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Articles

Characterization of the Interaction of Phospholipase A_2 with Phosphatidylcholine−Phosphatidylglycerol Mixed Lipids†

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ABSTRACT: The first requirement in the hydrolysis of phospholipid bilayers by phospholipase A_2 is the interaction of the enzyme with the bilayer surface. The catalytic ability of phospholipase A_2 has been shown to be extremely sensitive to the topology of the bilayer to which it binds and hydrolyzes. Phospholipid bilayer properties and composition such as unsaturation, charge, and the presence of reaction products are known regulators of the catalytic activity of phospholipase A_2 toward the phospholipids and influences the binding of enzyme to the membrane. We show in this paper that the effect of increased anionic lipid results in enhanced binding that can be described quantitatively in terms of a simple phenomenological model. However, the interaction with anionic lipid does not singularly dominate the thermodynamics of binding, nor can the lag phase observed in the time course of hydrolysis of large unilamellar vesicles simply be the result of limited interaction between the enzyme and the bilayer. Furthermore, we show that phospholipase A_2 from *Akgistrodon piscivorus piscivorus* can exist in at least two bilayer-bound states and that the absence of a fluorescence change upon mixing the enzyme with lipid bilayers does not necessarily indicate the absence of an interaction.

The interaction of proteins and enzymes with membranes is often the initial step in a signal transduction cascade (*1*−*5*). Binding initiates events that result in phenomena such as cell proliferation, cell mobility, inflammation, and immune response. Thus, understanding those properties of the protein and membrane that temper the interaction between the two can lead to a better understanding of how signal transduction pathways can be regulated.

There is strong evidence that anionic phospholipids modulate the interaction between membranes and proteins such as protein kinase C (*3*) and phospholipase A_2 (PLA_2)[1] (*6*, *7*). Furthermore, PLA_2 is known to be sensitive to the topology of the surface with which the enzyme interacts. Indeed, the enzyme has been shown to be very active toward micellar systems (*8*) and sensitive to vesicle curvature (*9*,

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[1] Abbreviations: PLA_2, phospholipase A_2; AppD49-PLA_2, phospholipase A_2 from *Akgistrodon piscivorus piscivorus*; DPPC, dipalmitolyphosphatidylcholine; DPPG, dipalmitolyphosphatidylglycerol; POPC, palmitoyloleoylphosphatidylcholine; POPG, palmitoyloleoylphosphatidylglyercol; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DPPS, dipalmitoylphosphatidylserine; dansyl-PE, *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; X_{DPPG}, mole fraction of DPPG; K_D, dissociation constant; K_A, association constant; k_{off}, off rate.

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10), unsaturation of the phospholipids (*11*), and a variety of membrane perturbants (*7*). In fact, the hypothesis that "the quality of the interface" or the membrane surface to which the enzyme interacts dictates the activity of the enzyme has been central in many models proposed to describe the behavior of PLA_2 toward membranes (*7*, *9*, *12*−*14*).

Phospholipase A_2 is very sensitive to regions of high bilayer curvature that can be achieved by allowing hydrolysis to proceed, resulting in the accumulation of reaction products and compositional phase separation. PLA_2 instantaneously hydrolyzes highly curved small unilamellar vesicles (SUV) but can exhibit significant lag times before the onset of rapid hydrolysis on large unilamellar vesicles (LUV). The exogenous addition of reaction products reduces the lag time before the burst for PLA_2 on dipalmitoylphosphatidylcholine (DPPC) LUV. Lysophospholipids are known to induce regions of high radius of bilayer curvature, and fatty acids, when ionized, may result in the formation of regions of negative charge density that attract positively charged PLA_2. Although increased negative charge density results in increased affinity of PLA_2 to lipid bilayers (*6*, *7*), the effect of reaction products can be mostly attributed to the physical character of lysopholipids (*15*, *16*).

The conclusion that small mole percentages of anionic phospholipid or any other negatively charged lipid-soluble component can improve the binding of proteins to membrane surfaces is rooted in a number of early observations (*17*). The interaction of PLA_2 with binary mixtures of anionic and zwitterionic phospholipids is believed to strengthen with increasing concentrations of anionic phospholipid. The interaction of PLA_2 with these binary phospholipid mixtures is often marked by a change in the intrinsic fluorescence of the enzyme. This absence of a fluorescence change when PLA_2 is incubated with pure zwitterionic LUV bilayers led to the suggestion that little protein binding occurs under such circumstances (*7*). In this paper, we will quantitatively assess the binding of PLA_2 isolated from the venom of *Akgistrodon piscivorus piscovorus* (AppD49-PLA_2) to binary lipid bilayers composed of DPPC and dipalmitoylphosphatidylglycerol (DPPG), using direct fluorescence binding assays and equilibrium and kinetic competition assays to clarify the issue. Our results will show that phospholipase A_2 binds moderately well to pure DPPC LUV and that the absence of a fluorescence change is not necessarily indicative of a lack of an enzyme−lipid interaction. Furthermore, at moderate to high concentrations of anionic lipid where fluorescence changes are observed, the enzyme likely exists in more than one bound state. These results support the contention that the lag phase observed during the hydrolysis of LUV is not the result of a weak interaction. Rather, the slow production of reaction products is required to promote a change in the topology of the bilayer that results in a change in the bound state of the enzyme leading to higher catalytic efficiency.

EXPERIMENTAL PROCEDURES

Materials. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Purity of lipid stocks was determined by calorimetric analysis of lipid dispersions and thin-layer chromatography. For zwitterionic lipids, 1−2 μL of stock solution was spotted on a plate prewashed with 65:35 $CHCl_3$−MeOH and run in 60:30:10 $CHCl_3$−MeOH−H_2O. For anionic lipids, plates were run in 70:25:8:4 $CHCl_3$−MeOH−acetic acid−H_2O. No further purification was necessary. Crude venom of *Agkistrodon piscivorus piscivorus* was purchased from Sigma Chemical Co. (St. Louis, MO). The monomeric, aspartic acid 49 isozyme was isolated from the dimeric and lysine 49 forms as previously described (*18*, *19*). The purified protein was stored at −20 °C as a lyophilized powder. Stocks of AppD49 were typically prepared in 4 mg/mL stocks in water or 50 mM KCl. These concentrated protein stocks were used within 3 months when stored at 4 °C. However, the lifetime of the 4 mg/mL stocks could be lengthened to 6−9 months by storing 100 μL aliquots at −20 °C. Stocks were also prepared in 50 mM KCl to stabilize protein. These concentrated stocks were diluted 1:10 for use in binding experiments and further diluted to 1:100 and 1:1000 for enzymatic activity measurements. Before dilution and concentration determination, the original stock was centrifuged at 10 000 rpm to precipitate any aggregates. The enzyme concentration was determined from its absorption at 280 nm, with $\epsilon = 31\,000\ M^{-1}\ cm^{-1}$ (*19*). *N*-[5-(Dimethylamino)naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE) was purchased from Molecular Probes, Inc. (Eugene, OR) and used without further purification. Dansyl-PE was obtained as a lyophilized powder and was dissolved in 1:1 $CHCl_3$−MeOH and stored in the dark at −20 °C. It was used within 3 months.

Lipids: Sample Preparation. All lipid samples were stored at −20 °C in $CHCl_3$. For long-term storage, unopened lipid−$CHCl_3$ vials were stored at −70 °C as received from the manufacturer. Generally, lipid stocks were warmed to room temperature where the stocks clarified. Stocks of DPPG and dipalmitoylphosphatidylserine (DPPS) required gentle heating in a 55 °C bath to achieve clarification. Homogeneous lipid aliquots were gently warmed under a stream of nitrogen or argon and lyophilized for 12 or more hours. Binary mixtures of lipids were mixed in chloroform, gently warmed, and rotated in a rotovac under vacuum, and lyophilized for 12 or more hours. The dried lipid film was hydrated at 55−60 °C in various buffers, resulting in a dispersion of multilamellar vesicles (MLV). In the preparation of binary codispersions of MLV, the dispersions were heated to 60 °C and cooled to room temperature four or more times. The buffer used depended upon the type of experiment. For most binding experiments, lipids were suspended in 10 mM borate, 50 mM KCl, and 1 mM NaN_3, pH 8, containing either 1 mM or 10 mM EGTA. In binding and kinetic experiments of longer time duration or with lipid containing high mole fractions of anionic lipid where hydrolysis could have been a potential problem, 10 mM EGTA and, on occasion, 10 mM Hepes instead of borate buffer was used. No quantitatively significant differences between experiments performed in the different buffering systems were observed. LUV were prepared by extruding a MLV lipid dispersion at least 10 times though two 0.1 μm pore size Nucleopore polycarbonate filters (Costar, Cambridge, MA) using a high-pressure extrusion device from Lipex Biomembranes Inc. (Vancouver, BC) (*20*). During extrusion, the lipid suspension was maintained at least 10 °C above the phase transition of the lipid components. The resulting vesicles were characterized by quasi-elastic light scattering with a Nicomp model 370 submicron particle sizer (Santa Barbara, CA). Generally, the

size distribution was best fit with a Gaussian distribution with a mean size between 100 and 120 nm and a standard deviation of 20−40%. SUV were made by direct probe sonication of MLV with a sapphire-tipped probe sonicator (Misonix, Farmingdale, NY) for 8−12 min at 50% duty cycle. Sonication was always performed above the phase transition temperature of the lipid.

Lipid Vesicle Storage. Phospholipid LUV used for binding experiments were not used when older than 7 days. Stocks (20−40 mM) of lipids were used, and there was concern that the vesicles were aggregating at room temperature where they were stored. Therefore, prior to binding experiments, lipid vesicle stocks were heated to 50 °C and cooled to room temperature for three cycles and vortexed. SUV were kept at 55 °C to prevent fusion of vesicles and used within 24 h after sonication. Concentration of phospholipids was determined by the phosphate assay described by Bartlett (*21*).

Fluorescence Measurement—General. All fluorescence measurements were performed on the 8100 SLM fluorometer (Urbana, IL) with a T format and in the slow analogue mode. Samples were continuously stirred. The temperature of the sample was controlled with a circulating water bath (Forma Scientific) and recorded to a resolution of 0.02 °C with a Cole-Parmer digital thermometer 8502-16 immersed in the sample cell.

Fluorescence Measurements—Lipid Effects on the Intrinsic Fluorescence of PLA_2. Changes in the intrinsic fluorescence of AppD49-PLA_2 were monitored to examine the association of the protein with membrane. The excitation wavelength was 285 nm, and the emission was measured at 340 nm. The slit widths were uniformly 8 nm. In all equilibrium experiments, the protein concentration was 300 nM. The reaction volume was 2.5 or 2.0 mL. The buffer was thermally equilibrated before the addition of protein. The protein was added and allowed to incubate for 5−10 min, and the titration with phospholipid from concentrated stocks was then initiated. The contribution from light scattering at 340 nm due to the phospholipid was determined in control experiments for each vesicle preparation. The fluorescence change due to protein binding to the membrane was calculated by subtraction of the scattering contribution. The magnitude of the scatter contribution depended upon the affinity and the maximum fluorescence change. It varied from about 10% (high affinity) to 50% (low affinity) of the magnitude of the maximum fluorescence change.

Fluorescence Measurement—AppD49-PLA_2/Vesicle Competition Experiments with SUV. There is no detectable change in the intrinsic fluorescence of AppD49-PLA_2 when the protein interacts with DPPG−DPPC LUV containing <15% DPPG. A competition experiment was thus designed to estimate the degree of interaction between protein and LUV. The protein was first bound to DPPC SUV because of the associated fluorescence change upon binding and because their small size reduced light scattering effects. LUV were then titrated into the PLA_2−DPPC SUV mixture and the decrease in the intrinsic fluorescence of the protein demonstrated that the protein was disassociating from the DPPC SUV and binding onto the LUV.

The excitation and emission wavelengths and slit widths were as previously defined. A finite aliquot of SUV was added to a 2 mL preequilibrated sample of 300 nM AppD49-PLA_2 in 50 mM KCl, 10 mM EGTA, 1 mM NaN_3, and 10 mM borate, pH 8. The contribution from light scatter at 340 nm due to the phospholipid was determined in control experiments and subtracted from the fluorescence decay curve.

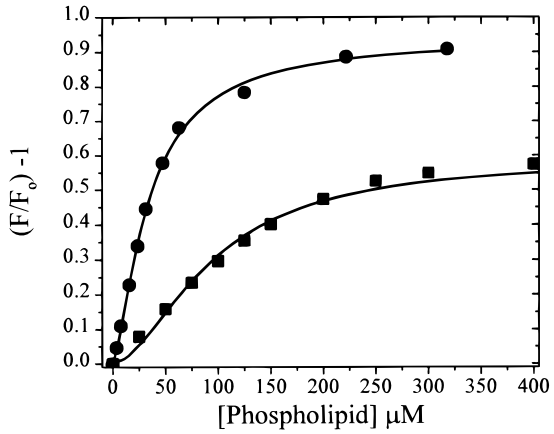

FIGURE 1: Relative change in the intrinsic fluorescence of AppD49-PLA_2 as a function of concentration of DPPG−DPPC LUV at 35.4 °C. F_0 is intrinsic fluorescence of the enzyme in the absence of phospholipid. Conditions: 50 mM KCl, 1 mM NaN_3, 10 mM sodium borate, pH 8, and 300 nM AppD49-PLA_2. X_{DPPG} = 0.2 (■) and 0.4 (●) are plotted as scatter plots and the solid lines were calculated from a least-squares fit by use of eq 1. The concentration of EGTA was 1 mM for X_{DPPG} = 0.2 and 0.4.

Fluorescence Measurements—AppD49-PLA_2/Vesicle Competition Experiments Using Fluorescence Energy Transfer. A 2 mL reaction volume of 50 mM KCl, 10 mM EGTA, 1 mM NaN_3, and 10 mM HEPES, pH 8, was equilibrated with a finite aliquot of DPPG−DPPC LUV doped with 2% dansyl-PE. The excitation wavelength was set at 280 nm and the change in emission at 510 nm was measured as aliquots of 300 nM AppD49-PLA_2 were added. Unlabeled vesicles at a concentration sufficient to cause dissociation of all or most of the protein from the dansyl-PE-labeled vesicles was added and the decay of fluorescence at 510 nm was monitored as a function of time. The time course of the decay was analyzed by least squares with an equation containing either one or two exponential terms.

RESULTS

Association of AppD49-PLA_2 with DPPG−DPPC LUV as a Function of the Mole Fraction of Anionic Lipid. The experimental procedure used to characterize binding of AppD49-PLA_2 to DPPG−DPPC LUV is as follows. Protein (300 nM) was incubated in a chelating buffer containing 1 mM EGTA to prevent hydrolysis from occurring within the time frame of the experiment (30 min). Lipid vesicles were then titrated into the thermally equilibrated protein−buffer mixture. The increase in the intrinsic fluorescence of the protein was used as the marker for the interaction of AppD49-PLA_2 with phospholipid bilayers. All incremental changes in fluorescence were corrected for light scattering. The DPPG−DPPC systems used in this study were extensively characterized by differential scanning calorimetry (data not shown and ref *22*).

Representative binding isotherms constructed for AppD49-PLA_2 interacting with DPPG−DPPC LUV at X_{DPPG} = 0.2 and 0.4 are plotted in Figure 1. The binding was performed at 35.4 °C, a temperature at which the lipids are in the gel

state. The minimum anionic composition required to induce a change in the fluorescence of PLA_2 appeared to be 15:85 DPPG−DPPC. The data in Figure 1 indicate that the maximum fluorescence change when protein is completely bound to phospholipid is a function of X_{DPPG}.

Binding isotherms were constructed as a function of total lipid added. Figure 1 shows a plot of data acquired after correction for light scattering. The data are plotted as relative fluorescence changes as a function of total phospholipid where F_0 equals to the fluorescence intensity of PLA_2 prior to phospholipid addition. The typical binding isotherm can be approximately described by a hyperbolic function. However, upon inspection of the complete set of data over the range of $X_{DPPG} = 0.15-1.0$, it became clear that a hyperbolic function failed to describe the binding isotherm at low lipid concentration when the total lipid was not in great excess of the total enzyme. Therefore, the free lipid concentration = $[\text{lipid}]_{total} - N[\text{enzyme}]_{total}$; where $N = 25-50$, the number of lipids per protein binding area. In these situations $[L]_{free} < [L]_{total} = X$ and a sigmoidal binding curve can result. A variation of the Hill equation was thus used to more exactly describe the AppD49-PLA_2 binding data:

$$Y = (aX^n)/(K_d{}^n + X^n) \quad (1)$$

K_D is the apparent disassociation constant; a is the maximum relative fluorescence intensity change of PLA_2 under saturating lipid concentration, and n is an apparent cooperativity term. This procedure was used throughout for self-consistency to obtain estimates of the lipid concentration at which 50% of the enzyme was bound. Although we fit the data with a cooperative term, we do not argue that binding of AppD49-PLA_2 to DPPG−DPPC LUV is necessarily cooperative.

Under certain experimental conditions and after very long times (>15 h), we were able to detect hydrolysis of DPPG−DPPC LUV by AppD49-PLA_2 for $X_{DPPG} > 0.5$. Although no hydrolysis was observed during the time required for a binding experiment, the concentration of EGTA was increased to 10 mM. Nevertheless, since the presence of reaction products is believed to improve binding of PLA_2 to bilayers, estimates of K_D were also obtained by use of AppD49-PLA_2 in which the active-site histidine of AppD49-PLA_2 was covalently reacted with *p*-bromophenacyl bromide (*23*). This form of the enzyme was found to be inactive. Upon comparing the binding properties of the modified protein to those of the wild-type enzyme, we found that for all mole fractions of DPPG the binding properties of the wild-type and the modified enzyme were the same (data not shown). Therefore, within the time frame of our binding experiments, little or no hydrolysis appears to be occurring, and the estimated values of K_D are valid for lipid preparations free of hydrolysis products.

Reversibility. It is important to show reversibility to validate the reported dissociation constants. Jain and Berg (*7*) have strongly argued that the binding of PLA_2 to anionic surfaces is very tight and should be essentially irreversible. In Figure 2, we show that the binding of AppD49-PLA_2 to pure anionic vesicles is reversible. AppD49-PLA_2 was bound to 20 μM 98:2 DPPG−dansyl-PE LUV and then competed off with a 40-fold excess of unlabeled DPPG LUV. The interaction between AppD49-PLA_2 and dansyl-PE-containing

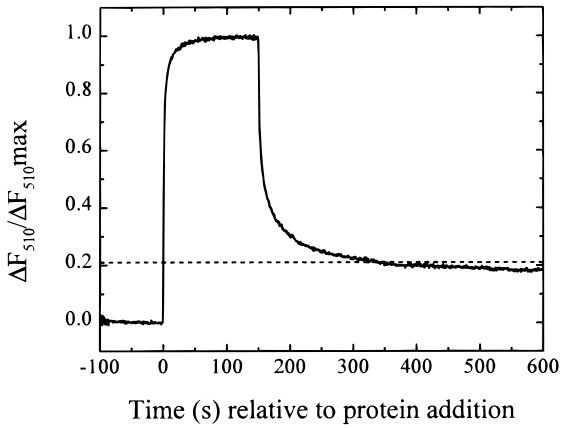

FIGURE 2: Reversibility of binding of AppD49-PLA_2 to 20 μM 98:02 DPPG−dansyl-PE LUV. Data are plotted relative the change in dansyl fluorescence observed upon the addition of 300 nM AppD49-PLA_2 at time zero. At 150 s, 800 μM 100% DPPG LUV is added. The horizontal dashed line at 0.217 marks the relative fluorescence intensity at 510 nm of 300 nM enzyme and 800 μM 100% DPPG LUV and confirms complete dissociation of protein from the labeled vesicles. Conditions: 10 mM sodium borate, pH 8, 10 mM EGTA, 50 mM KCl, 1 mM NaN_3, and 22 °C. Excitation = 280 nm. Emission = 510 nm.

vesicles was monitored by emission at 510 nm after the enzyme was excited at 280 nm. The dashed line in Figure 2 marks the apparent 510 nm emission intensity of AppD49-PLA_2 in the presence of 800 μM pure DPPG LUV and is the same as the final emission intensity following dissociation of PLA_2 from DPPG−dansyl-PE to unlabeled DPPG LUV. Hence, all protein disassociated from the dansyl-doped vesicles and was bound by unlabeled LUV demonstrating complete reversibility.

Binding of AppD49-PLA_2 to LUV Containing Less Than 15% DPPG. No change in the intrinsic fluorescence of AppD49-PLA_2 was detected when the enzyme was incubated with DPPG−DPPC LUV containing less than 15 mole % PG. The absence of a fluorescence change has been suggested to indicate a lack of binding (*7*). However, when AppD49-PLA_2 is incubated with DPPC LUV under hydrolysis conditions, a region of slow hydrolysis is observed. This lag phase precedes a burst in activity and its length is inversely proportional to the anionic lipid mole fraction. It has been hypothesized that initially the enzyme is weakly bound to the bilayer and that during this lag phase slow hydrolysis occurs, resulting in accumulation of reaction product and enhanced enzyme binding (*7*). However, Burack et al. (*6*) have demonstrated that a lag period can be induced under conditions where the enzyme is completely bound to a 2:8 DPPG−DPPC LUV. Thus, the presence of a lag phase is not proof of a weak initial interaction between enzyme and substrate. Evidence for an interaction between PLA_2 and 1:9 DPPG−DPPC LUV was observed when binding experiments were performed with AppD49-PLA_2 and 2:8 DPPG−DPPC LUV in the presence of finite concentrations of 1:9 DPPG−DPPC LUV. These experiments demonstrated that the apparent dissociation constant of PLA_2 and 2:8 DPPG−DPPC LUV decreased with increasing concentrations of 1:9 DPPG−DPPC LUV. It is thus clear that the binding of AppD49-PLA_2 to lipid bilayers containing small percentages of anionic lipid must be reevaluated.

The dissociation constant of AppD49-PLA_2 to pure DPPC SUV could be determined by monitoring the intrinsic

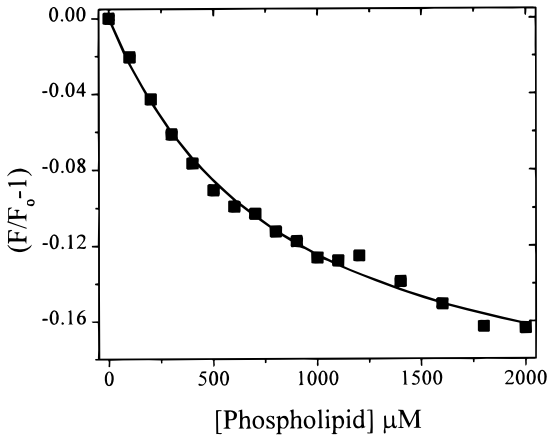

FIGURE 3: Measurement of the dissociation constant for AppD49-PLA_2 and 1:9 DPPG−DPPC LUV by competition binding experiments with DPPC SUV. Enzyme (300 nM) was incubated with 200 μM DPPC SUV before aliquots of 1:9 DPPG−DPPC LUV were titrated into the fluorescence cell. The decrease in the intrinsic fluorescence of AppD49-PLA_2 was monitored. Conditions: 50 mM KCl, 10 mM EGTA, 1 mM NaN_3, and 10 mM sodium borate, pH 8, 22 °C. Equation 2 was used to estimate a $K_D = 130$ μM for 1:9 DPPG−DPPC LUV. At the end of the experiment the concentration of phospholipid was 2 mM, resulting in a large light scattering contribution. These light scattering effects as well as dilution effect of the initial protein fluorescence signal were subtracted from the total fluorescence change.

fluorescence change of the enzyme and was found be on the order of 100 μM. Since the binding of AppD49-PLA_2 to DPPC SUV was associated with a change in the intrinsic fluorescence of the protein, that interaction was used to monitor binding of PLA_2 to LUV containing $X_{DPPG} \leq 0.1$ by the following rationale. The protein was first mixed with a fixed amount of DPPC SUV so that approximately 60% of the protein was bound to the SUV. Aliquots of 1:9 DPPG−DPPC LUV, for example, were then titrated into the protein−SUV mixture to compete the protein off the SUV and onto the LUV and the decrease in fluorescence was measured. Figure 3 shows the decrease in fluorescence as a function of the concentration of total 1:9 DPPG−DPPC phospholipid titrated, which was analyzed by use of

$$\frac{f^*}{f_0} = \frac{1 + K_1X}{1 + K_1X + K_2Y} \qquad (2)$$

Equation 2 represents the ratio of the fraction of protein bound in the presence of LUV (f^*) to the fraction of protein bound when only SUV are present (f_0). K_1 is the apparent association constant for AppD49-PLA_2 and DPPC SUV, and X is the molar lipid concentration of DPPC. K_2 is the apparent association constant for AppD49-PLA_2 and 1:9 DPPG−DPPC LUV, and Y is the molar lipid concentration of 1:9 DPPG−DPPC LUV. Given that $K_1 = 0.0078$ μM^{-1} and $X = 200$ μM, a $K_D = 330$ μM was calculated for 1:9 DPPG−DPPC LUV. It is thus clear that the enzyme can bind to a phospholipid bilayer without a significance change in fluorescence.

Summary of the Binding of AppD49-PLA_2 to DPPG−DPPC LUV. Although increasing the charge density on the membrane surface by increasing the mole percent of anionic lipid enhances the binding of PLA_2 to those surfaces, the binding is reversible at all compositions. Table 1 lists the values K_D, K_A, the error associated with K_A, and ln K_A with

Table 1: List of Mole Fraction DPPG and Average Binding Constants

mole fraction DPPG	K_D[a] (μM)	K_A (M^{-1})	± estimated error of K_A (M^{-1})	ln K_A
0.05	1000[b]	1000	400[c]	6.9
0.1	375[d]	2700	500	7.9
0.15	250	4000	800	8.3
0.175	225	4400	700	8.4
0.2	125	8000	2800[e]; 590[f]	9.0
0.3	70	14 000	2000	9.6
0.4	30	33 000	5000	10.4
0.5	24	42 000	6300	10.6
0.6	16	63 000	9400	11.0
0.7	8	125 000	19 000	11.7
0.8	4	250 000	50 000	12.4
0.9	5	200 000	40 000	12.2
1.0	5	200 000	40 000	12.2

[a] Determined from 3−6 direct equilibrium binding experiments except when noted. [b] Equilibrium competition studies only. [c] Error estimated to be the internal standard deviation multiplied by 2. [d] Equilibrium and kinetic competition binding studies. [e] Standard deviation for 38 experiments. [f] Standard error for 38 experiments.

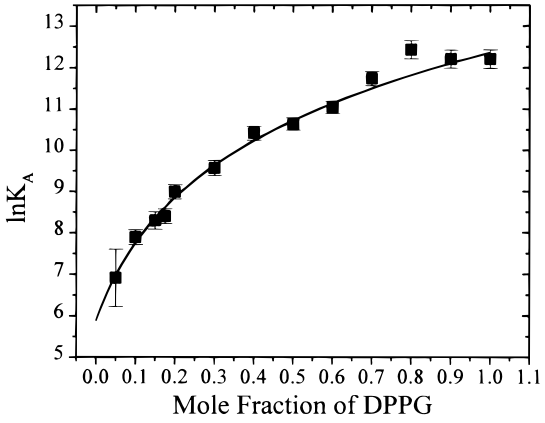

FIGURE 4: Analysis of binding of AppD49-PLA_2 to DPPG−DPPC LUV. Ln K_A is plotted as a function of the mole percent of DPPG. The line was a nonlinear least-squares fit to $\ln K_A = \ln K_0 + N \ln (1 + MX)$, yielding estimates of $\ln K_0 = 5.9 \pm 0.5$, $N = 2.7 \pm 0.2$, and $M = 10 \pm 4$. Data were analyzed as described by Johnson and Frasier (*49*).

their corresponding mole fraction of DPPG. The errors in the association constants are estimated to be 15−20% with the greatest error at higher percentages of anionic lipid, where the estimated K_D values are the smallest. The accuracy in estimating K_D is limited by the 300 nM concentration of protein used for these binding experiments. The calculated minimum concentration of phospholipid required to bind 50% of proteins is 3−8 μM assuming a protein-binding site of 25−50 lipids (*7*, *24*, *25*). Hence values for K_D that are estimated to be less than 4 μM are higher estimates of K_D.

The plot of ln K_A vs X_{DPPG} in Figure 4 clearly shows that the apparent association constant increases with increasing mole fraction of DPPG. The data in Figure 4 were fit to eq 3, which relates the apparent association constant K_A to the association constant of PLA_2 and DPPC LUV, K_0, and a binding polynomial characterizing the preferential binding to anionic lipid. The appropriateness of this representation has been discussed previously by McLaughlin and coworkers (*26*, *27*) to describe binding of peptides to mixed bilayer systems.

$$K_A = K_0(1 + MX)^N \quad (3)$$

Thus, AppD49-PLA_2 binding can be represented in terms of a preferential interaction with anionic lipid. Literal interpretation of this model is that the enzyme possesses N independent and equivalent lipid binding sites that interact with an affinity M-fold greater with anionic lipid than zwitterionic lipid. However, such literal interpretation of the model is probably not warranted. Its primary purpose is to provide a phenomenological model with some physical basis to quantitatively represent the binding of AppD49-PLA_2 to these binary lipid systems as a function of anionic lipid so that an estimate of the affinity to pure DPPC LUV can be obtained.

The affinity of PLA_2 for zwitterionic DPPC LUV has been debated for a number of years with the values of K_D ranging from 50 μM to 10 mM (*7*). The least-squares application of eq 4 yields a value for the association constant of 360 M^{-1} for PLA_2 binding to 100% DPPC LUV that is equivalent to a K_D of 2.8 mM. The preference for PLA_2 binding to DPPG over DPPC defined as M in eq 3 was calculated to be 10 ± 4. The stoichiometry term, N, was calculated to be 2.7 ± 0.2.

Analysis of Off-Rates of AppD49-PLA_2 from DPPG−DPPC LUV. We showed in Figure 2 that the binding of AppD49-PLA_2 to pure anionic phospholipids was reversible. Similar experiments were also performed with AppD49-PLA_2 and dansyl-PE LUV containing 0.1−0.9 X_{DPPG} to yield decay kinetics. In these experiments, an excess of unlabeled vesicles were added to an equilibrated labeled vesicle dispersion to remove a major fraction of the protein initially bound to the dansyl-doped 1:1 DPPG−DPPC LUV. In Figure 5, we have plotted the fluorescence decay of AppD49-PLA_2 from LUV containing $X_{DPPG} = 0.1$ (panel A), 0.2 (panel B), 0.5 (panel C), and 0.9 (panel D). These data have been corrected for lipid scattering effects and show that the interaction between AppD49-PLA_2 and the DPPG−DPPC LUV is reversible. The data in Figure 5 were fit to a single-exponential (dashed line) and to a two-exponential term (solid line) equation. An equation containing two exponential terms better fits the high X_{DPPG} data with a 7−10-fold improvement in the χ^2 compared to use of a single exponential term equation. No significant improvement in the fit of the data obtained with 1:9 DPPG−DPPC LUV and only a slight improvement in the fit of the data obtained with 2:8 DPPG−DPPC LUV was observed when two exponential terms were used. The measured off rate of the protein from 0.05:95 DPPG−DPPC LUV was faster than the detection limit of the experiment of about 1−2 s.

At the lower mole fraction of DPPG the single-exponential decay time was about 2−3 s, with the off rate always being faster for the vesicle system containing the smaller amount of DPPG. If it is assumed that the on rate constant is diffusion-controlled and identical in all cases, the relative magnitude of the equilibrium association constants can be calculated for these systems. Although the accuracy of the estimates is poor for such a purpose, it was found that comparison of the off rates for 1:9 and 2:8 DPPG−DPPC vesicles consistently yielded values for the relative association constants that were in reasonable agreement with those obtained from the equilibrium experiments.

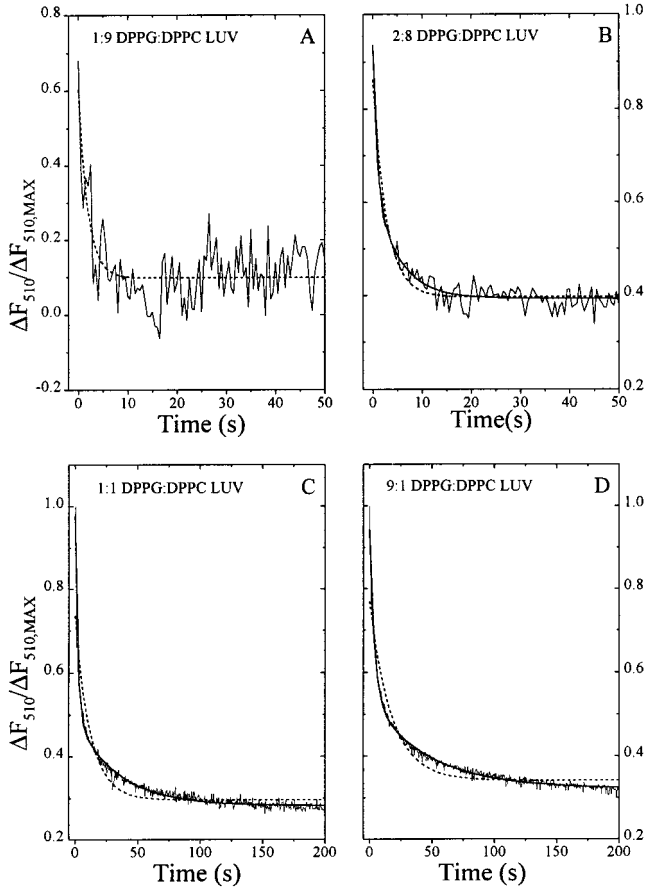

FIGURE 5: Measurement of the off rates of AppD49-PLA_2 from various compositions of DPPG−DPPC−0.02 dansyl-PE LUV. In all experiments, 300 nM AppD49-PLA_2 was preequilibrated with the dansyl-labeled vesicles prior to the addition of unlabeled DPPG−DPPC LUV. The decay of fluorescence for 100 μM 1:9 DPPG−DPPC LUV μM and for 50 μM 2:8 DPPG−DPPC LUV following addition of 500 μM 0.2:0.8 unlabeled DPPG−DPPC vesicles are shown in panels A and B, respectively. DPPG−DPPC−dansyl-PE 5 μM; (1:1:0.2, panel C) or 9:1:0.2 DPPG−DPPC−dansyl-PE (panel D) LUV had 500 μM 1:1 DPPG−DPPC vesicles added at time zero. The fluorescence decay at 510 nm follows the loss of the interaction between protein and dansyl-doped vesicles. The data were fit to an equation with one (dashed line) and two exponential terms (solid line). The fits of these data yield the following constants: for 1:9 and 2:8 DPPG−DPPC LUV, decay constants of 1.9 s^{-1} and 2.7 s^{-1}, respectively; for 1:1 DPPG−DPPC LUV, decay constants of 2.1 s^{-1} and 33 s^{-1} with relative amplitudes of 0.5 and 0.2; and for 9:1 DPPG−DPPC LUV, decay constants of 3.4 s^{-1} and 30 s^{-1} with relative amplitudes of 0.4 and 0.2. Conditions: 10 mM HEPES or sodium borate, pH 8, 10 mM EGTA, 1 mM NaN_3, and 50 mM KCl, 22 °C.

At high mole fractions of DPPC the data are not consistent with a single-exponential dissociation process. Detailed interpretation of these data is difficult since, for example, the two relaxation times could reflect direct dissociation of two distinct bound forms of the enzyme or dissociation following interconversion of two forms on the bilayer surface. This precludes comparison of the rate data to yield equilibrium information without further assumptions. It is nevertheless clear that these results are consistent with AppD49-PLA_2 having two distinct bound states, one with a decay time of 2−3 s and another characterized by an apparent decay time of 30−45 s. It is noteworthy that two off rates are observed in cases where the protein vesicle interaction is strongest and results in a change in the intrinsic fluores-

cence of the protein. However, only one off rate is observed when no fluorescence change of the protein is directly detected.

DISCUSSION

An increase in the X_{DPPG} or the surface charge density results in an increase in the affinity of AppD49-PLA_2 for DPPG−DPPC bilayers. We investigated binding of AppD49-PLA_2 to LUV by three general types of experiments depending upon the X_{DPPG}. For bilayers containing $X_{DPPG} \geq 0.15$, the change in the intrinsic fluorescence of the enzyme could be used directly. For bilayers composed of $X_{DPPG} \geq 0.5$, we monitored the intrinsic change of the active enzyme and an inactive, modified AppD49-PLA_2 and found that the affinities of both the wild-type and modified proteins for bilayers were identical. These latter experiments indicate that no significant reaction products were produced during the time frame of the binding experiments, which could have resulted in an overestimate of the K_D. We used a variety of fluorescence competition experiments to follow the dissociation of AppD49-PLA_2 from bilayers composed of $X_{DPPG} < 0.15$. The apparent dissociation constant can be represented as the product of an interaction (K_0) of AppD49-PLA_2 with DPPC LUV and a binding polynomial characterizing the preference for binding to anionic phospholipids. By extrapolation to $X_{DPPG} = 0$, a $K_D = 2.8$ mM for the interaction of AppD49-PLA_2 with DPPC LUV was calculated.

The apparent binding constants of AppD49-PLA_2 to LUV range over a factor of about 200 from pure DPPC to pure DPPG LUV. The apparent Gibbs free energy of binding AppD49-PLA_2 to pure DPPG LUV is estimated to be −7.2 kcal/mol of lipid and the apparent Gibbs free energy of binding of AppD49-PLA_2 to pure DPPC LUV is −3.6 kcal/mol of lipid. Since both quantities contain a cratic entropy term, the unitary Gibbs' free energy for binding to pure DPPC vesicles is more negative than −3.6 kcal/mol of lipid and thus the interaction to the zwitterionic surface is stronger than the enhancement achieved by replacing them with anionic lipids. Although binding does have an electrostatic component, another thermodynamic component, likely of a hydrophobic or van der Waals type, must be playing a major role in the interaction.

The interaction of peptides with bilayers composed of varying mole fraction of anionic phospholipid has been examined by McLaughlin and colleagues (*26*, *27*) who found approximately a 3 orders of magnitude enhancement in the association constant of peptides to bilayers attributed to the incorporation of anionic phospholipid. This is the same order of magnitude enhancement reported here for AppD49-PLA_2. Their extrapolated value for the interaction of peptides with pure zwitterionic lipid surfaces is approximately 100 mM (*26*), which is almost 2 orders of magnitude poorer than the binding of AppD49-PLA_2 to pure zwitterionic surfaces. The MARCKS and MARCKS-related peptides that are enriched in lysine and arginine residues have been shown to bind via electrostatic interactions to membranes containing anionic phospholipids. However, these peptides were also shown to have a weak association with zwitterionic surfaces, suggesting a nonelectrostatic component to the interaction (*28*). This suggestion is further supported by their ability to bind to anionic bilayers in the presence of 0.5 M KCl (*29*). However, it is difficult to make direct comparisons between the peptide−bilayer and protein−bilayer interactions since proteins have more complexity. A protein with a well-defined active site or intramolecular interactions may not have the degrees of freedom afforded a peptide to accommodate optimal binding.

The applicability of eq 3 to describe binding as a function of X_{DPPG} has been interpreted to indicate positive cooperativity due to loss of dimensionality. This is simply not the case; the formulation involves no cooperativity. The apparent cooperativity arises from the fact that the "specific" ligand (in this case DPPG) resides in one phase of the system (the bilayer) whereas the protein exists in both the aqueous and bilayer phases. If three-dimensional phases comprised the system, but only one contained the ligand, identical behavior would be observed.

The distinction between ionic and the other thermodynamic forces involved in the interaction is not clear. However, hydrophobic forces and van der Waals interactions are likely to play a significant role (*28*). There are a number of hydrophobic residues that flank the active site of PLA_2. Some of these hydrophobic residues have been shown to be conserved and essential since upon their mutation the enzyme failed to bind to the bilayer interface or failed to bind substrate in the active site (*30*, *31*). The presence of tryptophan in the interfacial recognition site of PLA_2 may contribute to the binding of PLA_2 to a neutral bilayer (*32*). Indeed, Baker et al. (*32*) engineered a V3W mutant of human group II secreted PLA_2 that showed a 250-fold improvement in activity toward PC vesicles, which was suggested to be the result of increased interfacial binding.

Natural membranes are generally zwitterionic with relatively small mole percentages of a variety of anionic phospholipids. Hence, the hydrophobic component of binding most likely controls the basal level of binding, which can be enhanced by an electrostatic component as dictated by the mole fraction of anionic phospholipid. For PLA_2, the hydrophobic interaction may be the result of partial insertion of enzyme into the bilayer. The observation that monolayers exposed to high surface pressure resulted in no observed hydrolysis supports the proposal that insertion of PLA_2 into the bilayer is part of the catalytic mechanism (*33*). Dehydration of the interface between PLA_2 and the bilayer may also play a role in binding and has been used to explain the blue shift of the tryptophan emission spectra that results upon protein binding (*34*, *35*).

Burack et al. (*6*) proposed two modes of binding within the context of an activation model, and the current results strongly support the existence of two bound states of AppD49-PLA_2. First, the presence of interactions between AppD49-PLA_2 and DPPG−DPPC LUV that do and do not result in an intrinsic fluorescence change of the enzyme shows that the enzyme has at least two modes of binding to the membrane surface. These two modes of binding were further evident upon inspection of the competition off-rate data. We could describe the nonfluorescent interaction between PLA_2 and 1:9 DPPG−DPPC with a single-exponential decay term, whereas the dissociation of PLA_2 under conditions where an intrinsic fluorescence change of the enzyme was observed required two exponential terms to fit the data.

The secondary and tertiary structure of PLA_2 is highly conserved among the small, secreted PLA_2 family despite sequence dissimilarities and is therefore assumed that any conformational changes that accompany "activation" of PLA_2 occurs for all in this family. The fluorescence change of AppD49-PLA_2 may be an indication of a conformational change of the enzyme or a change in its mode of binding. NMR and Fourier transform infrared (FTIR) results have shown that pancreatic PLA_2 undergoes conformational changes, particularly in the N-terminal region, when PLA_2 interacts with substrate, with the most marked changes seen with aggregated substrate and transition-state analogues and not with monomeric substrate (*36*, *37*). However, the N-terminal region of AppD49-PLA_2 is well-defined in solution, unlike pancreatic PLA_2 (*38*). However, for both enzymes, the N-terminal helix is involved in the binding of enzyme to membrane surfaces and perhaps, via a hydrogen-bonding network, plays a role in catalysis (*38*). The N-terminal helix of PLA_2 is believed to play a role in the binding of pancreatic PLA_2 to membranes and the amino acid composition of this helix may also govern the functional differences among members of this family (*39*). Dekker et al. (*36*) showed that when pancreatic PLA_2 binds to micelles, certain residues are shielded from solvent while other aromatic residues showed changes in the 2D nuclear Overhauser effect (NOE) spectra. These latter changes could have been due to changes in hydrogen bonding, orientations of aromatic ring, or small changes in the backbone conformation (*36*). Peters et al. (*40*) compared the conformation of soluble PLA_2 and micelle-bound PLA_2 and found that the enzyme is more flexible in solution. When PLA_2 binds to a membrane, hydrogen bonds may form to "lock" the enzyme into a conformation that allows the substrate to enter the active site, resulting in the observed interfacial activation (*40*, *41*).

FTIR results and the results of amide hydrogen/deuterium exchange have shown that upon interaction with membranes the existing α-helices of AppD49-PLA_2 became more flexible and new α-helices may be created at the expense of irregular and/or β-structure as the extent of β-sheet is reduced (*42*). Kennedy et al. (*37*) detected a reduction in the amide I/II ratio by FTIR structural analysis in bovine and porcine pancreatic PLA_2 when the protein binds to micelles, and the marked differences suggests a conformational change between the two environments. Tatulian et al. (*42*) showed that soluble AppD49-PLA_2 was more resistant to deuterium exchange than the membrane-bound form. This result implies the secondary structure of the membrane-bound form is less stabilized by intramolecular hydrogen bonding compared to the free enzyme. However, the detailed structural variation among interfacial binding, binding of enzyme to the bilayer surface, and binding of lipid into the active site remain undefined.

AppD49-PLA_2 prefers to bind to binary mixtures of PG−PC unsaturated phospholipids in a manner correlated with headgroup spacing and negative surface charge density. A 2−3-fold preference was detected for AppD49-PLA_2 binding to gel state phospholipids compared to fluid-phase phospholipids when $X_{DPPG} \leq 0.3$. The phospholipid possessing a PG headgroup may prefer a more expanded fluid phase, which would allow for greater spacing between the charged headgroups than the gel phase (*43*). AppD49-PLA_2 was also found to bind 5−10 fold more tightly to fluid-phase unsaturated phospholipids (mixtures of POPG−POPC and DOPG−DOPC) than to their fluid-phase saturated phospholipid counterparts that have the same headgroup. This is probably related to the area per lipid, which increases with acyl chain unsaturation (DOPC, 72.2 $Å^2$ compared to DPPC, 62.9 $Å^2$) (*44*).

The relationship between the binding of PLA_2 to lipid vesicles and hydrolytic activity is a complex one. A lag period of slow hydrolysis preceding a rapid increase in activity can be observed with zwitterionic LUV, LUV containing anionic lipid, and even smaller unilamellar vesicles. In the first case the length of the lag period is very dependent upon temperature, reaching a minimum near the gel−fluid transition temperature (*45*). Monte Carlo simulations of this transition suggest that the length of the lag is quantitatively related to the amount of boundary lipid between gel and fluid domains (*46*). Furthermore, the onset of the burst in activity occurred at a constant, small amount of hydrolysis and upon phase separation of the reaction products from the bulk lipid (*15*). In the second case, Burack et al. (*6*) were able to induce a lag in DPPG−DPPC (2:8) LUV hydrolysis by increasing the lipid concentration and reducing the Ca^{2+} concentration under conditions where all the enzyme was initially bound. In the third case, Gheriani-Gruszka et al. (*47*) showed, using unilamellar vesicles containing a constant fraction of cholate, that the length of the lag phase increased with vesicle size. These results all point to the conclusion that attributes of the vesicle structure, particularly a high radius of curvature and structural or compositional heterogeneity, play a major role in determining the activity of the enzyme on the bilayer surface.

In light of these results we are forced to conclude that the single form of the enzyme initially bound to zwitterionic vesicles and both forms initially bound to anionic vesicles are inactive or, at best, of low activity. This is underscored by several observations regarding Ca^{2+} affinity. Bell and Biltonen (*9*) suggested that the initial interaction of AppD49-PLA_2 with zwitterionic SUV did not change with the presence of calcium. However, Lathrop and Biltonen (*48*) argued that the affinity for Ca^{2+} is about 40-fold greater for the active enzyme−zwitterionic SUV complex than for the enzyme in solution. More recently, Lathrop and co-workers (in preparation) showed that the affinity of the enzyme or enzyme−substrate complex increased by about 3 orders of magnitude upon activation (i.e., at the burst.) Thus, while the fluorescence change distinguishes between two bound forms, it does not, by itself, distinguish between active and inactive enzyme. Although AppD49-PLA_2 can exist in at least two distinct states on bilayer surfaces, the final "activation step" remains to be clarified.

Summary. Our binding results describe quantitatively how increased surface charge density enhances the binding of AppD49-PLA_2 to DPPG−DPPC bilayers. The magnitude of the fluorescence change upon AppD49-PLA_2 interaction with lipid bilayers is related to charge density in a complex fashion. Importantly, we show that the absence of an intrinsic fluorescence change in AppD49-PLA_2 does not mean a lack of an interaction. The observation that AppD49-PLA_2 interacts with bilayers with or without an intrinsic fluorescence change argues that the enzyme has at least two distinct modes of binding. The existence of two modes of enzyme

binding is supported by dissociation kinetic experiments for the interaction between AppD49-PLA_2 and DPPG−DPPC LUV that results in a change in the protein fluorescence at 340 nm. In these cases the dissociation apparently involves two decay processes. However, for interactions of AppD49-PLA_2 that exhibit no change in protein fluorescence, the data are best described by a single exponential. The difference in the change in binding Gibbs energy for AppD49-PLA_2 to DPPC LUV compared to DPPG LUV is estimated to be about −3.6 kcal/mol of lipid. However, the electrostatic interaction between the anionic lipid and AppD49-PLA_2 is not the sole thermodynamic driving force for lipid bilayer binding; there must also be a significant nonionic thermodynamic component. Our observations suggest that while binding of AppD49-PLA_2 to lipid bilayers is sensitive to the surface charge density on the membrane, lipid headgroup spacing, acyl chain unsaturation, and the identity of the phospholipid headgroup are also important. We are unable to directly integrate protein conformational changes and membrane fluctuations into our analysis, but these undoubtedly also play roles in the interaction of the protein with membranes. Once the protein is initially bound, the PLA_2 likely undergoes a conformational change, which results in an activated protein species that readily binds substrate into the active site and has an increased affinity for calcium. In some cases this requires the presence of a minimal amount of lysolecithin or equivalent species in the bilayer to act as a trigger for a change in bilayer topology.

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REFERENCES

1. Dennis, E. A., Rhee, S. G., Billah, M. M., and Hannun, Y. A. (1991) *FASEB J. 5*, 2068−2077.
2. Liscovitch, M., and Cantley, L. C. (1994) *Cell 77*, 329−334.
3. Newton, A. C. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 1−25.
4. Serhan, C. N., Haeggstrom, J. Z., and Leslie, C. C. (1999) *FASEB J. 10*, 1147−1158.
5. Roberts, M. F. (1996) *FASEB J. 10*, 1159−1172.
6. Burack, W. R., Gadd, M. E., and Biltonen, R. L. (1995) *Biochemistry 34*, 14819−14828.
7. Jain, M. K., and Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127−156.
8. Dennis, E. A. (1978) *Adv. Exp. Med. Biol. 101*, 165−175.
9. Bell, J. D., and Biltonen, R. L. (1989) *J. Biol. Chem. 264*, 225−230.
10. Honger, T. E. R., Dibble, A., Burack, W. R., Allietta, M. M., and Biltonen, R. L. (1995) Vesicle Morphology Modulates Phospholipase A2 Activity, *Biophys. J. 68*, a218 (abstract).
11. op den Kamp, J. A. F., Kauerz, M., and Van Deenen, L. L. (1975) *Biochim. Biophys. Acta 406*, 169−177.
12. Verger, R., Mieras, M. C., and de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023−4034.
13. Jain, M. K., Ranadive, G. N., Yu, B. Z., and Verheij, H. M. (1991) *Biochemistry 30*, 7330−7340.
14. Biltonen, R. L., Heimburg, T. R., Lathrop, B. K., and Bell, J. D. (1990) *Adv. Exp. Med. Biol. 279*, 85−103.
15. Burack, W. R., Yuan, Q., and Biltonen, R. L. (1993) *Biochemistry 32*, 583−589.
16. Henshaw, J. B., Olsen, C. A., Farnbach, A. R., Nielson, K. H., and Bell, J. D. (1998) *Biochemistry 37* (30), 10709−10721.
17. Bangham, A. D., and Dawson, R. M. C. (1959) *Biochem. J. 72*, 486−492.
18. Maraganore, J. M., Merutka, G., Cho, W., Welches, W. K., Ezdy, F. J., and Heinrikson, R. L. (1984) *J. Biol. Chem. 259*, 13839−13843.
19. Scott, D. L., Achari, A., Zajac, M., and Sigler, P. B. (1986) *J. Biol. Chem. 261*, 12337−12338.
20. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
21. Barlett, G. R. (1960) *J. Biol. Chem. 234*, 446−450.
22. Findlay, E. J., and Barton, P. G. (1978) *Biochemistry 17*, 2400−2405.
23. Volwerk, J. J., Pieterson, W. A., and de Haas, G. H. (1974) *Biochemistry 13*, 1446−1454.
24. Romero, G., Thompson, K., and Biltonen, R. L. (1987) *J. Biol. Chem. 262*, 13476−13482.
25. Zhou, F., and Schulten, K. (1996) *Proteins: Struct., Funct., Genet. 25* (1), 12−27.
26. Mosior, M. and McLaughlin, S. (1992) *Biochim. Biophys. Acta 1105* (1), 185−187.
27. Ben Tal, N., Honig, B., Peitzsch, R. M., Denisov, G., and McLaughlin, S. (1996) *Biophys. J. 71* (2), 561−575.
28. Jacobs, R. E. and White, S. H. (1989) *Biochemistry 28* (8), 3421−3437.
29. Bahr, G., Diederich, A., Vergeres, G., and Winterhalter, M. (1998) *Biochemistry 37*, 16252−16261.
30. Ghomashchi, F., Lin, Y., Hixon, M. S., Yu, B. Z., Annand, R., Jain, M. K., and Gelb, M. H. (1998) *Biochemistry 37* (19), 6697−6710.
31. Lee, B. I., Yoon, E. T., and Cho, W. (1996) *Biochemistry 35* (13), 4231−4240.
32. Baker, S. F., Othman, R., and Wilton, D. C. (1998) *Biochemistry 37* (38), 13203−13211.
33. Verger, R., Rietsch, J., van Dam-Mieras, M. C., and de Haas, G. H. (1976) *J. Biol. Chem. 251*, 3128−3133.
34. Jain, M. K., and Vaz, W. L. (1987) *Biochim. Biophys. Acta 905*, 1−8.
35. Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., and Sigler, P. B. (1990) *Science 250*, 1541−1546.
36. Dekker, N., Peters, A. R., Slotboom, A. J., Boelens, R., Kaptein, R., Dijkman, R., and de Haas, G. H. (1991) *Eur. J. Biochem. 199*, 601−607.
37. Kennedy, D. F., Slotboom, A. J., de Haas, G. H., and Chapman, D. (1990) *Biochim. Biophys. Acta 1040*, 317−326.
38. Jerala, R., Almeida, P. F., Ye, Q., Biltonen, R. L., and Rule, G. S. (1996) *J. Biomol. NMR 7* (2), 107−120.
39. Forst, S., Weiss, J., Blackburn, P., Frangione, B., Goni, F., and Elsbach, P. (1986) *Biochemistry 25* (15), 4309−4314.
40. Peters, A. R., Dekker, N., van den Berg, L., Boelens, R., Kaptein, R., Slotboom, A. J., and de Haas, G. H. (1992) *Biochemistry 31*, 10024−10030.
41. van den Berg, B., Tessari, M., Boelens, R., Dijkman, R., de Haas, G. H., Kaptein, R., and Verheij, H. M. (1995) *Nat. Struct. Biol. 2* (5), 402−406.
42. Tatulian, S. A., Biltonen, R. L., and Tamm, L. K. (1997) *J. Mol. Biol. 268* (5), 809−815.
43. Loosley-Millman, M. E., Rand, R. P., and Parsegian, V. A. (1982) *Biophys. J. 40* (3), 221−232.
44. Tristram-Nagle, S., Petrache, H. I., and Nagle, J. F. (1998) *Biophys. J. 75* (2), 917−925.
45. Lichtenberg D., Romero, G., Menashe, M., and Biltonen, R. L. (1986) *J. Biol. Chem. 261*, 5334−5340.
46. Hoenger, T., Jorgensen, K., Biltonen, R. L., and Mouritsen, O. (1996) *Biochemistry 35*, 9003−9006.
47. Gheriani-Gruszka, N., Almog, S., Biltonen, R. L., and Lichtenberg, D. (1988) *J. Biol. Chem. 263*, 11808−11813.
48. Lathrop, B. and Biltonen, R. L. (1992) *J. Biol. Chem. 267*, 21425−21431.
49. Johnson, M. L. and Frasier, S. G. (1985) *Methods Enzymol. 117*, 301−342.

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