Enzymic Formation of Glycerol 1:2-Cyclic Phosphate

By N. CLARKE and R. M. C. DAWSON

Department of Biochemistry, ARC Institute of Animal Physiology, Babraham,
Cambridge CB2 4AT, U.K.

(Received 11 December 1975)

Glycerol 1:2-cyclic phosphate is released simultaneously with glycerophosphate when kidney glycerophosphinicholine diesterase (EC 3.1.4.2) acts on glycerylphosphorylcholine and glycerylphosphorylethanolamine. The percentage of cyclic phosphate ester formed is increased at pH values below the optimum and is decreased when Mg^{2+} or Ca^{2+} is added to stimulate the reaction.

The isolation of inositol 1:2-cyclic phosphate as a metabolic intermediary in the catabolism of phosphatidylinositol by Dawson *et al.* (1971) immediately raised the question as to whether other cyclic derivatives could be formed by analogous reactions. In this respect we looked at other possible lipid substrates and could find no evidence for the formation of other cyclic phosphate derivatives, except a small amount of the cyclic form of inositol diphosphate formed when diphosphoinositide was being attacked by phosphodiesterase action. In agreement with the results of Lapetina *et al.* (1975), we found that triphosphoinositide did not produce a cyclic form of inositol triphosphate on enzymic attack by its diesterase, nor does phosphatidylglycerol yield glycerol cyclic phosphate when treated with phospholipase C (EC 3.1.4.3) (Michell & Allan, 1975).

We have now found that glycerol 1:2-cyclic phosphate can be formed as one of the reaction products during the action of glycerophosphinicholine diesterase (EC 3.1.4.2) on glycerylphosphorylcholine or glycerylphosphorylethanolamine substrates, which are intermediaries in the normal route of catabolism of phosphatidylcholine and phosphatidylethanolamine in tissues.

Materials and Methods

The diesterase was prepared from rat kidney cortex, and purified to remove alkaline phosphatase. The initial purification was based on the method of Baldwin & Cornatzer (1968), up to the production of the precipitate in 30-60% satd. $-(NH_4)_2SO_4$. The precipitate was directly dissolved in a solution containing 0.38% (w/v) sodium deoxycholate and 0.2M-glycine/NaOH buffer, pH9. The enzyme was collected by reprecipitating it in 7-21% satd. $(NH_4)_2SO_4$. This precipitate was dissolved in 0.2M-glycine/NaOH buffer, pH9, and the second

$(NH_4)_2SO_4$ precipitation repeated. The final precipitate was dissolved in 0.2M-glycine/NaOH buffer, and the solution dialysed for 24h against 0.02M-glycine/NaOH buffer. The resulting enzyme preparation contained virtually no alkaline phosphatase and represented a 34-fold purification.

Glycerylphosphorylcholine and glycerylphosphorylethanolamine were prepared by deacylation of phosphatidylcholine and phosphatidylethanolamine in methanolic 0.125M-LiOH, H_2O for 15 min at room temperature (20°C). After separation of the water-soluble reaction products the glycerylphosphoryl derivatives were isolated as described by Dawson (1956). When necessary, contaminating glycerol cyclic phosphate (less than 0.5%) was removed by preparative paper ionophoresis (pyridine/acetic acid buffer, pH3.6; 1h; 40V/cm).

The reference glycerol 1:2-cyclic phosphate was prepared by two methods. In one, phosphatidylcholine was subjected to mild alkaline methanolysis under conditions that resulted in an appreciable accumulation of D-glycerol 1:2-cyclic phosphate (Maruo & Benson, 1959; Brockerhoff, 1963), which was isolated from the reaction mixture by preparative ionophoresis. The second method involved shaking disodium β -glycerophosphate pentahydrate (Sigma Chemical Co., St. Louis, MO, U.S.A.; 5g) with 19ml of butyric anhydride and 4ml of pyridine for 1h at 70°C. To the cooled reaction mixture was added 330ml of diethyl ether, and, after being left overnight at 0°C, the precipitate of 3-butyrylglycerol 1:2-cyclic phosphate was collected by centrifugation, and washed with diethyl ether. As required, glycerol 1:2-cyclic phosphate ammonium salt (DL) was generated by heating 0.55g of the butyryl ester in a glass pressure vessel with 10ml of 17M- NH_3 for 20min at 100°C. The ammonium salt was recovered by evaporation to near dryness in a 500ml flask *in vacuo* at 60°C. Methanol (5ml) was added and the evaporation continued, giving a white residue of ammonium glycerol 1:2-cyclic phosphate

(overall yield based on P content was 60%) mixed with some ammonium butyrate. The latter could be removed by precipitation of the cyclic ester (40 mg of P) from methanol (10 ml) with 90 ml of acetone.

Results and Discussion

In preliminary experiments glycerylphosphorylcholine (50 μ g of P) was incubated with 0.05 ml of rat kidney-cortex homogenate (1 g per 10 ml of water, dialysed overnight at 0°C against distilled water) and 0.15 ml of 30 mM-glycine/NaOH buffer, pH 8.9, for 25 min at 37°C. The reaction was stopped by the addition of 4 vol. of chloroform/methanol (2:1, v/v), and the upper phase was collected, decreased in volume and used for paper ionophoresis (acetic acid/pyridine buffer, pH 3.6; 40 V/cm; 2 h). Spraying with acid molybdate to detect compounds containing phosphorus showed unchanged glycerylphosphorylcholine near the origin, glycerophosphate (m_{R_f} 0.86),

Table 1. Effect of bivalent cations on glycerol 1:2-cyclic phosphate liberation from glycerylphosphorylcholine

The incubation mixture consisted of glycerylphosphorylcholine (8 mm), glycine/NaOH buffer, pH 8.9 (0.1 M), and 35 μ g of protein in a final volume of 0.2 ml. Incubation was for 2 h at 37°C.

Bivalent cation	Concentration (mm)	Glycerylphosphorylcholine decomposed (μ g of P)	Cyclic glycerophosphate formed (%)
Mg^{2+}	0	11.7	58
	0.05	18.4	51
	0.2	22.9	47
	1	25.1	32
	10	27.4	26
	20	24.8	24
Ca^{2+}	0	6.8	60
	0.05	8.6	45
	0.2	13.3	28
	10.0	14.2	28
Zn^{2+}	0.1	11.0	44
	1.0	12.4	34

Fig. 1. Effect of pH on glycerol 1:2-cyclic phosphate formation from glycerylphosphorylcholine

Glycerylphosphorylcholine (13 mm), $MgCl_2$ (5 mm), 35 μ g of enzyme protein in a series of glycylglycine/NaOH buffers (90 mm) in final volume of 0.22 ml. The incubation was for 100 min at 37°C. O, Glycerylphosphorylcholine decomposed; ●, % of glycerol 1:2-cyclic phosphate in phosphorus-containing reaction products. The result is typical of two similar experiments.

P_i and a further spot running slightly faster than P_i (m_{R_f} 1.04). When the experiment was repeated with the purified enzyme, which was free from alkaline phosphatase, virtually no P_i was produced, and only glycerophosphate and the unknown phosphorus-containing compound were formed.

The latter compound was isolated by preparative paper ionophoresis under the conditions described above. The strip of the unknown compound was located by spraying marker strips on either side and eluting it with water. It was identified as glycerol 1:2-cyclic phosphate by comparison with the two reference samples of this substance prepared as described above. On paper ionophoresis at pH 3.6 it migrated identically with the two reference preparations (m_{R_f} 1.04). All three compounds co-chromatographed on paper in phenol saturated with water/acetic acid/ethanol (10:1:1.2, by vol.) (R_F 0.48) and in ethanol/5.4 M- NH_3 (2:1, v/v) (R_F 0.64). On mild acid hydrolysis (0.05 M-HCl; 40°C) all three compounds decomposed with identical half-lives ($8 \pm \frac{1}{2}$ min), so that by 50 min virtually none of the original substances remained. Paper ionophoresis showed that the sole product was glycerophosphate. The extreme acid-lability of glycerol 1:2-cyclic phosphate has previously been reported (Ukita *et al.*, 1955), and is useful for eliminating the possibility that the enzymic product could be glycerol 1:3-cyclic phosphate, which would be much more stable (Khorana *et al.*, 1957).

In agreement with Baldwin & Cornatzer (1968), the pH optimum of the kidney glycerophosphinicholine diesterase was higher than that in liver

Fig. 2. Time-course of glycerol 1:2-cyclic phosphate (●) and glycerophosphate (○) release from glycerylphosphorylcholine

The incubation mixture consisted of glycerylphosphorylcholine (8.1 mM), $MgCl_2$ (10 mM), glycine/NaOH buffer, pH 8.9 (90 mM), and 35 μ g of enzyme protein in a final volume of 0.2 ml (temperature of incubation 37°C). The result was confirmed in a similar experiment.

(Dawson, 1956), being 9 in a glycine/NaOH buffer and above 9 in a glycyglycine/NaOH buffer (Fig. 1). The percentage of glycerol 1:2-cyclic phosphate in the reaction products increased at pH values below the optimum (Fig. 1). The addition of Mg^{2+} to the incubation mixture caused a stimulation of the rate of glycerylphosphorylcholine breakdown, as had previously been observed for the liver enzyme (Dawson, 1956), but at the same time there was a substantial decrease in the percentage of the substrate decomposed to the cyclic ester (Table 1). Ca^{2+} and Zn^{2+} produced similar effects (Table 1). When the time-sequence for the formation of both glycerophosphate and cyclic ester was followed it became clear that both products were liberated simultaneously. Thus there was no evidence that the glycerophosphate formed in the initial reaction, arose via glycerol cyclic phosphate as intermediate (Fig. 2).

Glycerol 1:2-cyclic phosphate was also formed during the decomposition of glycerylphosphoryl-ethanolamine, which was readily attacked by the enzyme preparation. Glycerylphosphorylglycerol and glycerylphosphorylserine were attacked only very slowly, but the cyclic ester was still formed. However, the cyclic ester was not detected with glycerylphosphorylinositol as substrate.

It seems likely that glycerol 1:2-cyclic phosphate has not previously been reported as a metabolic product of glycerylphosphorylcholine and glycerylphosphorylethanolamine decomposition because of its extreme acid-lability. This means that it is quickly hydrolysed in acid protein-denaturing agents, such as trichloroacetic acid. In addition, we have established that in tissues there is an Mg^{2+} -dependent phosphodiesterase that can rapidly open the ring and produce glycerophosphate. The activity of this enzyme could explain the decreased formation of cyclic ester observed with increased Mg^{2+} concentrations. In the present experiments no phosphorylcholine was detected when glycerylphosphorylcholine was decomposed by kidney homogenates, but this may be due to their high alkaline phosphatase content. Abra & Quinn (1975) have described the formation of phosphorylcholine from glycerylphosphorylcholine by brain homogenates, and our preliminary studies with a water extract of the same tissue have confirmed this finding.

References

- Abra, R. M. & Quinn, P. J. (1975) *Biochim. Biophys. Acta* **380**, 436–441
- Baldwin, J. J. & Cornatzer, W. E. (1968) *Biochim. Biophys. Acta* **164**, 195–204
- Brockerhoff, H. (1963) *J. Lipid Res.* **4**, 96–99
- Dawson, R. M. C. (1956) *Biochem. J.* **62**, 689–693
- Dawson, R. M. C., Freinkel, N., Jungalwala, F. B. & Clarke, N. (1971) *Biochem. J.* **122**, 605–607
- Khorana, H. G., Tener, G. M., Wright, R. S. & Moffat, J. G. (1957) *J. Am. Chem. Soc.* **79**, 430–436
- Lapetina, E. G., Seguin, E. B. & Agranoff, B. W. (1975) *Biochim. Biophys. Acta* **398**, 118–124
- Maruo, B. & Benson, A. A. (1959) *J. Biol. Chem.* **234**, 254–256
- Michell, R. H. & Allan, D. (1975) *FEBS Lett.* **53**, 302–304
- Ukita, T., Bates, N. A. & Carter, H. E. (1955) *J. Biol. Chem.* **216**, 867–874