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# The substrate requirements of phospholipase D

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**Abstract**

The hydrolysis rates of different diphosphates, compared with the one observed with natural phosphatidylcholine, are used to identify the molecular basis for phospholipase D (PLD) catalysis. Experimental data strongly support the idea that PLD is a rather generic phosphodiesterase with very wide substrate specificity and a net preference for lipophilic substrates. The presence of choline in the polar head is not required for activity although it improves hydrolysis efficiency. Choline esters are found to be substrates for PLD hydrolysis, but only with long chain fatty acids. 

*Keywords:* Substrate; Phospholipase D; Hydrolysis

## 1. Introduction

Bacterial phospholipase D (PLD) from *Streptomyces* PMF is an extracellular hydrolytic enzyme with a broad substrate specificity and high *trans*-esterification capacity in the presence of water as a (co-) solvent. This property is quite unique among hydrolytic enzymes, and essential in practical applications like the preparation of non-natural phospholipids (PL) or less abundant ones, starting from crude or purified phosphatidylcholine (PC) (**1**) [1,2]. The latter is the most abundant PL in nature. Of the many enzymes hydrolysing phosphoric esters, some of them for which regions having a high sequence homology has been recognised, are defined as belonging to the PLD superfamily. The over 85 proteins belonging to the superfamily share a repeating catalytically important motif $HxK(x)_4D$ [3].

Although data from different PLDs on PL hydrolysis have been collected, mechanism and substrate specificity for the bacterial enzyme is not completely defined. In analogy with other hydrolytic enzymes, the formation of a phosphoryl-enzyme intermediate has been postulated. This will undergo hydrolysis or *trans*-esterification according to several parameters that are not fully understood. In particular, no explanation at molecular level has been found for the *trans*-esterification capacity observed with high water activity (biphasic system). We recently showed kinetic evidence of the common phosphoryl-enzyme intermediate by measuring the PX/PA ratio for a

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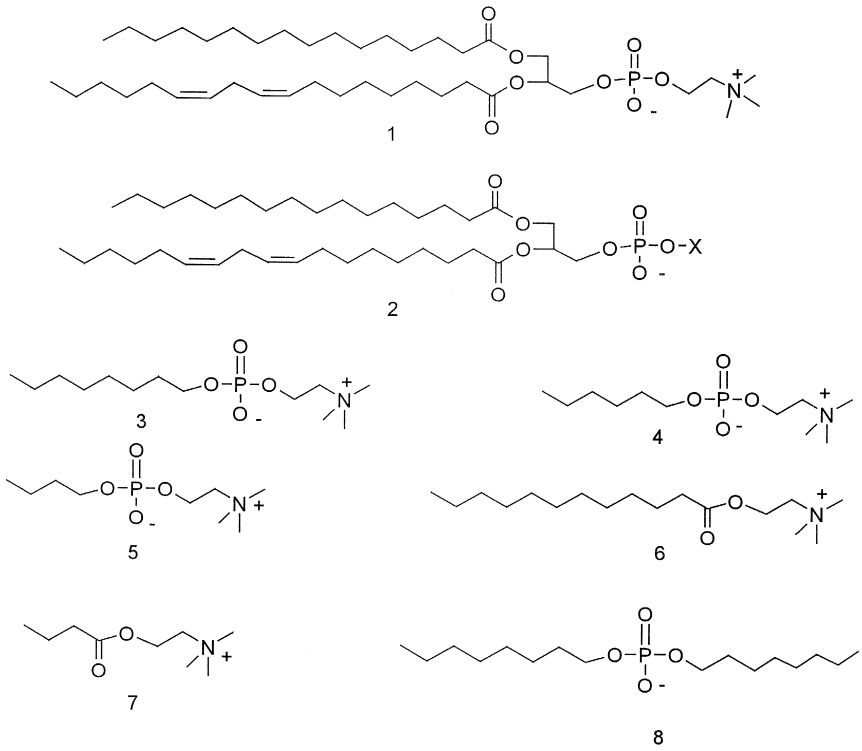



Scheme 1. Possible substrates for PLD.

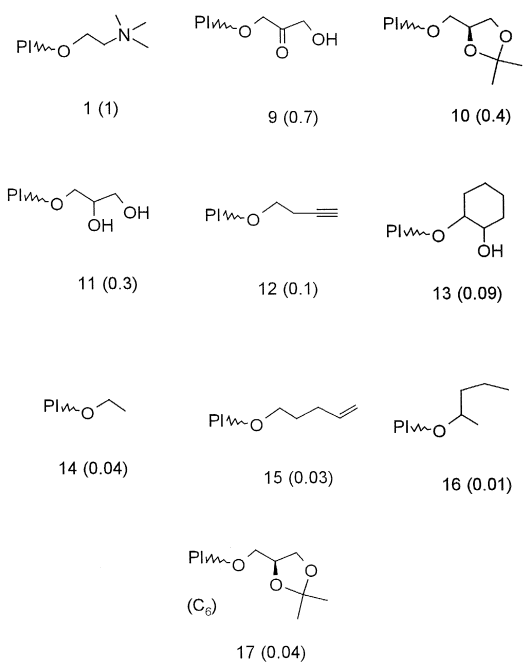
given nucleophile and different substrates [4]. The existence of the latter intermediate is also substantiated by studies on labelled material showing that a double inversion at the P atom occurs [5]. Recently, the first 3D structure of a PLD, namely PLD from *Streptomyces sp.* PMF [6] has been obtained [7]. From these data, the presence of a covalent enzyme-substrate intermediate is evident. A complete model of the catalytic step in PLD-catalysed hydrolysis has thus been proposed [8]. The collection and analysis of kinetic data on phospholipases are complicated from the fact that this group of enzymes, having the common feature of working on water-insoluble substrates, show an increased activity on substrates organised in aggregates, compared to soluble monomeric substrates. The kinetic data, therefore, depend on the particular phase in which the PL is found in the reaction mixture [9]. In this article, we report about the data concerning the hydrolysis rate of the structurally different PL or analogues reported in Scheme 1, with the aim of refining our knowledge on the substrate specificity of the enzyme, focusing on the effect of the nature of the polar head and acyl chains dimensions. Since our main interest is the exploitation of the synthetic possibilities offered by PLD in the transformation of phosphoric diesters, the conditions employed are those applied in preparative reactions, i.e. highest possible substrate concentration, biphasic system with non-water-soluble substrates, no use of detergents, whenever possible. We made no investigations concerning the phase of the PL or analogues in the reaction conditions.

## 2. Results and discussion

We prepared and considered as PLD substrates four different types of compounds in which one

important structural feature is varied at a time. Compounds of type **1**–**2** compare PL with a modified polar head (**2**) with the structure of the most abundant natural PL, **1** (Scheme 1). They are listed in Scheme 2. In compounds of type (**3–5**), the polar head of choline is conserved, while the other part of the molecule contains no longer a diacylglyceride moiety. Compounds (**6**) and (**7**) also, still present a choline polar head, but the phosphoric ester has been exchanged by a carboxylate. Finally, compound (**8**) is a symmetric diphosphate in which only the phosphate diester functionality present in glycerophospholipids is conserved.

PC (**1**) is considered the best substrate for PLD-catalysed hydrolysis. The apparent $K_m$ in a water–organic solvent biphasic system is around 2 mM. Most polar head modified PL have higher $K_m$ and lower turnover numbers. $K_m$ values are available for the most common natural PL, i.e. phosphatidyl serine (PS), phosphatidyl ethanolamine (PE) and phosphatidylglycerol (PG). The effective formation of a modified polar head PL in *trans*-esterification reactions from **1** is also due to the fact that the products are less readily hydrolysed than the starting material,




Scheme 2. Hydrolysis rates (in brackets) of polar head modified PL of kind **2** (Scheme 1) relative to **1** (2 mM, biphasic system).

thus allowing accumulation in the medium. Thus, parameters affecting *trans*-esterification efficiency are important for the synthetic relevance of the transformation. They are basically of four kinds:

- the binding of the PL substrate,
- the $K_m$ for the alcohol behaving as phosphatidyl acceptor,
- the hydrolysis rate of the initially formed product,
- the hydrolysis rate of the PL substrate.

Hydrolysis rates in the first group of compounds were measured from HPLC analysis of the reaction mixtures at different times and the data relative to **1** hydrolysis (taken as 1) at 24 h are reported in parenthesis in Scheme 2.

They clearly indicate that head polarity is apparently very much involved into recognition. Completely apolar *polar head* bearing PL like **14**, **15** and **16** are very slowly hydrolysed. They are nevertheless formed from **1** and an alcohol as phosphatidyl acceptor. The presence of an OH or oxygen atom in the head definitely increases reactivity towards hydrolysis. Interestingly, compound **17** is structurally related to **10** but with $C_6$ acyl chains, is about 10 times less reactive than the analogue with *natural chains* substitution pattern. As it will be seen from further examples, the role of the size of the acyl chains is confirmed from the hydrolysis rate of other analogues.

Alkylphosphocholines **3**, **4** and **5** have been prepared by standard methodologies as described in the experiment. Hydrolysis rates were measured from the determination of the released choline by an enzymatic method [10]. Hydrolysis conditions also differed from the first set of compounds just described in that it was performed in a one-phase system in the presence of surfactants. Compared with **1** hydrolysis in the same conditions (taken as 1), octylphosphocholine **3** was hydrolysed with a rate coefficient 0.8, *n*-hexyl (**4**) 0.4 and *n*-butyl (**5**) 0.2. The influence of the chain length is quite impressive also in this series. Phase properties of course can be at the origin of the observed differences, thus masking true substrate specificity. The absence of the choline head in compounds like **8** deprives the compound of reactivity. All the symmetric diphosphates with different

chain length tested (data not reported) resulted not hydrolysed (and not prone to *trans*-phosphatidylation). Although **1** is the best substrate for PLD hydrolysis, PLD from PMF cannot be defined as **1** specific. The idea that recognition of the PL as substrate derives from the presence of the charged choline itself does not find support either in the observation of the crystal structure of the PLD–PL complex since choline is immediately released during catalysis. In cholinesterases who are choline-specific enzymes and have as natural substrates choline carboxylates, it has been shown that the active site contains a specific binding domain for the quaternary carbon which is a requisite for substrate recognition [11,12]. This is proposed to occur through a π-interaction with the aromatic ring of tryptophane residues. On the contrary, the carboxyl group is not a recognition site but only a reactivity center [13]. We have prepared and submitted to PLD hydrolysis a few choline esters in order to check the presence of a cholinesterase activity in our enzyme. We found that lauroylcholine **7** is a good substrate for PLD hydrolysis, being transformed at a rate (0.75) similar to the one observed with natural **1**. However, butirrylcholine is transformed only at a very slow rate (0.06). Hydrolysis of choline carboxylates by PLD has not been observed before. Also, this experiment, in our opinion, points to the fact that the lipophilic chain, more than the choline head, is responsible for recognition.

As mentioned before, the crystal structure of the PL–PLD complex shows the direction in which the chains of the large lipophilic moiety point in the active site, leaving without apparent substrate–protein interaction the part of the polar head.

Although PLD *trans*-esterification can be used for the preparation of modified phosphocholines and even phosphonocholines [14] with good efficiency, a rather surprising finding in view of the large difference of those substrates with the natural ones was that *trans*-esterification with choline esters was not successful.

## 3. Conclusions

According to the present experiments, PLD PMF behaves as a rather generic phosphodiesterase with very wide substrate specificity and a net preference for lipophilic substrates. It has been suggested that, in a way similar with hydrolytic enzymes acting on water-insoluble substrates, PLD could also exert catalysis at the interface [15]. We demonstrated that in a biphasic system, PLD shows interfacial saturation kinetics (only PLD at the interface is active) [6]. The preference for hydrophobic substrates seems to support this view. However, it has been observed that water-soluble (short chain) PL are also substrates for PLD. Determination of true kinetic constants requires working below the critical micellar concentration, which is usually very low for long chain PL [16]. Those conditions are not of interest for the identification of parameters affecting preparative transformations.

## 4. Experimental

### *4.1. Preparation of PL **9**–**17** via PLD-catalysed trans-phosphatidylation reaction*

3-*sn*-Phosphatidylethanol (**14**). PC (**1**) (5 g) was dissolved in 50 ml of methylene dichloride and added to the aqueous phase (100 ml) containing 150 U of PLD from *Streptomyces* PMF, 1.03 g of NaOAc (0.1 M), 7.29 ml of ethanol (1 M) and adjusted to pH 5.6. The mixture was stirred by a mechanical stirrer at 200 rpm at 37°C. When **1** was totally transformed (20 min), the organic phase was separated and evaporated to give a residue of 4 g. The product dissolved in hexane (20 ml) was precipitated with 125 ml of chilled acetone to give **1** as a pale yellowish solid (3.8 g) of more than 95% purity as judged from HPLC analysis.

The same procedure was employed for the preparation of the other PL cited in the text.

### *4.2. Choline lauroyl ester **6***

Compound **6** was prepared as described in the literature [17]. β-Dimethylaminoethanol (4.4 g) in benzene (40 ml) is added dropwise over 30 min to a solution of lauroyl chloride (10 g) in benzene (100 ml) at 5°C. The reaction mixture is then warmed at 60°C to dissolve the residue. After allowing the

solution to cool spontaneously to RT, the white precipitate is separated by filtration. The solid (20 g) is then treated with ethanol (170 ml). An alcoholic solution of KOH 0.5 M (132 ml) is added dropwise, cooling the reaction mixture at 0°C over a period of 30 min. The mixture is left at 0°C and KCl is filtered on a sintered glass. The solvent is removed at reduced pressure. The free base (β-dimethylaminoethyl laurate) is obtained as a yellow oil (8.6 g).

Dissolved in 100 ml of anhydrous ethyl ether, 8 g of β-dimethylaminoethyl laurate are treated with 2 ml of $CH_3I$. The mixture is kept at −10°C for 1 h and the resulting white solid precipitate is removed by filtration. The solid recovered after solvent evaporation (6.11 g) is crystallised from acetone with some drops of methanol giving 3 g of a white solid product identified as the iodide of β-trimethylammoniumethyl laurate.

Freshly prepared AgCl (0.76 g, 5.29 mmol) is stirred with a solution of β-trimethylammoniumethyl laurate iodide (1.6 g) in 45 ml of $CH_3OH$ for 30 min. A yellow precipitate of AgI is formed and then filtered on a Buckner. The filtrate evaporated in vacuo gives **6** (1 g, 80.3%). $^1$H NMR 4.55 (t, 2H); 4.1 (t, 2H); 3.5 (s, 9H); 2.35 (t, 2H); 1.6 (m, 2H), 1.3 (m, 16H); 0.9 (t, 3H). [M + H] = 287.

### 4.3. Dioctyl phosphate (*8*)

Under nitrogen atmosphere at 0°C, 3.30 ml of anhydrous $POCl_3$ and 5 ml of anhydrous $Et_3N$ are mixed with 60 ml of anhydrous $CHCl_3$. A solution of octanol (9.36 g) in 100 ml of $CHCl_3$ is added slowly under stirring at 0°C. The mixture is stirred for 3 days at RT. After addition of iced water, complete removal of solvents gives an oily product (10 g), which is identified as **8** from the MS spectrum ($M^+$ 323).

#### 4.3.1. Butylphosphocholine (*5*)

Under nitrogen atmosphere at 0°C, 3.30 ml of anhydrous $POCl_3$, 5 ml of anhydrous $Et_3N$ and 60 ml of anhydrous $CHCl_3$ are mixed together. A solution of 2.13 g of butanol in 100 ml of $CHCl_3$ is added slowly. The mixture is allowed to stand at RT for 3 h, then cooled at 10°C. A solution of choline tosylate (10 g) in pyridine (26 ml) is added dropwise. The mixture is stirred for 3 days at RT. The mixture is then treated with iced water for 30 min and evaporated in vacuo. The residue is purified by flash chromatography. $^1$H NMR: 4 (2H, t); 3.8 (2H, t); 3.25 (9H, s); 2 (2H, q); 1.4 (2H, m); 1 (5H, m). $[M^+] = 240$.

Other alkylphosphocholines have been prepared in exactly the same way as previously described for the **5**. Hexylphosphocholine (**4**): $^1$H NMR: 4.1 (2H, t); 3.85 (2H, t); 3.3 (9H, s); 1.7 (2H, t); 1.35 (8H, m); 0.8 (3H, t). [M + H] = 268. Octylphosphocholine (**3**): $^1$H NMR: 4.3 (2H, t); 3.75 (2H, t); 3.3 (9H, s); 1.8 (2H, t); 1.5 (10H, m); 0.8 (3H, t). [M + H] = 296, [M + Na] = 318.

### 4.4. Hydrolysis experiments on compounds *3–5*

Alkylphosphocholine (50 mg) in 1.5 ml of toluene is mixed with 3 ml of an aqueous solution containing 3 U of PLD. The mixture is stirred at 37°C for 24 h. Aliquots of 20 μl are withdrawn at fixed time, warmed at 90°C for 5 min, centrifuged and the aqueous phase is diluted with water to a final volume of 134 μl and analysed with the enzymatic test.

### 4.5. Hydrolysis experiments on compound *8*

Dialkylphosphate (100 mg) in 1.2 ml of toluene are mixed with 4 ml of an aqueous solution containing 6 U of PLD, $CaCl_2$ 0.1 M and sodium acetate 0.1 M, pH 5.6. The mixture is stirred at 37°C for 24 h.

Aliquots of 20 μl are withdrawn at fixed time, warmed at 90°C for 5 min, centrifuged and the organic phases analysed by TLC.

### 4.6. Hydrolysis experiments on compounds *9–17*

PL (100 mg) in 1.2 ml of toluene are mixed with 4 ml of an aqueous solution containing 6 U of PLD, $CaCl_2$ 0.1 M and sodium acetate 0.1 M, pH 5.6. The mixture is stirred at 37°C for 24 h.

Aliquots of 20 μl are withdrawn at fixed time, warmed at 90°C for 5 min, centrifuged and the organic phases are evaporated at RT. The residues are then dissolved in hexane and analysed by HPLC.

### 4.7. Enzymatic assay

The assay is carried out using a Hewlett Packard 8453E spectrophotometer with thermostatic temperature control.

The amount of free choline is assayed by incubating at 37°C 134 μl of reaction solution, 67 μl of choline oxidase solution (50 U/ml Tris(HCl) 0.1 M, pH 8), 67 μl of peroxidase solution (100 U/ml Tris(HCl) 0.1 M, pH 8), 34 μl of $CaCl_2$ (100 mM in Tris(HCl) 0.1 M, pH 8), 700 μl of 4-APA/phenol reagent.

The 4-APA/phenol solution is prepared by mixing 8 ml of phenol solution (2 mg/ml in Tris(HCl) 0.1 M, pH 8) to 8 ml of 4-APA solution (3 mg/ml in Tris(HCl) 0.1 M, pH 8) and 84 ml of Tris(HCl) buffer 0.1 M, pH 8. The solution has to be made up fresh every day.

The amount of free choline is obtained from absorbance values by correlation with a calibration curve obtained by testing different aqueous solutions containing known amounts of choline chloride.

### 4.8. Hydrolysis experiments on compound **6**

The hydrolysis of choline carboxylate **6** is performed by incubating at 37°C in a cuvette 134 μl of aqueous solution of substrate (0.22 mg), 17 μl of PLD, 67 μl of choline oxidase solution (50 U/ml Tris(HCl) 0.1 M, pH 8), 67 μl of peroxidase solution (100 U/ml Tris(HCl) 0.1 M, pH 8), 34 μl of $CaCl_2$ (100 mM in Tris(HCl) 0.1 M, pH 8), 700 μl of 4-APA/phenol reagent. The reaction is monitored over a period of 1 h, reading the absorbance values every 300 s.

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