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Action of phospholipase A_2 on bilayers. Effect of fatty acid and lysophospholipid additives on the kinetic parameters

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Action of pig pancreatic phospholipase A_2 on the ternary codispersions of diacylphosphatidylcholine, 1-acyllysophosphatidylcholine and fatty acids is examined. The binding and kinetic constants are found to be the same under a variety of conditions. These parameters and the catalytic turnover number change with the phase-transition temperature of the ternary codispersions, and optimal binding, kinetic and catalytic constants are seen in the phase-transition range where an equilibrium exists between laterally separated phases. The effect of changing the structure of any of the three components is also via a change in the phase-transition temperature of their ternary codispersions. These observations suggest that the binding of pig pancreatic phospholipase A_2 to the defect sites on the substrate interface determines the substrate concentration dependence of the initial rate of hydrolysis, and the catalytic turnover by the bound enzyme also depends upon the phase state of the bilayer. An additive-induced stabilization of the defects in the substrate bilayer is postulated to account for the enhanced binding of the enzyme to the bilayer.

Introduction

Action of phospholipase A_2 on phospholipid bilayers is regulated by their ability to bind to the substrate interface [1]. Binding of pig pancreatic phospholipase A_2 to bilayers is postulated to occur at the organizational defects in the bilayer interface [2,3]. Such defects in bilayers of diacylphospholipids are, for example, induced by additives such as *n*-alkanols [4], freshly added lysophospholipids [5] and lysophospholipid + fatty acid [6,7]. In order to characterize the role of the additives in activating the action of pig pancreatic phospholipase A_2 on the ternary codispersions, we have studied the binding and the kinetics of hydrolysis of the codispersions containing various fatty acids and lysophospholipids.

Materials and Methods

Phospholipase A_2 from pig pancreas was a gift from Professor DeHaas. Diacylphospholipids (Avanti, Calbiochem), monoacylphospholipids (Avanti, Sigma, Medmark), dialkylphospholipids (Medmark) and fatty acids (Sigma) were purchased. Purity of these lipids was checked by thin-layer chromatography and by differential scanning calorimetry of their aqueous dispersions. Occasionally, these preparations showed presence of impurities, and they were purified on silica gel column.

Vesicles were prepared by dispersing a dry film of phospholipids in 0.1 M KCl in a bath-type sonicator (Sonicor SC-50T). Other experimental details are described elsewhere [2,4,5–7]. Typi-

cally, the initial rates of hydrolysis were measured from the pH-stat titration curve obtained in 5 ml of 0.1 M KCl/10 mM $CaCl_2$ (pH 8.0) by initiating the reaction with 0.5 μg phospholipase A_2 [6]. Titration efficiency of the released fatty acid was determined under all the conditions examined in this paper, and was found to be the same. The initial rates vs. substrate concentration data was fitted to the various types of plots by linear or nonlinear regression analysis on a microcomputer. The substrate concentrations and K_m values refer to the concentration of diacylphospholipid in the ternary codispersions. The V_m values are given as μmol/min per mg protein (I.U.).

Binding of phospholipase to dialkylphospholipid dispersions was studied by monitoring the increase in the fluorescence intensity of the enzyme at 333 nm. As described elsewhere [6], the fluorescence titration curve was fitted by nonlinear regression analysis to obtain the apparent dissociation constant (nK_d) for the enzyme + lipid complex. An estimated uncertainty of $\pm 30\%$ is expected in the measurement of K_m and nK_d values because different preparations of the ternary codispersions aggregate over 1–4 h time period, as determined by the turbidity measurements. This alters the effective substrate interface available for the binding. This problem is minimized if the vesicles are stored at a temperature 20°C above the end of the phase-transition range, where phase separation is not observed.

Results

Effect of changing the mole fraction of lysophosphatidylcholine + fatty acid on the kinetic parameters

Elsewhere we have shown that the initial rate of hydrolysis of dimyristoylphosphatidylcholine shows a biphasic dependence upon the mole fraction (X_p) of lysophospholipid + fatty acid (added in 1 : 1 mole ratio) in the ternary codispersions [6]. The initial rate of hydrolysis reaches its maximum when the mole fraction (X_p) of the additives is approx. 0.2. The latency period also decreases and becomes zero above $X_p \simeq 0.1$. The initial rate of hydrolysis above $X_p \simeq 0.15$ is essentially the same as the steady-state rate of hydrolysis observed at the end of the latency period seen at lower X_p. The initial rates exhibit a linear dependence on the enzyme concentration. A maximum of about 65% of the substrate present in the sonicated vesicles is hydrolyzed, suggesting that only the substrate present in the outer monolayer of the vesicles is hydrolyzed under these conditions [8].

The initial rate of hydrolysis of the ternary codispersions ($X_p > 0.1$) is zero order, and it exhibits a hyperbolic dependence upon the substrate concentration containing a constant mole fraction of the additives. The K_m and V_m values obtained from double-reciprocal, Cornish-Bowden, Eadie-Scatchard, Dixon, and Hanes-Woolf plots of the initial rate vs. the substrate concentration data are given in Table I. These plots suggest that a Michaelis-Menten type of saturation kinetics is obeyed, and it is consistent with the suggestion [6] that the substrate concentration dependence is due to the equilibrium binding step of the enzyme (E) to the substrate interface (I):

$$E + I \rightleftharpoons E^* + S \rightleftharpoons E^*S \rightarrow E^* + P$$

The kinetics of hydrolysis depends upon the concentration of the bound enzyme (E*) which binds the substrate in the interface and the resulting Michaelis-Menten complex leads to the catalytic turnover to form the products.

Pig pancreatic phospholipase A_2 has very low but measurable (< 5%) affinity for the vesicles of pure ditetradecylphosphatidylcholine [6]. The binding increases with the mole fraction of the products in the ternary codispersions. Thus, the

TABLE I

KINETIC PARAMETERS FOR THE ACTION OF PIG PANCREATIC PHOSPHOLIPASE A_2 ON THE TERNARY CODISPERSIONS CONTAINING DIMYRISTOYLPHOSPHATIDYLCHOLINE + 1-PALMITOYLLYSOPHOSPHATIDYLCHOLINE + PALMITIC ACID (100:22:22, MOLE RATIOS)

Type of plot	K_m (μM)	V_m (μmol·min^{-1}·mg^{-1})
Double-reciprocal	225	550
Cornish-Bowden	210	500
Eadie-Scatchard	214	470
Dixon	242	530
Hanes-Woolf	211	500

number of putative binding sites in the ternary codispersions containing a given mole fraction of the product would be proportional to the total substrate concentration. The apparent dissociation constant (nK_d) and the Michaelis-Menten kinetic constants (K_m) for these codispersions are expected to be similar as already demonstrated earlier for other substrates [6] at a single mole fraction of the additive. The variation of the kinetic constants K_m and V_m of the pig pancreatic phospholipase A_2 for dimyristoylphosphatidylcholine codispersions containing varying mole fractions of the additives (1-palmitoyllysophosphatidylcholine + palmitic acid in 1 : 1 mole ratio) is shown in Fig. 1. The K_m value is about $220 \pm 20\%$ μM for $X_p < 0.4$. The corresponding data could not be obtained at low ($X_p < 0.1$) mole fractions of the additive, because the latency period is not zero and therefore the initial rates do not follow zero-order kinetics [7].

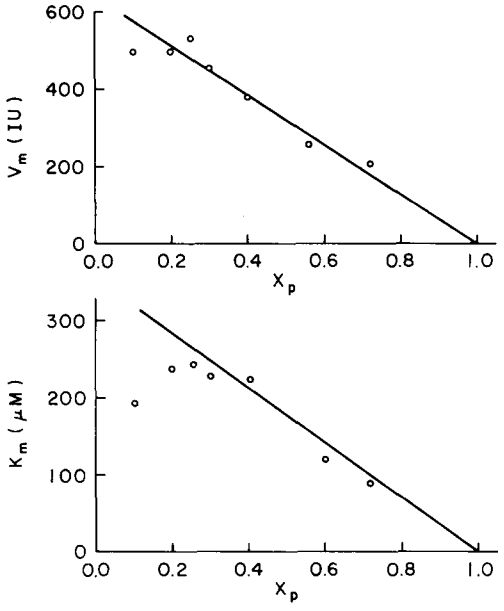

Fig. 1. V_m (top) and K_m (bottom) for the hydrolysis of dimyristoylphosphatidylcholine vesicles containing varying mole fractions (X_p) of 1-palmitoyllysophosphatidylcholine + palmitic acid (1 : 1 mole ratio). The initial rate of hydrolysis was measured by pH-stat titration curve initiated by 0.5 μg pig pancreatic phospholipase A_2 added to 5 ml of 100 mM KCl/10 mM $CaCl_2$ (pH 8.0) at 30°C). Appropriate concentrations of the ternary codispersions were added and equilibrated before addition of the enzyme. The K_m values refer to the concentration of dimyristoylphosphatidylcholine. The V_m values are expressed as μmol/min per mg (I.U.).

It is interesting to note that both K_m and V_m decrease linearly at higher X_p, that is, both the binding and the catalytic steps are influenced by the higher mole fractions of the additive. This is consistent with both the 'hopping' and the 'scooting' mechanisms [2–4]. However, for the reasons described elsewhere [7], we have interpreted our data in terms of the 'scooting' mechanism, that is, after initial binding to the substrate interface, the enzyme remains in the interface for several catalytic turnover cycles [1,6,7]. The K_m values decrease because these are expressed in terms of the dimyristoylphosphatidylcholine concentration. If expressed in terms of the total interface concentration, the calculated K_m value at $X_p = 0.6$ would be, for example, 270 μM instead of 110 μM. Thus, binding depends only on the surface area of the modified substrate interface. The V_m values decrease linearly, with X_p. This would be expected if during the catalytic cycle the bound enzyme (E*) had nonproductive random encounters with the additives. The proportion of the productive encounters of the enzyme with the substrate will be proportional to the mole fraction of the substrate in the interface. Thus an extrapolated value of about 700 I.U. for V_m of this enzyme is in close agreement with the values of specific activity observed with comparable long-chain substrates in detergent micelles [9].

Effect of varying lysophospholipid or fatty acid in the ternary codispersions

The activating effect of the additives in the ternary codispersions could be due to a specific interaction of the additives with the enzyme. In order to discern such effects, we measured K_m and V_m values for the codispersions of several fatty acids (Table II) and lysophospholipid analogs (Table III). For all these structurally diverse additives at $X_p = 0.18$, the K_m values are typically in the 150–260 μM range, and the V_m values are in the 300–500 I.U. range. A lack of any obvious dependence of K_m or V_m on the structure of the additives further reinforces the conclusion from the preceding section that the additives primarily stimulate the action of phospholipase A_2 by modulating the organization of the bilayer rather than by any high-affinity specific interaction of the monomer additives with the enzyme. The activat-

TABLE II

EFFECT OF FATTY ACIDS ON THE KINETICS OF HYDROLYSIS OF THE TERNARY CODISPERSIONS

Conditions: $X_p = 0.18$; Temp., 30°C. PC, phosphatidylcholine.

DimyristoylPC + 1-palmitoyllysoPC +	V_m (μmol·mg^{-1}·min^{-1})	K_m (μM)
Lauric acid	533	106
Palmitic acid	472	258
Stearic acid	608	120
Archadic acid	583	135
Ricinelaidic acid	400	140
Petroselenic acid	300	180
Olic acid	525	140
Vaccenic acid	330	240
Linolenic acid	290	180
Palmitoleic acid	420	180
Eladic acid	306	230
Linoleic acid	320	180
Phytic acid	498	150
Hexadecanol	383	115
Tetradecanol	690	374

ing effect is probably due to a solute-induced phase separation [5,6]. Since all these measurements with varying fatty acid or lysophospholipids (Tables II and III) were made at $X_p = 0.18$, it is quite likely that the mole fractions of these additives for the optimal phase separation are somewhat different [5]. This is reflected only slightly in the shape and position of the phase-transition profiles of the ternary codispersions of dimyristoylphosphatidylcholine with different additives (data not shown; see, however, Ref. 10). Indeed, such differences could account for the 2-fold range observed for the K_m and V_m values for the ternary codispersions of dimyristoylphosphatidylcholine containing structurally different additives (cf. Tables II and III). The apparent dissociation constants for the complex of pig pancreatic phospholipase A_2 with a few ternary codispersions are also given in Table III. The K_d values at 30°C are in the 200–300 μM range, which is the same as is seen for the K_m values (cf. Table III). Thus, the substrate concentration dependence of the kinetics of hydrolysis is essentially due to the binding equilibrium to the ternary codispersions.

TABLE III

EFFECT OF CHANGING THE LYSOPHOSPHOLIPIDS ON THE KINETIC (K_m AND V_m) AND BINDING (nK_d) PARAMETERS

Conditions: $X_p = 0.18$; temp., 30°C. PC, phosphatidylcholine, PG, phosphatidylglycerol.

Dimyristoyl PC + palmitic acid +	V_m (I.U.)	K_m (μM)	nK_d (μM)
1-Palmitoyl-2-methylPC	260	220	290
Octadecylphosphoethanolamine	405	200	
Octadecylphosphocholine	370	120	
Octadecylphosphoglycerol	470	211	
1-Palmitoyl-2-phosphocholineglycol	400	248	200
1-HexadecyllysoPC	410	140	
1-Palmitoyllyso-β-PC	376	200	
1-PalmitoyllysoPC	472	258	240
1-Palmitoyl-2-deoxyPC	380	190	300
1-StearoyllysoPC	480	211	
1-MyristoyllysoPC	460	240	230
1-LauroyllysoPC	562	240	
1-PalmitoyllysoPG	385	188	
1-ElaedoyllysoPC	412	150	

Effect of the phase properties on the kinetic parameters

One of the possible explanations of the 2-fold range in the kinetic parameters for the various ternary codispersions (cf. Tables II and III) is that these differences arise from a subtle difference in the phase properties. In the homologous codispersions, this possibility is tested by measuring the kinetic parameters as a function of temperature. As shown in Fig. 2, the K_m and V_m for the four mixtures of the substrate and products containing tetradecanoyl and hexadecanoyl chains, exhibit a complex temperature dependence. The endothermic phase transition of the four lipid codispersions is broad and complex but the transition occurs over a characteristic temperature range. Multiplicity of the phase-transition peak suggests that all these codispersions exhibit a phase separation over a fairly broad temperature range. As shown in this figure, V_m increases more than 4-fold in the phase-transition temperature range, suggesting that the catalytic turnover is strongly dependent upon the temperature. The V_m for the same substrate molecule in the various lipid mixtures can be up to 3-fold different, depending upon the phase state of the lipid codispersions. In all the cases we have examined, a maximum in the V_m values is seen when the two phases coexist. Similarly, the K_m

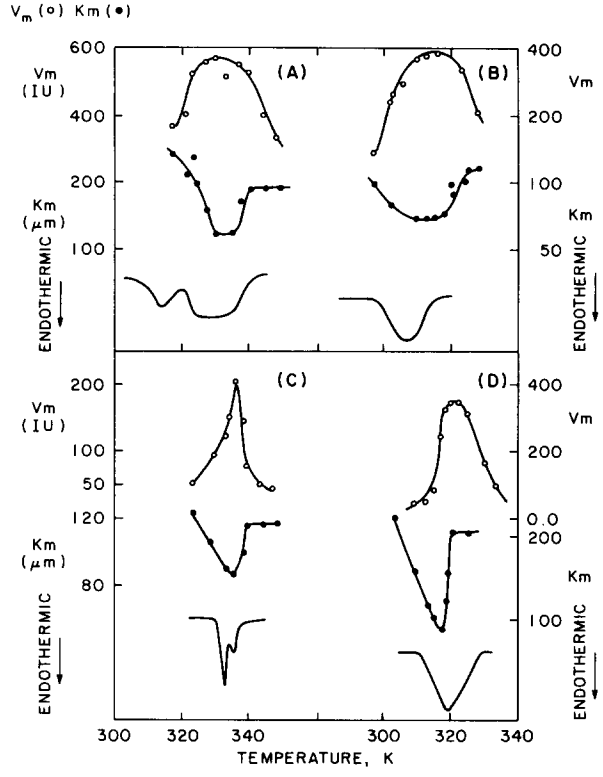

Fig. 2. V_m (top), K_m (middle) and the phase-transition profile (bottom) of the ternary codispersions containing dipalmitoylphosphatidylcholine (C and D) or dimyristoylphosphatidylcholine (A and B) with $X_p = 0.18$ mole fraction of 1-palmitoyllysophosphatidylcholine + palmitic acid (A and C), or 1-myristoyllysophosphatidylcholine + myristic acid (B and D). Other conditions as given in the legend to Fig. 1.

values decrease in the phase-transition range, as is expected if the enzyme binds preferentially to the defect sites present at the boundaries of the phase-separated domains. The apparent dissociation constants for the four mixtures prepared with the corresponding dialkylphosphatidylcholine analogs are given in Table IV. Once again, one observes a significant correlation between K_m and the apparent binding constants.

Effect on the binary codispersions of diacylphosphatidylcholines

All the possible binary combinations of didecanoyl-, ditetradecanoyl-, dihexadecanoyl- and dioctadecanoylphosphatidylcholines were examined for their phase-transition characteristics, their susceptibility to phospholipase A_2, and their (for the ether analogs) ability to bind phospholipase A_2. The results (not shown here) show that even under the conditions where a phase separation is observed by calorimetry, a long latency period is observed and little or no change in fluorescence intensity of phospholipase A_2 is induced by these binary codispersions. Thus, irrespective of their phase properties, the binary codispersions of diacylphosphatidylcholines do not bind pig pancreatic phospholipase A_2.

TABLE IV

APPARENT DISSOCIATION CONSTANTS (nK_d IN μM) FOR THE COMPLEX OF PIG PANCREATIC PHOSPHOLIPASE A_2 WITH THE TERNARY CODISPERSIONS

The chainlengths of dialkylphosphatidylcholine, 1-acyllysophosphatidylcholine and fatty acids are indicated at the top of the four columns. $X_p = 0.18$ in all the cases.

Temp. (°C)	14:14:14	14:16:16	16:14:14	16:16:16
15	216	195	210	1200
20	260	196	250	950
25	240	239	270	1200
30	200	210	270	900
40	360–420		300	190
50	400–600		262	1200

Discussion

The kinetic parameters, K_m and V_m, for the hydrolysis of the ternary codispersions by pig pancreatic phospholipase A_2 are found to depend upon the mole fraction of the additives, upon the temperature, upon the structure of the substrate, and only slightly upon the structure of lysophospholipid and the fatty acid. Within the resolution of our technique, these effects are related to the phase properties of the ternary codispersions. Optimal effects, that is lower K_m and higher V_m, are seen in or around the temperature range where the ordered and disordered phases are shown to coexist by differential scanning calorimetry. These observations confirm the observations of Op den Kamp et al. [11] that the optimal activity of pig pancreatic phospholipase A_2 is observed in the vicinity of the phase-transition temperature. However, binding of the enzyme to the dispersions of dialkylphosphatidylcholines

(alone or as binary codispersions) is poor even at their phase-transition temperatures. It implies that the phase separation in the product containing ternary codispersions is qualitatively different than the phase separation in the binary codispersions of diacylphosphatidylcholines [12,13]. One of the possible explanations is that the structurally different additives stabilize or increase the lifetime of the defects in a bilayer such that the residence time of the enzyme in the bilayer increases, and thereby increase V_m and decrease K_m. In the bilayers of diacylphosphatidylcholines, the residence time of the enzyme is small and therefore the action of the enzyme may be only by the 'hopping' type of mechanism.

The observations summarized in the preceding section essentially rule out any role of a specific interaction between the enzyme and the additives in the activation of the rate of hydrolysis. We have interpreted [6] these observations on the basis of a model that has been used to account for the kinetics of hydrolysis of diacylphospholipids in monolayers by pig pancreatic phospholipase A_2 [1]. It invokes binding of the enzyme to the substrate interface as the first step, followed by catalytic step mediated by the bound enzyme to hydrolyze the substrate molecules at the interface. In this model, it is postulated that the enzyme remains bound to the interface during several catalytic cycles. Thus, the substrate concentration dependence of the rate of hydrolysis arises exclusively from the initial binding step, because the substrate concentration that determines the formation of the Michaelis-Menten complex (ES) in the interface is constant and equal to the substrate concentration or the mole fraction in the interface. For obvious reasons, the substrate concentration dependence predicted by this model could not be tested in monolayers. However, as predicted by this model, we have found that the kinetic constant K_m and the apparent dissociation constant for the enzyme + interface complex are equal under a variety of conditions.

Both K_m and V_m exhibit their optimum values for hydrolysis in the phase-separated bilayers as in the ternary codispersions of the substrate. However, neither the binding nor the initial rates of hydrolysis without the latency period are seen in the dispersions of pure diacylphospholipids nor with the binary codispersions of homologous diacylphospholipids that exhibit phase separation by calorimetry [11,12]. Possible origin of such effects is being examined. One of the possibilities is that the lifetimes of the defects at the phase boundaries in the presence of the impurities are long enough so that phospholipase can remain bound over several catalytic cycles. The other possibility, although unlikely, is that the product-containing ternary codispersions have a unique type of substrate interface.

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