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Kinetic Characterization of Phospholipase A_2 Modified by Manoalogue†

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ABSTRACT: Manoalogue, a synthetic analogue of the sea sponge-derived manoalide, has been previously shown to partially inactivate the phospholipase A_2 from cobra venom (Reynolds, L. J., Morgan, B. P., Hite, E. D., Mihelich, E. D., & Dennis, E. A. (1988) *J. Am. Chem. Soc. 110*, 5172) by reacting with enzyme lysine residues. In the present study, the inactivation of the phospholipases A_2 from pig pancreas, bee venom, and cobra (*Naja naja naja*) venom by manoalogue was studied in detail. Manoalogue-treated enzymes were examined in the scooting mode on vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol. Here, the native enzymes bound irreversibly to the vesicles and hydrolyzed all of the phospholipids in the outer monolayer without leaving the surface of the interface. All three manoalogue-treated enzymes showed reduced catalytic turnover for substrate hydrolysis in the scooting mode, and the modified enzymes did not hop from one vesicle to another. Thus, inactivation by manoalogue is not due to the decrease in the fraction of enzyme bound to the substrate interface. This result was also confirmed by fluorescence studies that directly monitored the binding of phospolipase A_2 to vesicles. A chemically modified form of the pig pancreatic phospholipase A_2 in which all of the lysine ϵ-amino groups have been amidinated was not inactivated by manoalogue, indicating that the modification of lysine residues and not the amino-terminus is required for the inactivation. Several studies indicated that the manoalogue-modified enzymes contain a functional active site. For example, studies that monitored the protection by ligands of the active site from attack by an alkylating agent showed that manoalogue-modified pig phospholipase A_2 was capable of binding calcium, a substrate analogue, lipolysis products, and a competitive inhibitor. Furthermore, relative to native enzymes, manoalogue-modified enzymes retained significantly higher catalytic activities when acting on water-soluble substrates than when acting on vesicles in the scooting mode. Intact manoalogue had no affinity for the catalytic site on the enzyme as it did not inhibit the enzyme in the scooting mode and it did not protect the active site from alkylation. Pig pancreatic phospholipase A_2 bound to micelles of 2-hexadecyl-*sn*-glycero-3-phosphocholine was resistant to inactivation by manoalogue, suggesting that the modification of lysine residues on the interfacial recognition surface of the enzyme was required for inactivation. Previous fluorescence studies have shown that the phospholipid interface in contact with the bound enzyme becomes desolvated and this process may be required for efficient interfacial catalysis in the scooting mode. In the case of the manoalogue-modified pig phospholipase A_2, fluorescence studies indicated that the binding of the enzyme to the substrate interface was not accompanied by desolvation of the microinterface in contact with the bound enzyme. Certain properties of manoalogue-modified phospholipase A_2 are reminiscent of the proenzyme form of the pig pancreatic enzyme in that they both have significant catalytic activities when acting on soluble substrates and both bind to negatively charged vesicles but in a catalytically nonproductive manner.

Manoalide is a marine natural product first isolated from a sea sponge *Luffariella variabilis* and shown to have antibacterial activity (de Silva & Scheuer, 1980) (Scheme I). Later studies showed that this terpenoid had analgesic and antiinflammatory properties and suggested that the compound

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[1] Abbreviations: AMPA, derivative of pig pancreatic phospholipase A_2 in which all of the lysine residues have been converted to amidines; diC_6-thioPM, 2,3-bis(hexanoylthio)propylphosphomethanol lithium salt; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol lithium salt; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol lithium salt; HDNS, dansylated hexadecylphosphoethanolamine; 2H-GPC, 2-hexadecyl-*sn*-glycero-3-phosphocholine; MJ33, 1-hexadecyl-3,3,3-trifluoroethyl-*sn*-glycero-2-phosphomethanol; PLA2, phospholipase A_2.

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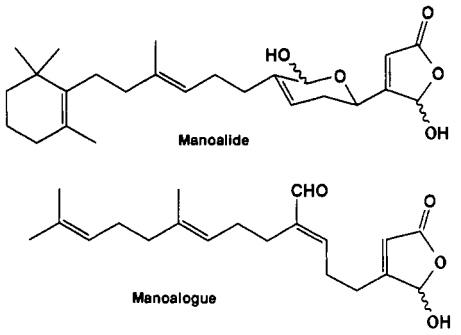
Scheme I



blocked the production of eicosanoids prior to the action of cyclooxygenase on arachidonic acid (Burley et al., 1982; Blankemeier & Jacobs, 1983; Jacobs et al., 1985). Since the production of arachidonic acid in cells is thought to require the action of phospholipase A_2 (PLA2),[1] a possible inhibition of this enzyme by manoalide was hypothesized. Indeed, studies by Jacobs and co-workers showed that manoalide was able to inhibit the action of bee venom PLA2 in vitro and to suppress the neurotoxicity of β-bungarotoxin (De Freitas et al., 1984). Later studies on the inhibition of PLA2 were reported (Lombardo & Dennis, 1985; Bennett et al., 1987; Deems et al., 1987).

Inhibition of PLA2 by manoalide is time-dependent and long-lasting, suggesting a covalent modification of the enzyme. Amino acid analysis of the manoalide-modified cobra venom PLA2 (Lombardo & Dennis, 1985) and protection from enzyme inactivation by exogenous lysine (Bennett et al., 1987) suggested that lysine residues were being modified, probably by nucleophilic addition of an amino group to one or more of the carbonyl groups present in the inactivator. Synthetic analogues of manoalide were prepared in order to define the pharmacophore for the inhibition of PLA2 (Reynolds et al., 1988; Glaser et al., 1989). Inhibition studies indicated that hydrocarbons containing only the 4-hydroxy-2-butenolide ring were inhibitory but the loss of activity was reversible (Deems et al., 1987). A compound termed "manoalogue" was prepared that contained the α,β-unsaturated aldehyde functional group of manoalide's hemiacetal ring in addition to a butenolide-containing hydrocarbon (Reynolds et al., 1988) (Scheme I). This compound was found to produce a time-dependent irreversible loss of PLA2 activity similar to that of manoalide. Reduction of the α,β-unsaturated aldehyde to the alcohol or methylation of the 4-hydroxy group of the butenolide ring produced compounds that were no longer irreversible PLA2 inactivators. These results suggested that both aldehydes present in manoalide (open form) are required for irreversible addition; however, the exact structure of the adduct remains to be elucidated. Reynolds et al. (1988) have shown that manoalogue modified 3–4 lysine residues in the cobra venom (*Naja naja naja*) enzyme and one of these is probably lysine 6. It is not yet known whether the active site or the interfacial binding site is being modified. Manoalide and manoalogue induce a partial loss of PLA2 activity and the degree of inactivation depends on the source of enzyme. Treatment of 25 nM bee venom PLA2 with 30 nM manoalide resulted in a nearly complete loss of enzymatic activity (Glaser et al., 1989), whereas the enzyme from cobra venom showed a maximum degree of inhibition of 50% even with micromolar concentrations of inactivator (Lombardo & Dennis, 1985; Reynolds et al., 1988).

It is well known that for interfacial catalysis PLA2 must first bind to the phospholipid interface in a step that is distinct from the binding of a single phospholipid molecule to the active site of the enzyme (Verger & de Haas, 1976; Dennis, 1983; Jain & Berg, 1989). Most, if not all, of the PLA2s bind several orders of magnitude more tightly to interfaces composed of anionic rather than zwitterionic phospholipids (Jain et al., 1982, 1986a–d; Volwerk et al., 1986; Jain et al., 1991b). Fluorescence binding studies have shown that when the pig pancreatic PLA2 binds to the vesicles, the region of the interface that contacts the enzyme becomes inaccessible to the bulk aqueous phase (Jain & Vaz, 1987). The tight binding of PLA2 to vesicles composed of pure anionic vesicles such as DMPM (Jain et al., 1986a) or covesicles of phosphatidylcholine containing 5–20 mol % phosphatidic acid (Ghomashchi et al., 1991) allows the enzyme to operate in the scooting mode (Jain & Berg, 1989). Here, the bound enzyme undergoes several thousand catalytic cycles without leaving the surface of the vesicle. This tight enzyme–vesicle association can be disrupted by increasing the salt concentration in the buffer, which suggests that electrostatic forces play a major role in interfacial binding (Jain et al., 1986b). Since a number of lysine residues are commonly found on the interfacial binding surfaces of numerous PLA2s (Scott et al., 1990a,b; White et al., 1990; Renetseder et al., 1985; Ramirez & Jain, 1991), it is possible that these residues play a role in the interfacial anchoring. This raises the possibility that the addition of manoalide or its analogues to one or more key lysine residues on PLA2 may interfere with the binding of the enzyme to the interface. Yet a second possibility is that the modified enzyme is still capable of interfacial attachment but that its turnover within the interface is somehow "crippled" either because of a defective catalytic site or perhaps because the interaction between the enzyme and the interface occurs in an abortive manner. These aspects of the inhibition at interfaces have not been previously addressed. The study of PLA2 acting on vesicle substrates in the scooting mode provides a powerful method to distinguish interfacial and catalytic site binding and to probe the nature of the enzyme–interface interaction. Thus, an examination of manoalogue-inhibited PLA2 acting in the scooting mode seemed warranted and is the topic of this study.

Materials and Methods

Materials. Manoalogue was synthesized as described previously (Reynolds et al., 1988). Stock solutions were prepared in dimethyl sulfoxide and stored indefinitely at −20 °C since TLC analysis showed no degradation after several months. Porcine pancreatic PLA2 and its proenzyme were purified as described by Niewenhuizen et al. (1974). The cobra venom enzyme was purified as described (Hazlett & Dennis, 1985) from *Naja naja naja* venom (Pakistan variety, Miami Serpentarium, Salt Lake City, UT). Bee venom PLA2 was obtained from Boehringer. AMPA was obtained as a generous gift from Dr. A. J. Slotboom (State University of Utrecht). The concentration of the pig enzyme was estimated from the absorbances at 280 nm by using an $E_{1\%}$ of 13 (Niewenhuizen et al., 1974). The $E_{1\%}$ values for the cobra and bee enzymes were estimated to be 23 and 13, respectively, by using the amino acid composition data (van den Berg et al., 1989) and the standard absorbance values at 280 nm for Trp (4.2), Tyr (1.8), and Phe (0.4). The following compounds were synthesized as described: DMPM (Jain & Gelb, 1991); DTPM (Jain et al., 1986a); 2H-GPC (Ries, 1989); diC$_6$-thioPM (Yuan et al., 1990); HDNS (Jain & Vaz, 1987); MJ33 (to be published). ω-Bromo-4-nitroacetophenone was from Sigma.

Preparation of Manoalogue-Inactivated Enzymes. Large amounts of manoalogue-inactivated PLA2s for spectroscopic

and protection from alkylation studies were prepared as follows. PLA2 (about 1 mg) was dissolved in 5 mL of 10 mM Tris-HCl, pH 8.0. Manoalogue was added from a stock solution in dimethyl sulfoxide to give the desired final concentration. The mixture was left to stand at room temperature. The exact conditions for each experiment are given in the figure legends. Inactivated enzyme solutions were concentrated to a final volume of about 0.3 mL with a Centricon 10 ultrafiltration device (Amicon). Solutions of inactivated enzymes were used immediately or stored frozen (−20 °C) for future use.

Fluorescence Binding Studies. Binding of modified PLA2s to phospholipid vesicles was studied by monitoring either the resonance energy transfer from tryptophan residues on the PLA2s to the fluorescent probe HDNS present in the vesicles or by directly monitoring the fluorescence emission of the HDNS probe without energy transfer (Jain & Vaz, 1987). All measurements were carried out on an SLM 4800S spectrofluorimeter. Solutions contained vesicles of DTPM (0.2 mg) and 4 μg of HDNS in 1.5 mL of 10 mM Tris-HCl, 0.5 mM $CaCl_2$, pH 8.0. Excitation was at 280 or 332 nm, and emission was at 450 to 550 nm, and the slit widths were 4 nm for both. The fluorescence of the solution was monitored following the addition of increasing amounts of native or manoalogue-treated PLA2s.

Miscellaneous Procedures. Kinetic analysis of PLA2 in the scooting mode on DMPM vesicles was carried out as detailed previously using the pH-stat method (Jain et al., 1986a; Jain & Gelb, 1991). Analysis of the competitive inhibitor MJ33 was carried out with large DMPM vesicles in the presence of 2.5 mM $CaCl_2$ as described (Jain et al., 1989, 1991a). HPLC analysis of modified PLA2s was conducted on a C4 reverse-phase column (Vydac 214TP54 Protein C4) with a solvent system gradient as follows: 98% solvent A (0.06% trifluoroacetic acid in water) plus 2% solvent B (80% acetonitrile, 20% of 0.05% trifluoroacetic acid in water) to 62% solvent A over 30 min, then to 25% solvent A over 25 min, and finally to 2% solvent A over 15 min. Under these conditions, all three native PLA2s eluted from the column between 30 and 35 min and the manoalogue-modified enzymes eluted 3–5 min later. Protection from alkylation studies were performed as described (Jain et al., 1991a). Briefly, native or inactivated PLA2 (30 μM) was incubated at 22 °C in 50 mM cacodylate buffer, pH 7.3, in the presence of 16 mM 2H-GPC, 0.8 mM ω-bromo-4-nitroacetophenone and other ligands when present. At various times, an aliquot containing typically 1–30 pmol of the enzyme was diluted into an assay mixture consisting of 280 μM DMPM, 2.5 mM $CaCl_2$, and 5 μg of polymyxin B in 4 mL, and the reaction velocity was monitored in a pH-stat (Jain et al., 1991c). PLA2 assays with the soluble substrate diC_6-thioPM were carried out by the chromogenic method with Ellman's reagent (Yuan et al., 1990). For all of these experiments, the compositions of the reaction mixtures are given in the figure legends.

Results

Inactivation Studies in the Scooting Mode on Large Vesicles. Analysis of PLA2 in the scooting mode has been described in detail elsewhere (Berg et al., 1991; Jain & Gelb, 1991; Jain & Berg, 1989; Jain et al., 1986a), and only a brief review of the concepts is given here. With vesicles of DMPM, PLA2s from a number of sources bind sufficiently tightly to the vesicles such that all of the phospholipids in the outer monolayer of the vesicles are hydrolyzed while the enzyme remains bound. The overall physical integrity of the hydrolyzed vesicle is retained with a one-to-one mixture of fatty acid and lysophospholipid making up the outer vesicle surface. The enzyme, the substrate, and the products of the lipolysis remain bound in the same vesicle and do not exchange with other vesicles. These experimentally verified constraints allow the kinetics of PLA2 to be studied in a rigorously quantitative fashion. The shape of the reaction progress curves (product vs time) under the conditions of at most one enzyme per vesicle depends on the size of the vesicles (Berg et al., 1991). With large vesicles (~100000–200000 lipids per vesicle), the surface concentration, or mole fraction, of substrate changes sufficiently slowly in time so that an initial constant velocity is observed that persists for several minutes. In contrast, the enzyme changes the composition of small vesicles (~10000 lipids per vesicle) relatively quickly compared to the situation in large vesicles so that the velocity or slope at each point along the curve is decreasing with time and the progress curve takes on a first-order appearance.

Large amounts of manoalogue-modified PLA2s were prepared by incubating 1-mg quantities of enzyme with an approximately 20-fold molar excess of manoalogue (300 μM). After various times, aliquots were diluted about 50000-fold into a pH-state vessel in order to measure the remaining PLA2 activity in the scooting mode. These assays were carried out with vesicles in the presence of a high calcium concentration (2.5 mM). Under these conditions, the sonicated vesicles fuse quickly to form large vesicles (Jain et al., 1986a). As discussed above, under these conditions a persistent initial velocity for the action of PLA2 in the scooting mode can be measured. Figure 1 shows that the initial enzymatic velocity decreased with the time of the preincubation of enzyme with inactivator. In the case of the bee venom PLA2, the activity decreased substantially during the first 20 h, and after 40 h the remaining activity was below 5%. With the enzyme from porcine pancreas, the activity decayed more slowly and reached a final value of about 6–8%. In this case, complete inactivation of the enzyme could not be achieved even when the manoalogue concentration in the preincubation mixture was increased to 400 μM after 48 h. Manoalogue also caused a time-dependent inactivation of the cobra venom PLA2, and the residual activity reached a limiting value of about 20%. Addition of a second portion of manoalogue did not lower this residual activity further. For all enzymes, the activity in the absence of manoalogue decayed by less than 5% over the time course of the experiment. The large amounts of inactivated enzymes prepared by this procedure were used in the fluorescence binding, the protection from alkylation, and the inhibition by MJ33 experiments described below.

For all three enzymes, the presence of remaining unmodified PLA2 was examined by HPLC analysis of aliquots taken from the preincubation mixture after the decrease in activity had ceased (Figure 1). In all cases, native PLA2 could not be detected. This result confirmed that the residual activity in the case of the cobra and pig enzymes was not due to the presence of small amounts of unmodified enzyme. In one experiment, HPLC analysis of the manoalogue-treated pancreatic PLA2 after the extent of inactivation reached 60% showed no native PLA2. This result indicated that complete inactivation of the porcine enzyme required the attachment of more than a single molecule of manoalogue per enzyme.

AMPA is a chemically modified form of the pig pancreatic PLA2 in which the ε-amino groups of all of the lysine residues have been converted to amidine residues. The amidine groups preserve the positive charge of the lysines, and AMPA hydrolyzes DMPM vesicles in the scooting mode with a turnover number that is identical with that of the native enzyme (Jain et al., 1986b). Since an amidine would not be expected to

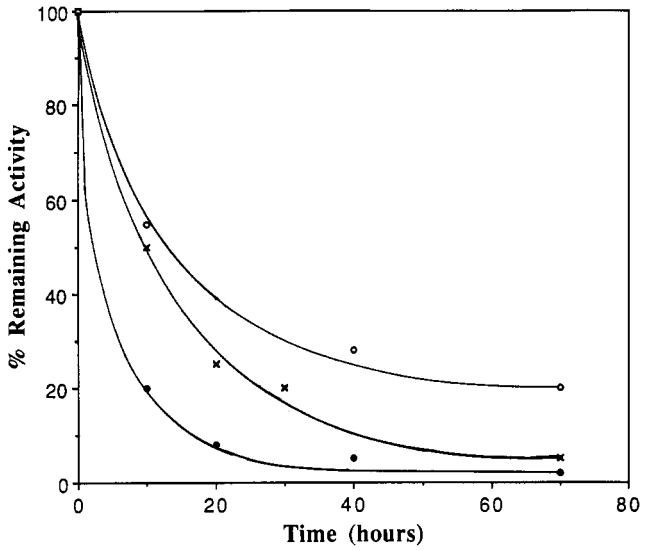


FIGURE 1: Kinetics of inactivation of PLA2 by manoalogue. Pig (x), cobra venom (O), and bee venom (●) enzymes were preincubated with an approximately 20-fold molar excess of manoalogue. At the times indicated, aliquots were withdrawn and diluted into a pH-stat assay containing 0.3 mM DMPM, 2.5 mM $CaCl_2$, and 1 mM NaCl, pH 8.0, at 21 °C. The percent remaining activity was calculated from the initial slopes relative to a control sample of enzyme in the absence of manoalogue. The turnover numbers of the enzymes in the absence of manoalogue under the above conditions are 240, 35, and 90 s^{-1} for the pig, cobra, and bee PLA2s, respectively.

undergo nucleophilic addition to the aldehydes present in manoalogue, it was of interest to examine whether manoalogue could inactivate AMPA. Treatment of AMPA with manoalogue under the conditions given in the legend to Figure 1 resulted in no loss of enzymatic activity (less than 5%) over a 5-h preincubation period when aliquots of the mixture were examined on DMPM vesicles as described in the legend to Figure 1. This result further illustrates that lysine modification is required for inactivation of PLA2 by manoalogue and that modification of the enzyme's amino-terminus (still present in AMPA) is not the reason for the inactivation seen with this agent.

Inactivation Studies in the Scooting Mode on Small Vesicles. In the next series of experiments, the enzymes were examined by the pH-stat assay in the scooting mode with small sonicated vesicles at a low calcium concentration (0.6 mM). Previous studies showed that, under these conditions, the small vesicles do not fuse over several hours (Jain et al., 1986a). As described above, the reaction progress curve with small vesicles had an essentially first-order character in which the velocity continuously decreased at all times along the curve. This is shown in Figure 2 for the action of all three PLA2s in the absence of manoalogue. In these experiments, the vesicle-to-enzyme ratio was about 5. Under this condition, the vesicles will either be devoid of enzyme or contain at most a single bound molecule of enzyme (i.e., according to the Poisson distribution for random and irreversible binding of the enzyme to the vesicles, the probability of having more than one enzyme per vesicle is less than 2%). Under these conditions, it has been rigorously shown that the enzymatic reaction ceases when only a small fraction of the total available substrate has been hydrolyzed (Jain et al., 1986a). This is because the enzyme does not hop to other vesicles and the intervesicle exchange of substrate and products is negligible. Thus, the reaction stops when all of the phospholipid in the outer monolayer of the enzyme-containing vesicles have been hydrolyzed.

The three different PLA2s at concentrations of 5 μM were preincubated with manoalogue (50 μM) and at various times

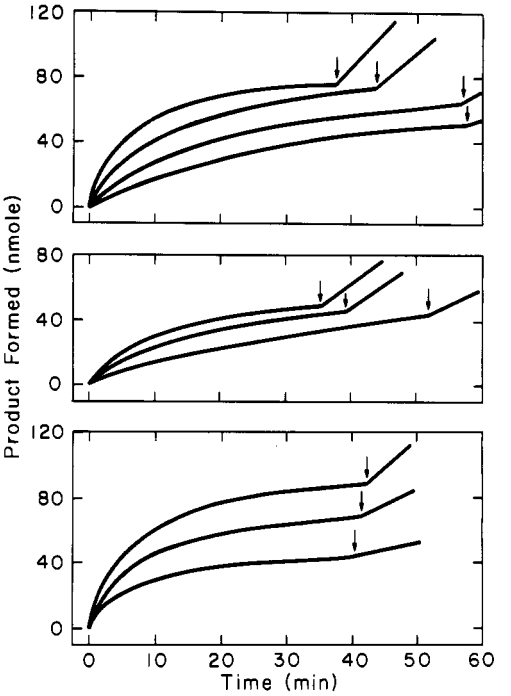


FIGURE 2: Kinetic studies of manoalogue-inactivated PLA2s on small vesicles. PLA2s (55 μg/mL) in 10 mM Tris-HCl, pH 8.0, were treated with manoalogue (50 μM). At various times (see below) an aliquot was added to an assay mixture consisting of 0.6 mg of DMPM in 4 mL of 0.6 mM $CaCl_2$ and 1 mM NaCl. The product formation was monitored in a pH-stat at 21 °C. The top curves are for the pig PLA2 (55 ng per curve) after 0, 15, 165, and 195 min of preincubation with manoalogue. The middle set is for the cobra venom enzyme (33 ng per curve) after 0, 30, and 250 min of preincubation. The lower set is for the bee venom PLA2 (80 ng per curve) after 0, 15, and 85 min of preincubation. The calcium concentration was increased to 2.5 mM by adding a small volume of 0.5 M $CaCl_2$ at the points indicated by arrows.

aliquots were diluted about 4000-fold into a pH-stat assay under the first-order conditions described above. For all three enzymes, the time needed to reach the end point in the reaction progress curve increased as the preincubation time with inactivator increased (Figure 2). As described under Discussion, this result suggested that a series of partially modified enzyme species were being sequentially formed. In the case of the cobra venom enzyme, the extents of reaction, defined as the amount of product formed at the end of the reaction, for the native and monoalogue-modified forms were similar (Figure 2, middle). In contrast, the extent of reaction for the pig and bee venom PLA2s decreased as the time of preincubation with manoalogue increased (Figure 2, top and bottom). These results are consistent with the partial inactivation of the cobra venom enzyme and the nearly complete inactivation in the case of the bee and pig PLA2s (Figure 1). In these experiments, it is also important to note that the numbers of phospholipids hydrolyzed per molecule of native monomeric PLA2 were 17900, 19500, and 16700 for the pig, cobra, and bee enzymes, respectively. These values correspond closely to the estimated number of phospholipids present in the outer layer of the DMPM vesicles of 14000 as previously described (Jain & Gelb, 1991; Jain et al., 1991b). This not only indicated that all three enzymes were catalytically active as monomers (Jain et al., 1991b) but the similarity in the extents of reaction of the native and manoalogue-modified cobra PLA2s established that all of the modified cobra enzyme was bound to the DMPM vesicles.

At the end of the reaction, the calcium concentration was raised to 2.5 mM, which allowed the enzymatic reaction to begin again (Figure 2). This reinitiation of the enzymatic

reaction has been proven to be due to the calcium-promoted fusion of the vesicles, which allows the bound enzymes to become exposed to new substrate (Berg et al., 1991). Now the reaction progress curve becomes linear for the action of the enzymes on these larger vesicles. The initial slope of these high calcium progress curves decreased with increasing preincubation time, consistent with the data in Figure 1. It is also important to note that, at low calcium, the extent of reaction in the scooting mode was never larger than the extent seen with the unmodified enzyme (Figure 2). If manoalogue-modified PLA2s were able to hop from vesicle to vesicle, all of the vesicles would have eventually become hydrolyzed and the extent of reaction would have been much larger than that seen with the native enzymes. The shapes of these reaction progress curves for manoalogue-treated enzyme are complex, since both unmodified and modified enzyme populations, each having a different first-order relaxation constant, are contributing to the total product formed. These results strongly suggest that it is the catalytic efficiency of the enzyme within the interface rather than the binding of the enzyme to the vesicles that is altered by manoalogue.

Binding of PLA2s to Vesicles. The binding of PLA2 to vesicles was independently examined by optical measurements in which the fluorescence resonance energy transfer between tryptophan residues on the enzyme and a fluorescent probe, HDNS, in the bilayer was monitored (Jain & Vaz, 1987). In the case of the pig PLA2, tryptophan 3 located on the interfacial binding surface of the enzyme undergoes essentially complete resonance energy transfer to the dansylated probe, which indicated that this amino acid residue is positioned less than 8 Å from the interface when the enzyme binds to vesicles (Jain & Vaz, 1987). In these experiments, vesicles of the nonhydrolyzable diether analogue DTPM were used so that the vesicles did not become hydrolyzed during the fluorescent measurements.

Figure 3A shows a titration experiment in which increasing amounts of pig PLA2 were added to a fixed amount of phospholipid vesicles (0.2 mg of DTPM containing 4 μg of HDNS), and the fluorescence energy transfer (emission of HDNS with excitation of tryptophan) was monitored. As reported in detail elsewhere (Jain & Vaz, 1987), the binding of the native PLA2 to the vesicles was observed as an increase in the fluorescence. This continued until sufficient PLA2 was added to completely cover the outer monolayer of all of the vesicles (Figure 3A). This occurred with about 50 μg of PLA2, which corresponded to about 50 phospholipid molecules per molecule of PLA2 (Jain et al., 1982). Since the initial part of the curve is linear, it is difficult to accurately estimate the value of the dissociation constant since the binding was so tight [the dissociation constant was less than 1 μM (Jain et al., 1982, 1986b)]. The energy transfer was observed only when the HDNS probe was present, and the probe dispersed in water did not show any energy transfer because it did not bind to the enzyme. In addition, no energy transfer was observed when enzyme was added to vesicles of DTPC, rather than DTPM, containing HDNS because the affinity of the pig PLA2 for zwitterionic vesicles is very weak (Jain & Berg, 1989; Jain et al., 1986b, 1982). These controls indicated that the energy transfer only occurred when the enzyme was bound to the vesicle interface and that the PLA2 did not promote the dislodgement of HDNS from the interface to form a water-soluble enzyme–HDNS complex.

That the manoalogue-modified pig PLA2 was capable of binding to DTPM vesicles is shown in Figure 3A. As with native PLA2, a binding isotherm was seen by the resonance energy transfer method. Again, the fluorescence increased up

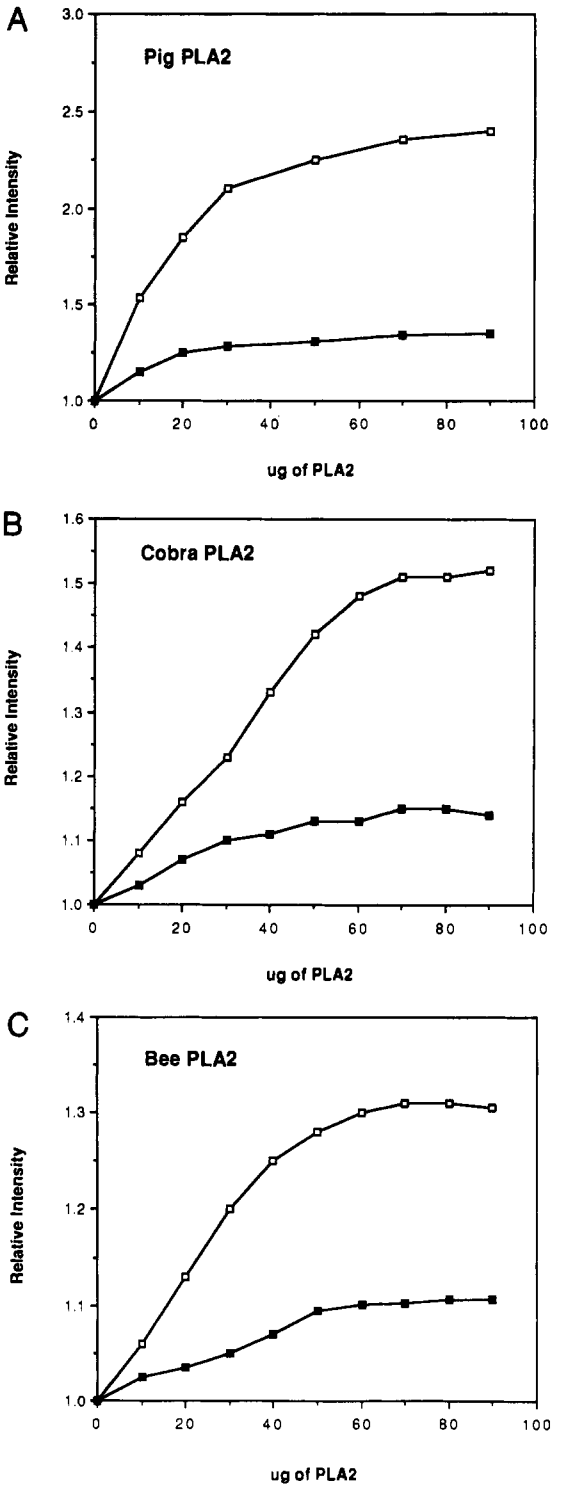


FIGURE 3: Direct vesicle binding studies of native and manoalogue-inactivated PLA2s: (A) pig PLA2; (B) cobra venom PLA2; (C) bee venom PLA2. Open squares denote native enzymes and closed squares denote enzymes modified with manoalogue as described in the legend to Figure 1. The binding was measured by fluorescence resonance energy transfer, and the details are given under Materials and Methods.

to about 50 μg of added PLA2. The fluorescence intensity after binding saturation was lower for the manoalogue-modified enzyme compared to native PLA2 (Figure 3A), and this was further investigated as described below. Results in Figure 3A would also occur if the energy transfer seen with the modified enzyme was due to the presence of approximately 20–30% of the enzyme in the unmodified form, with the latter producing the observed fluorescence change. This possibility

was ruled out on the basis of the fact that no native PLA2 in these samples was detected by HPLC analysis and the remaining enzymatic activity on large DMPM vesicles was less than 5% (Figure 1).

Similar results were obtained with the cobra venom (Figure 3B) and bee venom (Figure 3C) PLA2s. In these cases, the efficiency of energy transfer was much less than with the pig enzyme as judged by the smaller relative fluorescent intensity changes. In the case of the pig PLA2, the unique tryptophan is located on the surface of the enzyme that is in direct contact with the interface (Ramirez & Jain, 1991). The bee and cobra venom enzymes have two and three tryptophans, respectively, but not all of them are located on the interfacial binding surface (Scott et al., 1990a,b; White et al., 1990). Despite a smaller overall fluorescence response, it was clear from the data in Figure 3B,C that both manoalogue-modified cobra and bee venom PLA2s were able to tightly bind to DTPM vesicles and with the same binding stoichiometry as the native enzyme. As with the pig enzyme, the fluorescence emission intensity at binding saturation was less for the modified enzymes compared to the native forms.

To further understand the decrease in the fluorescence intensity for the manoalogue-modified PLA2s compared to native enzymes at binding saturation (Figure 3A–C), additional studies were carried out with the pig PLA2 since the fluorescence changes that accompany the binding of the enzyme to the vesicles have been extensively studied (Jain & Vaz, 1987). As shown in Figure 4, the binding of pig PLA2 to DTPM vesicles containing HDNS could also be studied by observing the emission of the HDNS probe with direct excitation of the probe at 332 nm (not resonance energy transfer) (Jain & Vaz, 1987). As observed in the resonance energy transfer experiments (Figure 3A), an increase in dansyl fluorescence was observed as native PLA2 was added until the vesicles became completely coated with enzyme (Figure 4). In the resonance energy transfer experiments, the increase in dansyl emission intensity is due to both the energy transfer from tryptophan 3 to HDNS and to the change in the polarity and/or the mobility of the HDNS probe due to the binding of PLA2 (Jain & Vaz, 1987). When the HDNS probe is directly excited at 332 nm (Figure 4), the increase in the dansyl fluorescence emission intensity is due only to the polarity/mobility changes. Again, manoalogue-modified pig PLA2 was capable of binding to the vesicles with a similar maximal binding stoichiometry as that seen with the native enzyme; however, the emission intensity at binding saturation was significantly lower for the modified enzyme (Figure 4).

In the case of the native enzyme binding to the vesicles, a portion of the increase in the dansyl emission is due to the desolvation of the HDNS probe accompanying the binding of the enzyme (Jain & Vaz, 1987). This can be seen from a comparison of the PLA2-induced fluorescence changes when the experiments are conducted in H_2O or D_2O solvent. The fluorescence emission quantum yield of HDNS in DTPM vesicles is higher in D_2O than that in H_2O due to a specific interaction of the excited state of the dansyl chromophore with its solvent cage (Stryer, 1966; Forster & Rokos, 1967). As shown in Figure 4, in the absence of PLA2, the fluorescence emission of HDNS is higher in D_2O than in H_2O, but the intensities in the two solvents became the same when sufficient native PLA2 was added to completely cover all of the HDNS probes in the vesicles. In other words, the total change in the emission intensity accompanying PLA2 binding was lower in D_2O than in H_2O. This results suggests that the dansyl chromophore becomes desolvated when the enzyme binds to the vesicle surface (Jain & Vaz, 1987). This desolvation is

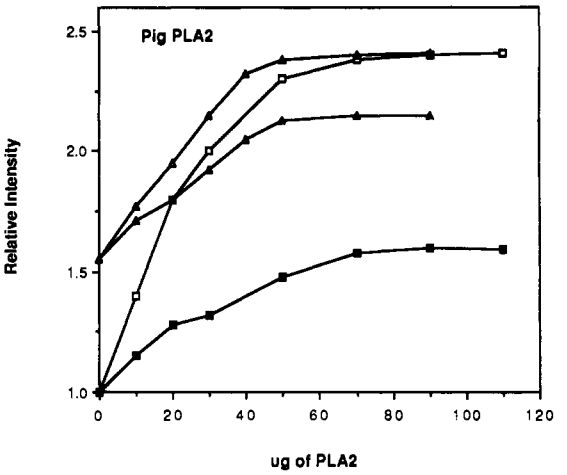


FIGURE 4: Binding of native and manoalogue-modified pig PLA2s to vesicles. In these studies, the binding was studied by monitoring the increase in the fluorescence emission of the HDNS probe present in the DTPM vesicles with direct excitation of the probe as described under Materials and Methods. Open squares and triangles are for the native pig PLA2 in H_2O and D_2O, respectively. Closed squares and triangles are for the manoalogue-modified pig PLA2 in H_2O and D_2O, respectively.

in part responsible for the increase in dansyl emission seen when the PLA2 binds to the vesicles as it relieves the solvent quenching of the fluorescence that occurs in H_2O. When the binding of the manoalogue-modified pig PLA2 was studied in D_2O, the total change in the emission intensity was similar to that observed in H_2O (Figure 4). This result clearly shows that when manoalogue-modified pig PLA2 binds to the vesicles, desolvation of the microinterface between the enzyme and the lipid occurs to a much lower extent than that observed with the native enzyme. Thus, the observed increase in dansyl emission intensity when the modified PLA2 binds to the vesicles must be due to factors other than desolvation. These studies in H_2O/D_2O explain in part why the dansyl emission is less for vesicles coated with manoalogue-modified versus native enzyme, as seen in the resonance energy transfer experiments (Figure 3) and in the direct dansyl excitation experiments (Figure 4).

Protection Studies on Manoalogue-Modified PLA2. The binding of ligands to the active site of PLA2 in the aqueous phase has been studied by monitoring the ligand-afforded protection of the enzyme to inactivators that covalently modify the catalytically critical histidine residue present in the enzyme's active site. (Verheij et al., 1980). These protection studies can be extended to examine the ligand binding to enzyme that is bound to a phospholipid–water interface (Jain et al., 1991a). In this case, use is made of the neutral surface diluent 2H-GPC. This compound forms micelles to which PLA2 binds but has very little affinity for the active site of the bound enzyme. The presence of an inactivator such as ω-bromo-4-nitroacetophenone leads to a time-dependent loss in the activity of the enzyme. In these experiments, the micelle concentration is sufficiently high (16 mM 2H-GPC) to ensure that all of the enzyme is bound. Under these conditions, the presence of ligands in the micelle that can bind to the active site of PLA2 will provide protection of the active site from alkylation with a resultant increase in the half-time for inactivation by the alkylating agent.

Since manoalogue-modified PLA2 is able to bind to the interface, it was possible to examine the dissociation constants for a variety of ligands interacting with the modified enzyme bound to micelles. The results are summarized in Table I for the pig PLA2 in which the half-times for inactivation by

Table I: Protection from Alkylation

Half-Times for Inactivation (min)[a]		
enzymes and additives[b]	native PLA_2	manoalogue PLA_2
E + 1 mM EGTA	5.0	19.3
E + 1 mM $CaCl_2$	21	439
E* + 1 mM EGTA	8	40
E* + 1 mM $CaCl_2$	55	798
E*·Ca + 0.1 mole fraction DTPM	390	~1500[d]
E*·Ca + 0.1 mole fraction products	436	~1500[d]

K_D Values[c]		
enzyme and additives	native PLA_2	manoalogue PLA_2
E·Ca	0.26 mM	0.05 mM
E*·Ca	0.16 mM	0.053 mM
E*·Ca·DTPM	0.025 mole fraction	~0.11 mole fraction[d]
E*·Ca·products	0.025 mole fraction	~0.11 mole fraction[d]

[a] Half-times were determined as described under Materials and Methods. [b] E and E* refer to enzyme in water or bound to 2H-GPC micelles, respectively. [c] Calculated from the half-times according to eq 1. [d] Because of the large half-times, these numbers are only approximate.

ω-bromo-4-nitroacetophenone are given for native and manoalogue-modified PLA2s both bound to 2H-GPC micelles or dissolved in aqueous solution. In these experiments, the remaining enzymatic activity of 8% for the fully modified enzyme (Figure 1) was monitored as a function of the time of incubation with the alkylating agent. Also given in Table I are the dissociation constants (K_d) for the ligand–enzyme interactions within the interface. Values of K_d were calculated from the half-times according to

$$\frac{1}{[1 - (t_o/t_L)]} = \frac{[(K_d/X_L) + 1]}{[1 - (k_L/k_o)]} \quad (1)$$

Equation 1 is a modified form of the standard Scrutton–Utter equation (Scrutton & Utter, 1965) in which the concentration of ligand is given by its mole fraction (X_L) (Jain et al., 1991a). Here, t_L and t_o are the half-times for enzyme inactivation in the presence and absence of a ligand, respectively. The constants k_L and k_o are the intrinsic inactivation rate constants for the reaction of enzyme saturated with ligand at its active site or in the absence of ligand, respectively. In a previous study, the ratio k_L/k_o was measured to be close to zero, i.e., the enzyme shows essentially complete protection from alkylation when saturated at its active site with a ligand (Jain et al., 1991a).

In Table I, E refers to the enzyme dissolved in water and E* refers to enzyme bound to 2H-GPC micelles. The first point that emerges is that the active site of the manoalogue-modified pig PLA2 is functionally intact. This is based not only on the fact that these enzymes are catalytically active but also on the fact that protection from alkylation was seen with calcium, the substrate analogue DTPM, and the products of DMPM hydrolysis (a 1:1 mixture of myristic acid and lyso-DMPM). Thus, all of these ligands were still capable of binding to the active site of the modified enzyme. It was clear that the access of the alkylating agent was somewhat retarded for the manoalogue-modified enzyme compared to native PLA2 since the half-time for inactivation increased about 4–5-fold following inactivation with manoalogue (Table I). This was true for the enzyme in solution or bound to micelles. This result served to illustrate that the 8% activity of the modified enzyme being monitored is not due to contaminating unmodified PLA2. Interestingly, the dissociation constant for the calcium cofactor was about 3–5-fold lower for the manoalogue-modified enzyme both in solution and bound to vesicles (Table I). This result ruled out the possibility that the lower catalytic activity for the manoalogue-modified PLA2 (Figures 1 and 2) was due to a lower fraction of the enzyme present in the calcium-bound form. Finally, for the enzyme bound to the micelles, the dissociation constants for the substrate analogue DTPM and the reaction products were somewhat higher, about 4-fold, for the manoalogue-modified enzyme relative to native pig PLA2; however, the error in these numbers was quite large due to the very long half-times observed with the modified enzyme in the presence of these ligands.

In a second type of experiment, the rate of alkylation of pig PLA2 bound to 2H-GPC micelles was investigated in the presence of increasing mole fractions of monoalogue added to the micelles immediately before or after ω-bromo-4-nitroacetophenone was added. This experiment was designed to monitor the affinity of intact manoalogue for the active site of PLA2. No decrease in the half-time for enzyme inactivation was seen, even when the mole fraction of monalogue added was 0.05. In addition, concentrations of manoalogue up to 0.05 mole fraction did not produce detectable inhibition of the hydrolysis of DMPM vesicles in the scooting mode by the pig PLA2. These results show that manoalogue does not bind to the enzyme's catalytic site.

In a third type of experiment, the pig pancreatic PLA2 was preincubated with manoalogue under the conditions given in the legend to Figure 1, except that 2H-GPC was included at a concentration of 16 mM to ensure that all of the enzyme would be bound to micelles (E* form). Under these conditions, no enzyme inactivation (less than 5%) was observed after a 10-h preincubation when aliquots of the incubation mixture were assayed on DMPM vesicles under the conditions given in the legend to Figure 1. For comparison, the enzyme in the absence of the 2H-GPC micelles was inactivated about 50% after a 10-h preincubation with manoalogue. This result indicated that the binding of the enzyme to the interface protected it from inactivation by manoalogue.

Studies with a Competitive Inhibitor. MJ33 is a phospholipid analogue that functions as a tight-binding competitive inhibitor of PLA2. It functions both as a protective agent for the alkylation of the pig PLA2 bound to 2H-GPC micelles and as a competitive inhibitor for the hydrolysis of DMPM vesicles in the scooting mode (Jain et al., to be published). The ability of this compound to inhibit both the native and manoalogue-modified PLA2s was compared. The pig and cobra PLA2s were treated with excess manoalogue under the conditions given in the legend to Figure 1, and the inhibition of the residual enzymatic activity by MJ33 was determined. These studies were carried out in large vesicles so that the initial velocities, i.e., at mole fraction DMPM close to 1, were being measured (Jain et al., 1989, 1991a). With the pig PLA2, the mole fractions of MJ33 required for 50% reduction of the initial velocities, $X_I(50)$, were 0.0034 for the native enzyme and 0.0068 for the manoalogue-modified PLA2. With the cobra PLA2, the $X_I(50)$ values were 0.0068 for both native and modified enzymes.

Inactivation Studies with Short-Chain Substrates. It is possible to study the action of PLA2s in solution on water-soluble substrates as long as special care is taken to insure that the enzyme and substrate do not combine to form a lipid–protein aggregate. For example, such aggregates form when the cobra venom PLA2 acts on certain short-chain phosphatidylcholine substrates or when the pig PLA2 operates on certain short-chain anionic substrates (Yuan et al., 1990, and references therein). However, it has previously been shown that the pig, cobra venom, and bee venom PLA2s hydrolyze diC_6-thioPM under certain conditions without the formation

of enzyme–lipid microaggregates (Yuan et al., 1990). With this substrate, the activity of manoalogue-modified PLA2s acting on soluble substrates could be studied. All three enzymes at a concentration of 3 μM were incubated with manoalogue (100 μM) for various times, and aliquots were diluted about 100-fold into an assay mixture containing diC_6-thioPM. The enzymatic activities were followed with Ellman's reagent to monitor the appearance of free thiol groups. The results are shown in Figure 5A. For both the pig and bee venom enzymes, about 70% of the initial activity was lost after about 3 h. Complete inactivation could not be achieved by adding an additional portion of manoalogue. Under these conditions, the activity of these enzymes acting on DMPM vesicles in the scooting mode was barely detectable, as seen in Figure 1. In contrast, treatment of the cobra venom PLA2 with manoalogue produced very little loss in activity (about 10%) when measured with the soluble substrate (Figure 5A). Further inactivation could not be achieved by doubling the concentration of manoalogue. Again, under these conditions, the activity of the cobra enzyme acting on DMPM vesicles was much lower (80% inhibited as seen in Figure 1).

There is some debate as to whether these PLA2s are really catalyzing the hydrolysis of diC_6-thioPM in a 1:1 enzyme–substrate complex or whether a small protein–lipid microaggregate is formed consisting of a few phospholipid molecules in contact with the enzyme's interfacial binding surface (Jain & Berg, 1989; Yuan et al., 1990). It is generally accepted that the proenzyme form of the pig PLA2 can hydrolyze monomeric substrates in a soluble 1:1 complex as this enzyme does not contain a functioning interfacial binding surface (Verger & de Haas, 1976). Thus, inactivation studies with manoalogue were also carried out on pro-pig PLA2. The results in Figure 5B show that like pig PLA2 about 80% of the activity on diC_6-thioPM is lost after preincubation with manoalogue.

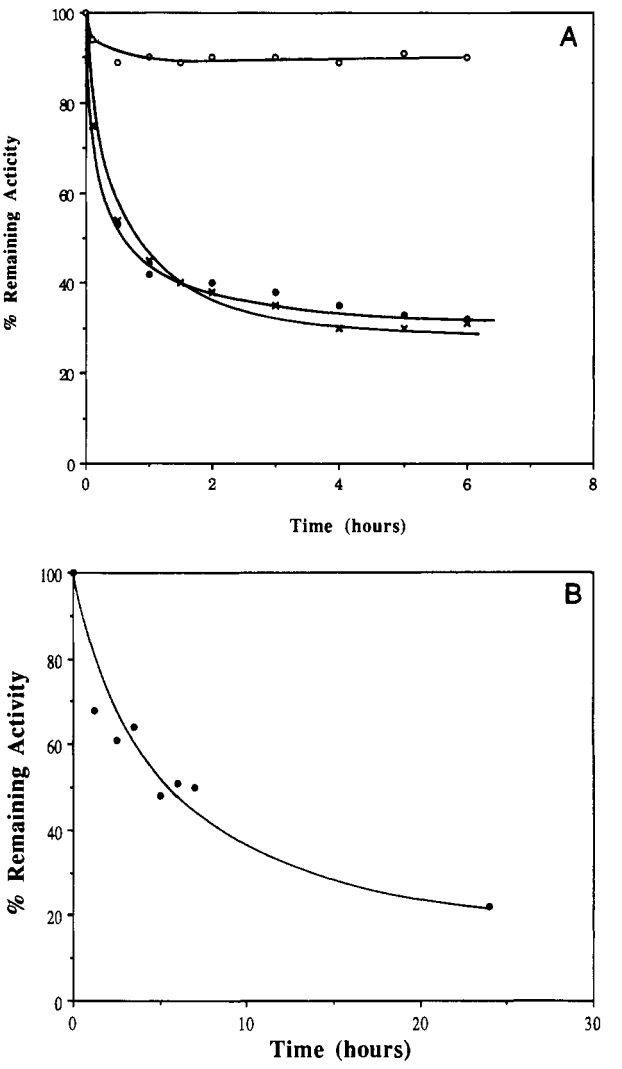


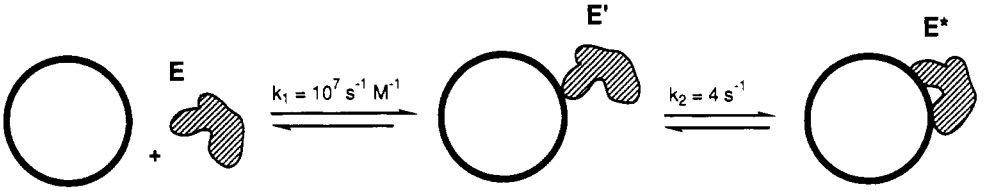
FIGURE 5: (A) Kinetic studies of manoalogue-inactivated PLA2s with the soluble substrate diC_6-thioPM. The cobra venom (○), pig (x), and bee venom (●) PLA2s, 45 μg each, in 1 mL of 10 mM Tris-HCl, pH 8.0, were treated with 100 μM manoalogue. At the indicated times, aliquots (typically 2–8 μL) were withdrawn and diluted into an assay mixture consisting of 0.3 mM diC_6-thioPM in 0.3 mL of Tris-HCl, 100 mM KCl, 10 mM $CaCl_2$, and 0.8 mM Ellman's reagent, pH 8.0. The absorbance at 412 nm was followed at 21 °C. The percent remaining activity was calculated from the initial reaction slope relative to controls in the absence of manoalogue. (B) Same as in panel A with 45 μg of the pig proenzyme.

Discussion

The idea that monoalogue-treated PLA2 is a poor catalyst because it is unable to bind to the substrate interface was discarded on the basis of the observations presented in this study. For example, in the case of the cobra venom PLA2, complete inactivation of the enzyme could not be achieved even with high levels of manoalogue present (Figure 1). This is consistent with the partial inactivation, measured as the initial rate of hydrolysis, when a mixed-micelle PLA2 substrate was used (Reynolds et al., 1988). Native cobra venom PLA2 binds irreversibly to DMPM vesicles (Jain et al., 1991b) and is therefore not able to hop from one vesicle surface to another. If the reduction in reaction velocity (Figure 1) was due to a decrease in the fraction of vesicle-bound enzyme (i.e., conversion from a scooting mode to a hopping mode) then the modified enzyme would be capable of hydrolyzing the outer monolayers of all vesicles present in the reaction mixture. The results in Figure 2 show that this is not the case since the extent of hydrolysis for native and manoalogue-modified cobra PLA2 were the same. Thus, the lower catalytic efficiency at the interface of the manoalogue-treated cobra venom PLA2 must be due to a lower intrinsic turnover number for the fully vesicle-bound enzyme. In the case of the pig and bee venom PLA2s, the reaction velocity became nearly undetectable after prolonged incubation with manoalogue. This result is consistent with a catalytic turnover number of near zero for the enzymes in the interface or a lack of binding of the water-soluble enzyme to the bilayer surface or a combination of both. However, direct binding studies using the fluorescence energy transfer technique revealed that all three enzymes were binding tightly to DMPM vesicles (Figure 3). Thus, like the cobra venom enzyme, the pig and bee venom PLA2s were also "crippled" in their abilities to catalyze the lipolysis reaction once bound to the substrate surface.

The reaction progress curves shown in Figure 2 deserve additional comment. With the bee venom and pig PLA2s, the extent of the reaction decreased as the preincubation time with manoalogue increased (Figure 2, top and bottom). This was expected since the turnover of the modified enzymes in the interface was near zero (Figure 1) and the lower reaction extents represented a decreasing fraction of the vesicles that contained a bound and unmodified PLA2. However, if the population of enzymes in the inactivation mixture consisted only of native and catalytically inactive species, then the extent of reaction would decrease but the first-order relaxation constant, characteristic for the native enzyme, would remain unchanged. The data in Figure 2 clearly show that the first-order relaxation constant also decreased during the

Scheme II



preincubation with manoalogue. This result implies that there are intermediate enzyme species being formed that contain perhaps one or two bound manoalogues and that these partially modified enzymes have a reduced but finite turnover number (i.e., first-order relaxation constant) within the interface. This result is consistent with the HPLC experiments showing that native pig PLA2 could not be detcted after the enzyme was only 60% inactivated with manoalogue. In the case of the cobra venom PLA2, the extent of reaction was not affected by manoalogue treatment (Figure 2, middle), but the time needed to reach the end point in the reaction increased during the preincubation with inactivator. This result is consistent with the data in Figure 1 showing that the cobra venom enzyme still retains considerable catalytic activity (20%) when fully modified by manoalogue. Thus, given enough time, both native and modified cobra enzymes will hydrolyze the same number of vesicles. These studies illustrate the power of the scooting mode protocol as this technique reveals characteristics of manoalogue-modified PLA2s that would not be readily discernible with other assays of interfacial catalysis.

Other investigators have proposed that enzyme aggregation within the interface occurs and leads to enzyme activation [see, for example, Menashe et al. (1985) and Pluckthun and Dennis (1985)]. Although manoalogue modification could potentially result in a decrease in enzyme aggregation within the interface, this cannot be the explanation for the inactivation seen in the present study. This is based on the fact that it has been rigorously shown in this study and previously that all three PLA2s are operating in the scooting mode on DMPM vesicles as monomeric enzymes (Jain et al, 1991b). Furthermore, in the case of the cobra PLA2, the extent of reaction on small DMPM vesicles was the same for native and manoalogue-modified enzymes (Figure 2). If the state of aggregation of these two enzymes were different, the number of vesicles hydrolyzed in the scooting mode would be different. This is because fewer vesicles will contain bound enzyme if the enzyme formed an aggregate.

Several experiments in the present study indicate that the manoalogue-modified enzymes contain an intact active site. All three PLA2s plus the proenzyme form of the pig PLA2 after treatment with manoalogue were still capable of hydrolyzing the water-soluble substrate diC_6-thioPM, although the turnover numbers were reduced to different extents compared to native enzymes (Figure 5). Protection from alkylation studies indicated that manoalogue-modified pig PLA2 was still capable of binding calcium, the substrate analogue DTPM, and the products of DMPM hydrolysis (Table I). In addition, the binding of the competitive inhibitor MJ33 to the enzyme was not significantly perturbed by manoalogue modification. The IC_{50} value for MJ33 with the pig enzyme increased only 2-fold after inactivation with manoalogue, and no change in the IC_{50} was detected with the cobra enzyme after treatment with manoalogue. In the case of the pig PLA2, the lower affinity of the active site for the substrate (estimated to be about 4-fold) cannot be the sole reason for the nearly complete inactivation of the enzyme when it is acting on DMPM vesicles (Figure 1). This is based on the fact that the K_m value for DMPM has a value of about 0.3 mole fraction (Jain et al., 1991a), and a modest (4-fold) increase in the value of K_m predicted for manoalogue-modified pig PLA2 would not be expected to lead to a nearly complete loss of enzyme activity with DMPM vesicles. In the case of the pig PLA2 dissolved in water or bound to 2H-GPC micelles, modification by manoalogue reduced the rate of alkylation of the active site histidine by about 4-fold. It is not clear from the present results whether manoalogue is producing a conformational change in the enzyme or is simply providing steric hindrance to the alkylating agent. The latter explanation is favored since these extracellular PLA2s contain multiple disulfide bridges and are therefore relatively conformationally rigid.

Interestingly, binding of the pig PLA2 to 2H-GPC micelles protected it from inactivation by manoalogue. This result suggests that the modification of lysine residues by manoalogue that lie on the interfacial recognition surface of the enzyme are required from the inactivation. In principle, manoalogue could partition into the micellar phase and react with the enzyme at the interface. Thus, the reasons for the complete protection from inactivation by interfacial binding remain to be determined.

Manoalogue-modified pig PLA2 showed a much higher catalytic activity on soluble diC_6-thioPM substrate compared to DMPM vesicles. This was also true for the bee venom enzyme and especially apparent in the case of the cobra venom PLA2 (Figure 5). These results illustrate that modification by manoalogue reduces the catalytic efficiency of the interfacial catalysis by vesicle-bound PLA2s much more significantly than the catalysis on water-soluble substrates. Importantly, the fluorescence binding studies (Figures 3 and 4) indicated that the quality of the interfacial binding for the manoalogue-modified enzymes was different compared to that of the native proteins. As discussed below, this observation provided insight into the mode of inactivation by manoalogue.

All of the observations reported in this study could be accommodated by the following model for the productive binding of PLA2 to the substrate interface (Jain & Berg, 1989). Fluorescence studies have indicated that native PLA2 binds to the phospholipid–water interface in a "suction cup-like" manner in which the surfaces of the enzyme and lipid that are in contact with each other are highly desolvated (Jain & Vaz, 1987; Ramirez & Jain, 1991). Stopped-flow fluorescence studies have proven useful in the kinetic analysis of the binding of PLA2 to the substrate interface (Jain et al., 1988). In these experiments, the intrinsic fluorescence of tryptophan 3, located on the interfacial binding surface of the pig PLA2, is increased when the enzyme binds to the interface. This provided a convenient way to monitor the interfacial binding immediately after the enzyme was mixed with either micelles or vesicles of nonhydrolyzable substrate analogues such as DTPM. Such studies demonstrated that the binding occurs in two kinetically distinct steps (Scheme II). A rapid increase in the fluorescence that accounted for about 30% of the total spectral change was seen, followed by a slower rise in the remaining fluorescent enhancement. The rate of the fast phase (the E to E′ step in Scheme II) depended on the concentration of vesicles and

occurred with a second-order rate constant of about 10^7 s^{-1} M^{-1}. This step likely represents a diffusion-limited encounter of the enzyme with the vesicle. The rate of the slow phase (E′ to E* step in Scheme II) was independent of both the vesicle (or micelle) and enzyme concentrations and occurred with a first-order rate constant of about 4 s^{-1}. It was proposed that this second step represents the "settling" of the bound enzyme onto the surface in a catalytically productive manner involving the desolvation of the contact surfaces. This was based on the observation that the pro-pig enzyme displayed the first phase of binding but not the second and by the fact that neither the vesicle surface nor the pro-pig PLA2 became desolvated on binding. Furthermore, it was proposed that the E* form of the enzyme but not the E′ form was catalytically active, possibly because the desolvation of the contact surfaces facilitated the dislodgement of a monomer of phospholipid substrate from the plane of the bilayer into the active site slot on the enzyme (Jain et al., 1988; Scott et al., 1990b). These latter suggestions are based on the observation that the turnover number of the pro-pig enzyme on DMPM vesicles [1.2 s^{-1} (Jain et al., 1988)] is close in magnitude to the rate constant for the E′ to E* conversion and is much lower than the turnover number for the native PLA2 (270 s^{-1}). In other words, the pro-pig PLA2 is able to bind to the interface to give E′, but the equilibrium for the conversion to the E* state is not favorable. The E to E* equilibrium is favorable for the native enzyme, and a rapid enzymatic turnover of thousands of substrate molecules can occur with the enzyme remaining in the E* state. In contrast, each turnover cycle with the pro-pig PLA2 involves a slow desolvation and a catalytic event, followed by resolvation.

In the case of the manoalogue-modified PLA2, one could propose that the conversion of E′ to E* is reduced compared to the native enzyme. This proposal is consistent with the observation that manoalogue-modified pig PLA2 can bind tightly to the vesicle interface but in a manner that does not promote the desolvation of the microinterface between the enzyme and the lipid surface (Figures 3 and 4). Thus, the modified enzyme is still able to bind to the interface, but the turnover number is lowered by the fact that not all of the enzymes are in the catalytically productive E* form. It is likely that positively charged amino acids on the interfacial binding surface through their interactions with the anionic head groups of the DMPM promote the desolvated interaction of the enzyme with the bilayer (Jain et al., 1986b). By modification of lysine residues, manoalogue may partially disrupt this step. This mode of inactivation suggested here is only possible for catalysis at interfaces, and this may explain why the manoalogue-inactivated enzymes displayed small losses of activity compared to native enzymes when acting on water-soluble substrates whereas the loss in activity on vesicles in the scooting mode was much larger (Figures 1, 2, 4, and 5). The modestly perturbed dissociation constants for calcium, reaction products, a substrate analogue, and a competitive inhibitor suggest that the E* forms of the manoalogue-modified and native enzymes have similar capacities to interact with ligands.

A final and important point that emerges from the present study is that manoalogue had no significant affinity for the active site of pig PLA2 since it provided no protection from alkylation by an active-site-directed probe nor did it function as a competitive inhibitor for the hydrolysis of DMPM in the scooting mode. It is still possible that manoalogue is interacting in a specific way with the surface of the enzyme and that this interaction promotes its reaction with surface lysine residues. However, this seems unlikely on the basis of the fact that manoalogue inactivated the three different PLA2s studied here and, although the cobra venom and pig PLA2s have somewhat similar surface topologies, the bee venom enzyme is structurally related to the other enzymes only in the vicinity of the active site (Scott et al., 1990a,b; White et al., 1990). Thus, although the manoalogue functions as a potent inactivator of PLA2 by a novel mechanism, lysine modification reagents that interact in a specific fashion with PLA2 will be required to improve the selectivity of this class of inactivators of interfacial catalysis.

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Activation of Methionine by *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: In the present work, we have examined the function of three amino acid residues in the active site of *Escherichia coli* methionyl-tRNA synthetase (MetRS) in substrate binding and catalysis using site-directed mutagenesis. Conversion of Asp52 to Ala resulted in a 10000-fold decrease in the rate of ATP–PP_i exchange catalyzed by MetRS with little or no effect on the K_m's for methionine or ATP or on the K_m for the cognate tRNA in the aminoacylation reaction. Substitution of the side chain of Arg233 with that of Gln resulted in a 25-fold increase in the K_m for methionine and a 2000-fold decrease in k_{cat} for ATP–PP_i exchange, with no change in the K_m for ATP or tRNA. These results indicate that Asp52 and Arg233 play important roles in stabilization of the transition state for methionyl adenylate formation, possibly directly interacting with complementary charged groups (ammonium and carboxyl) on the bound amino acid. Primary sequence comparisons of class I aminoacyl-tRNA synthetases show that all but one member of this group of enzymes has an aspartic acid residue at the site corresponding to Asp52 in MetRS. The synthetases most closely related to MetRS (including those specific for Ile, Leu, and Val) also have a conserved arginine residue at the position corresponding to Arg233, suggesting that these conserved amino acids may play analogous roles in the activation reaction catalyzed by each of these enzymes. Trp305 is located in a pocket deep within the active site of MetRS that has been postulated to form the binding cleft for the methionine side chain. Consistent with this, substitution of Ala for Trp305 resulted in a specific loss of affinity for methionine and an overall 100-fold decrease in k_{cat}/K_m for ATP–PP_i exchange. Comparison of the sequences in this domain of the Met, Ile, Leu, and Val synthetases shows that there is little homology between all members of this group but strong homology between enzymes specific for the same amino acids. This supports the idea that the region close to and including Trp305 forms part of the domain involved in discrimination of the methionine side chain by MetRS and suggests that corresponding sequences in the other enzymes play a similar role in distinguishing between the closely related side chains of this group of amino acids.

Accurate aminoacylation of tRNAs by aminoacyl-tRNA synthetases plays a crucial role in insuring the overall fidelity of protein synthesis. This accuracy is achieved by preferential binding of the enzymes to cognate substrates (amino acid and tRNA), by more rapid transfer of activated amino acid to cognate than noncognate tRNA species, and, in many cases, by additional proofreading steps which correct errors made in the initial amino acid activation and transfer reactions (eqs 1 and 2).

$$E + AA + ATP \rightleftarrows E{\cdot}AA{-}AMP + PP_i \quad (1)$$

$$E{\cdot}AA{-}AMP + tRNA \rightleftarrows AA{-}tRNA + AMP + E \quad (2)$$

The structural basis for selection of the cognate amino acid by an aminoacyl-tRNA synthetase has been determined for only one synthetase to date. *Bacillus stearothermophilus*

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