Recent genetic and structural studies have shed considerable light on the mechanism by which secretory phospholipases A₂ interact with substrate aggregates. Electrostatic forces play an essential role in optimizing interfacial catalysis. Efficient and productive adsorption of the Class I bovine pancreatic phospholipase A₂ to anionic interfaces is dependent upon the presence of two nonconserved lysine residues at sequence positions 56 and 116, implying that critical components of the adsorption surface differ among enzyme species (Dua, R., Wu, S.-K., and Cho, W. (1995) J. Biol. Chem. 270, 262–268). In an effort to further characterize the protein residues involved in interfacial catalysis, we have determined the high resolution (1.7 Å) x-ray structure of the Class II Asp-49 phospholipase A₂ from the venom of Agkistrodon piscivorus piscivorus. Correlation of the three-dimensional coordinates with kinetic data derived from site-directed mutations near the amino terminus (E6R, K7E, K10E, K11E, and K16E) and the active site (K54E and K69Y) defines much of the interface topography. Lysine residues at sequence positions 7 and 10 mediate the adsorption of A. p. piscivorus phospholipase A₂ to anionic interfaces but play little role in the enzyme’s interaction with electrically neutral surfaces or in substrate binding. Compared to the native enzyme, the mutant proteins K7E and K10E demonstrate comparable (20-fold) decreases in affinity and catalysis on polymerized mixed liposomes of 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine and 1,2-bis[12-(lipoyloxy)dodecanoyl]-sn-glycero-3-phosphoglycerol, while the double mutant, K7E/K10E, shows a more dramatic 500-fold decrease in catalysis and interfacial adsorption. The calculated contributions of Lys-7 and Lys-10 to the free energy of binding of A. p. piscivorus phospholipase A₂ to anionic liposomes (–1.8 kcal/mol at 25 °C per lysine) are additive (i.e., –3.7 kcal/mol) and together represent nearly half of the total binding energy. Although both lysine side chains lie exposed at the edge of the proposed interfacial adsorption surface, they are geographically remote from the corresponding interfacial determinants for the bovine enzyme. Our results confirm that interfacial adsorption is largely driven by electrostatic forces and demonstrate that the arrangement of the critical charges (e.g., lysines) is species-specific. This variability in the topography of the adsorption surface suggests a corresponding flexibility in the orientation of the active enzyme at the substrate interface.

Phospholipases A₂ (PLA₂; EC 3.1.1.4)¹ catalyze the hydrolysis of the fatty acid ester in the 2-position of 3-sn-phospholipids and are found both in intracellular and secreted forms (for recent reviews, see Refs. 1–4). The enzymes act at the lipid-water interface with a preference for organized lipids (micelles and vesicles) that is often orders of magnitudes greater than that shown for dispersed substrate. Calcium-mediated catalysis appears to involve two kinetically and structurally distinct steps (5, 6). Adsorption of PLA₂ to the interface precedes and is independent of substrate binding to the active site. Secretory PLA₂-s are small proteins (14 kDa) that can be classified into at least four groups based on structural differences (7, 8). Class I (exocrine pancreas and elapidae and hydrophodiae snake venoms) and Class II (mammalian nonpancreatic and crotalidae and viperidae snake venoms) enzymes are highly homologous (see Fig. 1) and have been extensively studied. Crystallographic and NMR analyses of multiple secretory PLA₂-s have implicated a common interfacial adsorption surface that is structurally distinct from the active site (2, 9). The proposed interfacial adsorption site is located on a flat external surface which incorporates a ring of hydrophobic and cationic residues. Recent genetic studies have shown that the adsorption of the Class I bovine pancreatic PLA₂ to anionic interfaces is driven primarily by a few cationic residues, particularly Lys-56 and Lys-116 (10). These residues are not conserved among PLA₂-s, indicating that many of the key determinants of interfacial adsorption differ among enzyme species.

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1The abbreviations used are: PLA₂, phospholipase A₂; App-D49, Asp-49 PLA₂ (App-D49) and Lys-49 PLA₂, and a dimeric PLA₂. Among these PLA₂-s, App-D49 has served as the prototype for a Class II phospholipase from snake venoms.
II PLA₂ (11–13). Previous work has shown that App-D49 strongly prefers anionic interfaces to zwitterionic ones and that Lys-7 and Lys-10 are involved in this selectivity (12, 14–16). Lysines 7 and 10 lie near the carboxyl end of the amino-terminal helix within a cationic patch that also includes Lys-11 and Lys-16 (see Fig. 2). This patch lies at the periphery of the proposed interfacial adsorption surface. At least two other lysines (Lys-54 and Lys-69), which lie on the other side of the enzyme in close proximity to the substrate binding site, also appear to play influential roles in interfacial catalysis. In particular, Lys-69, which is highly conserved among Class II secretory PLA₂s, has been shown to interact with the sn-3 phosphate oxygen of transition state analogs (17). In an effort to further characterize the structural determinants underlying interfacial kinetics, we have determined the crystal structure of App-D49 and analyzed the impact of selected mutations within the interfacial adsorption surface (i.e., E6R, K7E, K10E, K11E, and K16E). The side chains at sequence positions 54 and 69 were also altered to determine the effect of these substitutions on both interfacial adsorption and head group selectivity (54E and 69Y). From the results it is clear that although the adsorption of both the bovine enzyme and App-D49 to anionic interfaces is largely dependent on lysine residues, the locations of these critical residues are quite different. This variability in the topography of the adsorption surface may have an important role in determining the specific orientation of the enzyme at the substrate interface.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-Dioctanoyl-sn-glycero-3-phosphocholine (diC8PC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (dOPE) were obtained from Avanti Polar Lipids (Alabaster, AL); 1-hexadecanoyl-2-(1-pyridinium)glycerol-3-phosphocholine (pyrene-PC), -ethanolamine (pyrene-PE), and -phosphoglycerol (pyrene-POE) were purchased from Molecular Probes (Eugene, OR); and 1,2-bis[12-(tolyloxy)decenoxy]sn-glycerol-3-phosphocholine (BLPC) and -glycerol (BLPG) were synthesized as described previously (18). Large unilamellar liposomes of BLPG (BLPC) were prepared by multiple extrusions of a phospholipid dispersion in 10 mM Tris-HCl buffer (pH 8.4) through a 0.1-μm polycarbonate filter in a Liposofast microextruder (Avestin; Ottawa, Ontario) and then polymerized in the presence of 10 mM dithiothreitol (19). Phospholipid concentrations were determined by phosphate analysis (20). Nonlipid reagents were obtained from the following sources; fatty acid-free bovine serum albumin, Bayer Inc. (Kankakee, IL); guanidinium chloride and guanidinium isothiocyanate, Fisher; restriction enzymes, T4 ligase, T4 polynucleotide kinase, and isopropyl β-D-thiogalactosidase, Boehringer Mannheim; and oligonucleotides, Midland Company (Midland, TX).

**Construction of Mutant Phospholipase A₂ Genes**—The App-D49 gene, synthesized based on the corrected amino acid sequence of App-D49 (21), was a generous gift from Dr. Gordon Rules of the University of Virginia. The coding region for App-D49 was subcloned into a pET-21a vector (Novagen; Madison, WI) and designated as pSH-app. This synthetic gene carries the Asn to Ser mutation (N1S) at the amino terminus to facilitate the removal of the initiator Met by an endogenous nus to a Sculptor generator. The space group was determined to be P1 with unit cell dimensions of a = 39.89 Å, b = 53.10 Å, c = 39.10 Å. The collected data were processed with X-RED (2005). The search model was created by pruning nonhomologous side chains from one subunit of the refined coordinates of the App-D49 dimer (80% sequence homology). The rotation function yielded two strong peaks consistent with the expected presence of two independent molecules in the crystallographic asymmetric unit. The intermolecular translation function was also sharp generating a solution.
TABLE I
Data collection statistics for the crystalline monomeric D49 PLA$_2$ from the venom of A.p. piscivorus

| Resolution shell | Average intensity | Average error | Coverage | Normal $\chi^2$ | Linear $R$ | Square $R$ |
|------------------|------------------|---------------|----------|----------------|------------|------------|
| 99.0–2.73        | 24,633.0         | 676.7         | 95.7     | 2.171          | 0.036      | 0.039      |
| 2.73–2.16        | 6,519.6          | 335.9         | 90.6     | 2.129          | 0.061      | 0.064      |
| 2.16–1.89        | 2,766.4          | 316.9         | 81.1     | 1.877          | 0.108      | 0.101      |
| 1.89–1.72        | 1,233.6          | 290.8         | 77.4     | 1.813          | 0.209      | 0.190      |
| 1.72–1.59        | 510.4            | 295.5         | 74.5     | 2.075          | 0.329      | 0.310      |
| 1.59–1.50        | 623.2            | 301.7         | 71.8     | 2.052          | 0.452      | 0.388      |
| All              | 6871.8           | 381.0         | 81.8     | 2.079          | 0.048      | 0.042      |

Average bond angle (degrees) 0.031 0.050 2.448
Average bond angle (Å) 0.8 116.7
Plane restraint (Å) 0.018 0.040
Chiral-center restraint (Å$^3$) 0.588 1.000
Nonbonded contact restraints (Å$^6$) 0.178 1.000
Multiple-Torsion Contact 0.400 2.000
Possible Hydrogen Bond 0.339 2.000
Conformational torsion angle restraint (Å$^3$) 4.7 30.0

* The values of sigma are the input estimated standard deviations that determine the weights of the corresponding restraints during refinement.

With excellent packing. Refinement with the computer programs PROFFT and X-PLOR rapidly reduced the R factor while maintaining reasonable stereochemistry (Table II). When necessary, the coordinates were manually rebuilt with the graphics program FRDO. Two hundred and sixty-six water molecules were assigned based on density in the Fourier difference maps, hydrogen-bonding potentials, and refined temperature factors of less than 45 Å$^2$ at full occupancy. A second group of 52 less well defined water molecules refined to temperature factors ranging from 46 to 65 Å$^2$. The distal portions of solvent-exposed side chains (e.g. lysines) accounted for the majority of the protein disorder.

Kinetic Measurements—PLA$_2$-catalyzed hydrolysis of polymerized mixed liposomes was performed at 37°C in 2 ml of 10 mM HEPES buffer, pH 7.4, containing 0.1 mM pyrene-containing phospholipids (1 mol %) inserted into 9.9 μM BLPG, 2 μM bovine serum albumin, 0.16 mM NaCl, and 10 mM CaCl$_2$. Enzyme concentrations were adjusted to keep the half-life of the reaction below 5 min (e.g. [wild type] = 1–10 nm and [K/E/K16E] = 0.1–1 μM). Progress of the reaction was monitored as an increase in fluorescence emission at 378 nm using a Hitachi F4500 fluorescence spectrometer with an excitation wavelength of 345 nm. The spectral bandwidth was set at 5 nm for both excitation and emission. The pseudo-first-order rate constant was determined from the nonlinear least squares analysis of the reaction progress curves (10, 18, 19). This was divided by the enzyme concentration to obtain the apparent second-order constant, $k_{cat}$/[$E$]$_{app}$. The kinetics of PLA$_2$-catalyzed hydrolysis of diC$_{18}$PC and diC$_{18}$PE micelles were measured in the presence of 0.5 mM phospholipid, 0.16 mM NaCl, and 10 mM CaCl$_2$. Enzyme concentrations between 1–10 nM. The time course of the phospholipid hydrolysis was monitored with a computer-controlled Metrom pH-stat (Brinkmann) in a thermostated vessel. Under these conditions, the hydrolysis of diC$_{18}$PC (and diC$_{18}$PE) followed first-order kinetics, since the substrate concentrations remained lower than the apparent $K_a$ values.

Liposome-Protein Binding Measurements—The adsorption of the wild type and mutant enzymes to polymerized liposomes was examined fluorometrically. Typically, the fluorescence emission (excitation at λ = 280 nm) of the protein solution (0.5 μM) at 345 nm ($F_{\text{polym}}$) was measured at equilibrium in 2 ml of 10 mM HEPES buffer, pH 7.4, containing 0.1 to 100 μM BLPG polymerized liposomes and varying concentrations of NaCl and CaCl$_2$. An initial decrease in protein fluorescence was observed due to the adsorption of the protein to the cuvette wall. The final fluorescence emission was recorded after the adsorption was complete. Protein concentrations were, therefore, corrected for this loss and the liposome solution was added after the protein signal stabilized. To minimize potential artifacts due to both the inner filter effect and the scattering by BLPG polymerized liposomes, each observed $F_{\text{polym}}$ value was background-corrected against the $F_{\text{free}}$ value observed in the presence of the same concentration of BLPG polymerized liposomes. BLPC polymerized liposomes have the same spectroscopic properties as BLPG polymerized liposomes but App-D49 has an extremely low affinity for them (apparent dissociation constant > 1 mM) (14). The relative fluorescence change ($F_{rel}$) for each phospholipid concentration was calculated as ($F_{\text{max}}$ - $F_{\text{polym}}$)/$F_{\text{max}}$. Each experiment was repeated at least twice with similar results.

RESULTS

Crystal Structure of App-D49 and the Design of App-D49 Mutants—App-D49 is a typical Class II PLA$_2$ with high sequence homology to other members of this class (Fig. 1). The enzyme was crystallized in the absence of added calcium ion and, as expected, both independent molecules in the crystallographic asymmetric unit lack discernible metal ions (Fig. 2). Instead, a single water molecule replaces each of the primary calcium ions and makes comparable hydrogen bonds to a carboxylate oxygen of App-49 and three backbone carbonyls (Tyr-28, Gly-30, and Gly-32). Least squares superimposition of the α-carbon traces of App-D49 and its dimeric counterpart from A.
Structure and Function of Phospholipase A₂

Fig. 1. Comparison of the amino acid sequence of App-D₄⁹ with those of typical Class I and Class II sPLA₂s. Asterisks are used to identify residues that are homologous to those found in the sequence of App-D₄⁹. The numbering system used is based upon the homologous core developed by Renetseder et al. (43). Black rectangles appear above residues involved in coordinating the primary calcium ion; open rectangles denote conserved residues of the catalytic network, and the essential His-48 appears with an ion; appear above residues involved in coordinating the primary calcium ion; and the carboxyl terminus (residues 123–131). The residues focused on in this study (Glu-6, Lys-7, Lys-10, Lys-11, Lys-16, Lys-54, and Lys-69) can be visualized in their entirety.

The side chains of Lys-7, Lys-10, Lys-16, and Lys-54 are all well resolved in the electron density maps and are positioned to their respective Cα atoms, whereas Arg-6, Lys-11, and Lys-69 can be visualized in their entirety.

The dimensions of the crystalline App-D₄⁹ are approximately 22 × 30 × 42 Å with 50% of the structure α-helix and 10% β-sheet. The two long anti-parallel β-helices that form the backbone of the molecule (residues 37–54 and 90–108) are secured by a series of disulfide bridges. The active site residues (His-48, Arg-59, Tyr-73, and Asp-99) occupy the base of a short internal hydrophobic cavity or cleft that opens onto the external surface. The disposition of productively bound substrate within the active site has been shown through the co-crystallization of several PLA₂s with transition state and substrate analogs (17, 23576). and electrostatic data (37), suggests that the interfacial adsorption surface surrounds and incorporates the external opening of the hydrophobic channel.

All of the mutant proteins were expressed and refolded as efficiently as the wild type (data not shown), indicating similar thermodynamic stability. Retention times for the refolded wild type App-D₄⁹ and the native enzyme were identical on a CM-Sepharose column. The mutant enzymes, including the double mutant (K₇E/K₁₀E), migrated as expected for their altered surface charges. The CD spectra of the native, wild type, and the mutant proteins were indistinguishable (Fig. 4) as were estimates of α-helical content based on θ₂₂₂ values (~50%), indicating that the mutations did not induce any gross structural change in protein conformation. The preservation of tertiary structure is consistent with the surface locations of the altered side chains.

Kinetic Properties of App-D₄⁹ and Mutants—A major advantage of the polymerized mixed liposome system over other PLA₂ kinetic systems is that it allows an unambiguous distinction between phospholipids interacting with the active site of PLA₂ (i.e., hydrolyzable inserts) and ones interacting with the interfacial binding site of PLA₂ (i.e., polymerized matrix) (18). Thus, it is possible to separately determine the head group specificity of the interfacial adsorption surface and the active site. Furthermore, it is also possible to separately analyze the effects of protein and phospholipid modification on interfacial adsorption and substrate binding (10, 18, 33). Using this approach, we measured the kinetic properties of wild type and mutants toward a wide variety of polymerized mixed liposomes. Two zwitterionic phospholipids, pyrene-PC and pyrene-PE, and anionic pyrene-PG were used as inserts in the anionic BLPG polymerized matrix to determine the phospholipid head group specificity of the active site of each enzyme. Apparent second-order constants, (k₅₀/Kₐ)ₜₐₚ, determined for these proteins are summarized in Table III. Although (kₛ/Kₛ)ₜₐₚ has no obvious physical meaning, it is a useful parameter for comparing overall interfacial activity of wild type and mutants (10, 33). In agreement with a previous report (22), recombinant wild type App-D₄⁹ showed essentially the same activity and head group specificity toward polymerized mixed liposomes as native App-D₄⁹. The active sites of both enzymes prefer anionic phospholipids to zwitterionic ones by ~3.5-fold. This is a modest value when compared to the high (up to 5,000-fold) adsorption preference of App-D₄⁹ for anionic interfaces (18). Of note, App-D₄⁹ has comparable activities toward pyrene-PC and pyrene-PE substrates indicating that the enzyme cannot distinguish between the two zwitterionic head groups. Thus, the ratio of enzyme activity (pyrene-PG/pyrene-PC) can be used to describe the anionic phospholipid preference of each protein (see Table III).

The cluster of lysines lying adjacent to the carboxyl end of the amino-terminal α-helix is geographically remote from the catalytic site (e.g., ~15 Å from His-48). Mutation of these residues to Glu had no appreciable effect on the enzyme’s head group preference. These mutations did, however, significantly decrease adsorption and catalysis on polymerized mixed liposomes. When compared to the wild type, K₇E and K₁₀E demonstrated an approximately 20-fold drop in activity toward pyrene-PC/BLPG polymerized mixed liposomes. In contrast, K₁₁E and K₁₆E displayed modest 3.8- and 2.6-fold decreases, respectively. The rate decrease for the double mutant (K₁₇E/K₁₀E) was 500-fold on polymerized mixed liposomes and approximated the product of the decreases for the individual mutations. To investigate the effect of mutations on the binding of App-D₄⁹ to electrically neutral interfaces, we measured the activities of wild type and mutants using zwitterionic diC₈PC
micelles. These micelles were chosen because they provide neutral interfaces and yet are a good substrate for App-D49 due to their loose interfacial packing density. When assessed using these micellar substrates, none of the amino-terminal mutations caused a significant drop in activity. Eventhe K7E/K10E mutant showed 83% of wild type activity. This suggests that Lys-7 and Lys-10 play an essential role in binding to anionic interfaces but are dispensable in adsorbing to electrically neutral interfaces or in substrate binding.

Many secretory PLA₂s contain a cationic residue at position 6 in addition to those at position 7 and 10. If the enzymatic activity of App-D49 toward substrate aggregates is simply proportional to its electrostatic affinity for the interface, one would expect that the E6R mutant with additional positive charge in the vicinity of Lys-7 would be more active on anionic polymerized mixed liposomes. Surprisingly, the E6R enzyme displayed a 4-fold decrease in activity toward all polymerized mixed liposomes. This inhibition was not due to a disruption of the catalytic apparatus, since the activity of E6R toward electrically neutral diC₈PC micelles was only modestly affected (i.e. a 13% decrease). The decrease in activity suggests, however, that this mutant binds to the BLPG polymerized mixed liposomes in...
a suboptimal mode. The activity of PLA$_2$ toward anionic interfaces is, therefore, not merely proportional to the net charge of the interfacial adsorption surface.

To identify the origin of App-D49’s small, but definite, preference for anionic head groups, two lysines (Lys-54 and Lys-69) that are located close to the active site were mutated, and the resulting proteins’ activities were measured on polymerized mixed liposomes and micelles. If the active site of App-D49 prefers pyrene-PG to pyrene-PC because of electrostatic repulsion between the lysine side chain(s) and the choline head group, one would expect that K54E and K69Y would have enhanced activities toward pyrene-PC while showing the same activities toward pyrene-PG as the wild type. On the other hand, if the preference for pyrene-PC derives from favorable interactions (e.g., hydrogen bonding) between the lysine(s) and the glycerol head group, the mutants should show reduced activity on pyrene-PG but not on pyrene-PC. As it turned out, the K54E mutation had little effect on the enzyme activity toward pyrene-PC (15% decrease), pyrene-PG (20% decrease), or micellar dC$_{16}$PC (17% decrease). This mutation did, however, result in a 2-fold enhancement of activity on pyrene-PE in both polymerized mixed liposomes and micellar dC$_{16}$PC. In the case of dC$_{16}$PE, the (K$_{cat}$/V$_{max}$) values for wild type and the K54E mutant were 4.5 x 10$^{-1}$ s$^{-1}$ and 1.0 x 10$^{-2}$ s$^{-1}$, respectively. These results suggest that Lys-54 does not directly interact with productively bound phospholipids, but a glutamate side chain at this position might potentially form a favorable electrostatic contact with the ammonium ion of phosphatidylethanolamine. The activity of the K69Y mutant toward both pyrene-PC and pyrene-PE lipids in polymerized mixed liposomes was identical to the wild type, indicating that the Lys-69 side chain has a negligible effect on the productive binding of zwitterionic head groups. The mutation did, however, decrease the enzyme activity toward pyrene-PC/dBLPG by approximately 3-fold, rendering K69Y nonselective for the phospholipid head group. The modest nature of these changes underlines the equivalent abilities of the tyrosine and lysine side chains in coordinating the sn-3 phosphate of a productively bound phospholipid. At the same time, the decrease in activity toward the pyrene-PC substrate suggests that the e-ammonium group of Lys-69 may form hydrogen bond(s) with the glycerol head group which are not achievable with the phenolic hydroxyl of Tyr-69 (Fig. 5).

**Binding of App-D49 and Mutants to BLPG Polymerized Liposomes**—To delineate the relationship between the enzyme activity and the interfacial adsorption affinity of mutants, we measured their binding to BLPG polymerized liposomes. We previously showed that nonhydrolyzable BLPG polymerized liposomes could be used to determine the dissociation constant for PLA$_2$-liposome complex (18, 19). The n and K$_p$ values (Table IV) determined for the wild type and mutant enzymes were derived from curve fittings of the respective isotherms (Fig. 6). All of the proteins showed analogous saturation binding curves, indicating similar binding modes. Under the conditions in which the kinetic measurements were performed (10 mM Ca$^{2+}$ and 0.16 M NaCl), the wild type enzyme showed a high affinity for anionic BLPG liposomes comparable to that reported for the native App-D49 (nK$_p$ = 0.7 μM) (19). Calcium ion had no effect on this binding (Fig. 6). When compared to the wild type, the mutant enzymes displayed a wide range of K$_p$ values with only minor variation in n (n ~ 40). In general, the relative binding affinity of mutants to BLPG polymerized liposomes paralleled their relative enzymatic activity toward pyrene-PC/dBLPG polymerized mixed liposomes. For example, K7E, K10E, and K7E/K10E had 50-, 45-, and 500-fold increases in K$_p$, respectively. In the case of K7E/K10E, for which the binding saturation could not be reached with BLPG concentration up to 200 μM, the K$_p$ value was determined from a curve fitting assuming n = 40. Also, K11E and K16E showed 2-fold increases in K$_p$, whereas K54E and K69Y had essentially the same K$_p$ as wild type. For mutants of amino-terminal lysines, these results indicate that their reduced activities are primarily due to the decreases in interfacial adsorption affinities and again underscore the essential roles of Lys-7 and Lys-10 in the interfacial adsorption of App-D49. For K54E and K69Y, the binding data confirm that their altered substrate selectivity is solely due to changes in substrate binding. From these kinetic and binding data, it appears that wild type and mutants all bind to anionic BLPG-based polymerized mixed liposomes in the same productive mode and that tighter interfacial binding will lead to higher interfacial activity of enzyme. A notable exception was E6R which showed 3-fold increase in binding affinity despite its 4-fold decrease in activity. For this mutant, the enhanced binding to anionic interfaces is consistent with an increase in net positive charge in the vicinity of the critical Lys-7 and Lys-10. Therefore, the decrease in enzyme activity for this mutant again suggests that it might bind to the BLPG polymerized mixed liposomes in a suboptimal mode. Finally, we measured the effect of ionic strength of the medium on the adsorption of wild type and mutants to BLPG polymerized liposomes. As summarized in Table IV, the binding of wild type was significantly diminished in the presence of 1 M NaCl, whereas the mutants with lower interfacial binding affinities were less sensitive to the ionic strength. In the presence of 1 M NaCl, the wild type protein and the K7E and K10E mutants showed comparable binding affinities for BLPG polymerized liposomes. Furthermore, the nK$_p$ values for these enzymes were smaller than that of the K7E/K10E mutant only by an order of magnitude.
demonstrating the electrostatic nature of the interfacial adsorption of App-D49 to anionic BLPG polymerized liposomes.

DISCUSSION

Tertiary Structure of App-D49 and Mutants—The three-dimensional structure of App-D49, crystallized in the absence of calcium ion, shows the structural features typical of other Class II PLAs. The catalytic site, calcium-binding apparatus, and hydrophobic channel are identical to those described previously. Based on analogy with other PLAs, the interfacial adsorption surface of App-D49 surrounds the opening of the hydrophobic channel and includes residues 2 (Leu), 3 (Phe), 6 (Glu), 19 (Met), as well as portions of the calcium-binding loop. App-D49 is notable for its relatively large number of cationic residues with a correspondingly high pI (9.5). These charges are arranged asymmetrically with maximal positive charge potential in the region of the putative interfacial adsorption site, which is consistent with its role in liposome binding.

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Table IV

| Enzymes       | 0.16 M NaCl | 1 M NaCl, nKd |
|---------------|-------------|---------------|
| Wild type     | 40 ± 5      | (2.0± 0.3) × 10⁻⁸ | (1.0± 0.2) × 10⁻⁵ |
| E6R           | 38 ± 4      | (3.0± 0.5) × 10⁻⁹ | ND¹ |
| K7E           | 42 ± 5      | (5.0± 0.2) × 10⁻⁷ | (2.0± 0.3) × 10⁻⁵ |
| K10E          | 41 ± 5      | (6.0± 0.3) × 10⁻⁷ | (2.0± 0.4) × 10⁻⁵ |
| K7E/K10E      | 40 ± 5      | (5.0± 0.3) × 10⁻⁸ | (3.0± 0.7) × 10⁻⁴ |
| K11E          | 38 ± 4      | (1.7± 0.2) × 10⁻⁷ | ND¹ |
| K16E          | 45 ± 5      | (1.0± 0.1) × 10⁻⁷ | ND¹ |
| K54E          | 36 ± 5      | (2.3± 0.3) × 10⁻⁸ | ND¹ |
| K69Y          | 40 ± 5      | (2.5± 0.3) × 10⁻⁸ | ND¹ |

¹ ND, not determined.

Fig. 5. The interaction of a transition-state analog (t-1-O-octyl-2-heptylphosphonyl-sn-glycero-3-phosphoethanolamine) with the active site of the Class I PLA₂ from the venom of N. n. atria (A). Class II PLA₂s, including App-D49, substitute a lysine residue for the tyrosine at sequence position 69. The K69Y mutant has essentially the same activity as the wild type enzyme toward PC and PE substrates but shows a 3-fold drop in activity toward PG substrate. One explanation for this finding is that the ε-ammonium group of Lys-69 forms additional hydrogen bonds with phospholipid head groups, especially with PG whose hydroxyl group can function as hydrogen bond acceptors (B). Such an interaction would not be achievable by the phenolic oxygen of Tyr-69 or with PC and PE as substrate.

Fig. 6. A, the binding isotherms of wild type (○) and mutants including E6R (●), K7E (△), K10E (▲), K7E/K10E (●), K11E (■), and K16E (○). B, the binding isotherms of wild type (○), K54E (△), and K69Y (●). Also shown in panel B is the binding isotherm of wild type in the absence of Ca²⁺ (◆). Protein concentrations were 0.5 μM in 10 mM HEPES buffer, pH 7.4, containing 0.16 M NaCl and 10 mM CaCl₂. See “Experimental Procedures” for the definition of F_rel. The solid lines indicate theoretical curves constructed using Equation 1 with n and Kd values determined from nonlinear least squares analyses.
surface (Fig. 7B). This distribution of positive charge potential is in sharp contrast to that observed for bovine pancreatic PLA2. The residues chosen for mutation (Glu-6, Lys-7, Lys-10, Lys-11, Lys-16, and Lys-69) lie on the perimeter of the proposed interfacial adsorption surface. Orientations of the enzymes are similar to that depicted in Fig. 2. The proposed interfacial adsorption surface is oriented in the plane of the page with its free surface facing the reader. Most of the side chains undergoing mutation (Lys-7, Lys-10, Lys-11, Lys-16, and Lys-69) can be seen in this view. Anionic residues are colored red, cationic residues blue, hydrophobic residues green, and cysteines yellow. B, electrostatic representations of the surface shown in A. Both representations are contoured similarly. Note the distinct cationic patch at the right base which is composed of Lys-7, Lys-10, Lys-11, and Lys-16. The former two residues are particularly important in the adsorption of App-D49 to anionic surfaces.

Interfacial Adsorption of App-D49—App-D49 normally shows high enzymatic activity toward anionic phospholipid aggregates but extremely low activity toward densely packed zwitterionic phospholipid monolayers and bilayers (12, 14, 38). As a result, the activation of App-D49 on zwitterionic aggregates generally requires the formation of anionic lipid domains containing a reaction product such as a fatty acid (38). The contributions of Lys-7 and Lys-10 to the free energy of binding of App-D49 to anionic BLPG polymerized liposomes can be estimated using the equation 
\[ \Delta G^0 = R \cdot T \cdot \ln \left( n \cdot K_w \right) \] for wild type PLA2, where
\[ n \cdot K_w \] for mutant. Under standard conditions with the concentration of free phospholipid set at 1 M, each lysine contributes approximately \(-1.8\) kcal/mol at 25 °C. This calculated value compares well with the known value (\(-1\) to \(-3\) kcal/mol) for electrostatic interactions between two oppositely charged residues in proteins (39) and with our previous mutational experiments with the bovine pancreatic PLA2 (10). Based on these structural and functional data, it is probable that the enzyme’s helical surface containing Lys-7 and Lys-10 lies parallel to the membrane during interfacial adsorption. Since the contributions of Lys-7 and Lys-10 to the free energy of interfacial binding/adsorption appear additive \((-3.7\) kcal/mol at 25 °C), there is, at most, limited synergy in the binding of the two lysines to anionic interfaces (40). The strong electrostatic effect on interfacial adsorption is supported by the striking correlation between adsorption and the ionic strength of the medium. Other contributions to the binding energy presumably come from a number of weaker electrostatic and hydrophobic interactions (for instance, see Maliwal et al. (32)). The presence of calcium ion does not appreciably affect the interfacial adsorption of App-D49, consistent with the minor effect that an additional calcium ion has on the protein’s global electrostatics (37).

Lys-7 and Lys-10 are clearly the dominant residues directing the interfacial adsorption of App-D49. This is in contrast to our previous finding (10) for the bovine pancreatic PLA2, where Lys-56 and Lys-116 are critical residues confirming that the determinants of interfacial adsorption are specific to the species of PLA2 being studied. Our results also dispute the notion that the interfacial adsorption of secretory PLA2 is driven by a large number of weak interactions which cannot be specifically blocked. Whether the variability in interface topology translates into orientational differences at the interface remains.

FIG. 7. The interfacial adsorption surface of the crystalline monomeric D49 PLA2 from the venom of A. p. piscivorus: comparison with bovine pancreatic PLA2. In each panel, the bovine pancreatic PLA2 is shown on the left. A, space-filling representations of the proposed interfacial adsorption surface. Orientations of the enzymes are similar to that depicted in Fig. 2. The proposed interfacial adsorption surface is oriented in the plane of the page with its free surface facing the reader. Most of the side chains undergoing mutation (Lys-7, Lys-10, Lys-11, Lys-16, and Lys-69) can be seen in this view. Anionic residues are colored red, cationic residues blue, hydrophobic residues green, and cysteines yellow. B, electrostatic representations of the surface shown in A. Both representations are contoured similarly. Note the distinct cationic patch at the right base which is composed of Lys-7, Lys-10, Lys-11, and Lys-16. The former two residues are particularly important in the adsorption of App-D49 to anionic surfaces.
that the either in polymerized mixed liposomes or in micelles indicates small (2-fold) increase in activity of K54Et toward PE substrates. The change in enzyme activity toward PC or PG substrates. The integrity of the amino-terminal helix is essential for the high catalytic efficiency associated with interfacial catalysis. Residues 2, 5, and 9, which contribute to the inner walls of the hydrophobic channel, are invariant among PLAs. Mutation of residues in this region (Glu-6, Lys-7, Lys-10, Lys-11, and Lys-16) demonstrates a critical role for Lys-7 and Lys-10 in the enzyme’s interaction with anionic phospholipids.

uncharacterized since hydrophobic groups also play key roles in optimizing the interaction. The introduction of an additional charge to the amino-terminal α-helix by the E6R mutation modestly ($\Delta \Delta G^0 = -1.1 \text{kcal/mol at } 25^\circ C$) increases the affinity of App-D49 for anionic interfaces. This enhanced adsorption, however, does not facilitate productive-mode substrate binding to the active site since E6R has lower activity toward anionic polymerized mixed liposomes than the wild type. This reduction in activity may stem from electrostatic repulsion between Arg-6 and Lys-7 leading to a nonoptimal orientation of the lysine side chain. Alternatively, the side chain of Arg-6 may mechanically interfere with the proper orientation of the enzyme at the interface.

Roles of Lys-54 and Lys-69—Residues 53 through 58 lie at the carboxyl terminus of the first anti-parallel α-helix. This section of the enzyme lies under the calcium-binding loop and adjacent to the region occupied by the head group of productively bound substrate. The charge and orientation of side chains in this region have been shown to influence the head group selectivity of PLAs. For example, Lys-53 of the bovine pancreatic PLA2 (10) and Arg-53 of the porcine pancreatic PLA2 (41) appear to be responsible for the anionic phospholipid preferences of these enzymes. Glu-56 of the Class II human nonpancreatic enzyme makes a direct stabilizing contact with the ammonium ion of the PE head group in the x-ray structure of the transition-state analog complex (17). The side chain of App-D49’s Lys-54 points toward bulk solvent and, consistent with this orientation, its mutation to Glu did not significantly impair the enzyme at the interface.

The calculated decrease in substrate binding energy due to this putative electrostatic interaction is correspondingly small; 0.4 kcal/mol at 25 °C ($\Delta \Delta G^0 = -R^T \ln (k_w/(K_m)_\text{app} / (k_w/(K_m)_\text{app} \text{ for wild type})$). The crystal structures of several transition-state and substrate analog complexes have shown that both the Tyr-69 of Class I PLAs and the Lys-69 of Class II enzymes form a hydrogen bond with the pro-S nonbridging oxygen of the sn-3 phosphate (17, 29) (see Fig. 5). We suggest that the ε-amino group of Lys-69 may be able to form additional hydrogen bonds with phospholipid head groups, especially with PG whose hydroxyl groups can function as hydrogen bond acceptors (Fig. 5B). Such an interaction would not be achievable by the phenolic oxygen of Tyr-69 or with PC and PE as substrate. This would explain why K69Y has the essentially the same activity as wild type toward PC and PE substrates but shows a 3-fold drop in activity toward PG substrate. Our recent data indicate that the Lys-69 of App-D49 and of the human nonpancreatic PLA2 also interacts favorably with other anionic phospholipids (e.g. phosphatic acid and phosphatidylserine) that can serve as strong hydrogen bond acceptors (42). Class II secretory PLAs presumably show a modest degree of substrate selectivity due to the ability of Lys-69 to form a hydrogen bond(s) with anionic phospholipid head groups.

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