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ORIGIN OF THE LATENCY PHASE DURING THE ACTION OF PHOSPHOLIPASE A_2 ON UNMODIFIED PHOSPHATIDYLCHOLINE VESICLES

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The reaction progress curve for the action of pig-pancreatic phospholipase A_2 on dimyristoylphosphatidylcholine vesicles is characterized under a variety of conditions. The factors that regulate the rate of hydrolysis during the presteady-state phase determine the latency period. The results demonstrate that the accelerated hydrolysis following the latency phase of the reaction progress curve is due to the product-assisted binding of the enzyme to the substrate bilayer by chaning the number of bindings sites and therefore the binding equilibrium. A critical mole fraction of products appears to be formed in the substrate bilayers before the steady-state phase of hydrolysis begins. The latency phase shows a minimum at the phase-transition temperature of the substrate vesicles; however, we did not observe a significant binding of the enzyme to pure substrate bilayers even at the phase-transition temperature. The rate of binding of the enzyme is found to be fast and the rate of desorption of the bound enzyme is very slow compared to the latency phase. The rate of redistribution of products between substrate bilayers is rather slow. These observations demonstrate that during the latency phase of the action of phospholipase A_2, a critical mole fraction of products is formed in the substrate bilayer.

Introduction

Action of phospholipase A_2 on vesicles of diacylphosphatidylcholines follows a complex reaction progress profile [1,2]. It is characterized by an initial burst of product release, which after a latency period is followed by a fast steady-state rate of hydrolysis. Earlier we proposed that such an autocatalytic reaction progress curve could result from a product-induced modification of the substrate bilayer, which leads to an increased binding of the enzyme [3]. This is consistent with the suggestion that the activity of phospholipase A_2 on the organized substrate is regulated by the quality of the interface [4]. One of the predictions of this hypothesis is that a modification of the quality of interface, for example by incorporation of products into bilayers, leads to an increased binding of the enzyme to the substrate interface. Indeed, we have demonstrated a rapid binding of pig-pancreatic phospholipase A_2 to bilayers of nonhydrolyzable substrate analogs containing lysophosphatidylcholine and fatty acid [3]. Such a binding of the enzyme to bilayers is not observed in the absence of both the products of hydrolysis. The product-assisted binding of the enzyme to the ternary codispersions is due to an increase in the number of binding sites that shift the binding

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Abbreviations: Dimyristoylphosphatidylcholine (DMPC), 1,2-di(tetradecanoyl)-*sn*-glycero-3-phosphocholine; lysophosphatidylcholine, 1-tetradecanoyl-glycero-3-*sn*-phosphocholine; dialkylphosphatidylcholine or diether-PC_{14}, 1,2-di(tetradecyl)-*rac*-glycero-3-phosphocholine.

equilibrium, rather than by changing the rate of binding. In this paper we report studies that demonstrate that the conditions that regulate the rate of hydrolysis during the presteady-state phase, and therefore the mole fraction of the products in the substrate bilayers, determine the latency phase. Thus the latency phase is the period during which a critical level of products is accumulated in the substrate bilayers.

Materials and Methods

Dimyristoylphosphatidylcholine was purchased from Calbiochem and used without further purification. Lysophosphatidylcholine was prepared by the action of phospholipase A_2 in wet diethyl ether. Phospholipase A_2 from pig pancreas was prepared as described elsewhere [5]. 1,2-Di(tetradecyl)-*rac*-glycero-3-phosphocholine (diether-PC_{14}) was synthesized as reported [6]. All these lipids were judged to be homogeneous by thin-layer chromatography in $CHCl_3/CH_3OH/H_2O$ (65:25:5, v/v).

Vesicles were prepared by dispersing a film of phospholipids in 0.1 M KCl in a bath-type sonicator (Sonicor SC-50T) as described elsewhere [1]. These preparations were routinely 'annealed' by allowing them to equilibrate for 90 min at 55°C and were used after allowing them to stand for more than 10 h at room temperature. This protocol was found to be important to obtain reproducible results, especially for the early phases of the reaction progress curve. The progress of the hydrolytic reaction of phospholipase A_2 was monitored by automatic titration of the released fatty acid with NaOH (about 1 mM) in a pH-stat titrator [1]. All titrations were done under nitrogen in 5 ml of 0.1 M KCl/10 mM $CaCl_2$ (pH 8.0) at the indicated temperature unless otherwise stated. 5% H_2O_2 was used to wash the titration assembly between consecutive assays in order to destroy any residual activity that could be adsorbed to the electrodes or the reaction vessel. Unless stated otherwise, most experiments were initiated by addition of enzyme (5–10 μl of a stock solution of pig-pancreatic phospholipase A_2 in 1 mM Tris-HCl, pH 8.0). Under these conditions no imbalance in pH due to enzyme addition was found. Enzyme units are referred to the activity of the enzyme on egg yolk substrate, at 37°C, pH 8.0, and in the presence of 0.11% of deoxycholate [5]. One unit corresponds to the hydrolysis of 1 μmol substrate per min. Specific activity of the enzyme was 1400 units/mg.

Results and Discussion

The reaction progress curve

The time course of proton release after the addition of pig-pancreatic phospholipase A_2 to dimyristoylphosphatidylcholine vesicles follows a complex reaction progress curve, the precise shape of which depends upon all the factors that regulate catalytic activity of the enzyme, such as enzyme, substrate and product concentration. Some of the reaction progress curves are shown in Fig. 1. Typically, the initial burst of proton release (called initial burst hereafter) is followed by a slow accelerating latency phase (called latency phase), which after a certain time (called latency period) is followed by a fast steady-state phase of hydrolytic activity. Generation of any of these phases of the reaction progress curve requires the presence of all the components necessary for the catalytic activity; it does not depend upon the order of addition

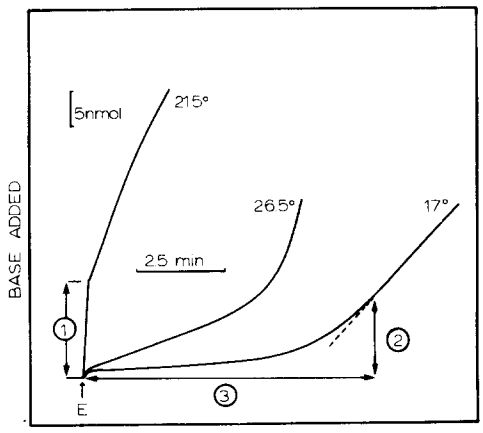

Fig. 1. The reaction progress curves for the hydrolysis of sonicated dimyristoylphosphatidylcholine (250 μM) vesicles by pig-pancreas phospholipase A_2 (0.25 μg/5 ml). All conditions identical except for the temperatures indicated in the graph. Reaction medium contained 100 mM KCl/10 mM $CaCl_2$ at pH 8.00. Initial burst (burst at $t=0$, ①), final or total burst (burst at $t=\tau$, ②), and the latency period (③) are defined as indicated.

of the components and is seen only after the last component is added [1]. Thus a typical reaction progress curve is multiphasic (see also Refs. 1, 7) and, as shown in Fig. 1, even the latency and the steady-state phase cannot be described by a simple first-order process. All these observations suggest that the delayed steady-state phase is not due to a slow penetration of the enzyme into the interface, as has been shown in kinetic analyses of the hydrolysis of monolayers [4], but, as shown below, the accelerated hydrolysis during the latency phase is due to a product-mediated binding of the enzyme to substrate bilayer.

All the experiments reported in this paper are based on the conclusion that the presteady-state phase of the reaction progress curve is not due to a slow penetration or a conformational change of the enzyme. This is demonstrated unequivocally by the observation that the latency phase in the binding and in the kinetics of hydrolysis is not seen with the ternary codispersions as substrate (Ref. 3, also see below). Similarly, the enzyme once bound to the interface does not readily exchange with a new substrate interface. This is best demonstrated by the experiments shown in Fig. 2, where the enzyme bound to the nonsubstrate ternary codispersions, diether-PC_{14} + lysophosphatidylcholine + fatty acid, cannot be exchanged readily with the substrate codispersions. Under these conditions, addition of vesicles of the hydrolyzable substrate alone or as ternary codispersions to the enzyme bound to the ternary codispersion of the nonhydrolyzable substrate analog (diether-PC_{14}) does not elicit any detectable hydrolysis in over 45 min after mixing (Fig. 2, curve A). If the two types of dispersion are premixed and the reaction is initiated with the enzyme, the hydrolysis ensues immediately (Fig. 2, curve B). These results demonstrate that the bound enzyme is not capable of exchanging with the newly added substrate dispersions; that the binding of the enzyme to the ternary codispersions of the substrate is complete in less than 30 s; and that the lack of activity of the bound enzyme (curve A, Fig. 2) is not due to an inhibitory effect of the diether-PC_{14}.

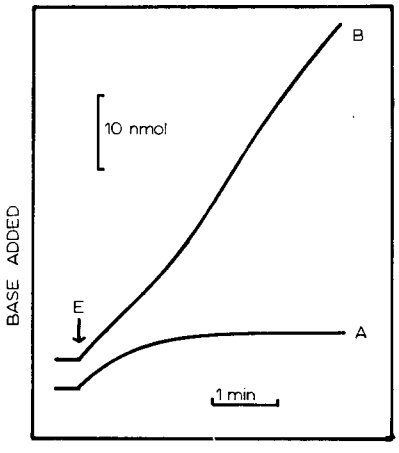

Fig. 2. Reaction progress curves for the hydrolysis of dimyristoylphosphatidylcholine by pig-pancreas phospholipase A_2. In curve A, the enzyme (0.35 μg) was preincubated for 3 min in the reaction vessel with the ternary codispersion of diether-PC_{14} (130 μM) + lysophosphatidylcholine + myristic acid (3 : 1 : 1). At the arrow, ternary codispersion of DMPC + lysophosphatidylcholine + myrisitic acid (3 : 1 : 1) was added to initiate the reaction. In B, both ternary codispersions were incubated for 3 min and the reaction was initiated by addition of the enzyme (arrow). Temperature was 30°C; other conditions are the same as in Fig. 1.

Effect of temperature on the reaction progress curve

As shown in Fig. 1, the reaction progress curves are drastically influenced by temperature; however, the various features of the curve seem to be influenced in different fashions. The effect of tem-

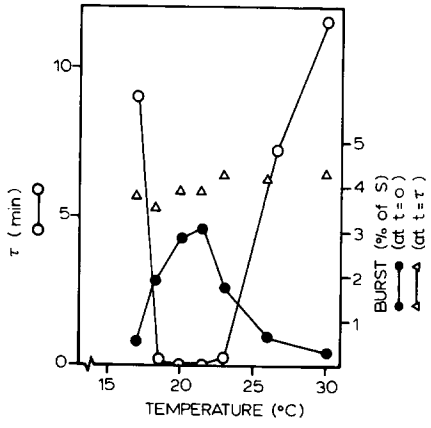

Fig. 3. The effect of varying temperature on the latency phase (○), initial burst (●), and total burst (△) of the reaction progress curve for the hydrolysis of dimyristoylphosphatidylcholine vesicles (250 μM) by pig-pancreas phospholipase A_2 (0.2 μg/5 ml). Reaction conditions as in Fig. 1. The burst is referred to as percentage of the total substrate hydrolyzed (percent S) during the burst phase (see Fig. 1 for details).

perature on the latency period, the initial burst, and the total burst are shown in Fig. 3. The latency period shows a minimum at the phase-transition temperature of the vesicles (20–22°C). The total amount of the substrate hydrolyzed at the beginning of the steady-state phase (total burst) is almost constant throughout the range of temperatures studied. In contrast, the initial burst shows a maximum at the phase-transition temperature. The steady-state rate of hydrolysis increases only slightly as a function of the temperature (data not shown). These observations show that a constant amount (about 5% of the total) of the substrate is hydrolyzed before a steady-state rate of hydrolysis is reached. The amount of substrate hydrolyzed in the presteady-state phase is the sum of the initial burst, which is maximal at the transition temperature, and of the amount of substrate hydrolyzed during the accelerating latency phase. Taken together, these observations suggest that product formation is required to achieve the steady-state phase of hydrolysis even at the phase-transition temperature.

A rationale for the observations described above can be found in the hypothesis that the enzyme binds to discrete sites on a bilayer, and that only the substrate present at the binding sites is hydrolyzed during the initial burst phase of the reaction progress curve [1,4]. Thus the initial burst would be at its maximum and the latency period at its minimum at the phase-transition temperature if either the number of binding sites is at its maximum at the phase-transition temperature, or if the rate of redistribution of the products is more rapid at the phase-transition temperature. The first explanation is essentially ruled out by direct-binding studies [3] in which we have demonstrated that there is no significant (less than 3%) binding of the enzyme to pure diether-PC_{14} vesicles at the phase transition temperature. Moreover, as shown below, one can observe a latency phase even at the phase transition temperature if the enzyme concentration is kept low. A more plausible explanation for the minimum in the latency period at the phase-transition temperature is that the rate of intervesicle exchange or of lateral diffusion of the products formed during the initial burst is most rapid at the phase-transition temperature. Once such a redistribution of the products takes place, more enzyme can bind to the interface at the newly created sites. The steady-state rate of hydrolysis is attained when the fraction of the bound enzyme reaches its maximum as determined by its equilibrium binding constant [3]. The possibility that the exchange of the bound enzyme to sites on other vesicles is rate limiting is ruled out on the basis of the results shown in Fig. 2 and discussed earlier.

Effect of substrate concentration on the reaction progress curve

Data summarized in Fig. 1 and 3 show that about 5% of the total substrate is converted to products at the end of the latency phase; in these experiments the substrate concentration is kept constant. This would imply that for the establishment of the steady-state rate of hydrolysis, either a critical mole fraction of products must be formed in the bilayer, or a critical concentration of products should be present in the aqueous phase. With certain assumptions, the former would be consistent with the possibility that the products in

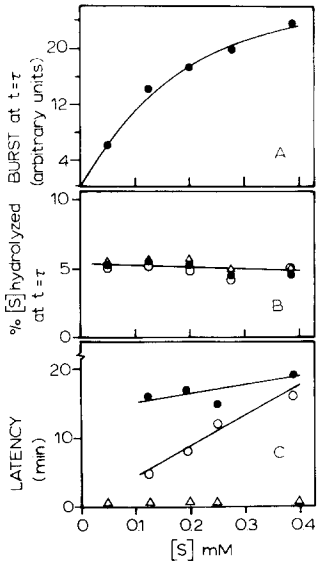

Fig. 4. The effect of varying the substrate concentration on (A) total burst, that is, the total substrate hydrolyzed at $t=\tau$; (B) the fraction of the total substrate hydrolyzed at the end of the latency period, and (C) the latency period obtained from the reaction progress curve for the hydrolysis of dimyristoylphosphatidylcholine vesicles by pig-pancreas phospholipase A_2 (0.25 μg/5 ml). In (A) temperature was 21.5°C. In (B) and (C), ○, 17°C; △, 21.5°C and ●, 30°C.

bilayer assist the binding; whereas the latter would suggest a product dependent activation of the enzyme in the aqueous phase. As shown in Fig. 4A, the total amount of products formed during the presteady-state phase of the reaction increases with the substrate concentration, however, the fraction of the total substrate hydrolyzed during this phase remains about 5% of the total substrate both below and above the phase-transition temperature (Fig. 4B). As shown in Fig. 4C, the latency period increases with the substrate concentration at 30 and 17°C, whereas no latency period is observed at the phase-transition temperature even at the lowest substrate concentration used. This suggests that it is not the concentration but the mole fraction of the products in the substrate bilayer that leads to an increased binding and therefore an increased rate of hydrolysis.

Effect of varying enzyme concentration on the reaction progress curve

The amount of product formed during the course of a reaction depends upon the amount of the enzyme bound to the interface [4]. Thus the latency period would decrease with increasing enzyme concentration because the fraction of the total enzyme bound to the pure substrate bilayer increases with the enzyme concentration. Indeed, as shown in Fig. 5, the latency period is inversely proportional to the enzyme concentration. It is particularly important to note that these experiments were done at the phase-transition temperature of the vesicles. In earlier experiments (Fig. 3) no latency is observed because higher enzyme concentrations are used. Dependence of the latency phase on the enzyme concentration demonstrates that the catalytic activity is required for incorporation of the enzyme. This is consistent with our working hypothesis that during the presteady-state phase a critical mole fraction of the products is formed, and the time required to produce it depends upon the amount of the enzyme bound initially.

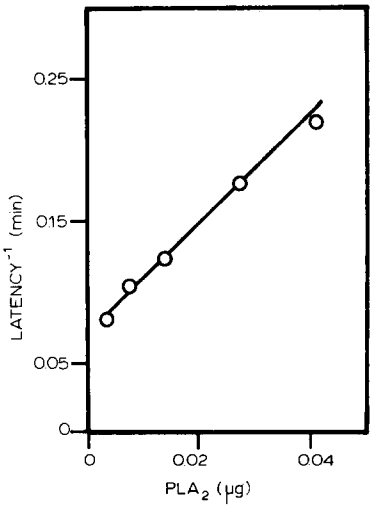

Fig. 5. The dependence of the latency period for the hydrolysis of DMPC by pig-pancreas phospholipase A_2 on the concentration of the enzyme. Dimyristoylphosphatidylcholine was 128 μM; temperature was 22°C.

Effect of the products of hydrolysis on the reaction progress curve

The experiments described so far demonstrate that about 5% of the total substrate is hydrolyzed at the beginning of the steady-state phase. The

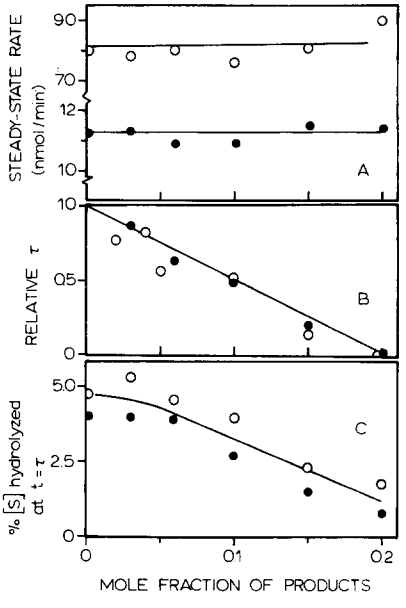

Fig. 6. The effect of varying the mole fraction of the products in dimyristoylphosphatidylcholine vesicles on the (A) steady-state rate of hydrolysis, (B) normalized relative latency period, and (C) percentage of the total substrate hydrolyzed until the beginning of the steady-state phase of the action of pig-pancreas phospholipase A_2. Dimyristoylphosphatidylcholine was 255 μM; enzyme concentration was 0.175 μg/5 ml. In all three figures, ○ and ● represent data obtained at 30 and 17°C, respectively.

effect of varying mole fraction of the products in the substrate vesicles was studied in order to examine directly the effect of the products on the reaction progress curve. As shown in Fig. 2, preincorporation of both the products of hydrolysis in the substrate vesicles eliminates the latency. Experiments not shown here demonstrated that incorporation of either one of the products into the substrate vesicles does not eliminate the latency phase. The results summarized in Fig. 6 show that the latency phase of the reaction progress curve is shortened in the presence of both the products of hydrolysis. Thus both the latency period (Fig. 6B) and the fraction of the substrate hydrolyzed until the beginning of the steady-state phase (Fig. 6C) decreases with increasing mole fraction of the added products. However, the steady-state rate of hydrolysis (Fig. 6A) remains constant at these various added mole fractions of products. This is because the total product concentration at the steady-state phase is probably similar under these conditons. It is particularly striking to note that the latency period disappears when about 20 mol% of the products is externally added to the substrate vesicles. However, as noted earlier, only about 5% of the nascently formed products is enough to bring about the steady-state phase of the reaction progress curve. This raises the possibility that the nascently formed products are more effective (compared to the added products) in incorporating the phospholipase A_2 into vesicles. One of the possibilities is that the premixed products are uniformly distributed in the two monolayer halves of a bilayer. In contrast, the nascently formed products are present only in the outer half of the bilayer [7]. Since about 67% of the total substrate is present in the outer monolayer of the vesicles, the effective mole fraction of the products in the outer monolayer at the beginning of the steady-state phase is about 9%. This still leaves a large discrepancy when compared to the value of 20% for the externally added products required for elimination of the latency phase.

In the calculations made above we have assumed that the lateral distribution of the premixed or the nascently formed products in the vesicles is more-or-less the same. This assumption would be correct if on the time scale of a typical reaction progress curve the nascently formed products were

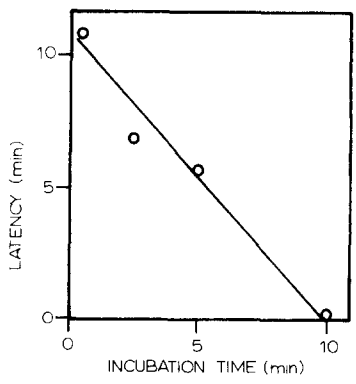

Fig. 7. Time dependence of the intervesicle exchange of products. Ternary codispersions of diether-PC_{14} (128 μM) + products (33 mol%) were incubated in the reaction vessel for 5 min and then dimyristoylphosphatidylcholine vesicles (128 μM) were added. The reaction was initiated by addition of enzyme at the end of the various incubation times (abscissa). Temperature was 30°C, enzyme concentration was 0.175 μg/5 ml. The decrease in the latency (ordinate) following the incubation of the dispersions indicates the extent of transfer of the products from the ternary diether dispersions to the substrate vesicles.

rapidly and completely equilibrated both within and inbetween the vesicle bilayers as they would be in the premixed ternary codispersions. One of the tests of a rapid and uniform redistribution of products would be that the reaction progress curve be first order, that is, the rate of formation of products be directly proportional to the rate of generation of the independent enzyme binding sites in the substrate bilayer. However, the reaction progress curve is not first order. This leads to the conclusion that either the nascently generated products are not readily distributed in bilayers in the same fashion as they are in the premixed ternary codispersions, or the nascently formed products in the vesicles are not readily exchangeable on the time scale of the reaction progress curve. While the first possibility is not completely excluded, the experiments described below support the second conclusion.

The time dependence of the exchange of products between vesicles of the substrate and of the substrate analog diether-PC_{14} is shown in Fig. 7. The latency period for the hydrolysis of the substrate vesicles decreases with the increasing preincubation time of the pure substrate vesicles with the ternary codispersions of the nonsubstrate diether-PC_{14}. In this experiment the rate of ex-

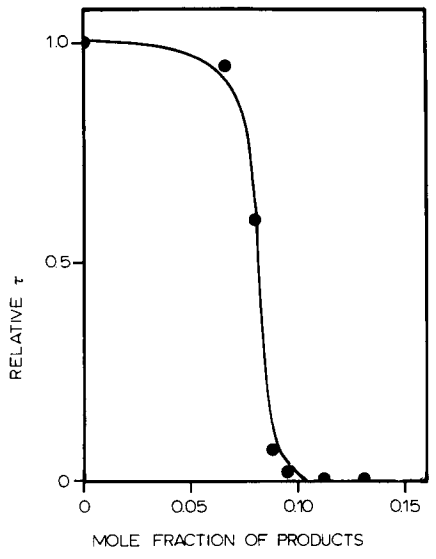

Fig. 8. Dependence of the rate of intervesicle exchange of products on the initial products concentration in the donor vesicles. Codispersions of diether-PC_{14} (64 μM) plus different mole ratios of products were incubated in the reaction vessel for 5 min, the time at which dimyristoylphosphatidylcholine (128 μM) was added. Incubation was continued for 10 min and the reaction was started by addition of enzyme. Temperature, 30°C; enzyme concentration was 0.175 μg/5 ml. The mole fraction values are those after equilibration with dimyristoylphosphatidylcholine. The relative latency period (τ) is normalized from the latency period ($\tau \approx 10$ min) observed in the absence of any added products.

change of the products between the diether-PC_{14} ternary codispersions and the substrate vesicles is slow, and the exchange is apparently complete in about 10 min. This interpretation is based on the assumption that the added enzyme distributes in the two types of vesicle in proportion to the number of the binding sites created by the products. As shown in Fig. 2, the rate of binding of the enzyme to the ternary codispersions is fast (half-time less than 10 s); therefore the dependence of the latency period upon the time of incubation of the vesicles is due exclusively to the rate of exchange of products.

The rate of exchange of products between vesicles could depend upon their mole fraction in the donor and acceptor vesicles. As shown in Fig. 8, preincubation of the ternary nonsubstrate codispersions containing varying mole fractions of products with the substrate vesicles for 10 min shows an abrupt change in the latency period. Interestingly, the latency period changes little between 0 to 7 mol% of products in the substrate after equilibration; however, the latency period decreases if longer preincubation periods are used. In contrast, no latency is observed at more than 10 mol% of products in the substrate. Taken together, the data in Fig. 7 and 8 demonstrate that the rate of exchange of products is quite sensitive to the mole fraction of the products in the donor and acceptor vesicles. Moreover, we believe that the abrupt change at 7 mol% is related to the critical amount of products formed at the end of the latency phase (cf. Figs. 3 and 4). One of the simplest explanations of these observations would be that incorporation of more than the critical mole fraction (about 5 mol%) of products in the substrate vesicles leads to a phase separation in bilayers, whereas at lower mole fractions the products are probably ideally mixed. A lower value for the critical mole fraction of nascently formed products is observed in the kinetic experiments because the rate of redistribution of the products is slow (cf. Fig. 7) and therefore, the nascently formed products may be present at higher local concentration in the substrate bilayer. Additional experiments have demonstrated that the rate of exchange of products from the ternary diether codispersions to dimyristoylphosphatidylcholine vesicles does not depend significantly upon the incubation temperature. Thus the minimum in the latency phase at the phase-transition temperature (cf. Fig. 3) cannot be ascribed to a faster redistribution of the nascently formed products.

General Discussion

The results reported in this paper demonstrate that the latency phase during the hydrolysis of dimyristoylphosphatidylcholine vesicles is dependent upon all the factors that regulate the catalytic activity of the enzyme. Thus the accelerating latency phase of the reaction progress curve is due to the product-assisted binding of the enzyme to the substrate bilayer. This is consistent with our earlier observations [3] that the ternary codispersions containing both the products bind the enzyme because they contain a greater number of binding sites.

The rate of binding of the enzyme to the ternary

codispersions is fast (half-time less than 30 s). This is manifested in the kinetics of hydrolysis of the ternary codispersions which shows no latency phase. Similar behavior is seen with detergent-dispersed substrate [4,5]. This raises questions about the reports that postulate that the binding of the enzyme to bilayer interfaces is rate limiting, and thus responsible for the latency phase. Menashe et al. [8] have assumed such a slow 'activation' step to account for the action of pig-pancreatic phospholipase A_2 on dipalmitoylphosphatidylcholine vesicles. their conclusions are based on an experiment in which the enzyme preincubated with the substrate below its phase-transition temperature shows no latency when the temperature is raised above the phase-transition temperature. Our results (cf. Fig. 6) demonstrate that a latency phase is not observed in the presence of products, and therefore one need not invoke an 'activation' step for the enzyme.

The product-dependent latency period exhibits a minimum at the phase transition temperature of the substrate (Fig. 3, see also Ref. 1). Thus the apparent 'initial' rate of hydrolysis (measured as a single time point) reaches a maximum at the phase -transition temperature [10–12]. These results have been explained by postulating that significantly more enzyme is bound initially to the substrate bilayer at the phase-transition temperature than above and below it. However, we have found no evidence for any significant binding of the pig-pancreatic phospholipase A_2 to diether-PC_{14} vesicles at, below, and above the phase-transition temperature [3]. Moreover, the latency phase can be observed even at the phase-transition temperature of the substrate vesicles (Fig. 6). These results are best explained by the hypothesis that the redistribution of the products formed in the presteady-state phase is most rapid at the phase-transition temperature. Thus the availability of the enzyme binding sites is dependent upon the concentration and spatial distribution of the products, and the redistribution of the nascently formed products is probably fastest at the phase-transition temperature. Dependence of the rate of redistribution of products between bilayers on the phase properties of the bilayers adds a new dimension to our understanding of the phase properties of lipid/water systems. Full implications of this phenomenon and the mechanisms involved in interfacial transfer of lipid molecules are yet to be worked out.

Acknowledgements

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