Enzymes of Lipid Metabolism II

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MECHANISM OF INTERACTION OF PHOSPHOLIPASE A_2 WITH PHOSPHOLIPID SUBSTRATES AND ACTIVATORS

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I. INTRODUCTION

Phospholipase A_2 is one of the smallest and simplest enzymes of lipid metabolism (1). Over the last several years, our laboratory has been focusing on the mechanism by which it interacts with phospholipid in the lipid-water interface and achieves an extraordinary increase in activity over monomerically dispersed substrate. This increase is observed on both micelles of synthetic short chain phospholipid substrates and on mixed micelles with detergents such as Triton X-100. This requirement for an interface for maximal activity has puzzled enzymologists for years.

Key to our studies was the finding (2,3) that the phospholipase A_2 from cobra venom has both an activator site with minimal specificity for a phosphorylcholine-containing lipid and a catalytic site with little specificity for the polar group on the phospholipid. Kinetic experiments show that the enzyme acts best on aggregated substrates such as mixed micelles with surfactants or short fatty acid-containing phospholipids above the cmc. Recent kinetic experiments on synthetic PE derivatives show that at a minimum either the activator or the substrate phospholipid must be in a micelle to achieve activation (4). Activator lipids have been found to cause enzyme aggregation at monomer lipid concentrations using fluorescein-labeled enzyme (5) and gel chromotography (6). Experiments on an immobilized form of the enzyme show that preventing the aggregation of the enzyme prevents activation of the enzyme by activator lipids as well as optimal activity toward interfacial phospholipid substrates (7).

We have recently discovered that a new class of phospholipase A_2 inhibitors that shows anti-inflammatory activity *in vivo* inhibits cobra venom phospholipase A_2 activity toward PC as substrate, but activates the enzyme toward PE as substrate (8). The enzyme from cobra venom has now been compared with that from other sources (6). Together, our recent experimental results have provided support for many features of our "dual phospholipid model" (9) for the mode of action of phospholipase A_2. In this manuscript, our recent experimental data on lipid activation, surface dilution kinetics, and enzyme aggregation will be summarized and discussed in terms of the mechanism of activation and specific models will be considered in detail.

II. LIPID ACTIVATION

Phospholipase A_2 acts very well on PC as substrate and it acts very poorly on PE (10). In the presence of phosphorylcholine-containing lipids, however, PE becomes a very good substrate. In fact, the activation of PE hydrolysis by phosphorylcholine-containing compounds is saturatable (11). Recent kinetic studies (12) are also consistent with the idea that the enzyme has two sites, an activator site, which requires the phosphorylcholine group and a catalytic site, which does not have great specificity for the polar group.

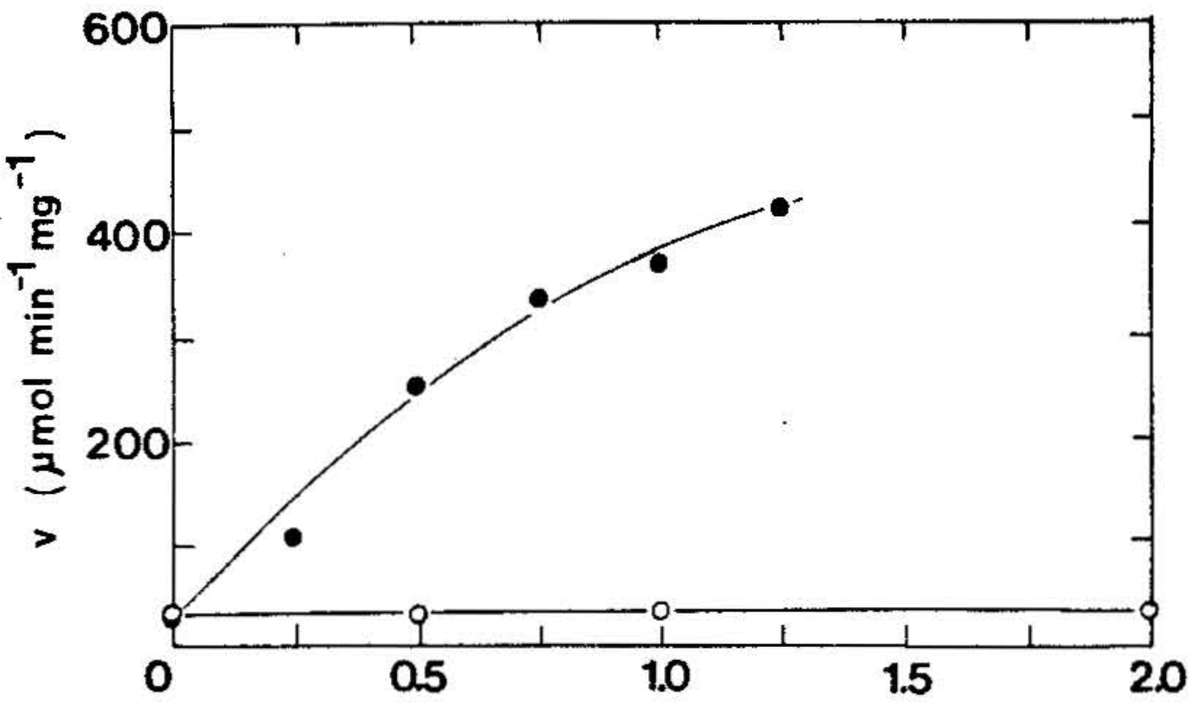


Figure 1: Specific activity of cobra venom phospholipase A_2 for the hydrolysis of PE (5 mM) in mixed micelles with Triton X-100 (20 mM) as a function of dodecyl phosphorylcholine (●) or N, N-dimethyl-N-tetradecyl-1-ammonio- propane-3-sulfonate (DTAPS) (O). Reproduced with permission from (6).

The specificity requirement for the activator is shown in Figure 1. Dodecylphosphorylcholine is an excellent activator whereas an analogue, a sulfobetaine with the same charge distribution and hydrophobicity is not an activator. Interestingly, monomeric PE derivatives are poor substrates even in the presence of Triton micelles. In the presence of monomeric PC activators, monomeric PE is still not activated as shown in Table I. Only in the presence of micelles and a phosphorylcholine-containing activator do we see activation. These and other experiments show that either the substrate or the activator must be interfacial. All of these results together give further support to the suggestion (2,3) that there is an activator site with minimal specificity for a phosphorylcholine-containing lipid and a catalytic site with little specificity for the polar group. In addition, we (4) now know that an interface is also required.

Table 1: Hydrolysis of Dihexanoyl PE[a]

Addition	Triton X-100 (Micelles)	Specific Activity (μmol min^{-1} mg^{-1})
None	-	20
	+	40
Dibutyrylcarbamoyl PC (Monomeric)	-	25
	+	280
Sphingomyelin	+	870

[a]Adapted with permission from data in (4).

III. SURFACE DILUTION KINETICS

According to our hypothesis of "surface dilution kinetics" (13-15), if the phospholipase binds a phospholipid in its activator site and then needs to bind a second phospholipid in its catalytic site, it must search in the two-dimensional interface for that second phospholipid. In other words, there is a second important concentration term in two dimensions of the interface. As one adds surfactant to the mixed micelle and dilutes the phospholipid in that surface, the concentration of phospholipid falls off and the enzymatic activity also falls off proportionately. This is indeed what was observed experimentally. These results are consistent with a two-step model as shown in the following equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ESS \xrightarrow{k_3} ES + Prod$$

[BULK] [SURFACE]

First the enzyme binds to a substrate phospholipid to form an ES complex. This depends on the bulk concentration of phospholipid. The ES complex then binds a second phospholipid substrate and this depends on a surface concentration term to form the ESS complex which is the Michaelis complex. We (12,16) have recently carried out a detailed kinetic analysis of this model using synthetic thio-containing phospholipids for which the activities could be very accurately measured. We carried out our experiments on PC and PE as well as activated PE. The results were found to be consistent with the model.

IV. ENZYME AGGREGATION

The enzyme from pancreas is a monomer at all concentrations whereas the enzyme from Crotalus venom is a dimer at all concentrations (6). The enzyme from N.Naja Naja cobra venom, which we have been studying, is intermediate in that it undergoes a rapid monomer-dimer equilibrium at moderate concentrations. At assay concentrations, it is a monomer; however, we

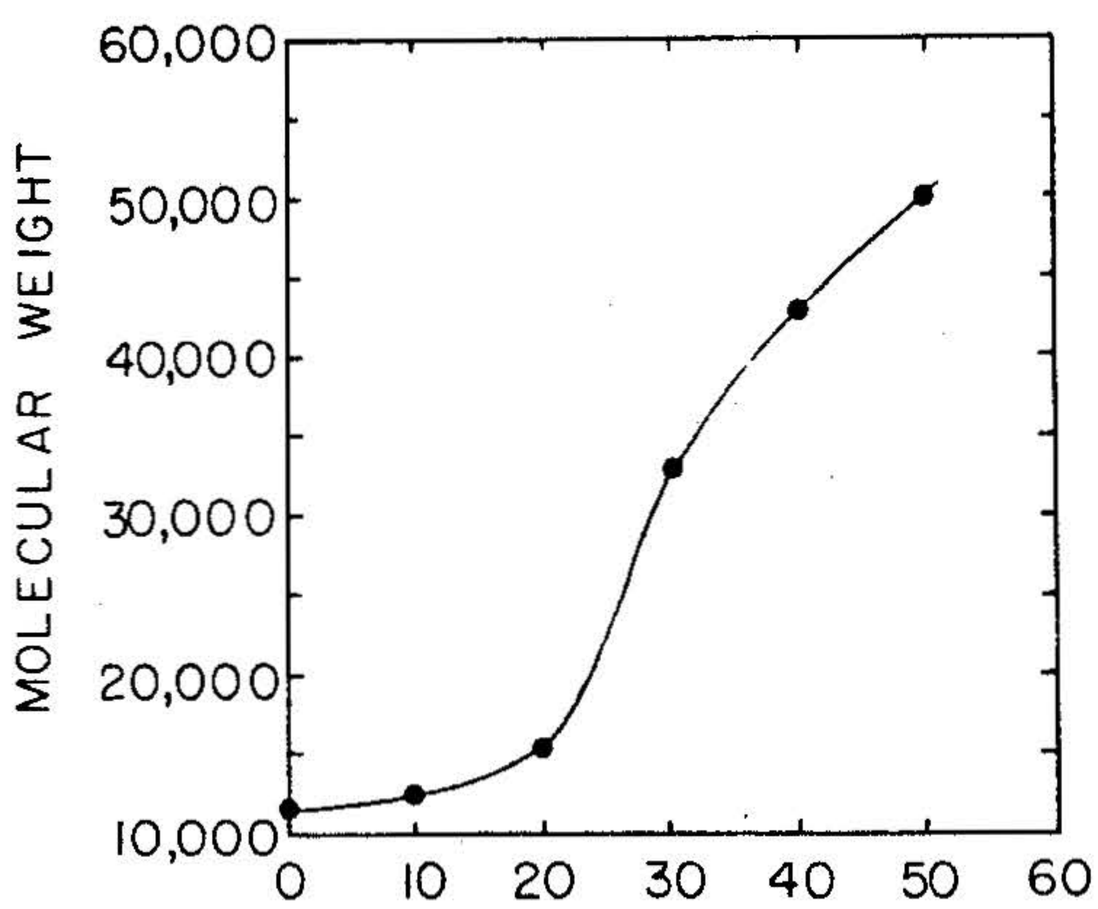


Figure 2: Apparent molecular weight of cobra venom phospholipase A_2 as a function of dodecyl phosphorylcholine concentration, as determined by gel chromatography on Sephadex G-75. The buffer contained 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, and dodecyl phosphorylcholine as indicated. Reproduced with permission from (6).

discovered that in the presence of lipid the enzyme aggregates. This is illustrated in Figure 2 for the very good activator dodecylphosphorylcholine which at concentrations far below its cmc causes a dramatic aggregation of the enzyme. Interestingly, this "activator" does not activate the hydrolysis of the monomeric substrate dibutyryl PC when the dodecylphosphorylcholine is below the cmc as shown in Figure 3.

In order to test the idea of "lipid-induced aggregation" (9) more directly, we recently prepared fluorescein-labeled phospholipase A_2 (5). The derivatized enzyme from N. naja naja cobra venom remained fully active and was employed in fluorescence polarization measurements where it gave an approximate molecular weight consistent with sequence data. As controls, the enzyme from pancreas, which is a monomer, and the enzyme from Crotalus, which is a dimer, gave consistent molecular weights. For the N. naja naja enzyme, polarization increased with increasing concentrations of diheptanoyl PC below the cmc at monomeric concentration. The experimentally observed polarization is consistent with a transformation from a monomer to dimer or larger aggregate. Similarly, with dodecylphosphorylcholine at monomeric concentrations, an increase in polarization was observed. Interestingly, the pancreatic enzyme and the Crotalus enzyme under these conditions remained monomeric and dimeric, respectively (5).

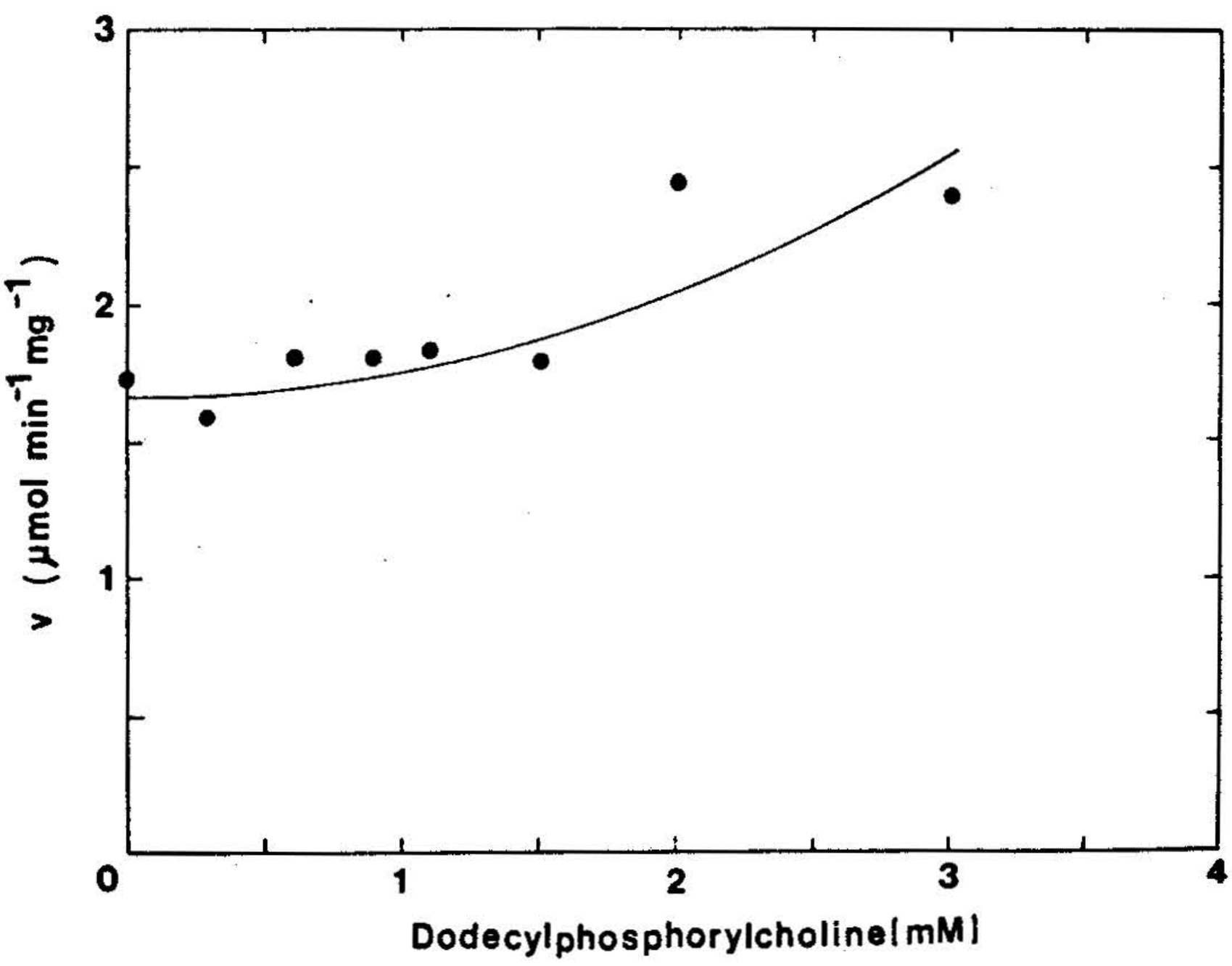


Figure 3: Specific activity of Cobra venom phospholipase A_2 for the hydrolysis of dibutyryl PC (5 mM) as a function of dodecylphosphorylcholine concentration. Standard pH-stat assay conditions (13,17) were utilized, except that no detergent was included.

As another approach to look at the requirement for aggregation, we (7) recently immobilized the enzyme in a manner which apparently prevents aggregation. The activity of the immobilized enzyme was identical to that of the soluble enzyme toward monomeric phospholipids below the cmc, but it did not increase above the cmc. Interestingly, the affinity of the immobilized enzyme was similar to the soluble enzyme toward micelles but the V_{max} was considerably less for the immobilized enzyme then for the soluble enzyme.

When PE is the substrate for the immobilized enzyme, phosphorylcholine-containing compounds are not able to activate it. In other words, the immobilized enzyme cannot be activated by either interface or phosphorylcholine-containing compounds, presumably because it is restricted from forming the proper aggregates.

We (8) have recently studied a natural product from sponge called manoalide which shows activity in vivo as a prostaglandin inhibitor. Modified enzyme is inhibited 50% toward long chain PC in mixed micelles with Triton X-100, diheptanoyl PC micelles, and diheptanoyl PE micelles in the presence of an activator lipid dodecylphosphoylcholine. Interestingly, the modified enzyme is actually activated by about the same factor toward PE substrates. This suggests that covalently bound manoalide is able to alter the specificity of the enzyme toward PE versus PC, possibly related to the manner in which activator molecules affect the enzyme.

V. ACTIVATION MECHANISMS

Recent results (6) with the phospholipase A_2 from bee venom, pancreas, Crotalus, and the cobra suggest that while the four phospholipases A_2 carry out the same catalytic reaction (1), differences in their interfacial interactions exist. We find that the dramatic activation of PE hydrolysis by phosphorylcholine-containing lipids (2,3,11) is unique to the enzyme from cobra venom among the enzymes examined. If the results found (6) with the other enzymes are viewed as controls for the assay and mixed micelle system, they support the contention that this activation is clearly not an experimental artifact. While the specific interactions may not be general for all phospholipases, they do allow insight into the general catalytic mechanisms involved. In particular, the dramatically increased reactivity of most lipolytic enzymes toward aggregated substrates may become susceptible to analysis through the cobra venom enzyme.

The activation can in principle occur through two different mechanisms: (i) It could occur via a change in the surface structure of the substrate, i.e. a long range or indirect effect of the activator on the substrate. This might result in a different affinity of the enzyme for the substrate, different mode of binding (potentially influencing k_{cat}), or a different rate of an on- or off-step. (ii) It could occur via a binding of the activator to the enzyme, i.e. a direct effect on either binding, catalysis, or the off-step.

We (11) have previously reported evidence against a change in the surface structure being responsible for the activation. Briefly, the extreme specificity of activators for the phosphorylcholine (polar) part of the activator molecule, but promiscuity on the hydrophobic part (2,11,12,16), the low surface molar fraction necessary to achieve activation and the observation that the cobra venom enzyme can be activated by soluble phospholipid analogs (11) all argue against an indirect activation via a change in surface structure, i.e. without the activator binding to the enzyme.

We conclude, therefore, that the activator must bind to the enzyme. We want now to distinguish detailed modes of activation, namely, how the activator achieves rate enhancements of the enzyme for PE hydrolysis. Four possible models have been developed and are presented in Figure 4.

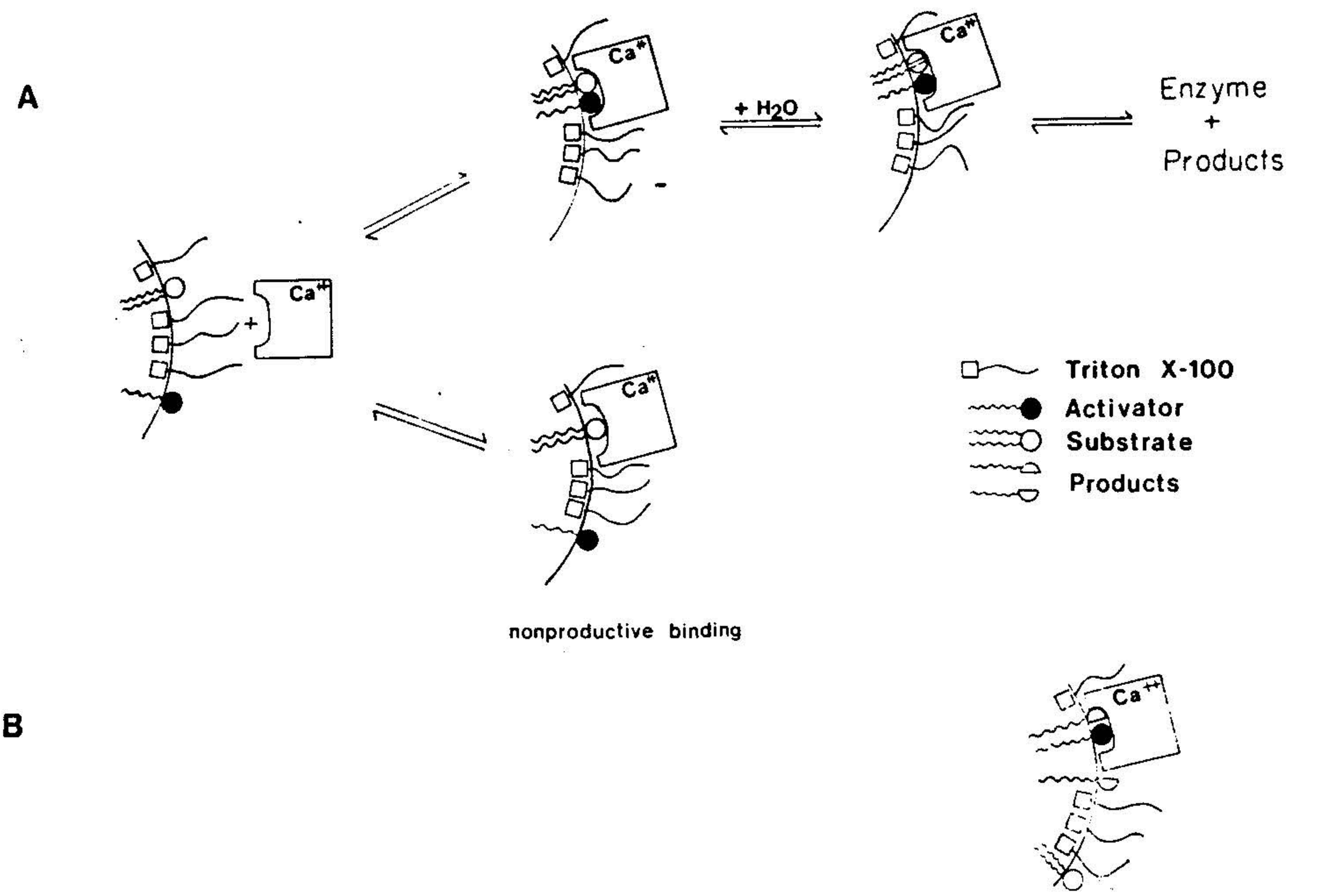


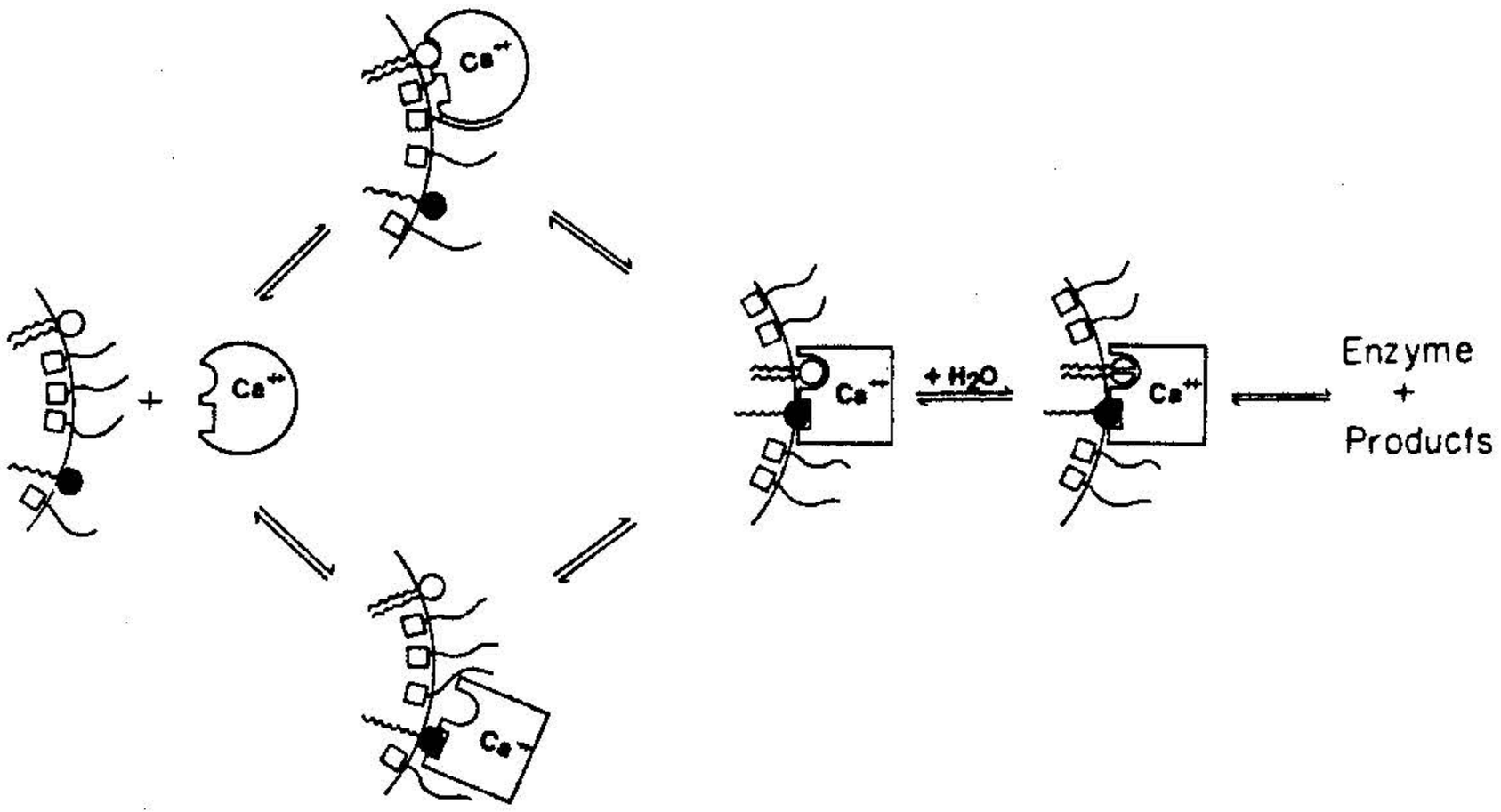
Figure 4: Possible simplified models for the activation of cobra venom phospholipase A_2 by phosphorylcholine-containing compounds. In all drawings, the binding of Ca^{2+} to the enzyme is assumed to precede the steps shown although this need not be the case. It is not implied in any of the mechanisms shown that the enzyme necessarily leaves the interface after each catalytic cycle.

(A) Productive binding model: The activator increases the amount of productive binding. Here this is assumed to not involve a conformational change of the enzyme, but merely a direct binding of the activator. A conformational change for the enzyme would imply models C or D.

(B) Product removal model: The activator facilitates product removal. In the model shown here, only one of the products is assumed to dissociate slowly from enzyme. Alternatively, the activator could assist in the removal of both products. Substrates, on the other hand, would not generally accelerate product removal as effectively as the activator would.

(continued)

C



D

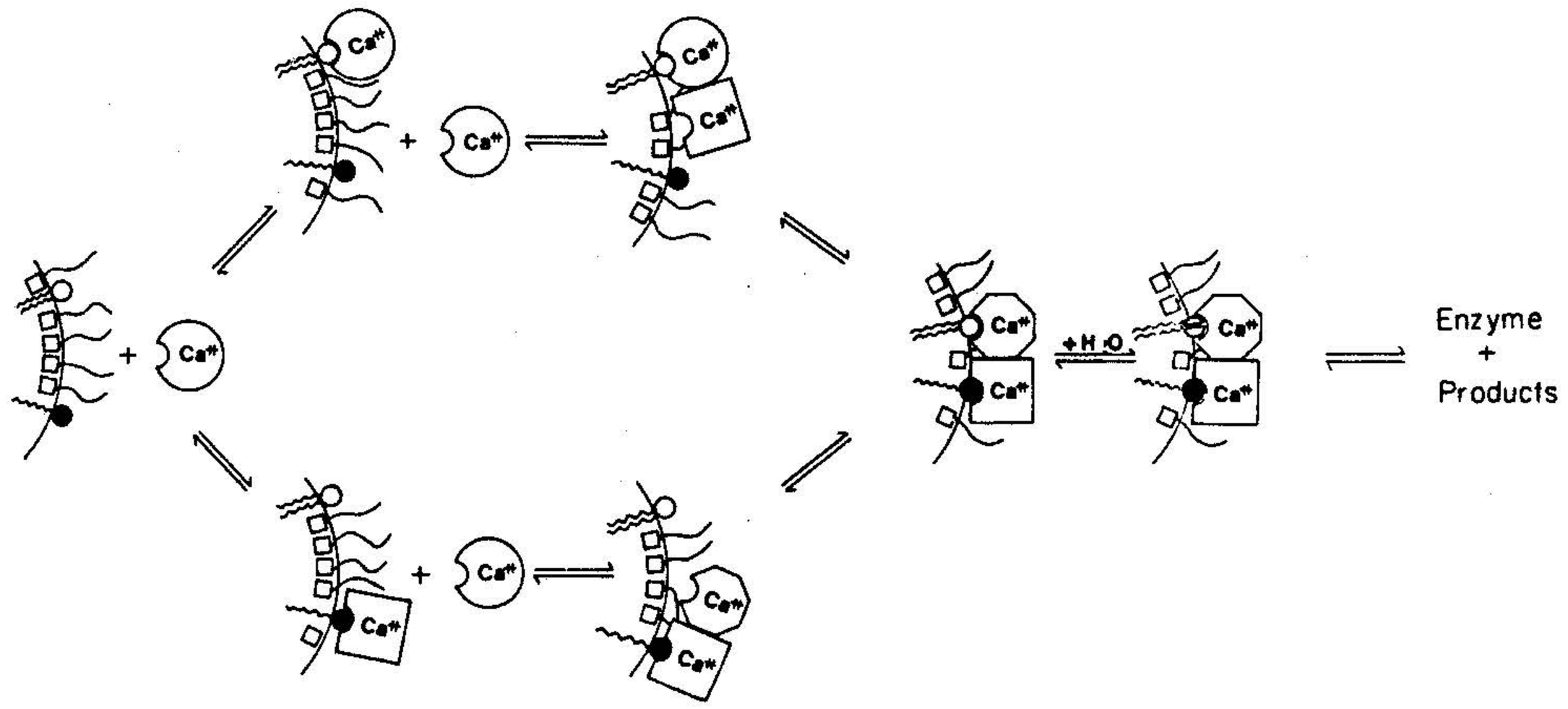


Figure 4: (C) Two site single subunit model: The enzyme monomer has two functional sites, an activator site and a catalytic site. The activator is assumed to cause a conformational change indicated by the transformation from a circle to a square. A random mechanism of binding of activator and substrate is assumed here. The numerous pathways in which the enzyme does not bind both activator and substrate to their correct sites are not shown, nor which of these would be nonproductive.

(D) Two site dimer model: Binding of enzyme to aggregated phospholipids or analogs is assumed to induce aggregation of the enzyme shown here as resulting in functional dimers. Binding of activator and substrate is assumed to proceed in a random fashion. The induced dimer as shown here would be functionally asymmetric. One subunit (square) may be responsible for the activation, while the other (octagon, when activated; circle, when not activated) may carry out catalysis. Alternatively, two micelle-bound enzyme molecules may dimerize directly or the small amount of enzyme dimer, which is present at equilibrium, may preferentially bind to the micelle. Only those pathways are shown, in which the dimer binds one molecule of substrate and one molecule of activator. A combination of models C and D would imply that each subunit carries binding sites for activator and substrate, as explained in the text.

VI. POSSIBLE MODELS FOR ACTIVATION

Binding and Product Effect Models: If the activator only increased the affinity of the enzyme for PE, no effect on V_{max} would be expected, contrary to the observations (2,11,12,16). If it increased the fraction of productive binding, as illustrated in Figure 4A, one would indeed raise V_{max}, but also Km by the same factor. While experimental limitations of the pH - stat system (17) have prevented us from obtaining accurate kinetic data over a sufficient concentration range with natural PE and PC, this problem was solved by carrying out such measurements with a thioester analog of PE (thio PE) (12) using a spectrophotometric assay. In this case, the relevant Km (12) is similar for thio PC, thio PE, and thio PE in the presence of activator, whereas V_{max} values differ greatly. This argues against the first model, strictly only for the thioester analogs, but since they behave identically to oxo-ester phospholipids in all respects examined, we believe this to hold for the latter as well.

Alternatively, one of the product release steps may be rate limiting for V_{max}. The activator may then accelerate this product release. The enzyme would then at least have to be able to bind activator and one of the products to accomplish this, which is not quite the same as requiring it to bind two phospholipid molecules simultaneously (Figure 4B). We have so far only partial information on the nature of the slowest elementary step and cannot yet rigorously rule out this mechanism. However, we can cite several circumstancial pieces of evidence that make this mechanism unlikely.

First, relying again on the more accurate rate determinations that have proven to be possible with the thio-phospholipid analogs (12,16), we note that the activation of thio PE hydrolysis by PC increases V_{max} and V_{max}/Km by approximately the same factor. This implies that the V_{max} limiting step corresponds to the highest overall transition state in the free energy profile (the V_{max}/Km limiting step). While not impossible, it would not be expected that this would be the transition state for the first of the product-release steps in a highly evolved enzyme catalyzing a strongly exergonic reaction (18) and it cannot be the transition state for any step following the first off-step (19).

Secondly, the inhibition by fatty acid is very weak when PC is the substrate and not detectable at all when PE is the substrate (6). Inhibition by lysophospholipid is detectable in neither case. This seems at first consistent with either the EP_1P_2 or EP_2 complexes (i.e. the enzyme with both or one product still bound) being of relatively high free energy relatively to $E + P_1 + P_2$ (i.e. the free enzyme) and therefore potentially implying a relatively high-lying transition state for one of the off-steps; indeed, potentially higher in PE than PC hydrolysis. If the slower off-step was really predominantly rate determining and if the activator stabilized the transition state of one of the off-steps by stabilizing either EP_1P_2 or EP_2, one would expect more product inhibition by fatty acid or lysophospholipid in PE hydrolysis when the enzyme is activated than when it is not activated. This is contrary to observation (6) and suggests that the fatty acid effects may rather be due to their binding to a second site, as elaborated below. We believe therefore, that the product release model is not consistent with the experimental data.

Conformational Change Models: A third alternative is that the activation might be brought about by a conformational change of the enzyme by the activator. This is shown schematically in Figure 4C. The conformational change could accelerate the hydrolysis step directly or itself be kinetically significant. These possibilities are in principle distinguishable by the measurements of kinetic isotope effects, but we do not yet have sufficient data bearing on this question.

The intrinsic susceptibility to ester hydrolysis of the sn-2 chain should not be different in PE and PC. Indeed, the nonenzymatic hydrolysis rates (20) are similar and most enzymes which we tested do not discriminate kinetically very strongly between these substrates. Recent experiments with manoalide in our laboratory (8) suggest, however, that a covalent modification of the enzyme from Naja naja naja inhibits the hydrolysis of a PC substrate, whereas it increases the rate on a PE substrate, suggesting either a somewhat different binding of these substrates to the catalytic site or to the postulated activator site of the enzyme.

The other enzymes which we investigated give no kinetic indication for a PC-specific second site that might specifically activate PE hydrolysis, but such a site may be involved in all the enzymes in the high-activity hydrolysis of aggregated substrates, albeit with somewhat different specificity in each case (see below). We (4) must require in this model that proper activation (i.e. binding to the activator site) can only occur when an interface is present, since we have found that a water-soluble phospholipid analog, which activates PE when an interface is present, acts as a competitive inhibitor, when water-soluble monomeric PE is used as substrate (4).

Aggregation Models: Finally, we must consider the likelihood that more than one subunit may be involved in the activation of the cobra venom enzyme. The tendency to aggregate is indeed one of the most notably different characteristics among the enzymes (1,5,6,21,22). The cobra venom enzyme is aggregated by low amounts of water-soluble substrate analogues (5,6) and only this enzyme is activated by those phosphorylcholine-containing substrates and analogs. The close analogue of dodecylphosphorylcholine, DTAPS, neither activates nor aggregates the enzyme under comparable conditions. The pancreatic enzyme, on the other hand, appears to be activated (6) and aggregated (23,24) by negatively charged surface active compounds. This model does not require the monomer to be inactive, only that the aggregated enzyme has increased activity. We have shown the simplest view in Figure 4D, where each subunit is depicted to bind only one lipid molecule: substrate or activator (similar to our earlier proposal (9,25)). While the aggregation of the cobra venom enzyme clearly proceeds beyond a dimer (5,6), we do not know whether it is dimers that aggregate to higher oligomers since in our experiments we have so far only obtained molecular weight averages.

The question then arises whether this aggregation is coincidental to activation or is a prerequisite for activation for cobra venom phospholipase A_2. Recent experiments (7) showed that the immobilized cobra venom enzyme, while having kinetic constants identical to the soluble enzyme toward monomeric substrates, shows highly reduced rates toward PC or PE in mixed micelles and, most importantly, cannot be activated toward PE by dodecylphosphorylcholine. Unfortunately, it is not easy to distinguish between an activator site that is blocked by the crosslinker and the prevention of enzyme aggregation in the immobilized state. We can, therefore, only state that the enzyme is aggregated in the activated state with micellar substrates.

Is aggregation then sufficient to bring about activation toward any substrate? We suggest that this is not the case since monomeric dibutyryl PC is not hydrolysed faster in the presence of dodecylphosphorylcholine below the cmc (<1 mM) (Figure 3). This suggests that the aggregated enzyme is of advantage only for micellar substrates. In agreement with this view is the observation (26) that the Crotalus enzyme, while always being dimeric, still hydrolyzes aggregated substrates with a much higher V_{max}. Finally, dodecylphosphorylcholine does not competitively inhibit the enzyme with respect to the monomeric substrate (6). (A fortuitous cancellation of activation and inhibition, of course, cannot be rigorously excluded). These facts are difficult to reconcile with each subunit binding only one lipid. It must also

be noted that in the crystal structure of the Crotalus dimer, it does not seem possible to bind substrate molecules in the same surface to both active sites simultaneously (27).

VII. CONCLUSIONS

From the experiments discussed herein it is clear that none of the four models as schematically drawn is consistent with all observations. Experimental data appear to not be consistent with models A and B but all of the results could easily be accomodated by a model that is a combination of Figure 4C and D. Thus it is possible that each subunit may bind more than one phospholipid or analogue, but activation (e.g. a necessary conformational change) is achieved only by aggregated lipid leading to aggregated enzyme. This may be common for all related phospholipases in that each phospholipase may have a different specificity for binding to its activator site. We (6) found that in the cobra venom enzyme, only the binding of phosphorylcholine-containing compounds leads to a strong activation, whereas negatively charged detergents and fatty acid products lead to a weak activation.

Similarly, for the pancreatic enzyme, we (6) have recently shown that a dramatic activation may occur only by negatively charged detergent-like "activators" or fatty acid products. This explains the long standing puzzle of why the pancreatic enzyme does not act on PC in Triton X-100 micelles without the presence of fatty acid product or crude egg yolk emulsions which contains negatively charged lipids. We would suggest that the activator site of this enzyme has a specificity for negatively charged lipids; this is fulfilled in vivo by the negatively charged bile salts which emulsify the lipid substrates during digestion. De Haas and coworkers (23,24) have recently observed an aggregation of the enzyme from porcine pancreas caused by negatively charged detergents to give enzyme aggregates which would support our suggestion of an activator site for that enzyme. For the cobra venom enzyme, we have presented evidence that phosphorylcholine-containing compounds both activate and aggregate the enzyme. These observations strongly point to a common activation mechanism for all phospholipases.

VIII. ACKNOWLEDGEMENT

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