was discarded, and the pellet was redissolved with the aid of a blender in 0.2 M Tris-HCl, pH 7.4, in the same volume as Fraction 1. The redissolved streptomyein pellet (Fraction 2, Table III) was clarified by a second centrifugation at 30,000 × g for 20 min and stored in an ice bath. To a portion of this fraction, MgCl2 (1.0 M) was added to give a final magnesium concentration of 20 mM. Ten percent sodium deoxycholate was then added to a final level of 0.57%, and the enzyme was then centrifuged for 20 min at 30,000 × g. The supernatant (Fraction 3, Table III) contained relatively little enzyme, while most of the activity could be recovered by resuspension of the magnesium deoxycholate precipitate in 0.1 M potassium phosphate buffer of pH 7.4 containing 0.5% Brij 58 (Fraction 4).

**Table III**

| Fraction         | Percentage of total synthetase activity | Percentage of total RNA | Enzyme specific activity (units/mg protein) |
|------------------|----------------------------------------|-------------------------|-------------------------------------------|
| 1. Initial 40,000 × g supernatant | %                                     | %                       |                                           |
| 2. Streptomyein precipitate | (100)                                 | (100)                   | 13                                        |
| 3. Supernatant after deoxycholate + Mg⁺⁺ | 76                                    | 82                      | 24                                        |
| 4. Precipitate after deoxycholate + Mg⁺⁺ | 50                                    | 11                      | 20                                        |

When [3H]-serine was used to label the cells, radioactive lipid accumulated in the hydroxylamine-treated cells at about the same rate as the control, indicating that hydroxylamine acts at the decarboxylase step, and not earlier in the sequence of steps leading to the incorporation of serine into lipid.

Thin layer chromatography as described under “Materials and Methods” revealed that the labeled lipid which accumulated in the hydroxylamine-inhibited cells was almost entirely phosphatidylserine. No radioactive phosphatidylserine could be detected in the lipid extract of the uninhibited cells, the small amount of radioactivity shown in Fig. 4 is apparently attributable to labelled serine.
to a trace contaminant of the labeled serine, which was, however, 99% pure by chromatographic analysis. It may be noted that the inhibition of the decarboxylase by hydroxylamine under these conditions is not complete, since the accumulation of carbonyl-labeled lipid levels off, and the radioactivity is "chased" by the addition of unlabeled serine to the medium.

These results show a very rapid decarboxylation of phosphatidylethanolamine in living cells of E. coli since after as little as 1 min significant amounts of phosphatidylethanolamine more than 1000 counts above the background level had been formed and accumulated in the inhibited cells, but had been completely decarboxylated in the uninhibited cells.

Intracellular Localization of Newly Synthesized Phosphatidylethanolamine—Since phosphatidylethanolamine synthetase and the decarboxylase appear to be localized on different intracellular structures, the experiment of Fig. 4 suggests that a rapid intracellular translocation could account for the efficient decarboxylation of the newly synthesized lipid. In an attempt to determine the location of the phosphatidylethanolamine which accumulates in hydroxylamine inhibited cells, such cells were disrupted and subjected to sucrose gradient centrifugation in the experiment of Fig. 5. Nearly all of the newly synthesized phosphatidylethanolamine was recovered in the membrane fraction, with very little in the region expected to contain ribosomes. The experiment, however, must be interpreted with caution, since redistribution of lipid among membrane fragments may take place during sonic disruption (15). A similar redistribution of phosphatidylethanolamine between ribosomes and membrane fragments may also take place. Even if this is the case, it can be concluded that the equilibrium greatly favors localization of the lipid product on the membrane, under conditions in which the synthetase is largely retained on the ribosomes.

![Graph showing intracellular localization of phosphatidyl[1-14C]serine newly synthesized in the presence of hydroxylamine. Cells of E. coli B were labeled by growth for 10 min in the presence of dL-[1-14C]serine and 10 mM hydroxylamine as in the experiment of Fig. 4, chilled in an ice bath, and sonically disrupted. A sample (0.4 ml) with a protein concentration of about 0.2 mg per ml was layered on a sucrose gradient, as described in Fig. 1 which also contained 10 mM hydroxylamine throughout. Fractions from the gradient were extracted with chloroform-methanol, and the lipid-soluble radioactivity was determined.](image)

Fig. 5. Intracellular localization of phosphatidyl[1-14C]serine newly synthesized in the presence of hydroxylamine. Cells of E. coli B were labeled by growth for 10 min in the presence of dL-[1-14C]serine and 10 mM hydroxylamine as in the experiment of Fig. 4, chilled in an ice bath, and sonically disrupted. A sample (0.4 ml) with a protein concentration of about 0.2 mg per ml was layered on a sucrose gradient, as described in Fig. 1, which also contained 10 mM hydroxylamine throughout. Fractions from the gradient were extracted with chloroform-methanol, and the lipid-soluble radioactivity was determined.

**DISCUSSION**

In this as in every other study of the intracellular distribution of enzymes, the possibility must be considered that the pattern observed in cell-free extracts may not correspond with that in the intact, living cell. Redistribution of enzymes may take place during disruption of the cell and handling of the extracts. For example, there is evidence that a ribonuclease often isolated in association with ribosomes is in fact a periplasmic protein which becomes adsorbed to ribosomes during disruption of the cell (16). RNA, however, is a substrate for this enzyme, and it can only be activated by procedures which tend to disrupt ribosome structure (17). Neither of these considerations apply to phosphatidylethanolamine synthetase. Furthermore, the apparent ribosomal localization of the enzyme is observed when the much gentler penicillin method is used to prepare cell-free extracts rather than sonic disruption. Nevertheless, the conclusion that the synthetase is a ribosomal enzyme is still subject to the reservations mentioned.

In rat liver, phosphatidylethanolamine is synthesized from phosphatidylethanolamine + L-serine in a reaction catalyzed by an "exchange" enzyme (18). This enzyme is localized in the endoplasmic reticulum, whereas phosphatidylethanolamine decarboxylase is entirely mitochondrial (19). Nevertheless, L-serine is rapidly converted to lipid and decarboxylated by the liver. The important studies of Wirtz and Zilversmit (20) and of McMurray and Dowson (21) have revealed the presence in liver of soluble proteins which rapidly carry out the reversible translocation of phospholipids such as lecithin from endoplasmic reticulum to mitochondrial membranes. Such an intracellular transport system would account for the rapid, mitochondrial decarboxylation of phosphatidylethanolamine synthesized on the endoplasmic reticulum. The experiment of Fig. 4 suggests the possible presence of such translocation proteins in E. coli. We cannot exclude the alternative, however, that the ribosomally bound synthetase exerts its action directly at the surface of the membrane in vivo. It is also possible that the small fraction of the synthetase not associated with the ribosomes may account for the total activity of the enzyme in living cells, although this seems quite unlikely.

The possible significance of the ribosomal localization of phosphatidylethanolamine synthetase deserves some consideration. Phosphatidylethanolamine is the principal membrane lipid of E. coli, and phosphatidylethanolamine synthetase catalyzes the first step in the branch of the pathway leading to phosphatidylethanolamine. It is obviously important for the cell to coordinate the synthesis of protein and of membrane lipids, but the mechanisms by which this is achieved are unknown. The localization of a key enzyme for the biosynthesis of lipids on ribosomes may be somehow linked to the joint regulation of protein and lipid synthesis.

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