Acta Chemica Scandinavica, 1993: 47: 971–973
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ACTA
CHEMICA SCANDINAVICA
ISSN 0904-213X

Phospholipid Transphosphatidylation Catalysed by Cabbage Phospholipase D in Emulsion Systems

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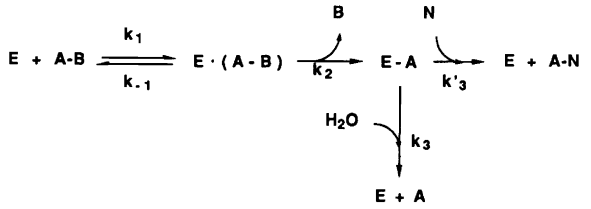
Orrenius, C., Norin, M., Persson, A., Hult, K. and Norin, T., 1993. Phospholipid Transphosphatidylation Catalysed by Cabbage Phospholipase D in Emulsion Systems. – Acta Chem. Scand. 47: 971–973.

Phospholipid modification catalysed by phospholipase D, partially purified from cabbage, has been performed in water–organic solvent emulsions. Phosphatidylcholine reacted with alcohols through nucleophilic substitution of the choline moiety (transphosphatidylation) to form new phospholipids or by hydrolysis, to form phosphatidic acid. The reactions were carried out in two systems based on aqueous buffer and either chloroform or diethyl ether. Products focused on were phosphatidylglycerol, in a preparative-scale reaction, and phosphatidylbutanediol for the study of reaction rates. *N*-(2-Hydroxyethyl)octanamide affected the product distribution by reducing the rate of hydrolysis more than the rate of transphosphatidylation.

Phospholipids are of great biological importance as structural elements of membranes. In addition, examples are known of phospholipid 'secondary messengers' in cellular signal transduction.[1] Enzymatic modification of phospholipids with phospholipase D[2] (PLD, EC 3.1.4.4) exchanges the terminal phosphoester moiety for a nucleophilic species through transphosphatidylation. A model for the competing reactions, transphosphatidylation and hydrolysis, was proposed by Juneja *et al.* in 1988[3] (Scheme 1).



Scheme 1. Proposed reaction pathways of phospholipase D catalysis (Juneja *et al*, 1988). Enzyme (E), phosphatidic acid/phosphatidyl moiety (A), alcohol substituent (i.e., choline) (B), nucleophilic alcohol (N).

A study of transphosphatidylation in different biphasic systems has been performed. The reaction mixture included an organic solvent, an aqueous sodium acetate buffer, a phospholipid substrate (phosphatidylcholine, PC), one or several nucleophiles and crude phospholipase D from savoy cabbage. In order to identify the optimum conditions for the formation of both natural and unnatural phospholipids the following parameters were varied: organic solvent, type of substrate, reaction temperature and time, nucleophiles as well as the amounts and concentrations of the components.

Commercial soybean lecithin (PC) has a mixed fatty acid composition and the physical appearance of a wax. In order properly to quantify the formation of the products in our investigation, it was necessary to use substrates with a specific fatty acid composition. However, dipalmitoylphosphatidylcholine (DPPC) is crystalline and only sparingly soluble in diethyl ether. It follows that the choice of solvent must be optimized regarding solubilization of the substrate and most successful transphosphatidylation (i.e., minimum of hydrolysis).

Materials and methods

Enzyme preparation. Partial purification of the enzyme was performed according to Lee *et al.*[4] (modified method of Davidson and Long[5]). One unit of hydrolytic activity is defined as the amount of enzyme that hydrolyses 1 nmol of dipalmitoylphospatidylcholine per minute at room temperature. The specific activity of the enzyme reparation was 180 units mg^{-1}. Other active enzymes that may have been present in the preparation were disregarded since no detectable side reactions occurred under the stated conditions.

Reaction procedure. The reactions were carried out in sealed 10 ml glass ampoules in an end-over-end incubator

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at room temperature. Phosphatidylcholine (DPPC, 5 mg or 1.75 mM) from Larodan (Malmö, Sweden) was dissolved in chloroform (2 ml) together with hydroxy-*N*-octanoylacetamide where applicable. Crude enzyme powder (5 mg) was added to 2 ml of sodium acetate buffer (0.2 M, 80 mM $CaCl_2$, pH 5.6). The nucleophile was added corresponding to 35 mM in the system (4 ml). The reaction was started by mixing the two phases. Samples of 100 µl emulsion were added to chloroform–water (450 + 450 µl) and the organic phase was analysed with a light-scattering detector after separation performed with an HPLC system, see below.

The preparative-scale reaction included soybean lecithin (5 g, 20 mM) in diethyl ether (200 ml), glycerol (5 ml) and crude enzyme (2.5 g) in buffer (150 ml). After vigorous stirring of the mixture for 2 h the phases were allowed to separate. The buffer fraction was washed with diethyl ether (3 × 100 ml) and the organic extracts were combined.

Separation and analysis. Substrate, products and *N*-(2-hydroxyethyl)octanamide were separated by HPLC (Shimadzu LC-6A) on a Lichrosorb Diol column (5 µm, 125 mm, 4.0 mm) at room temperature. An exponential gradient[6] of solution B in solution A, reaching 80 % (v : v) B after 25 min, was used.

Solution A: hexane–1-propanol–acetic acid–triethylamine 82 : 17 : 1.5 : 0.08 (v : v) and 40 mg of ammonium acetate per litre.

Solution B: 2-propanol–water–acetic acid–triethylamine 84 : 14 : 1.5 : 0.08 (v : v) and 80 mg of ammonium acetate per litre.

The total flow rate was 1 ml min^{-1} and a 100 µl loop set the loaded volume. A SEDEX 45 Light Scattering Detector at 43°C and 2 bar detected the compounds of interest.

Substrate (PC) and reference compounds phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) were obtained from Larodan in Malmö, Sweden.

Results and discussion

Preparative-scale reaction. Nucleophiles used in the diethyl ether reaction system to displace the choline moiety of PC were 1-propanol, ethanolamine, 1,4-butanediol and glycerol in order of decreasing reactivity. Ethylene glycol and 2-phenylethanol did not form any detectable transphosphatidylation products.

A gram-scale synthesis was performed with glycerol. The production of phosphatidyl glycerol (PG) from 5 g of commercial lecithin (i.e., PC) was achieved in diethyl ether–aqueous buffer with a 56% conversion into PG within 2 h. The products were quantitatively extracted with diethyl ether. Separating the desired product from phosphatidic acid (PA 29 %) and starting material (14 %) required the use of preparative-scale HPLC techniques (performed on a fraction of reaction mixture, courtesy of *Karlshamms Lipidteknik*, Nacka, Sweden). PA was formed in the hydrolytic side reaction.

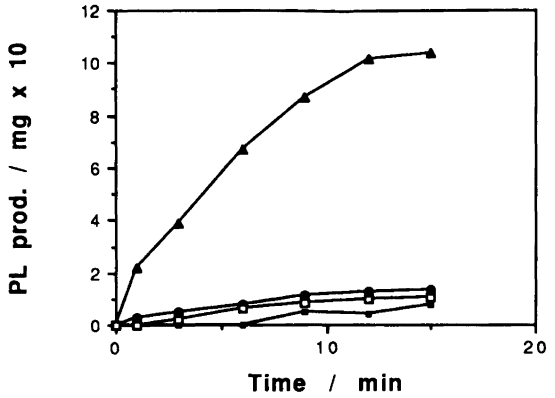

Fig. 1. Progressive curves of phospholipid production in chloroform. Several nucleophiles were present simultaneously in the reaction mixture. Nucleophiles and products were water affording phosphatidic acid ▲, 1,4-butanediol (phosphatidylbutanediol ●), glycerol (phosphatidylglycerol ■) and ethanolamine (phosphatidylethanolamine □). The total concentration of added nucleophiles was 35 mM.

Reactions in chloroform. Dipalmitoyl PC did not dissolve satisfactorily in diethyl ether or ethyl acetate. Chloroform however, achieved good substrate solubilization and was used despite an increased rate of hydrolysis observed in this solvent.

A comparison of the reaction systems, even though they differ in experimental design, showed 1-propanol to be more reactive in diethyl ether (reaction time 2 h, [PC] 7 mM)[7] and showed hydrolysis to predominate in the chloroform reaction system (15 min, 1.75 mM), where 1,4-butanediol was the most effective nucleophile for transphosphatidylation.

Transphosphatidylations were performed in chloroform with 1,4-butanediol, ethanolamine, glycerol, 1-propanol and 1-octanol. Fig. 1 illustrates progressive curves of the detectable reactions with all nucleophiles present in one incubation. Initial production rates of this experiment are given in Table 1. 1,4-Butanediol is shown to be the fastest reacting nucleophile, water excluded, in the chloroform system. For comparison, it should

Table 1. Initial rates of formation of the various phospholipids. Reaction performed with all alcohols present, 7 mM each, giving a total concentration of 35 mM. The activity of water is approximately 1 (55.6 M).

Nucleophile	Product	Initial rate/mg min^{-1}
Water	Phosphatidic acid	0.112
1,4-Butanediol	Phosphatidylbutanediol	0.014
Ethanolamine	Phosphatidylethanolamine	0.010
Glycerol	Phosphatidylglycerol	<0.002
1-Propanol	Phosphatidylpropanol	<0.002
1-Octanol	Phosphatidyloctanol	<0.002

be noted that the rate of transphosphatidylation and hydrolysis recorded with 35 mM 1,4-butanediol alone were 0.03 and 0.17 mg min^{-1}, respectively. Naturally, the rate of formation of phosphatidylbutanediol could be enhanced by excluding other nucleophiles (it was not possible to exclude water in this system since no detectable reaction occurs in organic solvent only) and by increasing the concentration of 1,4-butanediol (Fig. 2). The product distribution could be further manipulated through the addition of *N*-(2-hydroxyethyl)octanamide. The initial rate of hydrolysis showed a 70% decrease with 20 mM of this compound present in the reaction mixture. However, transphosphatidylation was also affected, but not to the same extent (50%), resulting in a higher yield of the desired product. Fig. 3 illustrates the effect of *N*-(2-hydroxyethyl)octanamide on product distribution. In experiments with other nucleophiles, a similar effect is apparent although kinetic data from these experiments do not allow a detailed analysis.

In conclusion, we have demonstrated that *N*-(2-hydroxyethyl)octanamide reduces the initial rate of hydrolysis more than the rate of formation of phosphatidylbutanediol (PBd). According to Scheme 1, changes in the relative concentrations of the nucleophiles would also affect product distribution. Changes in K_m or k_{cat} for the different nucleophilic species would have to be affected independently (e.g., the change in $K_{m,trans.}$) by *N*-(2-hydroxyethyl)octanamide to explain its observed effect. Otherwise, the factor of rate reduction would be equivalent for the two competing reactions. The reasons why *N*-(2-hydroxyethyl)octanamide exerts this effect are not readily apparent. It may, for example, change the distribution of water at the catalyst (aqueous)–substrate (organic solvent) interface and thereby alter the effective concentrations of the participating reactants. However, even without the specific knowledge of the mechanism of this *N*-(2-hydroxyethyl)octanamide interaction, the addition of *N*-(2-hydroxyethyl)octanamide may be useful for the improvement of preparative-scale transphosphatidylation reactions.

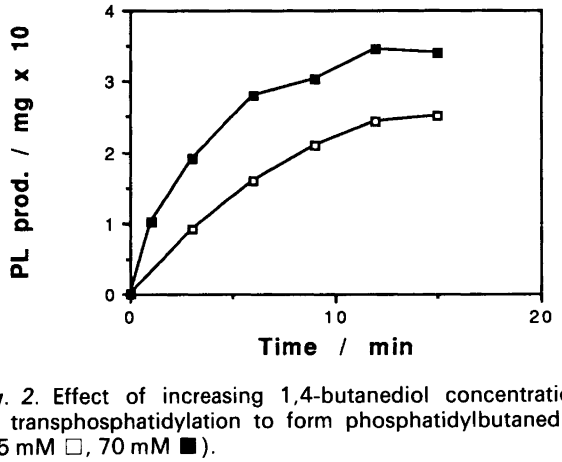

Fig. 2. Effect of increasing 1,4-butanediol concentration on transphosphatidylation to form phosphatidylbutanediol (35 mM □, 70 mM ■).

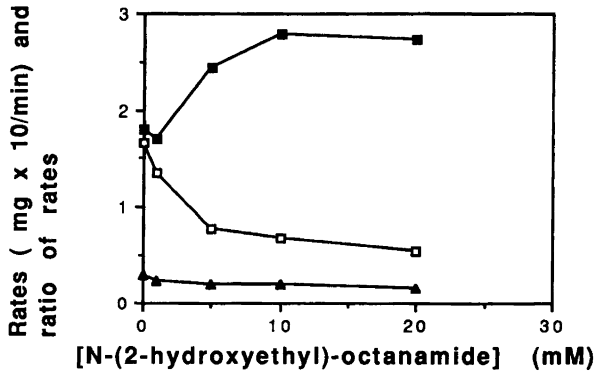

Fig. 3. Initial rates of hydrolysis (□) and phosphatidylbutanediol formation (▲) in chloroform with different *N*-(2-hydroxyethyl)octanamide concentrations (all reactions with 35 mM 1,4-butanediol). The ratio of transphosphatidylation rate (1,4-butanediol) to hydrolysis rate at the various *N*-(2-hydroxyethyl)octanamide concentrations is also shown (■).

Acknowledgments. Financial support from *Karlshamns Stiftelse för Vetenskaplig Forskning* and the Swedish Board for Technical Development (STU/NUTEK) is gratefully acknowledged.

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Received October 26, 1992.