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ARTICLE *in* BIOCHEMISTRY · MAY 1994

Impact Factor: 3.02 · DOI: 10.1021/bi00181a600 · Source: PubMed

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Characterization of Acylating and Deacylating Activities of an Extracellular Phospholipase A_2 in a Water-Restricted Environment†

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Received May 4, 1993; Revised Manuscript Received February 7, 1994®

ABSTRACT: The behavior of porcine pancreatic phospholipase A_2 (ppPLA2) in monophasic low-water media has been explored, for the first time, in a systematic manner. It has been investigated how a number of variables can modulate both acylating and deacylating activities of the enzyme, and several interesting, unexpected results are presented. Among the most relevant, when placing ppPLA2 in the water-restricted environment, are the following: (i) it displays a remarkable alteration of its specificity toward the substrate polar head relative to all-water medium; (ii) it is quite severely inhibited by lysophosphatidylcholine (LPC), which has important implications, particularly concerning its acylation activity; and (iii) it exquisitely discriminates between saturated and unsaturated long-chain fatty acids when esterifying them with LPC. Finally, it is also illustrated how these results can be exploited to optimize the catalytic performance of the enzyme in nonaqueous medium and obtain a nearly 30-fold increase in the yield of phosphatidylcholine synthesis with respect to previously reported data.

The last decade has witnessed the emergence and consolidation of an impactful area of research in enzymology, i.e., nonaqueous enzymology [for reviews, see, e.g., Martinek et al. (1986), Klibanov (1989), Dordick (1991a,b), Blinkovsky et al. (1992), and Gupta (1992)]. Interest in the field is based on the finding that enzymes placed in a water-restricted environment not only remain active but also can exhibit exciting, unusual properties not displayed in aqueous medium (Klibanov, 1989; Dordick, 1991a; Gupta, 1992), and has been spurred by the realization of the biotechnological potential of the use of biocatalysts in unconventional media, reflected in an explosive growth of literature [see Dordick (1991b) and references cited therein; Blinkovsky et al., 1992; Gupta, 1992]. However, our knowledge of the fundamentals of enzymology in organic solvents is still limited, and the need is repeatedly claimed for extending the spectrum of enzymes studied in an effort to both rationalize and optimize the catalytic function in organic media. It is expected that work in this direction will enrich our understanding of some structure–function relationships and of the interplay of factors modulating enzyme stability or specificity as a result of environmental changes.

Among the different experimental approaches developed to study the behavior of enzymes in low-water media, essentially two have obtained a wide acceptance, namely: (1) to incorporate the enzyme into a surfactant-based water-in-oil microemulsion (reverse micellar media) [for recent reviews, see, e.g., Luisi et al. (1988) and Garza-Ramos et al. (1992)]; and (2) to suspend the enzyme powder (optionally the enzyme can be adsorbed onto a support) in an anhydrous or nearly anhydrous organic solvent, i.e., in monophasic organic media [for recent reviews, see, e.g., Klibanov (1989), Dordick (1991a), and Russell et al. (1992)]. To be strict, this latter option is much better suited for the enzyme to be in a predominantly nonaqueous environment (Dordick, 1991a). In the last years, in addition to some efforts addressing fundamental aspects of enzymology in monophasic organic media [see, e.g., Zaks and Klibanov (1988), Burke et al. (1992), Ryu and Dordick (1992), Affleck et al. (1992), and Fitzpatrick et al. (1993)], a large number of varied applications have also been developed based on this strategy (Dordick, 1991a,b; Blinkovsky et al., 1992).

Enzymology in monophasic organic media seems particularly interesting not only because of a number of inherent advantages [listed in Dordick (1991a)] but also for the remarkable, novel properties the biocatalyst can potentially display under these conditions, some of the most relevant being the following: (i) the enzymes (proteins in general) typically exhibit a dramatically enhanced thermostability (Zaks & Klibanov, 1984) due to an increased conformational rigidity and the minimization of deleterious processes in which water is involved; (ii) they can manifest a "pH" (Zaks & Klibanov, 1988) or "ligand-memory" phenomenon, the latter also being commonly referred to as molecular (bio)impriting (Russell & Klibanov, 1988; Braco et al., 1990; Stahl et al., 1991), which can result in an enhanced catalytic rate or altered specificity; (iii) the shifting of the thermodynamic equilibrium in a desirable direction (to favor synthesis over hydrolysis) can be maximized; and (iv) interestingly, the possibility exists of exquisitely modulating the substrate specificity or enantioselectivity by changing the nature of the solvent, the so-called "medium (solvent) engineering" approach (Klibanov, 1989; Gupta, 1992).

In this context, lipases (and proteases) have been undoubtedly the enzymes devoted most attention in the last years both in basic and in applied studies, which paradoxically contrasts with the poor knowledge of the behavior of phospholipases, particularly in monophasic organic media. In fact, most of the work has been carried out with phospholipase D from different sources in biphasic or reverse micellar media, and has been mainly polarized to the synthesis or conversion of phospholipids [see, e.g., Juneja et al. (1989) and Testet-Lamant et al. (1992)]. This lack of information is still more remarkable in the case of phospholipase A_2 (PLA2)[1] (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4). PLA2s are a family of ubiquitous, highly conserved Ca^{2+}-dependent enzymes which catalyze the stereospecific hydrolysis of the 2-*sn* acyl ester

† This work was supported by Grant PB90-0578 from DGICYT (Spain). I.M. was a recipient of a long-term postgraduate fellowship from the Ministerio de Educación y Ciencia (Spain).

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

0006-2960/94/0433-4652$04.50/0

bond of 3-*sn*-phosphoglycerides, and are implicated in a number of physiologically relevant metabolic and regulatory processes (Waite, 1987; Jain & Berg, 1989). They are in general relatively small proteins (*ca.* 14 kDa), rather stable due to the large number of disulfide bridges in their structure. Although PLA2 can hydrolyze monomeric phospholipids, its activity toward aggregated substrates is greatly enhanced, a phenomenon commonly referred to as "interfacial activation" (Verheij et al., 1981; Jain & Berg, 1989).

A study of the basic properties of PLA2 in monophasic nonaqueous media seems interesting based on the following considerations. (i) PLA2s, like other phospholipases, are lipid/water interface-associated enzymes functioning in relatively hydrophobic (rather than true all-aqueous) natural environments, where water activity is diminished relative to bulk solution. Although this system seems particularly attractive because of the enzyme dependence on Ca^{2+} and of the amphiphilic, relatively bulky nature of the substrate (soluble in organic solvents), the behavior of PLA2 under nearly anhydrous conditions has not been explored systematically so far. Apart from a few isolated studies in reverse micellar systems (Misiorowski & Wells, 1974; Na et al., 1990), to the best of our knowledge only one recent report has shown that extracellular PLA2s can catalyze (though with very poor yields) the synthesis of phospholipids in a monophasic nonpolar medium (Pernas et al., 1990). (ii) On the other hand, the reasons for the apparent reluctance of PLA2s (as opposed to lipases) to catalyze acylation reactions, even in anhydrous media, have never been directly addressed. It is evident that a water-restricted environment seems ideally suited to get an insight into the requirements for the acylating activity. (iii) Since the natural substrates of PLA2 are rarely present as monomers in the aqueous phase due to their very low critical micelle concentration (cmc), typically lower than 100 pM, previous studies on the hydrolytic activity or specificity of the enzyme toward monomolecularly dispersed substrates have made use of short-chain phospholipids or phospholipid analogs [see, e.g., Verheij et al. (1981), van Oort et al. (1985a,b), Jain and Berg (1989), and Bhat et al. (1993)]. Interestingly, the nonaqueous approach provides us with an opportunity to investigate the behavior of PLA2 toward monomerically dispersed but long-chain, natural phospholipid substrates, virtually nonaligned and conformationally less restrained than in water. Furthermore, facilitated diffusion of the reaction products from the enzyme, one of the factors proposed to account for the enhanced hydrolytic activity of PLA2 at water-aggregated lipid interfaces (Waite, 1985), is expected to occur also in the water-restricted environment.

In the present work, we have undertaken characterization of the behavior of porcine pancreatic PLA2 (ppPLA2), as a widely studied model of extracellular phospholipase, in a water-restricted environment. A critical evaluation of both acylating and deacylating activities has been made regarding a number of variables related both to the enzyme and to the substrates. The results are discussed in light of current structural and functional knowledge on PLA2s.

[1] Abbreviations: cmc, critical micelle concentration; CL, cardiolipin; DDPC, didecanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; ppPLA2, porcine pancreatic phospholipase A_2; TLC, thin-layer chromatography; w_0, water/phospholipid mole ratio.

EXPERIMENTAL PROCEDURES

Materials. PLA2 from porcine pancreatic glands was purchased from Sigma Chemical Co. (St. Louis, MO) or obtained as a kind gift from Novo Industri A/S (Copenhagen, Denmark). Egg yolk lecithin (PC) was supplied by Merck (Darmstadt, Germany) and purified according to the method of Singleton et al. (1965). Egg yolk L-α-lysophosphatidylcholine (LPC), dipalmitoyl-L- and -D-α-phosphatidylcholine (L- and D-α-DPPC), didecanoyl-(DDPC), dimyristoyl-(DMPC), and distearoyl-L-α-phosphatidylcholine (DSPC), β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine, all fatty acids, natural-origin L-α-phosphatidylserine (PS), L-α-phosphatidyl-DL-glycerol (PG), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylinositol (PI), and cardiolipin (CL), as well as PG2000-200 controlled pore glass beads (mesh size 120–200), were purchased from Sigma. Tritium-labeled oleic acid (specific activity 10 Ci/mmol) was obtained from Amersham (U.K.). Thin-layer chromatography (TLC) plates as well as Triton X-100 and analytical-grade $CaCl_2$ were from Merck. All solvents used in this work were purchased from Merck and were of analytical grade or purer for enzymatic assays under nonaqueous conditions, and HPLC grade for chromatographic analyses. When referred to as "anhydrous", the solvents were desiccated with 3-Å molecular sieves (Merck) to bring their water content below 0.01%, as determined by optimized Fisher titration (Laitinen & Harris, 1975).

PLA2 Powder Optimization. Since the pH of the lyophilization buffer has been shown to modulate further enzyme activity in organic medium (Zaks & Klibanov, 1988), pH 8.0 was selected in the case of PLA2, as this value has been routinely used in previous assays of the pancreatic enzyme [see, e.g., Berg et al. (1991)]. For hydrolytic and synthetic purposes, PLA2 was typically optimized as follows. An aliquot of a freshly prepared enzyme solution (1 mL) was diluted (at 4 °C) with 29 mL of 10 mM Tris-HCl buffer, pH 8.0, containing 5 mM Ca^{2+}. Aliquots of this solution were then directly added to the tubes for the assay in organic solvent and freeze-dried overnight.

PLA2 Assays in Nonaqueous Medium. PLA2-catalyzed hydrolysis of PC in organic solvents was measured as follows. In a typical experiment, 1 mg of the optimized enzyme powder was suspended (in a stoppered 2-mL screw-capped scintillation vial) in 1 mL of a solution of PC in water-saturated chloroform. The suspension was immediately sonicated for 20 s and then shaken at 250 rpm at 25 °C. Periodically, 10-μL aliquots of the reaction mixture were withdrawn and assayed by high-performance liquid chromatography (HPLC) following the simultaneous disappearance of PC and the appearance of its lyso derivative. The kinetic course of hydrolysis of the series of phospholipids differing in the polar head was monitored by quantitative TLC analysis of aliquots of the reaction mixture taken at different incubation times. The eluent system consisted of chloroform/methanol/water (65:25:4). The area in each spot was integrated by means of a Kontron IBAS-2000 image analyzer system. For each phospholipid, the integrated densities at different times were converted to percentages relative to the density corresponding to the control nonhydrolyzed substrate spot.

For the PLA2-catalyzed synthesis of phosphatidylcholine in organic solvents, typically 0.2 mg of optimized enzyme powder was suspended in 1 mL of anhydrous toluene containing LPC and the fatty acid. Alternatively, LPC dissolved in anhydrous chloroform was first added to the enzyme, the solvent was rotary-evaporated, and then the powder was suspended in a solution of the fatty acid in anhydrous toluene. When tritium-labeled oleic acid was used in the synthesis of

phosphatidylcholine, the TLC-purified product was counted for radioactivity using an LKB 1217 Rackbeta liquid scintillation counter. Alternatively, for oleic acid as well as for the rest of the fatty acids, the synthesized phospholipid was purified by HPLC and quantified either by a phosphorus assay (Dittmer & Wells, 1969) or, following PLA2 hydrolysis in aqueous medium, by derivatization, extraction, and gas chromatographic (GC) analysis of the fatty acid released, in a similar manner as in Svensson et al. (1990). The results are in all cases the mean of triplicate independent measurements.

Immobilization of PLA2 on porous glass beads was carried out basically as reported for other enzymes (Braco et al., 1992) except that the beads were finally vacuum-dried for 1 h.

Thermal Inactivation. The kinetics of irreversible thermal inactivation of PLA2 in anhydrous *n*-octane were determined following a procedure essentially similar to that reported by Zaks and Klibanov (1988), except that a metal heating block was used for incubation at 100 °C. Briefly, suspensions of optimized enzyme powder in *n*-octane contained in sealed ampules were incubated at 100 °C for different time periods and then rapidly equilibrated at room temperature. The enzyme was next filtered and vacuum-dried, and its activity was assayed as usual in water-saturated chloroform at 25 °C.

HPLC Analysis. Complete separation of PC, LPC, and fatty acid was carried out on a Waters Resolve column (90-Å nominal pore size) using a Waters liquid chromatograph equipped with a Waters 410 differential refractometer. The column was eluted isocratically with acetonitrile/methanol/water (55:45:4, v/v/v) at a flow rate of 1.5 mL/min. For kinetic measurements, 10-μL aliquots of the reaction medium were dissolved in 90 μL of the eluent to stop the reaction, the mixture was centrifuged for 3 min at 12000*g*, and 50 μL of the supernatant was injected.

Assay of PLA2 Hydrolytic Activity in Aqueous Medium. Hydrolysis in water of PC and of other phospholipids differing in the polar head was monitored potentiometrically using a Crison MicroTT 2050 recording pH-stat system. In this case, the substrate was emulsified using Triton X-100 (detergent: phospholipid molar ratio of 2). Typically, 4 mL of a solution containing the emulsified phospholipid, 10 mM NaCl, and 5 mM Ca^{2+}, equilibrated at pH 8.0, was placed in a thermostated cuvette at 25 °C, and the reaction was monitored in the autotitration mode in a similar manner to that reported by Berg et al. (1991). Alternatively, HPLC was used to follow the reaction progress when the reaction took place in the same buffer as that used for optimization of PLA2 powder.

Unless stated otherwise, the experiments were carried out at 25 °C. The protein content was determined according to the method of Lowry et al. (1951). The Ca^{2+} concentration in PLA2-containing solutions was determined by atomic absorption spectroscopy using a Perkin-Elmer Model 5000 spectrometer. Other details of experimental conditions are given in the corresponding legends to figures.

RESULTS

Hydrolytic Activity of PLA2 in Monophasic Organic Media. It was decided to select water-saturated chloroform in all the experiments in this section as a convenient assay reaction medium because it readily solubilizes not only the phospholipid substrates but also (unlike many other solvents) their lyso derivatives, which should facilitate diffusion of the products from the enzyme's catalytic site. Although it was not the purpose of this work to establish a quantitative comparison of the PLA2 hydrolytic activity as a function of the solvent nature, it was verified that the enzyme was also active in other solvents (results not shown), both water-immiscible (e.g., water-saturated toluene, benzene, ethyl acetate, or diisopropyl ether) and water-miscible (e.g., tetrahydrofuran, acetonitrile, or acetone, containing different amounts of water).

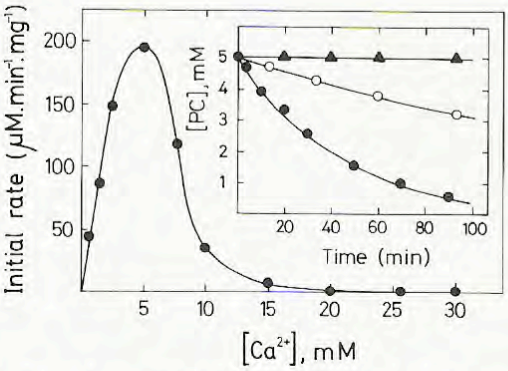


FIGURE 1: Dependence of the initial rate of PC hydrolysis (expressed as micromolar per minute per milligram of protein) catalyzed by ppPLA2 in water-saturated chloroform on the aqueous Ca^{2+} concentration prior to freeze-drying. (*Inset*) Time course of PC hydrolysis for different concentrations of Ca^{2+}: (●) 5; (○) 10; and (▲) 30 mM. Initial PC concentration was 5 mM.

Effect of Ca^{2+} Concentration. Given the absolute requirement for Ca^{2+} of ppPLA2, we first investigated the dependence of the enzymatic activity in nonaqueous medium on the cofactor concentration in the aqueous solution prior to lyophilization. The inset in Figure 1 shows, as an example, the time course of PLA2-catalyzed PC hydrolysis in water-saturated chloroform, for different Ca^{2+} concentrations. Fitting of the data to a first-order function yielded rate constant values of $(4.0 \pm 0.2) \times 10^{-4}$, $(8.0 \pm 0.2) \times 10^{-5}$, and 0 s^{-1} for 5, 10, and 30 mM Ca^{2+}, respectively. Under optimal conditions, conversion was complete in about 2 h. Figure 1 depicts the dependence of the initial rate of hydrolysis on Ca^{2+} concentration. Notice the presence of a maximum around 5 mM Ca^{2+} and the severe inhibition at moderately high cofactor concentration.

It was confirmed that preinactivation of PLA2 in aqueous solution (either by addition of EDTA or by boiling) prior to freeze-drying resulted in no detectable activity in organic solvent (no lyso-PC could be detected in the reaction medium even after overnight incubation). On the other hand, the enzyme retained in nonaqueous medium its selectivity of hydrolysis on the *sn*-2 acyl chain of the phospholipid (as verified by GC using β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine as a substrate), as well as its enantioselectivity (no activity was observed when D-α-DPPC was used as a substrate, whereas L-α-DPPC was effectively hydrolyzed). In all further experiments, the PLA2 powder was optimized by adjusting the aqueous Ca^{2+} concentration to 5 mM (see Experimental Procedures).

Effect of Substrate and Product Concentration. Next, the activity in water-saturated chloroform of PLA2 was assayed as a function of the substrate (PC) concentration. Surprisingly, a bell-shaped curve was obtained (see points in Figure 2A) showing a maximum around 30 mM PC and quite severe substrate inhibition at PC concentrations higher than 80 mM. Although inhibition by increasing PC concentrations has been previously reported in the case of phospholipase D in a biphasic system (Juneja et al., 1989), it was much less dramatic than that found for PLA2 in the present case.

In light of the bell-shaped dependence observed, we first tried to fit the data to a simple kinetic model of inhibition by high substrate concentration, where *only one* substrate molecule binds to the enzyme–substrate complex to form a nonproductive complex (note that the binding of one substrate molecule as a competitive inhibitor to the free enzyme does

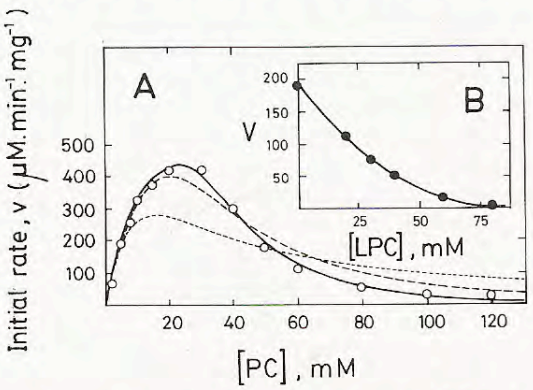
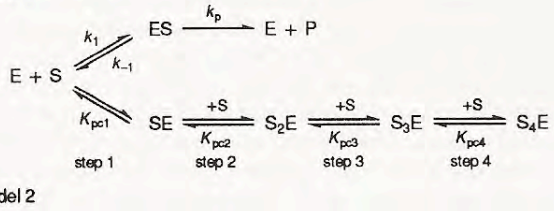


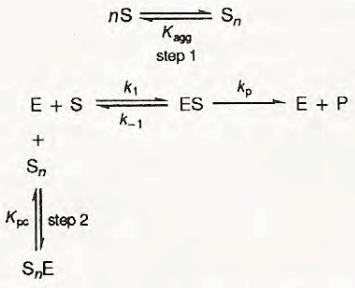
FIGURE 2: (A) Dependence of the initial rate of PC hydrolysis catalyzed by ppPLA2 in water-saturated chloroform (empty circles) on the initial substrate concentration. The enzyme powder was optimized according to Experimental Procedures. The curves correspond to theoretical velocity values obtained by nonlinear fitting of the experimental data to eq 1 (dotted line), eq 2 (dashed line), and eq 3 (solid line) of model 1 (see text). (B) Variation of the initial rate of PC hydrolysis with the concentration of LPC in the reaction medium. Initial concentration of PC was 5 mM.

not lead to a second-order term in PC concentration needed for bell-shaped dependence). In this case, the fitting was poor (not shown). This led us to further consider other more complex models involving two or more incorrectly bound PC molecules, in an attempt to better describe the experimental results. We present below two kinetic models of competitive inhibition by high substrate concentration: model 1, based on the sequential binding to the enzyme of several substrate molecules, and model 2, based on the reported ability of PC to self-associate, forming aggregates in organic solvent and particularly in chloroform (Datta et al., 1992). Both models take into account the particular nature and properties of the substrate in nonaqueous media (see Discussion) and, of course, are not intended to exclude other "mixed" or in general more complex mechanisms not considered in the present paper.

model 1



model 2



E, S, P, ES, SE, and S_n refer to free enzyme, free substrate, free product, productive enzyme–substrate complex, nonproductive enzyme–substrate complex, and (probably inverted-type) aggregate of n molecules of substrate, and k_1, k_{-1}, and k_p (catalytic constant) are rate constants. $K_m = k_{-1}/k_1$ is approximated to the Michaelis–Menten constant, K_{agg} denotes the dissociation constant for the substrate aggregation process, and the inhibition constants K_{pc1}, K_{pc2}, K_{pc3}, K_{pc4}, and K_{pc} are all defined as dissociation constants. Assuming steady-state conditions, the following velocity equations are easily obtained for **model 1**

$$v = V_{max}[S]/\{K_m + (1 + K_m/K_{pc1})[S] + (K_m/K_{pc1}K_{pc2})[S]^2\} \quad (1)$$

$$v = V_{max}[S]/\{K_m + (1 + K_m/K_{pc1})[S] + (K_m/K_{pc1}K_{pc2})[S]^2 + (K_m/K_{pc1}K_{pc2}K_{pc3})[S]^3\} \quad (2)$$

$$v = V_{max}[S]/\{K_m + (1 + K_m/K_{pc1})[S] + (K_m/K_{pc1}K_{pc2})[S]^2 + (K_m/K_{pc1}K_{pc2}K_{pc3})[S]^3 + (K_m/K_{pc1}K_{pc2}K_{pc3}K_{pc4})[S]^4\} \quad (3)$$

and for **model 2**

$$v = V_{max}[S]/\{K_m + [S] + (K_m/K_{agg}K_{pc})[S]^n\} \quad (4)$$

Note that in eq 1 only steps 1 and 2 are included, whereas in eq 2 and 3 steps 3 and 4 are added, respectively.

The curves in Figure 2A show, as an example, the least-squares nonlinear regression fits of the experimental data to eq 1 (dotted line), eq 2 (dahsed line), and eq 3 (solid line), all corresponding to model 1. It can be seen that the behavior of the system can be satisfactorily described by assuming the competitive binding of *more than two* (preferably four) PC molecules. Fitting of the data to eq 3 (solid line in Figure 2A) yielded V_{max} and K_m values of 1.1 ± 0.2 μmol min^{-1} (mg of protein)$^{-1}$ and 23 ± 3 mM, respectively, and K_{pc1} to K_{pc4} values of (2.3 ± 0.4) × 10^2, (2.4 ± 0.5) × 10^2, 21 ± 6, and 0.8 ± 0.3 mM, respectively. Alternatively, the system can also be satisfactorily described by fitting of the data to eq 4 (model 2) when $n = 3$ and preferably $n = 4$ (not shown, though the predicted curve for $n = 4$ was practically superimposable with the solid curve in Figure 2A). In this case, the estimated values for V_{max} and K_m were essentially identical to those obtained from model 1.

The V_{max} obtained in nonaqueous medium is about 1 order of magnitude lower than that reported for ppPLA2 in water using a monomeric diacylglycerophosphocholine as substrate (van Oort et al., 1985b) and is of the same order as those shown also for ppPLA2 in water for monomeric *n*-acylglycol lecithins (van Oort et al., 1985a) or a glycol thioester analog (Bhat et al., 1993). The lower rates in organic solvent relative to all-water medium for monomeric lecithin substrates are not surprising since it is known that placing enzymes in nonaqueous media generally causes a moderate drop in their activity.

Given the relative structural similarity between PC and LPC, one could wonder whether the enzyme activity was also significantly inhibited by this reaction product. Figure 2B shows that this was indeed the case, the inhibition being quite severe (notice that the hydrolysis rate in the presence of 80 mM LPC was almost negligible). Again, fitting of the data to product inhibition models where *only one* molecule of LPC binds to the enzyme was poor (not shown), very likely because several product molecules may be implicated in a more complex mechanism. On the other hand, inhibition by increasing fatty acid (palmitic) concentrations was much less pronounced than either by PC or by LPC.

Thermostability. It has been shown that, in general, enzymes (proteins) in nearly anhydrous media exhibit a dramatically enhanced thermostability, accounted for on the basis of both an increased conformational rigidity and a minimization of deleterious covalent processes involved in

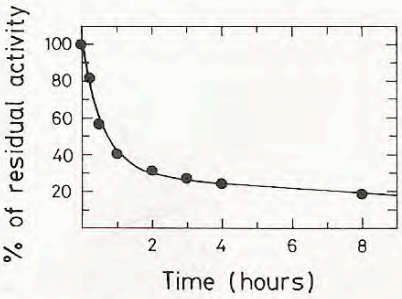


FIGURE 3: Thermostability of ppPLA2 in anhydrous *n*-octane at 100 °C. The results are expressed as the percentage of residual activity as a function of incubation time. The filled circles correspond to experimental data and the curve to the fitting of the data to a double-exponential function (see text).

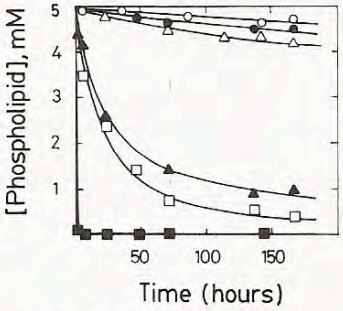


FIGURE 4: Time course of hydrolysis of different phospholipid substrates catalyzed by ppPLA2 in water-saturated chloroform. The initial phospholipid concentration was in all cases 5 mM. (○) PG; (●) PS; (△) PE; (▲) CL; (□) PI; and (■) PC.

irreversible inactivation. As Figure 3 depicts, this was also the case for PLA2, which showed a quite remarkable stability when incubated at 100 °C in anhydrous *n*-octane, in contrast with the almost instantaneous denaturation in an all-water medium at the same temperature (not shown). On the other hand, inactivation in the anhydrous solvent seemed to be described by a biphasic process (see Figure 3), a result similar to that recently reported by Ottolina et al. (1992) for lipoprotein lipase in anhydrous toluene at 90 °C. Indeed, when an irreversible inactivation model was used, the data could be satifactorily fitted to a double-exponential function (see curve in Figure 3) with rate constant values of $(5.0 \pm 0.7) \times 10^{-4}$ and $(1.8 \pm 0.6) \times 10^{-5}$ s^{-1}.

Substrate Specificity. It has been previously described that the hydrolytic activity of PLA2s from different sources is strongly dependent on the nature and physical state of the phospholipid substrate. In particular, ppPLA2 is known to exhibit a preference for acidic phospholipids relative to neutral ones in liposomes, monolayers, and detergent-containing systems (Pluckthun & Dennis, 1985; Thuren et al., 1987; Ghomashchi et al., 1991), as well as also at premicellar concentrations (van Oort et al., 1985b; Jain & Berg, 1989). In the present case, it was exciting to test whether PLA2 selectivity toward the phospholipid polar head would be altered in nonaqueous relative to all-water medium. Figure 4 shows the time course of hydrolysis of PC, PE, PI, CL, PS, and PG, by PLA2 in water-saturated chloroform. It can be seen that PC was rapidly converted (*ca.* 2 h) to its lyso derivative, whereas PI and CL were hydrolyzed much slower (>50 and >100 h, respectively, were needed for 80% conversion). As for PE, PG, and PS, even after 200 h of incubation conversion was lower than 20%. The order of preference in this low-water environment was PC $[(4.0 \pm 0.2) \times 10^{-4}] \gg$ PI $[(7.8 \pm 1.0) \times 10^{-6}] \gtrsim$ CL $[(4.5 \pm 0.8) \times 10^{-6}] \gg$ PE $[(3.1 \pm 0.3) \times 10^{-7}] >$ PS $[(2.0 \pm 0.3) \times 10^{-7}] \simeq$ PG $[(1.1 \pm 0.2) \times 10^{-7}]$. The numbers in brackets correspond to the first-order rate constant values (in s^{-1}) obtained by fitting of the data in Figure 4. Interestingly, this order markedly contrasts with that obtained in a parallel experiment in water (not shown) using phospholipid/Triton X-100 mixed micelles (mole ratio 1:2) as a substrate: PS > PG $\simeq$ CL > PI $\gg$ PC > PE, which is consistent with the order reported by Thuren et al. (1987) using pyrene phospholipids as substrates. Notice that in nonaqueous medium the hydrolysis rate constant can be significantly higher (up to 3 orders of magnitude) for PC than for anionic phospholipids, whereas in aqueous solution the rate is about 1 order of magnitude higher for anionic than for neutral substrates (Thuren et al., 1987).

Some additional results are worth being mentioned in relation to the hydrolytic activity of PLA2 in nonaqueous medium. To investigate the effect of the fatty acyl chain length of the substrate, the rate of hydrolysis in water-saturated chloroform of L-α-DSPC, L-α-DMPC, and L-α-DDPC was compared. In all cases, bell-shaped dependencies (similar to that in Figure 2A) of the initial rate on the substrate concentration were obtained, which allowed an estimation of V_{max} and K_m similarly as described above for PC. Interestingly, no major difference was found in the K_m relative to the PC value, whereas a marked increase in V_{max} was observed for the shortest substrate [2.7 ± 0.4 μmol min^{-1} (mg of protein)$^{-1}$ for DDPC] with respect to DMPC or DSPC [1.0 ± 0.3 and 0.8 ± 0.2 μmol min^{-1} (mg of protein)$^{-1}$, respectively]. The optimal temperature in water-saturated chloroform was around 40 °C, similar to that recently reported by Bhat et al. (1993) for ppPLA2 activity in water toward the monomeric form of a substrate analog. PLA2 powder proved to be stable in the reaction medium and could be reused (after filtration, washing with an anhydrous nonpolar solvent, and vacuum drying) several times without a significant loss of activity. On the other hand, immobilization of PLA2 onto controlled pore glass beads caused an increase, relative to the powdered enzyme suspension, in the rate of hydrolysis (*ca.* 4-fold). Since it has been shown that in organic solvent the properties of the support can influence the catalytic activity of the adsorbed enzyme (Adlercreutz, 1991), this behavior can be likely attributed to both increased water activity (hydrophylic support) and decreased internal diffusional limitations of the immobilized biocatalyst.

Synthetic Activity of PLA2 in Monophasic Organic Media. The above findings, particularly the marked inhibition of hydrolysis observed at moderately high substrate concentration and especially the severe inhibition by LPC, led us to a reconsideration of the conditions previously reported in the literature for the synthesis of PC catalyzed by PLA2 suspended in a nearly anhydrous, monophasic reaction medium (Pernas et al., 1990). A careful examination of different experimental variables was therefore carried out both to better understand the requirements for the acylating activity of PLA2 and to more rationally delineate the conditions for an improved conversion yield.

Effect of LPC and Fatty Acid Concentration. The inset in Figure 5A shows, as an example, the time course of PLA2-catalyzed esterification of oleic acid and LPC in anhydrous toluene, at several lysophospholipid concentrations. It can be seen that in all cases the reaction proceeds slowly, though at a rate much lower for moderate and high than for low LPC concentrations. No PC* (PC* denotes here the synthesized phospholipid) synthesis was observed in control experiments where no enzyme was added to the reaction mixture. Interestingly, when the initial rate of phospholipid formation was plotted as a function of LPC concentration (at constant

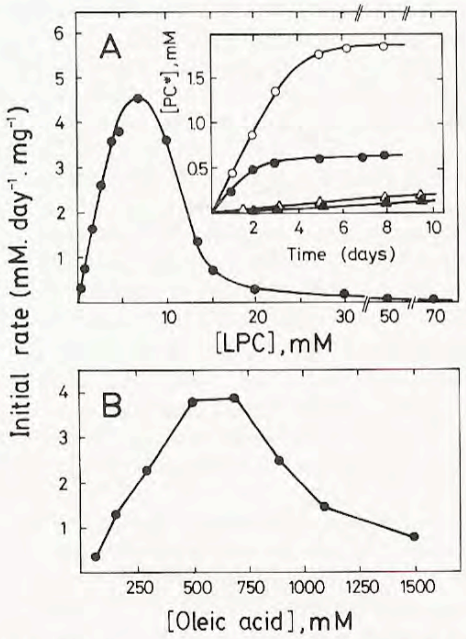


FIGURE 5: (A) Dependence on LPC concentration of the initial rate of esterification of LPC and oleic acid catalyzed by ppPLA2 in anhydrous toluene. Oleic acid concentration was 500 mM. (*Inset*) Time course of acylation at different LPC concentrations: (●) 3; (○) 7; (△) 30; and (▲) 70 mM. PC* denotes the synthesized phospholipid. (B) Dependence of initial acylation rate on oleic acid concentration. LPC concentration was 5 mM.

oleic acid concentration), a bell-shaped curve was obtained with a maximum aroud 7 mM LPC (Figure 5A), which shows how ppPLA2 acylating activity is indeed severely inhibited by the substrate (LPC). Again, it is not surprising that attempts to describe the experimental behavior using substrate inhibition models where *only one* LPC molecule can bind to the enzyme were unsuccessful. In this regard, efforts to fit the data to more complex inhibition mechanisms suggest the participation of several substrate molecules. On the other hand, in this experiment the conversion yield after a quite long incubation period (8 days) reflected a similar bell-shaped dependence on LPC concentration, with, e.g., *ca.* 1% conversion at 70 mM LPC. This is not surprising since at high LPC concentrations the esterification reaction is kinetically very impeded and far from equilibrium even after 1 week (as supported by additional experiments where samples were incubated for more than 1 month). Significantly, however, under optimum conditions (7 mM LPC and 500 mM oleic acid, see below), a 27% yield could be easily obtained *vs* the previously reported 1.3% using also the porcine pancreatic enzyme (Pernas et al., 1990).

The conditions for synthesis were also optimized with respect to the fatty acid concentration using oleic acid (Figure 5B). An optimum range of concentrations, from 500 to 700 mM, was obtained. When pure oleic acid was used as the reaction medium, no PC* synthesis could be detected. On the other hand, the reversibility of the reaction was demonstrated as follows. Addition of a small amount of radiolabeled oleic acid (1% of total acid concentration) to a sample in equilibrium (containing 7 mM LPC and 500 mM oleic acid, after 10-days incubation) resulted in the appearance of labeled PC*, as detected by TLC separation of lipids and fluorography of the plate.

Since there are few reports on the optimal temperature for enzymatic catalysis in predominantly nonaqueous media [see, e.g., Khmelnitsky et al. (1988) and Garza-Ramos et al. (1990)], the dependence of the ppPLA2-mediated LPC acylation rate on the incubation temperature was also studied. As can be seen in Figure 6, an optimum was obtained around 50 °C. However, carrying out the reaction at this temperature did not significantly improve the maximum yield achieved relative to room temperature.

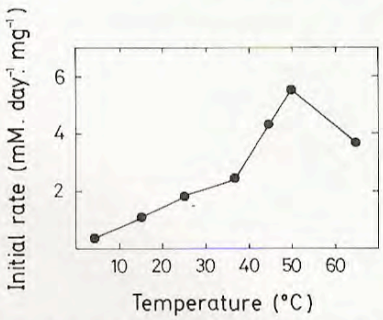


FIGURE 6: Dependence of the ppPLA2-mediated initial rate of LPC acylation in anhydrous toluene on the incubation temperature. LPC and oleic acid concentrations were 5 and 315 mM, respectively.

Table 1: Solvent Dependence of the Initial Rate of Esterification between LPC and Oleic Acid[a]

	rate (mM day^{-1} mg^{-1})		rate (mM day^{-1} mg^{-1})
n-octane	0.88	ethyl acetate	0.20
toluene	4.50	tetrahydrofuran	0.14
benzene	1.86	dioxane	0.17
chloroform	0.31	*tert*-amyl alcohol	0.12
ethyl ether	0.42	acetonitrile	0.00
isopropyl ether	0.14		

[a] Conditions: all solvents were anhydrous (see Experimental Procedures). LPC and oleic acid concentrations were 7 and 500 mM, respectively.

Effect of Organic Solvent. The acylating activity of PLA2 was screened in a number of anhydrous organic solvents both water-miscible and immiscible. The results are summarized in Table 1. It can be seen that the highest rates (and also the highest yields, not shown) were obtained for aromatic hydrocarbons, in agreement with the data from Pernas et al. (1990). However, in contrast with their results, PLA2 under optimal conditions indeed proved to be also active in many other solvents including chloroform. As expected, activity decreased as a general trend with solvent polarity (Zaks & Klibanov, 1988). Presaturating toluene with water increased the esterification rate but decreased the yield, which can be understood since water is a reaction product which can unfavorably shift the equilibrium. It is noteworthy that in anhydrous benzene the maximum yield obtained was as high as 35%.

Effect of Fatty Acid Chain Length and Unsaturation Degree. Next, the possibility was tested that fatty acids other than oleic acid could also be incorporated to LPC by PLA2. For this purpose, saturated fatty acids differing in chain length, as well as fatty acids of the same length but differing in the number of unsaturations, were assayed under the optimized conditions established above. The results in terms of esterification rate are summarized in Figure 7. Interestingly, for the saturated series the rate markedly decreased with increasing chain length, being almost negligible for stearic acid. However, for the same chain length, the presence of only one unsaturation (oleic acid) rendered the fatty acid a reasonably good substrate for the enzyme. On the other hand, increasing the number of unsaturations in the molecule did not result in a significant change in the esterification rate. Finally, when palmitic acid ethyl ester instead of palmitic acid was used as a substrate, no PC* synthesis could be detected after several

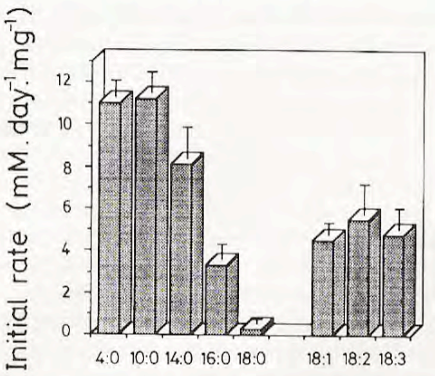


FIGURE 7: Comparison of the ppPLA2-mediated LPC acylation rates in anhydrous toluene for different saturated and unsaturated fatty acids: butyric (4:0), capric (10:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). LPC concentration was 7 mM. Fatty acid concentration was 500 mM.

days of incubation. In this respect, it is interesting to note that Svensson et al. (1990) recently reported that ppPLA2 immobilized onto Celite and suspended in water-saturated toluene was completely unable to catalyze the interesterification between PC and heptanoic acid.

DISCUSSION

In the present paper, we have undertaken the characterization of the behavior of ppPLA2 dispersed in a monophasic, microaqueous environment, a system so far unexplored in a systematic manner. The study has permitted us to extend our phenomenological knowledge of enzyme function in dehydrated environments by providing some interesting, quite unexpected results, particularly those concerning the reversal of PLA2 substrate specificity and the dramatic inhibition of activity (both acylating and deacylating) by LPC. This latter result has been taken into consideration to optimize the catalytic performance of the enzyme with synthetic purposes.

Porcine pancreatic PLA2 proved to be active in hydrolyzing PC in a great number of organic solvents both water-miscible and immiscible. Conditions for activity optimization involved freeze-drying from an aqueous solution not only at the appropriate pH (Zaks & Klibanov, 1988) but also containing an optimum amount of Ca^{2+} (see Figure 1), in agreement with previously reported data from *Naja naja* PLA2 (Pernas et al., 1990). The inhibition of activity in nonaqueous medium with increasing aqueous Ca^{2+} concentration has been suggested before to be due to the high ionic atmosphere created around the enzyme during the freeze-drying process (Pernas et al., 1990). Although this interpretation may be correct, in our opinion inhibition could also be the result of a "cofactor memory", based on the fact that we observed a similar though less pronounced effect by Ca^{2+} in aqueous solution (not shown), and that inhibition by moderate Ca^{2+} concentrations has been previously reported for this and other PLA2s (Verger et al., 1982; Thuren et al., 1987). On the other hand, PLA2 exhibited a markedly enhanced thermostability in anhydrous *n*-octane, as most proteins do in water-restricted environments because of their acquired much higher conformational rigidity. However, as has been recently pointed out (Garza-Ramos et al., 1990; Gupta, 1992), the increase in stability does not imply that the highest reaction rates in organic medium can be achieved at elevated temperatures (see Figure 6).

One interesting result of the present study is the severe inhibition of PLA2 activity observed at high concentrations of PC in the hydrolysis reaction (see Figure 2A) and, even more markedly, already at moderate concentrations of LPC in the synthesis reaction (see Figure 5A). This behavior was reproducible and occurred also in solvents other than those appearing in the figures. Furthermore, the different rate *vs* concentration bell-shaped curves obtained for a series of varied chain length synthetic PCs (see above) and for other tested natural phospholipids (e.g., PE or PI, not shown) are indicative of the dependence of inhibition on the nature of the substrate. An explanation at the molecular level for substrate inhibition is not immediate though. Recent NMR data have suggested that dipalmitoylphosphatidylcholine may form reverse micelles in chloroform, the cmc being 6 mM (Datta et al., 1992). The possibility of incorporation of a fraction of the enzyme to PC reverse micelles at concentrations well above the cmc seems unlikely on the basis of the very low water/phospholipid mole ratio (w_0) in the system. As it is known, extraction of solid protein is a difficult, very slow process particularly at low w_0 (Leser et al., 1986). Moreover, the enzyme powder clearly remained as a particulate suspension during the course of the reaction, and no protein could be detected by spectroscopic measurements in filtrates (through a 0.22-μm filter) of suspension aliquots taken at different times. In our opinion, one possible explanation for the inhibition phenomenon should be sought in the particular nature of both enzyme and substrate. In fact, there is strong evidence, based on X-ray crystallographic studies [see, e.g., Thunnissen et al. (1990), White et al. (1990), and Scott et al. (1991)], of the existence in PLA2s from different sources of a rather narrow hydrophobic channel (slot) flanking access of the substrate to the Ca^{2+}-containing catalytic site, a situation expected to be maintained in nonaqueous medium (Fitzpatrick et al., 1993) due to the protein-enhanced conformational rigidity. It could be suggested that at moderate or high substrate concentrations, an actual accumulation of PC molecules competing with each other at the channel entrance could result in a decrease in the effective hydrolysis rate. Notice also that the rigidity of the protein in the anhydrous environment may impose strict geometric restrictions for productive passage of the substrate in a correct orientation through the hydrophobic channel.

At this point, a brief comment needs to be made on the aforementioned models proposed to account for the observed substrate (PC) inhibition data (see Figure 2A and text). The considerable decrease in the model 1 inhibition constant values when going from K_{pc2} to K_{pc4} (see Results) could be interpreted as suggestive of a certain "cooperativity" in the binding of additional PC molecules to the enzyme pocket once two molecules are already bound (reflecting in a certain manner the known tencency of phospholipids to form aggregates in nonpolar media). It could be speculatively viewed as if at high substrate concentrations the presence of at least two PC molecules at the entrance of the pocket acted as a "nucleation" element to gather more concurring substrate molecules (a presumable mixed micellar-like aggregate of some protein residues and substrate molecules could be formed). On the other hand, the alternative (but not exclusive) possibility of previous formation of substrate aggregates of a few PC molecules acting as inhibitors is considered in model 2. It must be added that the experimental data could also be satisfactorily fitted to alternative models where two and preferably three substrate molecules inhibitorily bind to the enzyme–substrate complex (not shown). Anyway, all the assayed models describing the experimental behavior share in common the participation of several substrate molecules (simultaneously binding to PLA2) in the inhibition mechanism, which supports the above assumption of restricted access of the substrate (or release of products) *via* the active-site channel. Interestingly, this postulated tendency of the substrate to

aggregate at the entrance of the binding pocket is not surprising since it has been previously described that phospholipid-like anionic detergents at premicellar concentrations have a tendency to bind with high affinity to the lipid binding domain of pancreatic PLA2 (predominantly through electrostatic interactions with positively charged residues) and gather forming premicellar lipid–protein aggregates (van Oort, 1985a). Notice that in the low dielectric constant organic medium, interactions between enzyme residues and PC zwitterionic polar heads must be enhanced.

Given the relative similarity between PC and LPC, the same rationale could be easily extended to the inhibition by LPC observed in the esterification reaction (see Figure 5A). In this case, the catalytic event is obviously complicated by the requirement of a correct alignment of both an LPC and a fatty acid molecule in the binding pocket. This interpretation seems to be consistent with the results obtained for the synthesis rates using different chain length fatty acids (Figure 7): the shorter the chain length, the faster the esterification rate. In fact, the rate for stearic acid was extremely low, which could be plausibly ascribed to the greater difficulty of this long, flexible chain to travel through the channel and properly reach the active-site catalytic machinery. In this sense, the introduction in the fatty acid molecule of one or more unsaturations, and hence of some stiffness in the chain, should be expected to alleviate the steric restrictions and result in an enhanced rate of synthesis, as was indeed the case (Figure 7).

It is worth briefly considering the yield obtained in the synthesis of PC*. It must be emphasized that to the best of our knowledge in all the previous efforts using PLA2 in nonaqueous medium for the esterification of lyso-PC and fatty acid the yields were rather poor. For instance, with ppPLA2, the maximum yield was about 6% in an AOT reverse micellar system (Na et al., 1990) and 1.3% in a system containing a suspension of the enzyme (Pernas et al., 1990) (in this latter case, the LPC concentration proposed for maximal conversion in anhydrous toluene was 70 mM). Significantly, the yield in our hands increased to 35%, which means a 6-fold and a 27-fold increase relative to the micellar and the heterogeneous system, respectively. It has even been explicitly stated that esterification or transesterification of a phospholipid mediated by PLA2 is clearly not as facile as the lipase-catalyzed triglyceride conversion (Na et al., 1990). This difficulty might arise not only from the aforementioned steric restrictions imposed by the access channel to the phospholipase catalytic site but also from the different general mechanism of catalysis of both types of enzymes (Waite, 1985, 1987). The present effort to alleviate, at least in part, these drawbacks illustrates how in the particular case of PLA2 an indiscriminate increase in substrate concentration is rather counterproductive for maximizing the yield, even at quite long reaction times.

It has been previously shown for different enzymes that their substrate specificity or enantioselectivity can be altered in organic solvents relative to aqueous medium [see, e.g., Gololobov et al. (1992) and Tawaki and Klibanov (1992)]. Such a "medium (solvent) engineering" approach has been proposed as an alternative complementing the more widely used "protein engineering" strategies, to control a number of enzymatic properties (Klibanov, 1989). Although several efforts have been reported aimed at modifying PLA2 activity, stability, or substrate specificity by protein engineering [see, e.g., Noel et al. (1991) and Weiss et al. (1991)], this is the first time that a medium engineering-based, quite dramatic reversal of PLA2 substrate specificity is described. In the nonaqueous medium (see Figure 4), the hydrolytic activity of the enzyme toward a neutral, zwitterionic phospholipid such as PC was determined to be remarkably higher than toward anionic substrates, in sharp contrast to the behavior exhibited in water for the same substrates (Thuren et al., 1987), or even for phospholipid analogs at submicellar concentrations (van Oort et al., 1985b). Whereas a few attempts have been made for other enzymes (mainly proteases), trying to rationalize the observed changes in substrate specificity when placed in organic medium [see, e.g., Gololobov et al. (1992) and references cited therein], interpretation of the results in the case of PLA2 does not seem simple and should probably take into account a number of considerations. On the one hand, inspection of the results in Figure 4 seems to indicate that in nonaqueous medium the net charge of the substrate (negative vs zero) is no longer a crucial factor determining the enzyme's preference. In this sense, the reasons commonly invoked for the well-known marked preference for negatively charged substrates of ppPLA2 in aqueous medium (even below the cmc), based on a favored interaction (activation) of the enzyme with these substrates relative to neutral ones, do not seem to apply any more in the low-water environment. Also notice that once in nonaqueous medium ppPLA2 virtually lacks the opportunity to be "activated" either by a conformational change (enzyme model) or by a proper orientation of the substrate facilitating diffusion into the enzyme (substrate model) [see, e.g., Peters et al. (1992)]. The substrate size does not appear either to be a determinant factor. It cannot be disregarded, however, that the different dielectric screening of the medium or the effective hydration degree of the different polar heads could somewhat affect electrostatic interactions important for the partitioning of the substrate into the catalytic site. On the other hand, the altered order of preference in the anhydrous environment could arise, at least partly, from the apparently greater tendency of the more slowly hydrolyzed phospholipids to form inverted micellar aggregates in nonpolar solvents, which could reduce the actual concentration of monomolecularly dispersed substrate accessible to the enzyme. In fact, recent evidence from size-exclusion HPLC measurements (Bañó et al., 1988; and also unpublished results) suggests that, e.g., PS and PE form in nonpolar solvent (eluent) rather stable aggregates (present in the eluate after dilution through the column) with apparent cmc values lower than in the case of PC or CL. Anyway, any interpretation of the observed change in PLA2 specificity is complicated, taking into account the peculiar nature of both enzyme and amphiphilic substrates, and a dissection of the molecular reasons for this behavior deserves further investigation.

In conclusion, in this preliminary work we have explored how a water-restricted environment can modulate the catalytic properties of an extracellular PLA2. Thus, it has been shown that ppPLA2, when suspended in a monophasic low-water medium, displays a quite remarkable inversion in the order of preference toward the substrate polar head, is severely inhibited by LPC, and is able to acylate this substrate with an exquisite discrimination for long-chain fatty acids depending on whether or not they are unsaturated. It is hoped that these results will be stimulating for further studies (work is in progress in this direction), including other phospholipases, which may unveil novel facets in the catalytic properties of this type of enzyme, and enrich our understanding of the molecular basis of nonaqueous enzymology. On the other hand, since cellular PLA2s operate at membrane surfaces where water activity may be locally diminished, the question remains open as to whether these enzymes may under certain conditions catalyze transacylation reactions. This low-water approach could also be extended in the future to gain insight into the synthetic (acylating or transacylating) capabilities of

other enzymes, reported to be able to carry out CoA- and ATP-independent synthesis of phospholipids [see, e.g., Gross and Sobel (1982) and Kramer and Deykin (1983)].

Finally, from a practical standpoint, inspection of the results in Figure 4 shows that the nonaqueous medium offers a simple way to carry out selective hydrolysis of one given phospholipid in a mixture (e.g., PC in the presence of PE, PS, and PG) in reasonable times with the advantages of easy solvent removal and biocatalyst separation and reusability. It is also demonstrated that a nonexpensive PLA2 such as that from porcine pancreas can now be successfully exploited with more reasonable yields for the synthesis of tailored phospholipids (particularly cardiovascular disease-preventive polyunsaturated ones), of increasing interest in many industrial, food and medical applications (Szuhaj, 1989).

ACKNOWLEDGMENT

We express our thanks to Prof. L. Franco for his insightful comments and helpful discussions, and to Dr. M. C. Bañó for her suggestions.

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