Effects of Specific Fatty Acid Acylation of Phospholipase A_2 on Its Interfacial Binding and Catalysis†

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ABSTRACT: Monomeric phospholipase A_2 (PLA_2) from the venom of *Agkistrodon piscivorus piscivorus* (App-D49) was treated with 3-acyloxy-4-nitrobenzoic acids to acylate the ϵ-amino groups of two lysines (Lys-7 and Lys-10) in the amino terminal region. Resulting 7,10-diacylated-App-D49s, with acyl groups ranging from lauroyl to palmitoyl, spontaneously aggregated in solution. By contrast, 7,10-dioctanoyl-App-D49 existed as a monomer under the same condition. Kinetic and interfacial binding properties of diacylated enzymes indicated that they catalyzed the hydrolysis at the interface as a monomer. When compared to nonacylated App-D49, diacylated enzymes showed slightly increased activity or decreased activity toward monodispersed 1,2-dibutyryl-*sn*-glycero-3-phosphocholine, Triton X-100/1,2-dilauroyl-*sn*-glycero-3-phosphocholine mixed micelles, and small unilamellar vesicles (SUV) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Toward densely-packed liquid-crystalline phospholipid bilayers, such as large unilamellar vesicles (LUV) of POPC, however, diacylated enzymes exhibited a large increase in activity, which reached up to 250-fold for 7,10-dilauroyl-App-D49 ($(k_{cat}/K_m)_{app} = (1.0 \pm 0.02) \times 10^6$ M^{-1} s^{-1}). Measurements of the penetration of individual diacylated enzymes into 2-oleoyl-3-palmitoyl-*sn*-glycero-1-phosphocholine (i.e., D-POPC) monolayers indicated that the acyl groups enhanced the interfacial binding of protein by interacting with hydrocarbon moieties of phospholipids and that these hydrophobic interactions remained effective even when the phospholipid packing density was high. Furthermore, fluorometric measurements of the binding of diacylated enzymes to polymerized vesicles of 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine showed that the hydrophobic interactions increased the enzymatic activity toward LUV by accelerating the migration of enzyme molecules to vesicle surfaces. The analysis of the kinetic course of POPC LUV hydrolysis showed that diacylated enzymes as a catalyst were superior to nonacylated App-D49 in that they were not only more catalytically efficient but also able to catalyze more turnovers without being trapped in product-containing vesicles. In summary, the acylation of App-D49 by 3-acyloxy-4-nitrobenzoic acids provides a simple and convenient way of converting the enzyme into a highly active form toward densely-packed liquid-crystalline phospholipid bilayers, which might have potential industrial and biomedical applications.

Phospholipase A_2 (PLA_2,[1] EC 3.1.1.4) catalyzes the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids, liberating fatty acid and lysophospholipid [for reviews, see Verheij et al. (1981), Dennis (1983), and Waite (1987)]. Because the catalytic action of PLA_2 occurs at the lipid–water interface, its activity is sensitive not only to the chemical structure of phospholipid but also to the physical structure of the aggregate it forms. It has been shown that the interfacial organization of phospholipids (Op den Kemp et al., 1974; Kensil & Dennis, 1985; Jain et al., 1989; Burack et al., 1993) and the interfacial electrostatics (Apitz-Castro et al., 1982; Jain & Berg, 1989) greatly influence the interfacial binding and catalysis of PLA_2. Most PLA_2s cannot readily hydrolyze densely-packed monolayers and bilayers of zwitterionic phospholipid, such as phosphatidylcholine (PC). As a result, enzymatic hydrolysis often exhibits an anomalous kinetic pattern with a lag period up to several minutes. The lag period has been attributed to the slow penetration of PLA_2 (Verger et al., 1973), the dimerization of enzyme at the interface (Menasche et al., 1986; Bell & Biltonen, 1989), and the product activation by fatty acid (Apitz-Castro et al., 1982). The lag time can be substantially shortened near the phase transition temperature (T_m) of phospholipid substrates at which gel and liquid-crystalline regions coexist (Op den Kemp et al., 1974) or by the addition of a variety of hydrophobic or amphiphilic molecules that induce lateral phase separation of phospholipids (Jain & Berg, 1989; Burack et al., 1993). Independently, we (Cho et al., 1988a) and others (Tomasselli et al., 1989; Van der Wiele et al., 1988a,b; Lughtigheid et al., 1993) have shown that the fatty acid acylation of specific lysine(s) of PLA_2 converts the enzyme into a membrane-active form, which can readily hydrolyze densely-packed PC monolayers and bilayers. In particular, we showed that an artificial PLA_2 substrate, 4-nitro-3-octanoyloxybenzoic acid (NOB), specifically and quantitatively acylated Lys-7 and Lys-10 of Asp-49 PLA_2 from the venom of *Agkistrodon*

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[1] App-D49, Asp-49 PLA_2 from *Agkistrodon piscivorus piscivorus*; App-diC8, 7,10-dioctanoyl-App-D49; App-diC12, 7,10-dilauroyl-App-D49; App-diC14, 7,10-dimyristoyl-App-D49; App-diC16, 7,10-dipalmitoyl-App-D49; BLPC, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine; BLPG, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol; BSA, bovine serum albumin; DBPC, 1,2-dibutyryl-*sn*-glycero-3-phosphocholine; DL-LysoPPC, 1-palmitoyl-*rac*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; D-POPC, 2-oleoyl-3-palmitoyl-*sn*-glycero-1-phosphocholine; LUV, large unilamellar vesicles; LysoPC, lysophosphatidyl choline; NOB, 4-nitro-3-octanoyloxybenzoic acid; PC, phosphatidylcholine; PLA_2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicles; T_m, phase transition temperature.

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piscivorus piscivorus (App-D49) (Cho et al., 1988a). The resulting 7,10-dioctanoyl-App-D49 (App-diC8) exhibited the dramatically enhanced activity toward phospholipid monolayers when compared to nonacylated App-D49. Although the activation of PLA_2 by acylation might not be physiologically relevant, the acylation is potentially useful for preparing a highly active form of PLA_2 for biomedical and industrial applications (Labeque et al., 1993). To achieve a maximal activation of PLA_2 by means of acylation, one must first understand how covalently-attached acyl groups interact with phospholipids and how these interactions enhance the activity of parent enzyme molecule. Van der Wiele et al. (1988a,b) reported that palmitoylation of a specific lysine (Lys-10 or Lys-116) of the porcine pancreatic PLA_2 greatly activated the enzyme toward PC monolayers and intact erythrocyte membranes. By measuring the penetration of acylated protein into PC monolayers, they concluded that the rate increase was due to the improved penetrating power of acylated enzymes. On the basis of the high tendency of acylated PLA_2s to dimerize in solution, however, Tomasselli et al. (1989) argued that facilitated dimerization of acylated enzymes is responsible for the rate enhancement at the interface. More recently, Lugtigheid et al. (1993) reported that Lys-56-acylated porcine pancreatic enzymes can penetrate phospholipid monolayer more rapidly than the nonacylated enzyme, but they did not dimerize in solution. Since most of these studies were performed in the presence a particular type of interface, the observed behaviors of acylated enzymes might in part reflect unique properties of the interface. To better understand the effects of acylation on the interfacial binding and catalysis of PLA_2, we have acylated App-D49 with different acyl groups and studied the interactions of resulting acylated enzymes with a wide variety of model membranes including micelles, small and large unilamellar vesicles, polymerized vesicles, and monolayers. In this paper, we present kinetic and binding data showing that enhanced activity of acylated PLA_2's originates primarily from improved hydrophobic interactions that accelerate the interfacial binding step and that acylated PLA_2s are highly active as a monomer toward densely-packed liquid-crystalline phospholipid bilayers. By measuring the activity of acylated enzymes toward interfaces with different electrostatic properties, we have also assessed the relative importance of electrostatic interactions and hydrophobic interactions in the interfacial binding of PLA_2. Results described herein provide a new insight into how PLA_2 interacts with phospholipid vesicles.

MATERIALS AND METHODS

App-D49 was purified from lyophilized snake venom (Sigma) as described (Maraganore et al., 1984). Fatty acid-free bovine serum albumin (BSA) was from Miles Inc. Protein concentrations were determined by the micro bicinchoninic acid method (Pierce) (Smith et al., 1985). 1,2-Dibutyryl-*sn*-glycero-3-phosphocholine (DBPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids. 1-Palmitoyl-*rac*-glycero-3-phosphocholine (DL-lysoPPC) was from Sigma. 2-Oleoyl-3-palmitoyl-*sn*-glycero-1-phosphocholine (D-POPC) was synthesized from DL-lysoPPC. Briefly, 1-palmitoyl-2-oleoyl-*rac*-glycero-3-phosphocholine (DL-POPC), synthesized from the reaction of DL-lysoPPC with oleoyl chloride in the presence of 4-pyrrolidinopyridine (Sigma), was hydrolyzed by App-D49 to selectively remove L-POPC. D-POPC was separated from the hydrolyzed mixture by HPLC (Hitachi Instrument) using a LiChrosorb Silica 60 column (E. Merck) (0.4 × 25 cm) which was equilibrated and eluted with $CHCl_3/CH_3OH/H_2O$ (65:25:4 (v/v/v)). Purity of D-POPC was confirmed by measuring its specific rotation using L-POPC as a standard. Triton X-100 was obtained from Pierce. 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) was purchased from Molecular Probes. 1,2-Bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (BLPC) and 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol (BLPG) were prepared as described (Wu & Cho, 1993). Large unilamellar vesicles (LUV; an average diameter of 100 nm) were prepared by multiple extrusion through 0.1-μm polycarbonate filter (Millipore) in a microextruder Liposofast (Avestin, Ottawa, Ontario) (MacDonald et al., 1991). Small unilamellar vesicles (SUV; an average diameter of 25 nm) were prepared by sonication, followed by ultracentrifugation (New, 1990). The mean hydrodynamic radius of vesicles was determined by dynamic light scattering measurement as described (Budzynski et al., 1992). T_m for DMPC LUV was measured using a differential scanning calorimeter (Micro Calorimeter System). Phospholipid concentrations were determined by phosphate analysis (Kate et al., 1986). Mixed micelles of Triton X-100 and DLPC were prepared as described (Dennis, 1973). 4-Acyloxy-3-nitrobenzoic acids were synthesized from 4-hydroxybenzoic acid and acyl chlorides as described (Cho et al., 1988b; Cho & Kézdy, 1990).

Preparation of Acylated PLA_2. Octanoylated App-D49s were prepared by incubating App-D49 (5 μM) with NOB (2 mM) at 25 °C in 0.1 M Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$ and 0.1% acetonitrile (Cho et al., 1988a) until all the substrates were hydrolyzed. After the lyophilization of solution and the chromatographic removal of reagents using a Sephadex G-25 column (2.5 × 20 cm) equilibrated and eluted with 5% acetic acid, di- and monoacylated enzymes were purified by reverse-phase HPLC using a Vydac C-4 column (1 × 25 cm). The column was developed in a binary gradient system consisting of solvent A (0.15% aqueous trifluoroacetic acid) and solvent B (0.15% trifluoroacetic acid in acetonitrile/2-propanol (3:7, v/v)). Fractions corresponding to each peak were pooled and diluted with H_2O and lyophilized. Lauroylated App-D49's were prepared by the same protocol using 4-dodecanoyloxy-3-nitrobenzoic acids. For the preparation of myristoylated and palmitoylated App-D49's, the acylation method was modified. Briefly, enzyme (final concentration of 2 μM) was added to a solution of mixed micelles of Triton X-100 (2 mM) and 4-acyloxy-3-nitrobenzoic acid (0.5 mM) in 0.1 M Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$. The reaction mixture was then lyophilized, and Triton X-100 and organic compounds were washed with chloroform/methanol (2:1, v/v). The precipitated enzyme was collected by centrifugation and taken up with 5% aqueous acetic acid solution and further purified chromatographically as described above. Protein sequencing of acylated PLA_2's was performed by automated Edman degradation using Applied Biosystem Sequencer Model 477A. Acylated PLA_2's (0.5 nmol), labeled with tritiated fatty acid and purified by HPLC, were directly applied to the sequencer, and 20 cycles of Edman degradation were performed. HPLC analysis and radioactivity measurement of each PTH-amino acid were performed as described (Cho et al., 1988a).

Gel Filtration Chromatography. Apparent molecular weights of proteins were determined using a Superdex 75 column (1 × 30 cm; Pharmacia) connected to a FPLC system

(Pharmacia) equilibrated in a 50 mM Tris-HCl buffer solution, pH 7.4, containing 0.16 M NaCl and 10 mM $CaCl_2$. BSA, ovalbumin, carbonic anhydrase, α-chymotrypsinogen, cytochrome *c*, and ribonuclease A (all from Sigma) were used as molecular weight standards. The concentration of diacylated enzymes injected into the column was varied from 5×10^{-6} to 5×10^{-7} M, and protein peaks were monitored spectrophotometrically at 215 nm.

Kinetic Measurements. All the kinetic experiments were performed at 37 °C and at pH 7.4. Kinetics of PLA_2-catalyzed hydrolysis of vesicles were measured in the presence of 0.5 mM POPC, 0.1 mM BSA, 0.16 M NaCl, and 10 mM $CaCl_2$. BSA was added to relieve the product inhibition (Kupferberg et al., 1981). The hydrolysis of mixed micelles was performed in the presence of 2 mM Triton X-100, 0.5 mM DLPC, and 10 mM $CaCl_2$, and the hydrolysis of DBPC was measured with 1 mM DBPC, 0.16 M NaCl, and 10 mM $CaCl_2$. Time courses of the hydrolysis of phospholipids were monitored with a computer-controlled Metrom pH stat (Brinkmann) in a thermostated vessel. The completion of hydrolysis was confirmed by adding an additional amount of enzyme to the mixture after the reaction. To remove irreversibly absorbed protein from a pH electrode and a reaction vessel, they were frequently washed with 0.1 M HCl solution and 50% aqueous ethanol. In order to obtain kinetically meaningful rate constants for the comparison of enzymatic activities while avoiding complicated kinetic analyses, we employed the simplest possible kinetic model that is consistent with the observed kinetic patterns. Under the experimental conditions employed, all the PLA_2-catalyzed reactions exhibited apparent first-order kinetics. Thus, we treated enzymatic reactions as a simple bimolecular process as shown in eq 1:

$$E + S \xrightarrow{(k_{cat}/K_m)_{app}} E + P_1 + P_2 \qquad (1)$$

where E, S, P_1, P_2, and $(k_{cat}/K_m)_{app}$ represent free enzyme, phospholipid, fatty acid, lysophospholipid, and apparent specificity constant, respectively. Accordingly, the entire course of reaction was analyzed using a simple integrated first-order rate equation; i.e., $[P] = S_0(1 - e^{-kt})$ where k is a pseudo-first-order rate constant ($=(k_{cat}/K_m)_{app}E_0$) and E_0 and S_0 represent total enzyme and substrate concentration, respectively. Pseudo-first-order rate constants were directly proportional to enzyme concentrations within the range of enzyme concentration used; $(k_{cat}/K_m)_{app}$ was calculated by dividing the pseudo-first-order rate constant by enzyme concentration. According to the model shown in eq 1, $(k_{cat}/K_m)_{app}$ should contain information about the interfacial binding as well as catalytic steps. For all the kinetic experiments, enzyme concentrations were adjusted so as to keep the half-life of reaction below 5 min. The hydrolysis of POPC LUV by nonacylated App-D49 followed the first-order kinetics, but the amount of hydrolyzed phospholipids depended on the enzyme concentration. This made it difficult to directly compare the rate constant of nonacylated App-D49 with that of diacylated App-D49's which completely hydrolyzed all the available phospholipids even at low concentrations (see Results). To circumvent this ambiguity, the rate constant of nonacylated App-D49 was determined while maintaining its concentrations high enough to result in a complete hydrolysis of phospholipids.

Kinetic and Binding Studies with Polymerized (Mixed) Vesicles. Large polymerized vesicles of BLPC and polymerized mixed vesicles of pyrene-PC (5 mol%) and BLPC (or BLPG) were prepared as described (Wu & Cho, 1993, 1994). Kinetics of PLA_2-catalyzed hydrolysis of polymerized mixed vesicles were performed in 2 mL of 10 mM HEPES buffer, pH 7.4, containing 0.5 μM pyrene-PC (10 μM total phospholipids), 10 μM BSA, 0.16 M NaCl, 0.1 mM EDTA, and 10 mM $CaCl_2$. The sample was contained in a stirred, thermostated 1-cm path length quartz cuvette. The excitation wavelength was 345 nm, and the progress of reaction was monitored as an increase in fluorescence emission at 380 nm (F_{380}) using a Hitachi F4500 fluorescence spectrometer. Spectral band width was set at 5 nm for both exictation and emission. The hydrolysis of pyrene-PC/BLPC polymerized mixed vesicles by nonacylated App-D49 showed a biphasic kinetic pattern in which a rapid first-order reaction was followed by a slow zero-order reaction. Thus, we analyzed the course of reaction using an integrated rate equation: $F_{380} = \Delta F_{380}(1 - e^{-kt}) + k't$ where ΔF_{380} indicates a total fluorescence change at 380 nm and k ($=(k_{cat}/K_m)_{app}E_0$) and k' indicate apparent first-order and zero-order rate constants, respectively. The k values were directly proportional to enzyme concentrations within the range of enzyme concentration used; $(k_{cat}/K_m)_{app}$ was calculated by dividing the pseudo-first-order rate constant by enzyme concentration. For the hydrolysis of pyrene-PC/BLPG polymerized mixed vesicles by nonacylated App-D49, the zero-order reaction was negligible within the range of enzyme concentration used. Also, the hydrolysis of pyrene-PC/BLPC (or BLPG) by diacylated App-D49's showed simple first-order kinetics. Thus, these reactions were analyzed as described above.

Due to a strong absorption of BLPC molecule in the UV range (Wu & Cho, 1993), the tryptophan fluorescence emission of PLA_2 becomes largely quenched when it binds to the surface of BLPC polymerized vesicles (Wu & Cho, 1994). Based on this property, the binding of App-D49 to BLPC polymerized vesicles was measured by monitoring the change in the intrinsic protein fluorescence. The excitation wavelength was set at 280 nm, and the emission wavelength was 345 nm. Spectral band width was set at 5 nm for both excitation and emission. To avoid the inner-filter effect of BLPC molecule, its concentration was maintained as low as possible (<0.1 mM), and its signal was background-corrected before adding protein solution. Typically, the same concentration of protein (0.5 μM) was added to a solution containing 0.1–100 μM of BLPC, and the fluorescence intensity of protein (F_{345}) was measured after it reached an equilibrium value. Then, the relative fluorescence change (F_{rel}) for each phospholipid concentration was calculated as $(F_{max} - F_{345})/(F_{max} - F_{min})$ where F_{max} and F_{min} represent the protein fluorescence at 345 nm in the absence of BLPC and in the presence of an excess amount of BLPC, respectively. The F_{rel} values were plotted as a function of total BLPC concentration. For these studies, the apparent dissociation constant (K_d) was expressed as a bulk BLPC concentration and estimated from the binding isotherms, assuming that the binding of PLA_2 to vesicles is one-to-one and at rapid equilibrium.

Monolayer Measurements. Surface tension of solution in a circular Teflon trough was measured using a du Nouy ring attached to a computer-controlled Cahn electrobalance (Model C-32). The trough is similar to that described by Rebecci et al. (1992); i.e., 1 cm deep, 5 cm diameter trough has a 0.5 cm deep well for magnetic stir bar and a small hole drilled at an angle through the wall to allow an addition of protein solution. After the phospholipid monolayer was spread onto the subphase containing 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, and 0.16 M NaCl, the protein solution was added through the hole, and the change in surface pressure was measured as a function

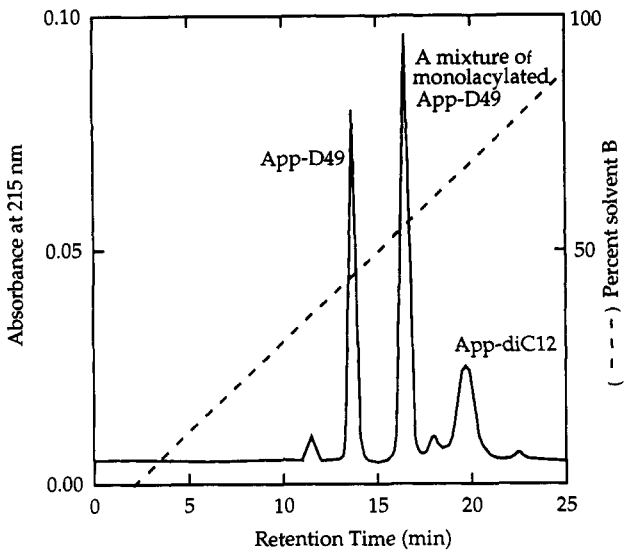

FIGURE 1: Representative chromatogram showing the separation of acylated enzymes by reverse-phase HPLC. See Materials and Methods for the condition of gradient elution.

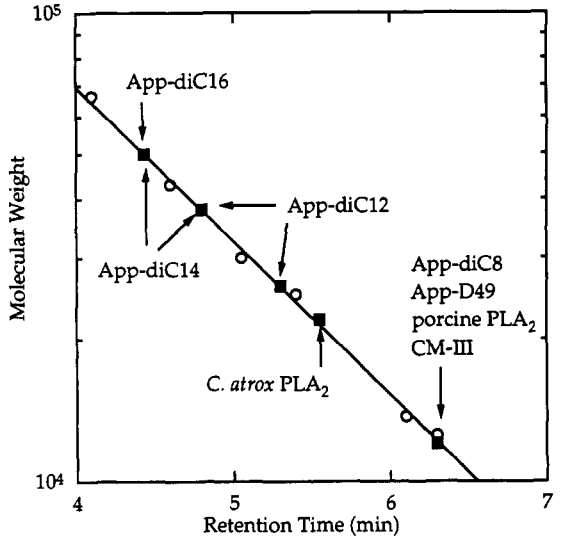

FIGURE 2: Determination of apparent molecular weight of nonacylated and diacylated App-D49's by gel filtration chromatography. See Materials and Methods for the elution condition. The solid line indicates a calibration line, and open circles and filled squares represent molecular mass standards and various PLA_2's, respectively. CM-III indicates a basic PLA_2 from *Naja mocambique mocambique*.

of time at 23 °C. Protein concentrations were maintained high enough (e.g., 0.15 μM) to ensure that a loss of protein by the adsorption to the wall is negligible.

RESULTS

Preparation and Characterization of Acylated App-D49's. We previously reported that acylation of App-D49 by NOB yielded App-diC8 as a major product that could be readily separated from monoacylated species by reverse-phase HPLC (Cho et al., 1988a). The reaction of App-D49 with 3-dodecanoyloxy-4-nitrobenzoic acid produced a similar result. However, the reaction of App-D49 with either 4-nitro-3-tetradecanoyloxybenzoic acid or 3-hexadecanoyloxy-4-nitrobenzoic acid resulted in a more complex mixture of acylated enzymes, which hampered the purification of a 7,10-diacylated form. Our modified method of acylation considerably improved the yield of the diacylated form, which in turn simplified its purification. A typical chromatogram for the separation of a mixture of acylated enzymes using a reverse-phase column is shown in Figure 1. Nonacylated App-D49, a mixture of monoacylated (7- or 10-acylated) forms, and the 7,10-diacylated form were eluted from the column in the described order. The identity of each major protein peak was estimated by the automated Edman degradation of its amino terminal 20 residues. When compared to monoacylated forms of App-D49, diacylated forms were easier to purify and chemically more homogeneous. For these reasons, we used only diacylated forms of App-D49 for these kinetic and binding studies. Overall yield of preparation of diacylated enzymes from nonacylated App-D49 ranged from 10 to 30%.

By means of gel filtration chromatography using a Sephadex G-50 column, we previously showed that octanoylated App-D49 acquired a tendency to dimerize in solution (Cho et al., 1988a). When the aggregation of acylated enzymes was re-examined under the defined condition, App-diC8 eluted as a single peak with an apparent molecular mass of 12 kDa whereas other diacylated enzymes containing longer acyl chains exhibited rather complex elution patterns. As illustrated in Figure 2, a single peak with an apparent molecular mass of 50 kDa was observed for App-diC16 whereas two peaks were shown for App-diC12 (26 and 38 kDa) and App-diC14 (38 and 50 kDa). Apparent molecular masses of monomeric PLA_2s including App-D49, porcine pancreatic enzyme (Sigma), and *Naja mocambique mocambique* PLA_2 (CM-III) (Sigma) were all estimated as 12 kDa, and that of a naturally occurring dimer, *Crotalus atrox* PLA_2 (Sigma), was 22 kDa. When measured by SDS–polyacrylamide gel electrophoresis, both nonacylated and acylated App-D49 showed a single band with an expected molecular mass of 14 kDa (data not shown). These results indicate that App-diC8 exists as a monomer in solution whereas other diacylated App-D49's form heterogeneous aggregates the molecular shapes of which are distinct from that of *C. atrox* PLA_2.

Activities of Diacylated App-D49's toward Various Substrates. In order to elucidate the mechanism by which the acylation affects the activity of PLA_2 at the interface, we measured the activity of diacylated App-D49s containing different fatty acids, ranging from octanoyl to palmitoyl, toward a wide variety of substrates. Diacylated enzymes with shorter acyl chains were not included because they are difficult to prepare and show significantly low activity toward various substrates (data not shown). Because the PLA_2-catalyzed hydrolysis of monodispersed DBPC (1 mM) would not involve the interfacial binding step, the enzymatic activity toward DBPC should only reflect the catalytic efficiency of enzyme. Toward DBPC, all the diacylated enzymes, whether monomeric or dimeric, were only slightly more active than nonacylated App-D49. It was thus evident that the acylation did not significantly affect the intrinsic catalytic activity of App-D49. Accordingly, changes in the enzyme activity toward aggregated substrates caused by acylation should be mainly due to changes in interfacial binding. We selected as PLA_2 substrates three types of aggregated phospholipids which greatly differ in their interfacial packing density, i.e., Triton X-100/DLPC mixed micelles, POPC SUV, and LUV, and measured the activity of diacylated enzymes toward these substrates.

As summarized in Table 1, the effect of acylation on the interfacial activity of App-D49 is highly variable depending on the physical state of phospholipid substrates. First, all the diacylated enzymes showed essentially the same activity as nonacylated App-D49 toward Triton X-100/DLPC mixed micelles, which are an excellent substrate for App-D49. Thus,

Table 1: Apparent Specificity Constant ($(k_{cat}/K_m)_{app}$)[a] Determined from PLA_2-Catalyzed Hydrolysis of Various Substrates

enzymes	DBPC $(k_{cat}/K_m)_{app} \times 10^2$ (M^{-1} s^{-1})	Triton X-100/DLPC $(k_{cat}/K_m)_{app} \times 10^7$ (M^{-1} s^{-1})	POPC SUV $(k_{cat}/K_m)_{app} \times 10^4$ (M^{-1} s^{-1})	POPC LUV $(k_{cat}/K_m)_{app} \times 10^4$ (M^{-1} s^{-1})
App-D49	3.5 ± 0.4	1.1 ± 0.1	240 ± 11	0.4 ± 0.02
App-diC8	4.5 ± 0.5	1.2 ± 0.1	300 ± 10	90 ± 0.8
App-diC12	4.7 ± 0.5	1.4 ± 0.1	150 ± 11	100 ± 1.5
App-diC14	4.8 ± 0.5	1.0 ± 0.1	43 ± 1.0	6.1 ± 0.05
App-diC16	5.0 ± 0.5	1.2 ± 0.1	7.0 ± 0.5	1.8 ● 0.01

[a] Values of $(k_{cat}/K_m)_{app}$ represent (mean values ± standard errors) determined from a minimum of three measurements. Experimental conditions for kinetic measurements and methods for the calculation of rate constants are described under Materials and Methods.

it appears that the acylation has little effect on the binding of App-D49 to these micelles. Second, diacylated enzymes showed either slightly higher (App-diC8) or lower activity (App-diC12, App-diC14, and App-diC16) than nonacylated App-D49 toward POPC SUV. Among diacylated enzymes, the activity monotonously decreased as the acyl chain length increased; 7,10-dipalmitoyl-App-D49 (App-diC16) showed only 3% of the activity of nonacylated enzyme. Third, diacylated enzymes exhibited a large increase in activity when compared to nonacylated App-D49 toward POPC LUV. The rate enhancement reached a maximum for App-diC8 (225-fold) and 7,10-dilauroyl-App-D49 (App-diC12) (250-fold) and sharply decreased as the acyl-chain length increased further. Furthermore, diacylated enzymes were able to hydrolyze more substrates than the nonacylated one during the hydrolysis of POPC LUV (see Figure 7 and the accompanying text). POPC LUV possess more densely-packed interfaces than POPC SUV and are a poorer substrate for App-D49. Thus, it appears that the acylation enhances the interfacial binding of App-D49 only to densely-packed phospholipids with which nonacylated App-D49 cannot interact efficiently.

To further investigate the relationship between the activity of diacylated App-D49's and the interfacial packing density of phospholipids, we measured the activity of nonacylated App-D49 and App-diC8 toward DMPC LUV as a function of temperature. DMPC LUV underwent a gel to liquid-crystalline phase transition (data not shown) at 25.5 °C, which is similar to the T_m value for DMPC SUV (ca. 24 °C) (Small, 1986). Due to the presence of fully saturated acyl chains, DMPC LUV have high interfacial packing density and consequently are an extremely poor substrate for most PLA_2's including App-D49 in both gel and liquid-crystalline phases. The hydrolysis is typically preceded by a long lag period, which can be substantially shortened near T_m, at which gel and liquid-crystalline regions co-exist (Op den Kemp et al., 1974). Nonacylated App-D49 (0.1 mM) showed essentially no activity up to 30 min toward DMPC LUV in the gel phase at 15 °C (data not shown) and slowly hydrolyzed the vesicles in the liquid-crystalline phase (37 °C) only after a long lag period (Figure 3). By contrast, App-diC8 at a much lower concentration (0.2 μM) was able to fully hydrolyze the vesicles in the liquid-crystalline phase without showing any lag period. Toward DMPC LUV in the gel phase, however, even App-diC8 (0.2 μM) did not show any detectable activity up to 30 min (data not shown). Together with the high activity of diacylated App-D49's toward POPC LUV that exist solely in liquid-crystalline phase above 0 °C, these results demonstrate the high activity of diacylated App-D49's toward densely-packed, liquid-crystalline phospholipid bilayers.

Binding of Diacylated App-D49's to Phospholipid Monolayers. If the dimerization is not the origin of the enhanced activity of diacylated enzymes, it should, *a priori*, derive from increased hydrophobic interactions between covalently-attached acyl chains and phospholipids. Hydrophobic interactions between protein and phospholipid bilayer are generally achieved by the penetration of protein into the hydrophobic core of bilayer. Thus, diacylated App-D49's are expected to exhibit an enhanced ability to penetrate if the acylation increases hydrophobic interactions. Phospholipid monolayers at the air–water interface are an excellent model membrane in which the penetration of protein into phospholipids can be sensitively monitored in terms of the change in surface pressure (π). Also, this technique allows an assessment of the effect of phospholipid packing density on the interfacial binding of protein because the packing density in the monolayer can be readily and accurately varied. The surface pressure in the outer monolayer of a 25-nm-diameter SUV of 1,2-dioleyol-*sn*-glycero-3-phosphocholine was estimated to be 15–20 dyn/cm (Schindler, 1980) whereas that of POPC LUV was 31–33 dyn/cm (Seelig, 1987). On the basis of these findings, monolayers of D-POPC, a nonhydrolyzable structural analog of L-POPC, at 15 and 30 dyn/cm, would be reasonable models for POPC SUV and POPC LUV, respectively. We measured the degree of protein penetration into phospholipid monolayers as a function of initial surface pressure of monolayers (π_o); a higher π_o value indicates higher phospholipid packing density. The change in π value ($\Delta\pi$) reached a plateau within ca. 5 min, and these $\Delta\pi$ values for diacylated and nonacylated App-D49's were plotted as a function of π_o of D-POPC in Figure 4. At low π_o (i.e., $\pi_o < 15$ dyn/cm), nonacylated App-D49, App-diC8, and App-diC12 resulted in a larger $\Delta\pi$ value than App-diC14 and App-diC16. As π_o increased, the penetration of nonacylated App-D49 decreased sharply and became

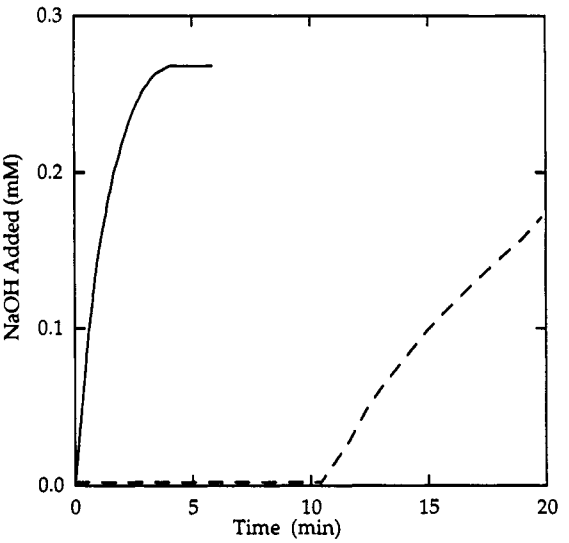

FIGURE 3: Kinetic courses of the hydrolysis of DMPC LUV by nonacylated App-D49 (dotted line) and by App-diC8 (solid line) at 37 °C and at pH 7.4. Reaction mixtures contain 0.5 mM DMPC, 0.1 mM BSA, 0.16 M NaCl, and 10 mM $CaCl_2$. Enzyme concentrations were 0.1 mM (App-D49) and 0.2 μM (App-diC8).

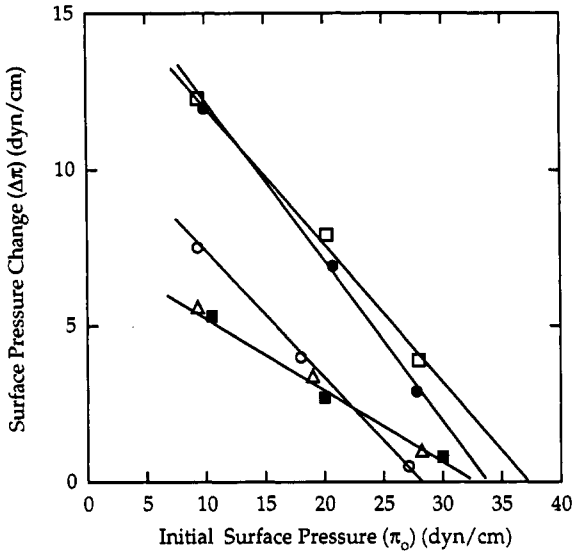

FIGURE 4: Effect of initial surface pressure of D-POPC monolayers on the penetration of nonacylated App-D49 (○), App-diC8 (●), App-diC12 (□), App-diC14 (■), and App-diC16 (△). The protein concentration in the subphase was 0.15 μM.

undetectable when $\pi_o > 30$ dyn/cm. By contrast, diacylated enzymes, App-diC8 and App-diC12 in particular, showed a significant penetration at high π_o values. Assuming that all the diacylated enzymes penetrate into monolayers by means of covalently-attached acyl groups, a larger $\Delta\pi$ value for the same protein concentration in the subphase will indicate a higher population of protein at the interface. Then, the relative degree of monolayer penetration displayed by diacylated App-D49's at 15 and 30 dyn/cm, respectively, is consistent with their relative activities toward POPC SUV and LUV, respectively. The penetration of nonacylated App-D49 into the monolayer at 15 dyn/cm is lower than expected from its high activity toward POPC SUV. It should be noted, however, that nonacylated App-D49 does not possess hydrophobic acyl groups and should thus penetrate the monolayer by a different mechanism. As a result, nonacylated App-D49 might be less efficient than diacylated enzymes in perturbing the monolayer even at the same surface concentration, thereby exhibiting smaller $\Delta\pi$ values.

Interactions of Diacylated App-D49's with Polymerized Mixed Vesicles. Polymerized mixed vesicles are a novel model membrane useful for measuring kinetic parameters for PLA_2–vesicle interactions (Wu & Cho, 1993). Also, polymerized vesicles are a nonhydrolyzable model membrane that can be used for determining thermodynamic parameters for PLA_2–vesicle interactions (Wu & Cho, 1994). Furthermore, polymerized vesicles allow the sensitive measurement of kinetics of interfacial binding of proteins. We measured interactions of nonacylated and diacylated App-D49's with pyrene-PC/BLPC polymerized mixed vesicles and BLPC polymerized vesicles. Results are summarized in Table 2.

Physical properties of BLPC LUV are similar to POPC LUV (Wu & Cho, 1993); e.g., they both exist in the liquid-crystalline phase at room (and higher) temperature. As expected from the structural similarity of two vesicles, the relative activity toward polymerized mixed vesicles among diacylated enzymes was similar to their relative activity toward POPC LUV. For all the diacylated enzymes, however, rate increases in comparison to nonacylated App-D49 were lower than observed with POPC LUV; the most active App-diC12 was ca. 60 times more active than nonacylated App-D49. This might be due to the restricted lateral diffusion of phospholipids

Table 2: Kinetic and Thermodynamic Parameters[a] Determined from Interactions of Nonacylated and Diacylated App-D49's with Polymerized (Mixed) Vesicles

enzymes	pyrene-PC/BLPC $(k_{cat}/K_m)_{app}$ × 10^4 (M^{-1} s^{-1})	pyrene-PC/BLPG $(k_{cat}/K_m)_{app}$ × 10^5 (M^{-1} s^{-1})	BLPC K_d (μM)
App-D49	0.1 ± 0.01	20 ± 1.0	>500
App-diC8	5.0 ± 0.2	10 ± 0.5	1.0 ± 0.05
App-diC12	6.0 ± 0.5	10 ± 0.4	ND[b]
App-diC14	1.2 ± 0.1	3.0 ± 0.1	ND
App-diC16	0.8 ± 0.1	2.0 ± 0.1	2.0 ± 0.25

[a] Values of parameters represent (mean values ± standard errors) determined from a minimum of three measurements. Experimental conditions for kinetic and binding experiments and methods for the calculation of parameters are described under Materials and Methods. [b] ND, not determined.

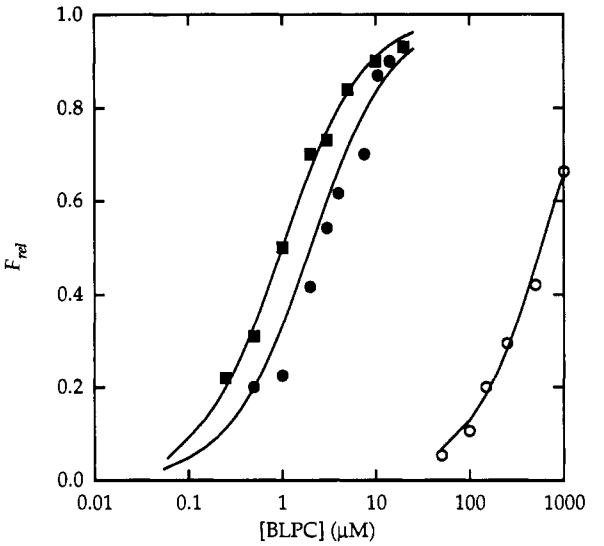

FIGURE 5: Dependence of relative fluorescence change (F_{rel}) of nonacylated App-D49 (○), App-diC8 (■), and App-diC16 (●) on the BLPC concentration. See Materials and Methods for the definition of F_{rel}. F_{rel} for nonacylated App-D49 was calculated assuming that its F_{min} value is the same as that of App-diC8 (or App-diC16). The binding was measured in 10 mM HEPES, pH 7.4, containing 10 mM $CaCl_2$ and 0.16 M NaCl at 37 °C. The solid lines represent theoretical curves constructed using K_d values.

in a polymerized matrix, which hinders the penetration of protein molecule. The binding isotherms of nonacylated App-D49, App-diC8, and App-diC16 to BLPC polymerized vesicles are shown in Figure 5, and individual apparent dissociation constants (K_d) determined from the isotherms are summarized in Table 2. The binding isotherms clearly illustrate that diacylated enzymes bind BLPC polymerized liposomes more tightly than nonacylated App-D49, which did not show a saturation in the presence of up to 1 mM BLPC. Quantitatively, however, the difference in K_d value is much larger than expected from their relative activity toward pyrene-PC/BLPC polymerized mixed vesicles. This implies that the interfacial binding of App-D49, whether acylated or not, is not a rapid equilibrium during the hydrolysis of pyrene-PC/BLPC polymerized mixed vesicles; i.e., the overall activity is not determined by the magnitude of equilibrium constant (e.g., K_d) but by the kinetics of interfacial binding. To further investigate the effect of acylation on the kinetics of interfacial binding of App-D49, we measured the time course of interfacial binding of the above three enzymes. As shown in Figure 6, the binding of App-diC8 to polymerized vesicles was slightly enhanced with an increase in ionic strength of medium. App-diC16 showed the same behaviors (data not shown). These

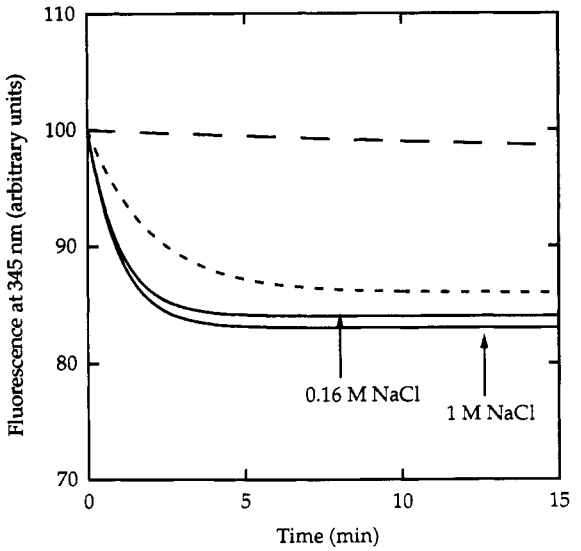

FIGURE 6: Kinetics of the binding of nonacylated App-D49 (broken line), App-diC8 (solid lines), and App-diC16 (dotted line) to BLPC polymerized vesicles. The binding was initiated by adding BLPC (final concentration of 150 μM) to the 1 μM enzyme solution in 10 mM HEPES, pH 7.4, containing 10 mM $CaCl_2$ at 37 °C. NaCl concentration was 0.16 M for nonacylated App-D49 and App-diC16 and varied from 0.16 to 1 M for App-diC8.

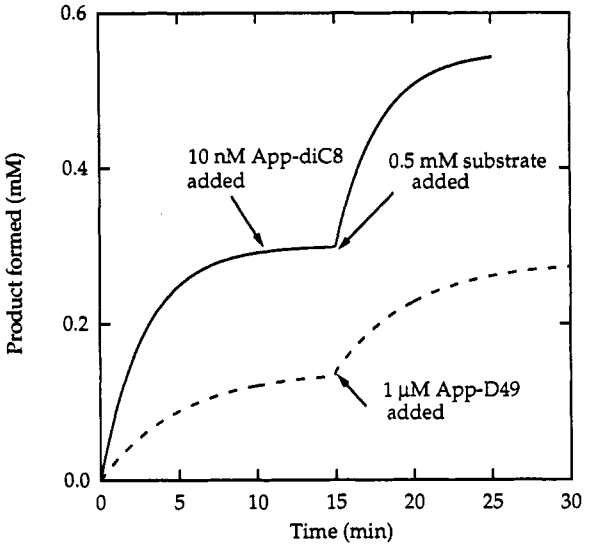

FIGURE 7: Kinetic course of the hydrolysis of POPC LUV by nonacylated App-D49 (1 μM; dotted line) and App-diC8 (10 nM; solid line). The amount of product formed was calculated from [NaOH] added by pH-stat taking into account the incomplete deprotonation of fatty acids on the vesicle surface due to electrostatic repulsion as described (Kupferberg et al., 1981). Note that only the phospholipids present in outer leaflets (ca. 60% of total phospholipids in POPC LUV) are conducive to PLA_2 hydrolysis.

results once again show the hydrophobic nature of interfacial binding of diacylated App-D49's. When compared at 0.16 M NaCl, under which condition all the kinetic measurements were made, rate constants for these apparent first-order processes estimated from their half-lives were 2, 0.5, and 0.04 min^{-1} for App-diC8, App-diC16, and nonacylated App-D49, respectively. The comparison of these values with their relative activity toward pyrene-PC/BLPC polymerized mixed vesicles in Table 2 reveals that the activation of App-diC8 originates primarily from the enhanced rate of the interfacial binding step.

Electrostatic vs Hydrophobic Interactions in the Interfacial Binding. Kinetic and binding studies described above indicate that two acyl groups attached to Lys-7 and Lys-10 of App-D49 provide the protein with hydrophobic anchors to densely-packed zwitterionic interfaces. Interestingly, the amino terminal α-helix of PLA_2 has been proposed to be involved in interfacial binding (Verheij et al., 1981). In particular, our recent studies (Wu & Cho, 1993; Dua & Cho, 1994) revealed that Lys-7 and Lys-10 of App-D49 are involved in the interaction with anionic interfaces. Thus, we measured the activity of nonacylated and diacylated App-D49's toward anionic pyrene-PC/BLPG polymerized mixed vesicles to examine if a neutralization of the two cationic lysine side chains by acylation reduces the ability of enzyme to interact with anionic interfaces. As shown in Table 2, nonacylated App-D49 preferred pyrene-PC/BLPG polymerized mixed vesicle to pyrene-PC/BLPC vesicles by a factor of 2000. By contrast, diacylated enzymes exhibited only ca. 20-fold activation toward pyrene-PC/BLPG polymerized mixed vesicles. Thus, a net decrease in enzymatic activity due to the neutralization of two lysines is 100-fold, which could be approximately translated into a loss in electrostatic interactions by $\Delta\Delta G = -RT \ln 100 = 2.7$ Kcal/mol at room temperature (i.e., 1.4 Kcal/mol for each lysine neutralization) (Fersht, 1985). This estimated value compares well with the known value for electrostatic interactions between two oppositely-charged residues in proteins (1–3 Kcal/mol) (Barlow & Thornton, 1983). Intriguingly, App-diC8 was 225-fold more active than nonacylated App-D49 toward POPC LUV due to increased hydrophobic interactions. Thus, it appears that hydrophobic interactions and electrostatic interactions can play the same role of driving the interfacial binding of PLA_2 with comparable efficiency.

Since the acylation neutralizes positive charges of the two lysines that are essential for the binding of App-D49 to anionic interfaces, one would expect that the kinetic course of the vesicle hydrolysis by diacylated enzymes will be distinct from that by nonacylated App-D49. During the hydrolysis of POPC LUV (or SUV) in the absence of BSA, nonacylated App-D49 was gradually inhibited as the reaction progressed. Kupferberg et al. (1981) reported that BSA relieved the product inhibition by sequestering lysophosphatidyl choline (lysoPC) desorbed from the vesicles which otherwise capture free PLA_2 molecules. Even in the presence of BSA, however, nonacylated App-D49 could not fully hydrolyze all the available phospholipids in the outer leaflets of POPC LUV; an addition of fresh App-D49 after the completion of the initial reaction resulted in further hydrolysis (Figure 7). Jain and Berg (1989) attributed this phenomenon to the trapping of cationic PLA_2 in the vesicles containing another reaction product, fatty acid, by electrostatic interactions (scooting mechanism). They also reported that the scooting mechanism was most significant when the enzyme-to-vesicle ratio was smaller than unity. Although we maintained this ratio greater than unity for all our kinetic experiments (a LUV particle is estimated to contain ca. 50 000 phospholipid molecules), it is conceivable that product-containing anionic domains of vesicles avidly capture all the cationic App-D49 molecules in the reaction mixture, thereby limiting the degree of hydrolysis. As is the case for nonacylated App-D49, the addition of BSA to the reaction mixture increased the amount of phospholipids hydrolyzed by App-diC8, which reached a maximum when the mole ratio of BSA to phospholipid is above 0.5 (data not shown). Other diacylated enzymes showed similar kinetic behaviors. In the presence of a saturating concentration of BSA, App-diC8 even at a lower concentration completely hydrolyzed available phospholipids; no further hydrolysis was observed when fresh enzyme was added (Figure 7). Most interestingly, we found

that App-diC8 was able to quantitatively hydrolyze POPC LUV when the substrates were added sequentially. Also, values of $(k_{cat}/K_m)_{app}$ for individual rounds of hydrolysis were essentially identical. These results indicate that App-diC8 can *rapidly* migrate from one vesicle to another during the course of hydrolysis without being trapped in product-containing vesicles.

DISCUSSION

An increasing number of cellular proteins are being found to undergo covalent modification by addition of long saturated fatty acids, mostly myristate and palmitate, which facilitates the interaction of proteins with the lipid bilayer and/or other membrane proteins. Studies described in this paper show that fatty acids covalently attached to PLA_2 can penetrate into the hydrophobic region of densely-packed zwitterionic phospholipid bilayers thereby anchoring the parent protein to the bilayer. Although the acylation of PLA_2 under physiologic conditions has not been observed, the study of the actions of diacylated App-D49's described herein can shed light into the mechanism by which the fatty acylation regulates the activity of cellular proteins. The acylated PLA_2's used in these studies are structurally unique in that they contain two acyl groups that are spatially close to each other. Thus, some of physical properties of diacylated PLA_2's presented herein might reflect their unique structural property.

The most noticeable property of acylated App-D49's is their tendency to aggregate in solution in order to sequester the hydrophobic acyl groups from water molecules. On the basis of the high tendency of acylated enzymes to dimerize in solution and the high activity of the naturally-occurring dimer, such as *C. atrox* PLA_2, it has been speculated that the acylation-induced dimerization enhances the interfacial activity of monomeric PLA_2's. Results described herein indicate, however, that the dimerization is not responsible for the high activity of acylated enzymes. First, the gel filtration chromatography showed that App-diC8 did not form a dimer at concentrations up to 5 μM, but diacylated enzymes with longer acyl groups readily aggregated under the same condition. Kinetic experiments showed, however, that App-diC8 was more active toward all the aggregated substrates than App-diC14 and App-diC16. The difference in the aggregation behavior of App-diC8 between this paper and the previous one (Cho et al., 1988a) might be due to the difference in protein concentration employed. Second, apparent molecular weights of aggregated diacylated enzymes were widely variable and significantly different from that of *C. atrox* PLA_2. Although we do not fully understand the mode of aggregation of diacylated App-D49's, the heterogeneity of protein aggregates indicates that the aggregation is a nonspecific process and that a structurally well-defined dimer cannot be the catalytically active form for diacylated enzymes. In general, the relative activity of diacylated enzymes toward a specific interface is correlated not to their tendency to aggregate in solution but to their binding affinity for the interface. Thus, the enhanced activity of diacylated enzymes should originate primarily from the improved interfacial binding.

Measurements of the binding of diacylated enzymes to phospholipid monolayers and to polymerized vesicles showed that the interfacial binding of diacylated enzymes is hydrophobic in nature. Because the hydrophobic interactions drive both the protein aggregation and the interfacial binding of diacylated enzymes, it is expected that in the presence of interfaces there will be a competition between these two processes. In this sense, the acylation-induced aggregation of App-D49 should be considered as an inhibitory process. For instance, the finding that the activities of some diacylated enzymes are only slightly higher than or lower than that of nonacylated one toward loosely-packed phospholipids points to the fact that the population of diacylated enzymes at the interface is comparable to or lower than that of the nonacylated enzyme of the same concentration due to the aggregation. This notion is also consistent with the monolayer penetration data at 15 dyn/cm. By the same token, higher enzymatic activity and binding affinity of diacylated App-D49's toward LUV and their ability to penetrate into densely-packed D-POPC monolayers imply that the population of the diacylated enzyme is higher at the interface than that of the nonacylated one. Our binding experiments using polymerized vesicles indeed showed that hydrophobic interactions between covalently-attached acyl groups and phospholipids enhance the interfacial binding affinity of the enzyme as witnessed by a decrease in apparent dissociation constant. Furthermore, the comparison of kinetic and thermodynamic parameters for the enzyme–vesicle binding indicates that enhanced activity of diacylated App-D49's toward LUV derives primarily from the accelerated association of enzyme molecules with the interface. As exemplified by the binding of nonacylated App-D49 to BLPC polymerized vesicles, the binding of most extracellular PLA_2's to densely-packed zwitterionic interfaces is extremely slow and is, in general, a rate-limiting step in the catalysis. As a result, the acceleration of this step by acylation shows a dramatic effect on the overall rate of catalysis. The situation might be somewhat different in the presence of loosely-packed interfaces to which both nonacylated and diacylated enzymes can bind relatively rapidly. As described under Results, the relative activity toward these interfaces might be determined thermodynamically by the relative population of enzyme at the surface. In this context, it should be noted that all the diacylated enzymes are still more active toward POPC SUV than toward POPC LUV, although the difference is much smaller than that observed for nonacylated App-D49.

Kinetic studies of the hydrolysis of pyrene-PC/BLPC and pyrene-PC/BLPG polymerized mixed vesicles by nonacylated and acylated App-D49's showed that hydrophobic interactions and electrostatic interactions could play the same role of anchoring the PLA_2 to interfaces with comparable efficiency. This notion is also supported by our recent finding that the rate of the binding of App-diC8 to BLPC polymerized vesicles is comparable to that of nonacylated App-D49 to BLPG polymerized vesicles (S.-K. Wu and W. Cho, unpublished observation). The main difference is, however, that hydrophobic interactions are sensitive to the interfacial organization of phospholipids whereas electrostatic interactions are sensitive only to electrostatic properties of phospholipids. In case of the hydrolysis of anionic vesicles by cationic App-D49, enzyme molecules can avidly bind the first vesicles they encounter and hydrolyze all the phospholipids in the outer layers, but they eventually become trapped because strong electrostatic interactions between the enzyme and the vesicle remain largely unchanged. Our results indicate that diacylated App-D49's with their hydrophobic anchors not only bind rapidly to vesicles, regardless of the electrostatic properties of vesicles, but also have a high intervesicular mobility. The high intervesicular mobility is primarily due to the neutralization of two lysines involved in the binding of App-D49 to anionic interfaces (Wu & Cho, 1993; Dua & Cho, 1994), which prevents diacylated App-D49 molecules from being trapped in anionic vesicles. Also, the high intervesicular mobility of diacylated enzymes

is attributed to a gradual decrease in hydrophobic interactions between the acyl groups and phospholipids with the progress of the hydrolysis as the accumulation of hydrolyzed product changes the physical state of vesicles. Taken together, the rapid interfacial binding and the high intervesicular mobility of diacylated App-D49's confer to them a great catalytic advantage over nonacylated App-D49; they are capable of catalyzing more turnovers with higher catalytic efficiency. In addition, the comparison of the kinetic behaviors of nonacylated and acylated App-D49's toward PC LUV in the presence of BSA gives new insight into how BSA relieves the product inhibition during the PLA_2-catalyzed hydrolysis of PC vesicles. It has been known that BSA can bind both products of the PLA_2 hydrolysis of PC, fatty acid and lysoPC. Kupferberg et al. (1981) found that fatty acid and lysoPC molecules remaining on the vesicle surface did not inhibit *C. atrox* PLA_2 but desorbed lysoPC (ca. 40% of total lysoPC) did. Consequently, the quantitative sequestering of desorbed lysoPC, which was achieved when the BSA concentration was higher than 25% of total PC concentration, fully relieved the inhibition. Under the same condition, however, BSA was not able to fully relieve the product inhibition of nonacylated App-D49. This difference originates presumably from different preferences of the two enzymes for anionic vesicles; nonacylated App-D49 greatly prefers anionic vesicles to zwitterionic ones whereas *C. atrox* PLA_2 shows only a marginal preference (Wu & Cho, 1993). Thus, it is expected that a much higher concentration of BSA will be necessary to fully activate App-D49. Under this circumstance, the activating effect of BSA would be attributed largely to its ability to remove fatty acid (and lysoPC) from their surfaces, thereby enhancing the intervesicular mobility of the enzyme. Finally, the involvement of Lys-7 and Lys-10 in interfacial binding explains why these two lysines are selectively acylated by 3-acyloxy-4-nitrobenzoic acid. We previously reported that the acylation of App-D49 by NOB was observed only when the concentration of NOB was higher than 0.5 mM. Although we showed that NOB up to its solubility limit (ca. 1.5 mM in the presence of 1.5% CH_3CN) did not form micelles, it has been recognized that PLA_2 can induce premicellar aggregation of substrate (Yuan et al., 1990). In the presence of such aggregates of anionic NOB molecules, Lys-7 and Lys-10 will extensively interact with NOB molecules. In view of the high intrinsic reactivity of the NOB molecule, an elongated exposure of the two lysines to NOB will lead to a quantitative acylation of their ϵ-amino groups.

In summary, the acylation of PLA_2 by 3-acyloxy-4-nitrobenzoic acids provides a simple and convenient method for converting PLA_2 into a highly active form toward densely-packed phospholipid bilayers. In case of App-D49, the acylation of PLA_2 by 3-acyloxy-4-nitrobenzoic acids is highly selective to two lysines, Lys-7 and Lys-10, in the interfacial binding site, and a maximal activation is achieved when the two lysines are acylated with fatty acids containing 8–12 carbon atoms. Depending on the nature of PLA_2, the location of acylation and the number of acyl groups incorporated will vary. Also, an optimal acyl chain length for each type of PLA_2 would be determined by the balance between the interfacial binding and the protein aggregation. Further studies using a wide variety of extracellular PLA_2's are in progress to evaluate the general applicability of acylation as a method to prepare highly activated PLA_2 derivatives. A recent study showed that immobilized PLA_2 contained in an extracorporeal shunt might be potentially used as a novel therapy for hypercholesterolemia (Labeque et al., 1993). Our results indicate that immobilization of diacylated App-D49's will produce a highly efficient catalyst for this type of biomedical applications particularly because the acylation protects critical lysines from undesired modification during the immobilization. Also, the inhibitory effect of protein aggregation on the enzymatic activity would be avoided by the immobilization. The immobilization of various diacylated App-D49's is currently underway.

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