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Biochemistry

Dual role of interfacial phospholipid in phospholipase A_2 catalysis

(interfacial binding/surface dilution/lipid-induced asymmetric dimer/half-site reactivity/mixed micelles)

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ABSTRACT **The results of crosslinking experiments with dimethyl suberimidate and gel filtration binding studies are used to delineate a detailed model for phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) action in the presence of Ca^{2+} on mixed micelles of Triton X-100 and phospholipid. Important features of the "dual-phospholipid" model are: (*i*) the use of the nonionic surfactant as an inert matrix that may influence lipid conformation but does not interact with the enzyme; (*ii*) the involvement of two lipid molecules in a single cycle of catalysis as an explanation for the "surface dilution" phenomenon; (*iii*) the requirement of an ordered reaction whereby divalent metal ion binds prior to phospholipid binding; and (*iv*) the induction by lipid substrate of an asymmetric dimer structure for the enzyme.**

Phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) is a small, water-soluble enzyme that hydrolyzes the fatty acid ester bond at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides. The main difference between the action of this enzyme and that of other esterases is that the phospholipase acts on substrates that are part of a lipid/water interface. Previous studies have suggested that the interface plays an important role in the enzymatic activity, although the precise function of the interface has not been elaborated (1). Extensive kinetic and chemical studies have been conducted in this laboratory (2, 3) on the action of cobra venom phospholipase A_2 (*Naja naja naja*) on mixed micelles formed from the nonionic surfactant Triton X-100 and long-chain phospholipids. A mechanism for the action of this enzyme on mixed micelles must explain two key observations. First, as the concentration of Triton relative to that of phospholipid is increased, enzyme activity decreases. This phenomenon, called "surface dilution," has been discussed as arising from association of enzyme with the surface of the mixed micelle and dilution of phospholipid in that surface (4). Second, phospholipase A_2 exhibits half-site reactivity in its modification by *p*-bromophenacyl bromide (5). The inactive enzyme derivative has 0.5 mol of histidine modified per mol of enzyme. Such behavior is unusual for an enzyme that exists as a monomer at assay concentrations (0.2 μg ml^{-1}) in the absence of substrate (3).

We have now used the results of crosslinking experiments with dimethyl suberimidate and binding studies using gel filtration to delineate a detailed model for phospholipase A_2 action. This model is novel in two important respects: (*i*) two phospholipid molecules are required, one to sequester the enzyme to the interface, the other for subsequent catalysis; and (*ii*) lipid substrate is essential for enzyme aggregation, and it is the resulting dimer unit that is the active form of the enzyme. Implications of this "dual-phospholipid" model will be discussed.

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EXPERIMENTAL PROCEDURE

Materials. Lyophilized cobra venom, *Naja naja naja* (Pakistan), lot no. NNPOL and NNP45-1Z, was obtained from the Miami Serpentarium. The phospholipase A_2 was purified by the procedure of Deems and Dennis (3) as modified by Roberts *et al.* (6). Enzymatic activity toward phosphatidylcholine in mixed micelles with Triton X-100 was followed by the pH-stat technique (7). Triton X-100 (Rohm and Haas), *n*-dodecyl octaethylene ether (Nikol Chemical), dipalmitoyl phosphatidylcholine (Calbiochem), L-α-glycerophosphorylcholine, phosphorylcholine, and dimethyl suberimidate dihydrochloride (Sigma) were used without further purification.

Gel Filtration Binding Studies. The equilibrium gel filtration technique of Hummel and Dreyer (8) was used with Sephadex G-25 (1 × 30 cm column) to measure the affinity of phospholipase A_2 for the small ligands phosphorylcholine and glycerophosphorylcholine. Phosphorus analyses (9) were used to determine ligand concentration. A similar technique using Sephadex G-100 was used to determine the affinity of enzyme for micelles and mixed micelles, which are larger structures than the enzyme. The column (1 × 50 cm) was equilibrated with phospholipase A_2 (0.04–0.09 mg ml^{-1}) in 0.05 M Tris·HCl, pH 8.0. A monomer concentration of detergent was generally present in the buffer. Various metal ions were included in the buffer. Surfactant (1 or 2 ml of 50–100 mM) as micelles or Triton-phospholipid mixed micelles (50 mM total concentration) were applied to the column. The concentration of enzyme eluted was measured by activity. The concentration of Triton eluted was monitored by its absorbance at 276.5 nm, and the concentration of phospholipid was determined by phosphorus analyses or from the radioactive profile of mixed micelles which contained trace amounts of [^{3}H]dipalmitoyl phosphatidylcholine (Applied Science). The binding constant to phospholipase was determined from the equal peak and trough areas as well as from the amounts eluted in the individual fractions in the lipid–protein peaks. The mixed micelle binding of *p*-bromophenacyl bromide-modified enzyme, which is inactive, was followed using the ^{14}C-labeled enzyme.

Crosslinking Experiments. Enzyme, 0.60–0.012 mg ml^{-1} in 0.05 M borate, pH 8.5, was incubated with 10 mM dimethyl suberimidate for 2 hr. The imidate was added from a stock solution prepared immediately before use. The reaction was stopped by the addition of 10 mM lysine. The effect of surfactant, metal ions, and mixed micelles on the aggregation state of the enzyme was determined by crosslinking in the presence of these materials. The crosslinked protein was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis done as described previously (3). A Gilford gel scanner was used to quantitate the amount of phospholipase present as monomer and crosslinked multimers.

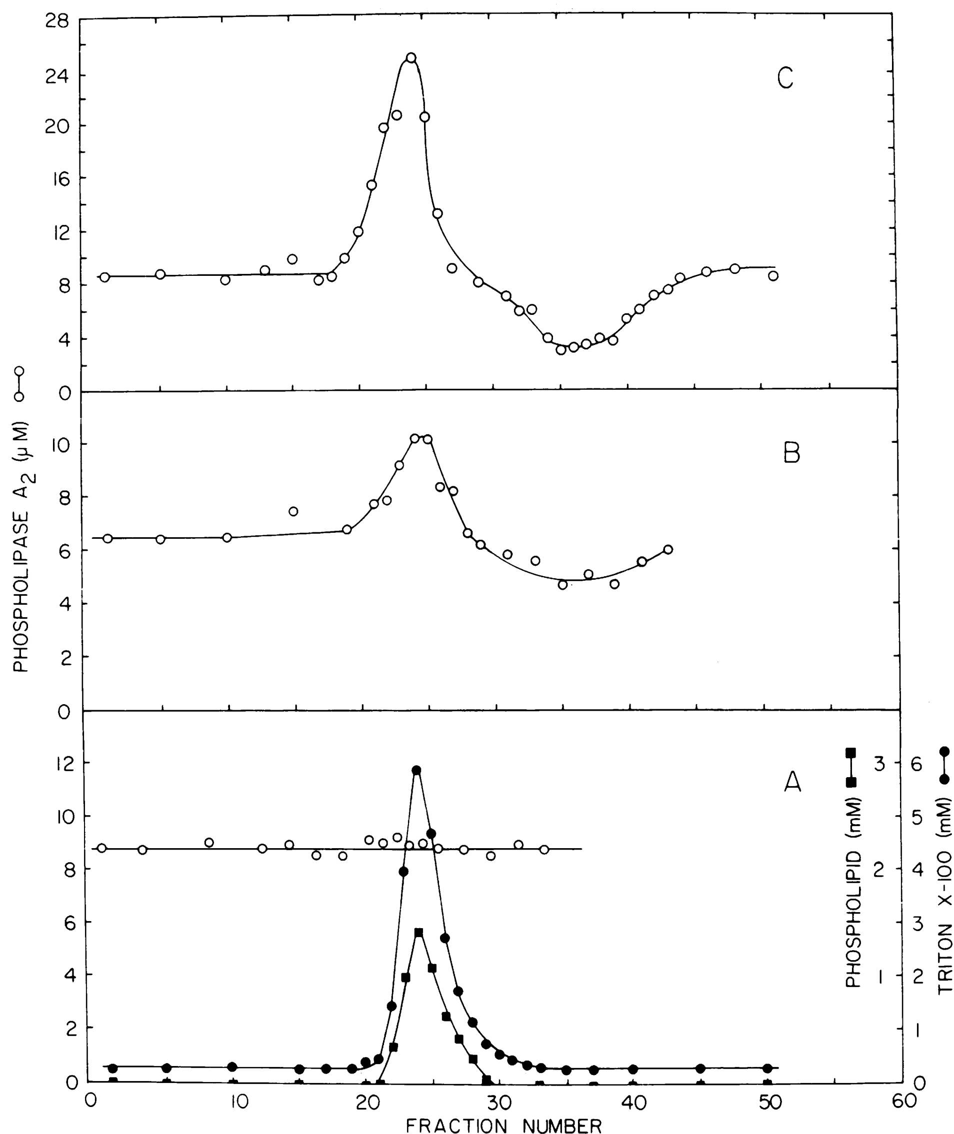

FIG. 1. Typical elution profiles used in measuring the affinity of phospholipase A_2 for Triton-phospholipid mixed micelles. A Sephadex G-100 column was equilibrated with enzyme, EDTA or Ba^{2+} ions, and 0.3 mM Triton X-100 in 0.05 M Tris·HCl, pH 8.0, at 40°. (*A*) A mixture (1 ml) consisting of 40 mM Triton X-100 and 9.5 mM dipalmitoyl phosphatidylcholine was applied to a column equilibrated with 8.7 μM enzyme and 5 mM EDTA. Elution profiles are shown for phospholipid (■), Triton (●), and enzyme (○). (*B*) The enzyme elution profile is shown for a column equilibrated with 10 mM Ba^{2+} and 6.2 μM enzyme with 2 ml of a mixture of 40 mM Triton and 10 mM dipalmitoyl phosphatidylcholine applied to the column. (*C*) The enzyme elution profile is shown for a column equilibrated with 8.7 μM enzyme and 5 mM EDTA with 1.4 ml of a mixture of 80 mM Triton and 9.6 mM palmitic acid applied to the column.

RESULTS

Gel Filtration Binding Studies. Examples of Sephadex G-100 column profiles of phospholipase A_2 interacting with micelles are shown in Fig. 1, and the results are summarized in Table 1. One striking feature of these binding studies is the lack of significant affinity of the cobra venom enzyme for pure Triton X-100 or *n*-dodecyl octaethylene ether micelles even when the enzyme is saturated with Ca^{2+}. A significant enzyme–micelle interaction is detected only when both phospholipid and metal ions are present along with the detergent. The apparent dissociation constant calculated for the Zn^{2+}–phospholipid–Triton–enzyme complex is about 1 mM in terms of bulk phospholipid concentration. This is about one-third that for the Ba^{2+}–phospholipid–Triton–enzyme complex. If metal ions are not bound to the enzyme, significant mixed micelle binding is not observed.

Enzyme modified with *p*-bromophenacyl bromide, though inactive, still binds to phospholipid–Triton X-100 mixed micelles in the presence of Ca^{2+}. A similar magnitude for the dissociation constant is observed, i.e., 1–2 mM in bulk phospholipid concentration. This derivative has 0.5 mol of histidine modified per mol of enzyme, and is thought to associate as functional dimers under assay conditions (5). The ability of modified enzyme to still bind phospholipid may provide evidence for a distinct phospholipid surface binding site separate from a phospholipid catalytic site.

The products of the reaction, fatty acid and lyso-phosphatidylcholine, when incorporated into Triton micelles, also bind to the enzyme. The binding constants are similar to that for the phospholipid mixed micelles; 1.0 mM for palmitic acid, 3.1 mM for lyso-phosphatidylcholine, but metal ion is not required for this binding.

Binding experiments by Sephadex G-25 chromatography show that the soluble zwitterionic portion of phospholipids does not by itself interact strongly with phospholipase A_2. No binding of phosphorylcholine or glycerophosphorylcholine in the

Table 1. Binding of various micelle systems to phospholipase A_2 as measured by gel filtration*

Enzyme (mg ml^{-1})	Metal ion	Micelle system (mol ratio)	Temperature (°C)	Dissociation constant† (mM)
0.07‡	10 mM Ca^{2+}	Triton	25	∞
0.04	10 mM Ca^{2+}	*n*-Dodecyl octaethylene ether	25	>120
0.06‡,§	10 mM Ca^{2+}	3:1 Triton/egg PC	25	1.8
0.09	5 mM EDTA	4.2:1 Triton/DP PC	40	>28
0.07	10 mM Ba^{2+}	4.5:1 Triton/DP PC	40	3.5
0.07¶	1 mM Zn^{2+}	4:1 Triton/DP PC	40	1.1
0.10	5 mM EDTA	8.3:1 Triton/palmitic acid	40	1.0
0.09	3 mM EDTA	5.4:1 Triton/lyso PC	40	3.1

* Unless specified, the buffer system contained enzyme in 0.05 M Tris·HCl, pH 8.0, and a monomer concentration of surfactant (0.3 mM Triton or 0.08 mM *n*-dodecyl octaethylene ether). DP refers to dipalmitoyl and PC to phosphatidylcholine.

† Dissociation constants are given in terms of the bulk concentration of phospholipid, fatty acid, or lyso-phospholipid, not in terms of the surface concentration in the mixed micelles. When the surfactant alone was used, the dissociation constant was calculated in terms of the bulk concentration of the surfactant.

‡ The column was not pre-equilibrated with a monomer concentration of Triton.

§ Enzyme modified with *p*-bromophenacyl bromide.

¶ The Zn^{2+}-mixed micelle system was studied in 0.05 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 7.0.

presence of Ca^{2+} could be detected within the experimental error of the columns. This would indicate that the binding must be at least a factor of 10 weaker than for the metal–phospholipid–Triton–enzyme complex.

Crosslinking with Dimethyl Suberimidate. *p*-Bromophenacyl bromide modification experiments strongly implied that in the presence of substrate phospholipase A_2 was a dimer or higher-order aggregate composed of asymmetric dimer units (5). When lipid is absent, the enzyme is a monomer at dilute concentrations (0.025 mg ml^{-1} or less) (3). Although Ca^{2+} and Ba^{2+} alter the monomer/dimer equilibrium somewhat, the enzyme is a monomer at assay concentrations in the presence of these metal ions (5).

Crosslinking experiments were undertaken to see if dilute phospholipase A_2 aggregates in the presence of substrate micelles. Dimethyl suberimidate was used as the chemical crosslinking reagent. The results are summarized in Table 2. At concentrations where physical studies show that the enzyme is a dimer, only a moderate amount of covalently linked enzyme is formed. The reaction conditions were in the extreme range of those suggested by Davies and Stark (10). Perhaps the bonding domains between phospholipase chains do not contain lysine residues in positions that can be crosslinked readily. When enzyme is diluted to 0.06 mg ml^{-1}, there is a negligible amount of crosslinking. When very dilute enzyme (0.01 mg ml^{-1}) is incubated with Triton, Ba^{2+}, or Ca^{2+}, there is no increase in crosslinked enzyme. When incubated with mixed micelles in the presence of EDTA, there is a slight increase in crosslinked enzyme, but small amounts of metal ion are probably still present and may be responsible for the slight increase. However, the addition of Ba^{2+} with mixed micelles causes a large increase in the relative amount of dimers and higher-order species. (It is not clear whether the trimers reflect functional aggregation beyond the dimer state or result from the crosslinking of reacted dimers.) Crosslinking in the presence of Zn^{2+} was not attempted, because at pH 8.5, where the crosslinking reaction is favored over suberimidate hydrolysis, Zn^{2+} is precipitated.

DISCUSSION

Enzymes that act on substrates that are localized in lipid/water interfaces can do so in two ways. The first is for a water-soluble enzyme to interact only with the substrate molecule at the surface; thus only the bulk concentration of substrate would influence the reaction. The second is for the enzyme to be sequestered in the interface, either as an integral or peripheral protein. In this case, the substrate concentration in the two-dimensional surface would be important. In the phospholipid/Triton X-100 mixed micelle system, hydrolysis by phospholipase A_2 depends on both bulk and surface phospholipid concentrations (2), i.e., there is a "surface dilution" phenomenon. This requires the enzyme to undergo at least two binding steps before catalysis occurs; during one of these binding steps the enzyme must be sequestered at the surface.

Such kinetic behavior could be explained by two simple models. In the first of these, the water-soluble enzyme binds to the interface itself, not to a particular substrate molecule. Once the enzyme is bound to the surface it then specifically binds a phospholipid molecule in its active site. In the second model, the enzyme interacts solely with substrate molecules. The enzyme binds to one phospholipid molecule and is sequestered to the surface. Another phospholipid molecule must be bound for catalysis to occur.

The only difference between these two models is the manner in which the enzyme is held to the interface. If the first model

Table 2. Effect of various mixed micelle systems on crosslinking of phospholipase A_2 by dimethyl suberimidate

Phospholipase A_2 (mg ml^{-1})	Triton (20 mM)	Phospholipid (4.8 mM)	Ba^{2+} (10 mM)	Relative ratio*: Dimer/monomer	Relative ratio*: Trimer/monomer
0.610	–	–	–	1.3	0.4
0.061	–	–	–	0.2	0.04
0.012	–	–	–	0.2	0.04
0.012	+	–	–	0.2	0.04
0.011	–	–	+	0.1	0.02
0.011	–	–	Ca^{2+}	0.2	0.02
0.011†	+	+	–	0.3	0.07
0.011	+	+	+	0.8	0.4

* The molecular weights of the monomer, dimer, and trimer, as determined by comparison with standards run on sodium dodecyl sulfate/polyacrylamide gels, were 11,200, 21,000, and 32,500, respectively. These ratios were obtained from the relative intensities of the stained bands. Duplicate experiments agreed within ±0.05.

† EDTA (2 mM) was included during incubation, but all metal ions may not have been removed.

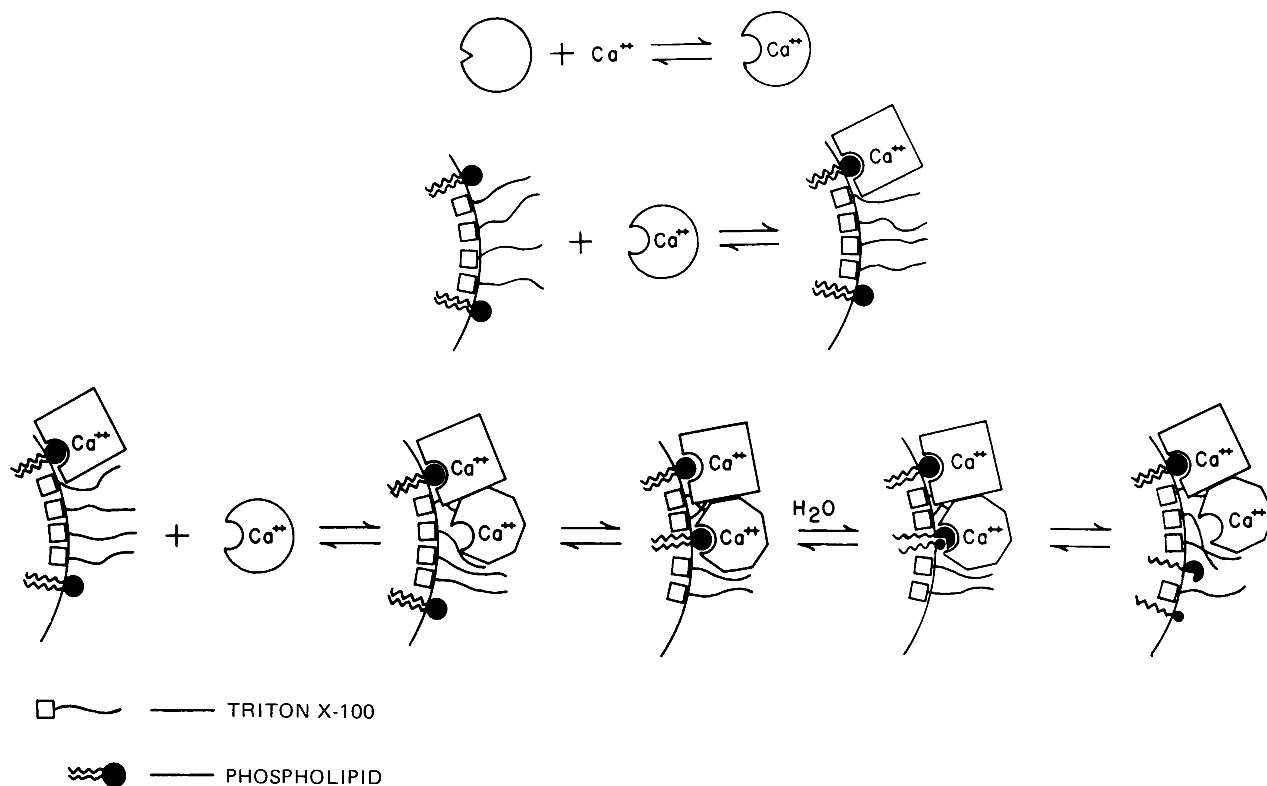

FIG. 2. Schematic diagram of the "dual-phospholipid" model for the action of phospholipase A_2 toward phospholipids contained in mixed micelles. Enzyme first binds Ca^{2+} and undergoes a conformational change that allows it to bind to phospholipid in the mixed micelle. The presence of interfacial phospholipid causes the enzyme to form an asymmetric dimer. One subunit of this dimer is responsible for binding to the interface via phospholipid, while the other hydrolyzes an accessible phospholipid. Lateral diffusion of phospholipid in the mixed micelle may be involved before the catalytic subunit binds phospholipid. Alternatively, two enzyme molecules bound to interfacial phospholipid may associate to form the asymmetric dimer directly. Once catalysis occurs, the products may diffuse away from the enzyme and either be retained in the mixed micelle or released into the solution.

is correct, the enzyme must be able to bind to an interface composed solely of neutral surface dilutors, i.e., an interface void of substrate, products, or inhibitors. For the second model, interfacial binding would be expected only when phospholipid is present in the micelle surface. We have attempted to differentiate between these two possibilities by examining interfacial binding with pure Triton X-100 micelles. Using gel chromatography, it was found that under no conditions could binding of the enzyme to the Triton micelle be observed. Furthermore, UV difference spectroscopy† indicates that monomers of Triton also do not interact with phospholipase A_2. This strongly indicates that Triton is not a competitive inhibitor of the enzyme and that it is in fact a neutral surface dilutor. Phospholipase A_2 also shows little affinity for micelles of *n*-dodecyl octaethylene ether, although it can bind monomers of that surfactant very tightly. All of these results cast strong doubt on the validity of the first model.

Phospholipase A_2 has an absolute requirement for Ca^{2+}; Ba^{2+} and Zn^{2+} are inhibitors, and UV difference spectroscopy shows that each binds at or near the active site, but in a slightly different way.† Interfacial binding of phospholipase is observed only when phospholipids and metal ions are present. Both types of binding studies show that metal is necessary before enzyme can interact with phospholipid. One would not expect, and there is no evidence for, an effect of divalent cation on the zwitterionic phosphatidylcholine (11). Thus it is reasonable to conclude that the binding mechanism is ordered with Ca^{2+} (or Ba^{2+} or Zn^{2+}) binding to the enzyme causing a conformational change that allows lipid binding. Neither phosphorylcholine nor glycerophosphorylcholine, soluble analogues of the polar portion of the lipid molecule, binds to phospholipase A_2, whereas micellar solutions of substrates or products bind to the enzyme with similar affinities. These results are consistent with the second model.

A problem with the "dual-phospholipid" model is that it may be difficult to envision both a distinct phospholipid surface-binding site and a phospholipid catalytic site on the same enzyme molecule because the cobra venom phospholipase A_2 is quite small (molecular weight 11,000). A more attractive mode of action for the phospholipase is as an asymmetric dimer. One subunit binds to the surface via lipid, while the other subunit hydrolyzes a second phospholipid molecule. Two types of experiments support this hypothesis. First, phospholipid induces the aggregation of enzyme, as shown by the dimethyl suberimidate crosslinking experiments. Aggregation occurs in the presence of a large excess of mixed micelles; there are roughly 260 mixed micelles for every enzyme subunit (assuming about 150 Triton and phospholipid molecules in a micelle and using the dissociation constant determined for the enzyme–phospholipid–Ba^{2+} complex), so that the dramatic increase in crosslinking should not merely reflect a concentration effect on the enzyme by the mixed micelles. Second, although the crosslinking experiments do not distinguish between symmetric or asymmetric dimers, chemical modification experiments with *p*-bromophenacyl bromide strongly suggest that an asymmetric dimer exists (5). Only 0.5 mol of histidine was modified per mol

† M. F. Roberts, R. A. Deems, and E. A. Dennis, unpublished data.

of enzyme, yet the derivative was inactive. An asymmetric dimer must be the active species of enzyme. Given these experiments, it is quite reasonable for the enzyme to exist at the interface as a dimer, each subunit binding phospholipid differently.

In summary, the most attractive model for phospholipase A_2 action on mixed micelles is presented in Fig. 2. The enzyme must first bind Ca^{2+} (or inhibitory metals such as Ba^{2+} or Zn^{2+}) before it can bind phospholipid. Once Ca^{2+} is bound, the enzyme binds one phospholipid molecule at the interface. This binding causes a conformational change in the enzyme that leads to dimerization. A second phospholipid is then bound by the dimer at a functional active site and catalysis occurs. These two phospholipid sites are quite distinct, since they occur on different enzyme molecules. One would expect lateral diffusion of phospholipid in the mixed micelle to be quite rapid. This may be the primary means for bringing the second phospholipid in contact with the functional catalytic subunit. Although the surfactant Triton X-100 acts as a pure surface dilutor in relation to the enzyme, it may in the process of solubilizing phospholipids also serve to alter phospholipid conformation. This could make the phospholipid more susceptible to phospholipase A_2 binding. It should be noted that other workers investigating phospholipase A_2 action have used micelles made of short-chain phospholipids, D-phospholipids, lyso-phospholipids, and zwitterionic detergents (12–14). These species are not neutral surface dilutors, but substrates or substrate analogues, and as such would make it difficult to differentiate the need for two phospholipid molecules. Finally, the lipid-induced enzyme aggregation may be a more general phenomenon and could occur with other water-soluble enzymes that act on substrates localized at an interface.

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