Continuous fluorescence assay for lecithin:cholesterol acyltransferase using a water-soluble phosphatidylcholine

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Abstract A water-soluble fluorescent phosphatidylcholine, 1,2-bis[4-(1-pyreno-butanoyl]-*sn*-glycero-3-phosphocholine (DPybPC) has been used to develop a sensitive, continuous assay for pure lecithin:cholesterol acyltransferase (LCAT) in solution. The monomeric substrate allowed us to examine the reaction of LCAT in the absence of a lipid/water interface in terms of the sensitivity of the enzymatic reaction to anions, ionic strength, apolipoproteins A-I and A-II, and a series of lysophosphatidylcholines and fatty acids. In contrast to the reaction of LCAT with aggregated phosphatidylcholines, the reaction of DPybPC with LCAT was not significantly affected by anions, ionic strength, nor apolipoproteins, indicating that these are only effectors of the interfacial reaction. Lysophosphatidylcholines and fatty acids inhibited LCAT in a chain-length-dependent manner below the critical micellar concentrations of these amphiphiles, indicating that the products of the LCAT reaction can bind to the enzyme and affect its kinetics even in the absence of an interface.—**Bonelli, F. S., and A. Jonas.** Continuous fluorescence assay for lecithin:cholesterol acyltransferase using a water-soluble phosphatidylcholine. *J. Lipid Res.* 1992. **33:** 1863–1869.

Supplementary key words phosphatidylcholine • lysophosphatidylcholine • fatty acid • apolipoprotein A-I • apolipoprotein A-II

The formation of cholesteryl esters in human plasma by the transfer of the *sn*-2 acyl chain of phosphatidylcholine (PC) to cholesterol is mediated by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) (EC.2.3.1.43). It is quite clear from previous work (1–4), that the nature of the lipid/water interface, as well as various modulators of the LCAT reaction (e.g., salt concentration, anions, apolipoprotein structure) can profoundly influence the apparent reactivity and substrate specificity of LCAT. The use of catalytically inert reconstituted HDL (rHDL) into which test lipids were incorporated has provided some information regarding the specific requirements of the active site in terms of substrate structure and the effects of salts and anions in vitro (2, 3). Recently, we used water-soluble fatty acyl esters of *p*-nitrophenol (PNP esters) as substrates for the LCAT reaction to study the hydrolysis of molecular substrates by this enzyme independently of the interactions of LCAT with the lipid/water interface (5). With this system, the effects of substrate acyl chain length, anions, salt concentration, and apolipoproteins on LCAT hydrolysis of PNP esters were examined. Because the molecular structure of the PNP esters differs substantially from that of phosphatidylcholines, it is possible that the observed effects of the modulators described in our previous study are unique to the PNP ester substrate system, and may not be the same for the hydrolysis of monomeric phosphatidylcholines.

We describe in this work a continuous fluorescence assay to follow LCAT hydrolysis of the fluorescent phosphatidylcholine 1,2-bis[4-(1-pyreno)-butanoyl]-*sn*-glycero-3-phosphocholine (DPybPC) at concentrations at which it exists in monomeric form, in order to demonstrate that the effects on LCAT hydrolysis of the PNP esters by external modulators previously described are not peculiar to that substrate system, but are properties of the interaction with water-soluble substrates in general. Furthermore, we have extended the study of the LCAT reaction toward water-soluble substrates by examining the effects of lysophosphatidylcholines and fatty acids on LCAT hydrolysis of DPybPC, to further elucidate the mechanism of LCAT inhibition by these compounds.

Abbreviations: PC, phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; rHDL, reconstituted high density lipoproteins; PNP esters, *p*-nitrophenol esters; DPybPC, 1,2-bis[4-(1-pyreno)-butanoyl]-*sn*-glycero-3-phosphocholine; lysoPC, lysophosphatidylcholine or lysolecithin; apoA-I and apoA-II, apolipoproteins A-I and A-II; CMC, critical micellar concentration; ANS, 8-anilino-1-naphthalenesulfonic acid; lysoPE, lysophosphatidylethanolamine; egg PC, PC from egg yolk; ether POPC, ether analog of palmitoyloleoyl phosphatidylcholine.

MATERIALS AND METHODS

Materials

1,2-Bis[4-(1-pyreno)butanoyl]-*sn*-glycero-3-phosphocholine (DPybPC) was obtained from KSV Lipids Oy, Finland, in sealed ampules under argon in an ethanol–toluene 1:2(v/v) solvent. The phospholipid concentrations in these solutions were determined by the phosphate assay method of Chen, Toribara, and Warner (6). Working stock solutions of DPybPC were prepared in spectroscopic grade methanol (Aldrich Chemical Co.) and stored under N_2 at −18°C during the course of the experiments. Methanol was chosen as the solvent for the working stock solutions due to its miscibility with water, allowing the direct addition of the substrate to the reaction mixture. The DPybPC working stock solutions were stable for approximately 1 month under these conditions, as assessed by excimer fluorescence intensity. No decomposition, as determined by TLC analysis using a solvent system of chloroform–methanol–water 65:25:4, could be detected in control solutions during the time in which the stock solutions were being used in the kinetic measurements.

The lysophosphatidylcholines (lysoPC): 1-caproyl, 1-capryl, 1-lauroyl-*sn*-glycero-3-phosphocholine and 1-lauroyl-*sn*-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids. The sodium salts of caproic, caprylic, lauric, and myristic acids, as well as the lysophosphatidylcholines, 1-palmitoyl and 1-stearoyl-*sn*-glycero-3-phosphocholine, were purchased from Sigma Chemical Co., St. Louis, MO. The lysophosphatidylcholines were checked for purity by thin-layer chromatography on silica gel 60 F254 glass-backed plates (E. Merck) using a solvent system of chloroform–methanol–water 65:25:4. The spots were detected by spraying the plates with 8% H_2SO_4 in ethanol and charring at 150°C. Since the lysophosphatidylcholines appeared pure by this method, they and the fatty acids were used as supplied. The salts used in this work were reagent grade chemicals obtained from Malinckrodt.

Purification of proteins

Human LCAT was purified by methods described previously (7) from 2 liters of blood plasma donated by the Champaign County Blood Bank, Regional Health Resource Center. The purity (>95%) was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie Blue staining using the Pharmacia Phast system. The stock enzyme solutions containing 50–97 μg/ml LCAT were stored at 4°C under N_2 in 10 mM Tris, 5 mM EDTA, pH 7.6 buffer. The specific activity of the LCAT stock solutions was determined by using egg PC, ^{14}C-labeled cholesterol, and apoA-I rHDL (7) as substrates. The average specific activity was 34 nmol cholesteryl ester formed/h per μg. The LCAT preparations retained approximately 90% of their activity over a period of 6 months. Apolipoproteins A-I and A-II (apoA-I and apoA-II) were prepared from fresh human plasma by a procedure similar to that described by Edelstein, Lim, and Scanu (8). Prior to use, the lyophilized proteins were solubilized in 3 M guanidine HCl and extensively dialyzed against 10 mM Tris-HCl, 0.15 M NaCl, 0.01% EDTA, and 1 mM NaN_3, pH 7.4 buffer. The concentrations of the apolipoprotein solutions were determined by the method of Lowry et al. (9) using bovine serum albumin as the standard. Reconstituted ether POPC rHDL particles containing ether POPC and apoA-I (in molar ratios of 80:1) were prepared by the cholate dialysis method (10) as were the rHDL substrates used in the assays during the preparation of the enzyme.

Enzymatic reaction

The esterase activity of LCAT was measured by following the increase in fluorescence intensity due to the formation of 4-(1-pyreno)butanoic acid and 1-[4-(1-pyreno)butanoyl]-*sn*-glycero-3-phosphocholine, the products of DPybPC hydrolysis, over time. The increase in fluorescence intensity is due to the separation of the two pyrene groups which, in the intact DPybPC molecules, result in quenching of monomer fluorescence. Typical reaction mixtures contained 0.03 to 0.07 μg/ml LCAT, 8×10^{-8} M DPybPC added from the methanol stock solution (3% methanol, v/v, final concentration), 4 mM 2-mercaptoethanol, 10 mM Tris-HCl, 150 mM NaCl, 0.01% EDTA, pH 7.4 buffer, with the indicated concentration of apolipoprotein, lysoPC, or fatty acid in a total volume of 1 ml. In the studies of salt and anion effects, the Tris-HCl buffer contained no NaCl. The Tris-HCl buffer solutions were purged with N_2 for 45 min before use to prevent the oxidation of the substrate by dissolved O_2 during the time course of the assay (11). LCAT was added to initiate the reaction after equilibrating the solution at the selected temperature. The time course of the reaction was followed by measuring the increase in pyrene monomer fluorescence of the hydrolysis products at 398 nm in a 1.0 cm pathlength fluorescence cuvette using a computer-controlled Perkin-Elmer MPF 66 spectrofluorometer. The excitation wavelength was 332 nm and the excitation and emission slit widths were 5 nm. The increase in fluorescence intensity was followed for 5 min after the addition of LCAT; the reaction proceeded in a linear fashion for up to 15 min. No increase in the fluorescence intensity at 398 nm was noted in the absence of LCAT for periods of up to 15 min. Temperature was regulated to within ± 0.1°C of the selected temperature by circulating water through the cuvette holder using a Lauda RM6 circulating water bath. Initial velocities were expressed as changes in fluorescence intensity per min and were obtained from the slope of the reaction time course as generated by the MPF 66 computer. The hydrolysis of DPybPC by LCAT could be detected at substrate concentrations as

low as 10^{-8} M. Initial velocities were proportional to substrate concentrations up to 5×10^{-7} M, but decreased at higher concentrations of the substrate possibly due to inner filter effects or to enzyme saturation. The initial velocity of DPybPC hydrolysis, as determined by the increase in fluorescence intensity per minute, increased linearly with increasing LCAT concentration over the range of 0.03 μg/ml to 0.1 μg/ml enzyme. Bovine serum albumin in concentrations as low as 20 μg/ml increased the monomer fluorescence emission at 398 nm at least 5-fold. This increase in fluorescence intensity was instantaneous and did not increase further over a period of 10 min. No effect was noted on the excimer emission at 480 nm. However, because the increase in fluorescence intensity of the monomer emission in the presence of BSA obscured the subsequent LCAT reaction, BSA was excluded from all reaction mixtures.

Critical micellar concentrations

Critical micellar concentrations (CMC) of the lysoPCs used in this study were determined by using 8-anilino-1-naphthalenesulfonic acid (ANS) (Sigma Chemical Co.) as described by De Vendittis et al. (12) under the reaction conditions of the assay described in the previous section. **Table 1** lists the CMC determined in this work compared to values obtained in the literature. The CMC of DPybPC was taken to be 7.6×10^{-6} M as determined by Hendrickson and Rauk (11).

RESULTS

Anions at concentrations of 0.1 M have profound effects on the reaction rates of LCAT with interfacial rHDL substrates (3, 14) but have no effect on the hydrolysis of the water-soluble substrate, *p*-nitrophenol butyrate (PNPB), by LCAT. **Fig. 1** shows that hydrolysis of monomeric DPybPC by LCAT was similarly unaffected by the presence of 0.1 M concentrations of F^-, Cl^-, and Br^- anions. A 35% decrease in the reaction rate is noted in the presence of 0.1 M NO_3^-. The effects of I^- and SCN^- were not examined because of the quenching of pyrene fluorescence (15) by these ions; the apparent decrease in the reaction rate due to NO_3^- is probably also the result of fluorescence quenching.

In **Fig. 2**, panel A, the effect of increasing the NaCl concentration on the hydrolysis of DPybPC by LCAT is demonstrated. After a small (30%) increase in the reaction rate from 0 to 0.1 M NaCl, concentrations of NaCl up to 0.4 M had no effect on LCAT hydrolysis of

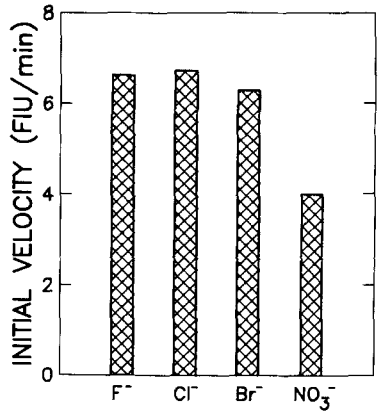

Fig. 1. Effect of anions on the hydrolysis rates of DPybPC by LCAT. The reaction mixtures contained 0.1 M concentrations of the anions, 0.025 μg/ml LCAT, 4 mM 2-mercaptoethanol, and 8×10^{-8} M DPydPC in 10 mM Tris-HCl, 0.01% EDTA, pH 7.4 buffer. The reactions were performed at 37°C. FIU are fluorescence intensity units.

TABLE 1. Critical micellar concentrations of lysophosphatidylcholines

LysoPC	CMC (M)	
	Present Work[a]	Literature[b]
C6 lysoPC	ND	0.7[c]
C8 lysoPC	ND	0.07[c]
C10 lysoPC	5.0×10^{-3}	7.0×10^{-3}
C12 lysoPC	5.0×10^{-4}	7.0×10^{-4}
C16 lysoPC	2.5×10^{-5}	7.0×10^{-6}
C18 lysoPC	5.0×10^{-6}	NA
C12 lysoPE	3.0×10^{-4}	3.3×10^{-4}

ND, not determined; NA, not available.

[a]Critical micellar concentrations (CMC) were measured using ANS as described in Materials and Methods (12) in 10 mM Tris-HCl, 150 mM NaCl, 1 mM NaN_3, 0.01% EDTA, pH 8.0 buffer at 37°C.

[b]The method for the determination of the CMC and the buffer system are described in reference 13.

[c]Estimated.

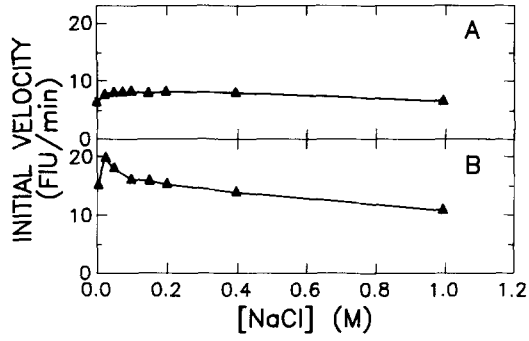

Fig. 2. Initial velocities of DPybPC hydrolysis by LCAT as a function of NaCl concentration. Measurements of DPybPC monomer hydrolysis (panel A) and hydrolysis in the presence of ether POPC-rHDL (panel B) were performed at 37°C in 10 mM Tris-HCl, 0.01% EDTA, 4 mM 2-mercaptoethanol, pH 7.4, with the indicated concentrations of NaCl and 8×10^{-8} M DPybPC. The LCAT concentration used for the hydrolysis of the monomeric DPybPC was 0.025 μg/ml and 0.1 μg/ml for the hydrolysis of DPybPC in the presence of 2.65×10^{-6} M ether POPC-rHDL particles (concentration of ether POPC). FIU are fluorescence intensity units.

DPybPC. A slight decrease in the reaction rate was observed at higher concentrations of NaCl (up to 1 M). These results compare closely to the minimal effects of NaCl on the enzymatic PNPB hydrolysis determined previously (5). In panel B of Fig. 2, the effect of increasing NaCl concentration on the LCAT hydrolysis of DPybPC in the presence of rHDL prepared with ether POPC (80:1 ether POPC/apoA-I) is demonstrated. From the increase in fluorescence intensity at 480 nm, it is estimated that approximately 30% of the DPybPC partitions into the rHDL particles. In this case the rate of DPybPC hydrolysis increases with NaCl concentration, reaching a maximum at 0.1 M NaCl and declining thereafter. This is in contrast with the pattern seen in the absence of the rHDL, and is almost identical to the pattern seen with increasing NaCl concentration for the LCAT transacylase activity when rHDL, prepared with POPC or with ether POPC, containing test lipids, are used as substrates (3, 14).

We have previously shown that apolipoproteins A-I, A-II, and C do not influence LCAT hydrolysis of the water-soluble PNP acyl esters (5). Similarly, we have found in this study that apolipoproteins A-I and A-II in solution did not affect DPybPC hydrolysis up to 100:1, apolipoprotein/LCAT molar ratios.

Lysophosphatidylcholines (16–19) and fatty acids (20, 21) have been shown by several laboratories to inhibit the reactions catalyzed by LCAT, although the exact mechanism is unknown. **Fig. 3** shows the effects of lysoPC with increasing acyl chain lengths on the hydrolysis rates of DPbyPC by LCAT. Inhibition occurs in a chain length-dependent fashion, with the longer acyl chain lysoPCs inhibiting at lower concentrations. For each lysoPC, greater than 90% inhibition of the LCAT hydrolysis of DPybPC occurs prior to its CMC. Fig. 3 also shows the effect of dodecanoyl phosphatidylethanolamine on the rate of DPybPC hydrolysis by LCAT. Evidently, the effect of lysoPE is the same as that of the corresponding lysoPC, indicating that the absence of the three bulky methyl groups in the headgroup has little effect on the inhibition of substrate hydrolysis by LCAT. This is consistent with the observation of Pownall, Pao, and Massey (2) that PE is only a slightly better substrate than PC in an ether POPC matrix, suggesting that a comparable inhibition should be seen with both lysoPC and lysoPE.

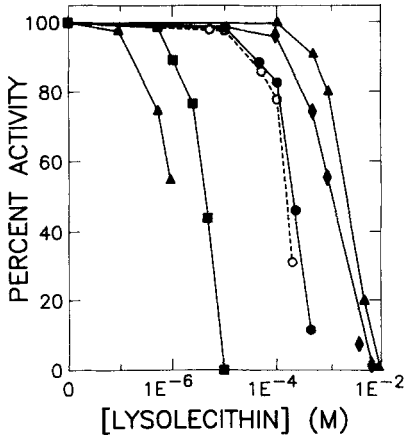

Fig. 3. Effect of lysoPC (lysolecithin concentration) on the rate of DPybPC hydrolysis by LCAT. Initial velocities of DPybPC hydrolysis were measured at 37°C in 10 mM Tris-HCl, 150 mM NaCl, 0.01% EDTA, pH 7.4 buffer, containing 8 × 10^{-8} M DPydPC, 0.075 μg/ml LCAT, 4 mM 2-mercaptoethanol, and the indicated concentrations of lysoPCs (from left to right): C-18 lysoPC (▲), C-16 lysoPC (■), C-12 lysoPC (●), C-12 lysophosphatidylethanolamine (○), C-8 lysoPC (♦), and C-6 lysoPC (▲). The average initial velocity of the control samples, expressed as the change in fluorescence intensity with time, was 12.8 fluorescence intensity units/minute.

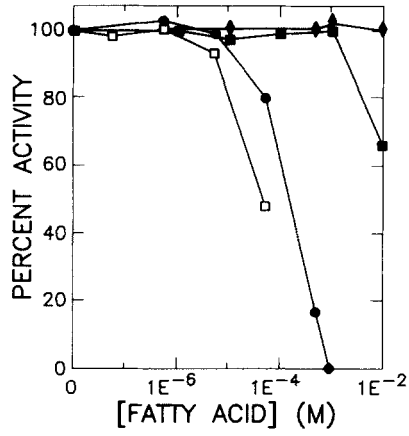

Fig. 4. Effect of fatty acids on the hydrolysis of DPybPC by LCAT. The rate of DPybPC hydrolysis was measured at 37°C in 10 mM Tris-HCl, 150 mM NaCl, 0.01% EDTA, pH 7.4 buffer, containing 8 × 10^{-8} M DPydPC, 4 mM 2-mercaptoethanol, 0.025 μg/ml LCAT, and the indicated concentration of sodium myristate (C-14) (□), sodium laurate (C-12) (●), sodium caprylate (C-8) (■), or sodium caproate (C-6) (♦). The average initial velocity of the control, expressed as the change in fluorescence intensity with time, was 6.1 fluorescence intensity units/min.

Fig. 4 shows how increasing the acyl chain length of a series of fatty acids (as their sodium salts) affects the rate of DPybPC hydrolysis. Caproic and caprylic acids have no effect on the hydrolysis of DPybPC by LCAT up to a concentration of 10^{-3} M. At concentrations greater than 10^{-3} M, caprylic acid inhibits DPybPC hydrolysis, with 30% inhibition occurring at 10^{-2} M. A significant degree of inhibition is observed for the medium chain length fatty acids, lauric and myristic acids, at lower fatty acid concentrations. Fifty percent inhibition of DPybPC hydrolysis occurs at 1.5 × 10^{-4} M laurate and 5.5 × 10^{-5} M myristate concentrations.

DISCUSSION

DPybPC was first synthesized by Hendrickson and Rauk (11) as a fluorescent substrate to monitor the activity of phospholipase A_2. These workers also noted that

molecular models of DPybPC and 1,2-bis-decanoyl-*sn*-glycero-3-phosphocholine showed that both phospholipids have the same extended length, and speculated that they have similar total hydrophobicities. Thus, in spite of the pyrene groups, DPybPC represents quite well the structure of the physiological phospholipid substrates of LCAT. Below its CMC DPybPC can be used to study the LCAT phospholipase reaction toward monomeric substrates by monitoring the hydrolysis of DPybPC in a continuous manner. Furthermore, because the DPybPC has a more physiologically relevant molecular structure compared to the PNP esters, the use of this substrate system complements the previous observations on the hydrolysis of water-soluble PNP esters by LCAT (5).

It is conceivable that phosphatidylcholines, because of their charged phosphate and choline groups, may interact with the active site of LCAT via charged amino acid residues that are necessary for proper positioning and stabilization of one or more chemical intermediates in the enzymatic reaction. In contrast, the PNP esters, which lack these charged groups, may not bind to LCAT in the same way and may not be susceptible to the ionic strength or composition effects as noted by us earlier (5). Therefore, to separate the effects of anions and increasing salt concentrations on the active site interactions of LCAT from their effects on the interaction of LCAT with a lipid/water interface, it is important to determine the effects of these factors on the hydrolysis of monomeric phosphatidylcholines by LCAT. In this study, we demonstrated that anions (F^-, Cl^-, and Br^-) do not affect the hydrolysis of DPybPC by LCAT, in contrast to what has been observed by Jonas et al. (3, 14) for the LCAT reaction on interfacial PC substrates, but in agreement with our findings when water-soluble PNP esters are used as substrates (5). We also observed that, despite the difference in chemical structure, the effect of increasing NaCl concentration on LCAT hydrolysis of both DPybPC and PNPB, although not identical, was quite similar. This is in marked contrast to the patterns and differences observed in the initial velocity versus NaCl concentration results for rHDL substrates composed of different PCs (3). Furthermore, it is interesting to note that the salt effects on the initial velocity of DPybPC hydrolysis by LCAT in the presence of ether PC-rHDL matrix are comparable to those observed by Jonas et al. (3) for the various PCs incorporated into the ether PC-rHDL matrix. This is probably the result of the partitioning of DPybPC into the ether PC-rHDL matrix and LCAT reaction with this substrate at the lipid/water interface. This is direct evidence that salt concentration affects the interaction of the enzyme with the interface. In addition, ionic strength has some influence on the substrate reaction at the active site of LCAT, as suggested by the observation for all substrates, both water-soluble and aggregated, that the reaction rates at 0 M NaCl are always lower than at higher salt concentrations. This effect does not seem to be of the same magnitude as that affecting the interfacial interactions, and may be due to a slight change in enzyme conformation such that access to the active site is decreased at low ionic strengths (22). The slight increase in initial velocity for the hydrolysis of the water-soluble substrates by LCAT, with increasing NaCl concentration, might be due to stabilization of the enzyme structure by nonspecific interactions with the ions, or to enhanced substrate binding at the active site by increasing the hydrophobic interactions between substrate and enzyme.

Both the transacylase and phospholipase reactions of LCAT are markedly stimulated by the presence of apoA-I (7, 16, 18) when PCs in vesicles or rHDL particles are the substrates; in fact, in the absence of apoA-I or other apolipoproteins the activity of LCAT (18, 23, 24) is essentially undetectable. However, we demonstrate in this study that neither apoA-I nor apoA-II, at molar ratios up to 100:1, apolipoprotein/LCAT, affected DPybPC hydrolysis, in agreement with the findings in our previous study of PNPB hydrolysis by LCAT (5). Thus, apolipoproteins are unable to enhance further the LCAT reaction toward water-soluble substrates. This suggests that the role of apolipoproteins is restricted to the activation of the LCAT reaction toward aggregated substrates (i.e., vesicles or rHDL) either by enhancing entry of the lipid from the aggregate into the enzyme active site, or by activating the interfacial lipid such that it can react with LCAT as efficiently as the water-soluble substrates.

LysoPC is a product of both the phospholipase and the transacylase reactions of LCAT. Free fatty acids are produced during the phospholipase reaction, and we have shown by monitoring the production of radiolabeled fatty acids, that significant quantities of free fatty acids are also produced during the transacylation reaction when rHDL containing DPPC and 5 mole% cholesterol are used as substrates (F. S. Bonelli, unpublished results). Several workers have demonstrated that the production of lysoPC during the transacylase reaction eventually inhibits the LCAT reaction, and that this inhibition can be relieved by serum albumin (18, 19). Little work has been done to date on the inhibition of purified LCAT by fatty acids; however, Rutenberg, Lacko, and Soloff (20) demonstrated that LCAT activity in human plasma could be inhibited by free fatty acid concentrations greater than 1 mM; similarly, Amr, Hamosh, and Hamosh (21) reported a relationship between increased free fatty acid concentrations and decreased LCAT activity in 5-week-old Sprague-Dawley male rats after the administration of Intralipid (an emulsion of phospholipids and triacylglycerols that produces large amounts of fatty acids upon lipolysis).

The mechanism of LCAT inhibition by both lysoPCs and fatty acids is still unclear, but there are several possibilities, including competitive inhibition at the active site, binding to an effector site elsewhere on the protein which

modulates catalysis, or binding to a region of LCAT that governs interaction of the enzyme with the interface or with apoA-I (25). An accumulation of these products at the lipid/water interface could also impair the interaction of LCAT with the interface or the activation by apoA-I by changing the properties of the interface. The reverse reaction could become significant in the presence of an interface, as high concentrations of the reaction products may occur locally. Finally, there is the possibility that premicellar concentrations of amphiphiles may induce LCAT aggregation affecting reaction kinetics, as has been shown by Hazlett and Dennis (26) for the dimerization of cobra phospholipase A_2 in the presence of monomeric PCs.

The inhibition of the enzymatic hydrolysis of DPybPC by lysoPCs demonstrated in this work indicates that these compounds can act directly on the enzyme to influence catalysis. The inhibition occurred below the CMC for each of the lysoPCs indicating that lysoPC monomers, rather than micelles (which could bind enzyme and inhibit DPybPC hydrolysis by reducing the amount of free enzyme available to react with the substrate (27), are responsible for the inhibition. This inhibitory effect is not the result of nonspecific detergent binding and denaturation of the enzyme as the inhibition occurs at relatively low concentrations of lysoPC, and lysoPCs are unlikely to bind cooperatively to proteins and to denature them (28). Our findings do imply that a hydrophobic region on LCAT is involved in binding of the lysoPCs as we found the longer acyl chain lysoPCs to be more potent inhibitors than the lysoPCs with the shorter acyl chains. Whether this region is the active site or an effector site elsewhere on the protein remains to be elucidated. However, the observation that palmitoyl lysoPC can inhibit PNPB hydrolysis by LCAT in a competitive manner provides evidence for binding of lysoPCs within the enzyme active site (29).

In this study, we also demonstrated that free fatty acids can influence the hydrolysis of DPybPC by LCAT through direct effects on the enzyme. As was the case for lysoPCs, fatty acids inhibit the hydrolysis of DPybPC in a chain-length-dependent manner, implicating a hydrophobic binding region on LCAT. A lower limit to the effectiveness of acyl chain length in inhibiting enzymatic hydrolysis apparently exists as caproic acid at concentrations up to 0.01 M had no effect on the enzymatic reaction.

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