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Phospholipid Biosynthesis in Some Anaerobic Bacteria

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We have identified and characterized enzymes of phospholipid synthesis in two plasmalogen-rich anaerobes, *Megasphaera elsdenii* and *Veillonella parvula*, and one anaerobe lacking plasmalogens, *Desulfovibrio vulgaris*. All three species contained phosphatidate cytidylyltransferase and phosphatidylserine synthase. Phosphatidylglycerophosphate synthesis was detected only in *D. vulgaris* extracts. Phosphatidylserine (diacyl form) was the major product of the phosphatidylserine synthase assay with particles from *M. elsdenii* or *V. parvula*. The amounts of phosphatidylethanolamine formed were very low. Only *D. vulgaris* particles had an active phosphatidylserine decarboxylase.

Interest in the structure and functions of biological membranes has generated intensive research into the biosynthesis of phospholipids in bacteria. E. P. Kennedy and his co-workers, studying *Escherichia coli*, have identified the following biosynthetic reactions (23):

$$\text{Phosphatidic acid} + \text{CTP} \xrightarrow{Mg^{2+}} \text{CDP-diglyceride} + PP_i \quad (1)$$

$$\text{CDP-diglyceride} + \textit{sn}\text{-glycerol-3-phosphate} \xrightarrow{Mg^{2+}} \text{phosphatidylglycerophosphate} + \text{CMP} \quad (2)$$

$$\text{Phosphatidylglycerophosphate} \rightarrow \text{phosphatidylglycerol} + P_i \quad (3)$$

$$\text{CDP-diglyceride} + \text{L-serine} \rightarrow \text{phosphatidylserine} + \text{CMP} \quad (4)$$

$$\text{Phosphatidylserine} \rightarrow \text{phosphatidylethanolamine} + CO_2 \quad (5)$$

Reactions 2 and 4 have also been shown to utilize dCDP-diglyceride. These reactions have also been found in other facultative and aerobic bacteria (5, 19, 21, 26). Because no comparable work had been done with anaerobic bacteria, we carried out a study of phospholipid-synthesizing enzymes in *Clostridium butyricum* and found that reactions 1 through 4 were present (25); however, phosphatidylserine decarboxylase activity was barely detectable (25). To determine if our results with *C. butyricum* were characteristic of anaerobic bacteria containing plasmalogens (10), we have studied several other species, including *Megasphaera elsdenii* and *Veillonella parvula*, both of which are rich in plasmalogens (22, 28), and *Desulfovibrio vulgaris*, which does not contain them (25). The lipid compositions of *M. elsdenii* and *V. parvula* are different from that of *C. butyricum* in that the former two species contain mainly serine and ethanolamine phosphatides, but no phosphatidylglycerol (22, 28). The lipids of *D. vulgaris* consist primarily of phosphatidylethanolamine and phosphatidylglycerol, with lesser amounts of cardiolipin and lysophosphatidylserine (18).

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MATERIALS AND METHODS

For the preparation of [^{32}P]phosphatidylserine, *M. elsdenii* was grown on a synthetic medium described by Forsberg (6). The medium contained the following (as described in reference 6): minerals 1, minerals 2, resazurin, glucose, amino acids (groups A, B, and C), volatile fatty acids, vitamins, and sodium carbonate. The phosphate of minerals 1 was reduced to 10% of the original, and it was replaced by an equivalent amount of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonate). Cells were grown in 500 ml of the low-phosphate medium containing 5 mCi of $^{32}P_i$. The washed cells were extracted according to Bligh and Dyer (2). The organic phase was concentrated and applied to a column (10 by 0.8 cm) of BioSil A (Bio-Rad Laboratories, Richmond, Calif.) treated with $NaHCO_3$ (20, 24). Elution was as follows: fraction 1, chloroform, 12 ml; fraction 2, chloroform-methanol, 7:1 (vol/vol), 15 ml; fraction 3, chloroform-methanol, 4:1, 15 ml; fraction 4a, chloroform-methanol, 7:2, 15 ml; fraction 4b, chloroform-methanol, 7:2, 20 ml; fraction 5, chloroform-methanol, 3:2, 15 ml; and fraction 6, chloroform-methanol, 1:1, 15 ml. Fraction 5 contained most of the phosphatidylserine, which was further purified by preparative thin-layer chromatography in

solvent system C (see below). This yielded 4×10^6 cpm of phosphatidylserine (>90% pure). Other radiochemicals and reagents were obtained as described previously (25).

Cells and culture conditions. *M. elsdenii* ATCC 17752 and *V. parvula* ATCC 10790 were grown as described previously (22, 28). *D. vulgaris*, obtained from R. Makula (University of Georgia, Athens), was grown on a high-sulfate medium (18). *M. elsdenii* and *D. vulgaris* cells were broken in a French pressure cell as described previously (9). *V. parvula* was first treated with lysozyme (1 mg/ml) and EDTA (10 mM) (11) in 0.05 M KPO_4 (pH 7.2)–0.01 M 2-mercaptoethanol at 37°C for 30 min and then broken by sonication (Bronson model 6575, setting 4) for 10 bursts of 1 min with intervals for cooling. The crude extracts were centrifuged at $4,500 \times g$ for 30 min to remove unbroken cells. The resulting supernatant was centrifuged at $150,000 \times g$ for 1 h. With *D. vulgaris* the $4,500 \times g$ supernatant was first centrifuged at $30,000 \times g$ for 1 h to remove iron sulfide particles, and the supernatant was centrifuged at $150,000 \times g$ for 1 h. The pellets were washed and prepared as described previously (25).

Enzyme assays. Phosphatidylserine synthase and phosphatidylglycerophosphate synthase were assayed by a nonspecific filter disk assay (8, 27). Phosphatidate cytidylyltransferase was assayed, using a nonspecific trichloroacetic acid preparation procedure (13). When the enzymatic products were to be further analyzed, lipids were extracted with butanol and analyzed as described previously (25). Assay mixtures were essentially as described in reference 25 except for the differences noted in Results.

Lipids were analyzed by thin-layer chromatography on silica gel HR (E. Merck AG, Darmstadt, Germany). The following solvent systems were used: (A) chloroform-methanol-acetic acid-water, 50:30:8:4 (vol/vol); (B) chloroform-methanol-acetic acid, 65:25:8 (vol/vol); and (C) chloroform-methanol-7 N NH_4OH, 60:35:5 (vol/vol). Identification of enzymatic products was based on the following: CDP-diglyceride comigrated with an authentic standard in solvent system A and served in situ as a substrate for phosphatidylserine formation (see below), and phosphatidylserine and phosphatidylethanolamine cochromatographed with authentic standards in solvent system C and were further analyzed by subjecting them to alkaline hydrolysis (4) and chromatographing the water-soluble products on paper in saturated phenol-water, 100:38 (wt/vol). Plasmalogen content was determined by acetic acid hydrolysis (12).

RESULTS

The formation of CDP-diglyceride from [5-^{3}H]CTP and phosphatidic acid was catalyzed by membrane particles from *M. elsdenii* and *V. parvula*. The reaction was dependent in both cases on phosphatidic acid (0.7 mM), Mg^{2+} (10 mM), and [5-^{3}H]CTP (0.6 mM) and linear with respect to protein up to 1 mg/ml. KCl (0.2 M) was found to stimulate the activity of *V. parvula* particles twofold, but had no effect on the activity of *M. elsdenii* particles. Triton X-100 stimulated both *V. parvula* particles (4-fold at 0.1%) and *M. elsdenii* particles (2.5-fold at 1.0%). The following apparent K_m values were determined from Hanes-Woolf plots (data not shown): *M. elsdenii*, 0.02 mM phosphatidic acid, 0.4 mM CTP; *V. parvula*, 0.07 mM phosphatidic acid, 1.0 mM CTP. Particles of *D. vulgaris* also produced CDP-diglyceride in the above assay system with 1.0% Triton X-100. This activity was not characterized further. The CDP-diglyceride formed by particles of *M. elsdenii* was tested for its further conversion to phosphatidylserine by incubating CTP, Mg^{2+}, phosphatidic acid, L-[3-^{14}C]serine, and particles. Table 1 shows the formation of phosphatidylserine from CDP-diglyceride made in situ.

The conversion of CDP-diglyceride to phosphatidylglycerophosphate in the presence of *sn*-[*U*-^{14}C]glycerol-3-phosphate (specific activity, 5 Ci/mol), Mg^{2+}, and particles was also studied. *D. vulgaris* particles appeared to catalyze the formation of phosphatidylglycerophosphate, phosphatidylglycerol, and cardiolipin, which were provisionally characterized by thin-layer chromatography (solvent system B). With particles from *M. elsdenii* or *V. parvula*, some radioactivity from *sn*-[*U*-^{14}C]glycerol-2-phosphate was incorporated into butanol-extractable material. However, on thin-layer chromatography it did not migrate with either phosphatidylglycerophosphate or phosphatidylglycerol.

The formation of phosphatidylserine from CDP-diglyceride and L-[3-^{14}C]serine was studied in particles prepared from each organism. Assay conditions were optimized, and linearity with respect to time and protein was established. Phosphatidylserine formation in each case required CDP-diglyceride, and with *V. parvula* particles the reaction was also dependent on added Mg^{2+} (optimal at 10 mM). With *M. elsdenii* and *D. vulgaris* particles, Mg^{2+} (10 mM)

TABLE 1. *Coupled phosphatidylserine formation from phosphatidic acid, CTP, and L-[3-^{14}C]serine with M. elsdenii particles*[a]

Assay	Phosphatidylserine formed (nmol)
Complete	4.10
– CTP	0.04
– Phosphatidic acid	0.03

[a] Assays contained, in a total volume of 0.2 ml, the following: Tris-hydrochloride (pH 8.0), 0.1 M; KCl, 0.2 M; phosphatidic acid, 0.7 mM; CTP, 0.525 mM; L-[3-^{14}C]serine (specific activity, 10 mCi/mmol), 1 mM; $MgCl_2$, 10 mM, Triton X-100, 0.1%; and *M. elsdenii* particles, 0.38 mg of protein. The mixture was incubated at 37°C for 45 min, extracted with butanol (24), and chromatographed in solvent system C.

was only stimulatory (3- and 1.6-fold, respectively). The effect of added Triton X-100 (0.1 to 0.2%) was also variable, with a fourfold stimulation for the *M. elsdenii* enzyme, twofold stimulation with the *D. vulgaris* enzyme, and no effect on the *V. parvula* enzyme. Table 2 lists the specific activities of phosphatidylserine synthase determined with particles from each microorganism at optimal conditions. The following K_m values were determined: *M. elsdenii*, 0.03 mM CDP-diglyceride, 1.2 mM L-serine; and *V. parvula*, 0.03 mM CDP-diglyceride, 0.37 mM L-serine.

The product of the phosphatidylserine synthase reactions with *M. elsdenii* or *V. parvula* particles was phosphatidylserine with barely detectable amounts of phosphatidylethanolamine. The phosphatidylserine formed with *V. parvula* particles was tested as a substrate in situ by adding *E. coli* particles possessing phosphatidylserine decarboxylase activity (25). This resulted in the conversion of 25% of the phosphatidylserine to phosphatidylethanolamine, compared with about 1% conversion in the absence of the *E. coli* particles. To test whether the products thus formed contained plasmalogens, both the phosphatidylserine and phosphatidylethanolamine were subjected to alkaline hydrolysis (4). No significant amounts of alkali-stable ether lipids were detected.

The apparent inability of particles from *C. butyricum* (25), *M. elsdenii*, and *V. parvula* to catalyze the decarboxylation of phosphatidylserine generated in vitro may have been the result of the absence of the plasmalogen form of phosphatidylserine, which has been suggested to be the in vivo substrate in *M. elsdenii* (22). To test this hypothesis, we isolated [^{32}P]phosphatidylserine from *M. elsdenii* (approximately 20% plasmalogen) and used it as a substrate in direct assays for the decarboxylase. The results (Table 3) showed significant production of phosphatidylethanolamine with particles from *E. coli* and *D. vulgaris*, less with *V. parvula* particles, and barely detectable amounts of the decarboxylation product with *C. butyricum* and *M. elsdenii*. An unfractionated *C. butyricum* cell extract gave similar results.

TABLE 2. *Specific activities of phosphatidylserine synthase*[a]

Species	Sp act of phosphatidylserine synthase (nmol/min per mg of protein)
M. elsdenii	0.35
V. parvula	0.56
D. vulgaris	0.66

[a] Assays were done by the filter disk method (8, 27) and contained L-[*U*-^{14}C]serine, 1 mM (specific activity, 10 Ci/mol); CDP-diglyceride, 0.056 to 0.095 mM; $MgCl_2$, 10 mM; Triton X-100, 0.1 to 0.28%; and protein, 1 to 2 mg/ml, in a total volume of 0.07 to 0.10 ml. Incubations were at 37°C (*M. elsdenii*, 3 min; *V. parvula* and *D. vulgaris*, 4 min).

TABLE 3. *Phosphatidylserine decarboxylase activities in particulate preparations from bacteria*

Species	Cell fraction	[^{32}P]phosphatidylserine decarboxylated (%) in assay[a]:	
		A	B
E. coli	Particles	17.4	25.2
D. vulgaris	Particles	7.4	17.3
C. butyricum	Particles	0.3	2.2
C. butyricum	Cell extract		1.2
M. elsdenii	Particles	1.3	1.5
V. parvula	Particles	3.5	

[a] Assay A contained potassium phosphate (pH 7.0), 0.2 M; Triton X-100, 0.3%; $MgCl_2$, 5 mM; [^{32}P]phosphatidylserine (derived from *M. elsdenii*), 130 μM, 8.8 × 10^4 cpm (specific activity, 4.46 × 10^3 cpm/nmol); and particles of *C. butyricum*, 250 μg; *M. elsdenii*, 200 μg; *V. parvula*, 200 μg; *D. vulgaris*, 250 μg; or *E. coli*, 200 μg. The assay volume was 0.1 ml, and incubation was at 37°C for 20 min. Assay B contained potassium phosphate, Triton X-100, and $MgCl_2$ as in assay A; [^{32}P]phosphatidylserine, 57 μM, 8 × 10^4 cpm (specific activity, 1.40 × 10^4 cpm/nmol); and particles of *C. butyricum, M. elsdenii,* and *D. vulgaris* as in assay A; *E. coli*, 240 μg; or *C. butyricum*, 410 μg of protein. The assay volume was 0.1 ml, and incubation was at 37°C for 40 min. In both assays the lipids were extracted with butanol (25) and analyzed by thin-layer chromatography as described in the text.

In view of the findings of Langley et al. (14) concerning the low level of phosphatidylserine decarboxylase in certain strains of *B. megaterium* and the sensitivity of the *B. megaterium* enzyme to low concentrations of Triton X-100 (>0.04%), we carried out additional assays for the enzyme in *C. butyricum* and in *M. elsdenii* particles, using phosphatidyl[*U*-^{14}C]serine prepared enzymatically (5). The highest activity measured in *M. elsdenii* particles was 0.0055 U/mg at 0.02% Triton X-100, which was 0.04% of the activity measured in *E. coli* particles. Similarly, the highest activity measured in *C. butyricum* particles, 0.009 U/mg at 0.02% Triton X-100, was 0.07% of the activity in *E. coli* particles. Thus, lower Triton X-100 concentrations had little effect on phosphatidylserine decarboxylase activity in particles from *M. elsdenii* and *C. butyricum*. Langley et al. (14) also found that 20% glycerol enhanced the activity of the *B. megaterium* decarboxylase. No effect of added glycerol (16%) was seen in experiments with *M. elsdenii* particles.

DISCUSSION

These studies on anaerobic bacteria extend our previous work with *C. butyricum* (25). *M. elsdenii* and *V. parvula* membranes were shown to catalyze the formation of CDP-diglyceride in the presence of CTP, phosphatidic acid, and Mg^{2+}. Particles from *M. elsdenii* and *V. parvula* were also capable of forming phosphatidylserine either from CDP-diglyceride generated in situ or from added CDP-diglyceride in the presence of L-serine. *D. vulgaris* particles formed phosphatidylserine from added CDP-diglyceride and serine. They were not tested for activity with CDP-diglyceride generated in situ. *D. vulgaris* was also provisionally shown to be capable of forming phosphatidylglycerol and cardiolipin from CDP-diglyceride and *sn*-glycerol-3-phosphate, but extracts of *M. elsdenii* and *V. parvula* did not catalyze these reactions. These findings are consistent with the presence of phosphatidylglycerol in *D. vulgaris* (18) and its absence in *M. elsdenii* and *V. parvula* (22, 28).

All four anaerobes that we have studied have substantial amounts of phosphatidylethanolamine in their membranes. In *C. butyricum* ATCC 6015, phosphatidyl-*N*-methylethanolamine, presumably formed from phosphatidylethanolamine, is also present (1). It is paradoxical that all efforts to demonstrate the decarboxylation of phosphatidylserine in vitro have shown, at best, only barely detectable levels of this enzyme in *C. butyricum* (25), *M. elsdenii*, and *V. parvula* membranes or whole cell extracts. *D. vulgaris*, in contrast, an anaerobe that does not have plasmalogens (18, 25), does have readily measurable phosphatidylserine decarboxylase activity in its membranes (Table 3). Similar results have recently been reported by Carman and Wieczorek (3). Working with *Clostridium perfringens*, another anaerobe that does not contain plasmalogens (10, 17), they have shown that the major product formed from CDP-diglyceride and L-serine in vitro is phosphatidylethanolamine, indicating the presence of an active phosphatidylserine decarboxylase.

The recent work of Langley et al. (14) with various strains of *Bacillus megaterium*, a gram-positive anaerobe, have shown that strains that have significant amounts of phosphatidylserine have very low levels of phosphatidylserine decarboxylase in vitro. One strain, Km (JW), had 0.5 to 1.5 U/mg (1 to 3% of optimum *E. coli* levels), which was increased to about 5.0 U/mg at 0.02% Triton X-100, compared with 1.5 U/mg at about 0.1% Triton X-100. In another strain, ATCC 13632, the activity could only be measured in extract from lysozyme-treated cells. When 20% glycerol was added to the assay, the highest activity observed was 0.026 U/mg, or about 0.05% of the activity of the *E. coli* enzyme. The activities in both strains were inhibited by hydroxylamine (5 mM). Langley et al. argue that the levels of phosphatidylserine decarboxylase observed are sufficient for the in vivo formation of phosphatidylethanolamine in these strains of *B. megaterium*, whereas such organisms as *E. coli*, with very small phosphatidylserine pools, have excessive amounts of decarboxylase. This conclusion receives support from studies on *E. coli* mutants in the decarboxylase with much lower enzyme levels which nevertheless have normal ratios of phosphatidylethanolamine to phosphatidylserine (23).

Whereas these arguments may apply with even greater force to *M. elsdenii* and *V. parvula*, which have larger amounts of phosphatidylserine than the strains of *B. megaterium* studied by Langley et al. (14), they do not apply to *C. butyricum*, which has a negligible pool of phosphatidylserine (1, 7). Yet it is clear that serine does serve as a precursor to lipid-bound ethanolamine and *N*-methylethanolamine in *C. butyricum* (7). Recent experiments in this laboratory show that the accumulation of [^{32}P]phosphatidylethanolamine is inhibited in *C. butyricum* growing in the presence of $^{32}P_i$ when hydroxylamine (10 mM) is added to growing cultures (H. Goldfine, unpublished data). However, in contrast to other organisms (14, 22), *C. butyricum* showed no accumulation of phosphatidylserine.

It is clear that more work must be done to unravel the pathways of both plasmalogen and phosphatidylethanolamine syntheses in anaerobes. As suggested by Prins et al. (22) these processes may be closely linked. The recent work of Lombardi et al. (15, 16) on *B. megaterium* ATCC 14581 also provides substantial evidence for the formation of phosphatidylethanolamine by an alternative pathway.

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