Interfacial Catalysis by Phospholipase A_2: Substrate Specificity in Vesicles†

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ABSTRACT: The binding equilibrium of phospholipase A_2 (PLA2) to the substrate interface influences many aspects of the overall kinetics of interfacial catalysis by this enzyme. For example, the interpretation of kinetic data on substrate specificity was difficult when there was a significant kinetic contribution from the interfacial binding step to the steady-state catalytic turnover. This problem was commonly encountered with vesicles of zwitterionic phospholipids, where the binding of PLA2 to the interface was relatively poor. The action of PLA2 on phosphatidylcholine (PC) vesicles containing a small amount of anionic phospholipid, such as phosphatidic acid (PA), was studied. It was shown that the hydrolysis of these mixed lipid vesicles occurs in the scooting mode in which the enzyme remains tightly bound to the interface and only the substrate molecules present on the outer monolayer of the target vesicle became hydrolyzed. Thus the phenomenon of scooting mode hydrolysis was not restricted to the action of PLA2s on vesicles of pure anionic phospholipids, but it was also observed with vesicles of zwitterionic lipids as long as a critical amount of anionic compound was present. Under such conditions, the initial rate of hydrolysis of PC in the mixed PC/PA vesicles was enhanced more than 50-fold. Binding studies of PLA2 to vesicles and kinetic studies in the scooting mode demonstrated that the enhancement of PC hydrolysis in the PC/PA covesicles was due to the much higher affinity of the enzyme toward covesicles compared to vesicles of pure PC phospholipids. A novel and technically simple protocol for accurate determination of the substrate specificity of PLA2 at the interface was also developed by using a double-radiolabel approach. Here, the action of PLA2 in the scooting mode was studied on vesicles of the anionic phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol that contained small amounts of ^3H- and ^{14}C-labeled phospholipids. From an analysis of the ^3H and ^{14}C radioactivity in the released fatty acid products, the ratio of substrate specificity constants (k_{cat}/K_{MS}) was obtained for any pair of radiolabeled substrates. These studies showed that the PLA2s from pig pancreas and *Naja naja naja* venom did not discriminate between phosphatidylcholine and phosphatidylethanolamine phospholipids or between phospholipids with saturated versus unsaturated acyl chains and that the pig enzyme had a slight preference for anionic phospholipids (2-3-fold). The described protocol provided an accurate measure of the substrate specificity of PLA2 without complications arising from the differences in binding affinities of the enzyme to vesicles composed of pure phospholipids.

Phospholipase A_2 (PLA2)[1] hydrolyzes glycerophospholipids at the *sn*-2 position to produce a free fatty acid and a lysophospholipid (Waite, 1987; Dennis, 1983; Volwerk & de Haas, 1981). The best characterized enzymes are those from animal digestive fluids and venoms (van den Bergh et al., 1989). Phospholipids containing most of the naturally occurring polar head groups, including choline, ethanolamine, inositol, serine, and glycerol as well as other molecular classes such as the cardiolipins and the phosphatidic acids, are hydrolyzed to some degree by these extracellular PLA2s (Christie, 1982). Since all of the naturally occurring phospholipids have essentially no solubility in water, the substrate for PLA2 will be present in an aggregated form. It is for this reason that previous studies on substrate specificity have been difficult to interpret. For example, the physical structure of the substrate aggregate will, in general, vary dramatically with the chemical structure of the phospholipid being studied. This, in turn, can modulate the observed substrate selectivities. In addition, the presence of additives, including detergents and organic cosolvents, can markedly affect the relative velocities for a series of PLA2 substrates. Even with vesicles of pure phospholipids, the substrate specificity results will vary dramatically depending on the fraction of PLA2 that is bound at the interface during the steady-state turnover. Thus it is claimed that any substrate

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[1] Abbreviations: DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate disodium salt; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol lithium salt; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate disodium salt; ^{14}C-DOPC, 1-[1-^{14}C]oleoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine; ^3H-DPPA, 1-palmitoyl-2-[9,10-^3H]palmitoyl-*sn*-glycero-3-phosphate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ^{14}C-DPPC, 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; ^3H-DPPC, 1-palmitoyl-2-[9,10-^3H]palmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; ^3H-DPPE, 1-palmitoyl-2-[^3H-9,10]palmitoyl-*sn*-glycero-3-phosphoethanolamine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol lithium salt; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; OHPC, 1-oleyl-2-hexadecyl-*sn*-glycero-3-phosphocholine; PA, phosphatidic acid; ^{14}C-PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PLA2, phospholipase A_2.

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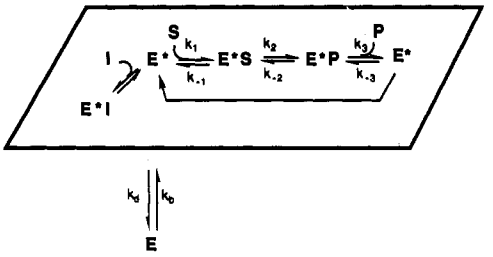
Scheme I: Kinetic Scheme Used To Describe Interfacial Catalysis by PLA2[a]



[a] Here the enzyme in the water phase, E, binds to the bilayer to give E*. The bound enzyme can bind a phospholipid molecule, S, and undergo catalytic turnover to produce the enzyme–product complex followed by release of products, P. Also shown is an inhibitor, I, within the interface that is capable of competing with S for the binding to E*.

specificity result with PLA2 can be obtained, depending on the conditions of the assay.

An understanding of the substrate specificity of PLA2 has important implications for the the role of these enzymes in controlling the breakdown of phospholipids in cell membranes. There are numerous efforts focused on understanding the PLA2-catalyzed release of arachidonic acid from the membranes of inflammatory cells since this fatty acid is the biosynthetic precursor of the eicosanoids (Irvine, 1982; Chilton, 1989). Possibilities ranging from a specific PLA2 acting on a single molecular phospholipid species to those invoking the release of fatty acids from the 2-position of all membrane phospholipids by a substrate-indiscriminate PLA2 have been considered. Although the purification of a number of intracellular PLA2s have been reported, the enzyme(s) responsible for the liberation of the arachidonic acid has (have) not yet been conclusively identified.

A key feature of the action of PLA2s on lipid bilayers that has important consequences for the study of substrate specificity is the demonstration that the binding of the enzyme to the interface is a distinct step from the binding of a phospholipid molecule to the catalytic site (Jain & Berg, 1989; Dennis, 1983; Verger & de Haas, 1976). This is illustrated in the scheme in Figure 1 of Berg et al. (1991) (reproduced in Scheme I in this paper) and explored in detail in Jain et al. (1991a). Here, the enzyme can be either in the aqueous phase (E) or bound to the substrate bilayer (E*). The steps shown in the box of Scheme I represent a Michaelis–Menten mechanism for the action of PLA2 within the interface. The amount of enzyme bound to the substrate interface will depend on the type of phospholipid used in the assay. What is really required is a procedure for the quantitative analysis of the relative velocities for a series of phospholipid substrates that is free from the distortions caused by a differential amount of bound enzyme. In this study the substrate specificity was monitored by studying the action of PLA2 in the scooting mode on covesicles of a mixture of phospholipids on which all of the enzyme remains tightly bound to the substrate bilayer. In this way, the enzyme is allowed to "choose" between different substrates while remaining at the interface.

Materials and Methods

DMPA, DOPA, OPPC, and DPPC were obtained from Avanti Polar Lipids. OHPC was obtained from Calbiochem. DMPM was synthesized from DMPA as described (Jain & Gelb, 1991). DTPC and DTPM were prepared as described (Jain et al., 1986a). ^{14}C-DPPC (55 mCi/mmol) and ^{14}C-DOPC (105 mCi/mmol) were obtained from Amersham. ^{3}H-DPPC (58 Ci/mmol) and ^{14}PAPC (53 mCi/mmol) were obtained from New England Nuclear. PLA2s from porcine pancreas and *Naja naja naja* venom (Miami Serpentarium, Pakistan variety) were purified as described (Niewenhuizen et al., 1974; Hazlettt & Dennis, 1985).

Synthesis of ^{3}H-DPPA. Unlabeled DPPC (2.0 mg) was mixed with 15 μCi of ^{3}H-DPPC (58 Ci/mmol) in 200 μL of chloroform. The solution was vortexed to dissolve all of the lipids, and the solvent was removed with a stream of N_2, and then the product was dried in vacuo for 3 h. Ether (0.8 mL) and 1.6 mL of buffer (200 mM sodium acetate, 80 mM $CaCl_2$, pH 5.6) were added. The solution was magnetically stirred vigorously for several minutes to give a homogeneous solution. Lyophilized cabbage phospholipase D (2 mg, Boehringer) was added. The mixture was vigorously stirred in a tightly capped tube, and the progress was monitored by TLC on silica ($CHCl_3$/MeOH/H_2O, 66:33:4; R_f DPPA, 0.5; R_f DPPC, 0.3; or with acetic acid/H_2O/$CHCl_3$/acetone/MeOH, 12:7:40:15:13; R_f DPPA, 0.7; R_f DPPC, 0.4). After 5 h, an additional 2 mg of enzyme was added, and the mixture was stirred overnight at room temperature. The ether was removed with a stream of N_2, and 1 mL of 0.5 M EDTA and 1.5 mL of $CHCl_3$/MeOH (2:1) were added. The mixture was vortexed and centrifuged to separate the layers. The lower phase was transferred to a new tube and the aqueous phase was extracted once more with $CHCl_3$/MeOH (2:1). The combined organic phases contained pure ^{3}H-DPPA (specific activity of approximately 5 Ci/mol). This solution was stored at −20 °C.

Synthesis of ^{3}H-DPPE. Unlabeled DPPC (3.0 mg) was mixed with 50 μCi of ^{3}H-DPPC (58 Ci/mmol) in 0.5 mL of $CHCl_3$. The solvent was removed with a stream of N_2, and then the product was dried in vacuo for 3 h. 2-Butanol (50 μL) was added to dissolve the lipids, followed by 0.5 mL of ether and 0.4 mL of buffer (200 mM sodium acetate, 80 mM $CaCl_2$, pH 5.6). A solution of ethanolamine (0.15 mL, 1 M in buffer) was added followed by 2.2 mg of cabbage phospholipase D. The mixture was vigorously stirred for 5 h in a tightly capped tube. The ether was removed with a stream of N_2, and 1 mL of 0.5 M EDTA and 1 mL of $CHCl_3$/MeOH (2:1) were added. The mixture was vortexed and centrifuged to separate the layers. The upper phase was extracted once more with $CHCl_3$/H_2O/MeOH (3:47:48), and the organic layers were combined. Solvent was removed with a stream of N_2, and then the product was dried in vacuo for 1 h. The residue was taken up in a few drops of $CHCl_3$/MeOH (2:1) and applied to a TLC plate (Merck silica, 0.25 mm thick, 20 × 20 cm, activated at 110 °C for 30 min). The plate was developed with $CHCl_3$/MeOH/7 M NH_4OH (65:30:4). The lipids were visualized with iodine vapor, and the region containing the DPPE was scraped from the plate. The product was eluted with $CHCl_3$/MeOH/H_2O (65:30:5) by soaking the silica gel in solvent for 30 min and filtering through glass wool. The filtrate was washed with water, and the layers were separated by centrifugation. The lower phase was dried with a stream of N_2 and then dried in vacuo for 2 h. The residue of pure ^{3}H-DPPE (approximate specific activity 10 Ci/mol) was taken up in $CHCl_3$/MeOH (1:1) and stored at −20 °C.

Standard Procedure for Double-Radiolabeled Substrate Specificity Studies in the Scooting Mode. Unlabeled DMPM (6 mg), tritiated substrate (approximately 4×10^6 cpm) and carbon-14 substrate (approximately 2×10^6 cpm) were mixed together, and a small volume of $CHCl_3$/MeOH (1:1) was added to produce a homogeneous solution. This insured that the lipids will be uniformly distributed in vesicles. The solvent was removed with a stream of N_2 and finally by placement in vacuo for several hours. Distilled water (0.6 mL) was added

and the suspension frozen and sonicated to make small vesicles as described (Jain & Gelb, 1991). The hydrolysis of the vesicles was carried out with 60 μL of this stock solution in 4 mL of 0.6 mM $CaCl_2$ and 1 mM NaCl at 21 °C in a pH-stat as described (Jain & Gelb, 1991). The reaction was started by the addition of PLA2 (typically 0.4 μg). After reaching the desired point in the reaction progress curve (typically at the end of the reaction and at the half-way point), the mixture was transferred to a glass tube and 2 mL of $CHCl_3$/MeOH (2:1) was added followed by 1 mL of $CHCl_3$. The mixture was vortexed and centrifuged to separate the layers. The aqueous layer was extracted two additional times with half as much solvent (1 mL of $CHCl_3$/MeOH, 0.5 mL of $CHCl_3$). The extracts were combined and dried down with a stream of N_2. The residue was taken up in a small volume of low-boiling petroleum ether/ether/acetic acid (70:30:1), and the solution was applied to a small column of silica gel in a glass pasteur pipet. The column was washed with 3–4 mL of the same solvent to completely elute the fatty acids while leaving the phospholipids bound to the silica. The solvent was removed with a stream of N_2, and then the product was dried in vacuo, scintillation fluid was added to the residue, and the radioactivity in the tritium and carbon-14 channels was measured in a scintillation counter.

The fraction of carbon-14 radioactivity appearing in the tritium channel was determined by processing a sample of carbon-14 oleic acid as described above. This fraction was used to correct the measured radioactivity in the two channels in order to obtain the cpm of tritium and carbon-14 in each sample. The radioactivity in a small aliquot of the original substrate stock solution was also determined by scintillation counting, and this number was used to determine the amount of tritium and carbon-14 cpm present in the PLA2 reaction mixture. Background reactions in which enzyme was omitted were processed as described above. This analysis indicated that less than 1% of the total radioactivity present in the sample was present as free fatty acid.

Gas Chromatography Analysis of the Released Fatty Acids. Aliquots from the pH-stat reaction of PLA2 on phospholipid vesicles were subjected to free fatty acid analysis as follows. An aliquot of the reaction mixture was mixed with an equal volume of 0.1 M EDTA and 2 M NaCl, at pH 7.0. A volume of 88% formic acid, equal to 7% of the total aqueous volume, was added. The mixture was extracted with two equal volumes of ethyl acetate (with vortexing and centrifugation to separate the layers). The extracts were combined and concentrated with a stream of N_2. Ether (0.5 mL) and methanol (50 μL) were added to the residue, and a few drops of diazomethane in ether (prepared for 1-methyl-3-nitro-1-nitrosoguanidine, Aldrich) were added to ensure a persistent yellow color. After 30 min, the solutions were concentrated with a stream of N_2, taken up in pentane, and analyzed by capillary gas chromatography.

Enzymatic Hydrolysis of Mixed PA/PC Vesicles. The vesicles of mixed lipids were prepared by evaporating a mixture of their solutions in $CHCl_3$/MeOH (1:1). The dried film was hydrated and then sonicated in a bath-type sonicator as described (Jain & Gelb, 1991). Reaction progress curves for the hydrolysis were monitored on small sonicated vesicles as described in Berg et al. (1991). The contents of the reaction mixtures are given in the figure legends. The reaction was initiated by adding 1–5 μL of an aqueous solution of PLA2 to the vigorously stirred reaction mixture without cavitation (Jain & Gelb, 1991). The progress of the reaction was monitored by pH-state titration with 2 mM NaOH. For some samples, the production of lysophospholipids was analyzed by HPLC. In those cases, the reaction mixture was extracted with an equal volume of $CHCl_3$/MeOH (2:1). An aliquot containing 50–200 μg of the lipid mixture in the organic layer was injected directly for HPLC. The analysis was conducted on a silica gel column (Lichrosorb SI-100 SU, 250 × 0.6 mm, Alltech) according to the procedure described by Christie (1985). The mobile phase was a gradient (20 min) from pure chloroform to a mixture of 92% methanol, 7% of 28% aqueous ammonia, and 1% chloroform. The eluted phospholipids were detected with a mass detector equipped with a linearizer (Varex, Mark II).

The binding of PLA2 to phospholipid vesicles was monitored as a change in the fluorescence of tryptophan 3 of the pig enzyme as described (Jain et al., 1982, 1986b). Typically, a 7 μM PLA2 solution (1.5 mL containing 1 mM $CaCl_2$, 10 mM Tris, pH 8.0) was titrated with a stock solution (20 mg/mL) of vesicles or covesicles until a maximum change in the fluorescence was observed (up to 3-fold at 333 nm, excitation at 292 nm). The change in the intensity was plotted as a function of the lipid concentration as shown in Figure 6 of Jain and Jahagirdar (1985).

Results

Substrate Specificity Studies with PLA2: Theoretical Considerations. The approach we have taken for analyzing the substrate specificity of PLA2 is to study the kinetics of action of the enzyme on covesicles of a mixture of phospholipids in the scooting mode (Jain et al., 1986a; Jain & Berg, 1989; Berg et al., 1991). The rationale for this is given in detail in the appendix to Berg et al. (1991), and the reader is referred to this document in order to get an appreciation for the relevant parameters that must be constrained in the kinetic analysis of PLA2 on vesicles. Briefly, the following conditions must be satisfied: (1) the enzyme must be tightly bound to the vesicles during the course of the lipolysis to avoid complications arising from the differential binding of PLA2 to vesicles of pure phospholipids (described below); (2) there should be an excess of vesicles over enzymes so that there is at most one enzyme per vesicle otherwise vesicles with more enzyme bound will be farther in their reaction progress than those with fewer bound enzymes and an appropriate average based on the statistical distribution would have to be carried out; (3) the intervesicle exchange of lipid substrates and products should be slow on the time scale of the hydrolysis of vesicles, otherwise the rate of lipid exchange could influence the observed substrate specificity; (4) the vesicles should not fuse during the experiment, otherwise the substrates will be replenished by fusion of enzyme-containing vesicles with vesicles that contain no bound enzyme. In short, one would like to study the substrate specificity of PLA2 in the interface rather than the rates of these other processes. Thus these other processes must be eliminated so that they don't contribute to the observed substrate selectivity. It may be noted that the elimination of these parallel kinetic processes does not influence the underlying mechanism for interfacial catalysis (steps shown in the box of Scheme I).

Scooting Mode Hydrolysis of Mixed PA/PC Vesicles. Figure 1A shows the reaction progress curves (product versus time) for the action of the pig PLA2 on vesicles containing various mol % of DOPA in OPPC. All of these experiments were carried out in the presence of 2.6 mM $CaCl_2$. With pure OPPC vesicles, the hydrolysis is barely measurable, whereas in the presence of 4% DOPA the initial rate of hydrolysis increased over 50-fold (Figure 1A). The apparent activation of the hydrolysis of zwitterionic vesicles containing anionic additives was consistent with previous studies with vesicles of

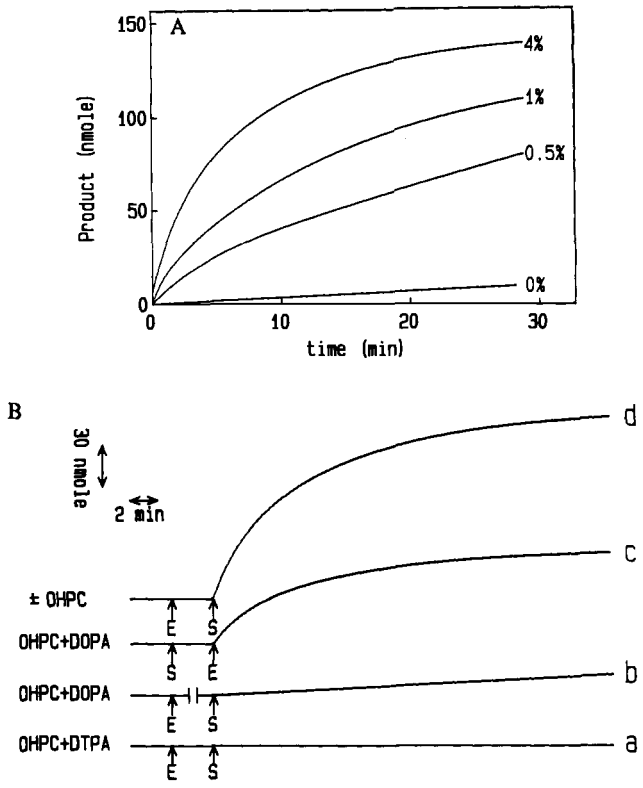


FIGURE 1: (A) Reaction progress curves for the hydrolysis of covesicles of 0.8 mg of OPPC containing varying mol % DOPA (0, 0.5, 1, and 4 mol %) by pig PLA2 (30 pmol) in 4 mL of 2.6 mM $CaCl_2$, 1 mM NaCl, pH 8.0 at 22 °C. (B) Reaction progress curves for the hydrolysis of 96:4 OPPC + DOPA covesicles (donated by S) added last to mixtures of pig PLA2 (denoted by E) (30 pmol) and the following lipids: (curve a) OHPC + DTPA (96:4) covesicles; (curve b) OHPC + DOPA (96:4) covesicles; (curve c) OHPC + DOPA (96:4) and the enzyme was added last; and (curve d) OHPC vesicles. Other conditions are given in the text. The order of addition of components is shown by the arrows in the figure.

PC containing saturated acyl chains in which a prolonged lag was observed before the onset of the steady-state phase of hydrolysis (Upreti & Jain, 1980; Apitz-Castro et al., 1982). The lag was due to the fact that the PLA2 bound weakly to pure PC vesicles. As the anionic fatty acid product together with the lysophospholipid began to build up in the vesicles, more and more of the enzyme bound to the interface, and at the end of the lag phase the proportion of the bound enzyme reached a maximum steady-state value that depended on the variables that control the E to E* equilibrium (Jain et al., 1982, 1989b; Jain & Jahagirdar, 1985). A detailed study of this phenomenon has shown that when the amount of anionic fatty acid product reaches a critical mol %, it began to segregate in the surface of the bilayer (Jain & de Haas, 1981; Jain et al., 1989). This segregation of the anionic charges provides a high-affinity binding site for the enzyme to the vesicles (Yu & Jain, 1989; Jain et al., 1982, 1989), which in turn causes an apparent acceleration in the enzymatic turnover.

As shown in Figure 1A, the shape of the reaction progress curve for OPPC vesicles containing at least 4 mol % DOPA was characteristic of catalysis in the scooting mode. For example, the reaction ceased after about 30 min when only a small fraction of the total available substrate had been hydrolyzed. That the enzyme was not hopping between vesicles was most directly shown by the results in Figure 1B. In these experiments, PLA2 was first added to vesicles of the non-hydrolyzable diether analogues OHPC + DTPA or OHPC + DTPM (96:4). Subsequent addition of substrate vesicles composed of 4 mol % DOPA in OPPC did not result in the formation of products (curve a). If, however, the PLA2 was initially added to pure OHPC vesicles, addition of OPPC + DOPA (96:4) vesicles resulted in immediate hydrolysis (curve d). Similar results were observed when PLA2 was first added to vesicles of OHPC + DOPA (96:4), where the initial proton release is exclusively due to the hydrolysis of DOPA (curve b). However, no further hydrolysis was observed when co-vesicles of DOPA in OPPC were added. In contrast, when the enzyme was added to a mixture of these two covesicles, the reaction started immediately (curve c), although the extent of hydrolysis per enzyme was about half of that observed with the pure substrate vesicles (curve d). These experiments demonstrate that pig PLA2 was bound irreversibly to the mixed PA/PC vesicles. Experiments not shown here also demonstrated that 55–65% of the total substrate present in covesicles was hydrolyzed in the presence of sufficient PLA2 to ensure that every vesicle had at least one bound enzyme. This demonstrated that the integrity of the vesicles was maintained even after all of the substrate in the outer monolayer was hydrolyzed. The fact that the substrate molecules present as excess vesicles were not accessible to the bound PLA2 under the conditions where there was a 6-fold excess of vesicles (as in Figure 1A) ruled out the possibility of intervesicle exchange of the substrate and products. These results are similar to the action of PLA2 on vesicles of pure DMPM (Jain et al., 1986a; Jain & Berg, 1989; Berg et al., 1991).

The progress curves in Figure 1A were obtained with a vesicle to enzyme ratio of more than 5 so that there was at most one enzyme per vesicle. Under these conditions, the moles of product formed at the end of the reaction can be used to calculate the number of phospholipid molecules on the outer layer of the vesicles, N_s. This is because N_s is equal to the moles of product formed at the end of the reaction divided by the moles of PLA2 added [eq A11b, appendix to Berg et al. (1991)]. The values of N_s had a constant value of 4600 ± 300 for small sonicated vesicles of OPPC containing from 4 to 15 mol % DOPA (not shown). This result rules out the possibility that only one of the two substrates became hydrolyzed during the reaction. In the presence of 25 mol % DOPA or higher, there was a slow drift upward in the reaction progress curves that was noticeable toward the end of the reaction. Such a drift was negligible in the presence of less than 6 mM calcium and with vesicles that contained 15 mol % DOPA or less. The drift was most likely due to the slow fusion of vesicles, which provided a slow and continuous supply of fresh substrate. This interpretation was consistent with light-scattering studies (data not shown) in which detectable fusion occurred with vesicles that contained 25 mol % DOPA or more, whereas vesicles containing 15 mol % DOPA or less did not shown an increase in scattering intensity, even after 30 min in the presence or in the absence of PLA2 (data not shown).

The value of N_s obtained from reaction progress curves with 15 mol % DOPA in OPPC remained at 4600 ± 300 as the calcium concentration was increased from 2 to 50 mM (not shown). These results and the results in Figure 1 provided an appreciation of the range of conditions in which the whole reaction progress curve could be monitored without significant contributions from the fusion of vesicles, the exchange of the enzyme, or severe inhibition from the products formed by the hydrolysis of one of the components during the hydrolysis of the second component in the covesicles.

The results so far indicated that PLA2 catalyzed the hydrolysis of mixed phospholipid vesicles without leaving the surface of the bilayer and that the enzyme remained bound

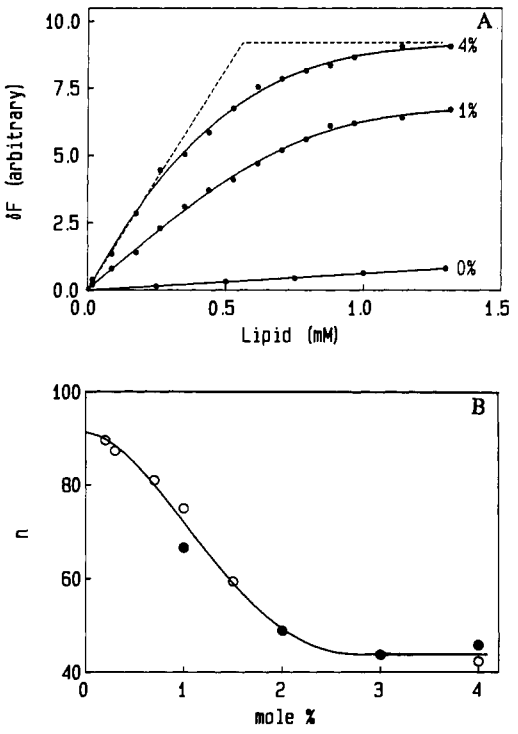


FIGURE 2: (A) Binding curves for pig PLA2 (7.7 μM) with increasing concentration of the covesicles of OHPC containing increasing mol % of DTPM (0, 1, and 4%) at 1.0 mM $CaCl_2$, 10 mM Tris, 22 °C, pH 8.0. The extrapolated lipid/protein (L/P) ratio for the binding of the enzyme (dotted lines) was used for the calculation of n as described in the text. (B) Value of n for the binding of pig PLA2 to OHPC vesicles containing varying mol % of DTPM (open circles) or DOPA (closed circles). Other conditions are as given in the legend to Figure 3.

even after all of the substrate in the outer layer of vesicles had been hydrolyzed. Similar behavior and N_s values were also observed with the PLA2s from *Crotalus atrox*, bee and *Naja naja naja* venoms [data not shown; however, see the fifth paper in this series, Jain et al. (1991b)]. Thus the phenomenon of scooting mode hydrolysis was not restricted to the action of PLA2s on vesicles of pure anionic phospholipids such as DMPM, but it also was observed with PLA2s from several sources on vesicles of zwitterionic lipids as long as a critical amount of anionic compound was present.

Binding of PLA2 to Mixed PA/PC Vesicles. Binding of the pig PLA2 to phospholipid vesicles can be monitored by fluorescence since the intensity of the tryptophan 3 residue was enhanced and was accompanied by a blue shift (from 345 to 333 nm) when the enzyme adsorbed onto the vesicle. This tryptophan residue is located on the surface of the enzyme that contacts the bilayer, and it becomes desolvated following interfacial binding (Jain et al., 1982; Jain & Vaz, 1987; Jain & Berg, 1989). Figure 2A shows that an increase in the fluorescence of a fixed amount of enzyme occurs following the addition of increasing amounts of vesicles. The added lipid consisted of the nonhydrolyzable ether analogue OHPC mixed with various mole fractions of DTPA, DOPA, or DTPM. In the absence of the anionic additive, essentially no binding of PLA2 to the OHPC vesicles was seen (Figure 2A). In the presence of greater than 0.3 mol % DTPM, significant binding of the enzyme was observed. These results provided strong evidence that the low enzymatic activity observed with vesicles of pure OPPC (Figure 1A) was due to the poor affinity of PLA2 to vesicles composed of pure zwitterionic phospholipids. These binding results were further confirmed by the resonance energy transfer method (Jain & Vaz, 1987) where the energy transfer from tryptophan 3 to a dansylated acceptor localized in the vesicles was observed only when OHPC vesicles contained DTPM (data not shown).

For the binding isotherms of type shown in Figure 2A, not only the initial slopes but also the overall change in the fluorescence intensity increased with increasing amounts of anionic lipid present in the vesicles. These binding curves were interpreted (Jain et al., 1982; Jain & Berg, 1989) according to the following equilibrium for the E to E* step:

$$E + IBS \rightleftharpoons E{\cdot}IBS$$

Here, E binds to a hypothetical interfacial binding site, IBS, present on the surface of the target vesicle. This binding is characterized by the dissociation constant K_D. Since the dimensions of E are small compared to typical vesicles, there will be many interfacial binding sites per vesicle. It is assumed that the enzyme molecules bind in an independent manner to all of the interfacial binding sites present in the sample. Furthermore, each interfacial site is composed of n lipid molecules so that the following relationship can be written.

$$[IBS]_T = \frac{[L]_T}{n}$$

Here, $[IBS]_T$ and $[L]_T$ are the total concentrations of interfacial binding sites and monomeric lipid molecules, respectively, present in the sample. Now, K_D can be expressed in the following manner:

$$K_D = \frac{[E][IBS]}{[E{\cdot}IBS]} = \frac{([E]_T - [E{\cdot}IBS])([L]_T/n - [E{\cdot}IBS])}{[E{\cdot}IBS]} \quad (1)$$

Defining $\delta F_{max} = C[E]_T$, where δF is the observed change in fluorescence intensity, δF_{max} is the value of δF at binding saturation, and C is the molar fluorescence change upon binding, one obtains

$$\frac{[E]_T}{[E{\cdot}IBS]} = 1 + \frac{nK_D}{([L]_T - n[E{\cdot}IBS])} = \frac{\delta F_{max}}{\delta F} \quad (2)$$

Here, nK_D is the apparent dissociation constant expressed in terms of the concentration of lipid monomer. The data in Figure 2A can be fitted to eqs 1 and 2 by nonlinear regression to obtain the values of K_D and n if the value of K_D is in the same range or larger than the concentration of the enzyme required for monitoring the signal. The curve-fitting procedures on the binding isotherms with covesicles containing more than 3% DTPM showed significant covariance between the estimates of n and K_D, which meant that the value of K_D is below about 1 μM. It was still possible to obtain an estimate of n by extrapolation of the initial linear region of the curves in Figure 2A to the maximum change in the emission intensity (dotted lines). The simplest interpretation of n is that it is the number of phospholipid molecules required for the binding of each enzyme molecule to the outer surface of the target vesicles. The calculations of n should take into consideration the fact that only the phospholipid molecules on the outer surface of the vesicles are available for the binding of PLA2. Therefore, as shown in Figure 2A, the lipid to enzyme mole ratio of 64 for the maximum change in the fluorescence intensity corresponds to n = 35–40 because in small vesicles only 60% of the phospholipid molecules are on the outer monolayer. As summarized in Figure 2B, the value of n was found to decrease as the mole fraction of the anionic lipid was increased in the vesicles, and above 3 mol % anionic lipid there was little effect on the value of n. In addition, as shown in Figure 2B, essentially identical results were seen when the dianionic lipid DOPA (closed circles) rather than DTPM (open circles) was

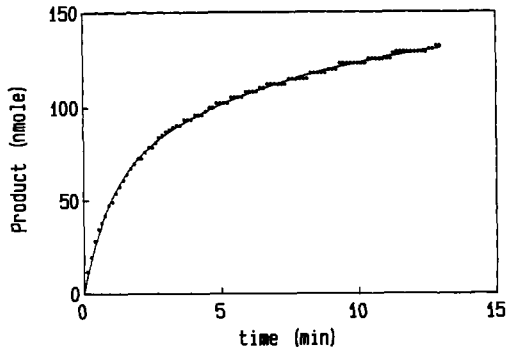


FIGURE 3: Reaction progress curve for the hydrolysis of the covesicles of DOPA + OPPC (15:85), 0.8 mg of total lipid, at 10 mM $CaCl_2$ with pig PLA2 (30 pmol). The theoretical curve for a two-exponential (four-parameter) fit is shown by the smooth line with the following values of the parameters: P_{max}^{PA}, 32 nmol; P_{max}^{PC}, 115 nmol; k_i^{PA}, 1.0 min^{-1}; k_i^{PC}, 0.14 min^{-1}. The standard deviations in these parameters were less than 30%. See the text for discussion.

used as the anionic component. On the basis of the data in Figure 2B, the important factor for binding may be the number of anionic lipids present in the vesicles rather than the total number of anionic charges.

The value of n obtained by a variety of techniques is between 30 and 60 for the micellar (Hille et al., 1983) as well as the bilayer (Jain et al., 1982, 1986b, 1991b) interfaces. As discussed elsewhere (Jain & Berg, 1989), a plausible interpretation is that when PLA2 binds to the interface, about 40 phospholipid molecules become inaccessible for the binding of other enzyme molecules. It may also be noted that the emission intensity of the bound PLA2 increased with increasing mole fraction of the anionic component (Figure 2A). This has not been investigated further, and the underlying mechanism is not clear; however, see Jain et al. (1986b) for a possible explanation.

Analysis of the Reaction Progress Curves For Mixed DOPA/OPPC Vesicles. The time course of the hydrolysis of small sonciated covesicles of OPPC + DOPA was further investigated under the conditions where there was at most one enzyme per vesicle and the fusion of vesicles did not occur on the time scale of the experiment. In previous studies (Jain et al., 1986a; Berg et al., 1991), the action of PLA2 on small DMPM vesicles at low calcium concentration displayed first-order reaction progress curves that can be completely described by the extent of hydrolysis, P_{max}, and a first-order relaxation constant, k_i, according to eqs A13 and A10 from the appendix to Berg et al. (1991).

$$P_t = P_{max}[1 - \exp(-k_i t)] \quad \text{(A13)}$$

$$N_s k_i = \frac{k_{cat}}{K_{MS}(1 + 1/K_p)} \quad \text{(A10)}$$

Here, P_t and P_{max} are the amounts of product formed at time t and infinity, respectively, K_{MS} is the Michaelis constant for the substrate, K_p is the dissociation constant for the lipolysis products, and k_{cat} is the maximal velocity per enzyme [see the appendix to Berg et al. (1991)]. With the mixed vesicles containing 15% DOPA in OPPC, the reaction progress curve could not be fitted to a single exponential (eq A13). A much better fit (Figure 3) was obtained if the curve was fitted to the sum of two exponential terms, one for each of the two substrates present in the covesicle.

$$P(t) = P_{max}^{PA}[1 - \exp(-k_i^{PA} t)] + P_{max}^{PC}[1 - \exp(-k_i^{PC} t)] \quad (3)$$

In eq 3, the PA and PC superscripted parameters refer to the reaction extents and relaxation constants for the PA and PC substrates. The solid line in Figure 3 shows the results of the fit of the observed reaction progress curve to eq 3 obtained by a nonlinear regression analysis with all four variables in the equation allowed to vary. The standard deviations for all four variables were less than 30%. Although the quality of the fit was acceptable, it is unlikely that this model used to fit the reaction progress curve is unique. In general, it is difficult to accurately judge the values of the four independent parameters in eq 4 from such a regression analysis. However, some qualitative features may be noted. The ratio of the amplitudes of the two components was approximately the same as the ratio of the two phospholipids that were present in the reaction mixture, and the hydrolysis of the minor component (DOPA) occurred with a shorter halftime than that for OPPC (Figure 3). From the regression analysis, an approximate ratio of $N_s k_i$ values for DOPA vs OPPC of 2 was obtained. These values for the turnover parameters were consistent with the gas chromatographic analysis of the fatty acids produced at different times in the reaction progress curve. At early points in the reaction progress curve (0.5–1 min), the ratio of oleic acid (from DOPA) to palmitic acid (from OPPC) was found to be 0.5 ± 0.1. Since the initial molar ratio of DOPA to OPPC was 0.18, this result was evidence that the DOPA was preferentially hydrolyzed compared to OPPC (if the ratio of the apparent rate constants for the hydrolysis of the two substrates were the same, the ratio of oleic acid to palmitic acid at early points in the reaction would be 0.18). From these values, it can be estimated that the ratio of k_{cat}/K_{MS} values for DOPA vs OPPC was approximately 3. This value was in agreement with the ratio of $N_s k_i$ values of 3–4 obtained from the regression analysis. At longer reaction times (5–10 min), the ratio of oleic acid to palmitic acid decreased as expected.

In previous studies with DMPM vesicles, a first-order reaction progress curve at low concentrations of calcium was seen (Berg et al., 1991). At higher calcium concentrations, the reaction progress curves had a significant zero-order, or linear, component at the beginning of the reaction that persisted for several minutes. This is because at calcium concentrations above 1 mM, the DMPM vesicles fuse to make larger vesicles. In large vesicles, the mole fraction of substrate will remain close to its initial value of unity for a longer period of time compared to small vesicles, and this accounts for the effect of the vesicle size on the shape of the reaction progress curves (Berg et al., 1991). In the present study, the mixed DOPA/OPPC sonicated vesicles do not fuse even in the presence of 10 mM $CaCl_2$. This is based on the fact that N_s was independent of calcium up to 50 mM and light-scattering studies failed to show an increase in the size of the vesicles with calcium concentrations up to 10 mM (not shown). In these small vesicles, an initial linear portion of the reaction progress curve was not seen (Figure 1) as expected. Thus, a first-order kinetic analysis, i.e., eq 3, was used to fit the data.

Two additional control experiments were also carried out. The idea that the PA was hydrolyzed in preference to the PC in covesicles was further investigated with covesicles containing one ether analogue and one substrate, such as DTPM + DOPC or OHPC + DOPA, where only one component was hydrolyzed. In these experiments, the extent of hydrolysis at the end of the reaction progress was determined in the presence of excess enzyme to ensure that all of the vesicles were acted on. For both mixed vesicles, approximately 60% of the total substrate was hydrolyzed. This demonstrated that there was no significant asymmetric distribution of the substrates between the inner and outer monolayer of the vesicles. This was a useful control as it showed that the relative rates of hydrolysis

of different phospholipids was not complicated by asymmetric distribution of components. This was further confirmed by HPLC analysis of the products formed from the hydrolysis of vesicles in which both substrates were present.

Substrate Specificity Studies Using the Double-Isotope Method. A technically simple and quantitatively more accurate approach for analyzing the substrate specificity of PLA2 in vesicles made use of a double-radiolabel approach in which one substrate was labeled with tritium and the other substrate with carbon-14. Analysis of the amount of tritium and carbon-14 radioactivity in the enzymatically generated product provided a means to measure the ratio of k_{cat}/K_{MS} values for the two substrates. This approach of carrying out a competitive substrate analysis is well known in enzymology (Segel, 1975; Fersht, 1985). As stated briefly above and in detail [appendix to Berg et al. (1991)], relative k_{cat}/K_{MS} values for the interfacial enzymology (steps within the box of Scheme I) are obtained only when the hydrolysis of vesicles was studied in the scooting mode with at most one enzyme per vesicle. Under these conditions, the ratio of k_{cat}/K_{MS} values for two competing substrates was derived as eq A42 in the appendix to Berg et al. (1991):

$$\frac{\ln [1 - N^3(t)/N^3(\infty)]}{\ln [1 - N^{14}(t)/N^{14}(\infty)]} = \frac{(k_{cat}/K_{MS})^3}{(k_{cat}/K_{MS})^{14}} \quad \text{(A42)}$$

Here, $N^3(t)$ is the number of tritiated products produced by the enzyme after time t and $N^3(\infty)$ is the number formed at infinite time after all of the substrate has been hydrolyzed. Like quantities are defined for the ^{14}C product. Equation A42 is valid regardless of the possible presence in the interface of competitive inhibitors such as products [appendix to Berg et al. (1991) and Segel (1975)]. Such species will only modulate the amount of free enzyme present at steady state and will not effect the competition between the two substrates for the hydrolysis by PLA2.

Now all that is needed is to cast eq A42 in a form that makes use of the experimentally determined quantities obtained by the double-radiolabel method. Vesicles of unlabeled DMPM were doped with small amounts of both ^3H-labeled and ^{14}C-labeled substrates. Unlabeled DMPM and the two labeled substrates will all compete with each other for the hydrolysis by PLA2, and the ratio for any pair of substrates is given by eq A42. Since radiometric methods were used to detect the products, the amounts of ^3H and ^{14}C products present at time t will be proportional to the cpm of ^3H and ^{14}C according to

$$\frac{\ln [1 - \text{CPM}^3(t)/\text{CPM}^3(\infty)]}{\ln [1 - \text{CPM}^{14}(t)/\text{CPM}^{14}(\infty)]} = \frac{(k_{cat}/K_{MS})^3}{(k_{cat}/K_{MS})^{14}} \quad (4)$$

Here, the superscripts 3 and 14 refer to ^3H and ^{14}C labels, respectively. The specific activities that link cpm to moles of product cancel out of eq 4.[2] Equation 4 is analogous to the situation for a water-soluble enzyme acting simultaneously on multiple substrates in bulk solution (Segel, 1975; Fersht, 1985). It is for this reason that k_{cat}/K_{MS} is often called the specificity constant for the action of an enzyme on a particular substrate.

Table I: Substrate Specificities of PLA2 Determined by the Double-Radiolabel Approach[a]

radiolabeled substrates	PLA2	$(k_{cat}/K_m)^3/(k_{cat}/K_m)^{14}$
^3H-DPPC vs ^{14}C-DPPC	pig	1.1 ± 0.1[b]
^3H-DPPE vs ^{14}C-DPPC	pig	1.2 ± 0.1
^3H-DPPA vs ^{14}C-DPPC	pig	3.0 ± 0.2
^3H-DPPC vs ^{14}C-PAPC	pig	1.7 ± 0.2
^3H-DPPC vs ^{14}C-DOPC	pig	2.1 ± 0.3
^3H-DPPC vs ^{14}C-DPPC	*Naja naja naja*	1.0 ± 0.1
^3H-DPPE vs ^{14}C-DPPC	*Naja naja naja*	0.9 ■ 0.2
^3H-DPPA vs ^{14}C-DPPC	*Naja naja naja*	0.6 ± 0.2

[a] All studies were carried out in vesicles of DMPM at 21 °C in 4 mL of 0.6 mM $CaCl_2$, 1 mM NaCl, pH 8.0. [b] The values represent the average ± standard deviation from three independent determinations.

As described under Materials and Methods, vesicles of DMPM containing small amounts of ^3H- and ^{14}C-labeled substrates were prepared. The reaction was monitored in a pH-stat at pH 8.0 in the presence of 0.6 mM $CaCl_2$ at 21 °C. At various points along the progress curve, typically at the end and at the half-way point, the reactions were stopped and the amount of ^3H- and ^{14}C-containing fatty acids analyzed. In these experiments, the product being monitored in the pH-stat was mainly unlabeled myristic acid from DMPM; however, by selectively monitoring the amount of radiolabeled fatty acids produced by scintillation counting, the ratio of k_{cat}/K_{MS} for the two radiolabeled substrates can be determined according to eq 4. In order to verify that both radiolabeled substrates were completely hydrolyzed at the end of the pH-stat reaction, the sample was analyzed just after the utilization of titrant ceased as well as 30 min later. The amount of ^3H and ^{14}C formed was the same in both cases. This also demonstrated that fusion of the vesicles or hopping of the enzyme to other vesicles did not occur in the experiment. Such events would have increased the amount of radiolabeled products produced at the longer reaction times. It was also possible to use the initial cpm of ^3H and ^{14}C present in the reaction mixture as a measure of the amount of labeled fatty acids produced when all of the substrates had been hydrolyzed. This applied of course only to those cases where all of the radiolabel was present in the fatty acid at the 2-position of the phospholipid. Initial cpm values would be useful when examining substrates that are hydrolyzed over many hours, rather than minutes.

As a control to check the methodology, DMPM vesicles containing ^3H-DPPC and ^{14}C-DPPC were analyzed as described above. From the ratio of the cpm in the products at both the midpoint and endpoint of the pH-stat titration curve, the ratio of the k_{cat}/K_{MS} values for these two substrates was found to be 1.1 ± 0.1 (Table I). Of course for two substrates of identical chemical structure, a ratio of the k_{cat}/K_{MS} values of unity was expected and this was observed.

By use of the double-label method, the ratio of k_{cat}/K_{MS} for various pairs of phospholipid substrates was determined with the PLA2s from pig pancreas and *Naja naja naja* venom. Both PLA2s have been shown to operate in the scooting mode on DMPM vesicles (Jain et al., 1991b). The results are given in Table I. It can be seen that both PLA2s showed almost no preference for DPPC versus DPPE. The pig PLA2 showed a 3-fold preference for the anionic substrate DPPA over the zwitterionic substrate DPPC, whereas the cobra venom enzyme had a slight preference, 1.7-fold, for zwitterionic lipids. A few measurements were done to compare the effect of changes in the fatty acid composition of the substrate on the hydrolysis

[2] Equation 4 is similar but not identical to the standard equation for heavy-atom competitive isotope effects determined by the same double-label method (O'Leary, 1980). For heavy-atom isotope effects, the ratio of the k_{cat}/K_{MS} values for the two isotopes will be close to unity. In this case, it is valid to consider only the *ratio* of ^3H to ^{14}C label in the product as a function of the reaction progress. Furthermore, the isotope ratios can be determined with a high degree of precision necessary for heavy-atom isotope effect studies. In the present study where the ratio of k_{cat}/K_{MS} values may be much different than unity, it is necessary to determine the *total* amount of ^3H and ^{14}C products formed at various times in the reaction progress. Although not as precise as $^3H/^{14}C$ ratios, total cpm of ^3H and ^{14}C products can be determined with sufficient precision to obtain the ratio of k_{cat}/K_{MS} values for the two substrates.

by the pancreatic PLA2. Here, phospholipids containing unsaturated fatty acids such as arachidonic acid (PAPC) and oleic acid (OOPC) were slightly preferred (2-fold) over DPPC. It is clear from the data in Table I that, at least for the substrates examined in the present study, both enzymes hydrolyze phospholipids with different head-group structures and fatty acid compositions with remarkably small preferences. It is also clear that the enormous (>50-fold) differences seen in the rate of hydrolysis of pure PC vesicles versus the PC in mixed PC/PA vesicles shown in Figure 1 was almost completely due to the differences in binding affinities of the enzyme for these different vesicles. In addition, the 3-fold preference of the pig PLA2 for PA over PC seen in the double-label experiments with DMPM vesicles agreed well with the 2-fold preference seen with the PA/PC covesicles described above.

Discussion

In this series of six papers, the action of PLA2s on phospholipid vesicles in the scooting mode has been studies in detail. Although this processive enzymatic behavior was first noted on vesicles of pure anionic phospholipids (Jain et al., 1986a), the work presented here showed that scooting occurs on covesicles of a number of different types of phospholipids as long as a critical amount of anionic amphiphile was present. This effect was not specific for PA because several other anionic additives promoted the high-affinity binding of PLA2 to the interface, including phosphatidylglycerol and fatty acids [not shown here; however, see Jain et al. (1982, 1989b)]. With vesicles of pure PC, most of the PLA2 is in the aqueous phase, and the enzyme began to favor the interface only after the products of the lipolysis, one of which is anionic, began to accumulate in the vesicle. This resulted in a latency period in the reaction progress (Jain & Berg, 1989). In numerous studies of the action of PLA2s on PC substrate, it was difficult to know whether the reported initial velocities represent the early part of this lag phase or whether they reflected the specific activity of the fully membrane-bound enzyme. The tight association of PLA2 with vesicles composed of predominantly PC reported here casts doubt on other mechanisms that have been suggested for the rate acceleration during the latency period such as enzyme acylation (Cho et al., 1988) and the percolation model (Menasche et al., 1985). In the fifth paper in this series (Jain et al., 1991b), it was also shown that the scooting phenomenon was seen with several different PLA2s, demonstrating that the tight association of these enzymes to interfaces containing anionic phospholipids was a general property of PLA2s. The transition from hopping mode to scooting mode hydrolysis induced by the presence of anionic phospholipid additives also explains the earlier observations from this laboratory on the activating effects of PA on the hydrolysis of PC vesicles by the PLA2 from human platelets (Apitz-Castro et al., 1981). A shift in the E to E* equilibrium is also likely to be the explanation for the activating effects of a number of different anionic phospholipids on the hydrolysis of PC vesicles by a high molecular weight PLA2 from macrophages (Leslie & Channon, 1990).

In this paper the issue of substrate specificity has been examined for the hydrolysis of a variety of phospholipids by PLA2. For any enzyme, what is meant by the phrase substrate specificity? It refers to a comparison of enzymatic velocities on a collection of substrates. In other words, when an enzyme is acting simultaneously on a collection of substrates, which substrates are preferentially converted to their corresponding products? In a mixture of competing substrates, the ratio of enzymatic velocities for any pair of substrates in the mixture is given by eq 5, which is derived in standard texts on enzyme kinetics [for example, Fersht (1985)].

$$\frac{v^1}{v^2} = \frac{(k_{cat}/K_{MS})^1}{(k_{cat}/K_{MS})^2}\frac{[S^1]}{[S^2]} \quad (5)$$

Equation 5 gives the ratio of velocities for conversion of substrate S^1 vs substrate S^2 to products as a function of the ratio of these two substrates present at some point in time in the mixture of multiple substrates. Equation 5 was derived under the assumption that all of the enzyme species present in the solution were in steady state, which is almost always the case. One additional virtue of eq 5 is that it is true even in the presence of inhibitors such as the reaction products or other competitive inhibitors. Such species only reduce the velocities for both substrates by the same factor so that the ratio of velocities remains unchanged. Equation 5 is true for *any pair* of substrates present in a mixture of any number of competing substrates. In many cases, the absolute value of k_{cat}/K_{MS} is not measured for a particular substrates, although it can be obtained from the first-order relaxation constant k_i (Jain & Rogers, 1989). This would involve a measurement of both the k_{cat} and the K_{MS} values for the substrate under study. Rather, the ratio of k_{cat}/K_{MS} values for two different competing substrates is determined by using eq 5. It is this approach that was utilized in the present study on the substrate specificity of PLA2.

In order to apply eq 5 to the case of PLA2 acting on phospholipids, special care must be taken to insure that the results can be interpreted in a meaningful way. This has been discussed in detail in the appendix to Berg et al. (1991) and summarized above under Results. One of the major problems that must be considered is that the enzyme will have different binding affinities to vesicles of pure phospholipids. For example, the pig PLA2 bound weakly to vesicles composed solely of PC lipids (Jain et al., 1982, 1986b), whereas the enzyme bound essentially irreversibly to vesicles of the anionic phospholipid DMPM (Jain et al., 1986a). As a result, the initial velocity for the hydrolysis of pure PC vesicles was extremely low, and the reaction was activated more than 50-fold when small amounts of anionic additives, such as PA, were added (Figure 1). This activation by PA was due to an increase in the binding of the enzyme to the interface (Figure 2). When the amount of PA in the PC vesicles exceeded about 4 mol %, the enzyme operated in the scooting mode in which all of the substrate in the outer monolayer of the vesicles became hydrolyzed and the enzyme remained tightly bound to the surface. Thus the phenomenon of scooting, which was originally described in vesicles composed solely of DMPM (Jain et al., 1986a), was also observed in vesicles of PC containing anionic additives such as PA.

When the enzyme hydrolyzed mixed DOPA/OPPC vesicles in the scooting mode (Figure 4), quantitation of the amounts of oleic (from DOPA) and palmitic acid (from OPPC) by gas chromatography showed that both phospholipids were hydrolyzed at comparable rates. If the enzymatic reaction was sampled at early times in the progress curve, the ratio of products formed and the ratio of DOPA to OPPC initially present in the vesicles was used to estimate the ratio of k_{cat}/K_{MS} values for the two substrates according to eq 5. Such an analysis showed that there was a slight preference for the hydrolysis of DOPA vs OPPC (ratio of k_{cat}/K_{MS} values of 3). Thus it is concluded that the greater than 50-fold difference in the initial velocities for the hydrolysis of PC vesicles vs PC/PA covesicles was due almost entirely to differences in the amount of bound PLA2 with the two different vesicles substrates. When the enzyme was confined to a PA/PC co-

vesicle and was allowed to "choose" between the two substrates in a competitive fashion, one found that the difference in substrate preference was only 3-fold in favor of PA. Further studies showed that ethanolamine- and choline-containing lipids were hydrolyzed at similar rates by both the pig and cobra venom enzymes and that the presence of unsaturated fatty acids in the substrate did not produce a dramatic effect on the selectivity.

A second approach was developed using a double-radiolabeling methodology to determine the ratio of k_{cat}/K_{MS} values for a pair of substrates. In this procedure two phospholipid species, labeled in the *sn*-2 fatty acid with either ^3H or ^{14}C, were added in small quantities (typically 0.1 mol % or less) to vesicles of unlabeled DMPM. PLA2 hydrolyzed these vesicles in the scooting mode, and all three substrates competed in the reaction with the enzyme. The ratio of k_{cat}/K_{MS} values for the two labeled substrates was computed by measuring the amount of ^3H and ^{14}C radioactivity in the fatty acid products at the end of the reaction and at the half-way point according to eq 4. This method was technically simple to carry out and was much more accurate and sensitive than either the curve-fitting process used for the PC/PA covesicles (Figure 3) or the gas chromatography analysis of these covesicles at early reaction times. With the double-radiolabel method, the ratio of k_{cat}/K_{MS} values for DPPA to DPPC was found to be 3. This value is similar to that obtained for DOPA vs OPPC in the PA/PC covesicles and again showed that the enzyme had only a slight preference for PA over PC. This was in marked contrast to the differences in initial velocities seen with pure PC vesicles compared to PC/PA covesicles (Figure 1). It is also interesting to note that this 3-fold preference for PA vs PC was the same whether the enzyme was operating on vesicles composed of 15% PA in PC or on vesicles of mainly DMPM. The important point is that the enzyme was completely bound to the interface in both cases. These studies led to the following conclusion. *Initial velocity studies with PLA2 on vesicles composed of pure phospholipids are more a measure of the differences in binding affinities of the enzyme to the different vesicles than they are of the true substrate specificity of the enzyme. In order to obtain meaningful substrate specificity information about PLA2, one must carry out a competitive analysis in which the enzyme has the opportunity to hydrolyze a mixture of phospholipids while remaining tightly bound to the interface. In this way the relative* k_{cat}/K_{MS} *values for a collection of substrates can be determined.*

In the mixed DOPA/OPPC vesicles, it is possible that there is lateral phase separation of the two phospholipids and this may have an effect on the substrate specificity. However, it is unlikely that DOPA phase separates in the OPPC matrix since phospholipids with unsaturated fatty acyl chains almost always form uniform mixtures (Jain, 1983). The results in Table I indicate that the pig and cobra PLA2s show only modest preferences for the different phospholipids examined. These observations are consistent with recent heavy-atom isotope effect studies (Ghomashchi et al., 1991) showing that the interfacial k_{cat}/K_{MS} for the hydrolysis of DMPM vesicles is given mainly by the rate constant for the binding of a phospholipid to the active site of PLA2, i.e., k_1 of Scheme I. This rate constant describes the process of dislodging the substrate from the plane of the lipid bilayer into the active site cavity of the vesicle-bound enzyme. Since this is a physical step, values of k_1 may be similar for the substrates of similar hydrophobicity that were examined in this study (Table I). Although the ease of substrate removal from the bilayer may be a major factor in controlling the substrate specificity in vesicles, it cannot be the only factor. This is because the preference for DPPA vs DPPC substrates displayed a reversal when different PLA2s were used (3-fold for pig vs 0.6-fold for cobra).

The methods developed in this paper for the analysis of substrate specificity for PLA2 can be compared to previous studies. For the purpose of discussion, it was convenient to group these other approaches into eight categories that can be individually addressed.

(*i*) *Studies with Vesicles without Normalization of the Amount of Bound Enzyme.* Numerous reports in which the relative activity of the PLA2 was measured on vesicles composed of pure phospholipid classes have been carried out [for example, Ballou et al. (1986), Leslie et al. (1988) and Mizushima et al. (1989)]. These studies are difficult to interpret because they do not control for the amount of enzyme bound to the vesicles.

(*ii*) *Studies with Vesicles under Apparent* V_{max} *Conditions.* A better approach is to measure the initial enzymatic velocity as a function of increasing vesicle concentration. An apparent V_{max} will be obtained when all of the enzyme is bound to the vesicles. This may not be the true maximal velocity for a particular substrate, i.e., when all enzyme is saturated in its catalytic site with substrate, but rather the velocity observed when the mole fraction of substrate in the vesicle is unity. In this way a comparison of apparent V_{max} values for different substrates can be obtained [for example, Lister et al. (1989)]. However, there are some problems with this approach. First, in some cases, for example the pig PLA2 acting on pure PC vesicles, the binding of the enzyme is so weak that it is impossible to estimate the apparent V_{max} for the hydrolysis with experimentally obtainable bulk concentrations of vesicles (Jain et al., 1982, 1986b). Second, one is most interested in determining the relative k_{cat}/K_{MS} values for a series of substrates rather than relative k_{cat} values since, as described above (eq 5), only the former parameter contains the relevant information about substrate specificity. Although apparent K_{MS} values were usually determined by measuring initial velocities as a function of the concentration of vesicles, this constant is a measure of the binding affinity of PLA2 to the substrate vesicle under study and is not the same as the interfacial K_{MS} used in eq 5 for the interaction of the substrate with the enzyme in the interface. Third, even when all the enzyme is bound, the apparent V_{max} corresponding to the initial velocity at a mole fraction of substrate of unity will be obtained. Since the interfacial K_{MS} values will in general be different for different substrates, the enzyme in the interface may not be saturated with substrate in its catalytic site for all substrates being examined.

(*iii*) *Studies with Vesicles Containing Optimal Amounts of Additives.* In many cases where the binding of PLA2 to PC vesicles is weak, the E to E* equilibrium can be made more favorable by including additives such as alkanols (Jain & Cordes, 1973; Apitz et al., 1980). The optimal amounts of additives required for a given system depend on the structures of the additive and phospholipid as well as on the source of the PLA2. Under these conditions at optimal mole fractions of additives, a large bulk concentration of substrate is often not needed to obtain an apparent V_{max} by shifting the E to E* in favor of E*. Again, as described in (ii) above, this method gives apparent V_{max} values at or near a mole fraction of substrate equal to 1 rather than relative k_{cat}/K_{MS}.

(*iv*) *Studies with Phospholipids Dispersed in Detergent Micelles.* Numerous studies on the substrate specificity of

PLA2 in mixed micelles in which the phospholipid substrate was dispersed in detergent micelles have been carried out [for example, Roberts et al. (1978)]. In one study, the action of the PLA2 from *Naja naja naja* venom on mixtures of egg PC and egg PE in Triton X-100 micelles indicated that the PE substrate was hydrolyzed in slight preference to the PC substrate (Roberts et al., 1979). This result is consistent with the similar rates of hydrolysis of DPPE vs DPPC seen in the study (Table I). The activity of the pig PLA2 was studied with a variety of mixed micelle substrates in which the surface charge of the particle was altered by using differently charged phospholipids and/or detergents (Volwerk et al., 1986). In general it was found that much higher activity was seen with mixed micelles that were predominantly negatively charged, and it was suggested that the pig PLA2 had a preference for binding to negatively charged interfaces. The activation of the pig PLA2 acting on Triton X-100 mixed micelle substrate by the addition of anionic additives such as oleic acid or sodium dodecyl sulfate has been attributed to the requirement for a negatively charged surface for binding of the pig PLA2 (Pluckthun & Dennis, 1985). Again, because the relative enzymatic velocities are distorted by the differential binding of PLA2 to the mixed micelles, the essence of substrate specificity (eq 5) is lost. In addition, a second concern must be addressed in studies employing mixed micelles. In any attempt to obtain relative k_{cat}/K_{MS} values by using a steady-state kinetic treatment in mixed micelles, it must be assumed that the exchange of compounds (substrates, products, and enzyme) among the ensemble of mixed micelles was fast on the time scale of the depletion of substrate in a single mixed micelle (of the order of 40 ms). Only in this limit will the distribution of all of the components in the particles be in equilibrium, will all enzymes "see" a similar environment during the course of the reaction, and will the rate of the reaction not be modulated by relative rates of intermicelle exchange of different phospholipids. This has been discussed more fully in the discussion and the appendix in Berg et al. (1991) and in Jain et al. (1991c). In general, it will be difficult to validate this fast exchange limit for every PLA2 substrate under study. There is limited data on the rates of exchange of phospholipids in various detergent micelles. However, in a recent study (Fullington, 1990), the half-times for the equilibration of long-chain phospholipids such as fluorescently labeled DPPE among the ensemble of mixed phospholipid/detergent micelles were found to be in the range of 0.2–40 s with concentrations of components similar to those used in PLA2 kinetic studies on mixed micelles. This rate was sufficiently slow that it could influence the rate of enzymatic turnover of mixed micelle substrates. If this is true, different velocities measured with different substrates in mixed micelles could be more of a reflection of the differences in intermicelle exchange between classes of phospholipid substrates rather than differences in k_{cat}/K_{MS} values for these molecules.

A second problem with mixed micelles is that it is not always clear whether the detergent functions as a neutral diluant or whether it has an affinity for the enzyme and therefore functions as an inhibitor. Some PLA2s [for example, Lister et al. (1989)] were strongly inhibited by detergents such as Triton X-100. Slootboom et al. (1976) studied the action of PLA2 in mixed micelles under conditions where the "quality of the interface" was maintained nearly constant. This meant that the total amount of phospholipid in the mixed micelles was held constant so that the fraction of enzyme bound to the interface would not change. Thus, the surface concentration of one substrate was varied by changing its mole fraction at the expense of a second substrate, both dispersed in Triton X-100. However, even when the amount of bound enzyme is controlled, meaningful results in mixed micelles will only be obtained if the rate of substrate replenishment was not a controlling factor.

(*v*) *Studies with Pure Phospholipid Micelles.* Relative velocities for the hydrolysis of a variety of short-chain phospholipid micelles in the absence of detergents have been carried out [for example, de Haas et al. (1971) and van Oort et al. (1985)]. In some of these studies, the bulk concentration of micelles was sufficiently high to ensure that all enzyme was bound to the interface. Even so, as with mixed micelles, it is still possible that the rate of substrate replenishment may limit the overall enzymatic turnover.

(*vi*) *Studies with Monomolecularly Dispersed Short-Chain Phospholipids.* In theory, some information about substrate specificity for PLA2 could be obtained by examining the kinetic properties of short-chain substrates in water below their critical micelle concentrations. However, with many substrates, anomalous results were seen due to the formation of enzyme–lipid microaggregates. For example, the pig PLA2 formed microaggregates with certain anionic substrates (van Oort et al., 1985a, 1985b; Jain & Berg, 1989), anomalous kinetics were seen with the same enzyme operating on certain PC substrates (Volwerk et al., 1979), and many of the venom PLA2s form aggregates with PC substrates (Yuan et al., 1990; Bukowski & Teller, 1986; Plukthun & Dennis, 1985; van Eijk et al., 1983).

(*vii*) *Studies on Mixed Phospholipid Covesicles in the Hopping Mode.* The action of PLA2 on covesicles of two different zwitterionic phospholipids has been described in a few cases [for example, Hazen et al. (1990) and Schalkwijk et al. (1990)]. The advantage of this method is that regardless of what fraction of the enzyme was bound to the interface, the enzyme would "choose" between the two substrates according to the relative k_{cat}/K_{MS} values as given by eq 5. Thus, the effects of differential binding of enzyme to vesicles of different pure phospholipids will not appear. Although this approach is seductively close to an ideal solution to the problem, it is not free complicating factors. In these studies, it was likely that the enzyme was not tightly bound to the interface and was able to "hop" between vesicles. This situation may be problematic as discussed in the appendix to Berg et al. (1991). If the rate of intervesicle exchange of enzyme was not rapid, different enzyme molecules will be bound to vesicles of different composition. For example, one enzyme may be operating on a vesicle that was nearly completely hydrolyzed, while a different enzyme may be functioning in a vesicle that has had no enzyme previously and is therefore composed mainly of nonhydrolyzed substrate. Thus, as time goes on, each enzyme "sees" a different environment, and this introduces new components in the reaction progress that are determined by the intervesicle enzyme exchange rate and not by the catalytic rate constants for the action of the enzyme within the interface (eq 5). This is analogous to the situation that would arise in enzyme systems operating in water if the mixing of all components was not rapid on the time scale needed for a significant change in the percent of substrate converted to product [appendix to Berg et al. (1991)]. To quantitatively analyze this situation, it is necessary to carry out a statistical averaging of the various vesicles that have been hydrolyzed to different extents. Such an averaging would not be straightforward to carry out since the distribution of enzymes on the vesicles would not be random. Rather, the enzymes will tend to accumulate on vesicles containing the

highest mol % of products since the E to E* equilibrium is shifted toward E* as the interface becomes anionic. In theory, this problem can be minimized by allowing the reaction to proceed for a short while so that the composition of the vesicles does not change significantly. Under these conditions, regardless of to which vesicle the enzyme is bound, all enzymes will be in a similar environment. However, it is difficult to verify that this is indeed the case. For example, the reaction may be allowed to proceed until the amount of total product formed was only a few percent of the total phospholipid present in the solution. At this point it is possible that most of the vesicles have been "nibbled on" by enzymes that are operating in a fast hopping mode with each vesicle containing a few mol % of product. Alternatively, it may be the case that most of the product is present in relatively few vesicles that have been acted upon in a processive fashion by enzymes operating in a slow hopping mode. Thus, even under the condition of limited hydrolysis, the enzymes may separate into different vesicle environments, and this would greatly complicate the analysis. It is important to note also that these complications from hopping mode catalysis still occur when the bulk concentration of substrate vesicles is high enough to ensure that all enzymes are bound. Even when all enzymes are in the E* form, they will segregate into different environments if hopping occurs. This complicating situation can be eliminated by analysis of mixed phospholipid vesicles in the scooting mode as described in the present study. In this way all of the enzyme-containing vesicles will be hydrolyzed to the same extent, and all enzymes will be in a common environment at all points in time in the reaction progress. In this case, no statistical averaging is required since the overall product formation is given simply by the sum of the products from each of the enzyme-containing vesicles (eq A42).

(viii) Studies in the Scooting Mode on Vesicles of Pure Anionic Phospholipids. In an initial attempt to resolve some of the above-mentioned problems, Jain and co-workers measured the rates of hydrolysis of vesicles of different anionic phospholipids (Jain & Rogers, 1989). In this case, the enzyme was operating in the scooting mode, and the amount of bound enzyme was therefore normalized. Furthermore, with vesicles composed of several thousand phospholipid molecules, in contrast to micelles of only 10–100 molecules, and it was possible to measure the rate of the enzymatic reaction without the need for substrate replenishment. The relative velocities seen in the scooting mode with anionic vesicles were very similar to those reported here. One disadvantage of this previous method is that one is limited to the study of anionic phospholipids. *The use of the double-radiolabel approach allows the specificity of PLA2 toward any two phospholipids to be studied in a scooting matrix.*

To recapitulate, the analysis of substrate specificity of PLA2 by competitive substrate hydrolysis in the scooting mode allows the relative k_{cat}/K_{MS} values for various substrates to be determined. These values are all that is needed to fully described the substrate specificity according to eq 5. By use of covesicles of different phospholipids, the problems associated with different binding affinities of PLA2 to vesicles of different pure phospholipids are avoided. An additional and equally important requirement is that the PLA2 must scoot on the vesicles in order to avoid the problems of a statistical distribution of particles of differing reaction extents.

Finally, the question of the physiological importance of scooting for the breakdown of phospholipids in cells may be raised. Since the mechanism(s) for triggering the production of free arachidonic acid in cells is (are) not yet understood, one can only speculate. One possibility based on the studies presented in this paper is that the stimulation of inflammatory cells leads to an accumulation of anionic phospholipids, which may provide a high-affinity binding site for the attachment of PLA2 to the membrane. The production of phosphatidic acid, for example, occurs during the phospholipase C catalyzed breakdown of inositol lipids followed by phosphorylation of the generated diacylglycerol. The issue of membrane anchoring of the PLA2 may be irrelevant if the enzyme involved was an integral membrane protein regulated by components of the transmembrane signaling machinery (Burch, 1990). *Regardless of how the PLA2 gets to the membrane, the process of substrate selection will necessarily occur when the enzyme is bound to the interface.* Thus, factors that modulate the amount of membrane-bound enzyme in the cell, for example, calcium fluxes (Channon & Leslie, 1990), will serve to control the overall rate of phospholipid breakdown. The selectivity of the lipolysis will be a manifestation of the relevant kinetic parameters for the enzyme toward the various substrates involved. Thus, the measurement of relative k_{cat}/K_{MS} values for substrates using the scooting mode protocol described in this paper provides a convenient and generally applicable method to quantitate the substrate specificity of PLA2 in membranes.

The arguments presented in this paper are generally applicable to all enzymes that operate in membranes. Difficulties due to the "quality of the interface" in assessing the substrate specificity of other enzymes, for example, phosphatidylinositol-specific phospholipase C (Ryu et al., 1987; Wilson et al., 1984) have already been noted. Problems with mixed micelles discussed above apply to numerous studies on enzymes that operate on lipids that have been studied in detergents. In the case of PLA2, a special situation arises in which the entire outer monolayer of the vesicles can be hydrolyzed without loss of the physical structure of the vesicle. Although this is unlikely to be the case for all lipolytic enzymes, one could imagine carrying out competitive substrate analyses in which the competing substrates were simultaneously acted on when present in a matrix that was inert to the action of the enzyme under study. Meaningful information will be gained if care is taken to ensure that the enzyme is scooting on the interface.

Registry No. DPPC, 63-89-8; DPPE, 923-61-5; DPPA, 7091-44-3; PAPC, 35418-58-7; DOPC, 4235-95-4; DMPM, 60569-01-9; PLA_2, 9001-84-7.

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