Toward Understanding Interfacial Activation of Secretory Phospholipase A_2 (PLA_2): Membrane Surface Properties and Membrane-Induced Structural Changes in the Enzyme Contribute Synergistically to PLA_2 Activation

Suren A. Tatulian
Section of Biochemistry and Biophysics, Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045 USA

ABSTRACT Phospholipase A_2 (PLA_2) hydrolyzes phospholipids to free fatty acids and lysolipids and thus initiates the biosynthesis of eicosanoids and platelet-activating factor, potent mediators of inflammation, allergy, apoptosis, and tumorigenesis. The relative contributions of the physical properties of membranes and the structural changes in PLA_2 to the interfacial activation of PLA_2, that is, a strong increase in the lipolytic activity upon binding to the surface of phospholipid membranes or micelles, are not well understood. The present results demonstrate that both binding of PLA_2 to phospholipid bilayers and its activity are facilitated by membrane surface electrostatics. Higher PLA_2 activity toward negatively charged membranes is shown to result from stronger membrane-enzyme electrostatic interactions rather than selective hydrolysis of the acidic lipid. Phospholipid hydrolysis by PLA_2 is followed by preferential removal of the liberated lysolipid and accumulation of the fatty acid in the membrane that may predominantly modulate PLA_2 activity by affecting membrane electrostatics and/or morphology. The previously described induction of a flexible helical structure in PLA_2 during interfacial activation was more pronounced at higher negative charge densities of membranes. These findings identify a reciprocal relationship between the membrane surface properties, strength of membrane binding of PLA_2, membrane-induced structural changes in PLA_2, and the enzyme activation.

INTRODUCTION

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the *sn*-2 ester bond of glycerophospholipids and generates free fatty acids and lysophospholipids that serve as precursors for lipid-derived mediators with a wide range of biological activities (Gelb et al., 1995, 1999; Tischfield, 1997; Dennis, 2000). Many fatty acids themselves act as bioactive mediators (Goodfriend and Egan, 1997; Forest et al., 1997). Eicosanoids, the oxygenated metabolites of arachidonic acid, play key roles in normal and pathological cell functions including cell signaling, inflammation, allergy, apoptosis, and tumorigenesis (for recent review see Heller et al., 1998; Dennis, 2000). The other product of PLA_2, lysophospholipid, may be metabolized either to platelet-activating factor, which is known as a potent inflammatory and allergic mediator (Kume and Shimizu, 1997; Jackson et al., 1998), or to lysophosphatidic acid, a signaling molecule with mitogenic activities (Fourcade et al., 1998; Gennaro et al., 1999).

Secretory PLA_2s constitute a large family of structurally and mechanistically related enzymes with relative molecular masses of 13–16 kDa. They are widespread in various mammalian cells and tissues, as well as in snake, lizard, and insect venom, and are divided into several groups and subgroups based on their amino acid sequences, disulfide bonding patterns, tissue distribution, and functional properties (Heinrikson, 1991; Tischfield, 1997; Maxey and MacDonald, 1998; Dennis, 1997, 2000). These enzymes perform phospholipid hydrolysis using a His-Asp doublet plus a conserved water molecule as a nucleophile and a Ca^{2+} ion as a cofactor. Secretory PLA_2s undergo a substantial increase in their catalytic activity upon binding to the surface of phospholipid membranes or micelles (Pieterson et al., 1974; Verger and de Haas, 1976; Jain and Berg, 1989; Gelb et al., 1995, 1999). Studies on the molecular mechanism of interfacial activation of PLA_2s led to conceptually diverse interpretations of this effect. According to one interpretation (the substrate hypothesis), the physical properties of the membrane, including membrane fluidity, curvature, surface charge, and others were considered as major determinants of the activation of PLA_2 at the membrane surface. The other (enzyme hypothesis) was that conformational changes in PLA_2 are primarily responsible for the interfacial activation of the enzyme. Indeed, unequivocal evidence has been provided for the importance of the physical state of the aggregated substrate in the activation of secretory PLA_2s (Verger and de Haas, 1976; Thuren et al., 1984; Jain and Berg, 1989; Burack and Biltonen, 1994; Burack et al., 1993, 1995; 1997; Gelb et al., 1995, 1999; Berg et al., 1997). The abrupt increase of PLA_2 activity in the presence of zwitterionic phospholipid vesicles or micelles is preceded by a dormant period that can be reduced, or abolished, by modifying the physical properties of the aggregated substrate, for example, by increasing the anionic surface charge of the membranes (Jain et al., 1982, 1986, 1989; Apitz-Castro et

Received for publication 27 June 2000 and in final form 27 November 2000.

Address reprint requests to Dr. Suren A. Tatulian, Section of Biochemistry and Biophysics, Department of Molecular Biosciences, University of Kansas, 5055 Haworth Hall, 1200 Sunnyside Ave., Lawrence, KS 66045-7534. Tel.: 785-864-4008; Fax: 785-864-5321; E-mail: suren@ukans.edu.

0006-3495/01/02/789/12 $2.00

al., 1982; Volwerk et al., 1986; Burack and Biltonen, 1994). Strong effects of non-ionized fatty acids, lysophosphatidylcholine, cholesterol, diacylglycerol, or phosphatidylethanolamine on PLA_2 activity suggested that, apart from electrostatic effects, perturbations of the membrane structure by these agents are crucial for PLA_2 activation (Jain and de Haas, 1983; Bell and Biltonen, 1992; Bell et al., 1996; Henshaw et al., 1998; Liu and Chong, 1999).

Considerable efforts have been directed to the characterization of structural changes in PLA_2 involved in the enzyme activation. X-ray crystallography revealed similar structures of secretory PLA_2s with and without bound monomeric substrate analogs (Brunie et al., 1985; Scott et al., 1990a,b; White et al., 1990; Thunnissen et al., 1990; Cha et al., 1996; Sekar et al., 1997), which was considered as evidence against structural changes in the enzyme during its interfacial activation (Scott and Sigler, 1994). Small (~1 Å) structural changes in PLA_2s upon binding of monomeric inhibitors have been detected by several x-ray studies (Scott et al., 1991; Tomoo et al., 1994; Schevitz et al., 1995); however, these changes were considered by others as insignificant (Cha et al., 1996; Sekar et al., 1997). Interpretation of x-ray results in the context of interfacial activation of PLA_2s is not straightforward because PLA_2s are activated by binding to the surface of phospholipid bilayers or micelles but not upon binding of the monomeric substrate; the binding of the substrate to the active site of the membrane-bound enzyme might have quite different structural consequences. NMR experiments revealed that in porcine pancreatic PLA_2 (group IB) the N-terminal helix and the catalytically important residues His^{48} and Asp^{99} adopt a fixed conformation only in a ternary complex of the enzyme with an inhibitory substrate analog and dodecylphosphocholine micelles (Peters et al., 1992; van den Berg et al., 1995), which might be implicated in more productive enzyme-substrate complex formation (Yu et al., 1999). Evidence for a possible allosteric coupling between the interfacial adsorption and catalytic machinery of PLA_2s has also been provided by fluorescence spectroscopy. Distinct shifts in the intrinsic Trp fluorescence of PLA_2 has been detected during the activation of the membrane-bound enzyme (Jain and Maliwal, 1993; Bell and Biltonen, 1989; Burack and Biltonen, 1994; Burack et al., 1995). Combined site-directed mutagenesis and spectroscopic studies showed that substitutions of residues in the interfacial adsorption surface (i-face) of a pancreatic PLA_2 affect both the enzyme-substrate interaction constant (K_S allostery) and the rate constant of the catalytic turnover (k^*_{cat} allostery), implying an allosteric effect that propagates from the i-face to the catalytic residues of PLA_2 (Rogers et al., 1998; Yu et al., 1999). Although these data provide evidence that interfacial activation of PLA_2 may involve conformational changes in the enzyme, the nature of these conformational changes and their relation to the physical properties of membranes are not well understood.

Our earlier attenuated total reflection Fourier transform infrared (ATR-FTIR) studies identified modification of the α-helices in a group IIA PLA_2 upon binding to lipid bilayers (Tatulian et al., 1997). In this work, the advantages of ATR-FTIR spectroscopy have been further exploited to establish a relationship between the surface properties of membranes and membrane-induced structural changes in PLA_2. The data indicate that both the strength and cooperativity of PLA_2 binding to membranes, as well as PLA_2 activity, increase at higher negative surface potentials of membranes. Phospholipid hydrolysis by PLA_2 is followed by preferential removal of the lysophospholipid and accumulation of the fatty acid in the membrane that could modulate the enzyme activation either through increasing negative electrostatic potential at the membrane surface or by affecting the membrane morphology and stability. When PLA_2 was applied to bilayers composed of an equimolar mixture of dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidylcholine with fully deuterated acyl chains [$DP(d_{62})PC$], both lipids were hydrolyzed at similar efficiencies, indicating that membrane surface electrostatics, rather than specific recognition of acidic lipids by the enzyme, plays a major role in increased activity of PLA_2 toward negatively charged membranes. A correlation has been established between the induction of previously described modified helices in PLA_2 during interfacial activation (Tatulian et al., 1997) and negative surface charge density of membranes. These findings delineate a reciprocal relationship between membrane electrostatic properties, membrane binding strength of PLA_2, and membrane-induced structural changes in the enzyme that contribute to PLA_2 activation in a synergistic manner.

MATERIALS AND METHODS

Materials

The secretory PLA_2 has been purified from the venom of the snake *Agkistrodon piscivorus piscivorus* according to Maraganore et al. (1984) and was kindly supplied by Dr. R. L. Biltonen of the Department of Pharmacology of the University of Virginia School of Medicine. The lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and the other chemicals from Sigma (St. Louis, MO).

Preparation of supported membranes

Supported lipid bilayers for ATR-FTIR experiments were prepared on a $1 \times 20 \times 50$ mm^3 germanium internal reflection plate (Spectral Systems, Irvington, NY) using two different techniques. The plate was washed by chloroform and methanol and processed in an argon plasma cleaner (Harrick, Ossining, NY) immediately before use. The first technique involved preparation of a monolayer of phosphatidylcholine at the surface of an aqueous buffer (10 mM Tris/acetic acid, pH 5) in a Langmuir trough (model 611, Nima, Coventry, UK). The monolayer was deposited onto the germanium plate by slowly (~2 mm/min) withdrawing the plate from the aqueous phase vertical to the air/water interface. The plate with the monolayer was assembled in a perfusable liquid ATR cell. Vesicles of desired lipid composition were prepared either by sonication, using a

Branson tip sonicator, or by extrusion through 100-nm pore size polycarbonate membranes using a Liposofast extruder (Avestin, Ottawa, Canada). Vesicles were injected into the ATR cell that contained the germanium plate with the monolayer and incubated for ~1.5 h to allow the vesicles to spread on the lipid monolayer and yield supported bilayers. This was followed by gently flushing the ATR cell with buffer and washing the excess lipid out of the cell. The advantage of this method is that it can be used for preparation of either symmetric or asymmetric membranes, depending on the choice of the lipids for preparation of the monolayer and the vesicles. Its disadvantage is that the membrane leaflet facing the plate cannot include acidic lipids because in that case the monolayer does not efficiently adsorb to the germanium plate, probably due to electrostatic effects. The second procedure that has been employed in this study permitted preparation of supported bilayers containing acidic lipids in both leaflets. According to this procedure, sonicated phospholipid vesicles are prepared that contain ≥20% anionic lipid (e.g., phosphatidylglycerol) in a buffer containing ~5 mM $CaCl_2$. When the vesicles are injected into the ATR cell that contains a bare germanium plate and are incubated for ~1 h, a lipid bilayer is formed at the surface of the germanium plate that is presumably stabilized by Ca^{2+} bridges between the acidic lipids and the germanium plate, which is hydrophilized by argon ion plasma processing. After preparation of supported bilayers, PLA_2 was injected into the ATR cell and allowed to adsorb to the supported membranes for 5–10 min, followed by recording of ATR-FTIR spectra. Protein concentration was measured by the Bradford assay (Bradford, 1976).

ATR-FTIR experiments

ATR-FTIR experiments were carried out on a Nicolet 740 infrared spectrometer (Nicolet Analytical Instruments, Madison, WI) using a liquid-nitrogen-cooled mercury/cadmium/telluride detector at a nominal spectral resolution of 2 cm^{-1}. A four-mirror model 57 single-beam ATR system was used (Buck Scientific, East Norwalk, CT). Normally, 1000 scans were co-added to achieve a reasonably good signal-to-noise ratio of the spectra. The incident infrared light was polarized using a gold grid polarizer (Perkin-Elmer, Beaconfield, UK). To obtain spectra including both the lipid and the protein components in the sample, the single-beam spectra of the buffer in the ATR cell with the germanium plate were used as reference. The absorbance spectra of the membrane-bound protein in the pure form were obtained by using as reference the single-beam spectra of the supported membranes that were measured before injection of the protein. These latter spectra were free of any contributions of the lipid to the spectral regions of the protein absorbance bands. The measurements were preceded by extensive purging of the instrument with dry air to remove humidity (H_2O vapors) and CO_2 and to minimize their interference with the spectra.

Data analysis

The exponentially decaying evanescent field that is created at the germanium/membrane interface at each internal reflection of the infrared beam makes it possible to detect all membrane components, including the membrane-bound protein, while the molecules far from the membrane do not contribute to the ATR-FTIR spectra (Fig. 1). This makes the ATR-FTIR spectroscopy a uniquely well suited technique for quantitative characterization of protein binding to supported membranes, the enzymatic activity of PLA_2, selective hydrolysis of different lipid components in membranes, and dissociation of lipid hydrolysis products from the membrane.

The activity of PLA_2 toward the supported lipid membranes was evaluated based on a PLA_2-concentration-dependent decrease in the intensity of lipid absorbance bands, which was shown to result from the partial removal of the lipid hydrolysis products from the membrane. The methylene symmetric stretching bands were integrated between 2878 and 2830 cm^{-1} (or between 2111 and 2071 cm^{-1} for deuterated lipid acyl chains)

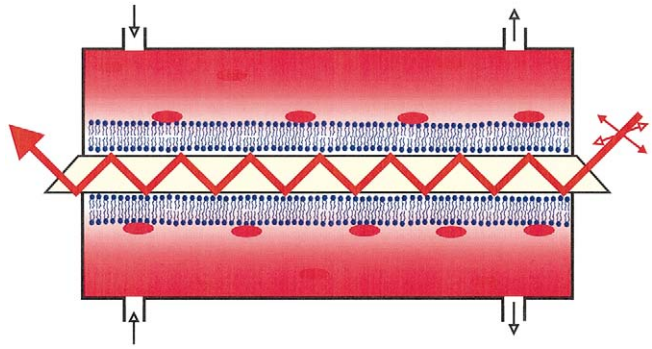

FIGURE 1 Schematic depiction of an ATR sample cell with an internal reflection plate (the yellow trapezoid in the center) that has lipid bilayers at both surfaces. The protein molecules are shown as red ellipsoids. The infrared beam is shown to enter the plate, perform several internal reflections, and exit the plate. The exponentially decaying evanescent field that is created at each internal reflection illuminates the membrane and the membrane-bound proteins whereas the molecules far from the membrane are "invisible" and do not contribute to the absorption spectrum. The outlet of one-half of the ATR sample cell is connected to the inlet of the other half, allowing for simultaneous perfusion of the whole cell.

and plotted as a function of PLA_2 concentration. The less intense symmetric methylene band was used because it, unlike its more intense asymmetric counterpart, is generated by an isolated vibrational mode and is free of Fermi resonance contributions (Rana et al., 1993). The removal from the supported membranes of the free fatty acid or the lysophospholipid were determined from changes in the olefinic CH stretching bands, which were integrated between 3023 and 2996 cm^{-1} (for lipids containing unsaturated *sn*-2 chains), and of the phosphate symmetric stretching bands integrated between 1106 and 1078 cm^{-1}, respectively. To estimate the removal from the membranes of PLA_2-generated free fatty acid for lipids with fully saturated hydrocarbon chains, selectively *sn*-1-chain deuterated lipids were used.

The protein/lipid (P/L) molar ratios in supported membranes were determined using the ratio of integrated intensities of the protein amide I and the lipid methylene stretching bands at perpendicular polarization of the infrared radiation, $A_{\perp P}$ and $A_{\perp L}$, which was corrected for corresponding molar extinction coefficients, ϵ_P and ϵ_L, and for the orientation factors, σ_i:

$$\frac{P}{L} = \frac{A_{\perp P}\sigma_L\epsilon_L n_L}{A_{\perp L}\sigma_P\epsilon_P n_P} \quad (1)$$

In Eq. 1, $\sigma_i = (S_i\sin^2\alpha_i)/2 + (1 - S_i)/3$, where i = P or L, S_i is the corresponding orientational order parameter, and α_i is the angle between the corresponding transition dipole moment and the molecular director. The subscripts P and L signify protein and lipid, respectively, n_P is the number of peptide bonds in the protein and n_L is the number of methylene groups in the lipid hydrocarbon chains. A value of $\epsilon_L = 4.7 \times 10^6$ cm/mol per CH_2 group of the lipid has been used (Fringeli et al., 1989). The amide I molar extinction coefficients of proteins depend on their secondary structure. A weighted average of $\epsilon_P = 5.7 \times 10^7$ cm/mol per peptide bond of PLA_2 was found assuming that the protein secondary structure incorporates 50% α, 10% β, and 40% irregular structure (Arni and Ward, 1996; Han et al., 1997) and using the corresponding integrated molar extinction coefficients (Venyaminov and Kalnin, 1990). The number of protein molecules per unit area of the membrane was determined using the protein/lipid molar ratio as

$$n = \frac{2P/L}{A}, \quad (2)$$

where A is the cross-sectional area per lipid molecule; $A = 50$ Å^2 was used for dipalmitoylphosphatidylcholine (DPPC) and DPPG (Seddon, 1993). PLA_2 binding to supported membranes was quantitatively characterized by plotting n against PLA_2 concentration and by describing these plots using a Langmuir-type adsorption isotherm supplemented with the Hill cooperativity coefficient:

$$n = \frac{NC^{\alpha_H}K^{\alpha_H}}{1 + C^{\alpha_H}K^{\alpha_H}}, \quad (3)$$

where N is the number of binding sites per unit area, C is the PLA_2 concentration, K is the apparent binding constant, and α_H is the Hill coefficient. The values of N were found from extrapolated intersections of the n/C versus n plots with the $n/C = 0$ line. The Hill coefficients and the dissociation constants ($1/K$) were determined respectively as the inverted slopes of the $\mathrm{Ln}(N/n - 1)$ versus $\mathrm{Ln}C$ plots and the PLA_2 concentrations corresponding to their intersections with the $\mathrm{Ln}(N/n - 1) = 0$ line, i.e., when $n = N/2$.

RESULTS

Quantitative characterization of membrane binding of PLA_2

Adsorption isotherms characterizing the binding of PLA_2 to supported bilayers containing DPPC in the lower (facing the germanium plate) leaflet and a 3:2 mixture of DPPC and DPPG in the upper leaflet at different ionic strengths were obtained by measuring the surface density of membrane-bound PLA_2, n, as a function of PLA_2 concentration (Fig. 2). As shown in Fig. 3 *A*, at low ionic strengths the n/C versus n dependencies were concave downward, indicating positive cooperativity in PLA_2 binding to negatively charged membranes (Cantor and Schimmel, 1980). The binding parameters K, N, and α_H were determined as described above and were used to calculate the theoretical curves of Fig. 2 using Eq. 3. The data of Table 1 and the curves presented in Fig. 2 demonstrate that at low ionic strengths the enzyme binding to membranes is saturable and cooperative. All three binding parameters, i.e., the binding constant, the density of binding sites, and the Hill coefficient, decrease at higher ionic strengths.

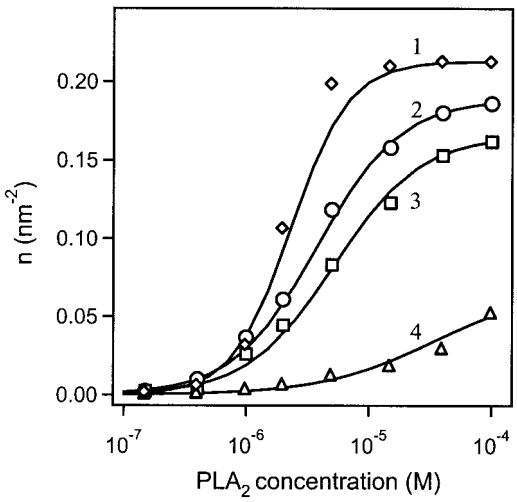

FIGURE 2 Binding of PLA_2 to supported membranes composed of DPPC at the lower leaflet and a 3:2 mixture of DPPC and DPPG at the upper leaflet. The buffer contained 5 mM Tris (pH 8.2), 0.5 mM EGTA, 1 mM NaN_3 plus 0, 0.01, 0.1, or 1 M NaCl (*curves 1–4*, respectively). The curves are simulated by Eq. 3 using the parameters summarized in Table 1.

Determination of PLA_2 activity by ATR-FTIR spectroscopy

Supported membranes that contained 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) in the lower (facing the germanium plate) leaflet and a 4:1 mixture of POPC and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) in the upper leaflet were prepared and flushed several times with buffer. After each flush, polarized ATR spectra were recorded as control measurements. This was followed by injection of PLA_2 that resulted in the appearance of a prominent amide I absorbance band, indicating binding of the enzyme to the membrane (Fig. 4). The intensity of the lipid methylene stretching band gradually decreased at each flush with buffer and then was stabilized, reflecting removal of excess lipid from the membrane. Binding of PLA_2 to the supported membrane was accompanied by a concomitant abrupt decrease in the intensity of the lipid signal (Fig. 5). This result is interpreted in terms of PLA_2-catalyzed lipid hydrolysis and dissociation of a fraction of the reaction products from the membrane. Several lines of evidence confirm this suggestion. First, before injection of PLA_2 the membrane was flushed with the buffer until the lipid signal was stabilized; i.e., additional flushes without PLA_2 did not affect the lipid signal (Fig. 5). Second, when PLA_2 was inhibited by EGTA, or when nonhydrolyzable lipids were used to prepare supported bilayers, such as dipalmitoylglycerol (DPG), dihexadecylphosphatidylcholine (DHPC) in combination with cardiolipin (CL) or arachidic acid (AA), PLA_2 did not cause any significant decrease in the lipid signal (Fig. 6). Third, partial inhibition of PLA_2 by $ZnCl_2$ (Mezna et al., 1994; Yu et al., 1998) substantially reduced the effect of PLA_2 on the lipid methylene band intensity (cf. Fig. 6 *E* and Fig. 7 *B*). These experiments demonstrate that the decrease in the lipid methylene stretching band intensity reflects PLA_2 activity that can be measured by ATR-FTIR spectroscopy.

Differential removal from the membrane of phospholipid hydrolysis products

Because the existing experimental evidence suggests that both products of phospholipid hydrolysis by PLA_2, i.e., the free fatty acid and the lysophospholipid, contribute to the activation of PLA_2 at the membrane surface, it was interesting to quantitatively determine whether one of the two products preferentially accumulates in the membrane and

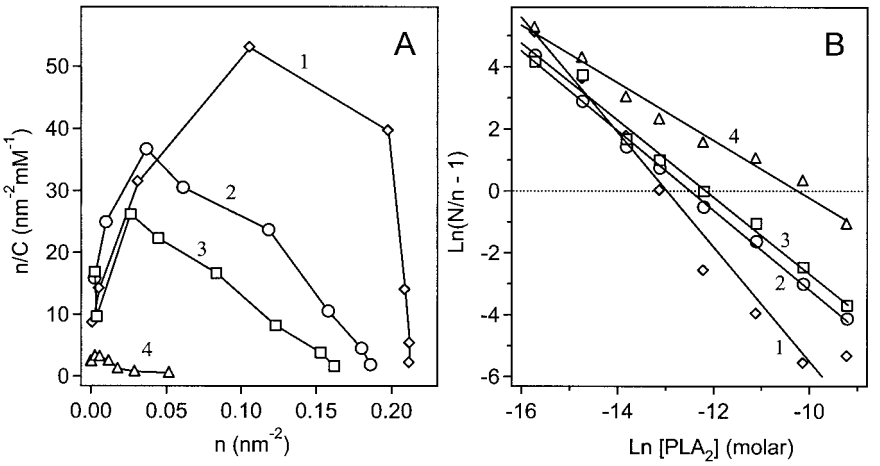

FIGURE 3 Scatchard plots that have been used to evaluate the parameters describing the binding of PLA_2 to supported bilayers containing 60 mol % DPPC and 40 mol % DPPG. Curves 1–4 correspond to NaCl concentrations 0, 0.01, 0.1, or 1 M added to the buffer: 5 mM Tris (pH 8.2), 0.5 mM EGTA, 1 mM NaN_3. The numbers of binding sites per unit area (N) were found from extrapolated intersections of the curves of A with the $n/C = 0$ line. The Hill coefficients (α_H) and the dissociation constants ($1/K$) were determined as the inverted slopes of the lines of B and the PLA_2 concentrations corresponding to the intersections with the line $\mathrm{Ln}(N/n - 1) = 0$.

plays a dominant role in the enzyme activation. Although partial removal of PLA_2 reaction products from phospholipid monolayers and bilayers has been demonstrated (Gericke and Hühnerfuss, 1994; Speijer et al., 1996; Callisen and Talmon, 1998), this question has not yet been answered.

The results of the action of PLA_2 on supported membranes of three different lipid compositions are presented in Fig. 7. In all three cases, the lipids contain a palmitic acid residue at the *sn*-1 position, but the *sn*-2 position is esterified by linoleic, oleic, and palmitic acids that contain two, one, and zero unsaturated olefinic (—HC═CH—) groups, respectively. The fractions of the total lipid components (i.e., the free fatty acid and the lysophospholipid) that remained in the membrane at each PLA_2 concentration, ΔA_{total}, were determined based on the integrated intensity of the symmetric CH_2 stretching band normalized relative to the corresponding intensity in the absence of PLA_2. To determine the differential removal from the membrane of the fatty acid and the lysophospholipid that results from phospholipid hydrolysis by PLA_2, the olefinic CH stretching band at 3005–3010 cm^{-1} was used as a marker for the fatty acid liberated from the *sn*-2 position of lipids containing unsaturated *sn*-2 chains (Fig. 7), whereas the phosphate PO_2^- symmetric stretching band at ~1090 cm^{-1} was used as a marker for the lysophospholipids (inset in Fig. 7). The normalized integrated intensities of respective absorbance bands were used as the fractions of retained *sn*-2 and *sn*-1 chains following phospholipid hydrolysis, i.e., $\Delta A_{sn\text{-}2}$ and $\Delta A_{sn\text{-}1}$. For lipids with unsaturated *sn*-2 chains, the following relationship was fulfilled: $\Delta A_{sn\text{-}2} + \Delta A_{sn\text{-}1} = 2\Delta A_{total}$. Therefore, for the membranes composed of DPPC and DPPG that lack olefinic groups, $\Delta A_{sn\text{-}2}$ was calculated as $2\Delta A_{total} - \Delta A_{sn\text{-}1}$. Data presented in Fig. 8 demonstrate that although the lipid hydrolysis is followed by partial removal from the membrane of both the liberated fatty acid and the lysophospholipid, the fraction of lysophospholipid that is removed from the membrane significantly exceeds that of

TABLE 1 Parameters characterizing PLA_2 binding to supported membranes composed of DPPC and DPPG at a 3:2 molar ratio at different ionic strengths

Ionic strength	K (M^{-1})	N (nm^{-2})	α_H
8 mM	4.3×10^5	0.213	1.85
18 mM	2.6×10^5	0.189	1.28
0.1 M	1.9×10^5	0.166	1.24
1.0 M	2.8×10^4	0.070	0.93

The buffer contained 5 mM Tris (pH 8.2), 0.5 mM EGTA, and 1 mM NaN_3. The ionic strength was adjusted by NaCl.

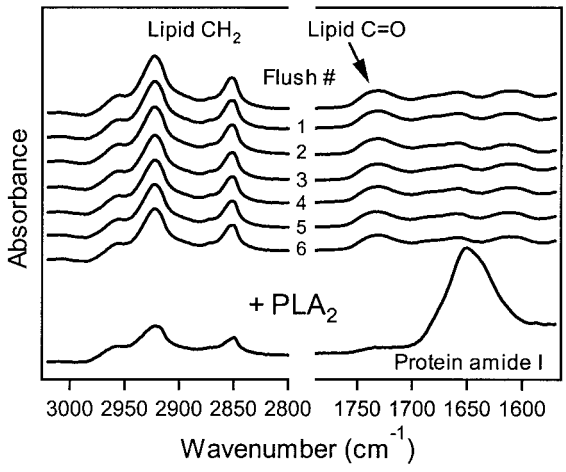

FIGURE 4 ATR-FTIR spectra of a supported bilayer containing POPC in the lower leaflet and a 4:1 mixture of POPC and POPG in the upper leaflet subjected to several flushes with buffer followed by injection of 5 μM PLA_2. The buffer contained 5 mM Hepes (pH 8.2), 100 mM NaCl, 15 mM KCl, 2 mM $CaCl_2$. The lipid methylene and carbonyl stretching bands and the protein amide I band are marked. Note a decrease in the lipid signal and appearance of a strong protein amide I signal following injection of PLA_2, indicating binding of the enzyme to the membranes and lipid hydrolysis.

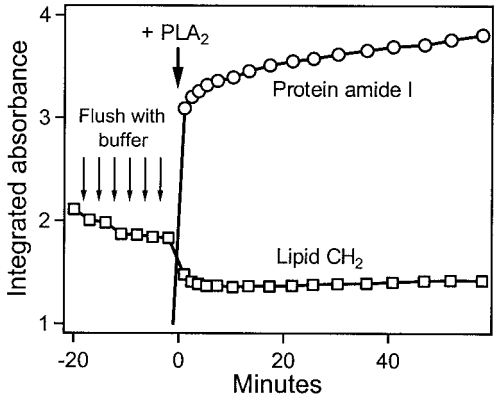

FIGURE 5 Dependence of the integrated intensities of the lipid methylene and the protein amide I bands on flushes with buffer and injection of 5 μM PLA_2, as described in Fig. 4.

the fatty acid, implying a predominant accumulation of the fatty acid in the membrane.

Because the olefinic stretching mode is not present in lipids with fully saturated hydrocarbon chains, and its intensity is low even in lipids containing chains with one or two double bonds, a second method has been used to assess the relative depletion of the fatty acid and the lysophospholipid resulting from lipid hydrolysis. Supported bilayers were prepared using 1-palmitoyl(d_{31})-2-palmitoylphosphatidylcholine in which the *sn*-1 chain is fully deuterated while the *sn*-2 chain is not. Substitution of methylene hydrogens by deuterium results in a >700-cm^{-1} shift of the methylene stretching modes toward lower frequencies, because of the heavier nuclear mass of deuterium (Fig. 9). Also, the CD_2 stretching mode is broader and approximately twofold weaker than the CH_2 mode due to the lower extinction coefficient of the former vibrational mode (Rana et al., 1993). The plots of the normalized integrated areas of the CH_2 and CD_2 symmetric stretching bands as a function of PLA_2 concentration showed that lipid hydrolysis is followed by a preferential removal of the *sn*-1 chain of DPPC (i.e., the lysophospholipid) whereas the *sn*-2 chain, which belongs to the free fatty acid, tends to stay in the membrane (Fig. 10). This result is consistent with the suggestion of the above experiments that the free fatty acid predominantly contributes to interfacial activation of PLA_2 by 1) increasing negative electrostatic potential at the membrane surface and/or 2) affecting the membrane morphology.

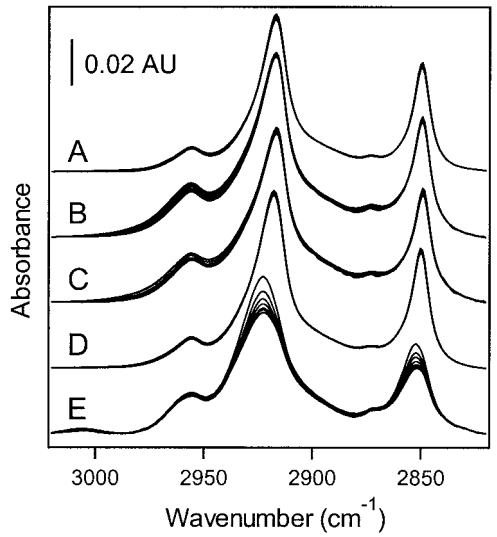

FIGURE 6 Lipid methylene stretching bands at different concentrations of PLA_2. The lipid composition of the asymmetric supported bilayers was as follows. (*A*) DPPC/(DPPC + DPPG) (4:1); (*B*) DPG/(DPG + CL) (9:1); (*C*) DPG/(DPG + AA) (4:1); (*D*) DHPC/(DHPC + AA) (4:1); (*E*) POPC/(POPC + POPG) (4:1). The buffer contained 5 mM Hepes (pH 8.2), 100 mM NaCl, and 15 mM KCl with the following additions: 0.5 mM EGTA (*A*), 2 mM $CaCl_2$ (*B–D*), and 2 mM $CaCl_2$ plus 0.5 mM $ZnCl_2$ (*E*). In each of five families of spectra, PLA_2 concentration was increased from 0 to 50 μM (from top to bottom). The spectra of DHPC/(DHPC + AA) (*D*) were more intense and were reduced by a factor of 2 to maintain proportionality; the others are presented as measured.

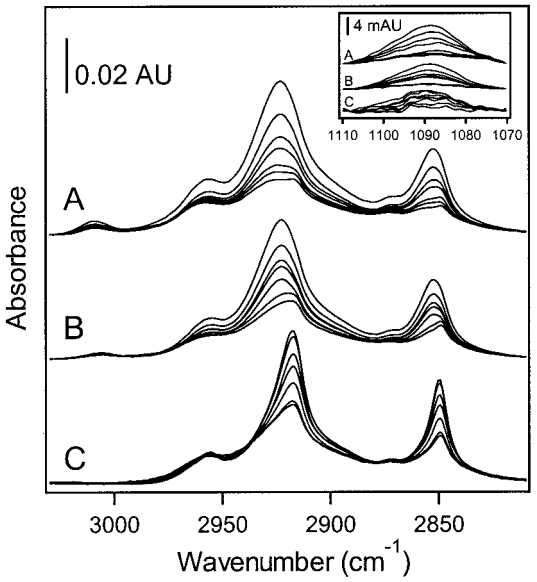

FIGURE 7 Lipid methylene (and phosphate, in the *inset*) stretching bands at different concentrations of PLA_2. The lipid composition of the asymmetric supported bilayers was as follows. (*A*) PLPC/(PLPC + PLPG); (*B*) POPC/(POPC + POPG); (*C*) DPPC/(DPPC + DPPG). In all three cases, the fraction of the acidic lipid in the upper leaflet is 20 mol %. The buffer contained 5 mM Hepes (pH 8.2), 100 mM NaCl, 15 mM KCl, 2 mM $CaCl_2$. In each of the three families of spectra, PLA_2 concentration was increased from 0 to 50 μM (from top to bottom).

Effect of the acidic lipid on PLA_2 activity

The effect of the acidic lipid in supported membranes on the activity of PLA_2 was studied by using bilayers composed of a mixture of POPC and POPG in which the fraction of POPG was increased form 0 to 0.5. The plots of the meth-

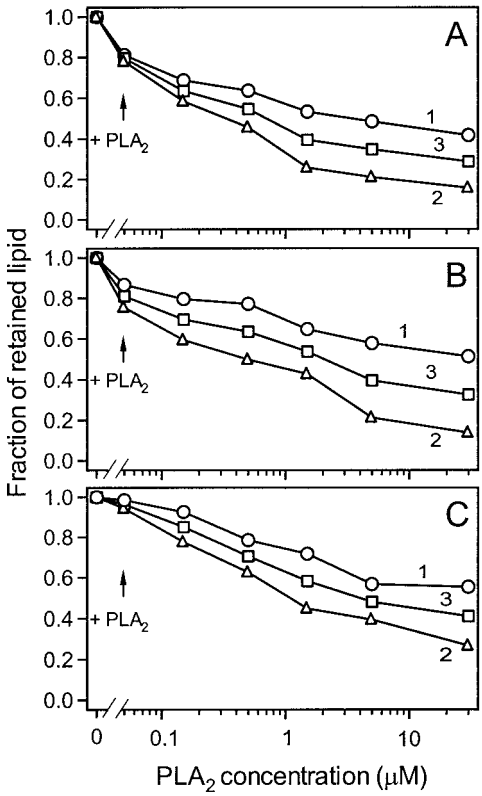

FIGURE 8 Normalized integrated intensities due to the total methylene stretching mode (□), *sn*-1 acyl chains (△), and *sn*-2 acyl chains (○) of the bilayers composed of PLPC/(PLPC + PLPG) in *A*, POPC/(POPC + POPG) in *B*, and DPPC/(DPPC + DPPG) in *C* as a function of PLA_2 concentration under conditions described in Fig. 7. For details, see the text.

ylene stretching intensities as a function of PLA_2 concentration indicated that PLA_2 exhibited higher activity toward membranes with higher fractions of the acidic lipid POPG (Fig. 11). To determine whether the correlation between PLA_2 activity and the membrane negative surface charge density is due to stronger electrostatic attraction between the cationic PLA_2 and negatively charged membranes or whether this effect results from stronger affinity of the acidic lipid to the enzyme active center, experiments were conducted on supported bilayers composed of 50% $DP(d_{62})PC$ with deuterated acyl chains and 50% unlabeled DPPG (Fig. 12). These membranes were prepared by using the method of direct spreading of sonicated vesicles onto the bare germanium plate, which ensured an equimolar content of the zwitterionic and acidic lipids in the membranes. Dependencies of integrated intensities of CH_2 and CD_2 symmetric stretching bands on PLA_2 concentration showed that both lipids were hydrolyzed at similar efficiencies (Fig. 13), indicating that the acidic lipid is not preferentially hydrolyzed by PLA_2. Instead, the greater activity of the enzyme toward membranes containing higher fractions of acidic lipids results from stronger binding of the enzyme to the surface of membranes with higher anionic surface charge.

Correlation between membrane surface properties and membrane-induced structural changes in PLA_2

Our earlier studies identified significant differences between the amide I bands of free and membrane-bound PLA_2 (Tatulian et al., 1997). The second-derivative spectrum of the free enzyme demonstrated a major component at ~1650 cm^{-1}, indicating a predominantly α-helical structure for the protein (Mendelsohn and Mantsch, 1986; Arrondo et al., 1993; Jackson and Mantsch, 1995), whereas the α-helical signal of the membrane-bound protein was split into two subcomponents at ~1658 and ~1650 cm^{-1}. Less stable α-helices are characterized by stronger carbonyl stretching force constants because of weaker helical hydrogen bonding and, consequently, their amide I vibrational mode occurs at higher frequencies (Dwivedi and Krimm, 1984). Therefore, the appearance of the higher-frequency signal in the α-helical region of the amide I band of PLA_2 is interpreted in terms of increased flexibility of the α-helices of membrane-bound PLA_2. The resolution-enhanced (second-derivative) amide I spectra of PLA_2 bound to supported membranes of POPC containing 0, 5, 20, and 50% POPG indicated a clear correlation between the intensity of the component at 1658 cm^{-1} and the fraction of the acidic lipid in the membrane (Fig. 14).

DISCUSSION

Strength and cooperativity of PLA_2-membrane interactions

Very high binding affinities have been reported for association of secretory PLA_2s with anionic phosphatidylglycerol surfaces, i.e., $K \approx 10^9\ M^{-1}$ and $K \approx 5 \times 10^7\ M^{-1}$ for human group IIA PLA_2 and AppD49, respectively (Han et al., 1997; Snitko et al., 1997). Electrostatic effects at least partly determine the high affinities of these PLA_2s for negatively charged membranes. Consistent with this, much lower binding constants ($<10^3\ M^{-1}$) have been measured for the binding of both enzymes to zwitterionic phosphatidylcholine vesicles (Han et al., 1997; Bayburt et al., 1993). The data presented in Figs. 2 and 3 and in Table 1 demonstrate that not only the apparent binding constant of AppD49 for anionic membranes but also the density of binding sites and binding cooperativity decrease when surface electrostatics is suppressed by high ionic strengths. Higher apparent binding constants at low ionic strengths are evidently due to electrostatic attraction between the cationic PLA_2 and negatively charged membranes. Binding of Na^+ ions to the acidic lipids in the membrane, which are probably involved in the creation of binding sites, may account

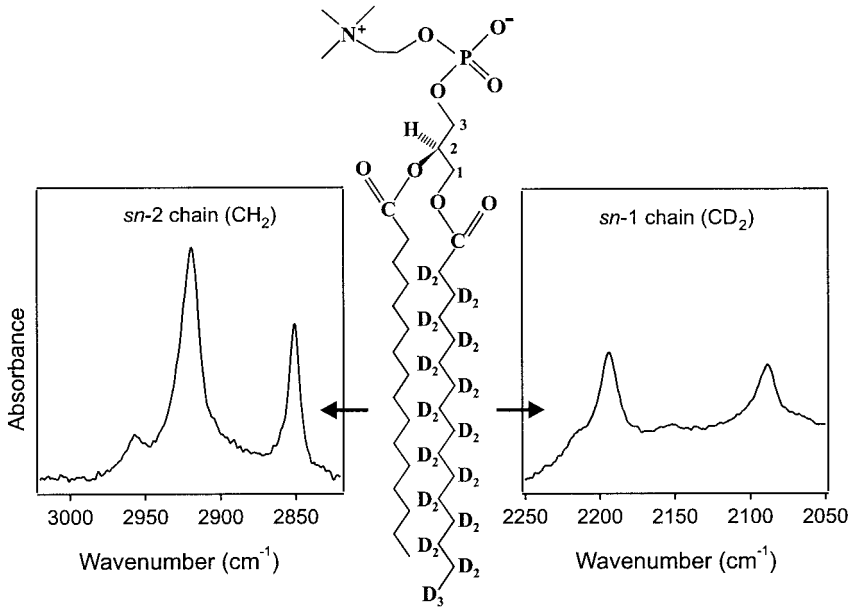

FIGURE 9 Methylene stretching bands of the *sn*-1 (right) and *sn*-2 (left) acyl chains of 1-palmitoyl(d_{31})-2-palmitoylphosphatidylcholine.

for the decrease in the binding site density at high NaCl concentrations (Tatulian, 1993, 1999; Tatulian and Biltonen, 1997). Increased binding cooperativity at low ionic strengths can be explained by hypothesizing that the enzyme forms dimers at the membrane surface; i.e., each membrane-bound enzyme induces the binding of another one for dimer formation. Dimerization of PLA_2 at the membrane surface may be facilitated by decreased electrostatic repulsion between the cationic enzyme molecules because of the negative surface potential of the membrane, an effect that would be more efficient at lower ionic strengths. Interestingly, the dimeric and monomeric isoforms of AppD49 are structurally similar to each other (Scott et al., 1994), but the dimeric isoform is acidic (excess charge at neutral pH is −1) whereas the monomeric form is strongly basic (excess charge is +6). This agrees with the hypothesis that electrostatic effects exerted by negatively charged membranes may facilitate dimerization of the monomeric enzyme at the membrane surface (see also Welches et al., 1993).

Role of phospholipid hydrolysis products in PLA_2 activation

The role of phospholipid hydrolysis products, the free fatty acid and the lysophospholipid, in PLA_2 activation is important for understanding 1) the mechanism of PLA_2 activation at the membrane surface in general and 2) the factors that make cell membranes susceptible to the action of PLA_2. It has been shown that phospholipid vesicles maintained their structural integrity upon complete hydrolysis of the lipid in their outer leaflet by PLA_2 (Jain et al., 1986; Berg et al., 1991; Bayburt et al., 1993), indirectly implying that most, if

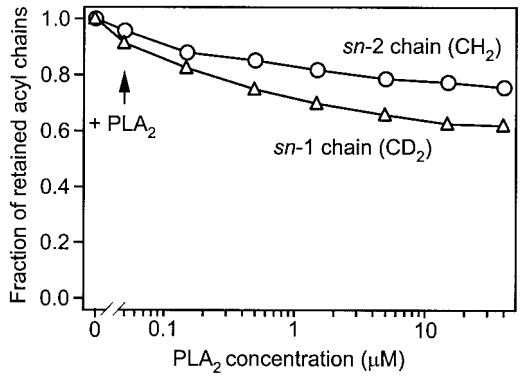

FIGURE 10 Normalized integrated methylene stretching intensities of the deuterated *sn*-1 and unlabeled *sn*-2 acyl chains of 1-palmitoyl(d_{31})-2-palmitoylphosphatidylcholine in supported membranes as a function of PLA_2 concentration in the presence of 2 mM $CaCl_2$.

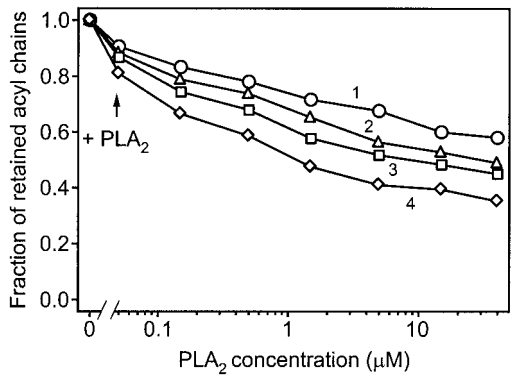

FIGURE 11 Normalized integrated intensities of methylene stretching bands of lipids in supported bilayers composed of POPC plus 0, 5, 20, and 50 mol % POPG (*curves 1–4*, respectively) as a function of PLA_2 concentration in the presence of 2 mM $CaCl_2$.

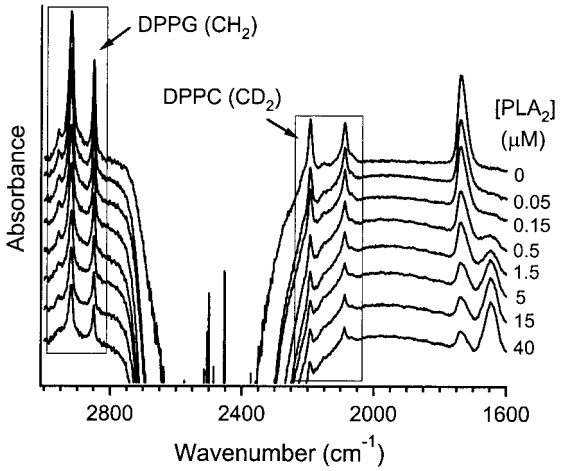

FIGURE 12 ATR-FTIR spectra of a supported membrane composed of an equimolar mixture of DPPG and DP(d_{62})PC as a function of PLA_2 concentration, as indicated, in the presence of 2 mM $CaCl_2$. The CH_2 stretching bands of DPPG and the CD_2 stretching bands of DPPC are indicated by thin rectangles.

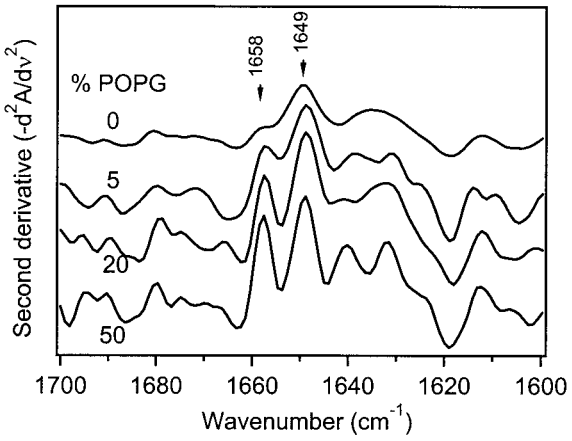

FIGURE 14 Second derivatives of the ATR-FTIR spectra in the amide I region of PLA_2 bound to supported membranes composed of POPC plus 0, 5, 20, and 50 mol % POPG, as indicated, in a buffer containing 5 mM Hepes (pH 8.2), 100 mM NaCl, 15 mM KCl, 2 mM $CaCl_2$. Note the increase in the intensity of the component at ~1658 cm^{-1} at higher fractions of the acidic lipid, POPG, in the membrane.

not all, reaction product stays in the membrane following phospholipid hydrolysis. The lipid degradation products in the membrane were further shown to promote PLA_2 activation by modifying the membrane structure and strengthening PLA_2-membrane interactions (Jain et al., 1982, 1986; Jain and de Haas, 1983; Apitz-Castro et al., 1982; Bayburt et al., 1993; Burack and Biltonen, 1994; Burack et al., 1997). For example, the binding affinity of human group IIA PLA_2 for phosphatidylcholine vesicles increased by three orders of magnitude in the presence of 18% reaction products in the membrane (Bayburt et al., 1993).

On the other hand, removal of a significant fraction of lipid hydrolysis products has been demonstrated by ellipsometry for bilayers supported on silicon discs (Speijer et al., 1996), by external reflection FTIR spectroscopy for monolayers at the air/water interface (Gericke and Hühnerfuss, 1994), and by cryo-transmission electron microscopy for unilamellar vesicles (Callisen and Talmon, 1998). The present results indicate that phospholipid degradation by PLA_2 is followed by dissociation from the membrane of a fraction of both the free fatty acid and the lysophospholipid and that the lysophospholipid is removed from the membrane to a significantly larger extent than the fatty acid (Figs. 8 and 10). This leads to the accumulation of the free fatty acid in the membrane, which would modulate membrane binding and activity of PLA_2 through electrostatic and/or morphological effects. In fact, an increase in the negative surface potential of both lipid vesicles and planar membranes has been observed in the presence of PLA_2 under catalytic conditions (Cherny et al., 1990, 1992). Addition of fatty acid, but not lysophosphatidylcholine, to lipid vesicles increased their negative zeta potential, suggesting that the PLA_2-induced negative surface potential may result from the accumulation of the fatty acid in the vesicle membranes (Cherny et al., 1992). It should be noted that although most of the liberated fatty acid stays in the membrane, a fraction of it partitions into the aqueous phase. Probably only an optimal amount of the fatty acid in the membrane is required for efficient lipolysis by PLA_2. Formation of 2:1 fatty acid/phosphatidylcholine complexes has been observed by several studies (Cevc et al., 1988, and references therein). At moderate fractions of PLA_2-generated fatty acid, complexes between intact phospholipid and fatty acid may form and serve as PLA_2 binding sites that are characterized by local negative curvature and increased anionic charge, although very high fractions of the fatty acid may inhibit PLA_2 activity by laterally segregating into negatively charged patches and electrostatically sequestering PLA_2 from its substrate.

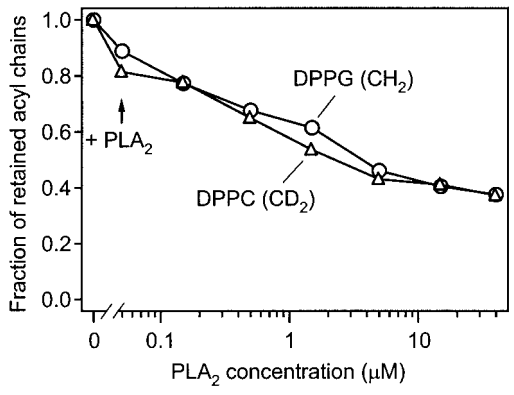

FIGURE 13 Normalized integrated intensities of methylene stretching bands of DPPG (○) and DP(d_{62})PC (△) as a function of PLA_2 concentration, calculated from the data of an experiment described in Fig. 12.

The other PLA_2 reaction product, the lysophospholipid, is also able to activate PLA_2 (Jain and de Haas, 1983; Bell et al., 1996; Henshaw et al., 1998). It should be noted that the present data indicate predominant, but not complete, removal of the lysophospholipid from the membrane following lipid hydrolysis. Disproportional removal from the membrane of the fatty acid and the lysophospholipid is likely to perturb the membrane structure and stimulate PLA_2 activation to a greater extent than in the case of proportional removal or preservation of both products. This is consistent with the observation that exogenous lysophosphatidylcholine reduces the ability of fatty acid to enhance interfacial activation of PLA_2 (Henshaw et al., 1998), probably by repairing the fatty-acid-induced structural irregularities in phospholipid membranes. These conclusions, which are drawn from the studies on model membranes, are consistent with the results obtained on cell cultures suggesting that proinflammatory cytokines render the membranes of the affected cells susceptible to the action of PLA_2 by modifying the structure of cell membranes (Murakami et al., 1998).

Role of acidic lipid in PLA_2 activity

Higher PLA_2 activity toward membranes with increased anionic surface charge has been observed by several earlier studies. Thus, porcine pancreatic PLA_2 had a two- to threefold preference for anionic lipids (Ghomashchi et al., 1991). AppD49, which was used in this study, exhibited a three- to fivefold preference for catalysis of anionic versus zwitterionic lipids (Han et al., 1997). When human group IIA PLA_2 was applied to polymerized phosphatidylglycerol vesicles containing 1 mol % pyrene-phosphatidylglycerol or pyrene-phosphatidylethanolamine, the anionic lipid was hydrolyzed 10 times faster (Snitko et al., 1997). On the other hand, no significant discrimination by this enzyme between acidic and zwitterionic lipids was detected by the double-radiolabel technique (Bayburt et al., 1993). Consistent with these latter results, the present data indicate that although the activity of AppD49 increased with increasing mole fraction of the acidic lipid in the membrane (Fig. 11), the enzyme did not demonstrate intrinsic preference for the anionic lipid (Fig. 13).

Correlation between membrane surface charge and membrane-induced structural changes in PLA_2

As described in the Introduction, studies on the interfacial activation of PLA_2 have been focused either on the role of the membrane surface properties or, in fewer cases, on the structural changes in PLA_2 caused by membrane binding, leading to the conceptually different substrate and enzyme hypotheses. The results of this work identify a correlation between the membrane surface electrostatics, the strength and cooperativity of membrane binding of PLA_2, membrane-induced structural changes in PLA_2, and PLA_2 activity. The data suggest that conformational changes do occur in PLA_2 during its interactions with membranes and that the membrane surface properties and structural changes in the enzyme contribute synergistically to PLA_2 activation. This synergistic mechanism of the interfacial activation of PLA_2 implies that the factors controlling membrane binding of PLA_2 determine structural changes in the enzyme that result in the activation of the enzyme. It should be emphasized that, as described in Tatulian et al. (1997), the structural changes upon membrane binding of PLA_2 occur under both catalytic and noncatalytic conditions. Therefore, these structural changes are likely to take place during the membrane-binding step of the complex process of interfacial activation of PLA_2, independent of the substrate binding to the active center. However, they are a prerequisite for the activation of PLA_2 at the membrane surface, provided there is calcium in the aqueous phase and a hydrolyzable lipid in the membrane. This is consistent with the notion that although the membrane binding of PLA_2 and the catalytic turnover are temporally dissociated and involve different residues, there is a close structural and functional coupling between them.

CONCLUDING REMARKS

Secretory PLA_2s are perhaps the most extensively studied enzymes that catalyze reactions at the lipid/water interfaces (Jain and Berg, 1989; Scott and Sigler, 1994; Mukherjee et al., 1994; Arni and Ward, 1996; Gelb et al., 1995, 1999; Dennis, 1997, 2000). However, certain aspects of interfacial activation of these enzymes, including the structural changes in the enzyme upon membrane binding and their correlation with the membrane physical properties, are still not well understood. The present study demonstrates that ATR-FTIR spectroscopy is uniquely well suited for investigating a wide range of problems pertaining to the activation of PLA_2 at the membrane surface. The data indicate a reciprocal relationship between the membrane surface properties, membrane binding strength of PLA_2, structural changes in the enzyme, and PLA_2 activity. This finding unifies the substrate and enzyme hypotheses of interfacial activation of PLA_2 and implies that both the membrane and enzyme factors are complementary and synergistic determinants of the activation of membrane-bound PLA_2. The surface properties of the membrane are indeed important for PLA_2 activation. But they are only a prerequisite for binding of PLA_2 to the membrane surface in a proper way, probably including the strength of binding, the depth of membrane insertion, and the orientation, which is required for the induction of the conformational changes in PLA_2 that ultimately activate the enzyme.

REFERENCES

Apitz-Castro, R., M. K. Jain, and G. H. de Haas. 1982. Origin of the latency phase during the action of phospholipase A_2 on unmodified phosphatidylcholine vesicles. *Biochim. Biophys. Acta.* 688:349–356.

Arni, R. K., and R. J. Ward. 1996. Phospholipase A_2: a structural review. *Toxicon.* 34:827–841.

Arrondo, J. L. R., A. Muga, J. Castresana, and F. M. Goñi. 1993. Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy. *Prog. Biophys. Mol. Biol.* 59:23–56.

Bayburt, T., B.-Z. Yu, H.-K. Lin, J. Browning, M. K. Jain, and M. H. Gelb. 1993. Human nonpancreatic secretory phospholipase A_2: interfacial parameters, substrate specificity, and competitive inhibitors. *Biochemistry.* 32:573–582.

Bell, J. D., and R. L. Biltonen. 1989. The temporal sequence of events in the activation of phospholipase A_2 by lipid vesicles. *J. Biol. Chem.* 264:12194–12200.

Bell, J., and R. L. Biltonen. 1992. Molecular details of the activation of phospholipase A_2 on lipid bilayers. *J. Biol. Chem.* 267:11036–11056.

Bell, J. D., M. Burnside, J. A. Owen, M. L. Royall, and M. L. Baker. 1996. Relationship between bilayer structure and phospholipase A_2 activity: interactions among temperature, diacylglycerol, lysolecithin, palmitic acid, and dipalmitoylphosphatidylcholine. *Biochemistry.* 35:4945–4955.

Berg, O. G., J. Rogers, B.-Z. Yu, J. Yoa, L. S. Romsted, and M. K. Jain. 1997. Thermodynamic and kinetic basis of interfacial activation: resolution of binding and allosteric effects on pancreatic phospholipase A_2 at zwitterionic interfaces. *Biochemistry.* 36:14512–14530.

Berg, O. G., B.-Z. Yu, J. Rogers, and M. K. Jain. 1991. Interfacial catalysis by phospholipase A_2: determination of the interfacial kinetic rate constants. *Biochemistry.* 30:7283–7297.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Brunie, S., J. Bolin, D. Gewirth, and P. B. Sigler. 1985. The refined crystal structure of dimeric phospholipase A_2 at 2.5 Å. Access to a shielded catalytic center. *J. Biol. Chem.* 260:9742–9749.

Burack, W. R., and R. L. Biltonen. 1994. Lipid bilayer heterogeneities and modulation of phospholipase A_2 activity. *Chem. Phys. Lipids.* 73: 209–222.

Burack, W. R., A. R. G. Dibble, M. M. Allietta, and R. L. Biltonen. 1997. Changes in vesicle morphology induced by lateral phase separation modulate phospholipase A_2 activity. *Biochemistry.* 36:10551–10557.

Burack, W. R., M. E. Gadd, and R. L. Biltonen. 1995. Modulation of phospholipase A_2: identification of an inactive membrane-bound state. *Biochemistry.* 34:14819–14828.

Burack, W. R., Q. Yuan, and R. L. Biltonen. 1993. Role of phase separation in the modulation of phospholipase A_2 activity. *Biochemistry.* 32:583–589.

Callisen, T. H., and Y. Talmon. 1998. Direct imaging by cryo-TEM shows membrane break-up by phospholipase A_2 enzymatic activity. *Biochemistry.* 37:10987–10993.

Cantor, C. R., and P. R. Schimmel. 1980. Biophysical Chemistry, Part III. W. H. Freeman, San Francisco.

Cevc, G., J. M. Seddon, R. Hartung, and W. Eggert. 1988. Phosphatidylcholine-fatty acid membranes. I. Effects of protonation, salt concentration, temperature and chain-length on the colloidal and phase properties of mixed vesicles, bilayers and nonlamellar structures. *Biochim. Biophys. Acta.* 940:219–240.

Cha, S.-S., D. Lee, J. Adams, J. T. Kurdyla, C. S. Jones, L. A. Marshall, B. Bolognese, S. S. Abdel-Meguid, and B.-H. Oh. 1996. High-resolution x-ray crystallography reveals precise binding interactions between human nonpancreatic secreted phospholipase A_2 and a highly potent inhibitor (FPL67047XX). *J. Med. Chem.* 39:3878–3881.

Cherny, V. V., V. M. Mirsky, V. S. Sokolov, and V. S. Markin. 1990. An electrostatic assay of phospholipase activity. *Biochemistry [Russian].* 55: 445–450.

Cherny, V. V., M. G. Sikharulidze, V. M. Mirsky, and V. S. Sokolov. 1992. Potential distribution on the lipid bilayer membrane due to the phospholipase A_2 activity. *Biol. Membr. [Russian].* 9:733–740.

Dennis, E. A. 1997. The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends. Biochem. Sci.* 22:1–2.

Dennis, E. A. 2000. Phospholipase A_2 in eicosanoid generation. *Am. J. Respir. Crit. Care Med.* 161:S32–S35.

Dwivedi, A. M., and S. Krimm. 1984. Vibrational analysis of peptides, polypeptides, and proteins. XVIII. Conformational sensitivity of the α-helix spectrum: α_I- and α_{II}-poly(L-alanine). *Biopolymers.* 2:923–943.

Forest, C., S. Franckhauser, M. Glorian, J. Antras-Ferry, D. Robin, and P. Robin. 1997. Regulation of gene transcription by fatty acids, fibrates and prostaglandins: the phosphoenolpyruvate carboxykinase gene as a model. *Prostagl. Leukotr. Essent. Fatty Acids.* 57:47–56.

Fourcade, O., F. Leballe, J. Fauvel, M.-F. Simon, and H. Chap. 1998. Regulation of secretory type-II phospholipase A_2 and of lysophosphatidic acid synthesis. *Adv. Enzyme Regul.* 38:99–107.

Fringeli, U. P., H.-J. Apell, M. Fringeli, and P. Läuger. 1989. Polarized infrared absorption of Na^+/K^+-ATPase studied by attenuated total reflection spectroscopy. *Biochim. Biophys. Acta.* 984:301–312.

Gelb, M. H., W. Cho, and D. C. Wilton. 1999. Interfacial binding of secreted phospholipases A_2: more than electrostatics and a major role for tryptophan. *Curr. Opin. Struct. Biol.* 9:428–432.

Gelb, M. H., M. K. Jain, A. M. Hanel., and O. G. Berg. 1995. Interfacial enzymology of glycerolipid hydrolysis: lessons from secretory phospholipase A_2. *Annu. Rev. Biochem.* 64:653–688.

Gennaro, I., J. M. Xuereb, M.-F. Simon, J. P. Girolami, J. L. Bascands, H. Chap, B. Boneu, and P. Sie. 1999. Effect of lysophosphatidic acid on proliferation and cytosolic Ca^{2+} of human adult vascular smooth muscle cell culture. *Thromb. Res.* 94:317–326.

Gericke, A., and H. Hühnerfuss. 1994. IR reflection absorption spectroscopy: a versatile tool for studying interfacial enzymatic processes. *Chem. Phys. Lipids.* 74:205–210.

Ghomashchi, F., B.-Z. Yu, O. Berg, M. K. Jain, and M. H. Gelb. 1991. Interfacial catalysis by phospholipase A_2: substrate specificity in vesicles. *Biochemistry.* 30:7318–7329.

Goodfriend, T. L., and B. M. Egan. 1997. Nonesterified fatty acids in the pathogenesis of hypertension: theory and evidence. *Prostagl. Leukotr. Essent. Fatty Acids.* 57:57–63.

Han, S. K., E. T. Yoon, D. L. Scott, P. B. Sigler, and W. Cho. 1997. Structural aspects of interfacial adsorption. A crystallographic and site-directed mutagenesis study of the phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. *J. Biol. Chem.* 272:3573–3582.

Heinrikson, R. L. 1991. Dissection and sequence analysis of phospholipase A_2. *Methods Enzymol.* 197:201–214.

Heller, A., T. Koch, J. Schmeck, and K. van Ackern. 1998. Lipid mediators and inflammatory disorders. *Drugs.* 55:487–496.

Henshaw, J. B., C. A. Olson, A. R. Farnbach, K. H. Nielsen, and J. D. Bell. 1998. Definition of the specific roles of lysolecithin and palmitic acid in altering the susceptibility of dipalmitoylphosphatidylcholine bilayers to phospholipase A_2. *Biochemistry.* 37:10709–10721.

Jackson, J. R., B. Bolognese, C. A. Mangar, W. C. Hubbard, L. A. Marshall, and J. D. Winkler. 1998. The role of platelet activating factor and other lipid mediators in inflammatory angiogenesis. *Biochim. Biophys. Acta.* 1392:145–152.

Jackson, M., and H. H. Mantsch. 1995. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* 30:95–120.

Jain, M. K., and O. G. Berg. 1989. The kinetics of interfacial catalysis by phospholipase A_2 and regulation of interfacial activation: hopping versus scooting. *Biochim. Biophys. Acta.* 1002:127–156.

Jain. M. K., and G. H. de Haas. 1983. Activation of phospholipase A_2 by freshly added lysophospholipids. *Biochim. Biophys. Acta.* 736:157–162.

Jain, M. K., M. R. Egmond, H. M. Verheij, R. Apitz-Castro, R. Dijkman, and G. H. de Haas. 1982. Interaction of phospholipase A_2 and phospholipid bilayers. *Biochim. Biophys. Acta.* 688:341–348.

Jain, M. K., and B. P. Maliwal. 1993. Spectroscopic properties of the states of pig pancreatic phospholipase A_2 at interfaces and their possible molecular origin. *Biochemistry.* 32:11838–11846.

Jain, M. K., J. Rogers, D. V. Jahagirdar, J. F. Marecek, and F. Ramirez. 1986. Kinetics of interfacial catalysis by phospholipase A_2 in intra-

vesicle scooting mode, and heterofusion of anionic and zwitterionic vesicles. *Biochim. Biophys. Acta.* 860:435–447.

Jain, M. K., B.-Z. Yu, and A. Kozubek. 1989. Binding of phospholipase A_2 to zwitterionic bilayers is promoted by lateral segregation of anionic amphiphiles. *Biochim. Biophys. Acta.* 980:23–32.

Kume, K., and T. Shimizu. 1997. Platelet-activating factor (PAF) induces growth stimulation, inhibition, and suppression of oncogenic transformation in NRK cells overexpressing the PAF receptor. *J. Biol. Chem.* 272:22898–22904.

Liu, F., and P. L.-G. Chong. 1999. Evidence for a regulatory role of cholesterol superlattices in the hydrolytic activity of secretory phospholipase A_2 in lipid membranes. *Biochemistry.* 38:3867–3873.

Maraganore, J. M., G. Merutka, W. Cho, W. Welches, F. J. Kézdy, and R. L. Heinrikson. 1984. A new class of phospholipases A_2 with lysine in place of aspartate 49. Functional consequences for calcium and substrate binding. *J. Biol. Chem.* 259:13839–13843.

Maxey, K., and J. MacDonald. 1998. PLA_2. A short phospholipase review. *Cayman Curr.* 8:1–5.

Mendelsohn, R., and H. H. Mantsch. 1986. Fourier transform infrared studies of lipid-protein interactions. *In* Progress in Protein-Lipid Interactions. A. Watts, and J. J. H. H. M. Depont, editors. Elsevier, Amsterdam. 103–146.

Mezna, M., T. Ahmad, S. Chettibi, D. Draines, and A. J. Lawrence. 1994. Zinc and barium inhibit phospholipase A_2 from *Naja naja atra* by different mechanisms. *Biochem. J.* 301:503–508.

Mukherjee, A. B., L. Miele, and N. Pattabiraman. 1994. Phospholipase A_2 enzymes: regulation and physiological role. *Biochem. Pharmacol.* 48:1–10.

Murakami, M., S. Shimbara, T. Kambe, H. Kuwata, M. V. Winstead, J. A. Tischfield, and I. Kudo. 1998. The functions of five distinct mammalian phospholipase A_2s in regulating arachidonic acid release: type IIA and type V secretory phospholipase A_2s are functionally redundant and act in concert with cytosolic phospholipase A_2. *J. Biol. Chem.* 273:14411–14423.

Peters, A. R., N. Dekker, L. van den Berg, R. Boelens, R. Kaptein, A. J. Slotboom, and G. H. de Haas. 1992. Conformational changes in phospholipase A_2 upon binding to micellar interfaces in the absence and presence of competitive inhibitors: a ^1H and ^{15}N NMR study. *Biochemistry.* 31:10024–10030.

Pieterson, W. A., J. C. Vidal, J. J. Volwerk, and G. H. de Haas. 1974. Zymogen-catalyzed hydrolysis of monomeric substrates and the presence of a recognition site for lipid-water interfaces in phospholipase A_2. *Biochemistry.* 13:1455–1460.

Rana, F. R., A. J. Mautone, and R. A. Dluhy. 1993. Combined infrared and ^{31}P NMR spectroscopic method for determining the fractional composition in Langmuir-Blodgett films of binary phospholipid mixtures. *Appl. Spectrosc.* 47:1015–1023.

Rogers, J., B.-Z. Yu, M.-D. Tsai, O. G. Berg, and M. K. Jain. 1998. Cationic residues 53 and 56 control the anion-induced interfacial k^*_{cat} activation of pancreatic phospholipase A_2. *Biochemistry.* 37:9549–9556.

Schevitz, R. W., N. J. Bach, D. G. Carlson, N. Y. Chirgadze, D. K. Clawson, R. D. Dillard, S. E. Draheim, L. W. Hartley, N. D. Jones, E. D. Mihelich, J. L. Olkowski, D. W. Snyder, C. Sommers, and J.-P. Wery. 1995. Structure-based design of the first potent and selective inhibitor of human non-pancreatic secretory phospholipase A_2. *Nat. Struct. Biol.* 2:458–465.

Scott, D. L., A. M. Mandle, P. B. Sigler, and B. Honig. 1994. The electrostatic basis for the interfacial binding of secretory phospholipase A_2. *Biophys. J.* 67:493–504.

Scott, D. L., Z. Otwinowski, M. H. Gelb, and P. B. Sigler. 1990a. Crystal structure of bee-venom phospholipase A_2 in a complex with a transition-state analogue. *Science.* 250:1563–1566.

Scott, D. L., and P. B. Sigler. 1994. Structure and catalytic mechanism of secretory phospholipase A_2. *Adv. Protein Chem.* 45:53–88.

Scott, D. L., S. P. White, J. L. Browning, J. J. Rosa, M. H. Gelb, and P. B. Sigler. 1991. Structures of free and inhibited human secretory phospholipase A_2 from inflammatory exudate. *Science.* 254:1007–1010.

Scott, D. L., S. P. White, Z. Otwinowski, W. Yuan, M. H. Gelb, and P. B. Sigler. 1990b. Interfacial catalysis: the mechanism of phospholipase A_2. *Science.* 250:1541–1546.

Seddon, J. M. 1993. Structural parameters of phospholipids. *In* Phospholipids Handbook. G. Cevc, editor. Marcel Dekker, New York. 909–938.

Sekar, K., S. Eswaramoorthy, M. K. Jain, and M. Sundaralingam. 1997. Crystal structure of the complex of bovine pancreatic phospholipase A_2 with the inhibitor 1-hexadecyl-3-(trifluoroethyl)-*sn*-glycero-2-phosphomethanol. *Biochemistry.* 36:14186–14191.

Snitko, Y., R. S. Koduri, S. K. Han, R. Othman, S. F. Baker, B. J. Molini, D. C. Wilton, M. H. Gelb, and W. Cho. 1997. Mapping the interfacial binding surface of human secretory group IIa phospholipase A_2. *Biochemistry.* 36:14325–14333.

Speijer, H., P. L. Giesen, R. F. A. Zwaal, C. E. Hack, and W. T. Hermens. 1996. Critical micelle concentrations and stirring are rate limiting in the loss of lipid mass during membrane degradation by phospholipase A_2. *Biophys. J.* 70:2239–2247.

Tatulian, S. A. 1993. Ionization and ion binding. *In* Phospholipids Handbook. G. Cevc, editor. Marcel Dekker, New York. 511–552.

Tatulian, S. A. 1999. Surface electrostatics of biological membranes and ion binding. *In* Surface Chemistry and Electrochemistry of Membranes. T. S. Sørensen, editor. Marcel Dekker, New York. 871–921.

Tatulian, S. A., and R. L. Biltonen. 1997. Electrostatically controlled binding of phospholipase A_2 (AppD49) to supported lipid bilayers. *Biophys. J.* 72:A217.

Tatulian, S. A., R. L. Biltonen, and L. K. Tamm. 1997. Structural changes in a secretory phospholipase A_2 induced by membrane binding: a clue to interfacial activation? *J. Mol. Biol.* 268:809–815.

Thunnissen, M. M. G. M., E. Ab, K. H. Kalk, J. Drenth, B. W. Dijkstra, O. P. Kuipers, R. Dijkman, G. H. de Haas, and H. M. Verheij. 1990. X-ray structure of phospholipase A_2 complexed with a substrate-derived inhibitor. *Nature.* 347:689–691.

Thuren, T., P. Vainio, J. A. Virtanen, P. Somerharju, K. Blomqvist, and P. K. Kinnunen. 1984. Evidence for the control of the activity of phospholipase A_2 by the physical state of the substrate. *Biochemistry.* 23:5129–5134.

Tischfield, J. A. 1997. A reassessment of the low molecular weight phospholipase A_2 gene family in mammals. *J. Biol. Chem.* 272: 17274–17250.

Tomoo, K., H. Ohishi, T. Ishida, M. Inoue, K. Ikeda, S. Sumiya, and K. Kitamura. 1994. X-ray crystal structure and molecular dynamics simulation of bovine pancreas phospholipase A_2-n-dodecylphosphorylcholine complex. *Proteins Struct. Funct. Genet.* 19:330–339.

van den Berg, B., M. Tessari, R. Boelens, R. Dijkman, G. H. de Haas, R. Kaptein, and H. M. Verheij. 1995. NMR structures of phospholipase A_2 reveal conformational changes during interfacial activation. *Nat. Struct. Biol.* 2:402–406.

Venyaminov, S. Yu., and N. N. Kalnin. 1990. Quantitative IR spectrophotometry of peptide compounds in water (H_2O) solutions. I. Spectral parameters of amino acid residue absorption bands. *Biopolymers.* 30:1243–1257.

Verger, R., and G. H. de Haas. 1976. Interfacial enzyme kinetics of lipolysis. *Annu. Rev. Biophys. Bioeng.* 5:77–117.

Volwerk, J. J., P. C. Jost, G. H. de Haas, and O. H. Griffith. 1986. Activation of porcine pancreatic phospholipase A_2 by the presence of negative charges at the lipid-water interface. *Biochemistry.* 25: 1726–1733.

Welches, W., I. Reardon, and R. L. Heinrikson. 1993. An examination of structural interactions presumed to be of importance in the stabilization of dimers based upon comparative protein sequence analysis of a monomeric and dimeric enzyme from the venom of *Agkistrodon p. piscivorus. J. Protein Chem.* 12:187–193.

White, S. P., D. L. Scott, Z. Otwinowski, M. H. Gelb, and P. B. Sigler. 1990. Crystal structure of a cobra-venom phospholipase A_2 in a complex with a transition-state analogue. *Science.* 250:1560–1563.

Yu, B-Z., J. Rogers, G. R. Nicol, K. H. Theopold, K. Seshadri, S. Vishweshwara, and M. K. Jain. 1998. Catalytic significance of the specificity of divalent cations as K^*_s and k^*_{cat} cofactors for secreted phospholipase A_2. *Biochemistry.* 37:12576–12587.

Yu, B.-Z., J. Rogers, M.-D. Tsai, C. Pidgeon, and M. K. Jain. 1999. Contribution of residues of pancreatic phospholipase A_2 to interfacial binding, catalysis, and activation. *Biochemistry.* 38:4875–4884.