Modulation of the Positional Specificity of Lecithin−Cholesterol Acyltransferase by the Acyl Group Composition of Its Phosphatidylcholine Substrate: Role of the *sn*-1-Acyl Group†

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ABSTRACT: Human lecithin−cholesterol acyltransferase (LCAT), which is normally specific for the *sn*-2 position of phosphatidylcholine (PC), derives a significant percentage of acyl groups from the *sn*-1 position, when *sn*-2 is occupied by 18:0, 20:4, or 22:6. We investigated the relative importance of the two acyl groups of PC in determining the positional specificity by first analyzing the cholesteryl esters formed in the presence of symmetric PCs labeled at *sn*-2. Both human and rat LCATs transferred exclusively the *sn*-2-acyl group from all symmetric PCs, including 18:0−18:0, and 20:4−20:4, showing that the presence of these fatty acids at *sn*-2 does not automatically alter the positional specificity. The role of the *sn*-1-acyl group was then tested by using PCs containing 20:4 or 18:0 at *sn*-2 and fatty acids of various chain lengths and unsaturation at *sn*-1. With 20:4 at *sn*-2 and saturated fatty acids of various chain lengths at *sn*-1, human and rat LCATs derived, respectively, 5−72% and 1−20% of the total acyl groups from the *sn*-1 position. However, the chain length of the *sn*-1-acyl did not correlate with its utilization by either enzyme. Various unsaturated fatty acids at *sn*-1 also were transferred by human LCAT at a higher rate (5−75% of total) than they were transferred by rat LCAT (0−21%). With *sn*-2-18:0 PCs, however, rat LCAT exhibited greater alteration in positional specificity (30−95% from *sn*-1) than human LCAT (15−83% from *sn*-1). These results show that while the primary determinant of positional specificity is the *sn*-2-acyl group of PC, the structure of *sn*-1-acyl significantly modifies it.

Most of the cholesteryl esters (CE[1]) in human plasma are derived from the action of lecithin−cholesterol acyltransferase (LCAT), which transfers an acyl group from the plasma phosphatidylcholine (PC) to free cholesterol (*1*, *2*). Although human LCAT has been shown to be generally specific for the *sn*-2-acyl group of PC (*3*), this specificity is not absolute, because a significant fraction of the acyl groups for CE synthesis are derived from the *sn*-1 position of certain PC species. For example we previously demonstrated that when the *sn*-2 position of PC is occupied by 20:4, 18:0, or 22:6, up to 80% of the acyl groups are derived from the *sn*-1 position and that the chain length of the *sn*-2-acyl chain is an important determinant of the positional specificity (*4*, *5*). However, we found that the degree of alteration in positional specificity (utilization of *sn*-1-acyl group) depended not only on the acyl group at *sn*-2 but also on the nature of the *sn*-1-acyl group. Thus, while human LCAT derived over 70% of the acyl groups from the *sn*-1 position of 16:0−18:0 PC, it derived only 18% from the *sn*-1 position of 18:1−18:0 PC (*4*). Since both the PCs contained 18:0 at *sn*-2, the difference is obviously due to the *sn*-1-acyl group. Similarly, while the *sn*-1 position of 16:0−20:4 PC contributed about 79% of the total acyl groups for CE synthesis, the *sn*-1 position of 18:0−20:4 PC contributed only 21%, showing again the influence of the *sn*-1-acyl group. Results of previous studies indicate that the *sn*-1-acyl group of the substrate has significant effect on the activity of the enzyme (*6*, *7*), but its possible effect on the positional specificity has not been investigated. Some of the differences in the observed positional specificity could be due to the presence of positional impurities in the substrates tested, because a preferential utilization of the *sn*-2-acyl from any contaminating isomer can distort the values obtained. However, we have corrected for the possible contamination by using the "best-fit curve" method and calculated the theoretical values for 100% pure substrates (*5*). Even after applying this correction, the contribution of the *sn*-1-acyl group from 16:0−20:4 PC was 50%, whereas the contribution from the *sn*-1 of 18:0−20:4 was less than 5%. The differences in positional specificity cannot be attributed to the differences in the fluidity of the substrates, because the matrix fluidity does not influence this property (*4*). Instead, it appears likely that the positional specificity is dictated by the structure of both the acyl groups, not just the *sn*-2-acyl group. To test this hypothesis, we prepared a series of synthetic PC substrates,

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[1] Abbreviations: CE, cholesteryl ester; FC, free cholesterol; FFA, free fatty acid; HPLC, high performance liquid chromatography; LCAT, lecithin−cholesterol acyltransferase; OPPC diether, 1-octadecenyl-2-hexadecyl-*sn*-glycerophosphorylcholine; PC, phosphatidylcholine; TLC, thin-layer chromatography.

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containing either 20:4 or 18:0 at the *sn*-2 position and various acyl groups differing in chain length and unsaturation at the *sn*-1 position, and determined their effects on the positional specificities of human and rat LCATs. The results show that while the primary determinant of the positional specificity is the *sn*-2-acyl group, the structure of the *sn*-1-acyl group significantly modifies this specificity.

MATERIALS AND METHODS

Materials. The 4-^{14}C cholesterol and 1-^{14}C labeled fatty acids were purchased from Dupont-NEN. Unlabeled PCs were purchased from Avanti Polar Lipids (Alabaster, AL). OPPC diether was obtained from Alexis Biochemicals Corp. (San Diego, CA). Human and rat LCATs were purified by the procedures described earlier (*4*). Because the positional specificity is not different for highly purified and partially purified LCAT (*5*, *8*), the phenyl sepharose eluates were used in most experiments. Apoprotein A-I was purified from human plasma high-density lipoproteins as described by Mills et al. (*9*), using a sephacryl S-200 column and 2 M acetic acid as the eluant.

Preparation of PC Substrates. Symmetric PCs labeled at the *sn*-2-acyl group were prepared by the acylation of the corresponding 1-acyl lyso PCs with 1-^{14}C fatty acid. The 1-acyl lyso PCs were first prepared from the unlabeled diacyl PCs by treatment with snake venom phospholipase A_2 and were used without chromatographic purification, to minimize the acyl migration. To 5 mg of unlabeled diacyl PC in 2 mL of diethyl ether, 1 mg of lyophilized venom (*Crotalus atrox*, Sigma Chemical Co.) dissolved in 100 μL of buffer (10 mM Tris, 30 mM borate, 1 mM $CaCl_2$, pH 7.4) was added, and the mixture was incubated at room temperature for 30 min with occasional vortexing. The ether was then evaporated off, and the total lipids were extracted (*10*) and immediately used for the synthesis of labeled PC. To the lipid extract, 1 mg of ^{14}C-labeled fatty acid containing 750 000 dpm was added, the solvent was evaporated under N_2, and the sample was further desiccated for 1 h under vacuum. Then, 10 mg of dicyclohexylcarbodiimide and 5 mg of (dimethylamino)pyridine were added, and the sample was again dried in a vacuum for 1 h. The sample was dissolved in 250 μL of anhydrous chloroform and incubated at 40 °C in a dry chamber under N_2 in the dark with stirring for 16 h. The solvent was evaporated, and the lipids were extracted (*10*) and separated on a silica gel plate with the solvent system chloroform/methanol/30% NH_4OH (50:50:1 v/v). The lipids were visualized by spraying with dichlorofluorescein (0.1% in 90% ethanol), and the spot corresponding to standard PC was scraped and eluted (*10*). The final product contained more than 98% of the label at the *sn*-2 position, as evident from the release of labeled fatty acid after treatment with snake venom phospholipase A_2.

Asymmetric PCs containing different fatty acids at the two positions were synthesized by a similar procedure, but the lyso PC was first purified by silica gel TLC before reacting with the desired unlabeled fatty acid. The positional purity of the asymmetric PCs was determined by gas chromatographic analysis of the fatty acid composition of lyso PC and free fatty acids released by snake venom phospholipase A_2 (*4*). The positional purity of the asymmetric PCs (84−99%) was generally lower than that of the *sn*-2 labeled symmetric PCs, presumably because of some acyl migration in lyso PC, which occurs during its purification by TLC.

Enzyme Assays. The LCAT activities were routinely assayed using proteoliposome substrates (*11*) containing PC: 4-^{14}C FC:apoprotein A-I at molar ratios of 250:12.5:0.8. To compare the efficacy of various substrates in a common matrix, proteoliposomes containing 90% OPPC diether and 10% test PC were used (*6*). The reaction mixture typically contained the proteoliposome substrate, 2−10 μg of partially purified enzyme, 5 mM mercaptoethanol, and 2.5 mg of bovine serum albumin in a final volume of 0.4 mL. After the reaction was allowed to progress for 1 h, the total lipid extract was plated on a silica gel TLC plate and developed with the solvent system of petroleum ether/ethyl acetate (85:15 v/v) after adding 50 μg each of unlabeled FC and cholesteryl oleate as carriers. The radioactivity in FC and CE was determined in a liquid scintillation counter, and the enzyme activity was calculated as the percent of FC esterified per hour.

Positional Specificity. The positional specificity of LCAT in the presence of symmetric (*sn*-2-acyl labeled) PCs was determined from the formation of labeled CE, FFA, and lyso PC after the reaction. The substrate used for these experiments contained labeled PC and unlabeled FC at the ratio of 10:1 to minimize the phospholipase A activity (instead of 20:1 in the usual assay). After the reaction, the total lipids were separated on silica gel TLC with a two-step solvent system. In the first step, the solvent used was chloroform/methanol/water (65:25:4 v/v) and the plate was removed after the solvent reached about 10 cm from the origin. After drying briefly in a fume hood, the plate was then developed in the second solvent (hexane/diethyl ether/acetic acid, 70:30:1 v/v) up to 1 cm from the top of the plate. The lipids were visualized by brief exposure to iodine vapors, and the radioactivity in the spots corresponding to lyso PC, PC, FFA, and CE was determined. The radioactivity in FFA and CE represents the hydrolysis and transfer of the *sn*-2-acyl group, whereas the radioactivity in lyso PC represents the hydrolysis and transfer of the *sn*-1-acyl group. Under the conditions of the assay less than 5% of the total product radioactivity was in FFA.

The positional specificity of LCAT in the presence of asymmetric PCs was determined by the HPLC analysis of labeled CE species formed, after incubation of the enzyme with the substrate containing unlabeled PC and labeled FC, as described before (*5*), with a few modifications. Briefly, the proteoliposome substrate containing the test PC and labeled FC was reacted with the purified enzyme for 2 h at 37 °C, and the lipids were extracted (*10*). The total lipid extract was filtered through a 0.45-μm filter, concentrated under nitrogen, and injected directly onto a C-18 reverse phase HPLC column running with the solvent system of acetonitrile/tetrahydrofuran/water (65:35:1.5 v/v). The flow rate of the solvent was 2.0 mL/min, and the column was maintained at 27 °C with the help of a column heater. The effluent of the column was passed through a radioactivity detector (Radiomatic Flo-one, Packard Instruments Co., Meriden, CT) with the following settings: scintillant-to-solvent ratio, 2:1; background subtraction, 25 dpm; update time, 10 s. The percent of radioactivity in CE species formed was calculated by electronic integration, using EZchrom software (Scientific Software Inc, San Ramon, CA).

Correction for Positional Impurities. To correct for the effect of contamination of positional isomers of PC on the specificity, the two isomers of a given PC (each of which was less than 100% positionally pure) were mixed in various ratios to give a series of mixtures with known positional composition. These mixtures were then incorporated into the proteoliposomes, and the labeled CE species formed were analyzed by HPLC. The data were then used to obtain the best-fit curves as reported earlier (*5*). Extrapolation of the curve to 100% purity gives the positional specificity in the presence of pure substrate.

Acyl Chain Length. The chain length of the *sn*-1-acyl group of PC was calculated using a computer model of the corresponding diacyl glycerol, as described by Applegate and Glomset (*12*), using Chem3D software (CambridgeSoft). The lengths were measured from the 021 oxygen of the *sn*-2-acyl group to the most distant hydrogen of the terminal methyl group of the *sn*-1-acyl chain. The van der Waals radii of the hydrogen atom and the 021 oxygen were added to the distance between the two atoms, to give the total effective length of the acyl chain.

RESULTS

Positional Specificity of Human LCAT in the Presence of Symmetric PCs. Our previous studies showed that the positional specificity of human LCAT is altered when the *sn*-2 position is occupied by 20:4 or 18:0 (*5*). To test whether the presence of these fatty acids at *sn*-2 automatically alters the positional specificity of human LCAT and whether the structure of the *sn*-1-acyl group also influences the specificity, we prepared symmetric PCs that contained identical fatty acids at both the positions and analyzed the CE synthesized by the enzyme in their presence. To trace the origin of the acyl group in the CE formed, we labeled the *sn*-2-acyl group with ^{14}C and determined the label in lyso PC, PC, FFA, and CE, after the enzyme reaction. The presence of radioactivity in lyso PC indicates the utilization of the *sn*-1-acyl group, whereas the presence of label in CE or FFA indicates the utilization of the *sn*-2-acyl group. Under the conditions of the assay, less than 5% of the total radioactivity in the products (lyso PC, CE and FFA) was present in FFA, showing that the phospholipase A activity is negligible. As shown in Table 1, human LCAT transfers almost exclusively the *sn*-2-acyl group from all the symmetric PCs tested, including 18:0−18:0 and 20:4−20:4 PCs. These results show that the natural preference of the enzyme is for the *sn*-2-position of PC, when there is no difference in the two acyl groups. They also demonstrate the importance of the *sn*-1-acyl group in determining the positional specificity, because the enzymes attack the *sn*-1 ester linkages of 16:0−18:0 PC and 16:0−20:4 PC (*5*) but not those of 18:0−18:0 PC and 20:4−20:4 PC.

Positional Specificity of Phospholipase A Activity of LCAT. The LCAT reaction is believed to take place in two distinct steps. The first step is formation of an acyl−enzyme intermediate with the hydroxyl group of Ser_{181} and the acyl group of PC, and the second step is the transfer of the acyl group to free cholesterol or other acyl acceptors such as lyso PC (*13*). In the absence of an acceptor, the acyl group is released as FFA (phospholipase A activity) (*14*). To ascertain whether the specificity for the *sn*-2 position is determined at the first or the second step, we repeated the above experiment with proteoliposomes containing no free cholesterol acceptor, when only the phospholipase A activity is expressed. In this case, the radioactivity in FFA measures the formation of the acyl−ester intermediate using the *sn*-2-acyl group of PC, whereas the radioactivity in lyso PC measures the utilization of the *sn*-1-acyl group. Since there is no lysophospholipase activity in the LCAT preparation used, all the counts in FFA are due to phospholipase A_2 activity and not due to the hydrolysis of any 2-acyl lyso PC. As shown in Table 1, with all the symmetric PCs tested, almost all the radioactivity released was in the form of FFA, with very little in lyso PC. These results show that the selectivity for the *sn*-2-acyl group from the symmetric PCs occurs at the formation of the acyl−enzyme intermediate not at the transfer of the acyl group to cholesterol. Similar results were obtained with rat LCAT (results not shown).

Table 1: Positional Specificity of Human LCAT in Presence of Symmetric PCs[a]

	% of label in products			
	cholesterol esterification		phospholipase A reaction	
PC substrate	lyso PC (*sn*−1)	CE + FFA (*sn*−2)	lyso PC (*sn*−1)	FFA (*sn*−2)
1-16:0-2-[1-^{14}C]-16:0 PC	1.00	99.00	0.99	99.10
1-18:0-2-[1-^{14}C]-18:0 PC	2.58	97.42	0.56	99.44
1-18:1-2-[1-^{14}C]-18:1 PC	4.49	95.51	0.12	99.88
1-18:2-2-[1-^{14}C]-18:2 PC	0.00	100.00	0.00	100.00
1-20:4-2-[1-^{14}C]-20:4 PC	1.72	98.28	2.11	97.89

[a] The results presented are representative of three experiments. For the cholesterol esterification reaction, the proteoliposome substrate contained labeled PC and FC in the molar ratio of 10:1, whereas for the phospholipase A reaction no FC was added. After the reaction, the products were separated on TLC and their radioactivity was determined. The total radioactivity in lyso PC, FFA, and CE was taken as 100%. The label in lyso PC represents the attack on the *sn*-1 ester linkage, whereas the label in CE or FFA indicates the attack on the *sn*-2 ester linkage.

Positional Specificity in the Presence of Asymmetric PCs: Effect of sn-1-Acyl Carbon Number. To determine the effect of *sn*-1 chain length on the utilization of 20:4 from the *sn*-2 position, we prepared several synthetic PCs, all of which contained 20:4 at *sn*-2 but saturated fatty acids of varying chain lengths at *sn*-1. These PCs were utilized as substrates for purified human or rat LCAT, and the labeled CE species formed were analyzed by HPLC. As shown in Figure 1 (top), the percent of 20:4 CE formed by human LCAT depended greatly upon the acyl group at *sn*-1. Thus, 20:4 CE constituted about 95% of the total CE formed in the presence of 18:0−20:4 PC but only about 28% of the total formed in the presence of 16:0−20:4 PC. However, with rat LCAT, 20:4 CE was the predominant product in the presence of all PCs. The positional purity of the PCs used, as measured by the percent of 20:4 in the FFA released by the snake venom phospholipase A_2, is also shown in this panel.

In a separate experiment, the effect of *sn*-1-acyl chain length on the enzyme activity was determined. To minimize the bulk-phase effects of the *sn*-1-acyl group, all the PCs were incorporated into proteoliposomes containing a common PC diether matrix (90% OPPC diether, 10% test PC) (*6*). The activity of human or rat LCAT as a function of the *sn*-

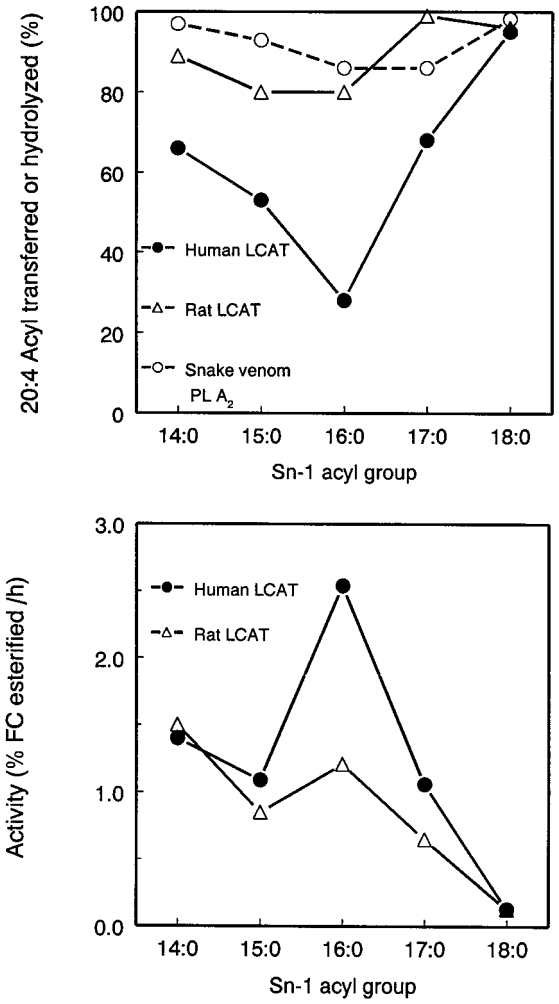

FIGURE 1: Effect of *sn*-1-acyl group on the positional specificity (top) and activity (bottom) of LCAT. (Top) Positional specifcities of human and rat LCATs were determined by the percent of 20:4 CE formed from the various *sn*-2-20:4 PCs. The PCs were incorporated into proteoliposomes without the diether PC matrix, and the CE species formed were determined as described in the methods section. The release of 20:4 by snake venom phospholipase A_2 (PL A_2) measures the positional purity of the various PC substrates. (Bottom) Enzyme activities in the presence of various *sn*-2-20:4 PCs. The PCs were incorporated into proteoliposomes containing a common inert matrix (90% OPPC diether, 10% test PC) and reacted with the purified enzymes for 1 h at 37 °C. The enzyme activities were calculated as the percentage of FC esterified per hour.

1-acyl carbon number is shown in Figure 1 (bottom). Human LCAT showed the highest activity when 16:0 was present at *sn*-1, and its positional specificity was also altered the most by this substrate. However, rat LCAT exhibited a decrease in enzyme activity with the increase in *sn*-1-acyl chain length from 14 to 18 carbons, in agreement with the results of Pownall et al. (*7*). The rat enzyme also showed much greater utilization of 20:4 from the *sn*-2 position of all substrates, indicating that its positional specificity is not altered significantly. In contrast, the positional specificity of human LCAT is markedly altered in the presence of all PCs except 18:0−20:4. Since the transfer of 20:4 from *sn*-2 correlated negatively with the enzyme activity, these results suggest that the activity of human LCAT in the presence of *sn*-2-20:4 PCs is a function of its ability to utilize the *sn*-1-acyl group.

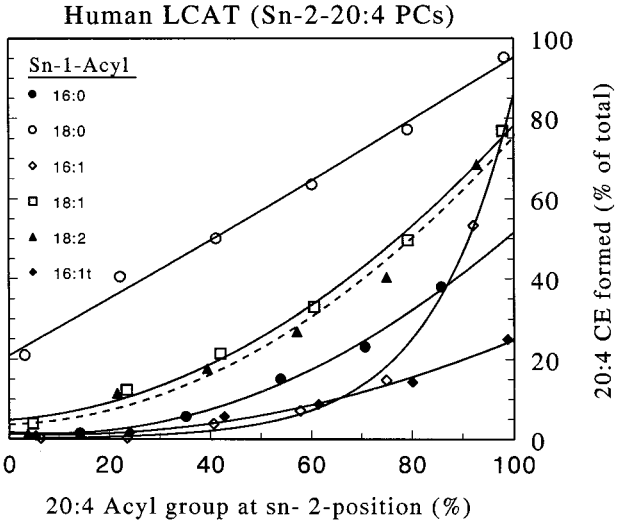

FIGURE 2: Positional specificity of human LCAT in the presence of *sn*-2 20:4 PCs. Correction for isomeric impurities. Labeled CE species formed by human LCAT in the presence of a series of *sn*-1-acyl-2-20:4 PCs of known positional purity were analyzed by HPLC, as described in the methods section. The percentage of 20:4 CE formed was plotted as a function of the percentage of 20:4 acyl at the *sn*-2 position of a given PC. The values were fitted to a best-fit curve using a computer program (SlideWrite Plus). Extrapolation of the curve to 100% 20:4 at *sn*-2 (right *Y* intercept) gives the specificity of the enzyme for *sn*-2 in the presence of pure substrate. If the enzyme showed absolute specificity for the *sn*-2 ester, this value would be 100%. The left *Y* intercepts show the formation of 20:4 CE from pure *sn*-1-20:4 PC isomers.

Isomeric Impurities and the Positional Specificity of LCAT. The results in Figure 1 show that the positional purity of the various *sn*-2-20:4 PCs used, as measured by the release of 20:4 by the snake venom phospholipase A_2, varied significantly from sample to sample. Since the presence of positional impurities can affect the results on positional specificity, we corrected for it by plotting best-fit curves, as described previously (*5*). In addition to 16:0 and 18:0, the most abundant acyl groups at *sn*-1 of plasma PC, we tested the effects of 16:1, 16:1-*trans*, 18:1, and 18:2, the unsaturated fatty acids which are also likely to be present at *sn*-1 (*15*). The best-fit curves for all the *sn*-2-20:4 PCs are presented in Figure 2, for human LCAT, and in Figure 3, for rat LCAT. The right *Y* intercept of each curve gives the percent of 20:4 CE formed from positionally pure PC (100% 20:4 at *sn*-2). The derived values for all *sn*-2-20:4 PCs are summarized in Table 2. For human LCAT, the lowest percentage of 20:4 CE formed was in the presence of 16:1 *trans*-20:4 PC (25%), whereas the highest percent of 20:4 CE was formed with 18:0−20:4 PC (95%). In accordance with our previous data (*5*), the enzyme derived about 52% of the acyl groups from the *sn*-1 position of pure 16:0−20:4 PC. The positional specificity of human LCAT was also altered when 16:1, 18:1, or 18:2 was present at *sn*-1, but to a lesser extent than that observed with 16:0 or 16:1-*trans*.

Unlike the human enzyme, rat LCAT utilized almost exclusively 20:4 (*sn*-2 position) from most PCs, except 16:1-*trans*−20:4 and 18:1−20:4 PCs, where about 21% and 15% of the acyl groups were derived from *sn*-1 position, respectively (Figure 3). These results show that rat LCAT utilizes 20:4 from *sn*-2 more efficiently than human LCAT, as expected from the substrate specificity studies (*7*). The effect of pure 20:4−18:0 PC isomer (open circles, left

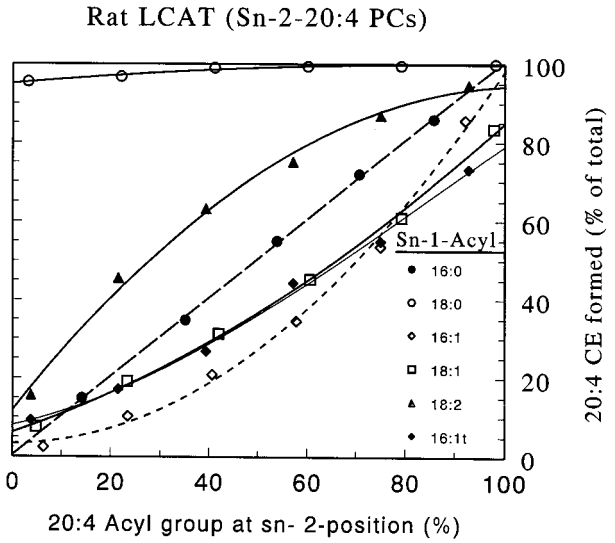

FIGURE 3: Positional specificity of rat LCAT in the presence of *sn*-2-20:4 PCs. Correction for isomeric impurities. Labeled CE species formed by rat LCAT in the presence of a series of *sn*-1-acyl-*sn*-2-20:4 PCs of known positional purity were analyzed by HPLC. The percent of 20:4 CE formed was then plotted as a function of the percentage of 20:4 at *sn*-2 of PC, and the values were fitted into a best-fit curve (SlideWrite Plus). Extrapolation of this curve to the right *Y* axis gives the theoretical positional specificity of the enzyme (for *sn*-2) in the presence of 100% pure *sn*-2-20:4 PC.

Table 2: Positional Specificities in Presence of Pure *sn*-2-20:4 PCs (Corrected for Positional Impurity)[a]

	20:4 CE formed (% of total CE synthesized)	
sn-1-acyl group	human LCAT	rat LCAT
16:0	51.6	100
16:1 trans	24.8	78.9
16:1 cis	85.7	98.0
18:0	95.2	99.7
18:1	78.1	84.9
18:2	75.2	94.2

[a] The right *Y* intercept values from Figures 2 and 3 are shown.

intercept) is noteworthy in that most of the acyl groups were derived from the *sn*-1 position of this PC, showing a complete switch in positional specificity of rat LCAT when 20:4 is present at *sn*-1. This suggests that the specificity of the enzyme for the acyl group (20:4) can overcome its natural preference for the *sn*-2 position.

Positional Specificity in the Presence of sn-2-18:0 PCs. Figures 4 and 5 show the best-fit curves in the presence of various *sn*-2-18:0 PCs for human and rat LCATs, respectively, and the right *Y* intercept values are given in Table 3. For both the enzymes, the alteration in positional specificity is more marked in the case of *sn*-2-18:0 PCs, compared to *sn*-2-20:4 PCs. Human LCAT transferred only 18%, 28%, and 41%, respectively, from the *sn*-2 position of 14:0−18:0, 16:0−18:0, and 18:2−18:0 PCs. However, when the *sn*-1 position was occupied by 18:1 or 20:4, about 80% of the acyl groups were derived from the *sn*-2 position (18:0). The alteration in positional specificity was even more dramatic for rat LCAT. Except for 18:1−18:0 PC, which contributed 70% of the acyl groups from *sn*-2 position for CE formation, all other PCs contributed less than 20% of the total acyl groups from *sn*-2. The stimulation of transfer of 18:0 from *sn*-2 by the presence of 18:1 at *sn*-1 is remarkable because

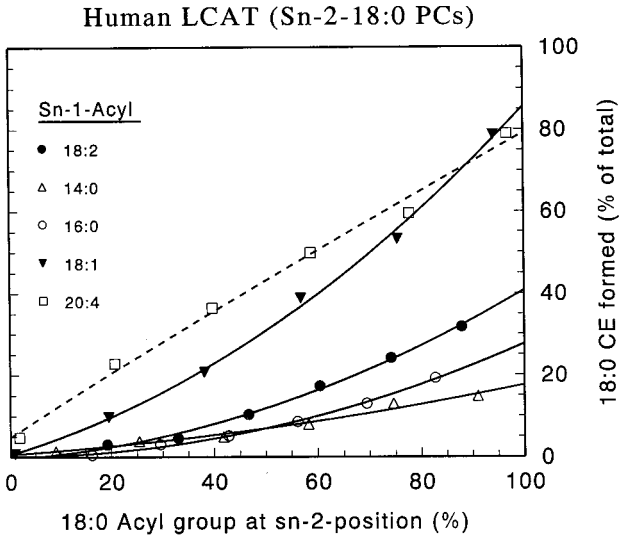

FIGURE 4: Positional specificity of human LCAT in the presence of *sn*-2-18:0 PCs. Labeled CE species formed by human LCAT in the presence of a series of *sn*-1-acyl-*sn*-2-18:0 PCs of known positional purity were analyzed, and the data were plotted as described in Figure 2. The right *Y* intercept of each line gives the specificity of the enzyme for the *sn*-2 position of pure *sn*-2-18:0 PC.

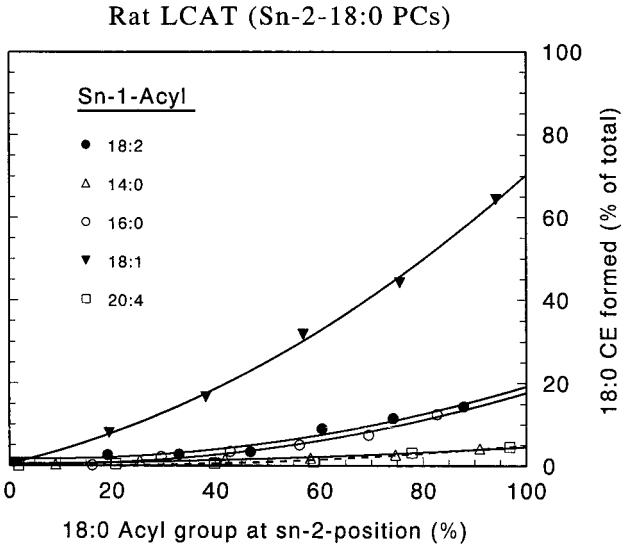

FIGURE 5: Positional specificity of rat LCAT in the presence of *sn*-2-18:0 PCs. Labeled CE species formed by rat LCAT in the presence of a series of *sn*-2-18:0 PCs of known positional purity were analyzed, and the data were plotted as described under Figure 2. The right *Y* intercept of each line represents the specificity of the enzyme for *sn*-2 in the presence of pure *sn*-2 18:0 PC.

Table 3: Positional Specificities in Presence of Pure *sn*-2-18:0 PCs (Corrected for Positional Impurity)[a]

	18:0 CE formed (% of total CE synthesized)	
sn-1-acyl group	human LCAT	rat LCAT
14:0	17.6	4.5
16:0	27.7	17.8
18:1	85.5	70.2
18:2	40.7	19.3
20:4	79.1	5.0

[a] The right *Y* intercept values from Figures 4 and 5 are shown.

normally both enzymes transfer 18:0 poorly from most substrates.

sn-1-Acyl Chain Length and Positional Specificity. Our previous studies showed an inverse correlation between the

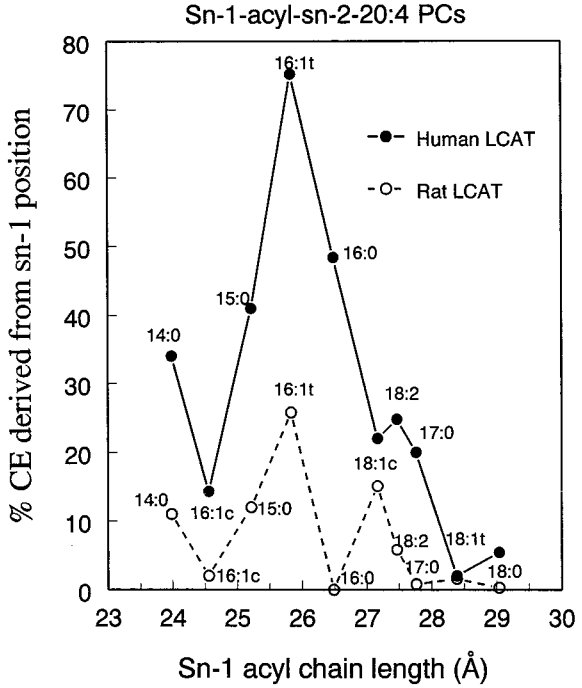

FIGURE 6: *sn*-1-Acyl chain length and positional specificity: *sn*-2-20:4 PCs. Utilization of *sn*-1-acyl group from the various *sn*-1-acyl-*sn*-2-20:4 PCs is plotted as a function of the chain length at *sn*-1. The transfer of the *sn*-1-acyl group from most PCs was calculated from Figures 2 and 3. Other values are from single PC substrates, whose positional purity varied from 86 to 97% (Figure 1). The value for *sn*-1-18:1-*trans* PC was taken from our previous study (*35*). Some of the *sn*-1-acyl chain lengths were obtained from published data (*12*), and others were calculated from the models constructed by Chem3D program, using the published coordinates (*12*).

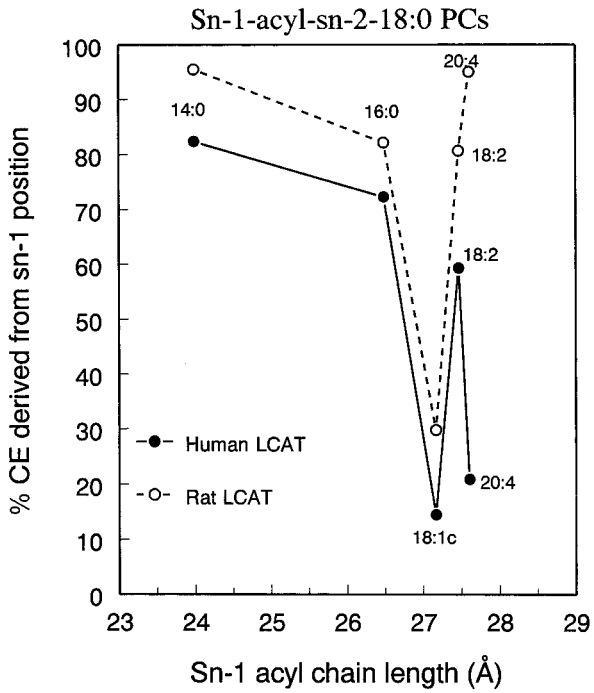

FIGURE 7: *sn*-1-Acyl chain length and positional specificity: *sn*-2-18:0 PCs. The transfer of an *sn*-1-acyl group from various *sn*-2-18:0 PCs is plotted against the chain length of the *sn*-1-acyl group. The values shown are calculated from the graphs in Figures 4 and 5.

chain length of the acyl group at *sn*-2 and its transfer to cholesterol by human or rat LCAT (*5*). To determine whether such a correlation exists for the transfer of *sn*-1-acyl group, we plotted the *sn*-1-acyl chain lengths as derived from computer modeling (*12*) against their utilization for CE synthesis, when the *sn*-2 is occupied by 20:4. As shown in Figure 6, the relationship between *sn*-1-acyl chain length (as measured from the 021 oxygen of the *sn*-2 ester group to the most distant hydrogen of the terminal methyl group of *sn*-1-acyl) and its transfer to cholesterol is not linear but instead exhibited a peak at about 26 Å, corresponding to 16:1-*trans* fatty acid. Acyl groups longer than 26 Å as well as shorter were transferred less efficiently by either human or rat LCAT. With all the PCs tested, rat LCAT derived a smaller percentage of acyl groups from the *sn*-1 position compared to human LCAT, showing its preference for the *sn*-2-20:4. However, when 18:0 occupied the *sn*-2 position (Figure 7), rat LCAT derived relatively more acyl groups from *sn*-1 compared to human LCAT. These results show that the positional specificity of human LCAT is altered more when *sn*-2 is occupied by 20:4, whereas that of rat LCAT is altered more when *sn*-2 is occupied by 18:0.

DISCUSSION

Although the esterification of cholesterol is the obvious physiological function of LCAT, this enzyme should actually be considered a specialized phospholipase A because its primary substrate is PC. However, in contrast to the phospholipases A from snake venom and pancreas, which show absolute specificity toward *sn*-2-acyl group of PC (*16*), and many lipases, which show absolute specificity for *sn*-1 position of PC (*17*), LCAT belongs to a unique class of phospholipases whose positional specificity is dependent upon the acyl group composition of the substrate PC. Our previous studies (*5*) showed that while the human LCAT transfers almost exclusively the *sn*-2-acyl group from some PCs (16:0−18:1, 16:0−18:2), it transfers exclusively *sn*-1-acyl from certain others (16:0-phytanoyl) and equally from both positions of yet others (16:0−20:4). A bacterial lipase−acyltransferase has been described that also exhibits flexible positional specificity with regard to its hydrolytic activity, although its transfer activity is limited to the *sn*-2 position (*18*). In contrast, both the hydrolysis and transfer reactions of LCAT are altered in a similar manner. The positional specificity of the bacterial enzyme, unlike that of LCAT, is not dependent upon the acyl group composition of PC.

The results presented here show that both human and rat LCATs attack exclusively the *sn*-2 ester linkage of all the symmetric PCs tested. Although the earlier study of Assmann et al. (*19*) suggested that up to 40% of the acyl groups for CE synthesis were derived from the *sn*-1 position of 18:2−18:2 PC, we did not find any transfer from the *sn*-1 position of this PC species. It is possible that the apparent transfer of the *sn*-1-acyl group in the aforementioned study is due to the presence of a positional impurity in the labeled substrate used, although these authors reported only 5% impurity. The labeled 18:2−18:2 PC used in the present study contained 99% of the label at *sn*-2. The preference of the enzyme for the *sn*-2 position of 18:0−18:0 PC and 20:4−20:4 PC shows the importance of the *sn*-1-acyl group in determining the positional specificity, because if the *sn*-1 is occupied by 16:0 (instead of 18:0 or 20:4), less than 50% of the total acyl groups are derived from the *sn*-2 (*5*) (Figures

2 and 4). These results therefore show that (*a*) the presence of 18:0 or 20:4 at *sn*-2 does not automatically result in the alteration of positional specificity and (*b*) the natural preference of both human and rat LCATs is for the *sn*-2 ester linkage if both positions are occupied by the same fatty acid. Conformational studies reveal that the *sn*-2-acyl chain shows a bend at the interface because it starts parallel to the interface, whereas the *sn*-1 chain starts perpendicular to the interface (*20*). Consequently, the *sn*-2 chain is shorter than the *sn*-1 chain by about 3.7 Å even when both chains are chemically identical (*12*), and this might partly explain the preference of the enzyme for the *sn*-2. It is also possible that since the *sn*-2 carbonyl is closer to the interface, it is more accessible to the enzyme or that the secondary ester is more susceptible to attack by LCAT compared to the primary ester. Since most physiological substrates of LCAT are asymmetric PCs, containing predominantly a saturated fatty acid at *sn*-1 and an unsaturated fatty acid at *sn*-2 (*15*, *21*, *22*), the altered positional specificity would have a significant impact in vivo. Comparative and epidemiologic investigations indicate that an increase in saturated CE and a decrease in 20:4 CE are correlated with increased susceptibility to atherosclerosis (*23*−*28*). Therefore, the positional specificity of LCAT toward *sn*-2−20:4 PCs may be important in determining the atherogenic risk.

The physicochemical basis for the effect of PC acyl group composition on the positional specificity of LCAT is yet to be established. In the case of the Ca^{2+}-requiring phospholipases, the metal ion appears to play a critical role in the proper orientation of the two acyl groups during the binding of the substrate to the active site (*29*), thus fixing the position at which the hydrolysis takes place. However, in the case of LCAT, which does not require a metal ion, the acyl group composition itself may dictate the binding alignment. Recent studies suggest that residue 149 of human LCAT (E149) may determine its substrate specificity toward different molecular species of PC (*30*, *31*). However, it is not known if this residue is also important in determining the positional specificity of the enzyme. It is likely that more than one residue of the enzyme is involved in the proper orientation of the PC substrate with respect to the active site nucleophile, because the data presented here show that both the acyl groups of PC interact with the enzyme, not just the acyl group being transferred. Even with respect to the substrate specificity, there is no obvious correlation between the size and hydrophobicity of residue 149 and fatty acid specificity (*30*, *32*), suggesting that other residues near it may also be important. Mutagenesis of residues topologically near E149 would be necessary to identify the putative determinants of positional specificity.

Since the enzyme attacks only the *sn*-2 position when the latter is occupied by certain acyl groups such as 18:1 or 18:2 or when a symmetric PC is the substrate, the primary determinant of the orientation appears to be the structure of the *sn*-2-acyl group, probably its chain length (*5*). The switch in the specificity of human LCAT to the *sn*-1 position occurs only when the *sn*-2 position is occupied by an extra long fatty acid such as 18:0, 20:4, or 22:6 and is usually accompanied by a decrease in the overall activity. We propose that under such a condition, the structure of the *sn*-1-acyl group becomes important in determining the activity as well as the positional specificity. Occupancy of the *sn*-1 position by acyl groups that are preferentially transferred by the enzyme (e.g., 16:0) increases the enzyme activity, and consequently, the alteration in positional specificity (% acyl groups contributed by *sn*-1) becomes more prominent. In contrast to human LCAT, the rat enzyme transfers the 20:4 acyl group readily, and hence, there is no significant alteration of its positional specificity in the presence of *sn*-2-20:4 PCs. However, in the presence of *sn*-2 18:0 PCs, both the enzymes exhibit altered positional specificity, the magnitude of alteration again depending upon the specificity of each enzyme toward the acyl group at *sn*-1. Although the chain length of the *sn*-2-acyl group is strongly correlated with the positional specificity of both human and rat LCATs (*5*), the chain length of the *sn*-1-acyl group showed no such correlation, suggesting that the interaction of the *sn*-1-acyl group with the active site is governed by other factors such as hydrophobicity and the number and position of double bonds.

The importance of the *sn*-1-acyl group in determining the positional specificity of LCAT is also evident in vivo. For example, our earlier studies showed that although dog LCAT alters its positional specificity in the presence of 16:0−20:4 PC in a similar way to human LCAT, dog plasma contains a relatively high concentration of 20:4 CE (21% of total) compared to that found in human plasma (7% of total) (*25*). This anomaly can be explained by the fact that dog plasma contains a high concentration of 18:0−20:4 PC (34% of total PC) compared to that found in human plasma (6% of total PC) (*22*). Since the positional specificity of neither enzyme is altered in the presence of 18:0−20:4 PC, dog LCAT synthesizes a high concentration of 20:4 CE. Recombination experiments showed that purified human LCAT also synthesizes a high percentage of 20:4 CE when incubated with heat-treated dog plasma as the substrate. Thus, the fatty acid structure at *sn*-1 of *sn*-2−20:4 PC greatly influences the formation of 20:4 CE in plasma. Since 20:4 CE appears to be the major source of arachidonate for many tissues (*33*, *34*), its synthesis in plasma may indirectly influence the formation of prostaglandins and leukotrienes in these tissues.

In summary, the data presented here show that the *sn*-1-acyl group plays a major role in determining the activity and positional specificity of LCAT when the *sn*-2 position is occupied by an extra long chain fatty acid such as 18:0, 20:4, or 22:6. The results also suggest that both the acyl groups of PC interact with the active site of the enzyme, although only one of them is transferred to FC. In contrast to the phospholipases A of snake venom and pancreas, the natural preference of LCAT for the *sn*-2 ester linkage can be overridden by its selectivity for certain fatty acids at *sn*-1.

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