Interfacial Catalysis by Phospholipase A_2: Evaluation of the Interfacial Rate Constants by Steady-State Isotope Effect Studies†

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Received September 17, 1990; Revised Manuscript Received March 26, 1991

ABSTRACT: The kinetics of hydrolysis of phospholipid vesicles by phospholipase A_2 (PLA2) in the scooting mode can be described by the Michaelis–Menten formalism for the action of the enzyme in the interface (E*). $E^* + S \rightleftharpoons E^*S \rightleftharpoons E^*P \rightleftharpoons E^* + \text{Products}$ The values of the interfacial rate constants cannot be obtained by classical methods because the concentration of the substrate within the lipid bilayer is not easily manipulated. In the present study, carbonyl-carbon heavy atom isotope effects for the hydrolysis of phospholipids have been measured in both vesicles and in mixed micelles in which the phospholipid was present in the nonionic detergent Triton X-100. A large [^{14}C]carbonyl carbon isotope effect of 1.12 ± 0.02 was measured for the cobra venom PLA2-catalyzed hydrolysis of dipalmitoylphosphatidylcholine in Triton X-100. In contrast, no isotope effect (1.01 ± 0.01) was measured for the action of the porcine pancreatic and cobra venom enzymes on vesicles of dimyristoylphosphatidylmethanol in the scooting mode. In a second experiment, the hydrolysis of vesicles was carried out in oxygen-18 enriched water. Analysis of the released fatty acid product by mass spectrometry showed that it contained only a single oxygen-18. All of these results were used to estimate both the forward and reverse commitments to catalysis. The lack of doubly labeled fatty acid demonstrated that the product is released from the E*P complex faster than the reverse of the esterolysis step. The small isotope effect in vesicles demonstrated that the E*S complex goes on to products faster than substrate is released from the enzyme. The relevance of these results to an understanding of substrate specificity and inhibition of PLA2 is discussed. In addition, the conditions placed on the values of the rate constants obtained in the present study together with results obtained in the other studies described in this series of papers have led to the evaluation of most of the interfacial rate constants for the hydrolysis of phospholipid vesicles by PLA2.

The kinetics of the hydrolysis of phospholipid vesicles by PLA2[1] can be described by the Michaelis–Menten formalism for the enzyme within the interface [Verger and de Haas (1976), Jain and Berg (1989), and Figure 1 of Berg et al. (1991) *Biochemistry 30* (first paper of six in this issue)]. In addition, the kinetic scheme must include a step in which the enzyme in the aqueous phase binds to the interface. The overall catalytic cycle occurs in two distinct steps. The enzyme in the aqueous phase, E, binds to the substrate interface to give E*. The enzyme in the interface then binds a molecule of phospholipid at the catalytic site to give the Michaelis complex, E*S, which goes on to generate the products of the hydrolysis and free enzyme in the interface, E* + P. Evidence for this scheme [from Figure 1 of Berg et al. (1991)] comes from studies such as those by Pieterson et al. (1974) where it was shown that alkylation of the active-site histidine with *p*-bromophenacyl bromide blocked the binding of monomeric phospholipids to the enzyme, but the alkylated PLA2 was still capable of binding to the interface. Additional evidence for the scheme comes from studies on the hydrolysis of phospholipid vesicles. According to the scheme, E* can be recycled within the interface, or it can dissociate and bind to a different interface after each catalytic cycle. These two extreme modes of interfacial catalysis have been termed "scooting" and "hopping", respectively, by Jain and colleagues (Jain & Berg, 1989; Jain et al., 1986). The fact that the enzyme has been observed to operate in the scooting mode, that is, to remain in the interface during several thousand catalytic cycles, rules out a simpler kinetic scheme in which the binding of the enzyme to the interface and the binding of the substrate to the catalytic site are the same step. If this latter mechanism were correct, the release of lipid products from the enzyme and desorption of the enzyme from the interface into the bulk solvent would occur in one step and the enzyme would have no tendency to scoot within the same vesicle. Prior to the present series of papers, the values of the interfacial rate constants in the scheme of Berg et al. (1991) had not yet been resolved under any conditions. The kinetics are not amenable to normal experimental techniques because the concentration of substrate within the lipid bilayer is not easily manipulated.

The action of PLA2 has also been studied on mixed phospholipid/detergent micelles in which a small amount of phospholipid substrate was dispersed into a micelle of nonionic surfactant such as Triton X-100 (Hendrickson & Dennis, 1984) or deoxycholate (Verger & de Haas, 1976). In this assay, the enzymatic velocity depended on both the bulk and surface concentrations of substrate. The enzyme in the aqueous phase first binds to a phospholipid in the mixed micelle followed by a second step in which the enzyme in the interface binds a second molecule of phospholipid (Hendrickson & Dennis, 1984). The binding of the first and second phospholipids is analogous to the binding of the enzyme to the

† This work was supported by Grant HL-36235 from the National Institutes of Health.
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[1] Abbreviations: DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol lithium salt; ^{14}C-DMPM, 1-myristoyl-2-[1-^{14}C]myristoyl-*sn*-glycero-3-phosphomethanol lithium salt; ^{3}H-DMPM, 1-myristoyl-2-[9,10-^{3}H(N)]myristoyl-*sn*-glycero-3-phosphomethanol lithium salt; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ^{14}C-DPPC, 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; ^{3}H-DPPC, 1-palmitoyl-2-[9,10-^{3}H(N)]palmitoyl-*sn*-glycero-3-phosphocholine; PLA2, phospholipase A_2.

0006-2960/91/0430-7298$02.50/0

substrate interface and the formation of the Michaelis complex, respectively [Figure 1 of Berg et al. (1991)].

In the present study, the carbonyl-carbon heavy atom isotope effects for the hydrolysis of phospholipids in both mixed micelles and in vesicles have been measured. This study was undertaken in order to probe the nature of the rate-limiting step in interfacial catalysis. In addition, the incorporation of oxygen-18 from solvent into the fatty acid product was also studied in order to probe the reversibility of the esterolysis step. As will be discussed below, this information was useful in the understanding of substrate specificity for lipolytic enzymes and in the design of more potent PLA2 inhibitors. These studies together with the first and third papers in this issue (Berg et al., 1991; Jain et al., 1991) have allowed the determination of many of the interfacial rate constants shown in Figure 1 of Berg et al. (1991).

Materials and Methods

Materials. DPPC was obtained from Avanti Polar Lipids. ^{14}C-DPPC (50 mCi/mmol) and ^{3}H-DPPC (40 Ci/mmol) were purchased from NEN Research Products. PLA2 from *Naja naja naja* venom (Pakistan variety, Miami Serpentarium Laboratories) was purified as described (Hazlette & Dennis, 1985), and the porcine pancreatic PLA2 was a generous gift from Professor Mahendra Jain (University of Delaware). DMPM was prepared as described (Jain & Gelb, 1991).

Synthesis of Radiolabeled Phospholipids. Radiolabeled samples of DMPM were prepared as follows. Unlabeled DMPM (40 mg) was added to ether (1 mL) containing 50 μL of buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0). Lyophilized *Crotalus adamanteus* venom (4 mg, Miami Serpentarium) was added, and the reaction mixture was stirred vigorously at room temperature for 2 h in a tightly capped tube. TLC with $CHCl_3$/MeOH/acetic acid (65:15:2) indicated that the reaction was completed. The ether was removed with a stream of air, and 10% MeOH in $CHCl_3$ (2 mL) was added followed by sufficient water to produce two phases. The aqueous phase was adjusted to pH 2–3 with 1 N HCl. The lower organic phase was transferred to a round bottom flask. The water layer was extracted with a second portion of $CHCl_3$/MeOH, and the combined organic layers were concentrated on a rotary evaporator at room temperature. The residue was taken up in $CHCl_3$ (2 mL) and treated with sufficient diazomethane (prepared from Diazald, Aldrich) in ether to produce a persistent yellow color. The solvent was removed on the rotary evaporator, and the residue was taken up in hexane (3 mL). The solution was dried over anhydrous $MgSO_4$ and filtered through Celite. The filtrate was concentrated in a tared vial with a stream of air. The oily residue was dried in vacuo for 1–2 h and weighed. For the ^{14}C-labeled DMPM, 50 μCi of [1-^{14}C]myristic acid (54 mCi/mmol, in toluene, NEN Research Products) was added. For the ^{3}H-labeled DMPM, 1 mCi of [9,10-^{3}H(N)]myristic acid (52 Ci/mmol in toluene, NEN Research Products) was used. The toluene was removed with a stream of air. Unlabeled myristic acid was added to give a total (labeled plus unlabeled) of 0.8 equiv of fatty acid (based on the amount of lysophospholipid used). Dicyclohexylcarbodiimide (2.6 equiv), *N,N*-dimethylaminopyridine (0.9 equiv, Aldrich) were dissolved in CH_2Cl_2(1.5 mL per 56 μmol of lysophospholipid), and the solution was chilled on ice. The cold solution was added dropwise to the mixture of fatty acid and lysophospholipid, and the vial was capped and stirred on ice for 5 min and then overnight at room temperature. The solution turned cloudy after a few minutes. The solution was filtered with a small Buchner funnel, and the filter cake was washed with a small amount of CH_2Cl_2. The filtrate was concentrated on the rotary evaporator, and the residue was dissolved in 1–2 mL of 10% ethyl acetate in low boiling petroleum ether. The soln. was loaded onto a silica column (1 × 15 cm, Merck, 230–400 mesh), and the column was washed with the same solvent followed by sufficient 20% ethyl acetate in petroleum ether to elute an impurity (R_f 0.3, 30% ethyl acetate in petroleum ether). The product (R_f 0.2, 20% ethyl acetate in petroleum ether) was eluted with 30% ethyl acetate in petroleum ether. Product-containing fractions were combined and concentrated on the rotary evaporator. The oily residue was transferred to a vial and dried in vacuo for 2 h. 2-Butanone (100 μL) and LiBr (5 mg) were added, and the vial was tightly capped and heated in an oil bath at 80 °C with stirring for 1.5 h. The solvent was removed with a stream of air. Acetone (1 mL) was added, and the solution was cooled at −20 °C overnight to precipitate the product. The mixture was centrifuged in a small plastic tube, the acetone was removed by pipet, and the solid was repeatedly washed with cold acetone and collected by centrifugation. The solid was dissolved in petroleum ether/ether/acetic acid (70:30:1) and applied to a silica column (1 × 15 cm). The column was washed with the same solvent (50 mL) followed by $CHCl_3$/MeOH/acetic acid (65:15:2) to elude the product. Product-containing fractions were pooled and concentrated first with a stream of air and then in vacuo. Stock solutions of labeled DMPM were made by dissolving the solid lipid in MeOH/toluene (1:1) and stored at −20 °C. The labeled DMPM was judged to be radiochemically pure by TLC analysis (R_f 0.5, $CHCl_3$/MeOH/acetic acid, 65:15:2).

[2-[U-^{14}C]Palmitoyl]DPPC was prepared as follows. [U-^{14}C]Palmitic acid (928 mCi/mmol, 18 μCi, NEN Research Products) and unlabeled palmitic acid (67 mg) were dissolved in CCl_4 (2 mL). Dicyclohexylcarbodiimide (27 mg) in CCl_4 (0.65 mL) was added, and the solution was kept in a tightly capped tube for 24 h at room temperature. The solution was filtered through a pasteur pipet containing a plug of glass wool. The solvent was removed on a rotary evaporator and acetone (1–2 mL) was added. The suspension was warmed to 40–50 °C to dissolve the solid. The solution was kept at room temperature for 2–3 h, and the acetone was removed from the crystals with a pipet. The solid was dried overnight in vacuo. Dry $CHCl_3$ (0.4 mL, distilled from P_2O_5 under argon) was added, and the solution was transferred to a flask containing 1-palmitoyl-*sn*-glycerol-3-phosphocholine (8 mg, Avanti Polar Lipids) and pyrolidino pyridine (2 mg, Aldrich) in dry $CHCl_3$ (0.1 mL). The flask was tightly capped, and the solution was stirred at 35 °C for 3 h and then room temperature overnight. TLC ($CHCl_3$/MeOH/acetic acid/H_2O, 25:15:4:2) showed that the lysophospholipid had completely reacted. The solution was loaded onto a silica column (1 × 20 cm) and washed with petroleum ether/ether/acetic acid (70:30:1) to remove fatty acid and anhydride. The product was eluted with $CHCl_3$/MeOH/acetic acid/H_2O (25:15:4:2), and the solvent was removed by rotary evaporation to give the product (7.7 mg, 65%), which was judged to be radiochemically pure by TLC on silica with the same solvent.

Isotope Effect Studies in Mixed Micelles. A stock solution of DPPC was prepared for isotope effect studies as follows. Unlabeled DPPC (74 mg) was added to MeOH/$CHCl_3$ (1:1) (1.86 mL). ^{3}H-DPPC (10 μCi) and ^{14}C-DPPC (2 μCi) were added. The solution was kept in a vial fitted with a teflon-lined cap at −20 °C. Aliquots of DPPC stock (100 μL) were transferred to small glass tubes, and the solvent was removed in vacuo for several hours. Mixed micelles were prepared by adding 1 mL of buffer (25 mM HEPES, 12 mM Triton X-

100, 10 mM $CaCl_2$, 0.1 mM EDTA, pH 7.0) to the lipid films and sonicating in a bath sonicator at 40 °C with occasional vortexing until the solutions become clear (typically 30–40 min). The tubes were placed in a water bath at 40 °C, and a small aliquot of cobra venom PLA2 was added. The amount of enzyme added was sufficient to hydrolyze about 20% of the DPPC in 30 min. The reactions were quenched by addition of 1 mL of $CHCl_3$/MeOH/acetic acid (2:4:1) followed by 0.5 mL of $CHCl_3$. The tubes were vortexed and allowed to sit on ice until the layers were fully separated (typically 30 min). The lower organic phases were transferred to clean tubes containing 10 μL of oleic acid. Scintillation counting of small aliquots of the top and bottom phases indicated that greater than 98% of the palmitic acid was extracted into the organic phase. Individual samples were loaded onto silica columns (1 × 5 cm). The columns were washed with petroleum ether/ether/acetic acid (70:30:1), and 5–6 1.5-mL fractions were collected. The fatty acid containing fractions were identified by TLC analysis of small aliquots by using the same solvent and visualizing the oleic acid with iodine vapor. The product-containing fractions plus one earlier and one later fraction were pooled in a scintillation vial. Most of the solvent was removed with a stream of air, and the residues were dried in vacuo for several hours. After elution of the fatty acid, the column was washed with $CHCl_3$/MeOH/acetic acid/H_2O to elute the remaining DPPC. The profile of eluted DPPC was determined by scintillation counting of small aliquots of column fractions. DPPC-containing fractions were pooled, and solvent was removed as described for the fatty acid. Some of the samples were treated with excess PLA2 to convert all of the DPPC to products. In this case, the experiment described above for the 20% conversion was scaled down 5-fold and 100 times as much enzyme was used. The enzymatic reactions were allowed to sit for several hours at 40 °C followed by a second addition of enzyme and a final overnight incubation. Control experiments in the absence of PLA2 showed that the amount of fatty acid produced was always less than 1% (typically 0.2–0.4%) of the total radioactivity present in the sample. For each isotope effect measurement, a minus enzyme control was performed at the same time, and the background cpm's were subtracted from the cpm's obtained in the presence of PLA2.

Double channel scintillation counting to determine the ^3H and ^{14}C was performed with a Beckmann LS 1801 counter with channel 1 recording greater than 99% of the ^3H counts and channel 2 recording less than 1% of the ^3H counts. The fraction of ^{14}C counts appearing in the ^3H channel was determined by counting [^{14}C]palmitic acid that was generated by enzymatic hydrolysis of ^{14}C-DPPC and worked up as described above for the mixed label. This fraction (31%) was used to obtain the actual $^3H/^{14}C$ ratio from the observed counts measured in channels 1 and 2. All vials were counted for 10 min in a cyclic repetitive fashion five to ten times. The fraction of phospholipid converted to products in the partial conversion samples was calculated from the total amount of radioactivity in the fatty acid fraction at both partial and complete substrate conversions. In all cases, $^3H/^{14}C$ ratios were determined from at least five independent experimental runs. The standard error values for the isotope effects were determined as described (Parkin et al., 1984), which takes into account the error of the counting and the standard deviation of multiple determinations.

Isotope Effect Studies in Vesicles. A stock solution of labeled DMPM for isotope effect studies was prepared by mixing appropriate amounts of unlabeled, ^3H-labeled, and ^{14}C-labeled DMPM to give a total lipid concentration of 4 mM with 8 μCi of ^3H and 3 μCi of ^{14}C in 1.7 mL of MeOH/toluene (1:1). Stock solutions were stored at −20 °C in vials fitted with teflon-lined caps. Vesicles were prepared by addition of DMPM stock (105 μL) to a small glass tube. The solvent was removed in vacuo for 30 min, and water (8.4 mL) was added. The lipid film was dislodged from the tube by brief sonication (typically 10 s) in a bath sonicator (Lab Supplies Model G112SPIT). The lipid suspension was frozen and then sonicated until the solution became clear (typically 2–3 min). The sonicated vesicles were stored at room temperature for up to several hours. Prior to an enzymatic hydrolysis, 4 mL of 0.3 mM $CaCl_2$ was placed in a reaction cup of a Radiometer ETS822 pH-stat maintained at 21 °C, and the pH was adjusted to 8.0. An aliquot of vesicle stock (200 μL) was added, and the pH was readjusted to 8.0. Cobra venom or porcine pancreatic PLA2 (190 μg/mL, 50 μL) was added, and the pH was kept at 8.0 by continuous titration with 3 mM NaOH. The vessel was maintained under an argon atmosphere to prevent CO_2 absorption. At the end of the titration, addition of more PLA2 did not produce further consumption of titrant, indicating that all of the vesicles were hydrolyzed. This represents the 100% conversion sample. Partial conversion samples were quenched when the reaction progress reached approximately 20% (based on the amount of titrant used). In this case, 5 times as much vesicle stock (1000 μL) was used so that the same amount of radioactive product was formed in both the partial and complete conversion samples. Additional details of the pH-stat assay have been reported (Jain & Gelb, 1991). The samples were quenched by mixing the reaction solution with 3 mL of $CHCl_3$/MeOH/acetic acid (2:4:1) followed by 1.5 mL of $CHCl_3$. The mixtures were vortexed in a glass tube and centrifuged to separate the layers. The lower phase was transferred to a clean tube containing oleic acid (10 μL), and the extraction was repeated twice. The combined organic extracts were concentrated to a small volume (0.5 mL) with a stream of air and applied to a silica column (1 × 10 cm). The column was washed with petroleum ether/ether/acetic acid (70:30:1), and the myristic acid containing fractions were identified by TLC of small aliquots. Product-containing fractions plus one earlier and one later fraction were pooled in scintillation vials, and solvent was removed with a stream of air and then in vacuo for several hours. Scintillation counting was performed as described above. Control experiments in the absence of PLA2 were performed as described above. In all cases, less than 1% (typically 0.3–0.5%) of the radioactivity present in the samples appeared as free fatty acid in the absence of enzyme.

Hydrolysis of DMPM in Oxygen-18 Water. The hydrolysis of DMPM in oxygen-18 water was carried out as described above for the isotope effect studies using the porcine pancreatic PLA2. The reaction mixture in the pH-stat contained 0.9 mL of DMPM (0.25 mM) and $CaCl_2$ (0.52 mM) in 88% oxygen-18 enriched water (Cambridge Isotopes). All of the vesicles were hydrolyzed by the addition of excess. The reaction was judged to be complete when the further addition of enzyme resulted in no further consumption of titrant. A portion of the reaction mixture (100 μL) was mixed with chilled 50 mM EDTA, pH 7.0 (100 μL). The mixture was extracted with 0.5 mL of chilled MeOH/$CHCl_3$/acetic acid (4:2:1). The lower layer was transferred to a clean tube and a solution of diazomethane in ether was added until the characteristic yellow color persisted. The solution was dried with a stream of N_2 and the residue was taken up in a small amount of ether. The methyl myristate was analyzed by

combined gas chromatography/mass spectrometry on a Hewlett Packard 5790A/5970A GC/MS.

Results

Isotope Effect in Mixed Micelles. Substitution of ^{14}C for ^{12}C at the carbonyl carbon of the fatty acid at the 2-position of a phospholipid may result in a primary kinetic isotope effect on the rate of PLA2-catalyzed ester hydrolysis. Since heavy atom isotope effects are small (typically less than 10%), they are usually measured by a competitive method. Here, the isotope effect is measured as change in the isotopic content of the product over the course of the reaction. In the present study, a double-label method is employed in which phospholipid containing ^3H in a position remote from the site of enzymatic hydrolysis is used to monitor the conversion of ^{12}C material into product. When this ^3H material is mixed with phospholipid labeled at the enzyme-susceptible carbonyl carbon with ^{14}C, the existence of a carbon isotope effect will cause a change in the $^3H/^{14}C$ ratio in the released fatty acid as a function of the fraction of phospholipid converted to product. The isotope effect can be determined from measurements of the $^3H/^{14}C$ ratios in the released fatty acid at a partial and complete conversion of substrate to product. In addition, the fraction of substrate converted to product in the partial conversion sample is also needed. The isotope effect can then be calculated according to the following equations (Parkin et al., 1984; O'Leary, 1980).

$$IE_{obs} = \frac{{}^3P_f/{}^{14}P_f}{{}^3P_1/{}^{14}P_1} \quad (1)$$

$$IE = \frac{\ln [1 - f(IE_{obs})]}{\ln (1 - f)} \quad (2)$$

Here, f is the fraction of substrate converted to product and ${}^3P_f/{}^{14}P_f$ and ${}^3P_1/{}^{14}P_1$ are the ratios of ^3H to ^{14}C in the product at fractional conversions of f and 1.0, respectively. Equation 2 allows calculation of the true isotope effect, IE, from the observed isotope effect, IE_{obs}, at fractional conversion of substrate to products f. The $^3H/^{14}C$ ratios were determined from the counts appearing in the ^3H and ^{14}C windows of the scintillation counter corrected for the counts from ^{14}C material that appeared in the ^3H window. To obtain the most accurate determination of the $^3H/^{14}C$ ratio, the amount of ^3H radioactivity that appeared in the ^3H channel was at least 3 times the amount of ^{14}C that appeared in the ^3H channel (Parkin et al., 1984).

All of the isotope effects measured in this study are given in Table I. By use of the double-label method, the isotope effect on the hydrolysis of DPPC by the cobra venom PLA2 in Triton X-100 mixed micelles was found to be 1.12 ± 0.02 (Table I). A control experiment was carried out in order to establish the validity of the approach described under Materials and Methods for determining heavy atom isotope effects. In this case, use was made of DPPC in which both labels are in isotope-insensitive positions. With this substrate, there should be no change in the isotopic content of the product as a function of fractional reaction extent. Rather than preparing a sample of DPPC that contained palmitic acid with a single ^{14}C label in a position remote from the carbonyl group, it was much less labor intensive to make use of commercially available palmitic acid that is uniformly labeled with ^{14}C in all carbons. In this case, only about 6% of the ^{14}C is at the carbonyl position, and the isotope effect should be essentially abolished. This was found to be the case. The isotope effect measured in this experiment was 1.01 ± 0.01, which indicated that there was no preferential selection of the ^3H substrate over the ^{14}C substrate.

Table I: Heavy Atom Isotope Effects for PLA2[a]

substrate[b]	enzyme	% substrate hydrolyzed[c]	isotope effect[d]
^{14}C-DPPC/^3H-DPPC/ Triton X-100	*Naja naja naja*	21	1.12 ± 0.02
(U-^{14}C)-DPPC/^3H-DPPC/Triton X-100	*Naja naja naja*	24	1.01 ± 0.01
^{14}C-DMPM/^3H-DMPM vesicles	*Naja naja naja*	18	1.00 ± 0.01
^{14}C-DMPM/^3H-DMPM vesicles	porcine pancreatic	26	1.01 ± 0.01

[a] All reported isotope effects were determined from five independent measurements. In each experiment, a minus enzyme control was carried out side by side. [b] See the text for details. [c] Listed are the average values of f for the five independent measurements. [d] The isotope effects were calculated from the observed isotope effects, IE_{obs}, and the f values according to eqs 1 and 2 in the text.

A potential problem with the isotope effect measurements in mixed micelles is that there is likely to be a small amount (less than a few percent) of radiolabel in the 1-position of DPPC resulting from acyl migration during synthesis. If the fraction of label in the 1-position is different for the ^3H-DPPC compared to the ^{14}C-DPPC and if the enzyme is able to release this label, the ratio of ^3H to ^{14}C in the released fatty acid in the 100% conversion sample may be distorted somewhat from the actual ratio of isotopes in the 2-position. Enzymatic release from the 1-position could potentially occur by a recombination of 1 → 2 acyl migration followed by enzymatic hydrolysis, especially during the prolonged reaction times with the larger amounts of PLA2 used in the 100% conversion samples. This concern can be addressed by evaluating the isotope effect from the ratio of ^3H to ^{14}C in the released fatty acid generated after high fractional conversion, i.e., f = 0.6–0.8. In this case the ratio of ^3H to ^{14}C was determined after incubating the DPPC/Triton X-100 mixture for 30 min in the presence of a larger amount of cobra PLA2 than was used in the 20% conversion samples. The incubation times for the low and higher conversion experiments were the same, and the possibility of release of fatty acid from the 1-position of DPPC was minimized. By a suitable manipulation of eqs 1 and 2, the value of IE can be calculated from the ratio of the IE_{obs} values obtained at significantly different f values. Such a ratio is independent of the ^3H to ^{14}C ratio in the fatty acid at f = 1.0. The ratio of IE_{obs} at f = 0.21 to IE_{obs} at f = 0.63 was used to calculate a value of IE = 1.11. Likewise, values of IE_{obs} at f = 0.21 and f = 0.75 yielded a value of IE = 1.10. These values of IE agree well with the value of 1.12 obtained with the 100% conversion data. This indicates that either the differential labeling at the 1-position and/or the enzymatic release from this position is not a significant factor in these experiments.

Isotope Effect in Vesicles. Vesicles made from radiolabeled DMPM were used to measure the carbonyl carbon isotope effect on the hydrolysis by PLA2. The isotope effect was determined exactly as described for the experiments with mixed micelles. In vesicles, the isotope effect was found to be 1.00 ± 0.01 for the cobra venom PLA2 and 1.01 ± 0.01 for the porcine pancreatic enzyme.

Hydrolysis of DMPM Vesicles in Oxygen-18 Water. Vesicles of DMPM in oxygen-18 water were hydrolyzed with the porcine pancreatic PLA2, and the released myristic acid was extracted and converted to the methyl ester by treatment with diazomethane as described under Materials and Methods.

The methyl myristate was analyzed for oxygen-18 content by combined gas chromatography/mass spectrometry. This experiment was carried out in 88% oxygen-18 enriched water. For the fatty acid methyl ester, the ratio of ion counts at $m/e = M + 2$ to that obtained at $m/e = M$ was 7.5, indicating that the fatty acid contained a single oxygen-18 from the solvent. No ion counts above background were observed at $m/e = M + 4$, indicating that very little if any doubly oxygen-18 fatty acid was produced. The sensitivity of the mass spectrometer detector was evaluated by repeating the analysis with 2% as much methyl myristate that was used in the oxygen-18 analysis. A signal over background at $m/e = M + 2$ was easily seen. Thus, it is estimated that the fraction of $M + 4$ material in the methyl myristate was significantly below 2%, which revealed that the released fatty acid contained a single oxygen-18. No doubly oxygen-18 labeled fatty acid could be detected.

Discussion

Isotope Effect in Mixed Micelles. The results given above indicate that the carbonyl-carbon heavy atom isotope effect on the PLA2-catalyzed hydrolysis of phospholipids is much larger in mixed micelles than it is in vesicles. The value of 1.12 for the isotope effect in mixed micelles with the cobra venom enzyme is close to the theoretical maximum for a carbon-14 primary isotope effect of approximately 1.15 (Klinman, 1978). Thus, although the value in mixed micelles is large, it is not an unreasonable number. It is not possible to compare the value of the isotope effect determined here to those from other enzymatic hydrolytic reactions since carbonyl-carbon isotope effects have not been reported for other hydrolases. O'Leary has measured the carbon-13 carbonyl-carbon isotope effects on the nonenzymatic hydrolysis of esters (Marlier & O'Leary, 1981; O'Leary & Marlier, 1979, 1978). The carbon-13 isotope effects on the hydrolysis of methyl benzoate under alkaline and acidic conditions are 1.04 and 1.03, respectively. By use of the Swain relationship (Swain et al., 1958), the carbon-14 isotope effect can be calculated from the carbon-13 isotope effect by raising the latter to a power of 1.9. Thus, the calculated carbon-14 isotope effects on the hydrolysis of methyl benzoate are 1.08 and 1.06 under alkaline and acidic conditions, respectively. Schowen has calculated the carbon-13 isotope effect on the hydration of formaldehyde, and a maximal value of 1.03 was obtained (Hogg et al., 1980).

Precise interpretation of the large carbonyl-carbon isotope effect measured in mixed micelles is difficult. The large effect indicates that there is a substantial change in the bonding to the carbonyl carbon on going from the ground state to the transition state. The value of this isotope effect alone does not help to distinguish whether the formation or breakdown of the tetrahedral intermediate is rate determining. The useful piece of information that emerges is that the carbonyl-carbon isotope effect can be large for the PLA2-catalyzed hydrolysis of esters and that the isotope effect decreases to unity when the enzyme hydrolyzes the substrate in vesicles. This large diminution of the isotope effect has significant implications on the kinetics of interfacial catalysis in vesicles as will be discussed further below.

It is important to note that the isotope effects measured in this study by the competitive double-label method are on k_{cat}/K_{MS}. The large isotope effect seen in the present study with mixed micelles does not establish that the chemical esterolysis step is the overall rate-determining step in the catalytic cycle. With mixed micelles, the observed steady-state reaction velocities are linear for several minutes (Hendrickson & Dennis, 1984). Since each mixed micelle contains only a few phospholipid molecules, the persistent steady-state velocities must be maintained by dynamic events that continuously bring enzyme in contact with fresh substrate. For example, the PLA2 could be rapidly sampling the entire substrate pool by jumping quickly from particle to particle. Alternatively, the enzyme may remain in a single mixed micelle for a period of time sufficient for exchange of substrate and products to occur. For long-chain phospholipids, such exchange would involve the collision between mixed micelles (Nichols, 1988). In the steady-state kinetic analysis of PLA2 operating on mixed micelles (Hendrickson & Dennis, 1984), it has been assumed that these dynamic events are all very fast compared to the catalytic turnover time of the enzymatic reaction. This assumption has not been verified, and it is possible that exchange of components among the ensemble of micelles may be partially controlling the overall steady-state enzymatic velocities that are observed. These aspects of the kinetics of PLA2 acting on micelles have been discussed in detail [Jain and Berg (1989) and the appendix in Berg et al. (1991)]. It is tempting to say that the large heavy atom isotope effect observed in the present study suggests that intermicellar exchange rates do not control the overall rate of the enzymatic reaction; however, this is incorrect. The isotope effect was measured by the competitive method, and even if the intermicellar exchange rate reduces the overall steady-state enzymatic velocity, this process would not have any effect on the relative selection of the ^{14}C versus the ^{3}H containing substrates. Thus the large carbonyl carbon isotope effect measured in this study provides no information about the contribution of the rates of intermicellar exchange to the observed reaction rates. Dynamics of micellar exchange would modulate an isotope effect on k_{cat}; however, there is no practical method for measuring such an isotope effect for PLA2.

Isotope Effect in Vesicles. Before the isotope effect in vesicles is interpreted, a brief discussion of the kinetics of PLA2 acting on vesicles of DMPM is required. A complete discussion is given in the first paper in this series (Berg et al., 1991). Jain and co-workers have shown that PLA2 operates on vesicles of anionic phospholipids, such as DMPM, in the so-called "scooting" mode (Berg et al., 1991; Jain & Berg, 1989; Jain et al., 1986). Here, the enzyme binds irreversibly to the vesicle and hydrolyzes all of the substrate in the outer layer without leaving the interface. In this way, the action of the enzyme within the interface [see the scheme in Figure 1 of Berg et al. (1991)] can be studied without the complications arising from the absorption and desorption of enzyme from the interface. With the low calcium concentrations (0.3 mM) used in the present study, the rate of vesicle fusion is negligible (Jain et al., 1986). Furthermore, and in contrast to mixed micelles, the kinetics of the enzymatic reaction in the scooting mode are not complicated by the exchange of substrate or product between vesicles since the intervesicle exchange rates are extremely slow and can be ignored (Jain et al., 1986; Berg et al., 1991). Thus, with vesicles of DMPM, it is possible to study interfacial catalysis in its "purest" form. In this case, only the steps within the box shown in Figure 1 of Berg et al. (1991) need to be considered.

The action of PLA2 on DMPM vesicles in the scooting mode has been shown to obey the standard Michaelis–Menten formalism (Jain & Berg, 1989; Berg et al., 1991). The reaction progress curve for the hydrolysis of small sonicated vesicles of DMPM in the presence of low concentrations of calcium (0.3–0.6 mM) is first order (Jain et al., 1986; Berg et al., 1991). In large vesicles, the formation of products at

early times changes the surface concentration of substrate (or mole fraction) relatively slowly, and a linear velocity that persists for several minutes is observed. In small vesicles, the steady-state concentration of E*S decreases relatively rapidly, and this causes the slope at all points along the reaction progress curve to decrease in time (Berg et al., 1991). Such a first-order progress curve can be completely described by equation A13 [from the appendix of Berg et al. (1991)].

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

Here, P_t is the amount of product molecules produced after time t, P_{max} is the amount of product molecules produced at the end of the reaction, and k_i is the first-order relaxation constant. If the vesicle-to-enzyme ratio is large (greater than 5 or so), vesicles will contain at most one bound enzyme. Under this condition, the value of P_{max} is simply equal to the moles of enzyme in the reaction mixture multiplied by the number of phospholipids in the outer layer of a vesicle, N_s [typically 13 000 in small unilamellar vesicles prepared by sonication of DMPM in water (Jain & Gelb, 1991)]. The constant k_i is defined by eqs A10 and A2c [from the appendix of Berg et al. (1991)].

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

$$\frac{k_{cat}}{K_{MS}} = \frac{k_1 k_2}{k_{-1} + k_2} \quad \text{(A2c)}$$

Here, K_{MS} is the interfacial Michaelis constant for the substrate, k_{cat} is the maximal reaction velocity per enzyme molecule, and $K_p = k_3/k_{-3}$ is the product dissociation constant. In the present case, the substrate concentration and interfacial K_{MS} are expressed as mole fractions [Appendix, Berg et al. (1991)]. Equation A13 is the usual integrated Michaelis–Menten equation adapted for lipolytic enzymes and under the conditions of first-order kinetics. In deriving eqs A10 and A2c, it was assumed that the rate constant for the reverse of the catalytic step k_{-2} is close to zero. This was validated in the present studies with the oxygen-18 results, as will be discussed below. One of the goals of this study was to probe the values of the interfacial rate constants given in Figure 1 of the first paper in this series (Berg et al., 1991). The usual methods for determining the kinetic rate constants cannot be applied in the present case since the concentrations of components in the interface cannot be easily manipulated [however, see the third paper in this series, Jain et al. (1991)]. As will now be discussed, the heavy atom isotope effect measured in the present study provides some insight into the relative values of the interfacial rate constants.

The heavy atom isotope effect was determined in the present study by the competitive method (^{14}C substrate competes with ^{3}H substrate). Here, the isotope effect is on k_{cat}/K_{MS}, and is given by (Northrop, 1977)

$$^{14}\left(\frac{k_{cat}}{K_{MS}}\right) = \frac{^{14}k_2 + C_f + C_r(^{14}K_{eq})}{1 + C_f + C_r} \quad (3)$$

In this equation, $^{14}(k_{cat}/K_{MS})$ is the observed isotope effect on k_{cat}/K_{MS}, $^{14}k_2$ is the intrinsic isotope effect, C_f is the forward commitment to catalysis, C_r is the reverse commitment to catalysis, and $^{14}K_{eq}$ is the carbon-14 isotope effect on the equilibrium constant for the reaction under study. The intrinsic isotope effect ($^{14}k_2$) is the isotope effect on the collection of elementary reaction steps that involve bond making and breaking with the heavy isotope. The meaning of the other terms in eq 3 have been discussed in detail elsewhere (O'Leary, 1989; Northrop, 1977). It is clear from eq 3 that the value of the observed isotope effect will, in general, be different than the value of the intrinsic isotope effect. This is due to the fact that enzymatic reactions always contain multiple steps and the isotope-sensitive steps may be masked by other steps in the reaction cycle.

For enzymatic reactions containing an irreversible isotope-sensitive step, C_r is zero and eq 3 can be greatly simplified. In the case of PLA2 acting in water, the reaction has never been found to proceed in the reverse direction, that is the resynthesis of a phospholipid ester from a fatty acid and a lysophospholipid has never been detected (however, see below). Actually, this does not say that the enzymatic hydrolysis of the ester is irreversible. This reaction could be reversible on the surface of the enzyme, but the resynthesized ester is never released to the free substrate pool and all of the enzyme substrate complex eventually partitions in the forward direction to generate the fatty acid and lysophospholipid products. To address this issue, we have carried out the hydrolysis of DMPM vesicles in oxygen-18 water. Enzyme-catalyzed attack of solvent water onto the ester will generate the lysophospholipid and a fatty acid containing a single oxygen-18, both bound to the enzyme. If the chemical step reverses, the newly formed ester will retain half of the oxygen-18 present in the fatty acid. Even if the ester is not released from the enzyme, the attack of a second molecule of oxygen-18 water may generate a fatty acid product that is doubly labeled with oxygen-18. The fact that no doubly labeled fatty acid was detected in the present study strongly suggests that the enzyme–substrate complex cannot be regenerated from the enzyme–product complex. In other words, the rate constant for the reversal of the chemical step [k_{-2} in the scheme in Figure 1 of Berg et al. (1991)] is negligible compared to the rate constant for the generation of free enzyme and free products from the enzyme-product complex [k_3, Figure 1 of Berg et al. (1991)]. This is precisely the definition of the reverse commitment to catalysis (O'Leary, 1989; Northrop, 1977), and it is concluded that the value of C_r is close to zero. PLA2-catalyzed hydrolysis of mixed micelles in oxygen-18 water has also been found to generate only singly labeled fatty acid product (Lombardo et al., 1986), demonstrating that C_r in mixed micelles is also close to zero.

Equation 3 can now be simplified under the condition that C_r is zero to give

$$^{14}\left(\frac{k_{cat}}{K_{MS}}\right) = \frac{^{14}k_2 + C_f}{1 + C_f} \quad (4)$$

It is clear from eq 4 that the observed carbon-14 isotope effect will be smaller than the intrinsic isotope effect if the forward commitment to catalysis is greater than 1. As described under Results, the observed isotope effect in mixed micelles is large and close to the theoretical maximum. This indicates that the observed and intrinsic isotope effects are close in value and that the value of C_f is less than 1. In contrast, the isotope effect in DMPM vesicles is not detectable, and this indicates that the value of C_f is much greater than 1. The forward commitment term C_f is defined as the rate constant for the isotope-sensitive step divided by the rate constant for the release of substrate from the enzyme–substrate complex [$C_f = k_2/k_{-1}$, Figure 1 of Berg et al. (1991)]. The value of C_f reflects the tendency of the enzyme–substrate complex to go forward to give the reaction products versus the release of substrate from the enzyme. From the present results it is concluded that $C_f \gg 1$ (i.e., $k_2 \gg k_{-1}$). Thus, in vesicles, once S binds to E*

to give E*S, this Michaelis complex tends to go forward to give E*P rather than backward to regenerate E* + S.

Equation A10 can now be simplified by using the condition that $k_2 \gg k_{-1}$ to give

$$N_s k_f = \frac{k_1}{(1 + 1/K_p)} \quad (5)$$

According to eq 5, the first-order relaxation constant for interfacial catalysis by PLA2 in small vesicles of fixed size, N_s, at low calcium concentrations is a function only of the rate constant for the binding of substrate to the catalytic site on the enzyme, k_1, and the product dissociation constant, K_p. The rate constant for the esterolysis step, k_2, is no longer a part of the interfacial turnover number. If a large carbon-14 isotope effect in vesicles was observed, this would have meant that C_f is less than unity. In this case, $k_{-1} \gg k_2$ and the interfacial rate constant would take on the form

$$N_s k_f = \frac{k_2}{K_s(1 + 1/K_p)} \quad (6)$$

In this case, the rate of esterolysis is small compared to the release of S from the E* and the Michaelis complex, E*S, is in equilibrium with E* and S with a dissociation constant for S given by the K_s term in eq 6. The turnover number would also be influenced by the value of k_2 (eq 6).

All of the kinetic parameters reported in this set of six papers have been examined by more than one independent method, and the values obtained were in good agreement. For example, the conditions on the interfacial kinetic rate constants determined in the present study can be compared to results obtained in the third paper in this series (Jain et al., 1991). Here, the ratio of the interfacial Michaelis constant to the substrate dissociation constant is given by

$$\frac{K_{MS}}{K_s} = \frac{(1 + k_2/k_{-1})}{(1 + k_2/k_3)} \quad (7)$$

These values of K_{MS} and K_s were determined in the third paper in this series to be 0.3 and 0.02, respectively (Jain et al., 1991). Thus, a ratio of K_{MS}/K_S of approximately 10 implies that $k_2 \gg k_{-1}$ (eq 7). This is consistent with the absence of an isotope effect (i.e., large forward commitment to catalysis) seen in the present study for the hydrolysis of DMPM vesicles.

An additional requirement on the rate constants can be obtained from a consideration of the overall equilibrium constant for the esterolysis reaction. In general, the hydrolysis of carboxylic esters at neutral pH in water lies far in the direction of the products. For the PLA2-catalyzed hydrolysis of DMPM vesicles, essentially all of the substrate in the outer monolayer of the vesicles can be hydrolyzed in the presence of a sufficient amount of PLA2 to ensure that every vesicle has at least one bound enzyme (Jain et al., 1986). This shows that the reaction equilibrium lies far in the direction of the fatty acid and lysophospholipid products. The overall equilibrium constant for the conversion of DMPM substrate to products is given by

$$K_{eq} = \frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} = \left(\frac{k_2}{k_{-2}}\right)\left(\frac{K_P}{K_S}\right) \quad (8)$$

The ratio of K_P/K_S was determined in the third paper in this series to have a value of about 1. Since the equilibrium constant for the reaction is large compared to unity, the ratio of k_2/k_{-2} must also be large compared to unity. This result plus the results of the isotope effect and the oxygen-18 studies lead to the following three conditions on the interfacial rate constants for the hydrolysis of DMPM vesicles by the pig PLA2.

$$k_2/k_{-1} \gg 1, \qquad k_2/k_{-2} \gg 1, \qquad k_3/k_{-2} \gg 1$$

These conditions were used in the first paper in this series to determine the values of most of the rate constants given in Figure 1 of Berg et al. (1991).

The results of the present study do not imply that k_{-2} is zero but rather that it is small compared to k_2 and k_3. It is expected that k_{-2} is finite since esterolysis reactions are generally reversible. Indeed, it has been recently shown that PLA2 can catalyze the synthesis of a phospholipid from a fatty acid and a lysophospholipid if the enzymatic reaction is conducted in organic solvent where the equilibrium constant in the esterolysis direction is not large (Pernas et al., 1990).

The results of the present study are helpful in understanding previous observations reported for the action of PLA2 on phospholipids. For example, on the basis of eq 5, it can be predicted that the substrate specificity of PLA2 acting in a vesicle interface will be controlled not so much by the chemical step, k_2, but by the kinetics of S binding to E*. Roberts and co-workers recently studied the action of PLA2 on bilayers of long-chain phospholipids containing small amounts of short-chain phospholipids such as diheptanoylphosphatidylcholine (Gabriel et al., 1987). In these mixed vesicles, the short-chain substrates were preferentially hydrolyzed. It is clear from the structure of the PLA2 that the substrate must become dislodged from the interface to bind in the catalytic site on the enzyme. This is because the catalytic residues of the enzyme lie in a binding cavity and are some 15 Å away from the surface of the protein, which lies on the interface (Verger & de Haas, 1976). Since the short-chain substrates are expected to become dislodged from the surface of the bilayer much more readily than the long-chain phospholipids, it is reasonable to propose that the rate constant for the dislodging of substrate, k_1, is controlling the substrate specificity of PLA2 according to eq 5. If the substrate selection were controlled by the equilibrium constant, K_s, according to eq 6, one would expect only small differences in the rate of hydrolysis of short-chain versus long-chain substrates. In a different study, it was recently found that short-chain phosphonate-containing phospholipid analogues are tight-binding inhibitors of interfacial catalysis by PLA2 in the scooting mode (Jain et al., 1989). In the same system, long-chain phosphonates were significantly weaker inhibitors (the IC_{50} values for inhibition dropped over 10-fold when the chain length of the inhibitor was changed from 8 to 16 carbons) (Jain and Gelb, unpublished results). Again, from the results of the present study, it is proposed that the ease of dislodging of the inhibitor from the interface is important in determining the degree of inhibition. In constrast, both short- and long-chain fluoro ketone-containing phospholipid analogues (Yuan et al., 1987) and phosphonate-containing phospholipid analogues (Yuan, 1990) were found to be equally potent as inhibitors of PLA2 when acting in mixed micelles. These observations are consistent with the view that the binding of S to E* is not the difficult step in mixed micelles where the forward commitment, C_f, is small.

The isotope effects determined in this study shed some light on the nature of the rate-determining step for the PLA2-catalyzed hydrolysis of vesicles. Two points emerge that may be of general application to the understanding of lipolytic enzymes. First, the substrate specificity of a lipolytic enzyme bound to a membrane interface may be controlled by the relative ease of removal of the various substrates present from the plane of the interface. Second, in designing potent in-

hibitors of interfacial catalysis, not only should the interactions of the inhibitor with the catalytic site on the enzyme be considered but the ease of dislodging the compound from the interface should also be stressed.

ACKNOWLEDGMENTS

We are grateful to Professors Mahendra Jain, Otto Berg, and Daniel Quinn for helpful discussions that made this work possible.

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