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Stereochemical and Positional Specificity of the Lipase/Acyltransferase Produced by *Aeromonas hydrophila*†

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Received December 23, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: *Aeromonas* species secrete a glycerophospholipid–cholesterol acyltransferase (GCAT) which shares many properties with mammalian plasma lecithin–cholesterol acyltransferase (LCAT). We have studied the stereochemical and positional specificity of GCAT against a variety of lipid substrates using NMR spectroscopy as well as other assay methods. The results show that both the primary and secondary acyl ester bonds of L-phosphatidylcholine can be hydrolyzed but only the *sn*-2 fatty acid can be transferred to cholesterol. The enzyme has an absolute requirement for the L configuration at the *sn*-2 position of phosphatidylcholine. The secondary ester bond of D-phosphatidylcholine cannot be hydrolyzed, and this lipid is not a substrate for acyl transfer. In contrast to the phospholipases, but similar to LCAT, the enzyme does not interact stereochemically with the phosphorus of phosphatidylcholine. In fact, the phosphorus is not required for enzyme activity, as GCAT will also hydrolyze monolayers of diglyceride, although at much lower rates.

Lipases are found throughout nature. All those which have been studied have been classified as members of the family of serine esterases, distinguished by their ability to act at lipid–water interfaces. They have recently attracted a great deal of attention, partly because of the important roles they play in lipid metabolism, as well as because of the many applications they may have in industry and medicine (Harwood, 1989). The glycerophospholipid–cholesterol acyltransferase (GCAT), released by *Vibrio* species, and lecithin–cholesterol acyltransferase (LCAT), found in mammalian plasma, may be grouped with the lipases as they each have small regions corresponding to the lipase consensus sequence in which their

† Supported by grants from the British Columbia Heart and Stroke Foundation and from the National Science and Engineering Research Council.

0006-2960/92/0431-4974$03.00/0 © 1992 American Chemical Society

active-site serines are located (Farooqui et al., 1988; Francone & Fielding, 1991; Thornton et al., 1988; Hilton & Buckley, 1991a). Although GCAT and LCAT have no apparent sequence homology outside the consensus region, they share several properties which distinguish them from other lipolytic enzymes. Most notably, even in aqueous systems they preferentially catalyze acyl transfer when suitable acyl acceptors are present. Phosphatidylcholine and cholesterol are the substrates most often studied; however, both enzymes will promote transfer from a variety of long-chain fatty acid esters to a wide range of alcohols, including any sterol which contains an equatorial hydroxyl group and a trans-fused A:B ring system. They catalyze hydrolysis at both the *sn*-1 and *sn*-2 positions of glycerophospholipids, yet acyl transfer is always only from the *sn*-2 position (Buckley, 1982, 1983; Aron, 1978; Subbaiah et al., 1985). Previous studies, using biochemical assays with synthetic mixed-chain or radiolabeled phosphatidylcholines, have shown that GCAT and LCAT each exhibit predominant 2-positional specificity for both lipolysis and acyl transfer but that this preference is not absolute (Buckley, 1983; Aron et al., 1978; Pownall et al., 1985). The stereochemistry of hydrolysis or acyl transfer has not been studied for either enzyme, although it has been demonstrated using synthetic chiral dipalmitoylthiophosphatidylcholine (DPPsC) that the interaction of LCAT at the phosphorus atom of phospholipid head groups is not stereospecific (Bruzik & Tsai, 1991; Rosario-Jansen et al., 1990). This is in contrast to various phospholipases A_2, which selectively hydrolyze the R_p isomer of DPPsC, and phospholipases C and D, which show a preference for the S_p isomer (Tsai et al., 1985; Bruzik & Tsai, 1991).

In spite of the many similarities between LCAT and GCAT, the microbial enzyme is far more easily purified and much more stable than its mammalian counterpart, so that its potential uses in medicine and industry are promising. Here we examine the stereochemical and positional specificity of GCAT by measuring its activity against a number of lipid substrates, using a variety of assay techniques including ^{31}P NMR spectroscopy, which has been particularly valuable in following the reaction of phospholipase A_2 with mixtures of phospholipids possessing different head groups (Roberts et al., 1979; Roberts, 1991).

Materials and Methods

Lipid Substrates. L-α-Dipalmitoylphosphatidylcholine (L-DPPC), D-α-dipalmitoylphosphatidylcholine (D-DPPC), L-α-dilauroylphosphatidylcholine (L-DLPC), DL-α-dilauroylphosphatidylcholine (DL-DLPC), 1,2-dilauroyl-*rac*-glycerol (DLDG), egg phosphatidylcholine, and cholesterol were obtained from Sigma. [4-^{14}C]Cholesterol (58 mCi/mmol) and L-α-dipalmitoylphosphatidyl[*methyl*-^{14}C]choline ([^{14}C]DPPC; 153 mCi/mmol) were purchased from Amersham or New England Nuclear. A mixture of the R_p and S_p isomers of L-α-dipalmitoylthiophosphatidylcholine (DPPsC) was a generous gift from Dr. Karol Bruzik, The Ohio State University. D-α-Dilauroylphosphatidylcholine (D-DLPC) was prepared by extensive digestion of DL-DLPC with *Naja naja* phospholipase A_2 (Sigma) as described by Bruzik et al. (1983) followed by recovery of the D-DLPC which remained by preparative thin-layer chromatography on silica gel G plates (Whatman) in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2). Phospholipid concentrations were determined by measuring phosphorus as described by Bartlett (1959).

Enzyme Preparation. Aeromonas hydrophila GCAT was overexpressed from its cloned structural gene in *Aeromonas salmonicida* strain CB3 pJT2 (Buckley, 1990) and purified from cell-free culture supernatants as described previously (Hilton et al., 1990).

Enzyme Assays. The acyl transfer and phospholipase activities of GCAT were measured in inverted micelles containing Triton X-100 as before (Buckley, 1982; Buckley et al., 1982). One assay is based on acyl transfer to radiolabeled cholesterol from phosphatidylcholine and the other on the production of radiolabeled lysophosphatidylcholine and glycerophosphocholine as a result of the hydrolysis of [*choline*-^{14}C]DPPC. Phospholipase activity was also followed by ^{31}P NMR spectroscopy as described below.

^{31}P NMR Spectroscopy. Spectra were recorded on a Bruker AMX-360 NMR spectrometer equipped with a 5-mm QNP probe. The chemical shifts were referenced to external 85% H_3PO_4. Spectra were recorded at 35 °C with a sweep width of 2000 Hz with 8K data points, usually 128 transients with a pulse width of 3 μs, and an acquisition time of 2.04 s. CPD decoupling was employed. Line broadening was −1 Hz and Gaussian broadening 0.05 Hz. Samples (5 μmol) were dissolved in 0.5 mL of 50 mM MOPS, pH 7.2, 0.25 mM EDTA, 35% D_2O, and 5% Triton X-100.

Monolayer Experiments. Measurements of hydrolysis of lipid monolayers were carried out at constant surface pressure at 25 °C as described previously (Hilton & Buckley, 1991b) using a KSV 2200 surface barostat fitted with a zero-order trough. Enzyme velocities were calculated by multiplying the lipid molecular density by the trough width and the asymptotic slope of the plot of barrier movement vs time. An adjustment was made for the surface density of the lipid, which was determined independently from surface pressure vs molecular area isotherms.

Results

GCAT Hydrolysis of L-DPPC Measured by ^{31}P NMR Spectroscopy. The digestion of L-DPPC in Triton X-100 micelles was monitored directly by ^{31}P NMR spectroscopy at two different concentrations of GCAT. This permitted simultaneous observation of GCAT's phospholipase and lysophospholipase activities. The results in Figure 1 show that hydrolysis of L-DPPC clearly proceeded more rapidly at the *sn*-2 position, resulting in production of 1-lyso-PC. However, hydrolysis at the *sn*-1 position also occurred, since 2-lyso-PC could be detected even at the earliest time points. As the reactions progressed (Figure 1, panel B), a third downfield resonance appeared, which corresponded to glycerophosphocholine, produced by GCAT-catalyzed hydrolysis of the lysophosphatidylcholines (Buckley, 1982). The amount of 2-lyso-PC diminished relative to 1-lyso-PC throughout the time period studied (Figure 2), indicating that it was more rapidly degraded than the 1 isomer. It would therefore seem that there is a preference for the secondary ester bond of both phosphatidylcholine (Figure 1) and lysophosphatidylcholine.

Hydrolysis of L-DLPC and D-DLPC by GCAT. To determine the stereospecificity of GCAT-catalyzed hydrolysis at the chiral *sn*-2 position of the phospholipid backbone, the enzyme's activity against the L and D isomers of PC was compared in two different assay systems. When GCAT was tested for its ability to hydrolyze monolayers of L-DLPC and D-DLPC, a 6-fold preference for the L isomer was demonstrated (Table I). Similar results were obtained by direct isolation and chemical analysis of the lyso-PC products of GCAT hydrolysis of L-DLPC and D-DLPC in Triton mixed micelles.

GCAT Hydrolysis of D-DPPC Measured by ^{31}P NMR Spectroscopy. In order to investigate the possibility that the hydrolysis observed with the D isomer in Table I represented

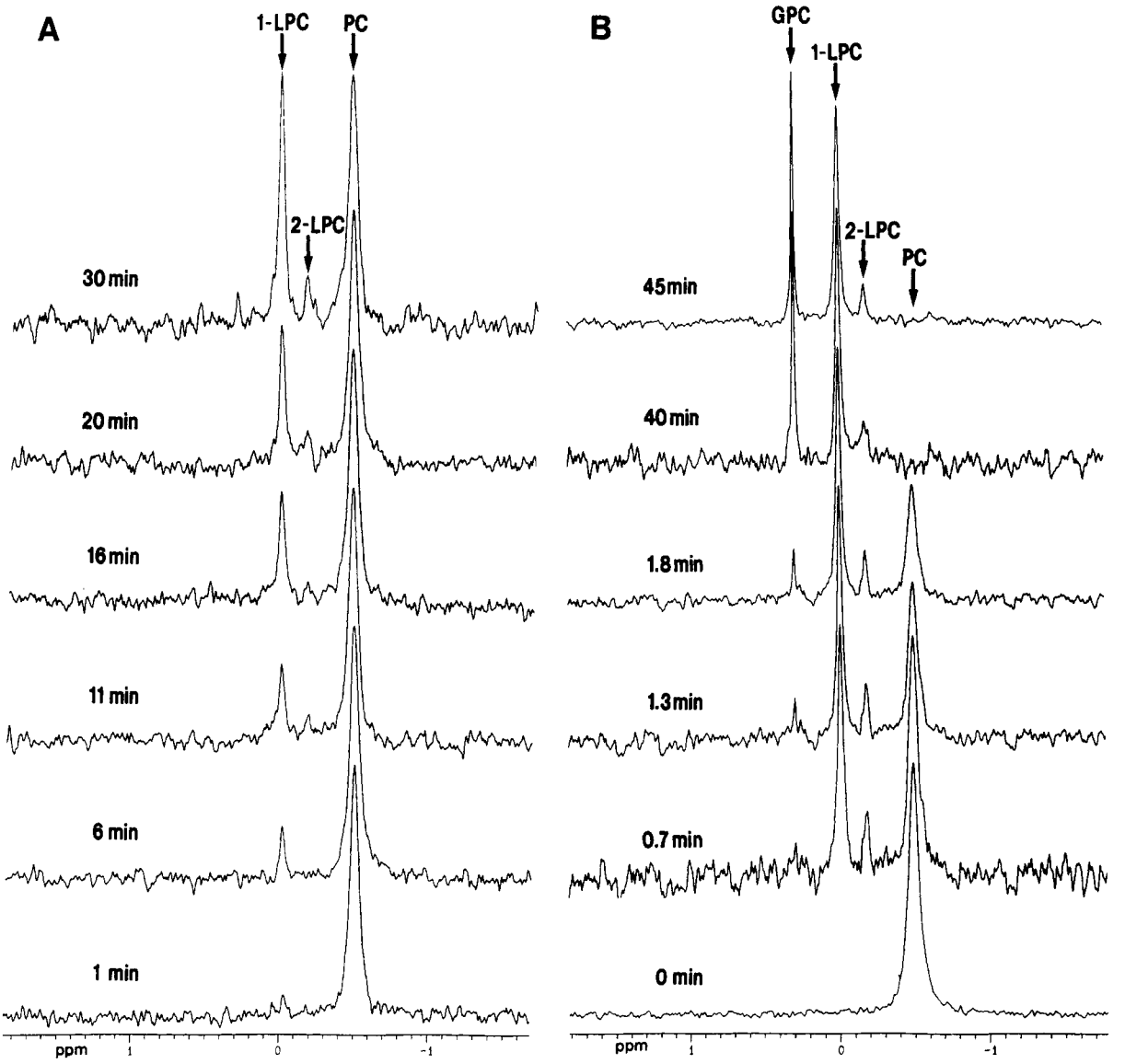

FIGURE 1: ^{31}P NMR spectra of the hydrolysis of L-DPPC by GCAT. Panel A, 1 μg of enzyme; panel B, 10 μg of enzyme. Peaks corresponding to phosphatidylcholine (PC), 2-acyllyso-PC (2-LPC), 1-acyllyso-PC (1-LPC), and glycerophosphocholine (GPC) are indicated by arrows. For experimental details, see Materials and Methods.

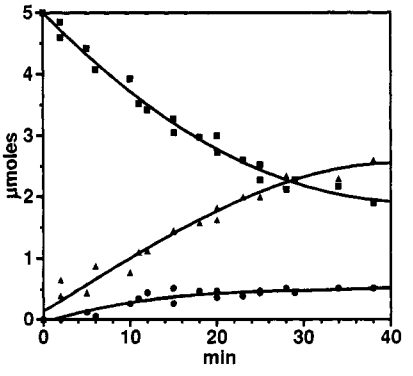

FIGURE 2: Time course of the breakdown of L-DPPC (■), 1-lyso-PC (▲), and 2-lyso-PC (●) by 1 μg of GCAT as followed by ^{31}P NMR spectroscopy.

fatty acid release entirely from the nonchiral *sn*-3 position of the molecule, hydrolysis of D-DPPC was also studied by ^{31}P NMR. The results, presented in Figure 3, are a striking contrast to those obtained with L-DPPC in Figure 1. They show that the enzyme may be completely unable to hydrolyze the ester linkage at the 2-position of D-DPPC. Virtually all of the lysophospholipid which was formed was the 2-acyl

Table I: Hydrolysis of L-DLPC and D-DLPC in Monolayers and in Triton Micelles

substrate	monolayer[a] (molecules/min)	micelle[b] (nmol of LPC)
L-DLPC	3.1×10^{15}	270
D-DLPC	0.5×10^{15}	82

[a] One microgram of GCAT enzyme was injected into the monolayer subphase as described under Materials and Methods. Results are the means of quadruplicate determinations which varied by less than 5%. [b] Each assay contained 0.5 μmol of PC and 2 μmol of Triton X-100 in 0.5 mL of 0.16 M KCl/20 mM Tris-HCl, pH 7.4. Incubations with 1 μg of GCAT enzyme were for 30 min at 40 °C. Results are the means of duplicate determinations which varied by less than 15%.

derivative, except at very late times when small amounts of 3-acyllysophosphatidylcholine could be detected, perhaps the product of spontaneous acyl migration from the 2-position.

Acyl Transfer by GCAT from L-DPPC and D-DPPC. The ability of GCAT to catalyze fatty acid transfer from the L and D stereoisomers of DPPC was compared using a Triton mixed micelle time course assay. No acyl transfer from D-DPPC to cholesterol was observed at any time points (Figure 4), indicating that the enzyme absolutely requires the L isomer for this reaction, in spite of the fact that it can catalyze hydrolysis

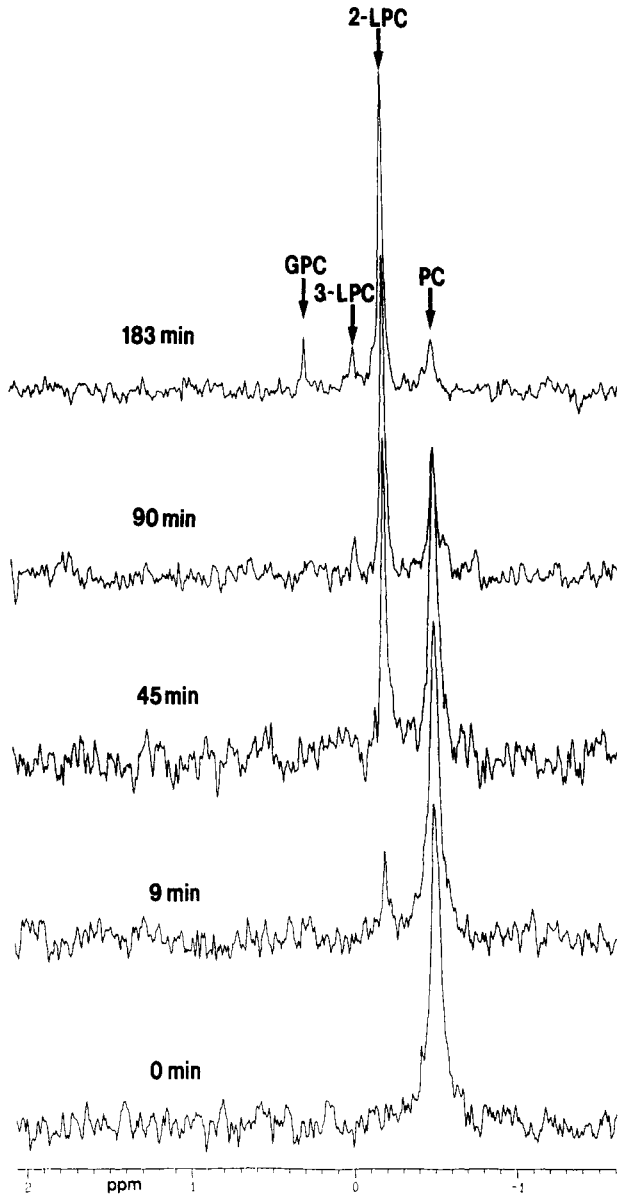

FIGURE 3: Hydrolysis of D-DPPC by 10 μg of GCAT as measured by ^{31}P NMR spectroscopy. Peaks corresponding to phosphatidylcholine (PC), 2-acyllyso-PC (2-LPC), 3-acyllyso-PC (3-LPC), and glycerophosphocholine (GPC) are indicated by arrows. For experimental details, see Materials and Methods.

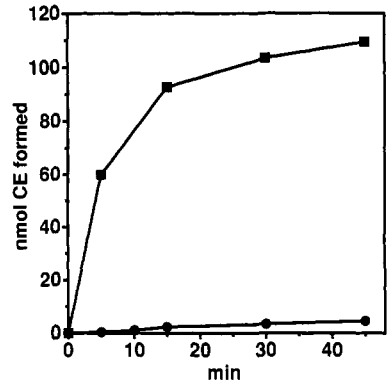

FIGURE 4: Time course of acyl transfer to cholesterol from L-DPPC (■) or D-DPPC (●). Each assay contained 0.5 μmol of DPPC, 0.25 μmol of cholesterol, 0.02 μCi of [^{14}C]cholesterol, and 2 μmol of Triton X-100 in 0.5 mL of 0.16 M KCl/20 mM Tris-HCl, pH 7.4. Incubation with 1 μg of GCAT was at 40 °C. Results are the means of duplicate determinations. CE, cholesteryl ester.

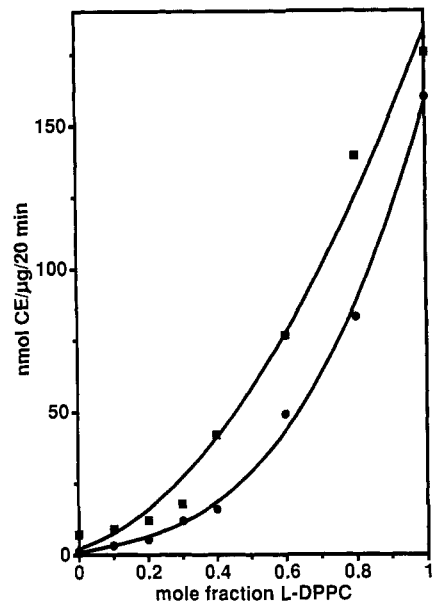

FIGURE 5: Effect of D-DPPC (■) and sphingomyelin (●) on acyl transfer from L-DPPC to cholesterol. Each assay contained a total of 0.5 μmol of phospholipid, 0.25 μmol of cholesterol, 0.02 μCi of [^{14}C]cholesterol, and 2 μmol of Triton X-100 in 0.5 mL of 0.16 M KCl/20 mM Tris-HCl, pH 7.4. Incubation with 1 μg of GCAT was for 20 min at 40 °C. CE, cholesteryl ester.

of the 3-position ester linkage of the D isomer (Figure 3).

Effect of D-DPPC and Sphingomyelin on Acyl Transfer from L-DPPC to Cholesterol. In view of the observation that D-DPPC was not a substrate for acyl transfer, it was of interest to determine how it would affect GCAT-catalyzed acyl transfer from the L isomer to cholesterol. Since we have shown previously that sphingomyelin is an inhibitor of the acyl transfer reaction (Buckley et al., 1982), it was included in the experiment for purposes of comparison. The results of the mixed micelle assays (Figure 5) show that D-DPPC acts in a similar fashion to sphingomyelin, inhibiting acyl transfer from L-DPPC to cholesterol.

Effect of D-DPPC and Sphingomyelin on the Phospholipase Activity of GCAT. Although sphingomyelin inhibits acyl transfer, it does not affect hydrolysis of PC (Buckley et al., 1982). We have interpreted this to mean that it does not compete with PC for a site on the enzyme but rather that by binding to cholesterol it may effectively reduce the concentration of steroid available for acyl transfer. The results in Figure 6 show that this may also be the case for D-DPPC. In contrast to the inhibition of acyl transfer observed in Figure 5, both D-DPPC and sphingomyelin had very little effect on the phospholipase or lysophospholipase activities of GCAT, as shown by the release of lyso-PC and glycerophosphocholine from L-DPPC in the assays presented in Figure 6A,B.

Hydrolysis of Chiral Thiophosphatidylcholine. In order to investigate any possible stereochemical constraints at the phosphorus atom of PC in its reaction with GCAT, the hydrolysis of a chiral mixture containing nearly equimolar amounts of the R_p and S_p isomers of DPPsC was followed by ^{31}P NMR (Figure 7). No preference was observed for either of the isomers, indicating that the interaction of GCAT with substrate does not involve stereospecific binding to the phosphate group.

Hydrolysis of Diacylglycerol Monolayers. The activity of GCAT on monolayers of dilauroyl-*rac*-glycerol (DLDG) was determined at a variety of lipid surface densities and compared to the activity against monolayers of DLPC, which we have studied previously (Hilton & Buckley, 1991). The results in Figure 8 clearly show that a phosphorus-containing head group is not essential for hydrolysis of glycerolipids by the enzyme

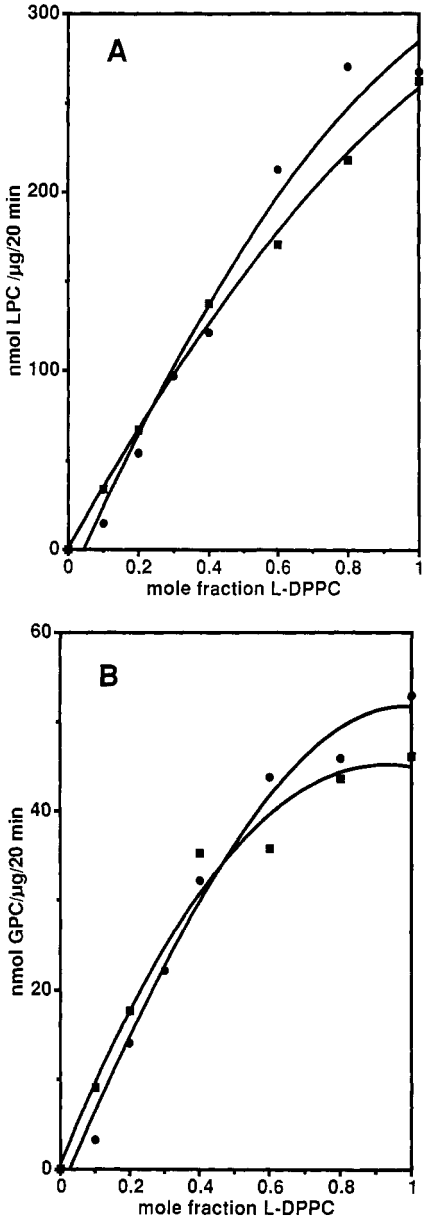

FIGURE 6: Effect of D-DPPC (■) and sphingomyelin (●) on hydrolysis of L-DPPC. Each assay contained a total of 0.5 μmol of phospholipid, 0.02 μCi of [^{14}C]DPPC, and 2 μmol of Triton X-100 in 0.5 mL of 0.16 M KCl/20 mM Tris-HCl, pH 7.4. Incubation with 1 μg of GCAT was for 20 min at 40 °C. Panel A, release of lyso-PC (LPC); panel B, release of glycerophosphocholine (GPC).

although the maximal enzyme velocity was far higher with DLPC (note the difference in scale and the amount of enzyme used with both substrates) and much higher surface densities of DLDG were required for measurable activity. Velocity decreased on either side of the maximum, producing a somewhat skewed bell-shaped curve not unlike those which have been obtained with lipoprotein lipase (Vainio et al., 1973) and pancreatic lipase (Rietsch et al., 1977) but quite different from the curve reported for the lipase of *Pseudomonas glumae* which does not show a maximum (Deveer et al., 1991). With DLPC and other phospholipids as substrates, we have observed lag periods, before the onset of hydrolysis, which depend on the surface pressure of the film (Hilton et al., 1991). No lag followed addition of the enzyme at any of the pressures we studied with diglycerides (data not shown).

DISCUSSION

Some lipolytic enzymes are specific for either the primary or the secondary ester bonds of their substrates. Pancreatic lipase is completely unable to attack secondary esters (Tattrie et al., 1958; Ransac et al., 1990), whereas phospholipase A_2 is absolutely specific for the 2-position of L-PC (van Deenen & de Haas, 1963). Other enzymes, such as the outer membrane phospholipase A of *Escherichia coli* (Horrevoets et al., 1989) and the lipase secreted by *Ps. glumae* (Deveer et al., 1991), are able to hydrolyze both kinds of esters, although they each favor the primary bond. The results presented here show that GCAT can also attack both ester linkages and that, as is the case with its mammalian analogue LCAT, it preferentially hydrolyzes the secondary ester.

Also like LCAT, and in contrast to the phospholipases, GCAT does not appear to interact specifically with the phosphate of PC. Thus, the results in Figure 7 show that there is no obvious difference in the rate of breakdown of the R_p and S_p isomers of DPPsC. Consistent with this result is the evidence that GCAT will catalyze hydrolysis of diacylglycerol monolayers (Figure 8), although at a much slower rate than phosphoglycerides, as has been demonstrated previously for LCAT (Pownall et al., 1985).

The action of GCAT on diacylglycerol monolayers is interesting in several other respects. In contrast to its action on phospholipids, there is no measurable lag time (the time taken by the enzyme to reach maximum velocity). This is similar to the behavior of lipoprotein lipase (Jackson et al., 1980) and suggests again that GCAT may lie somewhere between the phospholipases and true lipases. In addition, a higher molecular density was required for the onset of enzyme action, and the rate of increase in velocity with increasing DG surface density (Figure 8) appeared to be steeper than with PC.

The fact that D-DPPC is not a substrate for acyl transfer indicates not only that the L configuration at the 2-position is absolutely required for this reaction but also that the primary acyl groups of D- and L-PC (which are not asymmetric) cannot be transferred to cholesterol. This conclusion is supported by our earlier observation that 1-acyllyso-PC is a substrate for hydrolysis but not for acyl transfer (Buckley, 1983). Acyl transfer from the 1-position of L-PC which we previously reported (Buckley, 1983) may have actually occurred through the 2-position, by means of a lecithin–lysolecithin acyl transfer reaction such as that proposed by Fielding and Collet (1991) for LCAT.

Both LCAT and GCAT appear to be serine esterases, and this would suggest that the reactions they catalyze proceed by way of an acyl-enzyme intermediate. A single serine which is essential for activity has been identified within the lipase consensus sequence of both enzymes. Replacing this serine results in complete loss of the hydrolytic and acyl transfer activity of both LCAT and GCAT, an indication that both reactions occur at the same active site. How then is the positional specificity for acyl transfer obtained? If the first step in the attack at the 1- and 2-positions of L-PC results in the formation of the same acyl-enzyme intermediate, why can only the acyl group which came from the 2-position be transferred to cholesterol? Perhaps PC and cholesterol have to enter the active site of the enzyme together for acyl transfer to occur. Sphingomyelin and D-PC would inhibit acyl transfer, but not hydrolysis, by competing with L-PC for cholesterol as we have suggested earlier (Buckley, 1972). Once in the active site, cholesterol and PC may be oriented so that the carbonyl at the 2-position of PC is hydrogen-bonded to cholesterol, whereas the 1-position carbonyl is hydrogen-bonded to water. Hydrogen bonding of the 2-position carbonyl of L-PC to cholesterol has been suggested by several authors (Brokerhoff,

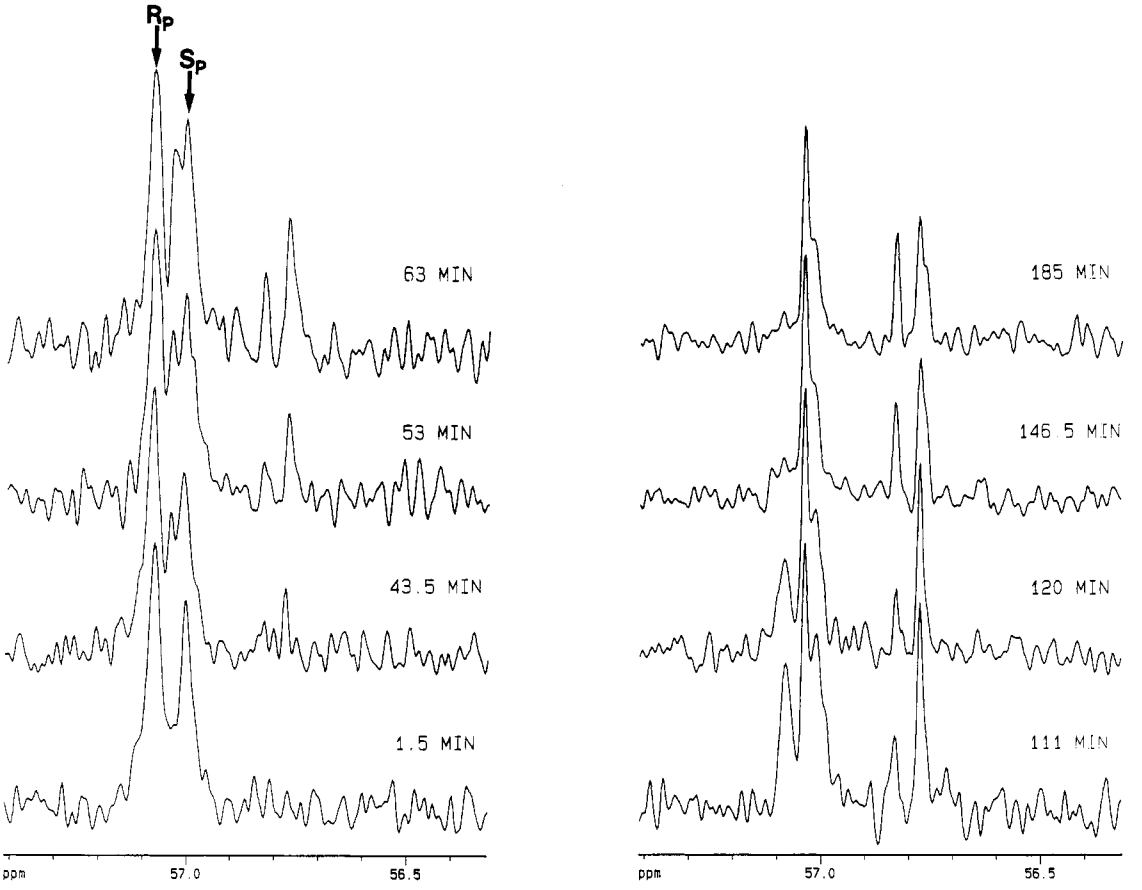

FIGURE 7: ^{31}P NMR spectra of the hydrolysis of (R_p,S_p)DPPsC by 1 μg of GCAT. For experimental details, see Materials and Methods.

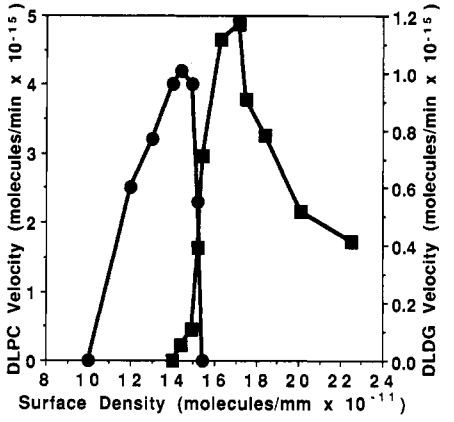

FIGURE 8: Hydrolysis of dilauroyl-*rac*-glycerol (DLDG) monolayers (■) and L-dilauroylphosphatidylcholine (DLPC) monolayers (●) by GCAT as a function of surface density. Velocities were determined as described under Materials and Methods. For DLDG, 30 μg of GCAT was used, and for DLPC, 3 μg. Each point is the mean of at least three separate determinations.

1974; Huang, 1976, 1977a,b). Formation of the acyl-enzyme intermediate may result in a transition state in which these hydrogen bonds are maintained, and its collapse could then lead to either hydrolysis or acyl transfer, depending upon whether water or cholesterol had contributed the hydrogen bond.

In summary, we continue to find similarities between the microbial enzyme GCAT and the mammalian plasma enzyme LCAT. We have shown that they possess very similar positional and stereochemical specificities, and it remains quite possible that these two enzymes, from such dissimilar sources, may have a common reaction mechanism. The fact that GCAT preferentially catalyzes acyl transfer in aqueous solution, and the absolute stereospecificity which it exhibits with secondary esters for hydrolysis or acyl transfer, makes it a particularly attractive tool in catalysis for synthetic purposes.

ACKNOWLEDGMENTS

We are indebted to Nana Gletsu and Chris Greenwood for skilled technical assistance and to Dr. K. Bruzik for providing us with a sample of (R_p,S_p)DPPsC. We also thank Dr. F. Pattus for critical discussion.

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Mechanism of Inhibition of Human Leukocyte Elastase by Two Cephalosporin Derivatives

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Received October 11, 1991; Revised Manuscript Received March 5, 1992

ABSTRACT: The cephalosporin derivatives L 658758 [1-[[3-(acetoxymethyl)-7α-methoxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-2-yl]carbonyl]proline *S,S*-dioxide] and L 659286 [1-[[7α-methoxy-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)thio]methyl]-5-thia-1-aza-(6*R*)-bicyclo[4.2.0]-oct-2-en-2-yl]carbonyl]pyrrolidine *S,S*-dioxide] are mechanism based inhibitors of human leukocyte elastase (HLE). The mechanism involves initial formation of a Michaelis complex followed by acylation of the active site serine. The group on the 3′-methylene is liberated during the course of these reactions, followed by partitioning of an intermediate between hydrolysis to regenerate active enzyme and further modification to produce a stable HLE–inhibitor complex. The partition ratio of 2.0 obtained for the reaction with L 658758 approaches that of an optimal inhibitor. These compounds are functionally irreversible inhibitors as the recovery of activity after inactivation is slow. The half-lives at 37 °C of the L 658758 and L 659286 derived HLE–I complexes were 9 and 6.5 h, respectively. The complexes produced by both inhibitors are similar chemically since the thermodynamic parameters for activation to regenerate active enzyme are essentially identical. The free energy of activation for this process is dominated primarily by the enthalpy term. The stability of the final complexes likely arises from Michael addition on the active site histidine to the 3′-methylene.

Elastases are potent serine proteases with the ability to degrade a number of proteinaceous components of connective tissues. Since the degradation of connective tissues is evident in the pathogenesis of a number of chronic inflammatory diseases, human leukocyte elastase (HLE, EC 3.4.21.37) has been implicated in disorders such as emphysema (Kaplan et al., 1973), atherosclerosis (Travis et al., 1980), and rheumatoid arthritis (Janoff et al. 1976).[1] The evidence[2] for the involvement of HLE in disease has led to the development of a number of synthetic inhibitors [for a review, see Stein et al. (1985)]. The observation that the benzylic esters of clavulanic

Scheme I

$$E + I \rightleftharpoons E \cdot I \longrightarrow \underbrace{(E - I)}_{\text{Acyl-Enzyme}} \longrightarrow \text{Further Modification}$$

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[1] This list is far from complete as a role for HLE has been suggested in a number of other inflammatory disease states [for example, see Davies et al. (1991)].
[2] For a concise discussion of the evidence, see Davies et al. (1991).

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