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Binding of phospholipase A_2 to zwitterionic bilayers is promoted by lateral segregation of anionic amphiphiles

Mahendra Kumar Jain, Bao-Zhu Yu * and Arek Kozubek **

Department of Chemistry, University of Delaware, Newark (U.S.A.)

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Catalytic action of phospholipase A_2 is appreciably influenced by the organization and dynamics of bilayers of glycerophosphocholines (Apitz-Castro et al. (1988) Biochim. Biophys. Acta 688, 341–348). However, such effects of the quality of the interface are not observed with bilayers of glycerophosphoryl methanol and other anionic phospholipids (Jain et al. (1986) Biochim. Biophys. Acta 860, 435–447). Such differences between the catalytic susceptibility of zwitterionic versus anionic bilayers are due to a large difference in the affinity of the enzyme for these interfaces. Binding to phospholipase A_2 to zwitterionic interfaces can be promoted in the presence of certain anionic additives. For example in the pre-steady-state phase of hydrolysis, segregation of the nacently produced products of hydrolysis could promote binding of phospholipase A_2 to regions of higher anionic charge density in the zwitterionic interface. In this paper we show that the dynamics of segregation of the nacently produced products of hydrolysis in zwitterionic bilayers can be readily followed by monitoring the fluorescence intensity of the cationic dye NK-529 (Yu and Jain (1989) Biochim. Biophys. Acta 980, 15–22). The fluorescence emission characteristics of NK-529 change appreciably due to self-quenching of the bound dye molecules as the fatty acid molecules segregate in the bilayer. The kinetics of segregation of fatty acids during the course of hydrolysis of bilayers of zwitterionic phospholipids by phospholipase A_2 exhibits an unequivocal correlation with a variety of phenomena that are observed during the transition from the pre-steady-state phase to the steady-state phase of hydrolysis in the reaction progress curves as a function of temperature and in the presence of lipophilic additives.

Introduction

Binding of phospholipase A_2 to phospholipid interfaces is a key step during the interfacial catalysis: the bound enzyme is considerably more active than the free enzyme in the aqueous phase acting on solitary monomeric substrates [1,2]. An understanding of the biophysical factors governing such an interfacial activation is beginning to emerge. For example, the dissociation constant for pig pancreatic phospholipase A_2 (PLA) bound to vesicles of anionic phospholipids is less than 0.1 pM [3], and the rate of intervesicle exchange of the bound enzyme is less than 0.00002 s^{-1} [4]. On the other hand, the dissociation constant for phospholipase A_2 bound to vesicles of zwitterionic phospholipids is more than 10 mM, and the apparent dissociation constant decreases by a factor of 100 to 1000 in the presence of the products of hydrolysis, i.e. lysophospholipids and fatty acid [5,6]. Such observations demonstrate that ionic interactions play a critical role in the binding of pig pancreatic phospholipase A_2 to the substrate interface [7]. Additional factors also modulate the affinity of PLA for interfaces. For example, the affinity of phospholipase A_2 for interfaces depends not only on the mole fraction of the anionic additives like the products of hydrolysis in vesicles of zwitterionic phospholipids, but also on the presence of other additives that influence the thermotropic phase transition properties of zwitterionic bilayers [8–10].

* Permanent address: Department of Physics, Peking University, Beijing, China.
** Permanent address: Department of Botany, Agricultural University, Wroclaw, Poland.

Abbreviations: LPC, 1-myristoyl-*sn*-glycero-3-phosphoryl choline; DMMe, 1,2-dimyristoyl-*sn*-glycero-3-phosphoryl methanol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphoryl choline; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphoryl choline; NK-529, 1,3,3,1′,3′,3′-hexamethylindocarbocyanine; Trp-3, tryptophan residue in the 3-position of PLA; PLA, phospholipase A_2 from pig pancreas.

Correspondence: M.K. Jain, Department of Chemistry, University of Delaware, Newark, DE 19716, U.S.A.

To interpret the product-induced binding of pig pancreatic phospholipase A_2 to zwitterionic interfaces we invoked a role of organizational defects as the putative sites for binding of PLA [5,6,9,11,12]. Some of the consequences of this hypothesis can now be elaborated further. Structurally different solutes in a bilayer induce isothermal phase change and lateral phase separation [8,13]. Therefore, a solute- or temperature-induced mismatch in the organization of the acyl chains in the bilayer could provide putative hydrophobic sites for the binding of phospholipase A_2. This interpretation can be ruled out by the observation that vesicles of single or a mixture of zwitterionic phospholipids, which do not ideally mix in a bilayer, do not bind PLA at, below, or above the phase transition temperature [6]. Such observations discount any direct role of the defects in the organization of the acyl chains introduced during the gel-to-fluid phase transition of zwitterionic bilayers as the preferential sites for the binding of phospholipase A_2, and they also rule out a similar role for the phase boundaries created by laterally phase-separated gel and fluid phases. Also consistent with this conclusion is the observation that the high-affinity binding of phospholipase A_2 to bilayers of anionic phospholipids is not noticeably influenced by their thermotropic or solute-induced gel-to-fluid phase transition [4] or the critical micelle concentration [7].

A somewhat different interpretation of the nature of the organizational defects that promote binding of phospholipase A_2 to bilayers, containing the products of hydrolysis, is that the lateral phase separation of fatty acids above a critical mole fraction creates a locally higher density of negative charges in the interface, and such regions are the sites for high-affinity binding of the enzyme for interfacial catalysis. Thus, anomalous kinetic effects in zwitterionic bilayers arise because binding of phospholipase A_2 to an interface requires a critical density of anionic charges, and the bound enzyme does not recruit uniformly distributed anionic charges required for the high-affinity binding. In this paper we provide direct experimental evidence for segregation of fatty acids in zwitterionic bilayers under a variety of conditions that also modulate the pre-steady-state phase of the kinetics of interfacial catalysis.

Materials and Methods

Specific details and experimental conditions used for the fluorescence measurements are described in the preceding paper [14]. Kinetic measurements were carried out by pH-stat titration at pH 8.0 in aqueous solution containing 6 mM $CaCl_2$ [5,6,11,12]. All reagents were the best grade available. Phospholipase A_2 from pig pancreas was a gift from Prof. G.H. DeHaas (Utrecht). Vesicles were prepared by suspending phospholipids in appropriate buffers followed by sonication in a bath type sonicator (Sonicor) for 2 to 5 minutes. Ternary codispersions used for the kinetic studies contain dimyristoyl-*sn*-glycero-3-phosphoryl choline (DMPC) with appropriate mole fraction of fatty acid and 1-myristoyl-*sn*-glycero-3-phosphoryl choline (LPC). The ternary codispersions prepared for the binding studies contain ditetradecyl-*sn*-glycero-3-phosphoryl choline (DTPC) with appropriate mole fraction of the products. Other experimental conditions are described in the figure legends, or in the text, or in the preceding paper.

Results

Reaction progress curves for the hydrolysis of sonicated unilamellar vesicles (SUV) of DMPC by pig pancreatic phospholipase A_2 are complex and exhibit several anomalous features [5,6,9–12]. For example, as shown in Fig. 1 (curve a) the steady-state hydrolysis of annealed vesicles precedes a latency period of low activity. At the end of the steady-state phase of activity all the available substrate (about 65% of the total) in the outer monolayer of all the vesicles in the reaction mixture is hydrolyzed. Elsewhere, it has been shown that the latency period disappears [5], and the equilibrium binding constant for PLA to DMPC vesicles increases over 100-fold [6], in the presence of a critical mole fraction of the products of hydrolysis. A change in the apparent affinity of the enzyme for the zwitterionic interface is not seen when the free fatty acid in the ternary codispersions is replaced by alkanols of comparable chain length. Similarly, codispersions of homologous diacylglycerophosphoryl cholines differing by more than four methylene residues, do not bind phospholipase A_2. Since these codispersions are phase-separated according to the usual biophysical criteria [13], such observations rule out a direct role of the phase-separation and gel-to-fluid thermotropic phase transition of the acyl chains in the binding of phospholipase A_2 to vesicles of zwitterionic phospholipids. These observations also suggest that the binding of phospholipase A_2 to the substrate interface is promoted by anionic charges in the interface arising from the formation and distribution of a critical mole fraction of the products of hydrolysis in the substrate vesicles.

Changes in the anionic charge distribution on the surface of DMPC vesicles that occur during the course of hydrolysis can be monitored spectrofluorimetrically. For example, as shown in Fig. 1 (curve b) the fluorescence emission intensity of the cationic dye NK-529 in the reaction mixture is altered appreciably when DMPC vesicles are hydrolyzed by phospholipase A_2, and the time-course of the fluorescence change closely follows the formation of products during hydrolysis. A comparison of the curves b and a (or of b′ and a′) shows

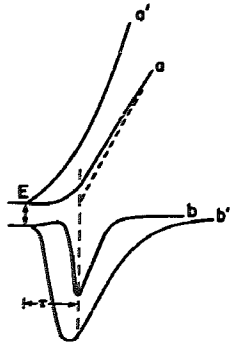

Fig. 1. Reaction progress curves for the proton release (curves a and a') and for the fluorescence change of NK-529 (5.2 μM) at 685 nm (curves b and b') during the hydrolysis of DMPC (0.59 mM) vesicles by pig pancreatic phospholipase A_2 (2 μg or 140 pmol) in 5 mM $CaCl_2$ at pH 8.0 and 21°C. The profiles a' and b' were obtained with unannealed vesicles which had been allowed to stand at 37°C for 30 min after sonication. Profiles a and b were obtained with annealed vesicles which had been allowed to anneal at 21°C for more than four hours. The reaction volume for titration of the proton release was 4 ml, and that for the fluorescence change was 2 ml and it also contained 10 mM Tris chloride. Excitation wavelength was 620 nm with excitation and emission slits at 4 nm.

that during the latency period there is only a slight increase in the fluorescence intensity which is accompanied by a slow rate of hydrolysis. At the end of the latency period, with the onset of the steady-state phase of hydrolysis, the fluorescence intensity decreases sharply, and then increases and reaches a maximum as the steady-state phase of hydrolysis progresses. The overall decrease in the fluorescence intensity at the onset of the steady-state phase of hydrolysis is between 10 and 25%. The latency period remains essentially unchanged with the dye concentrations below 8 μM, beyond which minor complications arise due to self-quenching of the dye in the aqueous phase. The latency periods for the pH-stat curves are defined as the intercepts on the time axis [5]. Similarly the latency period in the time profile for the fluorescence change is defined as the time to the minimum in the fluorescence change. Under a variety of conditions we found that these two latency period match to within 10% of their values. A small difference in the latency periods reported later in this paper arises mainly because some of the pH-stat titration experiments were done several years ago without NK-529 in the medium, or the exact experimental conditions are slightly different because no buffer is used in the pH-stat titrations. As we noted earlier [5,11] the latency periods are also very sensitive to the thermal history and the presence of undetectable trace impurities in vesicles. However, under the conditions where parallel experiments were done the correspondence between the two sets of latency periods is within the experimental error with no bias in favor of any one system.

The magnitude of the decrease in the fluorescence intensity at the end of the latency period as well as the shape of the profile for the fluorescence change depends upon several variables including the concentration of the dye, the enzyme to vesicle ratio, temperature, presence of other lipophilic additives, and the annealing conditions during the preparation of the vesicles. The same holds true for the pH-stat profiles. The observations reported here are quite specific for NK-529, and several other dyes (e.g. ANS, porphyrin) do not report comparable changes. With additional controls it was also shown that the changes in the fluorescence are not due to a direct effect of the dye on the enzyme, and that the aggregation of the dye in the aqueous phase (if any) does not contribute to the fluorescence changes that are characterized in this paper. For example, not only the ratio of the dye to the enzyme is well over 700 in the kinetic experiments, but the time-dependent fluorescence changes and the hydrolysis ceases immediately after the addition of EGTA (data not shown) which removes calcium ions that are required for the catalytic action of phospholipase A_2. Similarly, when hydrolysis is initiated by phospholipase A_2 from venom of *Naja melanoleuca*, the latency period disappears completely both in the profiles for proton release and those for the fluorescence change. Such observations also emphasize that the major changes in the fluorescence profiles relate to the change in the organization of the bilayer that occur up to the onset and during the beginning of the steady-state phase of hydrolysis of the vesicles, and that these changes are not necessarily a linear function of the extent of hydrolysis.

Our working hypothesis for the interpretation of the changes in the fluorescence intensity during the course of hydrolysis of DMPC vesicles is that the distribution of cationic dye NK-529 bound to vesicles follows the changes in the mole fraction and lateral distribution of the nacently produced fatty acids and lysophosphatidylcholine. Initially, formation of a small mole fraction of the products does not significantly alter the lateral distribution of the dye, presumably because the fatty acid molecules are randomly distributed and therefore there is a slight increase in the fluorescence intensity. When a critical mole fraction of the products is formed, the fatty acid molecules segregate. As a result of this segregation, the fluorescence intensity decreases due to self-quenching as the dye molecules bound to segregated fatty acids are closer to each other. With the increasing mole fraction of the products formed during the course of hydrolysis the average separation between the bound dye molecules increases, and this leads to an increase in the fluorescence intensity due to the loss of self-quenching. As shown in the preceding paper such a time-course of the fluorescence change is predicted from

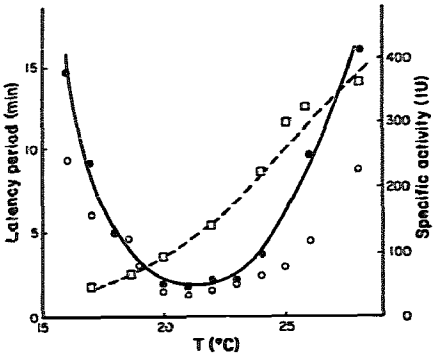

Fig. 2. Dependence of the latency period for the hydrolysis of DMPC (0.59 mM) vesicles (open circles) and for the simultaneous change in the fluorescence intensity of NK-529 (closed circles) as a function of temperature. The data for the specific activity in the steady-state phase of hydrolysis of annealed (squares) and unannealed (upper dotted line; data points not shown) DMPC vesicles is also shown. Other conditions as in Fig. 1.

the equilibrium measurements with ternary codispersions containing a varying mole fraction of the products (cf. Fig. 3 in Ref. 14).

The reaction progress curves for the hydrolysis of zwitterionic vesicles are influenced by a variety of factors [5,10,12], therefore we examined the effects of such factors on the time course in the pH-stat profile and that for the fluorescence changes of NK-529 bound to vesicles. As shown below the latency period in the fluorescence intensity profile (cf. curve b in Fig. 1) correlates well with the latency period in the reaction progress curve for the hydrolysis.

The thermotropic transition temperature of phospholipid vesicles is known to modulate the shape of the reaction progress curve for their hydrolysis by phospholipase A_2 [5,9,11]. As shown in Fig. 2, the latency period exhibits a minimum at the phase transition temperature, whereas the steady-state rate of hydrolysis increases monotonically with temperature. Also as shown in this figure a similar effect is seen for the latency period for the fluorescence change of NK-529: both below and above the phase transition temperature the latency period from the pH-stat profile and that for the fluorescence change are essentially identical. Under these conditions the latency period is not seen with PLA from venom of *Naja melanoluca* (data not shown). In conjunction with the results shown in Fig. 1 these results support the conclusion that the same changes in the bilayer organization regulate the time course for both of these processes. In the discussion section we have further elaborated on the genesis of the organizational changes that are responsible for these changes in the latency period as a function of temperature.

The effect of the thermal history of vesicles

The organizational factors that regulate the latency period for the hydrolysis or the fluorescence change are also altered by the thermal history of the vesicles after their formation by sonication. Elsewhere [5,11] we have shown that annealing of vesicles significantly alters the shape of the reaction progress curve for the hydrolysis of DMPC vesicles: the latency period increases, the initial burst decreases, and the steady-state rate of hydrolysis is somewhat slower in annealed vesicles. A comparison of apparent K_m and V_m for the steady-state phase of hydrolysis of annealed (0.63 mM and 122 IU) and unannealed (0.4 mM and 300 IU) vesicles suggests that the residence time as well as the apparent affinity of phospholipase A_2 for the unannealed vesicles is somewhat higher than it is for annealed vesicles. We have further investigated the possible origin of the organizational changes that occur during annealing. As shown in Fig. 1 (compare a and b with a′ and b′), similar changes are manifested in the time-course of the fluorescence and pH-stat profile; for the unannealed vesicles the latency period is considerably shorter and the change in the fluorescence intensity at the latency period is greater. Such observations suggest that self-quenching is more pronounced in unannealed vesicles, i.e. the bound NK-529 molecules are on the average closer to each other in unannealed vesicles than in annealed vesicles. This is also the case in DMPC vesicles in the absence of any products of hydrolysis, which implies that the time-averaged segregation of the bound dye molecules occurs in unannealed vesicles irrespective of the presence of the products. Very little is known about the organizational changes that occur during annealing. However, some characteristics of this phenomenon are beginning to emerge on the basis of the observations summarized below and interpreted on the basis of the experimental criteria developed above in terms of a difference in the latency periods.

Annealing of freshly sonicated vesicles requires slow cooling from about 50 °C. Vesicles annealed above the phase transition temperature (22 °C for SUV) do not behave like the vesicles annealed below the transition temperature. For example, for the data shown in Fig. 2, the vesicles are annealed at each temperature at which measurements were made. The time for annealing also depends upon the temperature of incubation. For example, the annealing time for a vesicle preparation at 15 °C (quenched from 55 °C) is well over 6 h. If however, the vesicle preparation is allowed to anneal at 21 °C for 30 min, further annealing at 15 °C can be accomplished in about 1 h. The possibility that annealing leads to fusion of small unilamellar vesicles (SUV) to form large unilamellar vesicles (LUV) is discounted by the observation that the vesicles annealed at 15 °C become unannelaed by heating to 55 °C for about 1 h; LUV can not 'de-fuse' to form SUV under these condi-

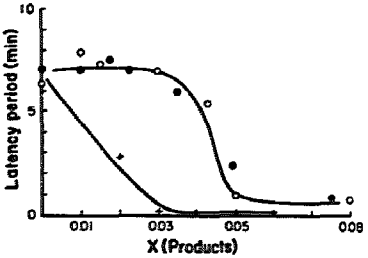

Fig. 3. Dependence of the latency period for the fluorescence change of NK-529 as a function of the mole fraction of DMMe(+) or (1:1) myristic acid + 1-myristoylglycerophosphocholine added to preformed vesicles of 0.59 mM DMPC (open circles). The latency periods from pH-stat profiles are shown as closed circles.

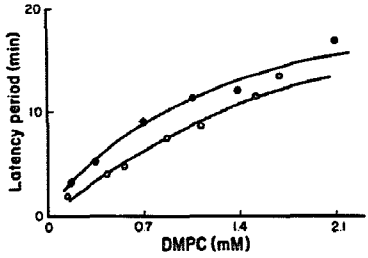

Fig. 4. Relationship between the latency period for proton release (filled circles) and for the fluorescence change (open circles) of NK-529 during the hydrolysis of PLA as a function of DMPC concentration. Other conditions as in Fig. 1.

tions. Similarly, we do not see any change in the light scattering properties of SUV during annealing. Also the time for annealing is considerably longer than the time required for gel-to-fluid phase transition. Based on such observations we suggest that the organizational characteristics that change on annealing are related to the inside-to-outside ratio of the phospholipid molecules in vesicles. We are trying to obtain a direct evidence to further support this suggestion.

The effect of the mole fraction of the products

Elsewhere [5] we have suggested that the changes leading to the steady-state phase of hydrolysis involve formation of a critical mole fraction of the products. In these experiments we showed that the products of hydrolysis codispersed with DMPC abolish the latency period at about 16 mol%, whereas the mole fraction calculated from the latency period is about 6–8 mol%. Now we have repeated these experiments under the conditions where the ternary codispersions are formed by adding LPC and myristic acid to preformed DMPC vesicles [14]. Under these conditions the products are present only in the outer monolayer of the substrate vesicles, and as shown in Fig. 3 the latency period is completely abolished when the mole fraction of the products approaches 6%. A similar effect is observed with 5 mol% 1,2-dimyristoyl-*sn*-glycero-3-phosphoryl methanol codispersed in DMPC vesicles. Similar changes can not be brought about by zwitterionic ionic phospholipids or by LPC + tetradecanol, however as shown elsewhere [5], about 10 mol% LPC added to preformed vesicles does cause a significant decrease in the latency period. These observations emphasize the importance of anionic additives as determinants for latency periods observed in the pH-stat and the fluorescence profiles.

The effect of the substrate concentration

The shapes of the pH-stat as well as the fluorescence change profile during the course of hydrolysis of DMPC vesicles change appreciably at different substrate concentrations. As shown in Fig. 4, the latency period for the onset of the steady-state rate of hydrolysis increases with increasing concentration of DMPC vesicles. Similarly, it is noted that at higher DMPC concentrations the minimum in the fluorescence profile is broader.

Our interpretation of both of these observations is based on the experiments described below which show that the rate of exchange of the products between the vesicles has the half-time of about 4 min. As shown in Fig. 5 (upper half) when dye bound to the product containing vesicles (S + P) is mixed with the substrate vesicles (S), there is an immediate increase in the fluorescence intensity because the free dye binds to the newly added interface. This is followed by a slow increase in the fluorescence intensity due to the release of self-quenching as the products achieve equilibrium distribution with the added substrate vesicles. This conclu-

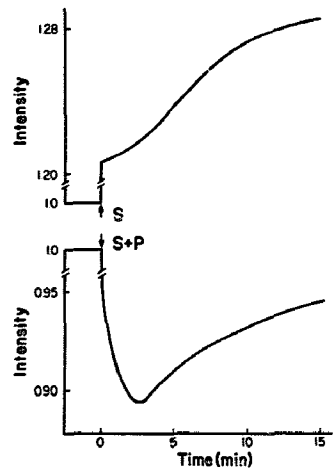

Fig. 5. The effect of the exchange of the products from ternary condispersions (0.52 mM) with 47% products added to preformed DMPC (0.59 mM) vesicles. DMPC vesicles were added to a mixture of NK-529 and ternary codispersions; (bottom) ternary codispersions were added to a mixture of NK-529 and DMPC vesicles.

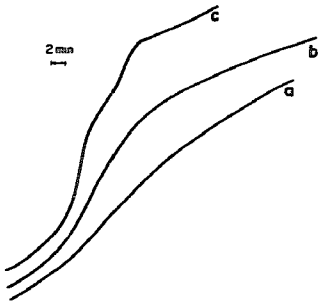

Fig. 6. Reaction progress curves for the hydrolysis of DMPC (1.4 mM) vesicles at 21°C with (from top) 0.168, 0.42, and 0.84 nmol phospholipase A_2. The pre-steady-state portion is shifted in order to emphasize the difference in the steady-state phase of the reaction.

sion is supported by the complementary experiment in which the tenrary (S + P) vesicles are added to a mixture of NK-529 and DMPC (S) vesicles. As shown in the bottom half of Fig. 5, under these conditions the fluorescence intensity decreases initially, and then increases slowly as the product exchanges from the ternary vesicles to DMPC vesicles. These experiments are done in the absence of any enzyme, and in both cases the half-time for the slow change in the fluorescence intensity, presumably due to exchange of the products, is about 4 min, or the first-order rate constant of about 0.25 min^{-1}.

A slow exchange of the products from hydrolyzed vesicles creates complex kinetic consequences when excess substrate vesicles are present. This is observed in the shape of the reaction progress curve as well as that of the fluorescence change during the steady-state phase of hydrolysis. At low concentrations of the substrate (where the enzyme to the vesicle ratio $\gg 1$) the dip in the fluorescence profile is sharp and the fluorescence intensity reaches a maximum and remains there. At $E/V \ll 1$, the increase in the fluorescence intensity after the minimum occurs in stages. Such a behavior would be expected if the vesicles to which the enzyme is initially bound are significantly hydrolyzed as would be expected because the residence time of the enzyme would increase on these vesicles.

Since the intervesicle exchange of the products occurs with the rate constant of 0.25 min^{-1}, it means not only that several minutes elapse before excess vesicles become susceptible to PLA, but also that the vesicles initially attacked by the enzyme become target for further catalysis by the enzyme. This is because the vesicles containing a higher mole fraction of the products and therefore a higher affinity for the enzyme [5,9]. Moreover, the slow exchange of the products makes excess vesicle less susceptible for hydrolysis. As shown in Fig. 6, this is seen as a secondary phase in the reaction progress curve from the hydrolysis seen only at higher enzyme to vesicle ratios. The role of excess vesicles in increasing the latency period is two-fold: initially they offer more sites for the action of PLA in the hopping mode, and in this phase the exchange of the products of hydrolysis would tend to lower their average mole fraction in the vesicles. This would not only increase the latency period, but the minimum in the fluorescence profile becomes shallower and broader.

The effect of lipophilic additives

Several lipophilic additives are known to modulate the reaction progress curve for the hydrolysis of DMPC vesicles by PLA. Such a modulation is a direct consequence of their action on the binding of phospholipase A_2 to the substrate interface [10,16]. A similar behavior is observed for the fluorescence intensity profile of NK-529 on bilayers in the presence of lipophilic additives. As shown in Fig. 7, *n*-octanol and oleic acid increase the latency period. As summarized in Table I, in the presence of 5 mol% lipophilic additives the latency periods increase appreciably. Although the general trend is the same, the latency periods obtained by pH-stat profile (experiments done in 1984 in the absence of

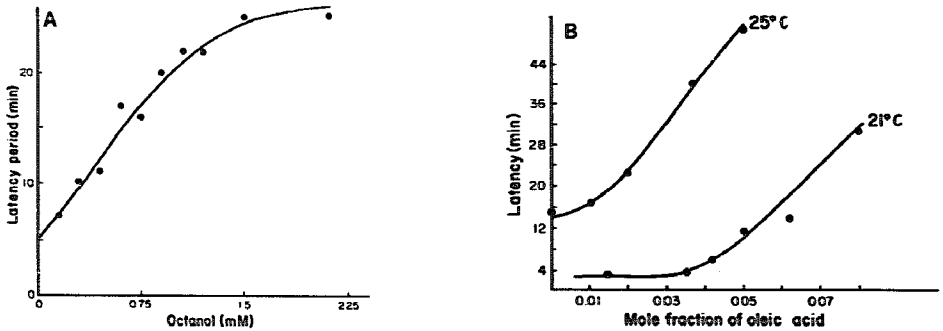

Fig. 7. (A) Dependence of the latency period for the fluorescence change of NK-529 as a function of the increasing concentration of *n*-octanol. (B) Dependence of the latency period for the proton release during hydrolysis of DMPC vesicles containing various mole fractions of oleic acid.

TABLE I

Latency periods for proton release (pH-stat) and for the fluorescence change of NK-529 during the hydrolysis of DMPC vesicles in the presence of 5 mol% additives at 21°C (pH 8.0).

Other conditions as given in Materials and Methods.

Additive	Latency period (min)	
	proton release	fluorescence change
None	3	3
1-Tetradecanol	1	1
trans-Tetradec-9-enol	5	6
cis-Tetradec-9-enol	22	9
Myristic acid	3	4
Palmitic acid	4	7
Stearic acid	4	3
Elaidic acid	5	14
Oleic acid	10	27
7,7-Dimethylicosadienoic acid	7	17

NK-529) are somewhat longer than those obtained from the fluorescence change. The ability of alkanols to increase the latency period is in the order tetradecanol < none < *trans*-tetradec-9-enol < *cis*-tetradec-9-enol. Since the partition coefficient of these solutes is large and comparable, the difference in their effects should be attributed to intrinsic differences in the ability of these solutes to influence lateral distribution of anionic charges due to segregation of the products of hydrolysis.

This conclusion is further substantiated by the fact that addition of unsaturated fatty acids also influence the latency periods (Table I), whereas saturated fatty acids have little or no effect. Since a solute can change the thermotropic transition properties of the bilayer, the effect of oleic acid on the latency period were also investigated as a function of temperature (Fig. 7B). At low mole fractions (below 0.01) the latency period increases significantly without a significant effect on the temperature range over which the transition occurs. It is also interesting to note that the steady state rate of hydrolysis shows an anomalous dependence on the presence of these additives (data not shown): under certain conditions activation is observed, whereas inhibition is observed under other conditions. Such a biphasic behavior would be expected if the interfacial binding equilibrium for the enzyme is shifted in the presence of these additives. These observations extend our earlier conclusion that lipophilic additives influence the interfacial binding equilibrium for PLA by modulating the ability of the products of hydrolysis to segregate in the substrate bilayer.

The effect of salts

Binding of pig pancreatic phospholipase A_2 to zwitterionic bilayers occurs only in the presence of anionic additive. The possibility of an ionic interaction of the

TABLE II

Latency periods and relative activities in the steady-phase of the reaction progress curve (pH-stat) in the presence of salts

The reaction vessel for both types of measurements contained 590 μM DMPC vesicles, 5.2 μM NK-529, 5 mM $CaCl_2$, and 0.8 μg phospholipase/2 ml at pH 8.0 and 21°C.

Salt	Latency period (min)		Relative activity (%)
	pH-stat	fluorescence	
None	1.6	2.5	100
0.1 M NaCNS	< 0.5	< 0.5	342
0.1 M NaCl	3.2	3.2	76
0.1 M Na_2SO_4	4.2	4.0	63

cationic microinterface of the enzyme with the anionic bilayer interface is supported by the effect of salts on the latency period and the steady state rate of hydrolysis. As summarized in Table II, the latency periods obtained from the pH-stat and the fluorescence change are virtually identical. Similarly, the steady-state rate of hydrolysis also decreases for the salts where an increase in the latency period is observed. This supports the conclusion that the ionic interactions are important for the events that controls the latency period of modulation the equilibrium binding of pig pancreatic phospholipase A_2 to substrate interface.

The effect of temperature on the equilibrium binding of phospholipase A_2

The observations sumarized so far demonstrate a remarkable correspondence between the binding of phospholipase A_2 or NK-529 to DMPC vesicles. Ionic

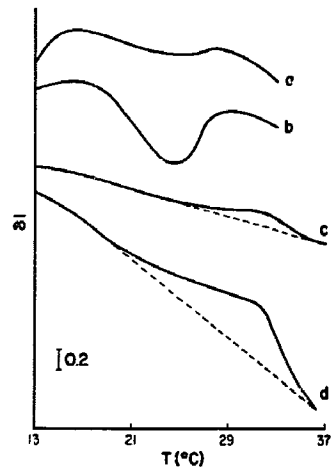

Fig. 8. The change in fluorescence intensity as a function of temperature. From top (a) DTPC vesicles + NK-529; (b) DTPC vesicles containing 6% products + NK 529; (c) DTPC vesicles with phospholipase A_2; (d) DTPC vesicles with 12% products and phospholipase A_2. Other conditions as given in the text.

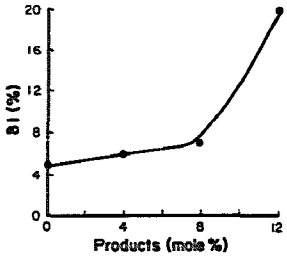

Fig. 9. The change in the relative fluorescence intensity of phospholipase A_2 at the phase transition of DTPC vesicles containing the indicated mole fractions of the products in the outer monolayer. Other conditions as given in the text.

interactions modulated by gel-to-fluid thermotropic or isothermal phase transition properties of the bilayer control the rapid equilibrium between the free and bound phospholipase A_2 or NK-529. This is probably, most directly demonstrated by the data shown in Fig. 8. In the top half of this figure is shown the change in the fluorescence intensity of NK-529 in DTPC vesicles alone or those containing mol% products in the outer monolayer. Only in the presence of the products a decrease in the fluorescence intensity is observed at the phase transition temperature. A similar experiment for the binding of phospholipase A_2 was carried out with vesicles of DTPC alone or with those prepared by cosonication of DTPC with 12% DMPC. As shown in Fig. 8 (bottom half), in the presence of excess phospholipase A_2 (after several hours) the fluorescence emission intensity due to Trp-3 from the enzyme shows an increase in the phase transition range. As discussed elsewhere [6,7] such an increase in the emission from Trp-3 is a direct measure of catalytically significant binding of phospholipase A_2 to bilayer. Similar, experiments were done at several mole fractions of the nacently formed products in DTPC vesicles. As summarized in Fig. 9 the transition temperature dependent increase in the Trp-3 fluorescence does indeed increase abruptly when the mole fraction of nacently produced products exceed 0.08 in the outer monolayer of DTPC vesicles.

Discussion

The results summarized in this paper help us elaborate on the nature of the binding of phospholipase A_2 to zwitterionic bilayers. Based on the kinetic and the direct equilibrium binding studies, elsewhere [5,6] we have shown that the affinity of pig pancreatic phospholipase A_2 for zwitterionic vesicles increases by three to four orders of magnitudes in the presence of the products of hydrolysis. A role of anionic charges in facilitating the binding of phospholipase A_2 [7], and a role of cationic charges in inhibiting the binding of phospholipase A_2 [10] to the interface, is also adequately demonstrated. In these studies it was apparent that the binding of phospholipase A_2 to zwitterionic bilayers is not observed until a critical mole fraction of the products is nacently formed or introduced during formation of the vesicles. Now we have further extended these observations to elaborate on the organizational factors that control catalytically meaningful binding of phospholipase A_2 during the course of hydrolysis of zwitterionic vesicles. Two of the most important factors are the presence of anionic amphiphiles in the interface, and their propensity to segregate in the bilayer.

The protocols developed in this and the preceding paper [14] provide a simple and versatile method to monitor segregation of anionic additives in zwitterionic bilayers. The results show that at the phase transition temperature the products of hydrolysis of DMPC by phospholipse A_2 are randomly distributed up to 0.03 mole fraction, beyond which segregation of fatty acids occurs. The data at hand does not demonstrate whether LPC is segregated or not, but its presence in the bilayer is necessary for the segregation of the fatty acid. Segregation of fatty acids creates a local increase in the anionic charge density. This promotes binding of phospholipase A_2, and therefore catalysis in the scooting mode [2,3,7]. In results we have shown that the segregation of fatty acids as monitored by the latency period for the proton release or the fluorescence change during the hydrolysis is appreciably altered under a variety of conditions: temperature, presence of additives, mole fractions, and relative amounts of the various components. The correlation between these two sets of observations during the equilibrium [14], as well as the steady-state conditions, demonstrates that a confluence of factors that influence the organization and dynamics of anionic charges in the interface influence the interfacial catalysis by modulating the equilibrium between the bound and free enzyme.

The underlying model that accounts for the kinetics of interfacial catalysis under a variety of experimental boundary conditions has been quantitatively elaborated elsewhere [2]. According to this model all the available data for the hydrolysis of zwitterionic vesicles can be described by the assumption that the binding of the enzyme to zwitterionic substrate vesicles is much weaker than the binding in the presence of the products of hydrolysis. For example, as shown in Fig. 10, it predicts an exponential relaxation after a time delay during which a sufficient amount of products will be formed to allow binding of the enzyme. The model can be elaborated further to predict other features of the experimental data.

Two other attempts have been reported in the literature to account for the shape of the reaction progress curve for the hydrolysis of zwitterionic vesicles. Both of these fall short of their goal, because the experimental boundary conditions do not adequately match the as-

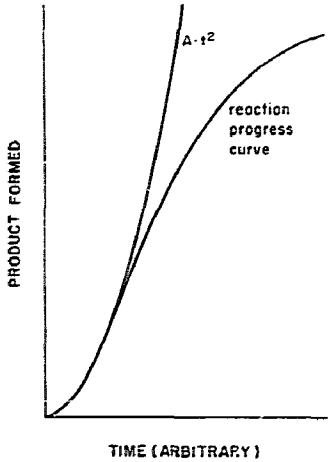

Fig. 10. Theoretical reaction progress curves for the hydrolysis of vesicles where formation of products promotes binding of PLA and the enzyme is readily exchangeable. The upper curve shows the relationship $A \cdot t^2$ (derived by Romero et al. [20]) based on completely different assumptions.

sumptions intrinsic in their theoretical treatments. For example, Tinker et al. [17–19] postulated that the latency period arises because the enzyme has a high affinity for the vesicles of pure substrate, and in this state the rate of catalytic turnover is assumed to be slow. According to this model, the catalytic turnover increases in the presence of the products of hydrolysis because the products promote desorption of the bound enzyme. Similarly, in developing their percolation model Biltonen et al. [20,21] have made an ad hoc assumption that the dimerization of phospholipase A_2 in the interface is a kinetically slow step in the pre-steady-state phase that ultimately leads to the burst of catalytic activity in the steady-state phase. These assumptions are inconsistent with the observation that the affinity of porcine phospholipase A_2 for zwitterionic vesicles is very low [6,7], and the kinetics of binding of the enzyme to the interface is rapid [4] when the binding equilibrium is favorable. As shown in Fig. 10, the shape of the reaction progress curve including the pre-steady-state phase can be adequately reproduced on the basis of the product-induced increase in the binding of PLA without any ad hoc assumption about the dimerization of phospholipase A_2 induced by fluctuations in the lateral organization of the bilayer as postulated by Romero et al. (1987) [20]. Similarly, one of the key assumptions of the percolation model, that only a critical mole fraction of additives leads to the loss of the latency period, can be questioned on the basis of some quantitative considerations. According to the percolation model the bound enzyme is able to dimerize more rapidly when it is able to move laterally over fluctuation-induced contiguous regions of a phase-separated bilayer. This is an obligatory step for dimerization of the enzyme in the interface. Contrary to the experimental observations reported in this and earlier papers [5,6,9,10], the percolation model does not require the presence of anionic charges on the additives that are phase-separated. Indeed, according to this model, phospholipase A_2 should readily bind bilayers in the gel-fluid coexistence region. As shown in Fig. 9 pig pancreatic phospholipase A_2 does not bind to bilayers of DTPC in the gel, fluid, or the coexistence region unless a critical mole fraction of the products of hydrolysis or other anionic phospholipids are also present. Even if it is assumed that the fluctuation-induced segregation of anionic amphiphiles is necessary for binding of the enzyme, the theoretically predicted mole fractions of additives that would lead to optimal percolation are in the range of 15 to 25 mol%. However, as shown in Fig. 3 the latency periods become zero in the presence of less than 4 mol% DMMe, that is a mole fraction at which contiguous regions cannot be reasonably established.

To recapitulate, the experimental boundary conditions elaborated in this and earlier papers [3–6,9–12] provide a reasonable basis for the interpretation of the complex kinetics of interfacial catalysis by phospholipase A_2. According to the general scheme for interfacial catalysis as developed in detail elsewhere [2], the origin of the latency period is in the shift of the equilibrium binding of the enzyme to the substrate interface, rather than in the intrinsic kinetics of binding of the enzyme to the interface. The equilibrium towards the bound form of the enzyme is modulated by the mole fraction and lateral distribution of the products of hydrolysis. The detailed molecular basis for the interplay of the phase properties and distribution of anionic charges is yet to be established, and we believe that the information provided by the spectral properties of NK-529 is an important first step in that direction.

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