Phytochemistry, 1980, Vol. 19, pp. 1661–1663. Pergamon Press Ltd. Printed in England.

IN VITRO SYNTHESIS OF PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLCHOLINE BY PHOSPHOLIPASE D

SUKHENDU B. MANDAL, PARIMAL C. SEN* and PARUL CHAKRABARTI

Department of Chemistry, Bose Institute, Calcutta 700009, India

(*Revised received 5 December 1979*)

Key Word Index—Phosphatidylinositol; phosphatidylcholine; synthesis; phospholipase D; egg yolk.

Abstract—Phosphatidylinositol (PI) was prepared from egg lecithin by a one-step transphosphatidylation reaction catalysed by phospholipase D in the presence of myo-inositol. Similarly phosphatidylcholine (PC) has been synthesized by the same technique from egg phosphatidylethanolamine using phospholipase D and choline chloride. The yield of PI was *ca* 25% and that of PC *ca* 28%. The transphosphatidylase function of phospholipase D offers a useful route for the synthesis of different classes of phospholipids.

INTRODUCTION

Phospholipase D is widely distributed in plants [1–3], high activities being present in sources such as Savoy cabbage, Brussels sprouts, spinach, carrot, cereal grains and cotton seed. It has not yet been detected in animal and bacterial sources. Phospholipase D is a hydrolytic enzyme and cleaves the terminal phosphate diester linkage of glycerophospholipids yielding phosphatidic acid (PA) [4]. It is active towards different phospholipids and its activity is reported [1, 5] to vary in the following order, phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidylserine (PS). Later it was found to be active on phosphatidylglycerol (PG) [6]. The enzyme acts readily on phosphoglycerides having the L-α-structure [7] but it will also attack substrates with the DL-α or the β-structure, although at much slower rates [1].

Besides the hydrolase activity, the enzyme also possesses transphosphatidylation activity, i.e. transfer of the phosphatidyl unit from PC to various acceptor molecules like glycerol, ethanolamine, methanol, ethanol and serine [8–14] with the formation of the corresponding phospholipids. The enzyme binds to the phosphatidyl unit of phospholipid and forms a complex. Then this complex transfers the phosphatidyl unit to the acceptor molecule. This may be represented as follows:

(a) PC + enzyme–SH

↓

Phosphatidyl–S–enzyme + choline

(b) Phosphatidyl–S–enzyme + R–OH

↓

Phosphatidyl–OR + enzyme–SH.

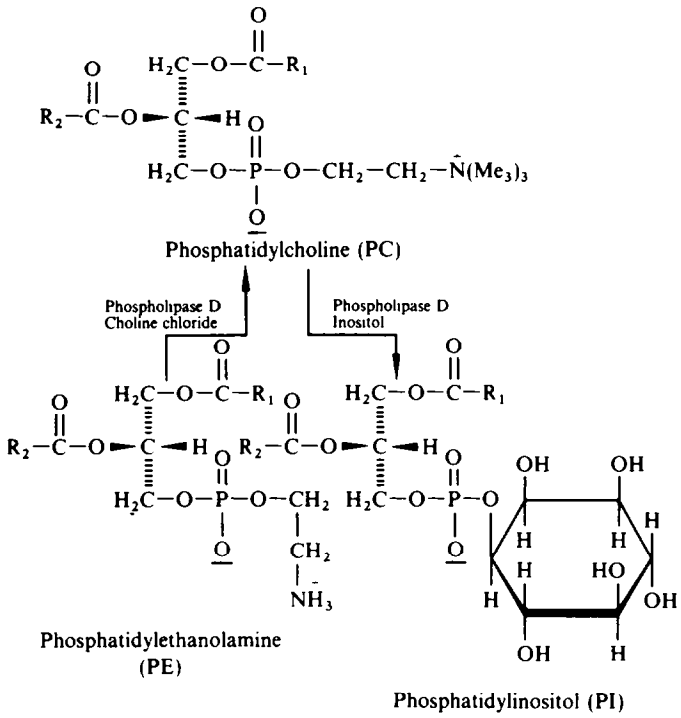
According to Dawson [13] the acceptor molecules should contain a primary hydroxyl group and be water soluble. However, glycollate, although possessing the above criteria, does not act as an acceptor molecule implying that the transferase reaction depends also on some other structural specificity.

Phospholipase D is active in the pH range 4–6. Generally acetate buffer (pH 5.6) containing $CaCl_2$ (40–100 mM) is used [8, 9]. The transferase reaction occurs simultaneously with hydrolase activity but with high alcohol concentrations the former predominates [13]. The discovery of the transphosphatidylation activity in addition to the classical hydrolytic activity of phospholipase D opens up a new route to synthesize different classes of phospholipids. PG [13], PS [8], and PE [9] have been synthesized from egg PC by this procedure. In the present communication we are reporting the enzymatic syntheses of PI and PC from egg PC and egg PE respectively. Myo-inositol and choline chloride have been used as acceptors in the synthesis of PI and PC respectively. The whole process is shown in Scheme 1.

RESULTS AND DISCUSSION

According to previous investigators [9, 12, 13], 90–95% of PC can be converted to phosphatidylmethanol, phosphatidylethanol, PG and PE at the optimal concentrations of the acceptor molecules, whereas the remaining 5–10% is converted to PA. However, in our work the percentage conversion of PC to PI is *ca* 25% and that of PE to PC *ca* 28%, the remaining being the summation of PA and the unreacted starting material, e.g. the hydrolytic reactions are not going to completion. In the first case *ca* 35% PC is unreacted, whereas in the second case unreacted PE is *ca* 36%. It appears that the hydrolase activity of phospholipase D is partially inactivated either by free inositol or PI formed in the reaction mixture, because after addition of a large

*Present address:—Upstate Medical Centre, New York 13210, U.S.A.



Scheme 1.

excess of enzyme only PA is obtained. Similar explanations may be given for the low conversion of PE to PC in which case unreacted starting material is also present.

Figure 1A shows that maximum PI is formed at 3–4 hr. Figure 2A shows that formation of PI is maximum when the inositol concentration is 20% wt/vol. and after that PI synthesis decreases with increase in inositol concentration. From Figure 1B it is apparent that maximum PC synthesis occurs after 3 hr. Figure 2B shows that maximum PC is formed when the choline chloride concentration is 35% wt/vol. After that no appreciable change is observed. In PI, phosphate and inositol were found to be in 1:1 molar ratio and in PC, phosphate and choline is in 1:1 molar ratio.

The present results confirm the previous observations on the transferase activity of phospholipase D. Our experiments further confirm the view that the same enzyme is responsible for both activities and the reaction is occur via a one-step transphosphatidylation reaction. It is further supported by the fact that no PI is formed when the reaction is carried out with PA as the starting material in the presence of inositol.

The structural specificity of the acceptor molecules to receive the phosphatidyl unit in the transferase reaction is perhaps not as rigid as suggested by previous investigators [13], because in the present work inositol which only contains secondary hydroxyl groups, could be used for the same purpose. However, inositol is a poor acceptor when compared with the primary alcohols. Therefore, *in vitro* syntheses of PG [13], PE [9], PS [8], diphosphatidylglycerol [24], PI and PC show that the transferase activity of phospholipase D offers a useful route for the synthesis of different classes of phospholipids.

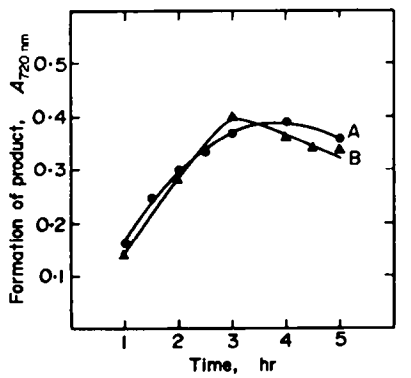


Fig. 1. A, formation of phosphatidylinositol (●----●) and B, formation of phosphatidylcholine (▲-▲) after different time intervals.

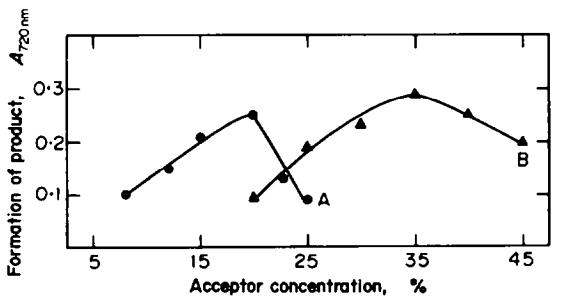


Fig. 2. A, formation of phosphatidylinositol (●——●) and B, formation of phosphatidylcholine (▲- ▲) at different inositol and choline chloride concentrations.

EXPERIMENTAL

Isolation of egg PC and PE. Crude phospholipid was prepared from egg yolk as described in ref. [15]. The total phospholipid was dissolved in a small vol. of $CHCl_3$ and then subjected to column (25 × 1.5 cm) chromatography over Si gel (60–100 mesh) for the separation of PC and PE. 11% MeOH in $CHCl_3$ elutes PE and 40% MeOH in $CHCl_3$ elutes PC. These were characterized by TLC (Si gel G) by spraying Dragendorff [16] and ninhydrin reagent [17] respectively and comparing R_f values with authentic standards. The developing solvent was $CHCl_3$–MeOH–H_2O (65:25:4).

Isolation of phospholipase D. The enzyme was isolated from spinach leaves according to the modified procedure of ref. [18]. Spinach leaves (70 g) were chopped and homogenized with 200 ml of H_2O. The homogenate was filtered through cheesecloth and centrifuged at 1000 *g* for 10 min. The cell debris was discarded and the supernatant incubated at 55° for 5 min to denature most of the other proteins, rapidly cooled to 0° and then centrifuged at 10000 *g* for 30 min. The supernatant was lyophilized and the powdered mass which contains mainly phospholipase D was stored at −20°.

Hydrolytic activity. For the routine assay of the hydrolytic activity of phospholipase D, protein content was estimated by the biuret method and egg PC was used as substrate. To a soln of PC (3 mg) in Et_2O (0.5 ml), acetate buffer (0.04 ml, 1 M, pH 5.6), $CaCl_2$ (0.04 ml, 1 M), protein (2.5 mg) in 0.6 ml H_2O and H_2O (0.32 ml) were added and the mixture incubated at 37° for 2 hr. The reaction was monitored by TLC and PC was completely converted to PA.

Transferase activity. Optimum time. Studies were carried out to find out the time for the maximum synthesis of PI. For this the following protocol was used: PC (3 mg) in Et_2O (0.5 ml), 20% inositol (0.06 ml) in acetate buffer (100 mM, pH 5.6), $CaCl_2$ (0.04 ml, 100 mM), protein (2.5 mg) in 0.7 ml H_2O and H_2O (0.2 ml). The reaction was carried out at 37° at different time intervals and at the end of the appropriate time each reaction was stopped by adding 0.1 ml of M HCl and the mixture extracted with $CHCl_3$. The solvent was evapd., the residue redissolved in a min vol. of $CHCl_3$ and PI was separated by prep TLC using the $CHCl_3$–MeOH 28% NH_4OH (13:7:1). The formation of PI was characterized by spraying with molybdenum blue [19] and orcinol reagent [20] and the product estimated colorimetrically [21]. Similarly in the synthesis of PC, egg PE (3 mg) was dissolved in 0.5 ml Et_2O. To this, 30% choline chloride (0.04 ml) in 1 M NaOAc buffer (pH 5.6), 0.04 ml of $CaCl_2$ (1 M), protein (5 mg) in 0.6 ml H_2O and (0.32 ml) H_2O were added. The different sets of reactions were carried out at 37° for different periods of time, stopped by acidification and extracted with $CHCl_3$. The product was separated by TLC (Si gel G, $CHCl_3$–MeOH–H_2O, 65:25:4) and characterized by spraying Dragendorff reagent and estimated colorimetrically [21].

Optimum acceptor concentration. Kinetic studies were carried out to find out the optimum concn of the acceptor molecules, i.e. inositol and choline chloride for the maximum product formation. Protocol used was the same as that above but with different inositol (8, 12, 15, 20, 23 and 25%) and choline chloride (20, 25, 30, 35, 40 and 45%) concns.

Large scale preparation of PI and PC. The transphosphatidylation reaction was scaled × 100 with optimum acceptor concn and time in each case. The products were separated by TLC using the solvent system cited earlier. Purity of the products, PI and PC was further checked by 2D-TLC using the solvent systems, (i) $CHCl_3$ MeOH–H_2O (65:25:4) and (ii) $CHCl_3$–MeOH 28% NH_4OH (13:7:1) in the first and the second direction respectively. Phosphate, inositol and choline content of the product were estimated colorimetrically.

Hydrolysis of PI: identification of inositol. PI (10 mg) was hydrolysed [22] with 0.2 M methanolic NaOH (0.5 ml) for 15 min. The product was neutralized with M HOAc. To this, were added 4 ml each of H_2O and $CHCl_3$–MeOH (9:1). After centrifugation the aq. layer was collected. The aq. layer was concd with C_6H_6, dissolved in a min. vol. of MeOH–H_2O (10:9) and further hydrolysed by refluxing with 2M methanolic HCl for 5 hr. The hydrolysate was concd to dryness *in vacuo* over KOH pellets. The residue was dissolved in a min. vol. of H_2O and free sugar was characterized by PC (descending) on Whatman No. 1 paper using inositol as authentic standard and the upper phase of the solvent system [23], Py–EtOAc–H_2O (2:5:5). The spots were detected by spraying first with 1% $AgNO_3$ in Me_2CO followed by 2% NaOH in EtOH.

Hydrolysis of PC: identification of choline. PC (10 mg) was subjected to the hydrolytic action of phospholipase D (7.5 mg) as described earlier. The aq. layer was concd and choline was characterized by PC (descending) on Whatman No. 1 paper using BuOH–diethylene glycol–H_2O (4:1:1) and choline as authentic standard. Detection was made by spraying with Dragendorff's reagent.

Acknowledgment—This work was supported by a grant from the Department of Science and Technology, Government of India

REFERENCES

1. Davidson, F. M. and Long, C. (1958) *Biochem. J.* **69**, 458.
2. Kates, M. (1960) in *Lipid Metabolism* (Bloch, K., ed.) p. 206. Wiley, New York.
3. Quarles, R. H. and Dawson, R. M. C. (1969) *Biochem. J.* **112**, 787.
4. Hanahan, D. J. and Chaikoff, I. L. (1948) *J. Biol. Chem.* **172**, 191.
5. Kates, M. (1956) *Can. J. Biochem. Physiol.* **34**, 967.
6. Haverkate, F. and van Deenen, L. L. M. (1965) *Biochim. Biophys. Acta* **106**, 78.
7. Kates, M. (1955) *Can. J. Biochem. Physiol.* **33**, 575.
8. Comfuris, P. and Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta* **488**, 36.
9. Smith, G. A., Montecucco, C. and Bennett, J. P. (1978) *Lipids* **13**, 92.
10. Benson, A. A., Freer, S. and Yang, S. F. (1965) *Int. Conf. Biochem. Lipids*, Noordwijk aan zee, The Netherlands.
11. Bartels, C. T. and van Deenen, L. L. M. (1966) *Biochim. Biophys. Acta* **125**, 395.
12. Yang, S. F., Freer, S. and Bensen, A. A. (1967) *J. Biol. Chem.* **242**, 477.
13. Dawson, R. M. C. (1967) *Biochem. J.* **102**, 205.
14. Lennarz, W. J., Bensen, P. P. M. and van Deenen, L. L. M. (1967) *Biochemistry* **6**, 2307.
15. Wells, M. A. and Hanahan, D. J. (1969) in *Methods in Enzymology Lipids* Vol. 14, (Lowenstein, J. M., ed.) p. 179. Academic Press, New York.
16. Beiss, U. (1964) *J. Chromatogr.* **13**, 104.
17. Patton, A. R. and Chism, P. (1951) *Analyt. Chem.* **23**, 1683.
18. Kates, M. (1969) in *Methods in Enzymology*, Vol. 14. *Lipids* (Lowenstein, J. M., ed.) p. 200. Academic Press, New York.
19. Dittmer, J. C. and Lester, R. L. (1964) *J. Lipid Res.* **5**, 126.
20. Neskovic, N., Sarlieve, L., Nussbaum, J. L., Kostic, D. and Mandal, P. (1972) *Clin. Chim. Acta* **38**, 147.
21. Ames, B. N. and Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 769.
22. White, D. C. and Frerman, F. K. (1967) *J. Bacteriol.* **94**, 1854.
23. Kates, M., Palameta, B., Perry, M. P. and Adams, G. A. (1967) *Biochim. Biophys. Acta* **137**, 213.
24. Stanacev, N. Z., Stuhne-Sekalec, L. and Domazet, Z. (1973) *Can. J. Biochem.* **51**, 747.