Biochimica et Biophysica Acta 860 (1986) 462–474
Elsevier

BBA 73236

Effect of the structure of phospholipid on the kinetics of intravesicle scooting of phospholipase A_2

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(Received December 17th, 1985)
(Revised manuscript received May 7th, 1986)

Key words: Phospholipase A_2; Substrate specificity; Kinetics; Phase property

Action of pig pancreatic phospholipase A_2 on vesicles of over 50 synthetic 1,2-diacylglycero-3-phosphate derivatives and analogs is examined in the absence of any additives. In general, shorter acyl chains and small substituents on the phosphate make a better substrate, while phospholipids with large apolar substituents are not hydrolyzed. The interfacial turnover rate constant for scooting kinetics, k_i, for the various phospholipids were from less than 0.1 to 1 per min. Intervesicle exchange of the bound enzyme is faster in vesicles of phospholipids with larger polar substituents, and it is promoted in the presence of anions like chloride, sulfate and thiocyanate. These factors lower the residence time of the enzyme on the bilayer and therefore effectively decrease the rate of hydrolysis. The apparent K_m for the enzyme in the interface of anionic phospholipids in the presence of salts is in the 40 to 100 μM range which is 3- to 7-times larger than the dissociation constants for the bound enzyme measured by fluorescence enhancement of Trp-3. The quantum yield of the bound enzyme in vesicles of the various lipids is found to be up to 4-fold different. It is suggested that this difference is due to the E* + S to E*S equilibrium, where E*S has higher fluorescence intensity. The role of calcium in generating the enzyme binding site at the anionic interface, the role of anion anchoring site on the enzyme, and the relationship between the catalytic efficiency and the fluorescence quantum yields are discussed.

Introduction

In the preceding papers of this series we have shown that the hydrolysis of the vesicles of DMPMe occurs predominantly in intravesicle scooting mode [1], and that an anion binding site involving the N-terminus region of the enzyme is implicated in interfacial anchoring of phospholipase A_2 [2]. In this paper we examine the effect of changing the second substituent on the phosphate

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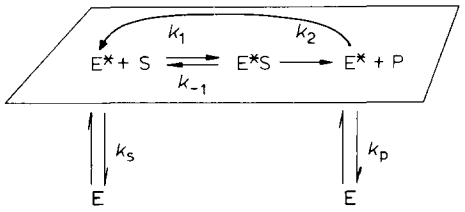
Abbreviations: DHPMe (ether), 1,2-dihexylphosphatidylmethanol; DMPMe (ester), 1,2-dimyristoylphosphatidylmethanol; DTPMe (ether), 1,2-ditetradecylphosphatidylmethanol; DMPC (ester), 1,2-dimyristoylphosphatidylcholine; DTPC (ether), ditetradecylphosphatidylcholine; lysoPC, 1-palmitoyllysophosphatidylcholine; NATA, *N*-acetyl tryptophanamide; OPN, *cis*-octadec-9-enylphosphocholine; PNPG, octadecylphosphoglycerol; N7, the N-terminus heptapeptide from pig pancreate phospholipase A_2; cmc, critical micelle concentration; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.



Scheme I. Scheme of interfacial catalysis.

in 1,2-diacylglycero-3-phosphate on the catalytic and binding characteristics of pig pancreatic phospholipase A_2. The results are interpreted in the general context of the model for interfacial catalysis implicit in Scheme I, where the enzyme in the aqueous phase (E) binds to the interface, and the catalytic turnover by the bound enzyme occurs by the steps shown in the box [1].

Materials and Methods

Phospholipase A_2 from pig pancreas was kindly provided by Professor DeHaas, and cyclopentanophospholipids (Nos. 43–46 in Table I) by Dr. Anthony Hancock. Action of phospholipase A_2 on sonicated aqueous dispersions of more than 50 phospholipids was examined. Their structure, thermotropic properties and source are given in Table I. New preparations of several phospholipids are described below. In all cases, lipid purity was judged to be better than 98% by thin-layer chromatography and by differential scanning calorimetry of their aqueous dispersions. All other procedures are described in the accompanying papers [1–3]. Typically, vesicles were prepared by dispersing a dry film or powder of sodium salts of phospholipids in distilled water in a bath type sonicator (Sonicor SC-50T). Reaction progress curves were obtained by pH-stat titration of the substrate in 4 ml of 0.3 mM $CaCl_2$, pH 8.0 at 30°C by initiating the reaction with 0.1 to 10 μg pig pancreatic phospholipase A_2 dissolved in the salt solution used for pH-stat titration [1]; as indicated in the text or in the figure legends, some studies were carried out at 45°C. Titration efficiency of externally added myristic acid at pH 8 was determined in the presence of several lipid dispersions and found to be $> 95\%$. For studies employing phosphatidic acid dispersions, the calcium chloride concentration was kept at approx. 0.075 mM. Special care is necessary in using phosphatidic acid vesicles as they tend to fuse and aggregate readily [3,4].

The aqueous dispersions of most lipids examined in this study were in the bilayer vesicle form. Sonicated dispersions of these lipids could be kept for at least 4–6 h as small unilamellar vesicles in a calcium free salt solution at 56°C. Dispersions of some of the lipids were in micellar form (Nos. 5, 5a, 31, 31a and possibly 18–20). Critical micelle concentrations of these lipids in the presence and in the absence of pig pancreatic phospholipase A_2 were obtained by monitoring the fluorescence of diphenylhexatriene. The thermotropic phase transition properties of most of the lipids are also summarized in Table I.

All fluorescence measurements were done on an SLM 4800S spectrofluorimeter. Binding of pig pancreatic phospholipase A_2 to vesicles or to micellar dispersions was measured by monitoring an increase in the fluorescence intensity at 333 nm (excitation 290 nm, slitwidths 4 and 4 nm) as a function of phospholipid concentration as described in detail elsewhere [2,5]. Fluorescence anisotropy was measured in the T-mode with excitation at 300 nm (4 nm slitwidth) and emission at 345 nm (16 nm slitwidth) with 5% potassium phthalate liquid filters.

Several lipids used in this study were purchased (Nos. 15, 16, DMPC from Avanti, Birmingham, AL, Nos. 2, 2a, 14, 14a, 16a, 71–75 from Medmark, Munich) or were gift from Professor Bittman (No. 62). Other lipids were synthesized by the procedures already published [6–9] or described below. The calcium salt of *O*(1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphoryl)epicholesterol was synthesized by a procedure analogous to that described [6,7] except that the coupling reaction between epicholesterol and the 1,2-diacyl-*sn*-glycerol cyclic enediol phosphotriester was allowed to proceed for four days at 25°C in anhydrous dichloromethane solution (2.0 mmol of reactants in 20 ml of solvent) in the presence of triethylamine (4.0 mmol). The compound had $[\alpha]_D = -7.7$ ($c = 2$, $CHCl_3$; 25°C). The phase transition properties of its aqueous dispersions are considerably different than those of the phosphatidylcholesterol analog [8]. The calcium salt of dimyristoylphosphatidylepicholesterol (No. 42) could not be readily

TABLE I

PHASE TRANSITION AND KINETIC PARAMETERS FOR ANIONIC DIACYLGLYCEROPHOSPHOLIPIDS (AT 35°C): $RCOOCH_2 \cdot (RCOO)CH \cdot CH_2OP(O)(OX)O^-$

Specific activities were measured in 0.1 M KCl at 35°C with 0.5 mM substrate vesicles (0.3 mM $CaCl_2$, pH 8.0). Protons released per substrate molecule (= H/S) in the presence of excess enzyme. *A* is the total moles of products formed by each mole of the enzyme at the end of the first-order phase of the reaction. n.d., not determined. 3′-TpT, thymine-5′-phospho-3′-thymine.

No.	RC	X	T_c (°C)/ΔH (kcal/mol) for		Specific activity (I.U.)	H/S	*A*
			Na salt	Ca salt			
1	12	H	20/7	42/2	~ 300	0.96	
2	14	H	52/7.5	62/7.5	~ 200	0.7	3600
2a	alkyl	H	55/8	–	–	–	
3	16	H	58/10	68	50	0.5	
4	18	H	63/14.5	82.5/9.5	~ 20		
5	6	CH_3	–	–	240	1.0	
5a	alkyl	CH_3	–	–	–	–	
6	14	CH_3	28/8.5	49/6	> 350	0.63	4300
6a	alkyl	CH_3	30/9.0	50/6.5	–	–	
7	16	CH_3	46/9	66/12	~ 120	0.5	
8	18	CH_3	57/12	75/12	40		
9	16	$CH_2CH_2C(CH_3)_3$	41/10	42/10	54	0.7	
10	14	$(CH_2)_3CH_3$	23/11	22.8/10	~ 100	0.6	1800
11	14	$(CH_2)_4CH_3$	21/11	25/12	~ 50	0.6	
12	14	$(CH_2)_7CH_3$	−3.5/4	28/3.5	< 9.1	–	
13	14	$(CH_2)_{11}CH_3$	54.3/8.5	35.9/14 49.1/6		–	
14	14	$-CH_2CH_2OH$	25/10	70/21	~ 50	0.55	2600
14a	alkyl	$-CH_2CH_2OH$	27/11	–	–	–	
15	14	$-CH_2CHNH_2COOH$	37/8	–	155	0.64	4800
16	14	$CH_2CHOHCH_2OH$	22.4/9.5	63.7/12/5	250	0.6	3800
16a	alkyl	$CH_2CHOHCH_2OH$	–	–	–	–	
17	16	$CH_2CHOHCH_2OH$	41.2/9.5		102	0.6	
18	14	5′-thymine	n.d.	n.d.	300	0.56	2600
19	14	3′-thymine	n.d.	73	~ 36	–	
20	14	3′-T_pT	n.d.	n.d.	~ 20	–	
21	16	-6-glucose	39.5	63/5.5	~ 30	–	
22	16	-6-(1-methylglucose)	53/10.5	65	~ 30	–	
23	16	-6-(triacyl-glucose)	–	67/7	~ 40	–	
24	16	-6-(tetraayl-glucose)	–	–	~ 20	–	
25	16	-6-(1-methyltriacyl-glucose)	38	54/6.5	13	–	
26	16	-6-(tribenzylglucose)	–	38/21	50	–	
27	12	cardiolipin	25.5	48.6/8.5	50	2.0	4400
28	14	cardiolipin	40	62.6/11	~ 60	1.3	
29	16	cardiolipin	54	78	120	0.72	
30	18	cardiolipin	54/30	–	~ 10		
31	6	*sn*-3,3-bisPA	–	–	30		
31a	alkyl	*sn*-3,3-bisPA	–	–	–		
32	12	*sn*-3,3-bisPA	52.3/14	41.6/15.5	23	1.7	
33	14	*sn*-3,3-bisPA	61.4/23	41.5/25	16	0.75	
34	16	*sn*-3,3-bisPA	74/26	50/28	7		
35	18	*sn*-3,3-bisPA	73.1/30	64.5/36	5		

Table 1 (continued)

No.	RC	X	T_c (°C)/ΔH (kcal/mol) for		Specific activity (I.U.)	H/S	*A*
			Na salt	Ca salt			
36	16	*sn*-3,1-bisPA	72.2/23	54/24	3		
37	16	*sn*-1,1-bisPA	74	50	NDH		
38	6	1,16-dihydroxy-hexadecane	–	–	NHD	–	
39	12	Cholesterol	–	32/4	NDH	–	
40	14	Cholesterol	–	38/7	NDH	–	
41	16	Cholesterol	–	47/11	NDH	–	
42	14	Epicholesterol	–	28-40 broad)	NDH	–	
Cyclopentanophospholipids							
43	16	1,2/3-3P$CH_2CH_2C(CH_3)_3$	45		n.d.	–	
44	16	1,2,3/O-1P choline	38		n.d.	–	
45	16	1,2/3-1P choline	36		n.d.	–	
46	16	1,2/3-3P choline	45		n.d.	–	
47	16	1,3/2-1P choline	46		n.d.	–	
48	16	1,2,3/O-2P choline	44		n.d.	–	
49	16	1,3/2-2P choline	42		n.d.	–	
50–56	16	1,3/2-1P (CH_2) choline $n = 3$–9	38–46		n.d.	–	

dispersed, and the hydrated sample exhibits a broad endotherm from 28 to 40°C.

Analytical data: $C_{116}H_{208}O_{16}P_2Ca \cdot 2H_2O$

Calcd.: C, 69.76; H, 10.70

Found: C, 69.92; H, 10.59

The sodium salt of *O* = (1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)-3,3-dimethyl-1-butanol (No. 9) was prepared by first reacting 1,2-di-*O*-palmitoyl-*sn*-glycerol and 2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphole in anhydrous ether containing triethylamine by the general procedure already described [7,9]. The coupling reaction between 3,3-dimethyl-1-butanol and the 1,2-diacyl-*sn*-glyceryl cyclic enediol phosphotriester was allowed to proceed for 36 h at 25°C in anhydrous tetrahydrofuran in the presence of triethylamine. Other steps were as described [7,9]. $[\alpha]_D = +4.9$ ($c = 5$, $CHCl_3$).

Analytical data: $C_{41}H_{80}O_8PNa$

calcd.: C, 65.22; H, 10.68; Na, 3.05

Found: C, 64.80; H, 10.59; Na, 2.93

An analogous procedure afforded the following compounds: *O*-(1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphoryl)-1-butanol (No. 10), -1-pentanol (No. 11), -1-octanol (No. 12), and -1-dodecanol (No. 13). The synthesis of analogous diplamitoylphosphatidyl alkanols by a different procedure has been described by Eibl [10]. The sodium salt of *O*-(1,2-di-*O*-tetradecyl-*sn*-glycero-3-phosphoryl) methanol (No. 6a) was prepared from 1,2-di-*O*-tetradecyl-*sn*-glycerol and 2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphosphole by the usual procedure [7,9]. The coupling step with methanol was carried out in anhydrous tetrahydrofuran for 18 h at 25°C in the presence of triethylamine. $[\alpha]_D = +1.0$ ($c = 5$, $CHCl_3$).

Analytical data: $C_{32}H_{66}O_6PNa \cdot 2H_2O$

Calcd.: C, 60.34; H, 11.07; Na, 3.61

Found: C 60.87; H, 10.81; Na 3.29

O-(1,2-Di-*O*-tetradecyl-*sn*-glycero-3-phosphoryl)choline (DTPC) was prepared from 1,2-di-*O*-tetradecyl-*sn*-glycerol and phosphorus oxychloride by a procedure patterned after that described by Brockerhoff and Ayengar [11]. The zwitterion was obtained as a powder upon stirring with acetone. $[\alpha]_D = +4.7$ ($c = 5$, $CHCl_3$); ^{1}H-NMR has signals at 3.45 ppm $(CH_3)_3N$ and at 1.35 ppm broad alkyl in the expected ratio.

Analytical data: $C_{36}H_{76}NO_6P \cdot 2H_2O$

Calcd.: C, 63.02; H, 11.75; N, 2.04

Found: C, 62.38; H, 11.40; N, 2.01.

The sodium salt of *O*,*O*-bis(1,2-di-*O*-hexanoyl-

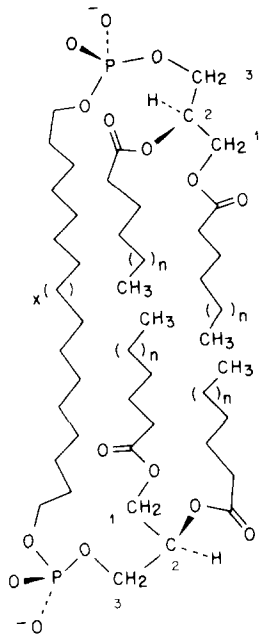


Fig. 1. Structure of *O,O*-bis(1,2-di-*O*-hexanoyl-*sn*-glycero-3-phosphoryl)-1,16-hexadecanediol, $x = n = 1$ (#38).

sn-glycero-3-phosphoryl)-1,16-hexadecanediol (No. 38, Fig. 1) was prepared by first reacting 1,2-di-*O*-hexanoyl-*sn*-glycerol and 2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphosphole in anhydrous ether containing triethylamine by the general procedure previously described [7,9]. The coupling reaction between 1,16-hexadecanediol (2.5 mmol) and the 1,2-diacyl-*sn*-glyceryl cyclic enediol phosphotriester (5.0 mmol) was allowed to proceed 4.5 days at 30°C in anhydrous tetrahydrofuran (30 ml) in the presence of triethylamine. Other steps were as described [7,9]. $[\alpha]_D = +9.34$ ($c = 3$, chloroform/methanol (1 : 1, v/v) at 20°C).

Analytical data: $C_{46}H_{86}O_{16}P_2Na_2 \cdot 2H_2O$

Calcd.: C, 53.27; H, 8.73; Na, 4.43

Found: C, 53.24; H, 8.58; Na, 4.77

Results

Effect of the head group of phospholipids on the rate of their hydrolysis by phospholipase A_2

All studies reported in this paper were done with pig pancreatic phospholipase A_2. Initial rates of hydrolysis of micellar and monomeric dispersions of dihexanoylphosphatidylmethanol (No. 5) and its bisphosphatidic acid analog (No. 31) were measured. As shown in Fig. 2 the rate of hydrolysis increases with the substrate concentration. For No. 5 the rate of hydrolysis is very slow at low concentrations and then it increases abruptly above 0.5 mM. This concentration is significantly lower than its critical micelle concentration (6 mM). However, as measured by DPH fluorescence (data not shown) the cmc is lowered in the presence of the protein. Which suggests that the protein and lipid form aggregates far below the cmc of the lipid alone. Formation of such aggregates can also be demonstrated, as shown in Fig. 2, by monitoring the intrinsic tryptophan fluorescence intensity of the enzyme as a function of dihexylphosphatidylmethanol (No. 5a) concentration. Such a large change in the fluorescence emission intensity of pig pancreatic phospholipase A_2 is observed only when the enzyme is bound to the interface. These observations on the hydrolysis of, and binding to, aggregates of dihexanoylphosphatidylmethanol show that its rate of hydrolysis is about 200 I.U. in the microaggregate or the micellar phase and about 10 I.U. in the preaggregate phase in which the enzyme presumably binds to

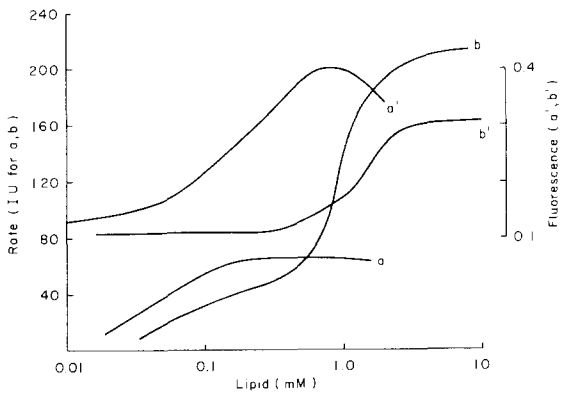


Fig. 2. Specific activity of pig pancreatic phospholipase A_2 (curves a and b) and its relative fluorescence emission intensity at 333 nm (curves a′ and b′) as a function of dihexanoylphosphatidylmethanol (No. 5, No. 5a curves b and b′) and tetrahexanoyl bis-phosphatidic acid (No. 31, No. 31a, curves a and a′). The ether analogs were used for binding studies. The actual data points are not shown for the sake of clarity, however, the quality of data is comparable to those shown in Fig. 4. All fluorescence measurements were done at 30°C, pH 8 in 0.1 M KCl, 5 mM $CaCl_2$, and 50 mM Hepes for the binding studies; the kinetic measurements were done in 0.3 mM $CaCl_2$.

monomeric substrate. The dispersions of tetrahexanoyl bisphosphatidic acid (No. 31) and its ether analog (No. 31a) also show a concentration dependent increase in the rate of hydrolysis and of the tryptophan fluorescence (Fig. 2). However, with this lipid we could not detect any 'monomeric' region for hydrolysis. The preaggregate region could not be detected by diphenylhexatriene fluorescence even in the absence of the protein, thus suggesting that its cmc is below 1 μM. This behavior of short chain anionic lipids is at considerable variance from the behavior of zwitterionic lipids which form lipid-protein aggregates and hydrolyze short chain homologs (data not shown, however, see Ref. 12) only above the cmc of the lipid alone. The observations summarized in Fig. 2 are consistent with the suggestion that the binding of monomeric anionic lipids to the enzyme promotes its affinity for the substrate interface.

Among the short acyl chain lipids, No. 38 (Fig. 1) is particularly interesting. It has two 1,2-dihexanoyl-*sn*-glycero-3-phosphate moieties coupled to hexadecane-1,16-diol. Like the aqueous dispersions of Nos. 5, 5a, 31 and 31a, the aqueous dispersions of No. 38 are micellar as indicated by sharp isotropic ^{31}P-NMR signal (data not shown). However, we could not detect any hydrolysis of the aqueous dispersions of No. 38 (specific activity $\ll$ 0.01 I.U., if any). This is remarkable because the enzyme apparently binds to these micelles and the monomerr lipid molecules have appropriate *sn*-3-phosphate stereochemistry in both halves of this bipolar molecule. As discussed later, this lack of hydrolysis shows that only amphipathic molecules can be hydrolyzed by phospholipase A_2; apparently bipolar molecules like No. 38 are not substrates.

Aqueous dispersions of most of the phospholipids listed in Table I form bilayer-enclosed vesicles on sonication. Some of these lipids (Nos. 13, 37, 39–56) did not show any indication of hydrolysis by phospholipase A_2 under the following conditions: at, below, or above their phase transitions temperature, at lower or higher salt or calcium concentration, and in the presence of additives like *n*-octanol. Apparently these compounds either can not serve as substrates, or their rate of hydrolysis under the conditions we have examined is significantly less than 10 nmol/min per mg enzyme. Kinetics of phospholipase A_2 action in the scooting mode on several other substrates could not be examined in detail because the specific activity of the substrate was too low. See Table I for the specific activity data.

Detailed kinetic investigation of the dianionic phosphatidic acid vesicles was hampered by the fact that its dispersions are relatively unstable over an 8- to 10-h time period, and that their rate of fusion and precipitation [3] is extremely sensitive to calcium concentration. Optimal hydrolysis of dilauroyl- (No. 1) and dimyristoylphosphatidic acid (No. 2) can be achieved at about 0.075 mM $CaCl_2$ when the substrate concentration is about 0.3 mM. However, it is very difficult to remove calcium bound to phosphatidic acid and thus precisely control the total concentration of calcium. Moreover, phosphatidic acid vesicles under some conditions undergo rapid fusion and size change in the presence of alkali metal salts. The ratio of calcium to phosphatidic acid in the bilayer appears to be critical. The presence of two ionizable groups also creates complications in the interpretation of the data. For all such reasons the results on phosphatidic acid are valid only within a limited range of conditions. The behavior of DMPA is probably ideal to observe a first-order reaction progress curve indicative of intravesicle scooting, because the rate of intervesicle exchange of the enzyme bound to phosphatidic acid vesicles is extremely slow, as would be expected for a small anion in the interface.

Kinetic parameters for the hydrolysis of several phospholipids by pig pancreatic phospholipase A_2 are summarized in Table II. For lipids with smaller head groups as in phosphatidic acid (Nos. 1–4), phosphatidyl methanol (Nos. 6–9), phosphatidylglycerol (No. 14), -serine (No. 15), -glycerol (Nos. 16, 17), and cardiolipin (Nos. 27, 28), a first-order reaction progress curve could be obtained even when the ratio of substrate vesicle to enzyme exceeded 5. The rate constant for interfacial turnover, k_i, for these lipids are typically less than 0.5 per min, thus suggesting that the rate of intervesicle exchange for the enzyme is considerably slower than this.

Based on the extent of hydrolysis by a given amount of enzyme, we have also calculated the number of lipid molecules in the outer monolayer

of the vesicles of the various phospholipids as 3000 to 5000. However, for some of these phospholipids the size of the vesicles is also dependent upon their history after sonication. For example, with DMPG (No. 16) the smallest vesicles with about 2000 lipid molecules in the outer monolayer are formed immediately after sonication, and these vesicles appear to be stable if kept at $t > 50°C$. If the vesicles are kept at 30°C, the size increases to $A = 4500$. Similarly, with 1-palmitoyl-2-oleoyl- and dioleoyl-phosphatidylglycerol $A = 3400$. These observations suggest that the size of vesicles and the number of lipid molecules in a vesicle does change significantly with the structure of the lipid molecules, as well as with the history of the vesicles. While these results are as expected, we are not aware of any other protocol with which the average number of lipid molecules in a vesicle has been measured directly. However, our observations are consistent with the current ideas about the packing constraints in highly curved vesicles [37–39].

In the presence of the excess enzyme, about 60% (H/S ratio 0.6) of the total substrate could be hydrolyzed. This is consistent with the hypothesis that these dispersions are in a vesicle form, and that only the substrate in the outer monolayer of the unilamellar vesicles is accessible to the enzyme. A strong tendency of the vesicles of anionic phospholipids to undergo fusion in the presence of $CaCl_2$ above 1 mM is also indicated by the observation that in fused vesicles about 50% of the total lipid is accessible and that the extent of hydrolysis at the end of the first-order phase of hydrolysis is considerably higher than it is for the vesicles before fusion, and the extent of hydrolysis increases with the time of fusion.

In the presence of salts the beginning of the first-order reaction progress curve degenerates into relatively linear steady-state rate of hydrolysis, and ultimately all the available substrate is hydrolyzed. Specific activities of the enzyme at 320 μM substrate concentration are given in Table II under the column 'before'. When salts are added at the end of the first-order reaction, the hydrolysis of the excess substrate is reinitiated which follows a linear course for several minutes. Specific activities measured under these conditions, that is, 'after' the formation of products, are given in Table II in the 'after' column. The specific activity data summarized in Table II illustrates an important point: the overall rate and the mode of hydrolysis of

TABLE II

THE k_i VALUES AND THE EFFECT OF ANIONS ON THE RATE OF HYDROLYSIS OF PHOSPHOLIPID VESICLES BEFORE FORMATION OF PRODUCTS AND AFTER FORMATION OF ANY PRODUCTS

Lipid No.	Temp (°C)	k_i (min^{-1})	Initial rate (μmol/min per mg enzyme)					
			Before			After		
			SO_4^{2-}	Cl^-	CNS^-	SO_4^{2-}	Cl^-	CNS^-
2	45	1.05	–	–	–	–	–	–
6	30	0.73	280	320	265	222	371	178
	45	0.76	–	–	–	–	–	–
7	45	0.33	100	103	78	188	94	27
8	45	< 0.05	48	48	19	–	–	–
9	45	< 0.1	0.7	12	17	–	–	–
10	30	0.1	41	46	55	–	–	–
14	30	0.30	65	52	58	58	46	41
15	45	0.46	88	142	142	–	–	–
16	30	0.22	49	46	47	41	57	27
17	45	0.32	10	116	84	202	75	65
18	30	micelles?	178	482	463	113	198	164
20	30	?	17	18	15	–	–	–
27	30	0.2	58	48	48	53	38	19
29	45	< 0.1	198	120	137	–	–	–
Tern	30	< 0.2	–	–	–	135	78	48

different substrates are very much dependent upon the presence of anions. Based on the arguments developed earlier [2], it may be postulated that these effects of anions are due to their ability to alter the rate of intervesicle exchange. A surprising aspect of these results is that the relative and absolute effects of anions also depend on the nature of the head group substituents. This is consistent with the suggestion that the anion binding site on the enzyme is involved in its anchoring to the anionic interface [2]. Fatty acid anions significantly alter the relative effects of the anions, presumably by competing with anions in the aqueous phase for the anion binding site on the enzyme during its intervesicle transfer.

The first-order phase was relatively insignificant or absent in the reaction progress curves of several phospholipids such as distearoylphosphatidylmethanol (No. 8), phosphatidylbutanol (No. 10) and its higher analogs (Nos. 11, 12), phosphatidylglucose derivatives (Nos. 18–26), and bisphosphatidic acids (Nos. 32–37). In some of these cases the rate of hydrolysis is so slow that the intervesicle exchange rate of the enzyme overtakes the rate of hydrolysis by intravesicle 'scooting'. In other cases the initial burst of hydrolysis by the scooting mode is significant, and is followed by a slower but linear steady-state phase of hydrolysis due to intervesicle exchange, which ultimately leads to the complete hydrolysis of all the accessible substrate. As shown earlier [1,2], such reaction progress curves arise under conditions in which the rate of intervesicle exchange of the enzyme is faster than the rate of hydrolysis by intravesicle scooting. This behavior is observed with those lipids that have relatively large substituents. We are trying to resolve such curves to obtain k_i for hydrolysis in the intravesicle scooting.

The structural feature of diacylglycerophosphates that appears to be absolutely necessary for the catalytic action is the '*sn*-3-chirality'. Among the lipids that show significant pseudo-zero-order kinetics dominated by intervesicle transfer of the enzyme, bisphosphatidic acids (Nos. 31–37) are of particular interest. For example, the enantiomeric 1,1-bisPA (No. 37) is not susceptible to hydrolysis. However, both halves of the molecule are hydrolyzed in *sn*-3,3-bisPA (Nos. 31–35),

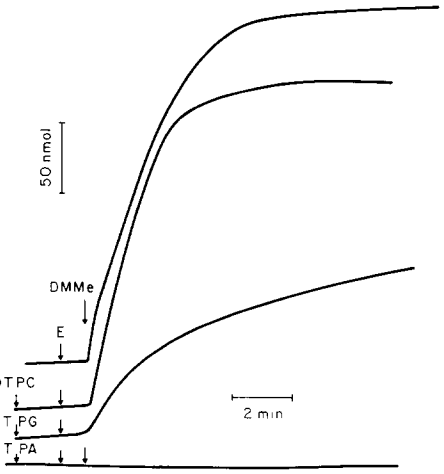


Fig. 3. Reaction progress curves for the hydrolysis of DMPMe (DMMe) (ester) (80 μM) added to a mixture of phospholipase A_2 (0.8 μg, E) with DTPC, or DTPG, or DTPA (80 μM each).

and only the sn-3- half is hydrolyzed in *sn*-3,1-bisPA. This is consistent with the suggestion that the chirality recognition function of the enzyme is associated with the active site rather than with the interfacial anchoring region [12–14]. As mentioned earlier, several lipids with smaller head groups exhibit reaction progress curves dominated by intravesicle scooting kinetics. One of the conditions for this is that the enzyme bound to the substrate interface does not readily become accessible to the excess substrate vesicles. This can be demonstrated in several different ways. For example the enzyme bound to DTPMe ether (No. 6a) vesicles is not accessible to DMPMe ester (No. 6) vesicles added afterwards unless salt is added [2]. Such exchangeability of phospholipase A_2 bound to DTPMe ether vesicles is different for different substrate vesicles as shown in Fig. 3. Based on such experiments the apparent affinity of the enzyme for vesicles increases in the order: phosphatidylcholine < bisphosphatidic acids < phosphatidylglycol < phosphatidylglycerol < phosphatidylmethanol < phosphatidic acid.

Binding of phospholipase A_2 to phospholipid interface

Binding phospholipase A_2 to bilayer vesicles results in an increase in the fluorescence anisotropy of Trp-3. As mentioned in the preceding paper the anisotropy of the semisynthetic enzymes

does not change noticeably. For phospholipase A_2 the increase in anisotropy is found to depend upon the concentration of the lipid and it approaches a maximum value that does not depend upon the nature of the lipid. Thus the anisotropy of phospholipase A_2 increases from 0.11 to 0.20 ± 0.02 in the presence of several phospholipids (Nos. 2a, 6a, 14a, 16a, DTPC, and 71, 72, 75). These results show that the increase in anisotropy is due to adsorption of the enzyme on vesicles, however, such a binding is not catalytically significant since the change in the anisotropy is the same in the presence of DTPC and DTPMe, both of which have very different catalytic susceptibility to phospholipase A_2. In terms of the Scheme I it would mean that the increase in anisotropy is due to binding of E, however, with this technique one can not distinguish between E* and E*S.

Yet another measure of the affinity of the enzyme for the interface can be obtained by the binding isotherms for titration of the enzyme with vesicles of the ether analogs of phospholipids (Fig. 4). As shown elsewhere [2,5], in these experiments the fraction of the enzyme bound to vesicles is proportional to the increase in the fluorescence intensity at 333 nm. The binding isotherm shown in Fig. 4 can be resolved by nonlinear regression analysis to obtain C, n and K_d, where K_d is the dissociation constant for the bound enzyme into the free enzyme and a site consisting of n lipid molecules [5]. C is expressed as the relative peak intensity (I_m/I_o) of the bound enzyme. It is probably more meaningful to examine the apparent dissociation constant values, $n \cdot K_d$, because the 'affinity' values obtained from the Michaelis-Menten type of kinetic analysis (K_m) are generally expressed as total monomeric substrate concentration.

The value of n can also be obtained independently. As shown in Fig. 5, the enthalpy of thermotropic transition of DTPMe ether (No. 6a) decreases with increasing mole fraction of the enzyme, and it approaches zero when the mole fraction of the enzyme in the lipid dispersions is about 0.025, that is the gel-to-liquid crystalline phase transition in DTPMe ether dispersions is not observed when the lipid to enzyme ratio is below 40 : 1. In these dispersions both sides of the bilayer are exposed to the enzyme, therefore the same limiting ratio for binding of the enzyme to the substrate in vesicles would reach when the enzyme to substrate mole ratio is 60. The value of $n = 60$ is consistant with the curve fitting parameters for the binding isotherm shown in Fig. 4. In fact the first part of this binding isotherm is linear as would be expected if the binding affinity is

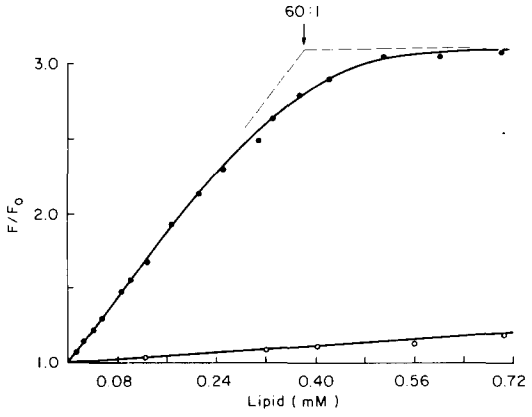


Fig. 4. Effect of DTPMe (upper curve) and DTPC (lower curve) on the relative fluorescence intensity of 6.2 μM pig pancreatic phospholipase A_2 at 333 nm at 30°C pH 8.0 in 0.1 m KCl, 10 mM Hepes and 0.3 mM $CaCl_2$ (10 mM $CaCl_2$ for DTPC). The Smooth line in the upper curve is the calculated line to fit the values of $n = 60$, $K_d = 0.3$ μM and $C = 3.1$.

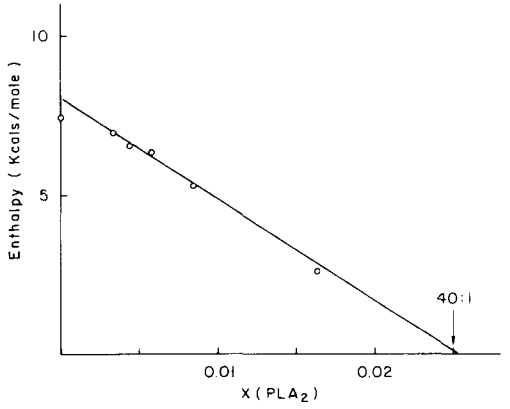


Fig. 5. Enthalpy of phase transition of DTPMe dispersions in the presence of pig pancreatic phospholipase A_2. The 35 μl sample for differential scanning calorimetry (on Mettlar 2000B at 2 Cdeg per min in a sealed aluminium pan) contained 4 μmole lipid with varying mole fractions of the enzyme ($x(PLA_2)$) in a buffer containing 200 mM Hepes and 0.5 mM $CaCl_2$ at pH 8.0. DTPMe was dispersed in the presence of phospholipase A_2.

high. By extrapolating the linear portion (as shown in Fig. 4) the maximum change in the fluorescence intensity is attained at lipid to protein mole ratio of about 60 : 1. This is a reasonable value of n since under the conditions for fluorescence measurements only the outer monolayer of the vesicles is exposed to the protein, and if a correction is made for this difference, the lipid to protein ratio for binding would be 40 : 1. One of the simplest, but not the only, physical interpretation of n would be that one enzyme molecule can somehow occlude and interfere with the cooperativity of the thermotropic transition of 40 substrate molecules in the interface. Physical studies (X-ray, electromicroscopy, carboxyfluoresceine trapping) show that the bilayer organization in these vesicles is not

TABLE III

BINDING PARAMETERS FOR PHOSPHOLIPASE A_2 TO AQUEOUS DISPERSIONS OF MONO- AND DIALKYLPHOSPHOLIPIDS

All binding curves were obtained by adding aqueous dispersions of phospholipids to 6.2 μM pig pancreatic phospholipase A_2 in 0.1 M KCl, 10 mM $CaCl_2$ (0.3 mM for acidic phospholipids), 20 mM Hepes at pH 8.0 and 30 °C. THe binding isotherms of type shown in Fig. 3 were resolved by nonlinear regression analysis to obtain $n \cdot K_d$ and C. In many cases considerable covariance was observed between n and K_d.

No.	CH_2 1	CH 2	CH_2 3	$n \cdot K_d$ (μM)	n	I_m/I_o	
57	Myristoyl	OH	phosphocholine	900	33	2.2	
58	Palmitoyl	OH	phosphocholine	250	42	2.3	
59	Stearoyl	OH	phosphocholine	170	40	2.1	
60	Oleoyl	OH	phosphocholine	1200	32	2.2	
						sn-1	1.50
61	$C_{16}H_{33}$-O	OH	phosphocholine	250	42		
						sn-3	1.40
62	$C_{16}H_{33}$-O	OH, CH_3(*rac*)	phosphocholine			1.38	
63	Palmitoyl	H	phosphocholine	400	42	2.2	
64	Palmitoyl	OH	-PO_3-O$(CH_2)_6 \cdot NMe_3$	170	20	1.7	
65	Palmitoyl	OH	-PO_3-O$(CH_2)_8 \cdot NMe_3$	150	20	1.45	
66	Stearoyl	H	-$PO_3 \cdot O(CH_2)_6 \cdot NMe_3$	210	20	1.55	
67	Palmitoyl	phosphocholine	H	150	30	1.65	
68	Palmitoyl	OH	phosphoglycerol	100	35	2.2	
69	Palmitoyl	OCH_3	phosphocholine	–	–	1.20	
						sn-1	1.73
70	Stearoyl	OCH_3	phosphocholine	–	–		
						sn-3	1.51
71	Tetradecylphosphocholine			330	35	2.6	
72	Hexadecylphosphocholine			160	42	2.6	
73	Octadecylphosphocholine			140	50	2.65	
74	*Cis*-octadec-9-enylphosphocholine			130	30	2.9	
75	Octadecylphosphoglycerol			120	40	3.5	
2a	DTPA ether (diC_{14})				44	1.31	
6a	DTPM ether (diC_{14})				30	3.15	
	D-DMPMe ether (diC_{14})				30	2.9	
14a	DTP glycerol ether (diC_{14})				112	2.15	
16a	DTP glycerol ether (diC_{14})				160	2.15	
37	*sn*-1,1-bisPA (tetrapalmitoyl)				120	1.25	
38	1,16-dihydroxyhexadecane				65	~1.95	
43	1,2/3-3P CH_2-CH_2 $C(CH_3)_3$				350	2.0	
	DTPC ether				> 2000 (?)	1.2	
	DTPC ternary mixture				180	1.95	

disrupted even when the lipid to protein mole ratio is 20 : 1 (to be published).

As summarized in Table III, the values of nK_d for most lipids are in a narrow range of 15 to 40 μM, even though some of the dispersions are miceller while others are bilayers. The major difficulty in obtaining these data is due to limitations intrinsic in the design of the experiments to generate binding isotherms of type shown in Fig. 4. The protein concentration is 5 to 10 μM, therefore the substrate concentration that would bind about 5% of the enzyme is already above the nK_d value. Other more sensitive methods need to be developed for measuring nK_d values less than 15 μM.

The values of relative peak intensities (I_m/I_o) of the bound enzyme show a significant difference in the various phospholipid interfaces. The uncertainty in this data is less than 5%. In all cases we have examined, phospholipase A_2 in micelles or bilayer exhibits essentially identical emission spectra with peaks at 333 nm. In these cases the relative peak intensities are probably directly related to the fluorescence quantum yield of the enzyme in the interface. However, we have not used the quantum yield values because these have a somewhat unquantifiable contribution from scattering at lower emission wavelengths.

Several interesting features of the relative intensity data summarized in Table III may be noted. The I_m/I_o values exhibit a relatively small dependence upon the chain length. The nature of substituent in *sn*-2 and 3 position appear to change the fluorescence intensity of the bound enzyme significantly, even though the shape of the emission difference spectrum does not change noticeably. Thus the analogs of 1-palmitoylphosphatidylcholine (compare Nos. 58, 61, 63, 64, 65, 67, 69, 72) exhibit a 250% difference in the emission peak intensities. Similarly, substitution of a hydrogen by methyl group in *sn*-2 position (Nos 62 and 69) can appreciably decrease the I_m/I_o ratio. Also the *sn*-1- and *sn*-3-phosphatidyl derivatives appear to give different I_m/I_o ratio even though nK_d values are essentially identical. These observations suggest that a specific interaction between the enzyme and the lipid molecules in the interface is responsible for the fluorescence quantum yield of the enzyme in the interface.

Even though the peak intensity of phospholipase A_2 in the various phospholipids changes by a factor of three, the average fluorescence lifetime (based on the three life-times and their amplitudes) are found to be within 50% of the values for phospholipase A_2 in aqueous phase (data not shown, however, it is comparable to the results shown in Ref. 3). As shown elsewhere [31] fluorescence quenching with brominated lipids is static, that is the fluorescence intensity decreases without any significant decrease in the life times. All these observations suggest that the increase in the fluorescence intensity of phospholipase A_2 on binding to the lipid interface is largely due to dequenching of Trp-3. In the light of the arguments developed in the preceding paper [2], it would imply that the dequenching of tryptophan fluorescence on binding to the interface, exhibits a strong dependence on the structure of the head group.

For phosholipids in a bilayer form there is significant variation in the relative peak intensities. Here again zwitterionic PC and anionic bis-PA show low I/I_o, while anionic lipids like DMPMe exhibit highest values comparable to those obtained with micellar alkyl phosphocholine dispersions. These observations imply head group specificity in inducing higher fluorescence emission. This could happen, for example, when a monomer substrate molecule in the interface binds to E* in the interface, and the fluorescence dequenching occurs due to formation of E*S. Thus, phospholipids that give highest fluorescence emission intensity are also more susceptible to hydrolysis by phospholipase A_2, as if they favor E*S form of the enzyme. On the other hand the increase in fluorescence anisotropy is due to the E* and E*S forms of the enzyme. This is also in accord with the observation that the fluorescence increase with ternary codispersions [5,15], as well as with other lipid interfaces [1,2,16,17] is a measure of catalytically meaningful binding of pig pancreatic phospholipase A_2 that is the formation of E*S. The significance of this hypothesis will also be discussed in the following paper.

Discussion

The observations reported in this paper pertain to the question of the substrate specificity of interfacial enzymes like phospholipase A_2. Even a

correlation of substrate structure with specific activities for hydrolysis under comparable conditions, or even under optimized conditions for each substrate is suspect because factors related to intervesicle exchange and the interfacial equilibrium (E* + S to E*S) can not be readily normalized. The usual kinetic parameters K_m and V_{max}, therefore, have little mechanistic significance in the context of the interfacial catalytic turnover in the intravesicle scooting mode. To a first approximation the approach utilized here provides a guide to permit some useful generalizations by comparing k_i and the effect of anions.

The size and polarity of the head group substituent is an important factor in determining the overall catalytic turnover in the interface. In general, the rate of hydrolysis decreases if the hydrophobicity increases either due to an increase in the chain length or the head group. Large polar head group substituents decrease the average residence time of the enzyme on the interface. One of the simplest interpretation of these observations is that the enzyme anchored to the interface, E*, extracts the monomer phospholipid from the bilayer to form E*S complex at the interface. If however, the substrate is anchored on the opposite side, as in No. 38 (Fig. 1), dislodging the monomer bipolar phospholipid from the aggregated particle to form E*S would be virtually impossible. Therefore, it will not be hydrolyzed as is indeed the case. This conclusion is also consistent with the fluorescence quenching experiments, according to which Trp-3 is accessible to water soluble quenchers for dynamic quenching, and also to 9- or 11-bromo substituents on the acyl chains [31]. Similarly, destabilization of monomer in the bilayer could account for a decrease in the catalytic turnover with increasing acyl chain length.

The specific activities obtained under apparent zero-order steady-state conditions, change appreciably in the presence of anions. This suggests that the interfacial anions somehow compete with anions in the aqueous phase for an anion binding site on the enzyme. Three possibilities may be considered: (a) the positive charge of the enzyme 'colloid' interacts with the negative surface charge, and anions in the aqueous phase determine the relative surface charge densities that regulate the interaction; (b) a specific anion binding site on phospholipase A_2 molecule can be occupied by anions in the interface or the anions in the aqueous phase; (c) a cationic site on phospholipase A_2 is actually created by a calcium bridge between the enzyme and an anion.

Relative polarizability of anions is an important factor in determining the anion binding selectivity [18], whereas monovalent cations in the aqueous phase modify the electrostatic interactions at the level of the double layer around the enzyme and the vesicle interfaces. Since lipids with a net negative charge but larger head groups (Nos. 15–26) exhibit shorter interfacial residence periods, and cations have little or no effects, the possibility 'a' can be essentially discarded. At this stage we favor possibility 'b', and it may also be noted that the possibilities 'b' and 'c' are not mutually exclusive.

Yet another role of interfacial anionic charge arises from the fact that divalent ions like calcium can alter the charge profile and the phase properties of bilayers. Such interactions [19–24] could induce instabilities or defects, and thus promote binding of the monomer substrate to anchored E*. Unfortunately, at this stage we can not directly measure contributions of such factors.

Phospholipase A_2 does not exhibit a large difference in its apparent binding affinity, nK_d, but a more significant change in the fluorescence quantum yield of phospholipase A_2 in the vesicle interface of the various anionic substrates. A particularly provocative suggestion, in the context of the general Scheme I of interfacial catalysis [29], is that it is not the binding equilibrium at the interface (E to E*) but the monomer binding equilibrium within the interface (E* + S to E*S) that ultimately determines the substrate specificity of the bound enzyme. This suggestion lends itself to experimental scrutiny if one assumes that the fluorescence peak intensity of the bound enzyme is substantially if not exclusively due to the E*S form rather than the E* form of the enzyme. It follows that the peak intensity will change not only with the structure and conformation of the substrate monomer [25–27] but it will also be correlated with the interfacial catalytic turnover rate constant, k_i. Data summarized in Tables I and III are consistent with both of these predictions, as well as with our earlier results which

suggest that the binding measured by the fluorescence change is catalytically meaningful [5]. This implies that the E* + S to E*S equilibrium for the various substrates is different. Thus it is interesting to note that the intervesicle exchangeability of the enzyme bound to DMPMe vesicles is substantially slower than that for the enzyme bound to bisphosphatidic acid interface. This suggests that the E*S to E* + S step is slower than the E* to E step in Scheme I. The observations in the following paper also support this conclusion.

Acknowledgements

We thank Professor G.H. DeHaas and Karl Koehler for very stimulating and useful discussions. This work was supported by PHS (GM29703 to M.K.J. and HL 23126 to F.R.).

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