Kinetic Characterization of *Escherichia coli* Outer Membrane Phospholipase A Using Mixed Detergent–Lipid Micelles

Anton J. G. Horrevoets,* Tilman M. Hackeng, Hubertus M. Verheij, Ruud Dijkman, and Gerard H. de Haas
Department of Biochemistry, State University of Utrecht, Transitorium 3, Padualaan 8, 3584 CH Utrecht, The Netherlands
Received June 14, 1988; Revised Manuscript Received August 26, 1988

ABSTRACT: The substrate specificity of *Escherichia coli* outer membrane phospholipase A was analyzed in mixed micelles of lipid with deoxycholate or Triton X-100. Diglycerides, monoglycerides, and Tweens 40 and 85 in Triton X-100 are hydrolyzed at rates comparable to those of phospholipids and lysophospholipids. *p*-Nitrophenyl esters of fatty acids with different chain lengths and triglycerides are not hydrolyzed. The minimal substrate characteristics consist of a long acyl chain esterified to a more or less hydrophilic headgroup as is the case for the substrate monopalmitoylglycol. Binding occurs via the hydrocarbon chain of the substrate; diacyl compounds are bound three to five times better than monoacyl compounds. When acting on lecithins, phospholipase A1 activity is six times higher than phospholipase A2 activity or 1-acyl lysophospholipase activity. Activity on the 2-acyl lyso compound is about two times less than that on the 1-acyl lysophospholipid. The enzyme therefore has a clear preference for the primary ester bond of phospholipids. In contrast to phospholipase A1 activity, phospholipase A2 activity is stereospecific. Only the L isomer of a lecithin analogue in which the primary acyl chain was replaced by an alkyl ether group is hydrolyzed. The D isomer of this analogue is a competitive inhibitor, bound with the same affinity as the L isomer. On these ether analogues the enzyme shows the same preference for the primary acyl chain as with the natural diester phospholipids. Despite its broad specificity, the enzyme will initially act as a phospholipase A1 in the *E. coli* envelope where it is embedded in phospholipids.

In the *Escherichia coli* cell envelope degradation and synthesis of lipids are strictly separated. Synthesis of phospholipids occurs exclusively at the inner or cytoplasmic membrane, as is the case for proteins and lipopolysaccharides. In the outer membrane only breakdown of lipids occurs (Albright et al., 1973; Bell et al., 1971). Several different catabolic activities have been found in the *E. coli* outer membrane, namely, phospholipase A1, phospholipase A2, and 1-acyl- and 2-acyllysophospholipase, as well as lipase activity (Albright et al., 1973). Biochemical studies indicate that all these activities presumably reside in one single enzyme, the so-called detergent-resistant phospholipase A1 (Patriarca et al., 1972; Doi et al., 1972; Doi & Nojima, 1973, 1974). This enzyme is a membrane-bound protein of 29 kDa, which can only be isolated from the envelope with the aid of detergents (Scandella & Kornberg, 1971; Nishijima et al., 1977; de Geus et al., 1986). The enzyme is located exclusively in the outer membrane [see, e.g., Albright et al. (1973)] although there are reports of an accumulation in the inner membrane/outer membrane attachment sites (Bayer et al., 1982). The function of the *E. coli* outer membrane phospholipase (OM PLA)[1] remains obscure since no appreciable turnover of outer membrane lipids can be detected in intact growing *E. coli* cells (Audet et al., 1974), implying that OM PLA is dormant in its natural state. Mutants lacking OM PLA grow as wild-type strains, whereas mutants which overproduce OM PLA do not exhibit increased membrane lipid turnover (Homma et al., 1984), stressing the fact that the enzyme is under strict regulation. High OM PLA activity can, however, be triggered by damage to the *E. coli* membrane brought about by, e.g., phage binding (Cohen et al., 1970), spheroplast formation (Patriarca et al., 1972), temperature shock (de Geus et al., 1983), or the action of detergent-like peptides like polymyxin B (Weiss et al., 1979).

Our interest in OM PLA was raised by two intriguing phenomena: first, the combination of several different lipolytic activities residing in one enzyme; second, the regulation mechanisms which must exist to restrict the membrane-bound phospholipase from destroying the membrane, since this lipid bilayer is not only the enzyme's natural environment but also its substrate.

Since previous studies on OM PLA (Scandella & Kornberg, 1973; Tamori et al., 1979) were severely hampered by the low abundance of the phospholipase in the *E. coli* envelope, we chose to clone *pldA*, the gene encoding OM PLA (de Geus et al., 1983). After overproduction of *pldA*, we were able to purify sufficient amounts of pure enzyme (de Geus et al., 1986) to investigate the properties of this enzyme in more detail. In this first characterization we set out to elucidate the substrate specificity of OM PLA, trying to evaluate the criteria that make a compound a suitable substrate for this enzyme.

Experimental Procedures

Materials. Triton X-100 and C12-sulphobetain were from Serva; sodium deoxycholate (DOC) was from Merck. Tweens 40 and 85 and *p*-nitrophenyl esters with different acyl chains were from Sigma. Intralipid 20% was obtained from Kabi-Vitrum (Stockholm, Sweden). All other chemicals were of analytical quality.

Lipids. Egg lecithin was purified from hen eggs according to standard procedures. The lecithin was converted to phosphatidylglycerol with phospholipase D as described by Comfurius and Zwaal (1977). Lecithin was converted to lysolecithin with phospholipase A2 (*Crotalus adamanteus*) or to diglyceride with phospholipase C (*Clostridium welchii*). *E.*

* To whom correspondence should be addressed.

[1] Abbreviations: OM PLA, *E. coli* outer membrane phospholipase; DOC, sodium deoxycholate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; cmc, critical micellar concentration; cac, critical activity concentration.

0006-2960/89/0428-1139$01.50/0

coli phosphatidylethanolamine was isolated from an *E. coli* lipid extract (PE IX, Sigma) on a CM-cellulose column (Comfurius & Zwaal, 1977). All other lipids were synthesized in our laboratory according to standard procedures. All lipids used in this study showed only a single spot upon thin-layer chromatographic analysis on HPTLC Kieselgel Platten (Merck) using as the solvent system chloroform–methanol–water (65:25:4 or 65:35:8 by volume) or ether–hexane (1:1 v/v).

Protein. The *E. coli* outer membrane phospholipase (OM PLA) was purified to homogeneity and freed from endogenous lipids as described by de Geus et al. (1986). The purified enzyme was stored as 1 mg/mL samples at −20 °C in a buffer composed of 10 mM Tris-HCl, pH 8.3, 2 mM EDTA, and 2.5 mM the detergent C12-sulphobetain. No decrease in specific activity was observed upon storage for several months.

Assays. Metal ion requirement was measured according to the chromogenic PLA assay (de Geus et al., 1986) with the modification that calcium was omitted from the buffer. Kinetic binding constants for Ca^{2+} and Sr^{2+} were determined by measuring the specific activity of OM PLA at different metal ion concentrations. Competitive binding constants for Ba^{2+} and Mg^{2+} were determined by measuring the binding of calcium in the presence of fixed concentrations of either metal ion. All data were analyzed by the use of Eadie–Hofstee plots.

pH dependence of hydrolysis was measured in two independent assays. One system comprised the use of the titrimeric assay as described below with the substrate 1-palmitoyl-propanediol-3-phosphocholine (1 mM). Alternatively, a modification of the chromogenic assay (de Geus et al., 1986) was used with the substrate *rac*-1,2-bis(hexanoylthio)phosphatidylcholine and a buffer composed of 50 mM Tris, 25 mM Hepes, and 50 mM sodium acetate.

Titrimetric Assay. Activity of OM PLA was measured by determining the free fatty acids released by hydrolysis of the substrate with a Radiometer titration set (PHM-84 research pH meter, TTT-80 titrator, ABU-80 autoburet, TTT-60 titration assembly, and a REC-80 servograph). Unless stated otherwise, the assay compartment contained 2.5 mL of buffer (5 mM Tris-HCl, pH 8.3, 10 mM $CaCl_2$) and 5 mM substrate solubilized with either 8 mM DOC or 12.8 mM Triton X-100. The substrate dissolved in methanol was premixed with the detergent before addition to the test buffer. The final methanol concentration never exceeded 5% and was shown to have no influence on hydrolysis rates. The assays were carried out under nitrogen at 25 °C. Routinely 5 or 10 μg of OM PLA was injected after a stable base line was obtained for 1–2 min. Activity was shown to be proportional to the amount of added protein in the range of 1–20 μg of OM PLA.

Positional Preference and Stereospecificity. OM PLA catalyzed hydrolysis of several mixed-acid phospholipids was followed in time, to determine whether the enzyme showed positional preference or stereospecificity when acting on phospholipids. Incubation was started by injecting 10 μg of OM PLA into a mixed micellar solution of phospholipid (5 mM) and DOC (10 mM) in a buffer composed of 50 mM Tris-HCl, pH 8.3, and 10 mM $CaCl_2$ at 25 °C.

In an alternative experiment OM PLA was incorporated into vesicles made by injection of a concentrated solution of 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine into the assay buffer at 37 °C to a final lipid concentration of 5 mM. No enzymatic hydrolysis could be detected even upon prolonged incubation. Hydrolysis was started by addition of DOC, Triton X-100, or ethanol to final concentrations of 10 mM, 12.8 mM, and 25 or 50% (v/v), respectively. At different time intervals 400-μL samples (2 μmol of lipid) were taken from the incubation mixture, and the reaction was quenched by the addition of 1 volume of chloroform–methanol–acetic acid (1:2:0.5 by volume), and 2 μmol of heptadecanoic acid was added as internal standard. The phases were separated by the addition of chloroform after which the components in the organic phase were separated on DC 60 Kieselgel Platten (Merck) with the elution solvent chloroform–methanol–water (65:25:4 by volume). After visualization by spraying with a Rhodamine G solution and UV light, the spots were scraped off and heated for 2 h at 70 °C in methanol containing 5% sulfuric acid, thereby converting all fatty acids to their methyl esters. After esterification water was added, and the fatty acid methyl esters were extracted with *n*-pentane. The pentane fractions were neutralized by washing with 50 mM $NaHCO_3$, dried on K_2CO_3, and taken to dryness under a stream of nitrogen. The fatty acid methyl esters were then dissolved in trimethylpentane for GLC analysis. The mixed-acid phospholipids were checked by fatty acid analysis both before and after phospholipase A2 hydrolysis. All compounds contained less than 5% of the positional isomer.

Competitive Binding. Relative binding constants for OM PLA substrates were determined by measuring the hydrolysis rates of these substrates in the titrimetric assay in the presence of the inhibitor *n*-octadecylphosphocholine. Substrate and inhibitor were mixed at varying molar ratios in methanol and premixed with Triton X-100 before addition to the assay buffer solution (5 mM Tris-HCl, pH 8.3, 10 mM $CaCl_2$). The fixed total lipid concentration of substrate plus inhibitor was 5 mM; Triton X-100 concentration was 12.8 mM. After a stable base line was obtained, 10 μg of OM PLA was injected. The initial hydrolysis rates were used to calculate the specific activity of OM PLA.

Results

Metal Ion Requirement. OM PLA absolutely requires calcium ions for its catalytic activity. When in the continuous chromogenic assay system EDTA is added in an equimolar amount to the calcium present, hydrolysis immediately stops. After injection of additional calcium, hydrolysis regains its initial rate. The dissociation constant of OM PLA for calcium ($K_{Ca^{2+}}$) was determined kinetically. Furthermore, it was checked whether the other alkaline earth metals magnesium, strontium, and barium could replace calcium as a cofactor. The OM PLA has a low dissociation constant for calcium of 10–15 μM with a maximal specific activity of 70 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Calcium can be replaced by strontium, albeit with a 10 times higher dissociation constant (120 μM) and a lower maximal hydrolysis rate (15 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). Neither magnesium nor barium could replace calcium as a catalytic cofactor. Eadie–Hofstee plot analysis of calcium binding in the presence of several fixed concentrations of magnesium or barium showed that these two ions are competitive inhibitors of calcium with dissociation constants of 0.9 mM (Mg^{2+}) and 0.3 mM (Ba^{2+}).

pH Dependence of Hydrolysis. The pH dependence of OM PLA catalyzed hydrolysis was checked in two different assay systems as described under Experimental Procedures. Both systems gave comparable results: OM PLA catalyzed hydrolysis exhibits an alkaline pH optimum between pH 8.0 and pH 9.0, with a sharp drop in hydrolysis rates below pH 8.0. The inflection point lies approximately at pH 7.0. Kinetic measurements were therefore routinely performed at pH 8.3.

Detergent-Dependence of Hydrolysis Rates. Preliminary studies in our laboratory showed OM PLA to have an ex-

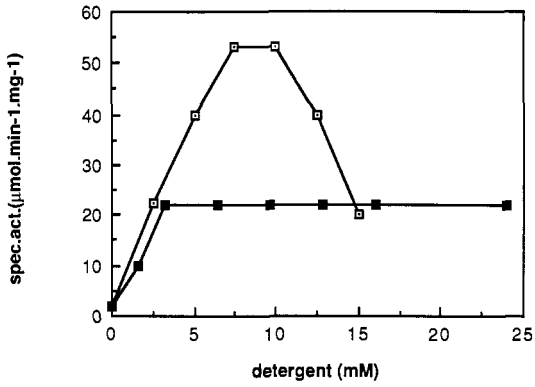

FIGURE 1: Influence of detergent concentration on egg lecithin hydrolysis by OM PLA. Conditions: titrimetric assay (see Experimental Procedures) at 5 mM egg lecithin concentration and varying concentrations of DOC (⊡) or Triton X-100 (■). The assay buffer consisted of 5 mM Tris-HCl, pH 8.3, and 10 mM $CaCl_2$; 10 μg of OM PLA was added to each test.

tremely low activity on intact phospholipid liposomes, although it readily can be incorporated into bilayers (data not shown). Activity could be triggered by the addition of detergents, the reason why we chose for this comparative study on substrate specificity the mixed micellar approach, thereby circumventing also differences in physical behavior of the various substrates. Since DOC and Triton X-100 give rise to morphologically quite different mixed micelles with phospholipids (Mazer et al., 1980; Dennis, 1974), both detergents were used in this study. The dependence of OM PLA catalyzed hydrolysis of egg lecithin on detergent concentration is shown in Figure 1. The data shown in Figure 1 were obtained at an egg lecithin concentration of 5 mM, but a similar detergent dependence was seen at egg lecithin concentrations ranging from 1 to 20 mM: both with DOC and with Triton X-100, optimal activity is reached at molar ratios of detergent to phospholipid between 1:1 and 2:1. These are in fact the known critical molar ratios for both DOC and Triton X-100, for incorporation of all bilayer phospholipid into mixed micelles (Mazer et al., 1980; Dennis, 1974). A striking difference between both detergents is that at molar ratios exceeding 2:1 DOC inhibits OM PLA activity, whereas in the presence of Triton X-100 hydrolysis rates are constant up to ratios of at least 5:1. Furthermore, initial hydrolysis rates at 1 mM egg lecithin concentration were the same as at 5 mM and higher concentrations, indicating that an egg lecithin concentration of 5 mM is indeed saturating for OM PLA. There does not seem to occur a "surface dilution" effect as observed for the snake venom phospholipases, when the ratio of Triton X-100 to phospholipid is increased (Deems et al., 1975). This indicates that the affinity of OM PLA for Triton X-100 compared to its affinity for egg lecithin must be very low. This may be illustrated by the observation that when the detergent C12-sulphobetain (a phospholipid analogue) was used to solubilize the egg lecithin, a decrease in hydrolysis rates was observed upon increasing the ratio of detergent to lipid (data not shown). Stability tests in which OM PLA was preincubated in detergent-containing assay buffer before the substrate was added showed OM PLA to be stable in Triton X-100 for at least 10 min whereas DOC readily and irreversibly inactivated OM PLA. This is also reflected in the time curves of hydrolysis, which are straight for Triton X-100 for at least 10 min, whereas rates in DOC started to level off within 5 min. Lysolecithin, a micelle-forming lipid, showed a different behavior than diacyl phospholipids. OM PLA is active on micelles of this monoacyllecithin alone, but Triton X-100 increased rates about 2-fold. DOC neither stimulated nor inhibited hydrolysis.

Positional Preference and Stereospecificity of OM PLA. Several enantiomeric mixed-acid phospholipids were used to check both positional preference and stereospecificity of OM PLA. The hydrolysis of phospholipids in mixed micelles with DOC was followed by determining the release of fatty acids and the accumulation of the corresponding lyso compounds as is shown in Figure 2.

From 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine mainly the ester bond at the primary position is hydrolyzed, resulting in an accumulation of myristoyllysolecithin, which

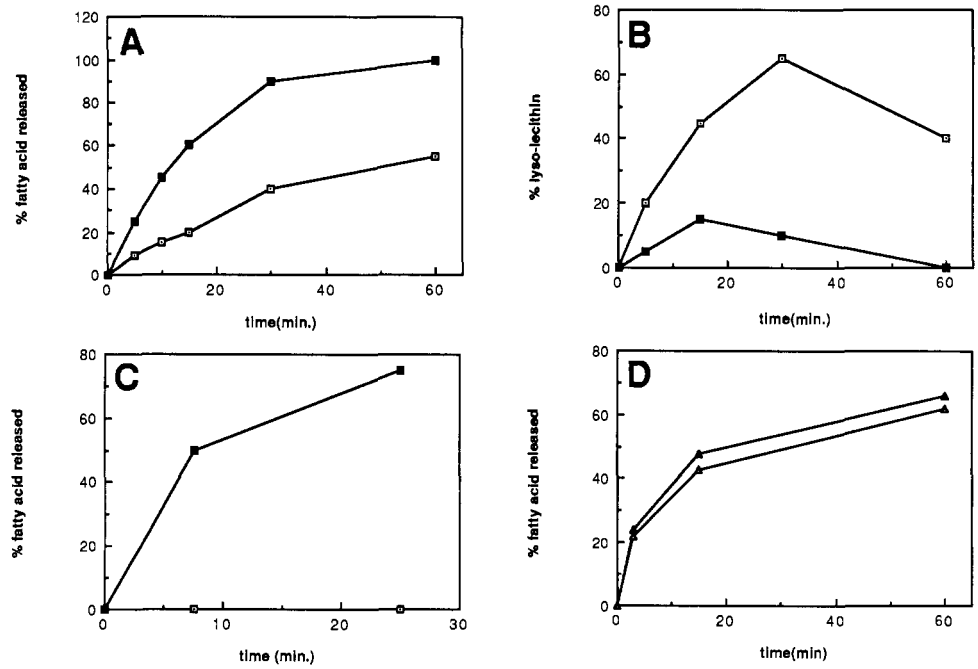

FIGURE 2: Hydrolysis of mixed-acid lecithins by OM PLA as a function of time. Plotted are the amounts of fatty acid as percentage of the initial amount of phospholipid. (Panel A) 1-Palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; (panel B) fatty acid composition and amounts of lysophospholipids resulting from the hydrolysis represented in panel A; (panel C) *rac*-1-palmitoyl-2-myristoylbutanetriol-4-phosphocholine; (panel D) 1-oleoyl-3-stearoyl-*sn*-glycero-2-phosphocholine. Symbols: (■) palmitic acid; (⊡) myristic acid; (△) oleic acid; (▲) stearic acid. For experimental details see Experimental Procedures.

is then slowly hydrolyzed, to glycerophosphocholine and fatty acid (Figure 2A,B). Since a significant amount of palmitoyllysolecithin is found, OM PLA exerts besides phospholipase A1 also phospholipase A2 activity. OM PLA clearly exerts also 1-acyl lysophospholipase and 2-acyl lysophospholipase activity since both compounds are readily hydrolyzed. To exclude effects caused by a possible acyl specificity of OM PLA, an identical experiment was performed with the positional isomer 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine as substrate. OM PLA showed the same preference for the primary acyl chain as presented in Figure 2A,B, indicating that this preference is not caused by acyl specificity of the enzyme. From Figure 2A,B an estimate can be made concerning the relative activities of OM PLA when acting on the different positions of a natural lecithin and on the resulting lyso compounds. Phospholipase A1 activity is about six times higher than phospholipase A2 or 1-acyl lysophospholipase activity. Activity on the 2-acyllysolecithin is somewhat lower than that on the 1-acyl lyso compound. The main difficulty in quantifying the lysophospholipase activities is the indistinguishability of hydrolysis of the 2-acyl lyso compound and hydrolysis of the same compound after migration of the acyl chain from the secondary to the primary position. This migration has been reported to be quite fast at pH 8.3 (Albright et al., 1973). To verify both 1-acyl and 2-acyl lysophospholipase activity of OM PLA more quantitatively, we tested the deoxy lyso analogues D/L-1-palmitoylpropanediol-3-phosphocholine and D/L-2-palmitoylpropanediol-1-phosphocholine in which no acyl migration is possible. OM PLA hydrolyzes the 1-acyl lysophospholipid two times faster than the positional isomer (Table II). If DOC is replaced by Triton X-100 or ethanol, similar results are obtained with only small differences in OM PLA lysophospholipase activity. A less complex picture is obtained with 1-palmitoyl-2-myristoylbutanetriol-4-phosphocholine as substrate (Figure 2C). Initially, OM PLA hydrolyzes only the primary ester bond of this substrate. The secondary acyl chain of the lyso compound is only released upon overnight incubation. This could indicate that acyl chain migration to the primary position has to occur before this lyso compound can be hydrolyzed.

After the positional preference of OM PLA was determined, a possible stereospecificity of the hydrolysis was checked. The already-mentioned 2-acyl lyso analogue D/L-2-palmitoylpropanediol-1-phosphocholine was completely hydrolyzed, indicating that OM PLA does not act stereospecifically on this monoacyllecithin. With a D-lecithin as substrate, the primary ester bond was cleaved with the same rate as in an L-lecithin, whereas the secondary acyl chain was released somewhat slower. In the isomeric β-lecithin 1-stearoyl-3-oleoyl-*sn*-glycero-2-phosphocholine, both acyl chains were released at the same rate (Figure 2D). Both oleoyl and stearoyl lysophospholipids were released in equal amounts and were hydrolyzed at a lower rate. Therefore, OM PLA is not stereospecific when acting on the primary ester bond of a lecithin, whereas hydrolysis of the secondary ester bond seems to be sensitive to the chirality of the substrate. Since OM PLA exerts both phospholipase A1 and A2 activities and also degrades the resulting lysolecithins, it is impossible to clearly distinct these activities with a natural diester phospholipid as substrate. Lecithins containing alkyl ethers are nearly indistinguishable from their diester analogues with respect to both structure (McKeone et al., 1986) and binding by water-soluble phospholipases (deBose et al., 1983). Therefore, substrates were tested in which either the primary or secondary ester bond was replaced by a nonhydrolyzable ether bond, to clearly distinct phospholipase A1 from phospholipase A2 activity. The substrate with a blocked secondary position, DL-1-palmitoyl-2-*O*-myristyl-*sn*-glycero-3-phosphocholine, is completely degraded to lysophospholipid. The positional isomer with a blocked primary position showed a different behavior: the L form, 1-*O*-myristyl-2-palmitoyl-*sn*-glycero-3-phosphocholine, was readily degraded by OM PLA whereas the D isomer was not hydrolyzed at all. The same stereospecificity of phospholipase A2 activity was observed when instead of an alkyl ether moiety either an amide-linked acyl chain or a benzyl group was present at the primary position.

Table I: Specific Activities of OM PLA Acting on Long-Chain Diacyl Substrates[a]

substrate	specific activity (μmol·min^{-1}·mg^{-1}) DOC	Triton X-100
egg lecithin	55	22
L-1-palmitoyl-2-myristoylglycero-3-PN[b]	55	23
L-1,2-dimyristoylglycero-3-PN	53	23
L-1,2-dilauroylglycero-3-PN	7	0
L-1,2-dicaproylglycero-3-PN	0	0
L-1-*O*-myristyl-2-palmitoylglycero-3-PN	65	20
D/L-1-palmitoyl-2-*O*-myristylglycero-3-PN	91	55
D/L-1,3-dipalmitoylglycero-2-PN	57	28
D/L-1-palmitoyl-2-myristoylbutanetriol-4-PN	50	16
phosphatidylethanolamine (*E. coli*)	60	35[d]
phosphatidylglycerol (from egg lecithin)	37	26[d]
diglyceride (from egg lecithin)	30	10[d]
Tween 85 (trioleate)	22[c]	28

[a] Assay conditions: titrimetric assay; 5 mM lipid and 8 mM DOC or 12.8 mM Triton X-100. [b] PN: phosphocholine. [c] No DOC present. [d] 24 mM Triton X-100.

To check whether the absence of OM PLA activity on the D isomer, 3-*O*-myristyl-2-palmitoyl-*sn*-glycero-1-phosphocholine, was caused by a lack of binding, hydrolysis rates were determined on mixtures of varying mole fractions of the L and D isomers at a fixed total lipid concentration of 5 mM in mixed micelles with 12.8 mM Triton X-100. Rates of L isomer hydrolysis decreased linearly with increasing mole fractions of the D isomer. Therefore, the D isomer, although not hydrolyzed by OM PLA, is bound with the same affinity as the L isomer. When rates were determined in mixtures of DL-1-palmitoyl-2-*O*-myristyl-*sn*-glycero-3-phosphocholine and DL-1-*O*-myristyl-2-palmitoyl-*sn*-glycero-3-phosphocholine, a difference was seen between the two positional isomers. The substrate with the alkyl ether moiety at the secondary position was bound about 2 times better and hydrolyzed 5 times more rapidly than the positional analogue with the alkyl ether on the primary position. Therefore, OM PLA distinguishes between the two acyl chains of a phospholipid, with a clear preference for the primary acyl chain.

Kinetic Analysis of OM PLA Catalyzed Hydrolysis. Hydrolysis rates of different substrates were determined according to the titrimetric assay. The data presented for OM PLA catalyzed hydrolysis of mixed micelles in Tables I and II only represent experiments performed at 5 mM substrate concentration. For all substrates presented, hydrolysis rates did not change at substrate concentrations ranging from 1 to 10 mM, indicating that a concentration of 5 mM is indeed saturating. The detergent dependence of OM PLA catalyzed hydrolysis was comparable to the data presented for egg lecithin in a previous section. For clarity, the substrates are divided in four classes: (1) long-chain diacyl lipids; (2) long-chain monoacyl lipids; (3) short-chain lecithins; (4) miscellaneous esters.

(*1*) *Long-Chain Diacyl Lipids.* Specific activities of OM PLA acting on long-chain diacyl lipids are listed in Table I.

Table II: Specific Activities of OM PLA Acting on Long-Chain Monoacyl Substrates

substrate	cac (mM)[a]	specific activity (μmol·min^{-1}·mg^{-1}) at cac[b]	specific activity (μmol·min^{-1}·mg^{-1}) Triton X-100[c]
egg lysolecithin	0.26	9.5	17.5
D/L-1-palmitoylpropanediol-3-PN[d]		18.5	18
D/L-2-palmitoylpropanediol-1-PN		0	8.5
D/L-1-palmitoylpropanediol-2-PN		10.5	10
(hexadecanoylthio)glycol-PN	0.03	70	52
palmitoylglycol-PN	0.13	35	16.5
myristoylglycol-PN	0.2	29	15
lauroylglycol-PN	0.6	17.5	0
caproylglycol-PN	1.5	15	0
monopalmitoylglycol		17.5	22
Tween 40 (monopalmitate)		38.5	15

[a] cac: critical activity concentration (see text for explanation). [b] Assay conditions: titrimetric assay; incubations started at 2 mM substrate concentration in the absence of detergents; maximal end rates (at cac) used to calculate the specific activity. [c] Activities at 5 mM substrate concentration and 12.8 mM Triton X-100. [d] PN: phosphocholine.

In the presence of Triton X-100 hydrolysis rates of substrates with acyl chains of more than 12 carbon atoms are quite similar, ranging from 10 to 55 μmol min^{-1} mg^{-1}. In general, each lipid gave about two times higher rates when DOC was used instead of Triton X-100, but again, a 5-fold variation in rates was observed. The negative phosphate group is not at all required since diglyceride as well as Tween 40 is hydrolyzed at rates comparable to those of phospholipids. This seems to indicate that the way of solubilization of the acyl chains of the substrate determines the hydrolysis rate and not the structure of the headgroup.

(2) *Long-Chain Monoacyl Lipids.* Specific activities of OM PLA acting on monoacyl lipids are listed in Table II. In mixed micelles with Triton X-100 rates are similar to those of diacyl lipids. The progress curves of the hydrolysis of these monoacyl substrates in mixed micelles were straight for at least 10 min. Also, it was confirmed that the reaction went to completion; i.e., eventually all substrate was hydrolyzed as calculated from the amount of NaOH added. In the absence of detergents these micelle-forming lipids give rise to peculiar phenomena. When OM PLA was injected at high substrate concentration, low initial rates were observed which increased gradually as more of the substrate was hydrolyzed (see Figure 3A). Hydrolysis abruptly stopped when a certain remaining substrate concentration was reached, which will be named hereafter the critical activity concentration (cac). The percentage of the total amount of the substrate than can be hydrolyzed by OM PLA is clearly related to the cmc of each individual substrate: substrates with shorter acyl chains will exhibit higher cmc values and as shown in Table II give rise to higher final remaining substrate concentrations, i.e., cac values. An addition of substrate immediately after reaching this abrupt stop of hydrolysis at the cac resulted in normal rates, indicating that OM PLA was still fully active. When the time interval between reaching the cac and the addition of more substrate was increased, hydrolysis rates decreased until finally no activity could be detected, even upon addition of an excess of monopalmitoylglycol. Apparently prolonged storage of OM PLA at these low amphiphile concentrations results in denaturation of this water-insoluble enzyme. The cause for this peculiar behavior seems to lie in the fact that these amphiphiles do not simply serve as substrates for OM PLA, but are also necessary to solubilize and stabilize the enzyme as illustrated by the following results obtained for palmitoylglycol-

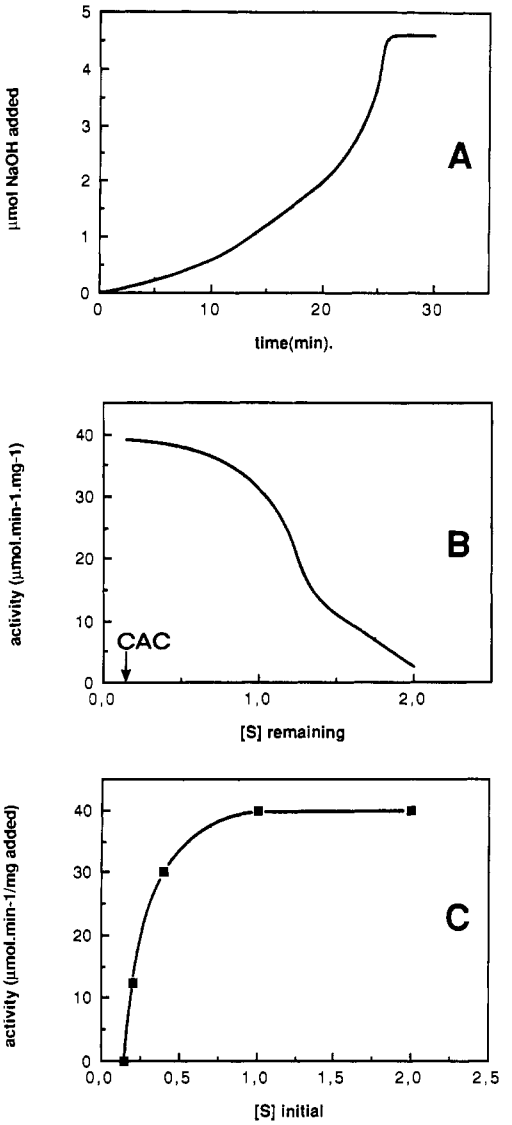

FIGURE 3: Influence of monoacyl phospholipid concentration on the activity of OM PLA. (Panel A) Progress curve of the hydrolysis of 5 μmol of palmitoylglycolphosphocholine at an initial concentration of 2 mM; (panel B) replot of panel A showing the activity of OM PLA as function of the remaining substrate concentration [cac: critical activity concentration (for explanation, see text)]; (panel C) maximal activity of OM PLA at the cac as a function of the initial palmitoylglycolphosphocholine concentration. Conditions: titrimetric assay without detergent; buffer of 5 mM Tris-HCl, pH 8.3, and 10 mM $CaCl_2$; 10 μg of OM PLA added per assay.

phosphocholine. (All other substrates gave comparable results.)

From Figure 3A one can calculate the specific activity of OM PLA as a function of the remaining substrate concentration, as deduced from the amount of added NaOH (Figure 3B). OM PLA activity is maximal at remaining substrate concentrations near the cac. One would therefore expect initial hydrolysis rates to be higher at lower initial substrate concentrations. When in the case of palmitoylglycolphosphocholine OM PLA was added to the test at decreasing initial substrate concentrations between 2 and 1 mM, initial hydrolysis rates were indeed higher at lower initial substrate concentrations as can be expected from Figure 3B. Final rates at the cac were nevertheless the same as is shown in Figure 3C. At initial substrate concentrations below 1 mM progress curves of the hydrolysis became almost straight as predicted by Figure 3B. However, final rates at the cac decreased with decreasing initial substrate concentration as depicted in Figure

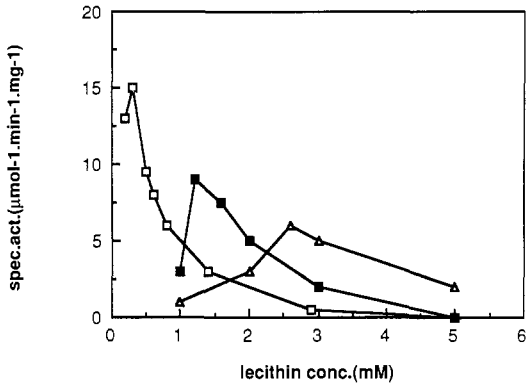

FIGURE 4: Influence of short-chain lecithin concentration on initial hydrolysis rates by OM PLA. Conditions were as follows: (□) L-1,2-Dioctanoyllecithin (cmc: 0.2 mM) and (■) L-1,2-diheptanoyllecithin (cmc: 1.5 mM): titrimetric assay without detergent with a buffer composed of 5 mM Tris-HCl, pH 8.3, and 10 mM $CaCl_2$; 10 μg of OM PLA added per assay. (△) *rac*-1,2-Bis(hexanoylthio)phosphatidylcholine (cmc: 1.2 mM): chromogenic assay with a buffer composed of 25 mM Tris-HCl, pH 8.3, and 10 mM $CaCl_2$; 0.2 μg of OM PLA added per assay. For further details see Experimental Procedures.

3C. This apparent contradiction between panels B and C of Figure 3 can be explained if one assumes that at lower initial substrate concentrations an increasing portion of the enzyme is denatured before it encounters a micelle, since at lower amphiphile concentration the concentration of micelles will necessarily be lower. This denaturation problem can be circumvented if the enzyme is added to the test at high initial substrate concentration. All enzyme is then readily incorporated in micelles, whereafter rates become maximal near the cac as shown above. Therefore the cac seems to represent the minimal substrate concentration required by OM PLA for solubilization and stabilization. The cac values and maximal hydrolysis rates of several substrates in the absence of detergent are listed in Table II. The effects of monoacyl lipid concentration on hydrolysis rates in the absence of detergent disappeared when 12.8 mM Triton X-100 was included in the assay mixture. Besides lysolecithins and glycollecithins, also Tween 40 and monopalmitoylglycol are readily hydrolyzed at comparable rates by OM PLA, indicating again that the structure of the headgroup of the substrate has little influence on hydrolysis rates in Triton X-100.

(*3*) *Short-Chain Lecithins.* Hydrolysis by OM PLA is very sensitive to the chain length of the substrate. Long-chain lecithins are readily hydrolyzed but dilauroylphosphatidylcholine is not hydrolyzed by OM PLA in Triton X-100 and only slowly in the presence of DOC (Table I). Dicaproylphosphatidylcholine is not hydrolyzed in the presence of either detergent. Bearing in mind the results obtained with the monoacyl compounds, we examined lecithins with even shorter acyl chains, which exhibit high cmc values compared to long-chains lecithins. Initial hydrolysis rates of these short-chain lecithins as a function of the substrate concentration are shown in Figure 4. OM PLA activity is maximal at substrate concentrations near the cmc values of these short-chain lecithins. Below the cmc of these lipids where only monomers are present, the enzyme is readily and irreversibly denatured since no hydrolysis could be detected after addition of excess monopalmitoylglycol. Above the cmc hydrolysis rates decrease with increasing substrate concentration. This decrease is not caused by an irreversible denaturation of OM PLA, since after addition of 5 mM monopalmitoylglycol hydrolysis rates were observed that were comparable to the rates of OM PLA acting on monopalmitoylglycol alone.

When long-chain and short-chain lecithins are mixed, both in the presence and absence of detergents, no detectable hydrolysis occurs of the short-chain component. This indicates that OM PLA has a high preference for lipids containing long acyl chains. Because OM PLA needs lipid aggregates for solubilization and stabilization, the enzyme will bind to short-chain lecithins but only when no long-chain lipids or detergents are present.

(*4*) *Miscellaneous Esters.* Since both monoacyl- and diacylglycerol as well as Tweens 40 and 85 are readily hydrolyzed, it was checked whether OM PLA can be classified as an aspecific esterase or lipase. *p*-Nitrophenyl esters of fatty acids with different chain lengths (4, 8, 12, and 16 carbon atoms) were not hydrolyzed by OM PLA, either in pure form or in the presence of several detergents including Triton X-100. No enzyme activity could be shown, even upon prolonged incubation, on triglycerides dispersed with arabic gum and/or detergents. Intralipid, composed of triolein droplets coated with a monolayer of lecithin, was stable in the absence of detergents whereas the presence of Triton X-100 in the incubation mixture resulted in a complete breakdown of only the lecithin. OM PLA therefore does not exert specific lipase or aspecific esterase activity.

Competitive Binding. Since all long-chain lipids gave comparable hydrolysis rates under our assay conditions, we checked whether OM PLA exhibits different affinities for these compounds. OM PLA, being a water-insoluble enzyme, cannot be subjected to normal Michaelis–Menten kinetic studies since it is denatured when not bound to aggregated amphiphiles. By measuring the hydrolysis rates at varying ratios of different substrates to a constant inhibitor, we determined kinetically the affinity of OM PLA for these substrates relative to its affinity for the inhibitor. Triton X-100 was used as an inert carrier to solubilize the lipids for the following reasons. OM PLA activity is very stable upon storage of the enzyme in micellar solutions of Triton X-100, indicating that this detergent is an excellent inert solubilizer of this originally membrane-bound enzyme. Although OM PLA clearly has an affinity for Triton X-100 since it is readily incorporated into micelles of this detergent, increasing the ratio of Triton X-100 to lipid does not influence the hydrolysis rates of long-chain substrates. From this we draw the conclusion that apparently the actual active site of OM PLA has a negligible affinity for Triton X-100 compared to its affinity for the long-chain substrates and inhibitor used in this study. In relation to this we recall the observation made in a previous section that the detergent C12-sulphobetain, which is structurally related to the substrates, does in fact compete for the active site of OM PLA in the presence of lipids. Triton X-100 forms optically clear solutions with all the substrates presented in this study at the given concentrations. We assume that by adding a 2.5-fold molar excess of Triton X-100 to lipid differences in physical behavior of the substrates caused by strong lipid–lipid interactions will be minimized. Micellar structure will then be equal at varying mole fractions of inhibitor and substrate. This is a reasonable assumption since the previous sections showed hydrolysis rates to be similar for all kinds of different substrates in the presence of a 2.5-fold molar excess of Triton X-100. The following scheme for competitive binding studies was developed, analogous to the scheme for water-soluble lipolytic enzymes (Verger & de Haas 1976):

In mixed micelles composed of lipids and a 2.5-fold molar excess of Triton X-100, hydrolysis rates were measured at a fixed total lipid concentration ([S] + [I]) with varying mole fractions of the substrate ([S]) and an inhibitor ([I]). As

inhibitor was chosen the substrate analogue *n*-octadecylphosphocholine, but essentially the same results were obtained with *n*-hexadecyl- or *n*-tetradecylphosphocholine. In this way we determined kinetically the relative binding constant (K_M/K_I) of OM PLA for each substrate (K_M) relative to its affinity for *n*-octadecylphosphocholine (K_I). The following equations were used to calculate K_M/K_I:

$$V_I/V_S = [ES]_I/[ES]_S \quad (1)$$

V_I and V_S are the rates in the presence and absence of the inhibitor. $[ES]_I$ and $[ES]_S$ are the amounts of enzyme bound to the substrate in the presence or absence of an inhibitor.

On account of the high affinity of OM PLA for lipids and its water insolubility as shown in the previous sections, we assume that all enzyme is bound to the mixed micelles under the experimental conditions employed. Since we assume that the active site of OM PLA has a negligible affinity for Triton X-100 compared to its affinity for the long-chain substrates and inhibitors used, all enzyme will be bound to either the substrate or the inhibitor:

$$\begin{aligned} [E]_{total} &= [ES]_S \quad \text{(in the absence of an inhibitor)} \\ [E]_{total} &= [ES]_I + [EI] \quad \text{(in the presence of an inhibitor)} \end{aligned} \quad (2)$$

The assay conditions were chosen as follows: total amount of protein was constant in all measurements: $[E]_{total}$ is constant. Total amount of lipid is constant: [S] + [I] = constant. The amounts of substrate [S] and inhibitor [I] are defined as mole fractions $1 - x$ and x.

Equations 1 and 2 then combine to yield eq 3. In the

$$V_I/V_S = \{1 + (K_M/K_I)[x/(1 - x)]\}^{-1} \quad (3)$$

hypothetical case that an inhibitor is bound with equal affinity as the substrate ($K_M/K_I = 1$), a straight line will be observed when the relative activity is plotted versus the mole fraction of this inhibitor (x). For such an inhibitor we define the mathematical relative activity as V_D/V_S, to discriminate it from the experimentally measured relative activity (V_I/V_S). We now define the ratio (R) of the mathematical relative activity (V_D/V_S) to the measured relative activity (V_I/V_S) using eq 4. Using eq 4 we can calculate K_M/K_I from the slope of the linear plot of R versus the mole fraction of inhibitor (x).

$$R = V_D/V_I = 1 + x\{(K_M/K_I) - 1\} \quad (4)$$

Figure 5A shows the measured points for several substrates and the inhibitor *n*-octadecylphosphocholine. From the slopes of a replot of these data (Figure 5B) one can calculate the relative binding constant (K_M/K_I) using eq 4. Monoacyl substrates with a large polar headgroup like lysolecithins and glycolphosphocholines, but also Tween 40, are bound with equal affinity as the inhibitor ($K_M/K_I = 1$). All α and β diacyl phospholipids tested as well as Tween 85 having K_M/K_I values ranging from 0.33 to 0.2, although for clarity Figure 5B shows only two examples. This means that diacyl compounds are bound with three to five times higher affinity than the monoacyl compounds when both contain large polar headgroups. Monopalmitoylglycol with its small nonionic headgroup seems to be an exception as shown by its K_M/K_I value of 0.11.

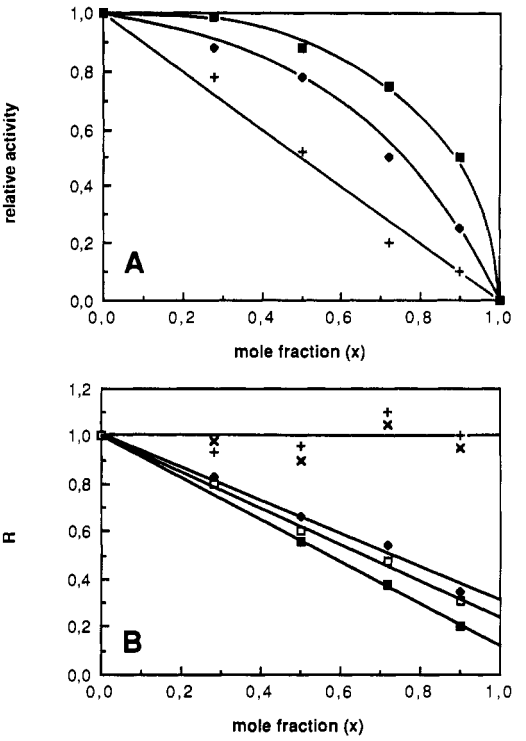

FIGURE 5: Relative activity of OM PLA on mixtures of substrate and the inhibitor *n*-octadecylphosphocholine. (Panel A) Relative activity at different mole fractions of *n*-octadecylphosphocholine (x) on the substrates: (+) lysolecithin; (◆) egg lecithin; (■) monopalmitoylglycol. For details, see Experimental Procedures. (Panel B) Plot of kinetic data according to eq 4 (see text) for the substrates shown in panel A and two additional substrates: (□) *E. coli* phosphatidylethanolamine; (×) palmitoylglycollecithin. R represents the ratio of the activities in the presence of a hypothetical inhibitor for which K_M/K_I equals 1 (V_D) and the measured values for *n*-octadecylphosphocholine (V_I); for further explanations, see text.

Discussion

OM PLA is in its natural state a membrane-bound enzyme. Whereas water-soluble lipolytic enzymes are characterized by a large increase in activity upon aggregation of their substrates, OM PLA actually needs these aggregates for maintaining the intact active protein conformation. In the absence of aggregates of lipids or detergents OM PLA is irreversibly denatured. This was shown by de Geus et al. (1986), who also showed that the specific activity of OM PLA depended on the detergent concentration at which the enzyme was stored. Activity was maximal when the protein had been stored at a detergent concentration near the cmc. This effect of detergent concentration on OM PLA activity was confirmed by us with several other detergents with the notable exception of Triton X-100. In the present study we transferred small amounts of OM PLA in single-chain detergent to a large excess of micelles of monoacyl substrates. This makes it possible to directly measure the dependence of the specific activity of OM PLA on lipid concentration. OM PLA activity is maximal at low substrate concentrations, i.e., the cac for monoacyl phospholipids or the cmc for short-chain lecithins, whereas higher substrate concentrations diminish OM PLA activity. It is clear that the activity of OM PLA is very sensitive to the concentration of micelle-forming lipids, especially at concentrations close to the cmc. The formation of micelles is a very dynamic process which includes phenomena like premicellar aggregation and changes in size and shape of the micelles dependent on amphiphile concentration [Allgyer and Wells (1979) and references cited therein; Johnson et al., 1981]. It has been shown that water-soluble snake venom phospholipases A2 are sensitive to changes in micellar structure at concentrations near the cmc of their substrates (Wells, 1974; Allgyer & Wells, 1979). From our results it seems clear that the lipids do not solely act as substrates for OM PLA but are also necessary to solubilize the enzyme in an active form. It is therefore not surprising that OM PLA is very sensitive to changes in micellar

structure. A possible explanation is that lipid structure influences the conformation of OM PLA. Direct evidence for a conformational transition in membrane-bound proteins caused by different lipid environments was provided for M13 coat protein with the use of ^{19}F NMR studies (Wilson & Dahlquist, 1985). Since in its natural environment, the *E. coli* envelope, OM PLA is only activated upon disturbance of membrane integrity, regulation of enzymatic activity via lipid structure could be involved in regulating the activity of this membrane-bound phospholipase.

The activity of OM PLA shows remarkably little variation on a broad range of substrates in the presence of DOC or Triton X-100 (cf. Tables I and II). Only lipids containing a hydrophobic part and a polar headgroup are readily hydrolyzed, but the charge and size of this headgroup seem to be of minor influence. The minimum substrate requirement for OM PLA can be given as where X stands for a polar head-

$$X\text{–}CH_2\text{–}O\text{–}CO\text{–}(CH_2)_{\geq 12}\text{–}CH_3$$

group, which can be small as in monopalmitoylglycol or large as in Tween 40. Since esters lacking a polar headgroup are not hydrolyzed, the function of the headgroup seems to be the orientation of the lipid in the micelle. Nonpolar esters like triglycerides or *p*-nitrophenyl esters of fatty acids show a random orientation in the hydrophobic core of the micelle, thus preventing productive binding by OM PLA. Competitive binding experiments with monoacyl substrates that contain bulky headgroups showed that binding appears to depend mainly on the hydrocarbon chain of the substrate since difference in headgroup composition and the lack of the ester bond in the inhibitor did not result in detectable differences in affinity of OM PLA.

The introduction of a second acyl chain in the substrate has two major effects. First, the binding of the substrate improves by a factor 3–5. This improved binding did not depend on headgroup composition and structure of the lipids and must therefore be ascribed to an interaction of OM PLA with the second acyl chain. A second effect is the induction of stereospecificity. Whereas both D and L forms of monoacyl phospholipids like lysophospholipids and deoxylysophospholipids are hydrolyzed, the introduction of a second acyl chain causes OM PLA to act stereospecifically on the secondary acyl chain. In general, stereospecificity can be explained by a three-point interaction of enzyme and substrate (Ogston, 1948). We propose that OM PLA binds diacyl phospholipids via both acyl chains. The polar headgroup, which may be very large, must for steric reasons be oriented away from the protein, thus pointing toward the lipid–water interface. Thus, OM PLA will hydrolyze in a stereospecific way esters of secondary hydroxyl groups attached to a chiral carbon. Esters of primary alcohols which are not attached to a chiral carbon atom will be hydrolyzed completely irrespective of the stereochemical conformation of the substrate. This is indeed observed for OM PLA: the enzyme hydrolyzes primary ester bonds of D- and L-α-lecithins but also both ester bonds of β-lecithins. D- and L-α-lecithins and -β-lecithins are all bound with equal affinity. Therefore, the mobility of the rest of the molecule relative to the bound primary acyl chain must be sufficient to make an interaction of the second acyl chain with the enzyme possible. In view of the different steric properties of these lecithins the interaction with the second acyl chain does not seem to occur at a distinct site but could merely constitute parts of the hydrophobic surface of this originally membrane-bound enzyme. OM PLA behaves thus quite different from secretory phospholipases A2. In these enzymes an interaction with the negative charge of the phosphate group and an acyl chain has been reported to be the cause of both site specificity and stereospecificity, not only for α- but also for β-lecithins (van Deenen & de Haas, 1963; Bonsen et al., 1972). We believe that the obvious lack of such an interaction with the negative phosphate group in OM PLA is the cause of the different behavior of this enzyme with respect to α- and β-lecithins. It is clear that OM PLA cannot be simply named a phospholipase since a negative phosphate group is not at all required for binding and catalysis. In the *E. coli* membrane OM PLA will nevertheless act as a phospholipase A1 when its activity is triggered, since the enzyme is embedded in phospholipids. The remaining lyso compound is diluted out by the initially abundant diacyl phospholipid and will accumulate as is indeed observed when OM PLA activity is triggered [see, e.g., Doi et al. (1972)].

OM PLA appears to be rather unique since no sequence homology is prominent with any phospholipase, lipase, or outer membrane protein (personal observations). However, since *Salmonella thyphimurium* contains a similar enzyme (Bell et al., 1971), OM PLA could represent a class of enzymes common to the envelopes of Gram-negative bacteria. OM PLA shares its broad substrate specificity and preference for primary ester bonds with several other bacterial and eukaryotic phospholipases A1 [see, e.g., Kucera et al. (1988)], but a prominent difference with these enzymes is the observed calcium dependence of OM PLA catalyzed hydrolysis. Like the water-soluble phospholipases OM PLA remains active in the presence of lipase/serine esterase inhibitors like diisopropyl fluorophosphate. The well-known inhibitor of water-soluble phospholipases A2, *p*-bromophenacyl bromide, readily inactivates OM PLA. This, together with the observed Ca^{2+} requirement and alkaline pH optimum of OM PLA catalyzed hydrolysis, might indicate a homology between the catalytic mechanism of OM PLA and the mechanism for water-soluble phospholipases as proposed by Verheij et al. (1980).

We have established that OM PLA has a very limited substrate specificity and indeed displays multiple lipolytic activities. To learn more about this interesting enzyme and its actual physiological role, we are performing studies to clarify the processes that regulate the activity of this membrane-bound enzyme. We have shown in the present study that the activity of OM PLA is very sensitive to the manner of presentation of the substrate. Similar importance of the presentation of the substrate on activity has been observed for mammalian membrane-associated phospholipases [see, e.g., Lenting et al. (1988)]. Studies on these interesting enzymes which appear to be involved in many important cellular processes are severely hampered by their low abundance in mammalian cells [see, e.g., Ulevitch et al. (1988)]. Since we have now available sufficient amounts of the membrane-bound OM PLA for detailed studies, we hope to elucidate the mechanisms that control the activity of this enzyme in phospholipid bilayers. The outcome of these studies might also contribute to the understanding of the regulation of mammalian membrane-associated phospholipases.

Registry No. PLA, 9043-29-2; DOC, 302-95-4; L-1-palmitoyl-2-myristoylglycero-3-PN, 69441-09-4; L-1,2-dimyristoylglycero-3-PN, 18194-24-6; L-1,2-dilauroylglycero-3-PN, 18194-25-7; L-1,2-dicaproylglycero-3-PN, 34506-67-7; L-1-*O*-myristyl-2-palmitoylglycero-3-PN, 95301-20-5; D/L-1-palmitoyl-2-*O*-myristylglycero-3-PN, 118043-52-0; D/L-1,3-dipalmitoylglycero-2-PN, 118138-66-2; D/L-1-palmitoyl-2-myristoylbutanetriol-4-PN, 118043-53-1; Tween 85, 9005-70-3; D/L-1-palmitoylpropanediol-3-PN, 18498-26-5; D/L-2-palmitoylpropanediol-1-PN, 118203-37-5; D/L-1-palmitoylpropanediol-2-PN, 118043-54-2; (hexadecanoylthio)glycol-PN, 60793-01-3; palmitoylglycol-PN, 39036-00-5; myristoylglycol-PN, 59540-28-2; lauroylglycol-PN, 5655-20-9; caproylglycol-PN, 41107-75-9; mono-

palmitoylglycol, 4219-49-2; Tween 40, 9005-66-7; Triton X-100, 9002-93-1.

References

Albright, F. A., White, D. A., & Lennarz, W. J. (1973) *J. Biol. Chem. 248*, 3968–3977.

Allgyer, T. T., & Wells, M. A. (1979) *Biochemistry 18*, 4354–4361.

Audet, A., Nantel, G., & Proulx, P. (1974) *Biochim. Biophys. Acta 348*, 334–343.

Bayer, M. H., Costello, G. P., & Bayer, M. E. (1982) *J. Bacteriol. 149*, 758–767.

Bell, R. M., Mavis, R. D., Osborn, M. J., & Roy Vagelos, P. (1971) *Biochim. Biophys. Acta 249*, 268–635.

Bonsen, P. P. M., de Haas, G. H., Pieterson, W. A., & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 270*, 364–382.

Cohen, L. W., Knipprath, W. G., & Freeman Allen, C. (1970) *Virology 41*, 430–435.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

de Bose, C. D., & Roberts, M. F. (1983) *J. Biol. Chem. 258*, 6327–6334.

Deems, R. A., Eaton, B. R., & Dennis, E. A. (1975) *J. Biol. Chem. 250*, 9013–9020.

de Geus, P., van Die, I., Bergmans, H., Tomassen, J., & de Haas, G. H. (1983) *Mol. Gen. Genet. 190*, 150–155.

de Geus, P., Riegman, N. H., Horrevoets, A. J. G., Verheij, H. M., & de Haas, G. H. (1986) *Eur. J. Biochem. 161*, 163–169.

Dennis, E. A. (1974) *J. Supramol. Struct. 2*, 682–694.

Doi, O., & Nojima, S. (1973) *J. Biochem. (Tokyo) 74*, 667–874.

Doi, O., & Nojima, S. (1974) *Biochim. Biophys. Acta 369*, 64–69.

Doi, O., Ohki, M., & Nojima, S. (1972) *Biochim. Biophys. Acta 260*, 244–258.

Homma, H., Chiba, N., Kobayashi, T., Kudo, I., Inoue, K., Ikeda, H., Sekiguchi, M., & Nojima, S. (1984) *J. Biochem. (Tokyo) 96*, 1645–1653.

Johnson, R. E., Wells, M. A., & Rupley, J. A. (1981) *Biochemistry 20*, 4239–4242.

Kucera, L. G., Sisson, P. J., Thomas, M. J., & Waite, M. (1988) *J. Biol. Chem. 263*, 1920–1928.

Lenting, H. B. M., Nicolay, K., & van den Bosch, H. (1988) *Biochim. Biophys. Acta 958*, 405–415.

Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601–615.

Mckeone, B. J., Pownall, H. J., & Massey, J. B. (1986) *Biochemistry 25*, 7711–7716.

Nishijima, M., Nakaike, S., Tamori, Y., & Nojima, S. (1977) *Eur. J. Biochem. 73*, 115–124.

Ogston, A. G. (1948) *Nature (London) 162*, 963.

Patriarca, P., Beckerdite, S., & Elsbach, P. (1972) *Biochim. Biophys. Acta 260*, 593–600.

Scandella, C. J., & Kornberg, A. (1971) *Biochemistry 10*, 4447–4456.

Tamori, Y., Nishijima, M., & Nojima, S. (1979) *J. Biochem. (Tokyo) 86*, 1129–1138.

Ulevitch, J. U., Watanabe, Y., Sano, M., Lister, M. D., Deems, R. A., & Dennis, E. A. (1988) *J. Biol. Chem. 263*, 3079–3085.

van Deenen, L. L. M., & de Haas, G. H. (1963) *Biochim. Biophys. Acta 70*, 538–553.

Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.

Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M., Puyk, W. C., Dijkstra, B. W., Drenth, J., & de Haas, G. H. (1980) *Biochemistry 19*, 743–750.

Weiss, J., Beckerdite-Quagliata, S., & Elsbach, P. (1979) *J. Biol. Chem. 254*, 11010–11014.

Wells, M. A. (1974) *Biochemistry 13*, 2248–2257.

Wilson, M. C., & Dahlquist, F. W. (1985) *Biochemistry 24*, 1920–1928.