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Kinetics of interfacial catalysis by phospholipase A_2 in intravesicle scooting mode, and heterofusion of anionic and zwitterionic vesicles

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In this and the following three papers we examine the kinetics of action of pig pancreatic phospholipase A_2 on vesicles of anionic phospholipids without any additives. The results provide the first unequivocal demonstration of interfacial catalysis in intravesicle scooting mode. In this paper we describe the conditions in which the action of pig pancreatic phospholipase A_2 on DMPMe (ester) vesicles in the absence of any additive commences without a latency. Under these conditions the free monomer substrate concentration is insignificant; the bilayer enclosed vesicle organization remains intact even when all the substrate in the outer monolayer has been hydrolyzed; the rate of intervesicle exchange and the rate of transbilayer movement (flip-flop) of molecules is negligibly slow; and the rate of fusion of vesicles is insignificant. Thus an enzyme molecule bound to one vesicle hydrolyzes all the DMPMe molecules in the outer monolayer of the vesicle by a first-order process with a rate constant of 0.6 per min at 30°C; or viewed another way, one enzyme molecule in a DMPMe vesicle can hydrolyze all the available substrate molecules at the rate of 3000 per min. At low anion concentrations excess substrate vesicles are not hydrolyzed unless the rate of intervesicle exchange of the bound enzyme is stimulated by anions in the aqueous phase. Higher calcium concentrations promote not only homofusion of DMPMe vesicles but also heterofusion of DMPMe and DMPC vesicles. It is proposed that calcium-induced isothermal lateral phase separation in DMPMe vesicles induces defects in the bilayer organization, and such defects are the sites for phospholipase A_2 binding and for heterofusion with DMPC (ester) vesicles which do not have such sites.

Introduction

Action of phospholipase A_2 on the aggregated form of its substrate, 1,2-diacyl-*sn*-3-glycerophospholipids is probably one of the best characterized examples of interfacial catalysis [1–5]. A general scheme of interfacial catalysis that adequately accounts for the major features of the action of the enzyme (E) in the aqueous phase on the substrate interface consists of the steps presented in Scheme I.

According to this Scheme, the catalysis occurs on the substrate interface, and the enzyme remains bound to the interface during several catalytic turnover cycles as represented by the cyclic

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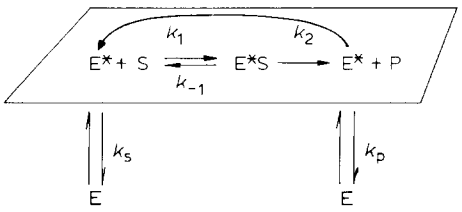
Abbreviations: DHPMe (ether), 1,2-dihexylphosphatidylmethanol; DMPMe (ester), 1,2-dimyristoylphosphatidylmethanol; DTPMe (ether), 1,2-ditetradecylphosphatidylmethanol; DMPC (ester), 1,2-dimyristoylphosphatidylcholine; DTPC (ether), ditetradecylphosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.



Scheme I. Scheme of interfacial catalysis.

reaction sequence in the box. E* is the enzyme bound to the interface, E*S is the Michaelis-Menten complex in the interface, which gives rise to E* and the products of hydrolysis. Scheme I was developed and tested for the action of pig pancreatic phospholipase A_2 on monolayers of medium chain phospholipids [1,6,7]. Due to intrinsic limitations of the monolayer system it is not possible to examine some of the key implications of Scheme I.

Implicit in Scheme I are two possibilities which have significantly different kinetic consequences. For example, after each or a few catalytic turnover cycles in the interface, E* could leave the interface and return to another site on the interface via the aqueous phase; that is, the intervesicle exchange of the enzyme is rapid and the enzyme in effect 'hops' from one interface to the other via the aqueous phase. The other extreme case would be that E* 'scoots' from one substrate molecule to the other without leaving the interface during several thousand catalytic turnover cycles [8,9]. In order to distinguish hopping from scooting it is necessary to have an understanding of the kinetics of the intervesicle exchange of the bound enzyme, E*. Two kinetically distinct steps leading to hopping may be considered: (a) the rate of desorption of E* in the interface of the pure substrate as determined by the rate constant k_s, and (b) the rate of desorption of the enzyme bound to the bilayer containing the products as determined by the rate constant k_p. The on-rate constants for both of these interfacial binding equilibria should also be considered; these rate constants are found to be relatively large (> 65 per s) under all the conditions we have examined (unpublished observations), and therefore essentially ignored from the present discussion. According to these considerations, in the intravesicle 'scooting' mode of catalysis, both k_s and k_p would be significantly smaller than the integrated rate constant for the hydrolysis of all the substrate in the interface, k_i. However, in the 'hopping' mode of catalysis, k_p and k_s would be of the same magnitude as the catalytic turnover rate constant.

The major goal of the studies reported in this and the next three papers [10–12] is to characterize the phenomenology and the experimental boundary conditions which determine the kinetics of intravesicle scooting and of intervesicle exchange of phospholipase A_2. In this paper we show that both k_p and k_s are at least 10000-fold smaller than the turnover number of over 3000 per min in the scooting mode of interfacial catalysis. Further implications of the scooting mode of hydrolysis by phospholipase A_2 will be examined in the accompanying papers where we have shown that the binding of phospholipase A_2 and its semisynthetic analogs to the interface occurs via an anion binding site, which is probably located in the interfacial recognition region (IRR) including the N-terminus of the enzyme [10]. It is also shown that vesicles of a variety of anionic phospholipids are hydrolyzed by scooting mode of catalysis [11], although the hopping mode becomes significant for bilayers of phospholipids with zwitterionic or larger anionic head groups. Inhibition of interfacial catalysis by nonhydrolyzable analogs suggests that the rate constant for the decomposition of E*S to E* + P (k_2) is larger than that for E*S to E* + S, k_{-1} [12]. A general discussion of the results in the broader context of interfacial catalysis is also given in the last paper of this series [12].

Materials and Methods

Phospholipase A_2 from pig pancreas was isolated as described elsewhere [13], and was kindly provided by Professor DeHaas (Utrecht). All the procedures used in this study have been described in detail elsewhere [2,3,8,14,15]. For example, action of phospholipase A_2 was monitored by automatic pH-stat titration (Radiometer pH-stat with Servograph REC61, ABU-13, PHM-62, TTT60, TTA60) with 3 mM NaOH under a stream of nitrogen in 4 ml aqueous phase containing, unless stated otherwise, 0.3 mM $CaCl_2$ at 30°C and pH 8.0. Whenever convenient, the reaction was ini-

tiated by adding the stock solution of the enzyme in water, and the pH-shift from the enzyme was negligible. Also under all the conditions the background drift was less than 5 nmol protons per min, that is less than 1% of the measured rates. The titration efficiency of the released fatty acid was calibrated with externally added fatty acids to vesicles of several zwitterionic and anionic phospholipid vesicles.

All kinetic studies reported in this paper were done on vesicles of DMPMe prepared by dispersing the dry sodium salt in distilled water in a bath type sonicator (Sonicor). Sonicated vesicles were stored at 56°C and an aliquot was added to the reaction mixture with a thermally equilibrated syringe. Vesicles prepared and stored in this manner do not exhibit any detectable degradation for well over 10 h, and their sizes remain unchanged as judged by centrifugation at $100\,000 \times g$ or by gel-filtration on Sepharose 2B. In the probe-containing vesicles the probe to phospholipid molar ratio was 8 : 92.

Gel-filtration of vesicles was carried out on Sepharose 2B (Pharmacia, exclusion limit 40 million) column (diameter 1 cm, void volume 10 ml) in 100 mM KCl and 3 mM Hepes at pH 8.0 and 25°C. Typically, a 0.15 ml lipid sample containing fluorescent labelled lipid or carboxyfluorescein was applied, and 1 ml fractions collected. The lipid eluted from the column was detected fluorimetrically in 0.1% Triton X-100 solution. Vesicles of DMPC, egg phosphatidylcholine, and DMPMe, as well as the products of homo- and heterofusion were gel-filtered to determine their size. The sonicated vesicles of these lipids eluted at 2.1-times void volume, whereas the products of fusion eluted in the void volume.

Kinetics of fusion were monitored by the method described by Hoekstra et al. [17] in which self-quenched octadecylrhodamine B (probe) containing vesicles were fused with probe free vesicles. Dequenching of the fluorescence of the probe occurs as the surface density of the probe decreases by mixing of lipid molecules during fusion. Typically, probe free vesicles (0.1 mM lipid) were mixed with probe containing vesicles in 20 : 1 ratio in a stirred thermostated cuvette containing 2 ml of 0.1 M KCl, 3 mM Hepes at pH 8.0, 30°C and calcium concentration as indicted in the text. Fusion was always initiated by adding the probe free vesicles to preequilibrated buffer. In some preliminary experiments fusion was also followed by tirbidimetry [16]. Qualitatively the results are similar to those obtained from the fluorescence dequenching method.

Steady-state fluorescence measurements were done on an SLM 4800S interfaced to an Apple IIe computer. Excitation was set at 560 nm and emission at 590 nm, and slitwidths 2 nm for both. Differential scanning calorimetry of lipid dispersions was done on Mettlar 2000B [2,18]. Typically, 4 μmol lipid in 35 μl buffer (100 mM KCl, 100 mM Hepes at pH 8.0) was scanned at the rate of 1 or 2 Cdeg/min, and a small correction for the scanning rate has been made in the reported values of T_m. The lipid dispersions were prepared by suspending a dry film or powder of lipid and then equilibrating it in sealed aluminum pan at 60 to 80°C for about 30 min. None of these samples were annealed at low temperatures to induce subtrasitions.

DMPMe was prepared from 1,2-dimyristoyl-*sn*-glycerol (8.2 nmol) and 2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphosphole (8.2 nmol) in anhydrous diethyl ether containing triethylamine (8.2 nmol) by the general procedure already described [19]. The condensation of the 1,2-diacyl-*sn*-glyceryl cyclic enediol phosphotriester with methanol (2 mol equivalents) was carried out in anhydrous tetrahydrofuran, in the presence of triethylamine (2 mol equivalents; 24 h, 25°C). Deprotection of the 1,2-diacyl-*sn*-glyceryl ((methyl) 3-oxo-2-butyl)phosphate, and conversion to the sodium salt of DMPMe was performed by the standard procedure [19].

$[\alpha]_D = +6.8$ ($c = 5$, $CHCl_3$, 25°C)

Calcd. for $C_{32}H_{62}NaO_8P$ (628.8): C, 61.12; H, 9.94; Na, 3.66

Found: C, 60.85; H, 10.10; Na, 3.50

DTPMe and DHPMe were prepared from the corresponding 1,2-dialkylglycerols.

Results

Eibl and co-workers [20,21] have described synthesis and properties of the aqueous disper-

sions of DMPMe. We have further characterized the vesicles of DMPMe and DTPMe, as well as the micelles of DHPMe. In aqueous dispersions DTPMe and DMPMe form lamellar bilayers as seen by wide-angle X-ray diffraction, freeze-fracture electron microscopy, and ^{31}P-NMR. The bilayer organization of these dispersions does not change in the dispersions of Ca · DMPMe (1 : 2) salt, although the chain melting endothermic transition temperature, Tm increases from 29°C for DMPMe (enthalpy 6.4 kcal/mol) and 31°C for DTPMe (6.8 kcal/mol) to 49 (7 kcal/mol) and 53°C (7.7 kcal/mol), respectively, for the stoichiometric sodium and calcium salts. Thus the properties of the aqueous dispersions of DTPMe and DMPMe analogs are very similar, as is the case for phosphatidylcholines [2,15,22].

Interfacial catalysis in intravesicle scooting mode

From the perspective of the present studies, one of the most interesting properties of DMPMe vesicles and liposomes is that in the absence of additives they are readily hydrolyzed by phospholipase A_2 without any latency. The high affinity of phospholipase A_2 for bilayers of DMPMe and other anionic lipids [11] contrasts with the behavior of vesicles of zwitterionic lipids like DMPC [2,15,23,25], and DPPC [8,24], which exhibit complex reaction progress curves in the absence of additives. As shown in Fig. 1, under certain conditions the reaction progress curve for hydrolysis of DMPMe vesicles is first order, and it can be completely described in terms of the intravesicle scooting mode of interfacial catalysis. We will also show that during the hydrolysis of DMPMe vesicles the complications due to the product induced binding of the enzyme, flip-flop, intervesicle exchange, fusion, and hydrolysis of monomeric substrate can be essentially eliminated by a judicious choice of the experimental conditions. These studies are extended in the accompanying papers [10–12]. The results show that the complexity of the reaction progress curves under a variety of conditions arises primarily due to intervesicle transfer of the bound enzyme promoted by anions and by fusion of vesicles.

The reaction progress curve for the hydrolysis of DMPMe vesicles at low salt concentration (less than 1 mM chloride) is first order (Fig. 1 curve a), and therefore can be completely described by two constants: A, the amplitude or the extent of hydrolysis, and k_i, the first-order rate constant obtained from a semilog plot. Such a behavior is normally expected for a reaction progress curve obtained at the substrate concentrations considerably below K_m. For the phospholipase A_2 + DMPMe vesicle system it can be readily shown

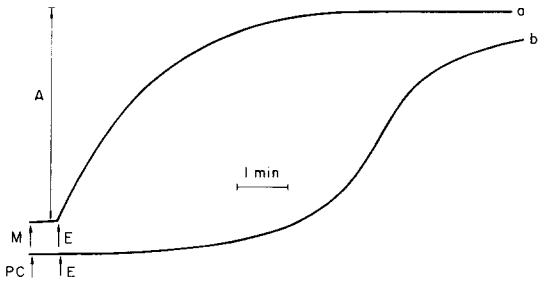


Fig. 1. Reaction progress curves for the hydrolysis of (a) DMPMe (ester) (M, 0.08 mM) and (b) DMPC (PC, 0.06 mM) vesicles by phospholipase A_2 (E) in 4 ml aqueous mixture at pH 8.0 and 30°C. Reaction was initiated by adding phospholipase A_2 to the stirred suspension of vesicles (stored at 57°C). The reaction mixture for DMPMe contained 0.3 mM $CaCl_2$ and 0.4 μg E, and that for DMPC contained 10 mM $CaCl_2$ and 1 μg E. For further details on the kinetics of the latency phase of DMPC vesicles see Ref. 15. The amplitude of hydrolysis (A) was obtained directly from the reaction progress curves.

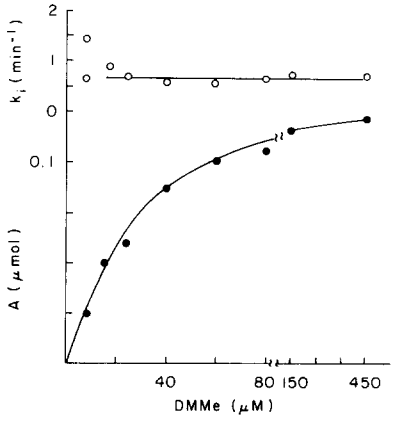


Fig. 2. Dependence of A and k_i on DMPMe ester (DMMe) concentration. Data were obtained by reaction progress curves of type shown in Fig. 1 (curve a); The first-order rate constant k_i was obtained from semi-log plots. Reaction is initiated by 0.4 μg (30 pmol) phospholipase A_2. Other conditions as in Fig. 1.

that k_i does not change noticeably even when the substrate and enzyme concentrations are altered several-fold either by dilution of the reaction mixture or by the addition of more substrate. Furthermore, as shown in Fig. 2, by changing the substrate concentration the amplitude of hydrolysis increases and reaches a maximum; also the rate constant k_i reaches a constant minimum value at higher substrate concentrations. Thus, in the presence of a high concentration of substrate vesicles and a constant enzyme concentration, only a constant amount of the substrate is hydrolyzed with a single first-order rate constant. Under a variety of conditions we have found that a maximum of 4300 mol of DMPMe in the form of sonicated vesicles can be hydrolyzed by each mole of phospholipase A_2, even when a 10-fold excess of the substrate vesicles is present.

The excess unhydrolyzed substrate at the end of the reaction progress curve can be hydrolyzed by adding more enzyme. The accessibility of the excess substrate vesicles to the enzyme in the reaction mixture is essentially the same whether the excess substrate is added at the beginning or at the end of the first-order reaction progress curve. The possibility that the initially added enzyme has been inhibited by the products or somehow inactivated during the first part of the reaction progress curve can be readily ruled out by the experiments described later, where it is shown that the hydrolysis of excess substrate vesicles can be reinitiated by added salts [10], or by inducing fusion of vesicles. These and other experiments [10–12] show that even in the presence of excess substrate vesicles hydrolysis ceases because the enzyme added initially is bound to vesicles, and that the bound enzyme does not exchange with excess substrate vesicles.

In the presence of excess substrate vesicles, the amplitude of hydrolysis also increases with the enzyme concentration. As shown in Fig. 3, the extent of hydrolysis changes with the enzyme concentration, and k_i remains constant as long as the substrate to enzyme mole ratio is more than 6000. These observations can be readily rationalized by the assumption that under the reaction conditions used in these experiments, there is little, if any, free enzyme in the aqueous phase, and that the enzyme bound to the substrate or to the product-

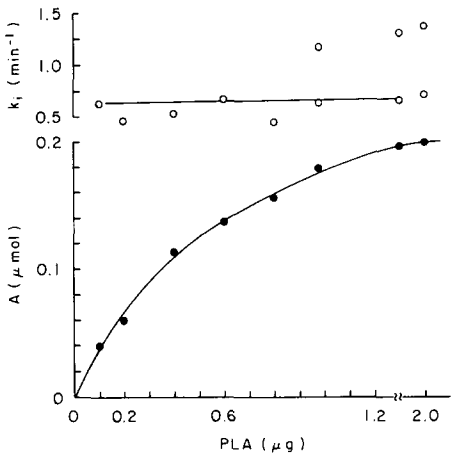


Fig. 3. Dependence of A and k_i on phospholipase A_2 (PLA) concentration. Data were obtained from the reaction progress curves of type shown in Fig. 1. Reaction mixture contained 0.32 μmol DMPMe (ester) as sonicated dispersions in 4 ml of 0.3 mM $CaCl_2$. Other conditions as in Fig. 1. At high enzyme concentrations the reaction progress curves were fitted to two simultaneous first-order constants.

containing vesicles (at the end of the reaction progress curve) is not readily exchangeable with excess substrate vesicles. Thus, for a given enzyme concentration the amplitude of hydrolysis will reach a maximum when the enzyme to vesicle stoichiometry falls below an integral number, n, that would reflect the number of enzyme monomers in a catalytically active aggregate form in the interface. The k_i values remain constant as long as the substrate to enzyme mole ratio is larger than 6000, which suggests that there are at least n enzyme molecules per vesicle under these conditions. The k_i values increase above the standard deviation when the substrate to enzyme mole ratio is less than 5000, which would be expected if there are more than n enzyme molecules per vesicle. At these lower substrate to enzyme mole ratios, the reaction progress curve is better described by two simultaneous first-order rate constants, 0.6 and 1.2 per min; the fraction of the substrate hydrolyzed by the slower process decreases when the substrate to enzyme ratio decreases below 6000, and it reaches zero at about 3500. This doubling of k_i will be expected if under these conditions the stoichiometry of the enzyme molecules per vesicle is n in some vesicles and $2n$ in other. Under, most of the conditions

used in this and the accompanying papers we have maintained the total substrate to the enzyme mole ratio more than 6000 so that the vesicle to enzyme ratio exceeds n, which as shown below is found to be one.

The value of n can be computed from the maximum number of moles of the substrate hydrolyzed by a mole of the enzyme. In a variety of experiments we have found that in the presence of excess sonicated vesicles, an enzyme molecule hydrolyzes a maximum of about 4300 substrate molecules at the end of the first-order reaction progress curve. Since only the substrate in the outer monolayer can be hydrolyzed with a large excess of the enzyme, it is reasonable to assume that there are 4300 substrate molecules in the outer monolayer of the vesicles to which one enzyme molecule is bound, and that the rate of transbilayer movement (flip-flop) of the substrate from the inner monolayer of the vesicle is negligibly slow on the time-scale of the reaction progress curves under consideration. Based on these assumptions it can be calculated that a vesicle containing 4300 DMPMe molecules of estimated cross-section area of 45 Å^2, will have a diameter of 200–250 Å, which is the same as the diameter of sonicated egg phosphatidylcholine or DMPC vesicles. If there are two enzyme molecules per vesicle, the calculated diameter of DMPMe vesicles would be about 190 Å. Indeed, the elution volume for DMPMe vesicles on Sepharose 2B is the same as the elution volume for phosphatidylcholine vesicles. These observations are consistent with $n = 1$ in the catalytically active state.

Evidence for a negligibly slow intervesicle exchange of the bound enzyme is obtained from the reaction progress curves shown in Fig. 4. When the enzyme is added to DTPMe vesicles (curve a), the enzyme is no longer accessible to the DMPMe vesicles added subsequently. If, however, the enzyme is added to a mixture of the vesicles of DTPMe and DMPMe (curve b), only a fraction of the total DMPMe is hydrolyzed by a first-order process with the same k_1 as observed with DMPMe vesicles alone. On the other hand, if the enzyme is added to the vesicles of premixed DMPMe and DTPMe (curve c), all the accessible DMPMe is hydrolyzed with a considerably smaller first order rate constant. Note that a 10-fold excess of the

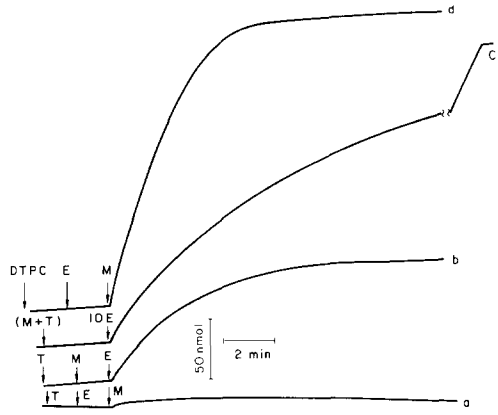


Fig. 4. Reaction progress curves for the hydrolysis of 0.32 μmol DMPMe (ester) (indicated by M) by 0.8 μg phospholipase A_2 (E) in the presence of 0.32 μmol DTPMe (ether) (T) or 1.2 μmol DTPC (ether) added in the sequence indicated. For example, for curve b the vesicles of DTPMe were mixed with vesicles of DMPMe, and the reaction was initiated by 0.8 μg E. On the other hand for curve c, vesicles were prepared by mixing DTPMe with DMPMe (1:1) and the reaction was initiated with 8 μg phospholipase A_2. Other conditions as in Fig. 1, and see text for further details.

enzyme is added in these experiments, significance of which is described later [12]. It is also relevant to compare curve a with curve d where it is shown that all the enzyme added to DTPC vesicles remains accessible to DMPMe vesicles that are added subsequently. By their very design the experiments summarized in Fig. 4 provide the strongest proof yet that the enzyme bound to DTPMe vesicles is not exchangeable. Moreover, the enzyme does not bind to DTPC as shown by the direct binding experiments [2,3]. It is also possible that the enzyme bound to DTPC ether vesicles is readily exchangeable, and such a binding is not catalytically meaningful (see also Refs. 3 and 15).

Intervesicle exchange in the presence of KCl

Intervesicle transfer of the bound enzyme is also promoted in the presence of anions. As shown in Fig. 4 (curve a) the rate of transfer of the enzyme from DTPMe vesicles to DMPMe vesicles is negligible. However, as shown in Fig. 5A, in the presence of 0.1 M KCl the hydrolysis of excess substrate by the enzyme bound to DTPMe vesicles (curve b) or by the enzyme bound to the product-containing vesicles (curve a) can be at-

tained with a short latency phase. From the latency periods in curves a and b, it can be shown that in the presence of 0.1 M KCl the k_p and k_s values are smaller than 0.75 and 0.34 per min, respectively. It may also be noted that, as shown in Fig. 5B, k_s does not depend significantly upon the enzyme concentration, whereas the steady-state rate of hydrolysis after the exchange increases linearly with the enzyme concentration. Such a behavior would be expected if the bound enzyme had equilibrated between the two vesicle populations. The results in Fig. 4 (curve a) show that the residence time of the enzyme on DTPMe vesicles is more than 20 min in the absence of salt, and as shown in Fig. 5A (curve b) the average residence time (reciprocal of the rate constant k_s) decreases to about 3 min in the presence of 0.1 M KCl. Similarly, the average residence time of the enzyme in the vesicles with all the substrate molecules hydrolyzed in the outer monolayer (reciprocal of k_p) is about 1 min. The significance of these residence times in terms of the k_i values will be elaborated on in the Discussion.

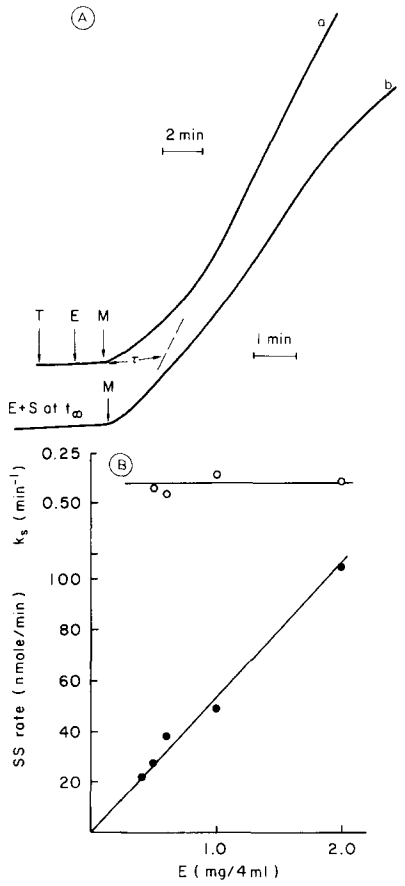


Fig. 5. (A) Curve a, reaction progress curve for the hydrolysis of 0.4 mM DMPMe (ester) (M) added to premixed 80 μM DTPMe (ether) (T) and 0.4 μg phospholipase A_2 (E). Curve b, reaction progress curve for hydrolysis of 0.4 mM DMPMe (ester) added to a mixture of 0.08 mM DMPMe and 0.4 μg phospholipase A_2 in which the hydrolysis has already ceased. Reaction mixture contained 0.1 M KCl and 0.3 mM $CaCl_2$, pH 8 at 30°C. The latency period was measured by extrapolating the steady-state phase of hydrolysis to the base-line, and the rate constants k_s and k_p are defined as reciprocal of the latency period. (B) Effect of the enzyme concentration on the (top) latency period and (bottom) on the steady-state rate of hydrolysis. Data were obtained from the reaction progress curves of type shown in Fig. 5A.

The apparent specific activity of the enzyme in the steady-state phase of the reaction progress curves (shown in Fig. 5A, and summarized in Fig. 5B) is 55 IU, compared to about 300 IU, if the apparent zero-order initial rates were measured with DMPMe vesicles alone in the presence of 0.1 M KCl. As discussed later [12] one of the reasons for this could be that the apparent affinity of the enzyme for DTPMe ether vesicles, K_s, is about 6-fold higher than K_m for DMPMe ester vesicles.

Homo- and heterofusion of vesicles

Formation of larger vesicles on fusion of DMPMe vesicles with themselves or with DMPC vesicles in the presence of calcium is indicated by gel filtration on Sepharose 2B and by the octadecylrhodamine B probe method [17]. Under conditions outlined in the Methods, sonicated vesicles of egg phosphatidylcholine, DMPMe and DMPC elute at 2.1-times the void volume in calcium-free buffer. This suggests that these vesicles have the same size, within the limits of resolution of this technique. In the presence of calcium, DMPMe vesicles fuse to form larger particles which elute in the void volume. According to these criteria DMPMe vesicles fuse not only with other DMPMe vesicles but also with DMPC vesicles in the presence of calcium. These conclusions are in accord with the results of fluorescence dequenching (see below) and turbidimetric measurements [16].

Yet another qualitative demonstration of increased size of fused vesicles is based on the observation that under certain conditions (cf. Fig. 4, curve d) an increase in the extent of hydrolysis

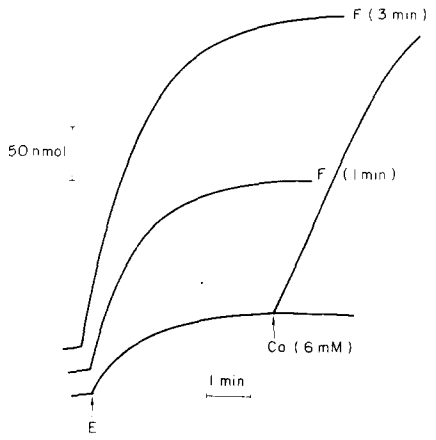


Fig. 6. Reaction progress curves for the hydrolysis of DMPMe (ester) vesicles: (bottom) DMPMe (0.32 mM) vesicles with 0.4 μg phospholipase A_2 in 0.3 mM $CaCl_2$; fusion is induced by adding 6 mM $CaCl_2$ at the end of the first-order reaction progress curve. (top) DMPMe (0.32 mM) vesicles that had been allowed to fuse for 3 or for 1 min in 1.5 mM $CaCl_2$ are then treated with EGTA to bring Ca concentration to 0.3 mM, and then treated with 0.4 μg phospholipase A_2. Higher proportion of the substrate is hydrolyzed when the fusion with calcium is allowed to proceed for a longer period of time or when higher concentrations of calcium are used. At calcium chloride concentrations greater than 6 mM the proportion of substrate accessible to phospholipase A_2 decreases well below 20%.

is observed during the first-order intravesicle scooting of phospholipase A_2. This is due to an increase in the average size of fused vesicles. As shown in Fig. 6, when the vesicle to enzyme ratio is about 5, only about 20% of the total accessible substrate is hydrolyzed before the reaction ceases. However, if the vesicles are allowed to fuse, a higher proportion of the accessible substrate is hydrolyzed, and ultimately that the proportion of the accessible lipid reaches 0.5 of the total lipid present in the reaction mixture. This behavior is expected if large unilamellar vesicles are formed in which the proportion of lipid in the inner and outer monolayers is equal. In these experiments the total amount of the accessible substrate is monitored by adding an excess of the enzyme so that each vesicle has at least one enzyme molecule. For sonicated vesicles the proportion of the accessible lipid is 0.63 of the total lipid present, and this proportion can be maintained for well over 10 h by keeping the vesicles at 57°C, under conditions precluding fusion.

When fusion is allowed to continue for long periods of time (more than 2 h at 1.5 mM calcium chloride) or when fusion is induced by higher concentrations of calcium chloride (more than 6 mM), the proportion of the accessible phospholipid decreases to a point far below 0.5 as if multilamellar liposomes are formed when the vesicles precipitate out. As expected, this change in the proportion of accessible phospholipid is not readily detectable by the fluorescence dequenching technique. The proportion of the total lipid accessible to phospholipase A_2 remains at or above 0.5 under all the conditions when fusion has occurred without any detectable precipitation. Experiments with phospholipase A_2 also suggest that the fused vesicles are not leaky enough to permit the transbilayer passage of phospholipase A_2, and that the inner monolayer of the vesicle is not exposed to the enzyme even when the enzyme is present during fusion.

Kinetics of fusion by dequenching of octadecylrhodamine B containing vesicles

The qualitative evidence for fusion of vesicles summarized in the preceeding section is substantiated by the fluorescence dequenching method [17]. As shown in Fig. 7, in 20 : 1 ratio DMPMe vesicles + DMPMe(rh) vesicles (the octadecylrhodamine-containing vesicles are represented as DMPMe (rh)), exhibit an increase in the fluorescence intensity in the presence of calcium ions. The total increase in the fluorescence intensity at the end of the fusion profile is typically about 7-fold, as expected on the basis of the self-quenching curve obtained by Hoekstra et al. [17]. The increase in the fluorescence of the probe stops almost instantaneously at any point along the fusion profile on addition of EGTA, however the increase in the intensity that has preceded the addition of EGTA is not reversed. Formation of larger vesicles during the course of the fusion profile of type shown in Fig. 7 is also indicated by an increase in turbidity, however, the time-course is somewhat different. Such a behavior is due to the fact that the dequenching and the turbidity change have different responses as a function of the underlying fusion events.

Fusion profiles of the type shown in Fig. 7 are a complex function of several parallel and sequen-

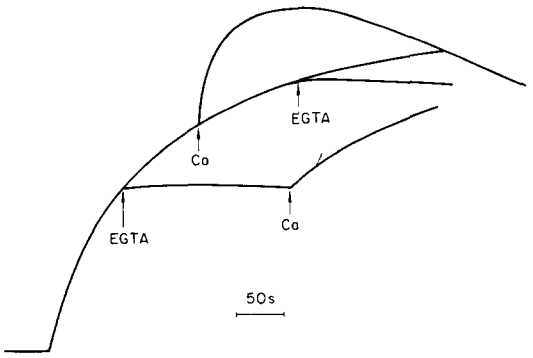


Fig. 7. Increase in fluorescence intensity of octadecylrhodamine B in DMPMe (ester) vesicles, DMPMe(rh) after mixing with probe-free DMPMe vesicles (0.15 mM) in the presence of 3.18 mM $CaCl_2$ at 30°C and pH 8.0. Addition of EGTA inhibits the increase, whereas addition of more calcium promotes the increase in the fluorescence intensity. When the calcium concentration is about 10 mM the maximum increase in fluorescence intensity is achieved faster, and then a decrease in fluorescence is accompanied by the appearance of precipitate.

tial events, and as such cannot be adequately described by a set of simple parameters. However, a relatively simple parameter can be devised to describe the initial fusion event on the basis of the following considerations. The increase in the fluorescence intensity of octadecylrhodamine containing vesicles on fusion is due to the mixing of lipid molecules from the probe-containing and the probe-free vesicles.

Self-quenched probe molecules are diluted, resulting in a linear increase in the fluorescence intensity. In this system, quenching or a decrease in the fluorescence intensity is a linear function of the concentration of the probe in the vesicle [17]. Under all of the conditions employed in this study the proportion of the probe free vesicles is 20-fold higher than that of the probe-containing vesicles. Therefore by design, the fusion profiles measure fusion only between a probe free and a probe-containing vesicle. Since the proportion of the probe-free vesicles is initially very large, substantially all of the fusion events involving the probe-containing vesicles will be with the probe-free vesicles. As fusion progresses, the probe-containing vesicles will encounter other probe-containing vesicles more often, thus kinetic complexities arise.

The fluorescence dequenching is not due to intervesicle exchange of phospholipid molecules,

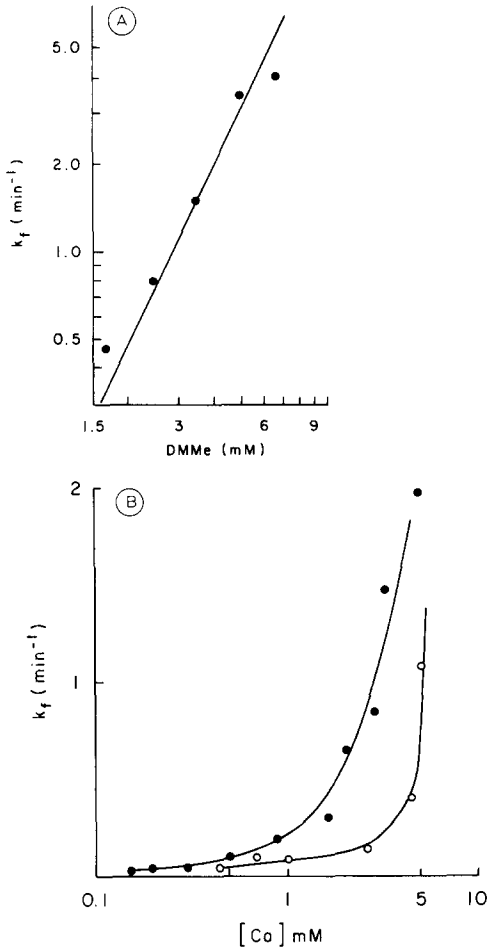


FIg. 8. (A) Dependence of k_f on DMPMe (DMMe) concentration. Conditions: 100 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 8.0, 30°C; homofusion was initiated with DMPMe vesicles. The line is drawn to slope = 2. (B) Dependence of k_f on Ca concentration for 0.15 mM DMPMe (●) and DMPC (○) vesicles.

because as shown in the following papers the fraction of the available substrate does not change appreciably with the time of incubation. Similarly, spontaneous intervesicle exchange of the probe can be ruled out by the fact that the rate of dequenching depends upon the calcium as well as the vesicle concentration. As shown in Fig. 8A, the rate of fusion shows a second order dependence on the vesicle concentration when the ratio of the probe-free and of the probe-containing vesicles is kept constant at 20. This is consistent with the assumption that the initial fusion event involves two vesicles. On the other hand dependence of k_f

on calcium concentration shows a threshold effect (Fig. 8B), whereby the rate of fusion increases abruptly at concentrations greater than 1.5 mM $CaCl_2$.

The extent of fusion and k_f were found to be essentially identical in 0.3 M KCl and in ^2H_2O. Fusion profiles for DTPMe and DMPMe vesicles are also identical. This absence of any difference in the rate of fusions serves as a very useful control for the experiments described in this series of papers in which it is assumed that deuterated water, anions, and ether linkage of the lipid to not appreciably perturb the interface. Similarly, k_f at 3 mM calcium is 1.5 per min, and less than 0.02 per min at 0.3 mM calcium, which means that under the conditions used for the kinetic measurements in this series of papers, the complications due to fusion are negligible.

Heterofusion or fusion of two different types of vesicles

Curve d in Fig. 4 shows that DMPC vesicles fuse with DMPMe vesicles at high Ca^{2+} concentrations. Heterofusion of DMPC vesicles with DTPMe vesicles containing phospholipase A_2 can be readily demonstrated by adding 6 mM $CaCl_2$, whereby a rapid hydrolysis of DMPC is observed. This is possible only in fused vesicles where DTPMe acts as an 'activator' in the mixed lipid vesicles by promoting interfacial binding phospholipase A_2. Intervesicle exchange of the enzyme from DTPMe vesicles to DMPC vesicles could not account for this hydrolysis, because DMPC vesicles are not hydrolyzed without an activator. This is also confirmed by the probe method. The fusion profiles of four different combinations of vesicle populations are shown in Fig. 9. Under identical conditions, the k_f for calcium induced fusion are in the order DMPMe + DMPMe(rh) 1.5 per min, DMPMe + DMPC(rh) 1.5 per min, DMPMe(rh) + DMPC 0.7 per min, DMPC(rh) + DMPC less than 0.05 per min at 3.18 mM calcium chloride. These results show that not only do the DMPMe vesicles fuse with other DMPMe vesicles but also with DMPC vesicles in the presence of calcium ions. Such heterofusion is not observed in the absence of calcium ions. The absolute rates for the first three combinations of vesicles are significantly higher because of the experimental design whereby only the fusion-mediated dilution of the probe in fused doublet vesicles is measured. In the first three cases formation of larger particles on fusion could also be demonstrated by gel filtration.

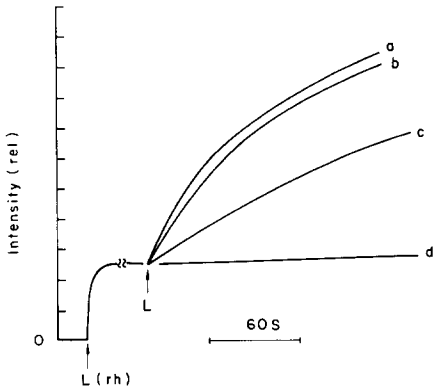


Fig. 9. Increase in fluorescence intensity vs. time. Vesicles 0.15 mM lipid containing 8 mol% octadecylrhodamine B were added at L(rh) to a buffer containing 0.1 M KCl, 3.18 mM $CaCl_2$, 3 mM Hepes at pH 8.0 and 30 °C. After 1 min vesicles of appropriate lipid (0.32 mM) were added. Only a part of the overall increase in the fluorescence is shown here. Curve a for DMPC(rh) + DMPMe, curve b for DMPMe + DMPMe(rh), curve c for DMPMe(rh) + DMPC, and curve d for DMPC(rh) + DMPC.

Discussion

Results in the preceding section show that the hydrolysis of DMPMe vesicles by phospholipase A_2 occurs by a first-order process under the conditions where intervesicle exchange of the bound enzyme, fusion of vesicles, and the rate of transbilayer movement of the lipid molecules in the bilayer are negligibly slow. These results are best interpreted by the postulate that the hydrolysis occurs in the intravesicle scooting mode. Several implications of this working hypothesis, within the framework of Scheme I, are elaborated on in this section and in the accompanying papers [10–12].

The catalytic turnover in the intravesicle scooting mode is rapid. As pointed out in the preceding section, whenever the vesicle to the enzyme ratio exceeds one, a maximum of 4300 substrate molecules are hydrolyzed by each molecule of phospholipase A_2. Since the first-order rate constant (k_i) for the hydrolysis of 4300 molecules in the outer monolayer of a vesicle containing one en-

zyme molecule is 0.6 per min, the catalytic turnover number in the intravesicle scooting mode of hydrolysis would be about 3000 per min. This is only a lower-limit estimate of the true catalytic turnover number of the enzyme molecule in the interface because the pseudo zero-order steady-state rate of hydrolysis is never attained. In a first order reaction progress curve resulting from catalysis in the scooting mode, the apparent rate decreases linearly with the decreasing mole fraction or interfacial concentration of the substrate. This could mean that the apparent affinity of the bound enzyme for the products in the bilayer is the same as it is for the substrate. Other possibilities are discussed in the last paper of this series [12].

The average catalytic turnover number of 3000 per min gives a lower-limit estimate of the interfacial rate constants k_1 or k_2, as > 3000 per min. Since k_s and k_p are considerably less than 0.5 per min, it is reasonable to surmise that far more than 6000 substrate molecules can be hydrolyzed during the residence time of the enzyme on the vesicle. These lower-limit values for the turnover numbers under different conditions are meaningful because they suggest that during the residence time of about 2 min in the presence of 0.1 M KCl, a single phospholipase A_2 molecule is capable of hydrolyzing a significant fraction of the phospholipid molecules in the monolayer half of a cell membrane.

As shown in this and the accompanying papers, the residence time of the enzyme on the bilayer interface decreases in the presence of anions in the aqueous phase. Similarly, anionic charge and organizational defects (instabilities) in the interface increase the catalytic turnover presumably by increasing the residence time of the enzyme. With long residence times, corresponding to a high affinity of the enzyme for the substrate interface, it will be virtually impossible to obtain pseudo-zero order initial rates of hydrolysis because the enzyme remains in the product-free environment for only a few turnover cycles.

With DMPMe vesicles essentially 98% of the reaction progress curve can be fitted to a single first order rate constant. However, it is likely that in large unilamellar vesicles or cell membranes where there are more than 50000 substrate molecules in a monolayer half, the bound enzyme could be exposed to a substantially product-free environment (say less than 20 mol% product) for a few minutes, and therefore a pseudo-zero-order rate of scooting could be attained. Under certain conditions (cf. curve c, Fig. 4) with large fused vesicles we have indeed obtained initial rates of hydrolysis corresponding to a turnover number of well over 10000 per min; however, the reaction progress curves remain first order.

On the other hand very short residence times resulting from large k_s or k_p, corresponding to a lower affinity for interface, probably describes the initial portions of the reaction progress curves for DMPC vesicles as shown in Fig. 1. Under these conditions the apparent catalytic turnover number is less than 10 per min; similarly, DTPC vesicles do not bind phospholipase A_2 in the absence of any additive [2]. It may also be pointed out that once a critical mole fraction of the product is formed in the vesicles, the binding affinity and therefore the residence time increases, and the average catalytic turnover number exceeds several thousand per min [2,3].

Obviously, a pseudo-zero-order initial rate of hydrolysis of a large number of vesicles by phospholipase A_2 will be achieved under conditions where intervesicle exchange occurs, but it is slow enough so that the residence time is large enough to hydrolyze a substantial proportion of the substrate molecules in a vesicle, say about 15%. An exact proportion will depend upon actual values of the various rate constants as well as the vesicle to enzyme ratio. But the residence time should be short enough so that the bound enzyme is on the average transferred to another vesicle before the reduced mole fraction of the substrate in the interface begins to retard the rate of hydrolysis. The residence time of the enzyme in a micellar form of the substrate is considerably reduced, however the rate of transfer of the enzyme from one particle to the other is also in effect substantially facilitated by a short life-time of micelles, where essentially all the monomers in most micelles are exchanged in less than 0.1 s. The concept of average residence time of the enzyme on the substrate interface could thus account, at least in part, for one of the most perplexing aspects of the interfacial catalysis, i.e., the same enzyme can exhibit over a 100-fold change in the rate of hydrolysis of the

same substrate depending upon the quality of the interface in which it is present (see Refs. 1–5 for several other examples).

The concept of the average residence time and of the intravesicle scooting of the enzyme are quite significant in evaluating the assay procedures and specific activity data for purification of intracellular phospholipase A_2. In crude biological materials, for example, the phospholipase A_2 activity is generally not detectable unless significant purification has been achieved [26–29]. This is probably because the enzyme bound to a lipid interface is not readily transferred to the externally added substrate interface. Moreover, membranes and other lipid constituents of serum and plasma could modulate the phase properties of the optimally activated externally added substrate vesicles. Thus the specific activity obtained with external substrate interface can not be used as a true measure of the degree of purification, unless it is assured that the enzyme has been completely transferred from the native interface to the added substrate. Several reports on intrinsic activators and inhibitors [30–32] of phospholipase A_2 in tissues are probably artifacts arising from the peculiar binding and exchange behavior of phospholipase A_2 with phospholipid interfaces. This situation would be particularly acute with detergent-free or nonmicellar assay systems, where the rate of intervesicle transfer of the bound enzyme, the sequence of addition of the substrate, enzyme, and calcium, as well as the time for the intervesicle exchange of the enzyme directly or by fusion of vesicles could give substantially different specific activities. Several intracellular phospholipase A_2 [33] appear to have little tendency for intervesicle exchange even in the presence of anions. In micellar systems interparticle exchange times would be considerably faster, and therefore these complications may be minimal.

The original goal of the fusion studies described in this paper was to establish the conditions under which the kinetics of action of phospholipase A_2 can be studied without complications from intervesicle transfer of the bound phospholipase A_2 by fusion of vesicles. The value of k_f is less than 0.02 per min for DMPMe vesicles in 0.3 mM Ca. Additional control experiments reported here also show that the rate of fusion does not depend upon deuterated water, anions and the enter analog of phospholipid. These results suggest that the bilayer interface is not appreciably modified under these conditions.

By default, as much as by design, our initial fusion studies led us to characterize a novel phenomenon of heterofusion. The results reported in this paper show that the fusion characteristics of DMPMe + DMPMe and of DMPMe + DMPC vesicles are essentially identical. This observation rules out a role of intervesicle bridging by calcium as the rate limiting step for the fusion process. Similarly, if precipitation of vesicles is taken as an indication of intervesicle Ca-bridging and consequent interlamellar dehydration, it should be possible to dissociate the fusion events from other effects of calcium on anionic vesicles. Unfortunately, at this stage there is no convenient method to quantitatively monitor changes related to precipitation of vesicles at low lipid concentrations. However, by monitoring the proportion of the total substrate accessible to phospholipase A_2 we have been able to show that during precipitation of vesicles with less than 5 mM $CaCl_2$, the proportion of the accessible substrate decrease drastically, and multilamellar structures are probably formed.

The whole range of observations described in this paper can be readily explained by the hypothesis that calcium induced isothermal phase separation in anionic phospholipids could generate organizational defects at which the hydrophobic region of the bilayer is somehow exposed enough to permit, not only the binding of phospholipase A_2 and other proteins [16,34,35], but also intrusion of complementary molecular features of the fusing vesicles. Thus, the kinetics of heterofusion process offers a unique opportunity to test the assumptions underlying theories of fusion. Our observations, for example, are not in accord with the suggestion that intervesicle bridging by calcium is a precondition for fusion [36]. Similarly, removal of water of hydration at the interface [37–39] can not be the reate-limiting step because the rate of hetero- and homodiffusion are essentially identical. Nachliel and Gutman [40] have also found that the water at the interface does not nociteably influence the rate-limiting step for interfacial transfer of protons. The activation

energies for desolvation of solutes at the bilayer interface is rather small, typically less than 3 kcal/mol [41]. On the other hand, the activation energies for homo- and heterofusion, about 25 kcal/mol (unpublished observations), suggest that the rate-limiting step involves the hydrophobic region of the bilayer. We believe that defects or regions of mismatch [42,43] in which hydrophobic regions are exposed to the aqueous phase act as sites for fusion as well as for incorporation of proteins [44]. Exposure of such hydrophobic regions could also destabilize the hydration layer, and thus intervesicle calcium bridging will not be necessary for dehydration.

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