Biochem. J. (1967) 102, 205

The Formation of Phosphatidylglycerol and other Phospholipids by the Transferase Activity of Phospholipase D

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(*Received* 1 *August* 1966)

1. Purified phospholipase D can catalyse the transfer of a 'phosphatidyl' unit from lecithin to various aliphatic alcohols such as glycerol, ethanolamine, methanol and ethylene glycol with the formation of the equivalent phospholipid. 2. The transferase reaction occurs simultaneously with hydrolase activity but at high alcohol concentrations the former predominates. 3. The acceptor molecule must contain a primary alcoholic grouping. 4. The chromatographic and ionophoretic mobilities of the deacylation products of many enzymically synthesized phospholipids are reported. 5. Enzymically prepared phosphatidylglycerol has been isolated in good yield. Chemical degradation showed that the 'phosphatidyl unit' of lecithin had been transferred predominantly to the α-hydroxyl groups of glycerol. 6. Water-soluble alcohols can markedly stimulate the liberation of choline from ultrasonically treated lecithin by phospholipase D. The stimulation is usually due to an increase in hydrolase activity although often the associated transferase activity contributes.

A. A. Benson, S. Freer & S. F. Yang reported to the 9th International Conference on the Biochemistry of Lipids, Noordwijk aan Zee (1965), the formation of phosphatidylethanol and phosphatidylmethanol when plant tissues containing phospholipase D were extracted with the cold alcohol. Before this we had noted that, when purified cabbage phospholipase D was incubated with lecithin in the presence of glycerol, some phosphatidylglycerol was formed as well as the usual metabolic product, phosphatidic acid (Dawson & Hemington, 1967). The characteristics of this transferase reaction of the enzyme have been examined in some detail. The reaction has been used to prepare phosphatidylglycerol, and evidence has been obtained that many aliphatic primary alcohols can act as acceptors with the formation of the corresponding phospholipid. During our investigations it has also been found that glycerol and some other aliphatic alcohols can greatly stimulate the activity of the enzyme towards an ultrasonically treated lecithin substrate. No evidence was obtained that this was directly related to the transferase activity of the enzyme.

METHODS

Preparation and assay of phospholipase D. The enzyme was prepared and partially purified by heat treatment and acetone precipitation by the method of Davidson & Long (1958). In some experiments the enzyme was further purified by gradient-density electrophoresis (Dawson & Hemington, 1967). Enzymic activity was assayed as described by Dawson & Hemington (1967) by measuring the liberation of choline from a lecithin substrate. Alternatively, the lipid products of the reaction (e.g. phosphatidic acid, phosphatidylglycerol) were determined by using alkaline degradation and single-dimensional ionophoresis at pH 3·6 (Dawson & Hemington, 1967) after the reaction had been terminated as described below.

Examination and estimation of the phospholipids formed by transferase activity. The reaction was stopped by shaking the incubation mixture with 5–6 vol. of chloroform–methanol (2:1, v/v). After centrifuging, the lower chloroform-rich layer was removed and washed with an equal volume of methanol–chloroform–0·01 M-$CaCl_2$ (45:3:47, by vol.). The lipid products of the reaction were degraded with alkali and the deacylation products were examined and estimated by two-dimensional paper chromatography and ionophoresis (Dawson, Hemington & Davenport, 1962).

Isolation of phosphatidylglycerol. Lecithin (130 μmoles) was incubated in 30 ml. of medium containing 14 μmoles of sodium dodecyl sulphate, sodium acetate–acetic acid buffer, pH 5·4 (0·014 M), $CaCl_2$ (0·04 M), glycerol (2 M) and 15 mg. of acetone-precipitated enzyme. After 50 min. at 27°, the mixture was shaken with 168 ml. of chloroform–methanol (2:1, v/v) and centrifuged. The lower chloroform-rich layer was collected and washed once with 0·5 vol. of chloroform–methanol–0·01 M-$CaCl_2$ (3:45:47, by vol.). The lower layer was evaporated to dryness in a rotary evaporator and the residue dissolved in 5 ml. of chloroform. The phospholipids were then chromatographed (Haverkate & van Deenen, 1965) on a silicic acid (Mallinckrodt) column (20 cm. long × 1·8 cm. diam.) poured in chloroform. The

column was eluted with (1) 300ml. of chloroform–methanol (97:3, v/v), (2) 300ml. of chloroform–methanol (47:3, v/v) and (3) 300ml. of chloroform–methanol (22:3, v/v); 26ml. fractions were collected. Two phosphorus-containing peaks emerged with maxima at fractions 15 and 23, i.e. on changing solvents from (1) to (2) and from (2) to (3). Thin-layer chromatography and alkaline methanolysis showed that the initial peak was largely phosphatidic acid and the other peak phosphatidylglycerol. The phosphatidylglycerol peak was collected and purified on a similar silicic acid column. The product was virtually homogeneous on thin-layer chromatography and glycerylphosphorylglycerol was the sole product on alkaline methanolysis (Dawson *et al.* 1962).

Periodate–dimethylhydrazine degradation. Glycerylphosphorylglycerol prepared from phosphatidylglycerol was degraded with periodate and dimethylhydrazine according to the procedures of Lecocq & Ballou (1964) and Brundish, Shaw & Baddiley (1965). Phosphatidylglycerol (144μg. of P) was deacylated with methanolic NaOH and the alkali was then removed with Amberlite IRC-50 (Dawson, 1960). The aqueous layer containing the glycerylphosphorylglycerol was evaporated to dryness and the residue treated with 0·3ml. of 0·1M-sodium metaperiodate for 90min. at room temperature. Excess of periodate was destroyed with 0·3ml. of 0·025M-ethylene glycol and the solution was treated with 0·45ml. of freshly prepared aq. 1% (v/v) 1,1′-dimethylhydrazine adjusted to pH6·0 with acetic acid. After being kept overnight (18hr.) at 37° the solution was extracted three times with 1ml. of chloroform. The aqueous solution was then examined by ionophoresis at pH3·6 (Dawson *et al.* 1962).

Thin-layer chromatography. Lecithin (R_F0·11), phosphatidic acid (R_F0·08) and phosphatidylglycerol (R_F0·35) were separated on silica gel H (E. Merck A.-G., Darmstadt, Germany) by using an S chamber (Parker & Peterson, 1965) and a solvent composed of chloroform–methanol–5·2N-NH_3 (65:30:5·7, by vol.). A solvent consisting of chloroform–methanol–water (28:7:1, by vol.) was also used for examining phosphatidylethanolamine (R_F0·5) and phosphatidylglycerol (R_F0·38).

Phospholipids. Phospholipids were prepared as follows: egg lecithin and egg phosphatidylethanolamine (Dawson, 1963); ^{32}P-labelled yeast lecithin (Bangham & Dawson, 1960); phosphatidic acid (Dawson & Hemington, 1967). Synthetic racemic 1-oleoyl-2-palmitoyl-phosphatidylglycerol was kindly given by Dr P. P. M. Bonsen.

RESULTS

Table 1 shows the results of initial experiments with the purified 'glycerinated' phospholipase D obtained by density-gradient electrophoresis. The phospholipids produced by the enzyme reaction were identified and determined by the procedure of Dawson *et al.* (1962) as well as by thin-layer cochromatography with the authentic phospholipids. As was to be expected phosphatidic acid was the main reaction product. However, some phosphatidylglycerol was formed in all experiments, the glycerol added in the form of glycerinated enzyme acting as an acceptor for the 'phosphatidyl' unit from the lecithin. In the presence of ethanolamine the overall reaction was inhibited, confirming a previous observation (Dawson & Hemington, 1967), but at the same time a small amount of phosphatidylethanolamine was formed. However, on adding L-serine or inositol no phosphatidylserine or phosphatidylinositol was produced and the overall reaction was not inhibited (Table 1). In these initial experiments a lecithin substrate activated with sodium dodecyl sulphate was used (Dawson & Hemington, 1967), but it was found that similar transferase activity occurred with a substrate of lecithin treated ultrasonically.

Many other aliphatic alcohols apart from glycerol and ethanolamine when added to the incubation medium could act as acceptors for the 'phosphatidyl

Table 1. *Transferase activity of phospholipase D*

The incubation medium (6ml.) contained lecithin (24·8μmoles) and sodium dodecyl sulphate (10μmoles) in 0·0125M-sodium acetate–acetic acid buffer, pH5·7, containing $CaCl_2$ (0·0365M); 0·5ml. of electrophoretically purified enzyme (Dawson & Hemington, 1967) was present. The mixture was incubated for 15min. at 27°.

	Additions to basic incubation medium	Phospholipids formed (μg. of P)				
		Phosphatidic acid	Phosphatidyl-glycerol	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Phosphatidyl-inositol
Expt. 1	Glycerol (0·47M)	156	61	—	—	—
	Glycerol (0·47M)+ethanolamine hydrochloride (0·1M)	40	14	17	—	—
	Glycerol (0·47M)+L-serine (0·063M)	156	59	—	0	—
Expt. 2	Glycerol (0·47M)	137	78	—	—	—
	Glycerol (0·47M)+ethanolamine hydrochloride (0·1M)	47	28	27	—	—
	Glycerol (0·47M)+inositol (0·1M)	160	104	—	—	0

unit' transferred from lecithin (Table 2). By isolating the phospholipids from such mixtures and subjecting them to alkaline degradation and thin-layer chromatography, evidence was obtained for the synthesis of phosphatidylmethanol, phosphatidylethanol, phosphatidylpropan-1-ol, phosphatidylethylene glycol, phosphatidylpropane-1,2-diol, phosphatidyldiethylene glycol and phosphatidylmonoacetin. The ionophoretic mobilities and R_F values in a phenol solvent of the glycerylphosphoryl derivatives produced by deacylation are reported in Table 2. On the other hand, propan-2-ol, sugars and hydroxy acids were unable to act as acceptor molecules (Table 2). Additional experiments made with [^{32}P]lecithin gave no evidence that the enzyme possessed any ability to catalyse an exchange reaction with other intact phospholipids such as phosphatidylethanolamine or phosphatidylinositol or to transfer a phosphatidyl unit to long-chain monoglyceride or diglyceride. In such experiments the lipid was admixed with the [^{32}P]lecithin in approximately equimolar proportions.

In the presence of glycerol the rate of formation of both phosphatidylglycerol and phosphatidic acid from ultrasonically treated lecithin was approximately linear with time (Fig. 1): 11% of the substrate had been decomposed at the end of the experiment. Fig. 2 shows the relationship between glycerol concentration and the relative formations of phosphatidic acid and phosphatidylglycerol from a mixed lecithin–dodecyl sulphate substrate. The addition of glycerol inhibited the overall liberation of choline, but the percentage of phosphatidylglycerol formed increased and became greater than that of the phosphatidic acid at about 1·5M-glycerol.

Structure of enzymically prepared phosphatidylglycerol. Phosphatidylglycerol was isolated from large-scale incubation mixtures in which lecithin and phospholipase D had been incubated in the presence of glycerol (see the Methods section). After deacylation and degradation of the glycerylphosphorylglycerol with periodate–dimethylhydrazine, ionophoresis at pH 3·6 showed the phosphorus to be in the form of inorganic phosphate (97%) and glycerophosphate (3%). A sample of phosphatidic

Table 2. *Ability of various aliphatic alcohols to act as acceptors in the transferase reaction*

The incubation medium contained 3·8 μmoles of ultrasonically treated lecithin/ml. of 0·05M-sodium acetate–acetic acid buffer, pH 5·4, containing $CaCl_2$ (0·029M); the amount of enzyme present was 0·13 mg. of protein/ml. in Expts. 1 and 2 and 0·26 mg. of protein/ml. in Expt. 3. The mixture was incubated for 15 min. at 27°, and the reaction was stopped by shaking with 5·5 vol. of chloroform–methanol (2:1, v/v). The solvents used were: (*a*) phenol (saturated with water–acetic acid–ethanol (50:5:6, by vol.); (*b*) pyridine–acetic acid–water (1:10:89, by vol.) (pH 3·6).

	Alcohol (X)	Phosphatidic acid formed (% of substrate)	Phosphatidyl-X formed (% of substrate)	Properties of deacylation product of phosphatidyl-X: R_F on chromatography in solvent (*a*)	Properties of deacylation product of phosphatidyl-X: M_{P_i} on paper ionophoresis in buffer (*b*)
Expt. 1	None	2·3	—	—	—
	Methanol (0·45M)	6·7	7·0	0·54	0·83
	Propanol (0·45M)	2·5	4·7	0·74	0·70
	Propane-1,2-diol (0·45M)	3·3	2·4	0·70	0·69
	Glycerol (0·45M)	4·0	6·4	0·45	0·63
	Ethylene glycol (0·45M)	4·6	7·6	0·49	0·77
	Sodium DL-malate (0·30M)	3·7	0	—	—
Expt. 2	None	2·4	—	—	—
	Glycerol (0·45M)	3·1	5·7		
	Propan-2-ol (0·45M)	1·2	0	—	—
	Diethylene glycol (0·45M)	5·3	1·2	0·70	0·70
	Ethanol (0·45M)	4·4	12·2	0·69	0·83
	Monoacetin (0·45M)	3·2	3·2		
	Sucrose (0·42M)	1·8	0	—	—
	Glucose (0·33M)	1·5	0	—	—
	Galactose (0·33M)	1·6	0	—	—
Expt. 3	None	2·8	—	—	—
	Sodium citrate (0·33M)	7·8	0	—	—
	Sodium DL-lactate (0·33M)	9·3	0	—	—
	Sodium glycollate (0·33M)	6·9	0	—	—
	Sodium DL-malate (0·30M)	7·4	0	—	—

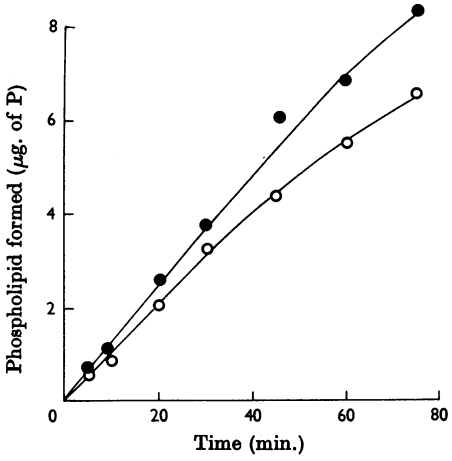

Fig. 1. Time-course of transferase and hydrolase activities of purified phospholipase D. The incubation medium contained ultrasonically treated lecithin (4·6 μmoles) as substrate and 0·2 ml. of electrophoretically purified enzyme (Dawson & Hemington, 1967) in a total volume of 1·2 ml.; glycerol (0·47 M was present); other details were as given in Table 1. ○, Phosphatidylglycerol; ●, phosphatidic acid.

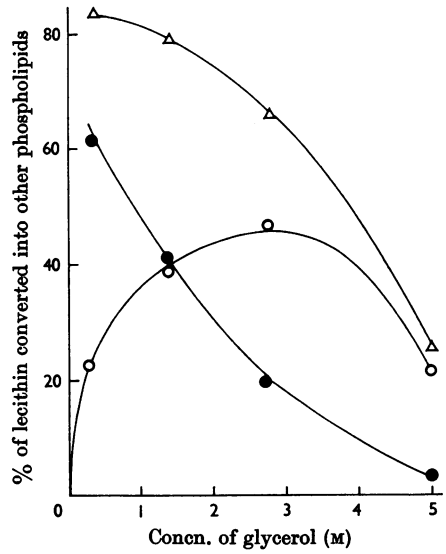

Fig. 2. Effect of glycerol concentration on the transferase and hydrolase activities of phospholipase D. The incubation medium contained lecithin (4·6 μmoles) as substrate, activated with sodium dodecyl sulphate (0·5 μmole), and 0·18 mg. of acetone-precipitated enzyme protein; other details were as given in Fig. 1. ○, Phosphatidylglycerol; ●, phosphatidic acid; △, phosphatidylglycerol + phosphatidic acid.

acid treated in the same way gave exclusively inorganic phosphate. This showed that the enzymically prepared phosphatidylglycerol had predominantly the phosphatidyl-α-glycerol structure.

Stimulation of the action of phospholipase D on ultrasonically treated lecithin by aliphatic alcohols. During the experiments described above it was noted that glycerol and many other aliphatic alcohols substantially stimulated the overall activity of phospholipase D (choline liberation) acting on an ultrasonically treated lecithin substrate (Table 2). However, this effect was by no means universal, and propan-2-ol and the sugars, sucrose, glucose and galactose were not active. Sometimes the stimulation was partly due to the transferase activity occurring but almost always there was an enhanced hydrolysis of the lecithin to phosphatidic acid (Table 2). For example, on adding glycerol to an ultrasonically treated lecithin system there was initially an increase in the hydrolysis rate, which then declined; the transferase activity reached a maximum at 0·7 M-glycerol and then also declined (Fig. 3). Hydroxy acids produced stimulation of the hydrolysis even though no transferase activity took place (Table 2).

In further experiments a suspension was prepared of mixed phospholipid particles containing lecithin together with 5 or 10 molar percentage of phosphatidylglycerol. After ultrasonic treatment such mixtures were hydrolysed by phospholipase D at

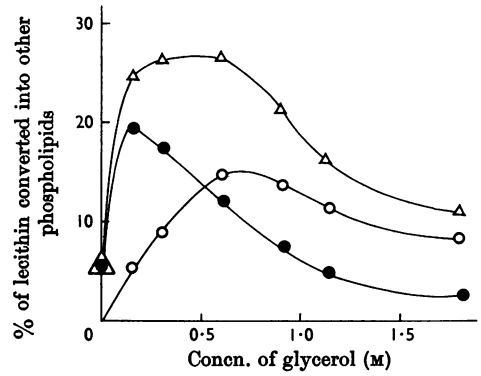

Fig. 3. Effect of glycerol concentration on the transferase and hydrolase activities of phospholipase D. The incubation medium (1·8 ml.) contained ultrasonically treated lecithin (6·9 μmoles) in 0·05 M-sodium acetate–acetic acid buffer, pH 5·4, containing $CaCl_2$ (0·029 M); 0·46 mg. of acetone-precipitated enzyme protein was present. The mixture was incubated for 20 min. at 27°. ○, Phosphatidylglycerol; ●, phosphatidic acid; △, phosphatidylglycerol + phosphatidic acid.

a substantially greater rate than ultrasonically treated lecithin alone. However, the increased rate was in fact less than that obtained in similar

experiments where an equivalent molar percentage of phosphatidic acid was added to the lecithin.

DISCUSSION

The present results confirm and extend the preliminary observations by A. A. Benson, S. Freer & S. F. Yang (see the introduction) on the transferase activity of phospholipase D. The enzyme is similar to many other hydrolytic enzymes such as β-glycosidases, proteinases and phosphatases, which can also show transferase activity (Morton, 1953). As with these enzymes the transferase activity of phospholipase D occurs simultaneously with normal hydrolysis of the substrate and presumably the same enzyme is responsible for both activities.

Clearly the structural requirements of the alcohol that accepts the phosphatidyl unit from lecithin are fairly specific; thus ethanolamine will accept whereas serine will not, and though propan-1-ol reacts propan-2-ol does not. The evidence in fact suggests that the transfer reaction is specific for compounds containing a primary alcohol group. Since monoacetin and diacetin can act as acceptors whereas long-chain monoglyceride and diglyceride admixed with the lecithin cannot, it seems likely that the primary alcohol needs to be water-soluble, possibly in order to obtain sufficiently high concentrations of acceptor molecules for the transferase reaction to be detected. However, the transferase reaction probably possesses other specificity since glycollate cannot act as an acceptor even though it is water-soluble and possesses a primary alcoholic grouping.

The conclusion that the enzyme has a specificity for the primary alcohol grouping is strengthened by the chemical degradation of the phosphatidylglycerol enzymically prepared from lecithin and glycerol. Since the original lecithin has the L-configuration and the α-structure (Baer, 1955), it is reasonable to suppose that the glycerol of the phosphatidyl unit transferred will continue to remain phosphorylated in the α-position. This means therefore that deacylation of the phosphatidylglycerol will produce αα′-glycerylphosphorylglycerol or αβ-glycerylphosphorylglycerol, depending on whether the phosphatidyl unit has been transferred to the α- or β-hydroxyl group of the receptor glycerol molecule. On oxidation of αα′-glycerylphosphorylglycerol with periodate and removal of the glycolaldehyde groups with dimethylhydrazine, inorganic phosphate would be the expected product, whereas αβ-glycerylphosphorylglycerol would yield glycerol 2-phosphate on similar treatment (Brundish *et al.* 1965). In the present case the deacylation product of the enzymically synthesized phosphatidylglycerol yielded 97% of inorganic phosphate and 3% of glycerol phosphate on periodate oxidation and dimethylhydrazine treatment. This indicates that the phosphatidyl unit has been predominantly, if not exclusively, transferred to the primary alcohol groups of the glycerol. The small amount of glycerol phosphate produced could, in fact, have arisen during the isolation of glycerylphosphorylglycerol through hydrolysis with phosphoryl migration.

The phosphatidylglycerol found in higher plants has a similar structure (Haverkate & van Deenen, 1965), but this is presumably synthesized by a different process analogous to that described by Kiyasu, Pieringer, Paulus & Kennedy (1963) in animal tissues and involving the dephosphorylation of phosphatidylglycerol phosphate. In fact it is doubtful whether the transferase reaction is of importance in the living plant cell owing to the high concentration of alcohol required to compete significantly with water for the 'phosphatidyl unit' combined in the enzyme–substrate complex. In this respect it is probably analogous to the transferase activity of phosphatases in the presence of high concentrations of alcohols (Morton, 1953), a process that appears to be of doubtful significance *in vivo*. Nevertheless, it is clear from the results presented in Figs. 2 and 3 that glycerol acts as a much better substrate for the enzyme than water. Thus when equal amounts of phosphatidic acid and phosphatidylglycerol are produced the reaction mixture contains 0·7–1·5 M-glycerol and approx. 48 M-water.

Though it is apparent that many aliphatic alcohols can stimulate the liberation of choline from ultrasonically treated lecithin by phospholipase D, there is no good evidence that this is directly related to the transferase activity of the enzyme. Whereas the simple mono-, di- and tri-hydric alcohols stimulate hydrolysis when at the same time phosphatidyl units are transferred to them, many hydroxy acids can stimulate hydrolysis without acting as acceptors (Table 2). With the stimulation invoked by glycerol there is no evidence from the relative rates of formation of phosphatidylglycerol and phosphatidic acid with time that the former is acting as an intermediary in the production of the latter. Presumably the hydrolysis of phosphatidylglycerol reported by Haverkate & van Deenen (1965) occurs under conditions different from those used in the present experiments (e.g. higher enzyme concentrations).

The observation that glycerol caused no stimulation of the choline liberation from a lecithin substrate activated with sodium dodecyl sulphate (Fig. 2) could indicate that it was having certain physicochemical effects on the ultrasonically treated lecithin sols. The size and shape of the particles in these sols can vary with the aqueous environment

(Attwood & Saunders, 1966). No evidence could be obtained that the formation of phosphatidylglycerol is necessary for such a physicochemical effect. Thus, though it is true that admixture of phosphatidylglycerol with lecithin before ultrasonic treatment increased the rate of hydrolysis, this stimulation was less than that produced by adding an equivalent amount of phosphatidic acid to the same system.

I am indebted to Miss Norma Hemington for valuable assistance.

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