THE INCORPORATION OF L-3-GLYCEROLPHOSPHATE INTO LIPID AND POLYGLYCEROLPHOSPHATE

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Summary: A membrane preparation from Bacillus subtilis ATCC 6051 incorporated L-3-glycerolphosphate into phosphatidylglycerol in the presence of CTP. Only the non-acylated glycerol moiety of the lipid was derived from L-3-glycerolphosphate substrate. Some polyglycerolphosphate was also formed, the amount depending upon the organism used.

Glycerol teichoic acids are derivatives of polyglycerolphosphate found in both cell walls and membranes of Gram positive bacteria (1). The synthesis of polyglycerolphosphate proceeds through CDPglycerol (2,3) arising from L-3-glycerolphosphate and CTP (4). Recent studies indicated that the wall teichoic acid is synthesized on a lipoteichoic acid carrier (5) while the lipoteichoic acid itself uses phosphatidyl glycerol as a precursor under in vivo conditions (6,7). As quoted by Kennedy (3), during a study on polyglycerolphosphate synthesis we noted that bacterial membrane preparations incorporated L-3-glycerolphosphate into a $CHCl_3$-extractable product as well as polyglycerolphosphate (8). This report shows that the lipid product was a phosphatidylglycerol compound.

MATERIALS AND METHODS

B. subtilis ATCC 6051 and B. stearothermophilus B65 were grown as described by Kennedy (3). Washed cells were suspended in 3 volumes of 0.1M Tris-HCl, pH 7.4 and disintegrated by stirring at maximum speed in a Sorvall Omnimixer homogenizer with No. 16 Ballotini glass beads (2 x wet wt of cells) at 0-5°C until 90-95% of the cells were broken, as judged by phase contrast microscopy (15-20 min). The glass beads were filtered on a sintered glass funnel. Cell walls were removed at

10,000 x g for 15 min and the supernatant was centrifuged at 35,000 x g for 90 min to precipitate the membranes. The membranes were washed once in 0.1M Tris-HCl, pH 7.4 and resuspended in the same buffer (final concentration 20-25 mg protein/ml) and stored at -15°C.

L-[1,3-^{14}C]Glycerolphosphate and CDP[1,3-^{14}C]glycerol were prepared as described (3). Radioactive samples were counted in a model 3214 Packard liquid scintillation counter with 10 ml of Bray's dioxan scintillation fluid. Radioactivity on paper chromatograms were detected by counting 1 cm strips of the paper in 10 ml of toluene scintillation fluid. Periodate oxidation and isolation of radioactive formaldehyde as the dimedone derivative were carried out as described by Kennedy (3). Paper chromatography was carried out with n-propanol-ammonia-water (6/3/1) (solvent A), ethanol-0.5M ammonium acetate, pH 3.8 (5/2) (solvent B) and ethanol-1M ammonium acetate, pH 7.5 (3/7) (solvent C).

RESULTS AND DISCUSSION

When [^{14}C]glycerolphosphate was incubated with bacterial membrane preparations in the presence of CTP, radioactivity was detected in a $HClO_4$-insoluble product. The reaction had an absolute dependence upon CTP which could not be replaced by ATP, UTP, GTP or TTP. In Table 1 we see that the reaction with B. stearothermophilus membranes was stimulated by a soluble enzyme fraction. This is consistent with the synthesis of CDPglycerol from CTP and L-3-glycerolphosphate by a soluble enzyme (4) followed by polyglycerolphosphate synthesis from CDPglycerol by the membranes (2,3). The absence of any stimulation by the soluble fraction of B. subtilis suggested that L-3-glycerolphosphate incorporation was not via CDPglycerol and the product formed might be different from that formed in the presence of added CDPglycerol.

The B. subtilis product from CDPglycerol was previously shown to be polyglycerolphosphate (2,8). It was readily extracted by 10% trichloroacetic acid (TCA) at 0°C for 18 hr but not by $CHCl_3$-methanol

TABLE 1. Localization of glycerolphosphate incorporating activity

Enzyme fraction	*B. stearothermophilus* Protein (mg)	*B. stearothermophilus* Glycerol incorporated (nmoles)	*B. subtilis* Protein (mg)	*B. subtilis* Glycerol incorporated (nmoles)
Cell-free extract	1.42	2.70	0.61	0.01
35,000 x g membranes	0.56	0.71	0.62	0.16
35,000 x g supernatant	0.80	0.17	0.42	0.00
35,000 x g membranes plus 35,000 x g supernatant	0.56 0.80	3.16	0.62 0.42	0.20
Boiled enzyme: 35,000 x g membranes plus 35,000 x g supernatant	0.56 0.80	0.00	0.62 0.42	0.00

The reaction mixture contained 30 umoles of Tris-HCl, pH 8.0, 10 umoles of $MgCl_2$, 0.5 umoles of EDTA, 1.5 umoles of CTP, 12 nmoles of [^{14}C]glycerolphosphate (35,520 dpm) and enzyme as shown, in a total volume of 0.35 ml. After incubation at 30°C for 30 min, 0.14 ml of 0.7N $HClO_4$ was added. The mixture was centrifuged at 10,000 x g for 15 min and the precipitate was washed with 3 x 1ml of 0.15N $HClO_4$ and then with 1 ml of water. It was dissolved in 0.6 ml of 1% NH_3 solution and counted.

(2/1) under reflux for 2 hr. In contrast, about 86% of the *B. subtilis* product from L-3-glycerolphosphate was extracted by $CHCl_3$-methanol (2/1) and was only slowly solubilized by cold 10% TCA. Successive 18 hr TCA extractions solubilized 12%, 15% and 17% of the original radioactivity. On chromatography in solvents A and B, the TCA-solubilized material was found to be mainly α-glycerolphosphate (84-89%) and glycerol (11-15%). Polyglycerolphosphate was immobile in these solvents. *B. stearothermophilus* product from L-3-glycerolphosphate was similarly made up of polyglycerolphosphate (87%) and lipid (13%).

The lipid product of *B. subtilis* was analysed by thin layer chromatography in a solvent system described previously (9); all the radioactivity was detected in the phosphatidylglycerol spot. Deacylation with 0.2N methanolic KOH at 37°C for 15 min (10) converted all the

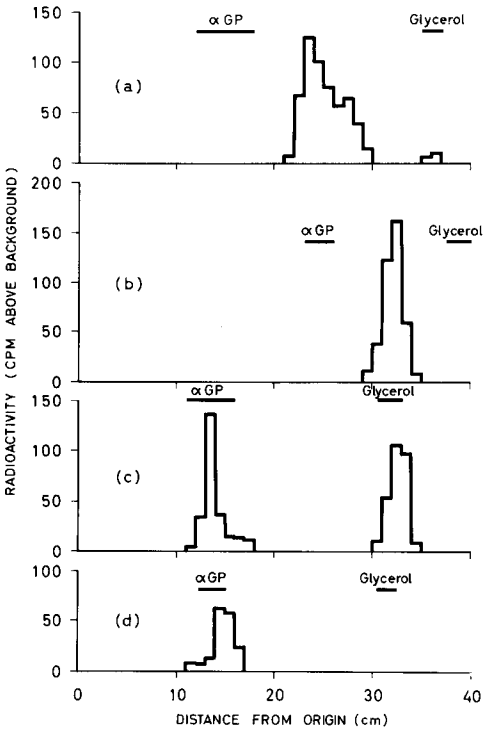

Fig. 1. Chromatography of phosphatidylglycerol degradation products. The radioactive lipid product of B. subtilis was treated as described in the text and the water-soluble products formed were chromatographed. Positions of standard α-glycerolphosphate and glycerol are shown.

radioactivity into a water-soluble compound with the chromatographic mobility of diglycerolphosphate (Fig. 1 a,b). When eluted and hydrolyzed in 1N HCl at 100°C for 10 min, equal amounts of α-glycerolphosphate and glycerol were obtained (Fig. 1c). As expected, periodate oxidation of the diglycerolphosphate (963 dpm) converted 51% of the radioactivity (491 dpm) to formaldehyde. The lipid (1,292 dpm) was also treated with 90% acetic acid at 100°C for 20 min (11) which converted 87% of the radioactivity (1,123 dpm) into a water-soluble compound identified as α-glycerolphosphate (Fig. 1d). The remaining 13% was intact lipid because it was converted into diglycerolphosphate when treated with 0.2N methanolic KOH as described above. This 90% acetic acid hydrolysis distinguishes phosphatidylglycerol from phosphatidylglycerolphosphate (11). Furthermore, since it released 87% of the radioactivity as

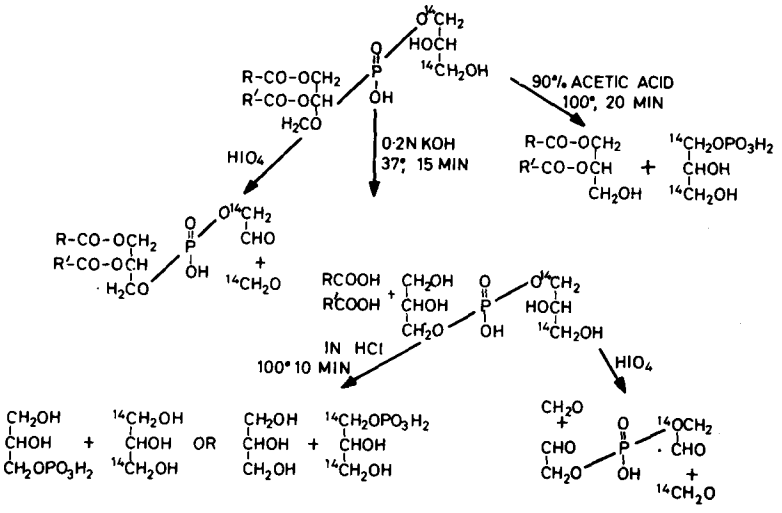

Fig. 2. Degradation scheme for phosphatidylglycerol. R and R' represent fatty acid chains.

glycerolphosphate, it is apparent that only the non-acylated glycerol moiety in the intact lipid was labelled (Fig. 2). This was confirmed by periodate oxidation of the lipid (1,800 dpm) which yielded 48% of the radioactivity (866 dpm) as formaldehyde. If both glycerol moieties were labelled the yield of radioactive formaldehyde would have been only 25%.

Although B. subtilis contains several related phospholipids (12) the incorporation of L-3-glycerolphosphate by B. subtilis membranes was largely into a phosphatidylglycerol compound. The level of incorporation was low but could possibly by increased under different conditions. In the present incubations, the limiting factor seemed to be the level of endogenous lipid to which the radioactive glycerolphosphate was transferred. The requirement for CTP may indicate an intermediate formation of CDPdiglyceride (13). Although under in vivo conditions, phosphatidyl glycerol is a precursor of cardiolipin (14-16) as well as lipoteichoic acid (6,7), the membrane incubations exhibited only the limited incorporation of glycerolphosphate into phosphatidylglycerol. It is interesting to note that in the synthesis of lipoteichoic acid chain from phosphatidylglycerol, Glaser and Lindsay (6) found that only the

glycerolphosphate moiety was used. In the present study, glycerolphosphate was incorporated only into the glycerolphosphate moiety of phosphatidyl glycerol. Clearly further studies on this system for the partial synthesis of phosphatidylglycerol would be useful.

REFERENCES

1. Knox, K.W., and Wicken, A.J. (1973) Bacteriol. Rev. 37, 215-257
2. Burger, M.M., and Glaser, L. (1964) J. Biol. Chem. 239, 3168-3177
3. Kennedy, L.D. (1974) Biochem. J. 138, 525-535
4. Shaw, D.R.D. (1962) Biochem. J. 82, 297-312
5. Fiedler, F., and Glaser, L. (1974) J. Biol. Chem. 249, 2690-2695
6. Glaser, L., and Lindsay, B. (1974) Biochem. Biophys. Res. Commun. 59, 1131-1136
7. Emdur, L.I., and Chiu, T.H. (1974) Biochem. Biophys. Res. Commun. 59, 1137-1144
8. Oo, K.C. (1965) Ph.D. Thesis, University of Otago, New Zealand
9. Oo, K.C., and Lee, Y.H. (1972) J. Biochem. 71, 1081-1084
10. Dawson, R.M.C. (1954) Biochim. Biophys. Acta 14, 374-379
11. Kiyasu, J.Y., Pieringer, R.A., Paulus, H., and Kennedy, E.P. (1963) J. Biol. Chem. 238, 2293-2298
12. Op den Kamp, J.A.F., Redai, I., and van Deenen, L.L.M. (1969) J. Bacteriol. 99, 298-303
13. Kennedy, E.P. (1961) Fed. Proc. 20, 934-940
14. Lee, Y.H., and Oo, K.C. (1973) J. Biochem. 74, 615-621
15. Short, S.A., and White, D.C. (1972) J. Bacteriol. 109, 820-826
16. Card, G.L. (1973) J. Bacteriol. 114, 1125-1137