Mechanism of Action of Bacterial Glycerophospholipid:Cholesterol Acyltransferase†

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ABSTRACT: The substrate requirements of a microbial phospholipid:cholesterol acyltransferase have been studied. A variety of hydrophobic esters are effective as acyl donors, yet acyl transfer is specifically from the 2-position when phosphatidylcholine is the substrate. This observation supports a model which requires hydrogen bonding between donor and acceptor for acyl transfer to occur. Sphingomyelin is not a substrate for the enzyme, nor does it inhibit enzyme-catalyzed phosphatidylcholine hydrolysis. In contrast, acyl transfer from phosphatidylcholine to cholesterol is inhibited by sphingomyelin. The results indicate that acyl transfer catalyzed by the microbial enzyme depends on the formation of a complex between the acyl donor and acyl acceptor. Sphingomyelin effectively competes with phosphatidylcholine by forming an inactive complex with cholesterol. The enzyme may potentially be used to measure the relative interaction between individual phospholipids and cholesterol in lipid mixtures.

The bacterial glycerophospholipid:cholesterol acyltransferase (GCAT)[1] recently characterized in this laboratory (Buckley, 1982; Buckley et al., 1982; MacIntyre et al., 1979; MacIntyre & Buckley, 1978) shares a number of features with the mammalian enzyme lecithin:cholesterol acyltransferase (LCAT). Like the plasma enzyme, GCAT will catalyze fatty acid transfer between phosphatidylcholine and cholesterol, and acyl transfer appears to be 2-position specific (Buckley et al. 1982). However, the microbial enzyme has much less stringent requirements for the acyl donor than does LCAT. Thus, all of the commonly occurring glycerophospholipids will function as substrates for acyl transfer (Buckley, 1982), and under some conditions the enzyme will catalyze removal of fatty acid from cholesteryl ester (unpublished observations), although it has not been established that the mechanism of the latter reaction is the same as the mechanism of acyl transfer.

In contrast to the rather nonspecific requirements for the acyl donor, the selectivity for the acyl acceptor exhibited by the bacterial enzyme is considerable. Cholesterol and other steroids with 3-β-OH's and trans A:B rings are good substrates, whereas those with 3-α-OH's or cis A:B rings will not act as acyl acceptors and actually inhibit acyl transfer from phosphatidylcholine to cholesterol (Buckley, 1982). The mechanism of this inhibition is particularly interesting as it may be due either to competition by these steroids with cholesterol for a binding site on the enzyme or to competition between the steroids and cholesterol due to their mutual ability to associate with the phospholipid acyl donor. The second possibility would lead to the conclusion that a specific interaction between the acyl acceptor and the phospholipid is required for acyl transfer to occur. If this were the case, the enzyme could potentially be used to measure the relative interaction between individual phospholipid classes and cholesterol, both in artificial lipid mixtures and in membranes.

In this paper, the structural requirements of the microbial acyltransferase for the acyl donor are further examined. In addition, evidence is presented which indicates that a specific interaction between acyl donor and acyl acceptor is required for enzyme-catalyzed acyl transfer to occur.

Materials and Methods

Materials. GCAT was purified from cell-free culture supernatants of *Aeromonas salmonicida* as described previously (Buckley et al., 1982). Bovine serum albumin (essentially fatty acid free), *p*-nitrophenyl esters, cholesterol, and individual phospholipids and phosphatidylcholine molecular species were obtained from Sigma. Each lipid was examined by thin-layer chromatography prior to use and discarded if less than approximately 99% pure. [4-^{14}C]Cholesterol (specific activity 58 mCi/nmol) was purchased from Amersham Corp., and 1-palmitoyl-1-[1-^{14}C]linoleoylphosphatidylcholine (56.5 mCi/nmol) was obtained from New England Nuclear.

Preparation of Substrates. Small unilamellar vesicles of the compositions described in the text were prepared by sonication followed by ultracentrifugation at 110000*g* for 1 h to remove undispersed lipid (Barenholtz et al., 1977). The actual amounts of individual lipids were determined quantitatively, and the liposomes were used immediately. Mixed micelles containing Triton X-100, phospholipids and cholesterol were prepared as described by Dennis (1973a,b). Unless otherwise specified each reported experiment is representative of at least two giving very similar results.

Incubation Conditions. Typical assay conditions have been described previously (Buckley, 1982). Incubations were normally carried out in 0.16 M KCl and 0.05 M Tris, pH 7.4. Reaction mixtures for the liposome assays also contained 1.4% (w/v) albumin. Reactions were terminated by the addition of chloroform/methanol (2:1 v/v), and the lipids were extracted as described earlier (Buckley et al., 1982).

Hydrolysis of Nitrophenyl Esters. Enzyme-catalyzed production of *p*-nitrophenol was measured continuously at 400 nm against a no-enzyme blank in a recording spectrophotometer or after extraction from the reaction mixture. The molar extinction coefficient was taken as 12000 (Shirai & Jackson, 1982). Reaction conditions were similar to those used with phospholipid–steroid mixtures.

Lipid Determinations. Cholesterol, fatty acids, and cholesteryl ester were separated by thin-layer chromatography

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[1] Abbreviations: CE, cholesteryl ester; CHOL, cholesterol; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; LPC, lysophosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; GCAT, glycerophospholipid:cholesterol acyltransferase; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Comparison of Egg Phosphatidylcholine and *p*-Nitrophenyl Esters as Substrates for GCAT-Catalyzed Acyl Transfer to Cholesterol

acyl donor[a]	concentration (mM)	cholesteryl ester produced (nmol)
p-nitrophenyl acetate	0.3	41.7
p-nitrophenyl acetate	0.75	63.2
p-nitrophenyl butyrate	0.3	50.0
p-nitrophenyl butyrate	0.75	75.4
p-nitrophenyl laurate	0.3	51.0
p-nitrophenyl laurate	0.75	59.5
phosphatidylcholine	0.3	32.8
phosphatidylcholine	0.75	56.5

[a] Each assay contained the specific amount of acyl donor, 0.5 μmol of [4-^{14}C]cholesterol, 2.0 μmol of Triton X-100, and the enzyme in 0.5 mL. Incubation time was 5 min.

in petroleum ether/ether/acetic acid (70:30:1). Phospholipids were separated by chromatography in chloroform/methanol/acetic acid/water (65:25:4:1). Plastic-backed plates (Polygram Sil G, Brinkmann) were used with radioactive lipids and glass-backed silica gel H (Merk) plates for subsequent chemical determinations. Lipids were located by brief exposure to iodine and either scraped from glass plates and eluted or cut from plastic plates and counted in Scintiverse II (Fisher Scientific). Fatty acid methyl esters were prepared as described by Christie (1982) following addition of cholesteryl pentadecanoate as an internal standard. Methyl esters were separated and quantitated by gas–liquid chromatography on 10% DEGS–PS on 80/100 Supelcoport (Supelco).

Other Procedures. Phospholipid phosphorus was measured by using the method of Bartlett (1959), following sample digestion with perchloric acid. Protein was determined according to the procedure of Peterson (1977).

Results

Because of the small degree of selectivity exhibited by the enzyme for individual phospholipid classes as acyl donors, and because cholesteryl ester itself appears to be a substrate under some conditions, it seemed possible, if not likely, that the enzyme would carry out hydrolysis or acyl transfer with any hydrophobic ester. In order to assess whether the glycerophosphate backbone was required for hydrolysis to occur, enzyme activity was studied with *p*-nitrophenyl esters as representatives of general neutral esterase substrates. The data in Figure 1 show that several *p*-nitrophenyl esters are hydrolyzed by the enzyme. The results also indicate that the acetate ester is more readily hydrolyzed than phosphatidylcholine and that both *p*-nitrophenyl butyrate and *p*-nitrophenyl laurate are even better substrates. When the data are expressed in double-reciprocal plots, however (not shown here), it is clear that the apparent V_{max}'s are similar and that it is the apparent K_m's that vary, perhaps due to differences in the physical properties of the substrates. The nitrophenyl esters were also effective donors for enzyme-catalyzed acyl transfer to cholesterol (Table I), although the data show that the large differences observed when acyl hydrolysis was studied (Figure 1) were not apparent.

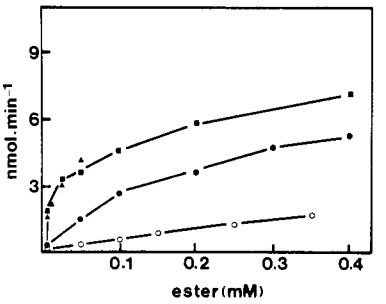

FIGURE 1: Hydrolysis of *p*-nitrophenyl esters and phosphatidylcholine. Reactions were carried out in 0.16 M KCl and 20 mM Tris-HCl pH 7.4. Triton X-100 (4 mM) was present when egg phosphatidylcholine containing [^{14}C]linoleoyl-PC was the substrate. (○) Phosphatidylcholine, (●) *p*-nitrophenyl acetate, (■) *p*-nitrophenyl butyrate, and (▲) *p*-nitrophenyl laurate.

These results indicated that the enzyme is a nonspecific esterase which can alternatively carry out acyl transfer in the presence of a suitable acyl acceptor. This lack of specificity was difficult to rationalize with the observation, reported earlier (Buckley et al., 1982), that acyl transfer to cholesterol is specific on the 2-position when PC is the acyl donor. The prior study was based on data obtained with PC containing an unsaturated fatty acid in the 2-position and a saturated fatty acid in the 1-position. Thus, it was possible, since we have shown a strong preference for unsaturated PC as an acyl donor (Buckley, 1982), and in the light of the lack of specificity demonstrated above, that the positional specificity which was observed earlier was due simply to the fatty acid preferences of the enzyme. In order to examine this possibility, the action of the enzyme on 1-palmitoyl-2-oleoyl-PC and on 1-oleoyl-2-palmitoyl-PC was compared. The results in Table II indicated that both species of PC are much more effective than DPPC and somewhat less effective than DOPC as acyl donors, adding strength to the earlier conclusion (Buckley, 1982) that the physical properties of the acyl donor are major determinants of reaction rate. Gas–liquid chromatographic analysis of the CE produced with both the two PC positional isomers confirmed our earlier finding that acyl transfer was 2-position specific. Thus, more than 80% of the fatty acid incorporated into CE was palmitate when 1-oleoyl-2-palmitoyl-PC was the substrate, and more than 80% was oleate when the positions of the fatty acids in PC were reversed (Table II).

The earlier observations that LPC was not a donor for acyl transfer and that epicholesterol and coprostanol inhibited the reaction, as well as our conclusion, confirmed here, that acyl transfer was 2-position specific (Buckley, 1982), all pointed to the possibility of a specific interaction between the phospholipid and cholesterol. In order to investigate this further, the influence of sphingomyelin on the GCAT-catalyzed reaction was examined. Sphingomyelin is not itself a substrate for the enzyme (Buckley et al., 1982), and the results in Figure

Table II: Positional Specificity of Acyl Transfer to Cholesterol from Phosphatidylcholine Isomers

phosphatidylcholine[a]	total CE produced (nmol)	nmol of		% FA from	
		cholesteryl palmitate	cholesteryl oleate	1-position	2-position
dioleoyl	382				
dipalmitoyl	60				
1-palmitoyl-2-oleoyl	315	45.4	269.6	14.4	85.6
1-oleoyl-2-palmitoyl	257	208.9	48.1	18.7	81.3

[a] Each assay contained 1.0 μmol each of phospholipid and [4-^{14}C]cholesterol in 1.0 mL containing 4.0 μmol of Triton X-100 and the enzyme. Incubation time was 15 min.

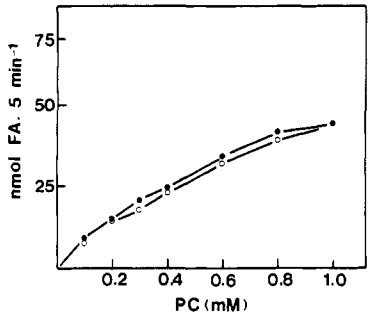

FIGURE 2: Effect of sphingomyelin on hydrolysis of egg phosphatidylcholine. Each assay contained 4 mM Triton and the indicated concentration of [^{14}C]phosphatidylcholine in 0.16 M KCl and 20 mM Tris-HCl, pH 7.4. When required, sphingomyelin was added so that the total concentration of phospholipid was 1.0 mM. (●) Without sphingomyelin and (○) with spingomyelin.

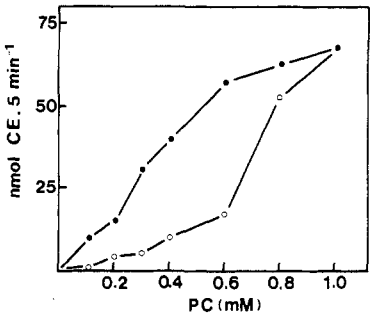

FIGURE 3: Effect of sphingomyelin on acyl transfer from egg phosphatidylcholine to cholesterol. Conditions were the same as those in Figure 2 except that each assay also contained 1.0 mM [^{14}C]-cholesterol.

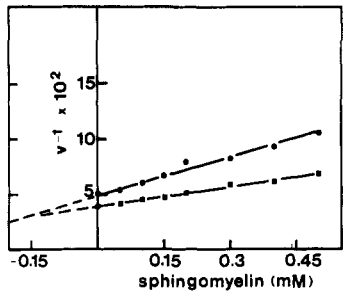

FIGURE 4: Inhibition of acyl transfer from phosphatidylcholine to cholesterol by sphingomyelin: effect of different PC:cholesterol ratios. Each assay contained the indicated concentration of sphingomyelin, 0.2 mM cholesterol, 4 mM Triton, 0.16 M KCl, 20 mM Tris-HCl, pH 7.4, and 0.4 or 0.6 mM egg PC. (●) PC:cholesterol ratio 2:1 and (■) PC:cholesterol ratio 3:1. Velocity is in nmol of CE·5 min^{-1}.

2 show that it does not inhibit the simple hydrolysis of PC in Triton micelles, indicating that it does not compete with PC for an attachment site on the enzyme. In contrast, however, a very large decrease in the production of CE was observed in comparable micelles which contained cholesterol and increasing amounts of sphingomyelin (Figure 3), demonstrating that sphingomyelin inhibits acyl transfer. The extent of inhibition was dependent on the relative amounts of PC and cholesterol, decreasing as the amount of PC increased (Figure 4). In addition, the degree of inhibition increased with decreasing amounts of cholesterol in the mixed micelles (Table III).

Because enzyme activity depends on the physical properties of the substrate, it was important to determine whether or not sphingomyelin was inhibiting simply because it was predominately saturated. The results in Table IV clearly indicate that this is not the case as the degree of sphingomyelin inhibition was the same in micelles containing egg PC or DPPC.

Table III: Effect of Cholesterol Concentration on Sphingomyelin Inhibition[a]

cholesterol (mM)	% inhibition[b]
0.2	52
0.4	45
0.6	34
0.8	25
1.0	21

[a] Reactions were carried out with the listed concentration of cholesterol in 0.16 M KCl and 20 mM Tris-HCl, pH 7.4, containing 1 mM egg PC and 4 mM Triton X-100. Half the reaction mixtures also contained 1 mM sphingomyelin. Incubation was for 5 min at 37 °C. [b] Calculated from the ratio of the amount of CE formed in the presence of sphingomyelin to the amount formed in its absence. Mean of two nearly identical experiments.

Table IV: Influence of Physical Properties of PC on Sphingomyelin Inhibition[a]

	% inhibition[b]	
sphingomyelin (mM)	egg PC–cholesterol	DPPC–cholesterol
0.2	10	12
0.4	19	17
0.6	28	29
0.8	32	30
1.0	38	38

[a] Sphingomyelin was present in reaction mixtures containing 1 mM egg PC or DPPC, 0.5 mM [4-^{14}C]cholesterol, 4 mM Triton, 0.16 M KCl, and 20 mM Tris-HCl, pH 7.4. Incubations were for 5 min at 40 °C. [b] Based on the amount of CE formed with egg PC or DPPC in the absence of sphingomyelin.

Table V: Effect of Sphingomyelin on Cholesteryl Ester Production in Liposomes and Liposome Mixtures

liposome mixture[a]	nmol of CE produced	% inhibition
DPPC–CHOL	16.4	
DPPC–CHOL + 0.2 μmol of SPH	15.3	10
DPPC–CHOL + 0.5 μmol of SPH	13.5	17
DPPC–CHOL + 0.2 μmol of SPH–CHOL	15.6	10
DPPC–CHOL + 0.5 μmol of SPH–CHOL	15.5	10
DPPC–CHOL containing 0.5 μmol of SPH	3.7	78

[a] Liposomes were prepared as described in the text. The molar ratios of lipids in the liposomes were the following: DPPC:CHOL, 1:0.5; SPH:CHOL, 1:0.5; DPPC:SPH:CHOL, 1:1:0.5. All assays contained DPPC liposomes with 0.5 μmol of PC. Where indicated sphingomyelin-containing liposomes were also present in the incubation mixture. Only the PC liposomes contained [4-^{14}C]cholesterol. Incubation was for 5 min at 40 °C.

Sphingomyelin may compete with cholesterol or with a PC–cholesterol complex for a site on the enzyme, thereby lowering the rate of acyl transfer, or it may compete with PC for cholesterol and thus lower the concentration of PC–cholesterol complex available for transacylation. Experiments using mixtures of liposomes were carried out in an attempt to distinguish between these possibilities. The results in Table V show clearly that neither sphingomyelin nor liposomes containing sphingomyelin and cholesterol have a large effect on CE production when present in the same assay with PC–cholesterol liposomes, whereas when sphingomyelin is incorporated into the PC–cholesterol liposomes at comparable concentrations during their preparation, it nearly completely

inhibits CE production. Thus, it would appear that neither sphingomyelin by itself nor sphingomyelin complexed to cholesterol appears capable of binding to the enzyme.

Discussion

Several features of the reaction catalyzed by GCAT must be explained in any proposed reaction mechanism. The enzyme is apparently able to use a variety of hydrophobic esters as acyl donors, yet it is 2-position specific when the ester is PC, and it will not catalyze the transacylation of 1-acyl-PLC, although it will carry out hydrolysis of this phospholipid. A variety of short-chain aliphatic alcohols will act as acyl acceptors, yet cholesterol and other 3-β-OH steroids with trans-fused (planar) A:B rings are the preferred acceptors, and steroids such as epicholesterol and coprostanol are inhibitors. One way of explaining these observations is to argue that the minimum requirement for enzyme-catalyzed acyl transfer is for a hydrophobic ester, hydrogen bonded to a potential acyl acceptor. If the hydrogen bond is to water, simple hydrolysis occurs. If it is to an alcohol, the result is transacylation. Cholesterol is presumably a good substrate because of its ability to interact maximally with the acyl donor through van der Waals interactions and because in doing so it can form the necessary hydrogen bond. Steroids with either an α C-3 hydroxyl or a puckered ring system may inhibit both the acyl transfer and PC hydrolysis because they can interact with the donor PC but not form a hydrogen bond, thus effectively removing the enzyme's substrate. LPC is not a substrate for acyl transfer although it is hydrolyzed by the enzyme, either because the single ester carbonyl is hydrogen bonded to water in LPC–cholesterol mixtures or because it is hydrogen bonded to the adjacent hydroxyl. This model, which suggests that the enzyme recognizes a complex between the phospholipid and cholesterol, is strengthened by the observation with sphingomyelin. This phospholipid is not a substrate for the enzyme, nor does it bind to the acyl donor site, as PC hydrolysis is not affected in its presence. This latter observation also supports the conclusion that inhibition is neither due to surface dilution of the PC nor due to an interaction between sphingomyelin and PC. This conclusion is also supported by the demonstration that the addition of sphingomyelin to PC–cholesterol micelles had no effect on snake venom phospholipase A_2 hydrolysis of PC (data not shown). It has little or no effect unless it is incorporated directly into PC–cholesterol liposomes, indicating it does not prevent the binding of the complex to the enzyme. The most obvious explanation for the inhibition observed with sphingomyelin is that it is due to the formation of a complex with cholesterol which effectively lowers the concentration of steroid available to accept a fatty acid from the donor PC. This explanation is consistent with the observation that the amount of sphingomyelin inhibition decreased as the amount of cholesterol increased in the mixed micelles (Table III).

The nature of the molecular bonding which gives rise to cholesterol–phospholipid complexes in natural and model membrane systems has not been convincingly established. There is little doubt that van der Waals interactions are essential features of the sterol–phospholipid association (Rothman & Engleman, 1972; Yeagle et al., 1977); however, a contribution of hydrogen bonding to complex stability remains a matter of considerable debate. Models for both direct and water-mediated hydrogen bonding have been proposed (Brokerhoff, 1974; Huang, 1976; Yeagle et al., 1976). Direct hydrogen bonding to the phospholipid carbonyls has been justified by model building (Huang, 1976, 1977), by the results of proton NMR investigations (Chaterjie & Brockerhoff, 1978), and by comparative studies using thiocholesterol (Parkes et al., 1982). In contrast, permeability studies with diether and dialkyl lipids indicate that the carbonyl oxygens are not necessary for cholesterol interactions (Clejan et al., 1979), and infrared and Raman spectroscopic investigations suggest that cholesterol does not hydrogen bond to the carbonyl groups of anhydrous PC (Bush et al., 1980). Recently, Presti & Chan (1982a,b) have proposed an alternate model to accommodate the conflicting data. They suggest that hydrogen bonds may be formed between cholesterol and the glycerol ester oxygen of phospholipids. Although the results reported here do not provide details of steroid–phospholipid hydrogen bonding, they do indicate that a hydrogen bond exists; otherwise acyl transfer, which appears to require hydrogen bonding between acceptor and donor, would not occur between cholesterol and PC. Because association between donor and acceptor is essential for transesterification, GCAT may potentially be used to measure the relative interaction of individual phospholipids with cholesterol both in artificial systems and in membranes.

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Registry No. GCAT, 68009-86-9; DPPC, 2644-64-6; DOPC, 10015-85-7; 1-palmitoyl-2-oleoylphosphatidylcholine, 6753-55-5; 1-oleoyl-2-palmitoylphosphatidylcholine, 17118-56-8; *p*-nitrophenyl acetate, 830-03-5; *p*-nitrophenyl butyrate, 2635-84-9; *p*-nitrophenyl laurate, 1956-11-2.

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