J. Membrane Biol. 55, 113–121 (1980)

The Journal of
Membrane Biology

Action of Phospholipase A_2 on Unmodified Phosphatidylcholine Bilayers: Organizational Defects are Preferred Sites of Action

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Summary. The hydrolytic action of the bee venom phospholipase A_2 on phosphatidylcholine bilayers is studied under a variety of conditions that introduce alterations in the packing, such as those induced by sonication, gel to liquid crystalline phase transition, and osmotic shock. Two phases of hydrolysis could be resolved under a wide range of experimental conditions. With the various forms of the bilayers one observes only a partial hydrolysis of the total available substrate during the first phase. However, the fraction of the substrate hydrolyzed in the first phase changes with the form of the available substrate, with the amount of the enzyme added, with the temperature, with the phase transition characteristics of the substrate, and by the sonication of the substrate. The second phase of hydrolysis is generally observed when a certain concentration of the products has been produced during the first phase of hydrolysis. These observations are interpreted to suggest that the bee venom phospholipase A_2 preferentially catalyzes hydrolysis of the substrate available at or near the defects in the organization of the substrate in the bilayers.

Introduction

The action of phospholipase A_2 (bee venom) on phospholipid bilayers is facilitated several hundredfold by a variety of additives [3, 12, 13]. We have shown [13] that the activating effect of alkanols is related to the free space introduced by alkanols in the bilayers. This implies that the action of phospholipases on the phospholipid bilayer in the absence of an additive may be related to the free space present in such bilayers. In this communication, we present observations that demonstrate that the action of bee venom phospholipase A_2 on phosphatidylcholine bilayers is facilitated under conditions that introduce defect structures, and therefore the free space, in the bilayers.

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Materials and Methods

Most of the methods and materials have been described previously [3, 12, 13]. L-α-dipalmitoylphosphatidylcholine was purchased from Calbiochem, and it was judged to be better than 99% pure by thin-layer chromatography and by differential scanning calorimetry of multilamellar vesicles formed from this lipid.

Liposomes were prepared as described earlier [3, 12]. These preparations are "annealed" by incubating the phospholipid dispersions 10 °C above their phase transition temperature for 30–45 min. All the liposome preparations normally used are annealed in this fashion. However, if the liposomes are prepared well below (15 °C) their phase transition temperature, the bilayers contain structural defects [4] as if the bilayer in such liposomes has not been annealed. The "unannealed" lipsomes were prepared by dispersing of film of L-α-DPPC by vigorous shaking on a vortex mixer. Occasionally, the dispersions were subjected to sonication for 5 sec in a bath-type sonifier. The phospholipid dispersion thus obtained have the appearance of a gel. The temprature of these unannealed liposomes (multilamellar vesicles) through the preparative procedure and storage was maintained at 10–20 °C.

The progress of the hydrolytic reaction catalyzed by bee venom phospholipase A_2 was monitored by an automatic titration of the released fatty acid with 1 mM 2-amino-2-methyl-1,3-propanediol by pH-stat titrator (Radiometer model TTT-60 equipped with ABU-13 with 250 µl burette, PHM-62, TTA-60). All the experiments were done in 5 ml reaction mixture under a stream of nitrogen saturated with water at the temperature of the reaction. With this set up we could routinely measure the rate of hydrolysis greater than 1 nmol/min with an accuracy of ± 1 nmol/min [*see* reference 12 for details]. The response time of the instrument to the externally added myristic acid was less than 5 sec under all the conditions tested. The reaction progress curves described in this paper were determined at about 10-fold higher enzyme concentration compared to that used in the preceding paper [13]. Under these conditions it was necessary to wash the enzyme absorbed on the electrodes with saturated KCl solution between the successive runs.

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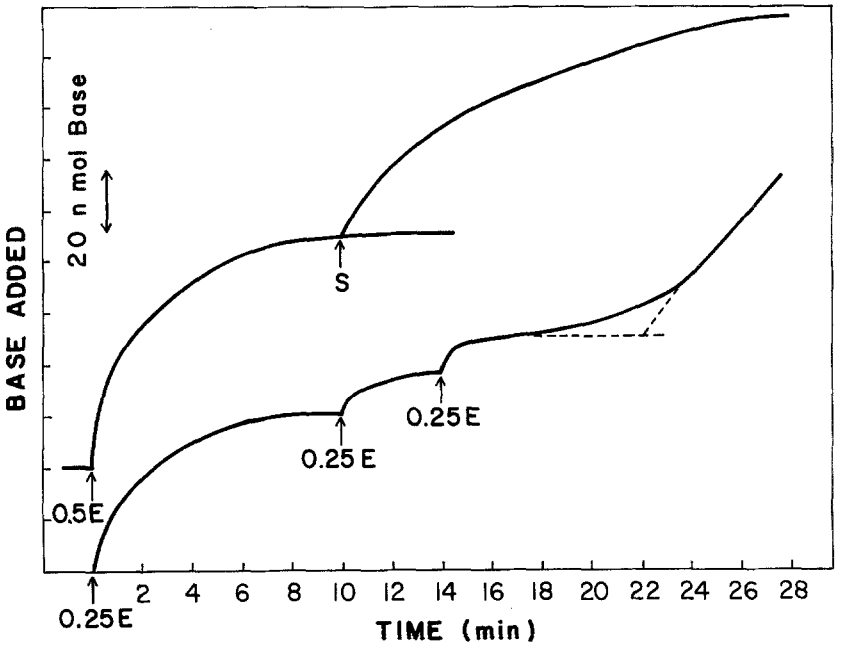

Fig. 1. The reaction progress curves for the hydrolysis of egg phosphatidylcholine (500 μM) in 5 ml of 6 mM $caCl_2$ at 37°, pH 7.4. *Bottom:* The reaction was initiated by 0.25 U enzyme. Two successive aliquots of 0.25 U enzyme were added after the reaction ceased. A second phase of hydrolysis is observed when the hydrolysis due to the addition of the third aliquot is completed. The procedure outlined by the dotted lines was used to determine the latency for and the products formed up to the second phase of hydrolysis. *Top:* The reaction was initiated by 0.5 U enzyme and a second aliquot (500 μM total) of the substrate was added when the reaction in the first phase ceased. (Such a proton release is not observed when the same amount of the substrate or the enzyme alone is added to the mixture.)

Results

The rate of hydrolysis of phosphatidylcholine bilayers was monitored continuously by titration of the released fatty acids without any additive or a bilayer modifying agent. Under these conditions, the kinetics of hydrolysis is complex. A typical reaction progress curve obtained following the addition of phospholipase to 500 μM egg phosphatidylcholine as unilamellar vesicles is shown in Fig. 1. It exhibits several interesting features. The burst in hydrolysis of the egg phosphatidylcholine unilamellar vesicles is considerably slowed down when less than 7% of the total available substrate in unilamellar vesicles is hydrolyzed. The reaction reaches a slow steady-state rate in about 5–7 min. The total number of protons (100 nmol) released before the hydrolysis reaches a slow steady-state level is more than 1000-fold higher than the total number of the enzyme molecules (<20 pmol) present in the reaction mixture. Such comparisons rule out the possibility that the observed proton release is due to a substrate induced deprotonation of the enzyme, or an enzyme induced deprotonation of the substrate in the bilayer. Similarly, the proton release is not observed when only the substrate or the enzyme is added to the mixture. Identical reaction progress curves were obtained whether the reaction is initiated by the substrate or the enzyme. However, for most of the experiments the reaction was initiated by the addition of the enzyme to the substrate preincubated at pH 7.4. These observations demonstrate that the initial phase of the proton release is not due to an imbalance of pH between the salt solution and the components added afterwards to the reaction mixture to initiate the reaction. This is also consistent with the observation that the addition of a second aliquot of the substrate or the enzyme induces further hydrolysis, the rate and magnitude of which are different. As shown in Fig. 1, the extent of hydrolysis by the second aliquot of the enzyme is smaller than that for the first aliquot, and the extent of hydrolysis by the second aliquot is independent of the time lapse between the first and the second aliquots. In contrast, the extent of hydrolysis by two successive aliquots of the substrate are similar, whereas the initial rates of hydrolysis are different. These experiments demonstrate that the reaction does not cease due to an inactivation of the enzyme, rather the residual phosphatidylcholine is not available to the enzyme as substrate.

In the reaction progress curves of the type shown in Fig. 1 the initial rate of hydrolysis is relatively fast such that the addition of the base could lag the generation of protons in the reaction medium. In order to evaluate the kinetics of hydrolysis we resolved the first part of the reaction progress curve. When the base is pre-added to the reaction mixture before the addition of the enzyme, a lag is observed between the addition of the enzyme and the beginning of the addition of the base. The duration of the lag depends upon the amount of the base pre-added to the reaction mixture. However, the reaction progress curve following the lag period is identical to the normal progress curve adjusted for neutralization of the pre-added base. Since the response time of the instrument and mixing time in the reaction vessel is less than 5 sec

and the rate of reaction does not change noticeably at pH 7.4 to 8.0 (the pH range observed when the base is pre-added to the reaction mixture), these results demonstrate that the proton release occurs at a measurable rate even during the first 10 sec. A semi-log plot of the residual available substrate as a function of time shows that the rate of hydrolysis is not a simple first-order process. Similarly, the reaction progress profile could not be satisfactorily curve fitted by nonlinear regression to the integrated Michaelis-Menten equation where it is assumed that (i) the total available substrate is equal to that given by the total extent of hydrolysis, or (ii) the products inhibit the reaction.

The observation that only a small fraction of the total available substrate ($<7\%$ in ULV) is hydrolyzed under conditions described above is quite interesting since more than 95% of the total available substrate is hydrolyzed in the presence of the optimal amounts of activating alkanols [3, 12]. This suggests that a slow steady-state rate of hydrolysis after ~ 5 min could not be simply due to a product inhibition. Thus the proton release of the type shown in Fig. 1 may represent an early event during the action of phospholipase A_2 on phosphatidylcholine bilayer. To further ascertain that the proton release of the type shown above arises from the hydrolytic action of the enzyme on the substrate in the bilayer, we studied the effect of the various components required for the reaction on the reaction progress curve. However, since the initial rate of hydrolysis could not be measured directly and the reaction progress curve could not be described by a simple rate expression, we have chosen for the following discussion to compare only the extent of hydrolysis under a variety of experimental conditions that lead to a controlled perturbation of the packing in bilayers.

Effect of Calcium on the Reaction Progress Curve

Calcium is required for the hydrolysis of phosphatidylcholine by phospholipase A_2 [14]. The effect of calcium in the medium on the reaction progress curve shows that the effect of calcium is on the extent of hydrolysis. The half-times for the reaction appear to increase slightly with the calcium concentration. When the enzyme dissolved in a calcium-free solution is added to unilamellar vesicles in a calcium-free medium, the extent of hydrolysis is about 10% of the value observed for the reaction conducted in a calcium (6 mM) containing medium. A slightly higher ($\sim 20\%$) extent of hydrolysis is observed when the enzyme preincubated in 10 mM Ca^{2+} is added to the substrate in a calcium-containing medium. The

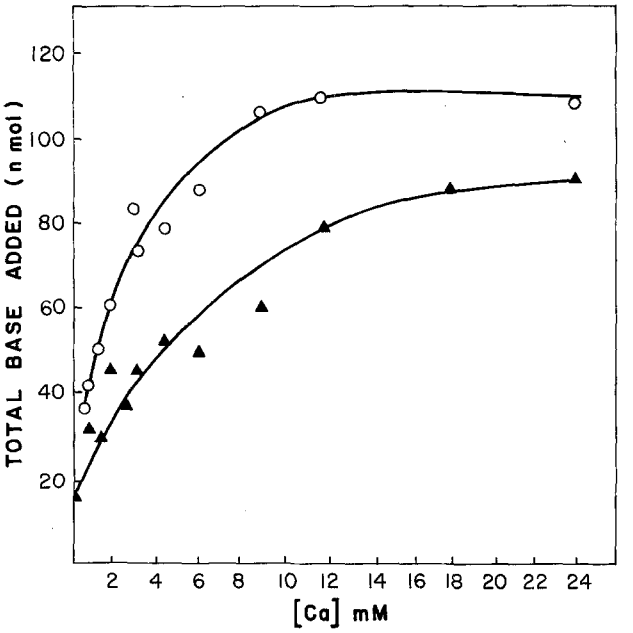

Fig. 2. Effect of calcium concentration on the extent of hydrolysis for bee venom phospholipase A_2 preincubated in a calcium-free (-▲-) and a calcium (10 mM) containing medium (-○-).

extent of hydrolysis as a function of the calcium concentration in the medium shows a hyperbolic dependence (Fig. 2). The maximal rate of hydrolysis depends upon whether the enzyme is preincubated in a calcium-free or a calcium-containing medium. The significance of this difference is not clear; however, it may be noted that a half-maximal activation is produced by ~ 2 mM calcium whether or not the enzyme is preincubated with calcium ions. Moreover, we have consistently observed some activity with the enzyme preincubated in a calcium-free medium in the absence of calcium ions in the reaction medium. A small (but above the background) proton release similar to that observed in the calcium-free medium was also observed in a medium containing 75 μM EGTA. These experiments suggest that the "calcium-free" enzyme has residual activity, and the extent of hydrolysis is affected by calcium ions.

Effect of Varying the Enzyme Concentration Upon the Extent of Hydrolysis

The total amount of the substrate hydrolyzed during the initial burst at a constant substrate concentration changes as a hyperbolic function of the amount of phospholipase A_2 added to the reaction mixture. (Figure not shown.) A double log plot of the initial burst *vs.* enzyme concentration shows that the amount of the total hydrolyzable substrate increases with the

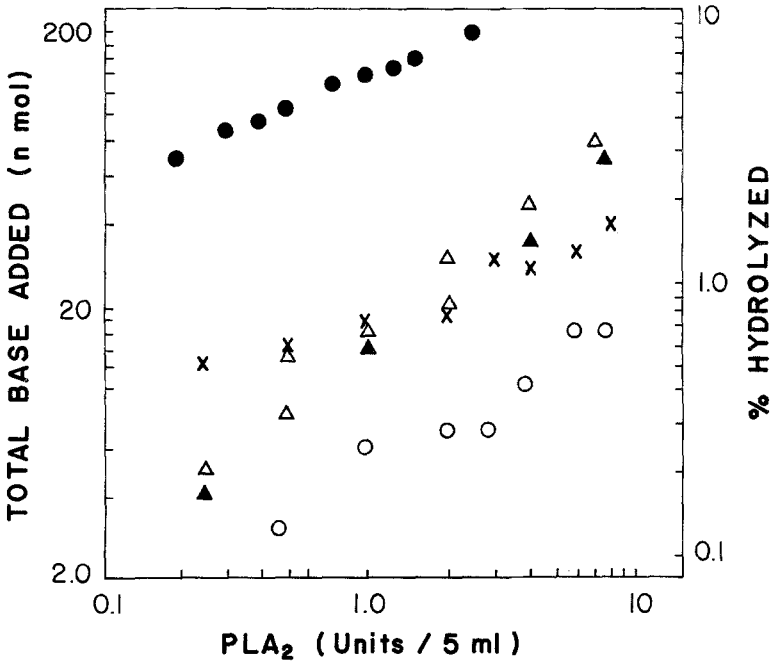

Fig. 3. The plots of the extent of hydrolysis as a function of phospholipase A_2 concentration (1300 U = 1 mg) for 500 μM osmotically-shocked egg phosphatidylcholine unilamellar vesicles (–●–); 500 μM osmotically shocked egg phosphatidylcholine multilamellar vesicles (–▲–); 5 mM osmotically intact egg phosphatidylcholine multilamellar vesicles (–△–); 500 μM annealed osmotically shocked DPPC multilamellar vesicles at 45° (–×–) and 33° (–○–). The ordinate to the right refers to % substrate hydrolyzed when the substrate concentration is 500 μM. Reaction conditions are the same as in the caption of Fig. 1.

enzyme concentration and it is different for different types of liposomes. Such observations suggest that only a small proportion of the total substrate in the bilayer is available for hydrolysis by the enzyme, and different proportions of the substrate are hydrolyzed in different forms of bilayer. Thus unilamellar vesicles and unannealed DPPC multilamellar vesicles are hydrolyzed to a significantly greater extent than the annealed multilamellar vesicles. Moreover, 5 mM osmotically intact egg phosphatidylcholine multilamellar vesicles release about the same number of protons as 500 μM shocked egg phosphatidylcholine multilamellar vesicles, suggesting that about 10-fold more surface may be exposed by osmotically shocking the egg phosphatidylcholine multilamellar vesicles [10, 12]. Since the slopes of the plots in Fig. 3 are approximately the same and considerably less than one, it may be argued that an increase in the extent of the substrate hydrolyzed depends upon the form of the bilayer, and it does not simply reflect an increase in the rate of hydrolysis that would accompany an increase in the enzyme concentration.

The reaction progress curves at high enzyme (>2 units/5 ml for egg phosphatidylcholine unilamellar vesicles) concentrations were found to exhibit a second phase of hydrolysis following a lag period after the first phase of proton release. The onset of the second phase depends not only upon the enzyme concentration, but also on the form of the bilayer and concentration of the substrate (*see* below). In fact, the second phase of hydrolysis appears to be initiated when a certain critical concentration of the products has formed during the first phase of hydrolysis. Thus under a variety of conditions the onset of the second phase is observed when >40 nmol (~8 μM) protons have been released during ~10 min of the first phase. We suspect that the second phase is initiated when the product concentration reaches a critical local concentration in the bilayer.

Effect of Phosphatidylcholine Concentration in Different Forms of Bilayers on the First Phase of Hydrolysis

Phospholipids can be dispersed into unilamellar vesicles and multilamellar vesicles, which have not only different proportions of the total phospholipid exposed to the external medium but they also have somewhat different organization in the bilayer [5, 9]. The amount of the substrate hydrolyzed changes as a hyperbolic function of the total substrate concentration in different forms of the bilayer (Figure not shown). A double log plot of the initial burst *vs.* substrate concentration (Fig. 4) shows that the shapes of these plots are not identical; they are not only shifted along the concentration axis but their slopes are also different. These observations demonstrate that the total amount of the substrate hydrolyzed is directly related to the available substrate in the bilayer, and the extent of hydrolysis depends

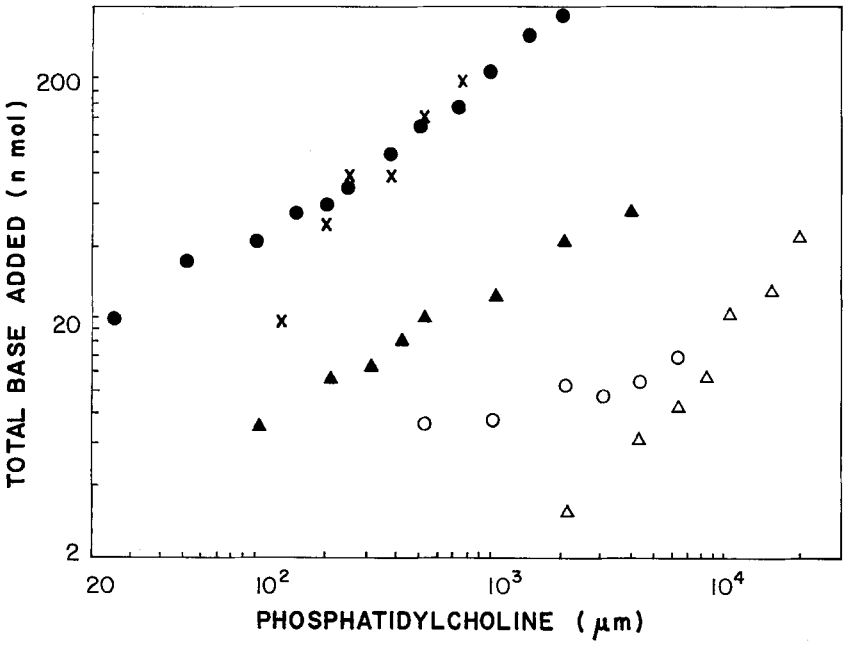

Fig. 4. The plots of the extent of hydrolysis as a function of the substrate concentration in osmotically shocked egg phosphatidylcholine unilamellar vesicles (–●–), shocked egg phosphatidylcholine multilamellar vesicles (–▲–), intact egg phosphatidylcholine multilamellar vesicles (–△–), unannealed intact DPPC multilamellar vesicles (–×–), and annealed shocked DPPC multilamellar vesicles (–○–). In all cases 0.5 U 5 ml of the enzyme was used to initiate the reaction at 37°.

upon the form of the bilayer. The amount of the total hydrolyzable substrate under these conditions is more than 1000-fold higher than the critical micelle concentration of phosphatidylcholine. Since the lipid molecules are more closely packed in multilamellar vesicles than in unilamellar vesicles, the difference in the extent of hydrolysis of multilamellar vesicles and unilamellar vesicles could be related to a difference in the packing in these bilayers. The unannealed bilayers are hydrolyzed to a greater extent than the annealed bilayers, presumably due to a larger proportion of defects in the unannealed bilayers.

Effect of Varying Phospholipase A_2 Concentration on L-α-*di-palmitoylphosphatidylcholine Bilayers*

One of the possible explanations of the partial hydrolysis of egg phosphatidylcholine bilayer by phospholipase A_2 is that the enzyme acts only on the phospholipid containing a specific acyl chain composition. The experiments in Figs. 3 and 4 show that this is not the case since the behavior of egg phosphatidylcholine liposomes is qualitatively similar to that of DPPC liposomes. The quantitative differences could be ascribed to the differences in the organization of these bilayers. The organization of DPPC in bilayer can be altered to a considerable extent simply by changing the temperature [1] or by preincubation of the dispersions below or above the phase transition temperature [4]. The reaction progress curves for dipalmitoylphosphatidylcholine in unannealed and annealed liposomes at 37 °C are presented in Fig. 5. The data demonstrates that the fraction of the total available DPPC in the various forms of bilayers is hydrolyzed to different extents under otherwise identical experimental conditions. Thus unannealed multilamellar vesicles and annealed unilamellar vesicles are hy-

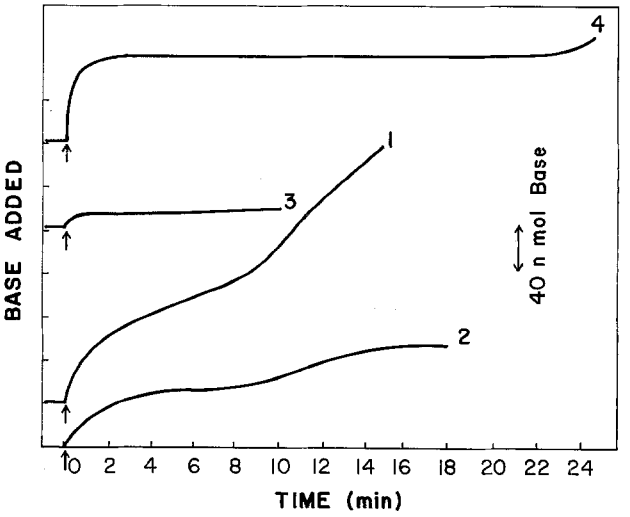

Fig. 5. The reaction progress curves for DPPC liposomes under various conditions (*1*) 500 μM intact unannealed multilamellar vesicles; (*2*) 50 μM osmotically shocked unannealed multilamellar vesicles; (*3*) 500 μM osmotically shocked annealed multilamellar vesicles; and (*4*) 500 μM osmotically shocked annealed unilamellar vesicles. 0.5 U enzyme was used to initiate the hydrolysis at 37 °C. The substrate hydrolyzed in initial burst is 2.8% for (*1*), 22% for (*2*), 0.4% for (*3*) and 3% for (*4*).

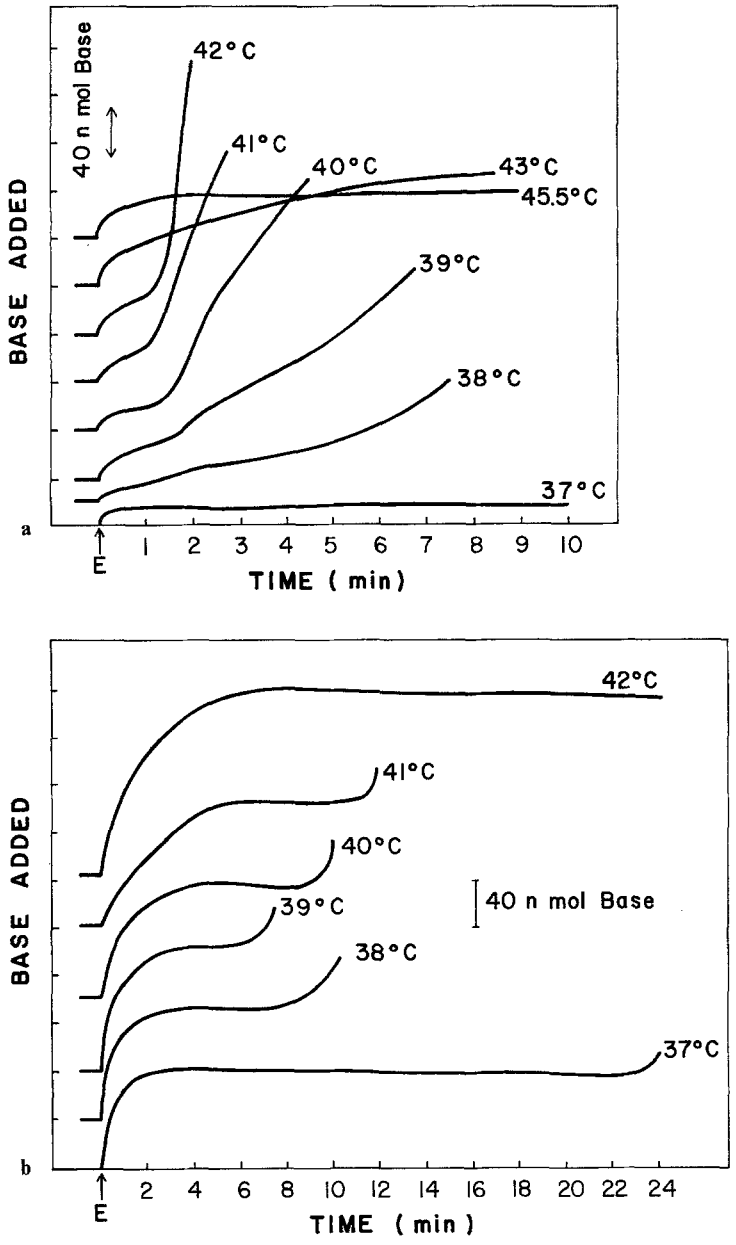

Fig. 6. The reaction progress curves for L-α-dipalmitoylphosphatidylcholine, (*a*) multilamellar vesicles and, (*b*) unilamellar vesicles at the various temperatures around the phase transition temperature of the liposomes. It may be noted that both the extent of hydrolysis in the first phase, and the lag period for the second phase change as a function of temperature.

drolyzed to the same extent, whereas the annealed multilamellar vesicles are hydrolyzed to a much smaller extent than the unannealed multilamellar vesicles. The unannealed multilamellar vesicles could be rapidly annealed by preincubating these at temperatures above the phase transition temperature. The reaction progress curves of such annealed preparations is identical to those that are annealed during the initial dispersion of the phospholipid.

To further establish the role of defect structures in the bilayers, we studied the effect of varying temperature on the extent of hydrolysis of bilayers by phospholipase A_2. The organization of dipalmitoylphosphatidylcholine bilayers undergoes an abrupt change at a characteristic phase transition temperature, 41.3 °C [1,5]. Since the gel-to-liquid crystalline phase transition is a highly cooperative process [2], it is thought that large domains of phospholipid molecules undergo such a transition simultaneously. It follows that the boundary region between the gel phase domains (regions of "mismatch" or the defect structures) would reach a maximum at the phase transition temperature [5, 6].

The reaction progress curve for the hydrolysis of DPPC bilayers show a strong temperature dependence. As shown in Fig. 6 the reaction progress curves for DPPC multilamellar vesicles, and unilamellar vesicles at the various temperatures in the vicinity of

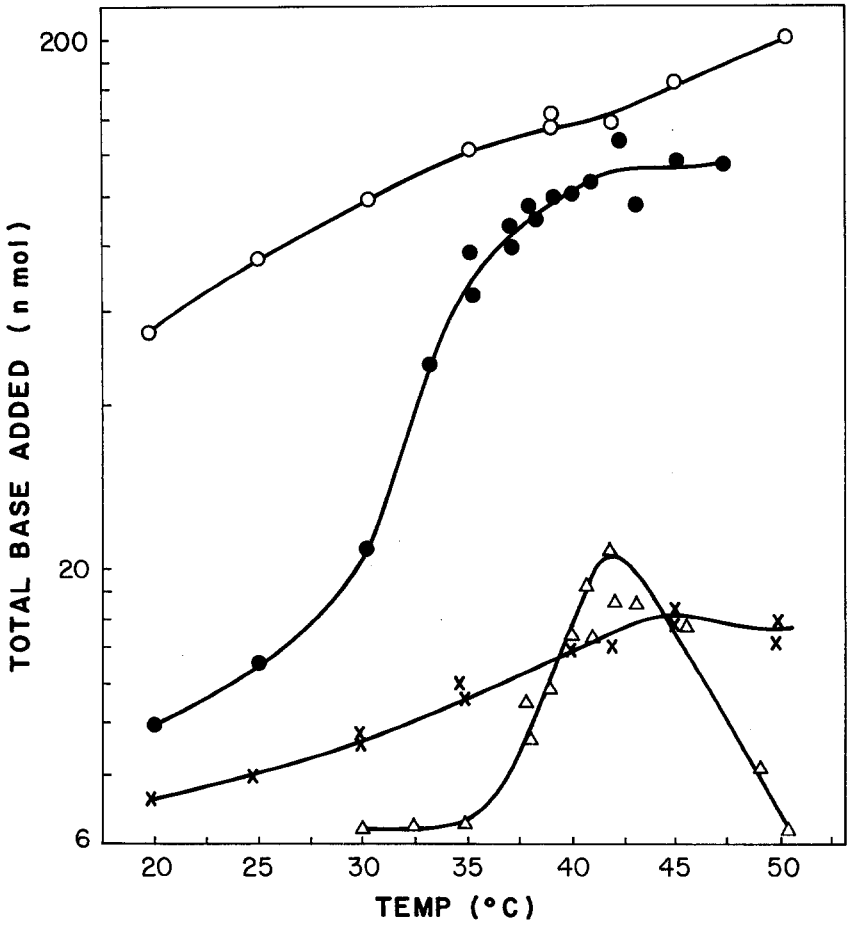

Fig. 7. The extent of hydrolysis of liposomes as a function of temperature. Osmotically shocked egg phosphatidylcholine (–○–) and egg phosphatidylcholine multilamellar vesicles (–×–), osmotically shocked annealed DPPC unilamellar vesicles (–●–) and multilamellar vesicles (–△–). In all cases the enzyme concentration was 0.5 U 5 ml, and the substrate concentration was 500 μM.

their phase transition temperature are qualitatively different. As plotted in Fig. 7, the proportion of the substrate hydrolyzed during the first phase of hydrolysis increases monotonically with the temperature for the various forms of liposomes prepared from egg phosphatidylcholine or DPPC. The liposomes prepared from egg phosphatidylcholine do not exhibit an abrupt increase in the extent of hydrolysis in the first phase. Also with egg phosphatidylcholine liposomes the second phase of hydrolysis was not observed for at least up to 20 min after the addition of the enzyme to initiate the reaction (data is not shown here). On the other hand, for the DPPC liposomes the extent of hydrolysis in the first phase changes abruptly near the transition temperature. As shown in Fig. 7 for DPPC unilamellar vesicles the transition is observed at 32–88°, whereas the corresponding transition for DPPC multilamellar vesicles is observed at 38–42°. A large scatter in the data at 38–43° for DPPC multilamellar vesicles is presumable due to a contribution from the second phase of hydrolysis, the lag period for which is drastically lowered at the phase transition temperature (*see* below).

The lag period for the second phase of hydrolysis shows a much stronger dependence upon the temperature. As shown in Fig. 6, for DPPC multilamellar vesicles the lag period is about 1 min at 41° and 42°, whereas at the lower (<37°) and the higher (>45°) temperatures the second phase is not observed for at least 10 min.Similarly, as shown in Fig. 6, the lag period for the apperance of the second phase reaches its minimum of ~6 min at 39 °C, and then it increases both below and above this temperature. These minima for both the unilamellar vesicles and multilamellar vesicles correspond to the phase transition temperatures observed by a variety of techniques [1, 5, 9]. Such a dependence of the extent of hydrolysis and the lag period upon the phase transition characteristics of a phospholipid bilayer provides a strong support to the suggestion that the extent of hydrolysis during the first phase depends upon the organizational defects in the bilayer. The observations presented here also confirm the reports that the hydrolysis of the liposomes by pancreatic phospholipase A_2 is drastically facilitated at the phase transition temperature [8, 15]. Thus, it seems that in general phospholipases require defective structures for penetra-

tion at the interface, and these defects are present at the boundaries of the gel-to-liquid crystalline phases. However, our data demonstrate that an early onset of the second phase of hydrolysis, plays a significant role in the phospholipase catalysis.

Discussion

In this paper we have characterized the experimental conditions and identified the early events under which phosphatidylcholine in bilayers is hydrolyzed by bee venom phospholipase A_2 in the absence of additives. The early part of the reaction progress curve is resolved into two components. The first phase of hydrolysis is found to depend upon the enzyme, substrate and calcium concentrations, as well as upon the various factors that modulate the organization of phospholipids in bilayers, such as temperature, sonication, and annealing. Several features of the hydrolysis in the first phase may be noted: only a small fraction of the total available substrate is hydrolyzed; the amount of substrate hydrolyzed varies with the amount and type of substrate organization, as well as to the enzyme and the caclium ion concentrations; the extent of hydrolysis increases with the disorder in the membrane as induced by sonication or thermotropic phase change; the apparent rate of hydrolysis (the half-time for the reaction progress curves for the burst) is remarkably constant (~ 1 min) under a variety of conditions; the substrate that is not hydrolyzed during the first phase by an aliquot of the enzyme is only partially hydrolyzed by the second aliquot of the enzyme; addition of a second aliquot of the substrate to the reaction mixture (in which hydrolysis has ceased) leads to additional proton release. Thus one of the most intriguing features of the first phase of hydrolysis is that the reaction slows down considerably even though a large fraction (95%) of the total substrate along with the catalytically active enzyme are present in the mixture at the end of the first phase. Since more than 90% of the total available substrate can be hydrolyzed under a variety of conditions where the substrate organization is modified by an activator [12, 13], we suggest that the cessation of the reaction at the end of the first phase is not a simple case of product inhibition.

Tinker and Wei [11] have proposed a generalized model for the heterogeneous catalysis by phospholipase A_2 on phospholipid bilayer. According to this model, after initial absorption of the enzyme it diffuses in the plane of the bilayer, and the second phase of hydrolysis is due to a product dependent desorption of the enzyme. This model predicts an initial burst of product formation followed by slower hydrolysis when the total substrate concentration is less than the "surface Michaelis constant". Since we have observed the burst kinetics over a wide range of total substrate concentration, it would mean that only a small fraction of the total substrate is available for the enzyme binding and that the fraction of the available substrate varies with the state of the bilayer. Our results suggest that the sites of action of the enzyme during the burst phase are the defect structures in the bilayer organization. Indeed, the observed extent of hydrolysis in the first phase is qualitatively the same as expected on the basis of the "fluidity" of the bilayer: liquid crystalline phase > gel phase; unilamellar vesicles > multilamellar vesicles; unannealed liposomes > annealed liposomes; egg phosphatidylcholine > dipalmitoylphosphatidylcholine liposomes. Moreover, if the defect structures in the bilayer are the preferential sites of action of phospholipase A_2, the proportion of the molecules in the defect regions should be related to the substrate hydrolyzed in the first phase. It may be a coincidence, but it is noteworthy that the proportion of the substrate hydrolyzed in dipalmitoylphosphatidylcholine multilamellar annealed vesicles is nearly the same (0.4%, legend to Fig. 5) as the number of molecules in the "mismatch" region between the cooperative units of the gel phase [6]. If there is a common basis for this similarity, it would imply that even in highly fluid membranes the defects are localized in the plane of the membrane rather than uniformly distributed in the plane of the bilayer.

The model presented by Tinker and Wei [11] and our interpretation has several implications. It suggests that phospholipase A_2 can diffuse or *scoot* in the plane of the membrane (path 2 in the terminology of Tinker and Wei [11]), or it can *hop* from one substrate to the other (path 1) through the aqueous phase. The relative contribution of these two paths depends upon the state of the bilayer. Thus defect structures favor scooting of the enzyme; and, the product modified bilayer favors the hopping of the enzyme. Optimal concentrations of lower alkanols appear to favor the hopping mechanism [13], whereas the higher alkanols could favor hopping or scooting depending upon the activator used and the phase properties of the resulting bilayer [13]. These interpretations are particularly relevant to the action of phospholipase A_2 on cell membranes. Thus, the distribution and density of defects in such membranes could play an important role in regulating the expression of the phospholipase activity, e.g., in the release of arachidonic acid for the synthesis of prostaglandins.

This work was supported by the National Institute of Health Research Grant GM 23122.

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Received 18 September 1979; revised 5 February 1980