Role of *sn*-2 Acyl Group of Phosphatidylcholine in Determining the Positional Specificity of Lecithin-Cholesterol Acyltransferase†

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ABSTRACT: Although human plasma lecithin-cholesterol acyltransferase (LCAT) is believed to be specific for the *sn*-2 position of phosphatidylcholine (PC), our recent studies showed that it derives a significant percent of acyl groups from the *sn*-1 position of certain PC species. To understand the physicochemical basis for this altered positional specificity, we determined the effect of *sn*-2 acyl group of PC on the enzyme activity and utilization of 16:0 from the *sn*-1 position by purified human and rat LCATs. Positional isomers of PC containing 16:0 at *sn*-2 were better substrates for human LCAT than the corresponding *sn*-1-16:0 isomers, whereas the reverse was true for rat LCAT. The positional specificity of human LCAT varied greatly depending on the nature of the acyl group at *sn*-2. The *sn*-1 contribution from various *sn*-1-16:0-2-acyl PCs for cholesteryl ester (CE) synthesis was 1.0% from 16:0−16:0, 1.4% from 16:0−20:5, 7.3% from 16:0−18:1, 47.0% from 16:0−20:3, 49.9% from 16:0−20:4, 54.9% from 16:0−22:6, and 72.3% from 16:0−18:0. There was a linear relationship between the percentage of 16:0 CE formed (from *sn*-1 position) and the acyl chain length at *sn*-2 position ($r = 0.94$). Rat LCAT also transferred some 16:0 from *sn*-1 position of 16:0−22:6, 16:0−20:3, and 16:0−18:0 PCs, but not from the other natural PCs tested. The phospholipase A activity of both LCATs in the presence of 16:0−20:4 PC showed the same positional specificity as CE synthesis, indicating that the specificity is determined at the formation of acyl−enzyme intermediate. These results show that the positional specificity of LCAT is influenced by the structure of PC, especially the chain length of the *sn*-2 acyl group.

LCAT, which transfers an acyl group from PC to free cholesterol, plays an important role in the metabolism of cholesterol in the plasma (Glomset, 1979; Jonas, 1991). Although the majority of the acyl groups for cholesterol esterification are known to be derived from the *sn*-2 position of PC, several studies have shown that the enzyme can also transfer the *sn*-1 acyl group of PC *in vitro* (Assmann et al., 1978; Aron et al., 1978). However, this apparent flexibility in the positional specificity is not observed with all the PC substrates, because the extent of *sn*-1 acyl transfer appears to be dependent on the acyl group composition of PC. Our earlier studies with purified human LCAT showed that while the enzyme preferentially transferred the *sn*-2 acyl group from most of the naturally occurring PC species, it transferred significant amounts of 16:0 from 16:0−20:4 PC and 16:0−22:6 PC (Subbaiah et al., 1992). This altered positional specificity was not due to changes in the matrix fluidity induced by the substrate and was not influenced by the nature of the apoprotein activator employed or by the PC:cholesterol ratio in the substrate, indicating that the molecular structure of PC is the determining factor. The enzyme purified from rat plasma, in contrast, transferred predominantly 20:4 from 16:0−20:4 PC. On the basis of these results, we postulated that when the *sn*-2 position of PC is occupied by long and bulky acyl groups, human LCAT transfers the *sn*-1 acyl group in preference over the *sn*-2 acyl group because of steric constraints at the active site. The active site of rat LCAT, on the other hand, is probably large enough to accommodate the arachidonoyl group, and therefore the positional specificity of this enzyme is not altered. However, a systematic study on the effect of the structure of the *sn*-2 acyl group on the positional specificity of LCAT has not been carried out. Furthermore, since most of the synthetic PC species employed as substrates contain small amounts of positional isomers as contaminants, one cannot rule out the possibility that the formation of 16:0 CE in the presence of 16:0−20:4 PC is due to a preferential utilization of the contaminating 20:4−16:0 PC by the enzyme. In the present study we investigated the effect of carbon number and unsaturation of the *sn*-2 acyl group on the transfer of 16:0 from the *sn*-1 position by human and rat LCATs. We have also corrected for the effect of positional impurity in each PC substrate by applying a best-fit formula, which was derived by employing mixtures of positional isomers of known purity. The results presented here show that the effective chain length of the *sn*-2 acyl group is more important than the carbon number or the number and position of double bonds in determining the positional specificity of the enzyme.

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

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MATERIALS AND METHODS

Enzymes. Human plasma LCAT was purified to homogeneity by the procedures described earlier (Subbaiah et al., 1980; Subbaiah et al., 1992). The final preparation had a specific activity of 10-12 nmol of cholesterol esterified/h per μg of protein in the standard proteoliposome assay, using egg PC as the acyl donor (Chen & Albers, 1982). Rat LCAT was purified from pooled rat plasma (Pel-Freez Biological Inc.) by the same procedure used for human LCAT. In most cases, however, the rat enzyme obtained after the phenyl Sepharose step was used without further purification. We did not find any difference in the substrate and positional specificities of pure and semipure LCAT preparations, in agreement with the results of Grove and Pownall (1991). Apoprotein A-I was purified from pooled human plasma as described earlier (Subbaiah et al., 1992).

Substrates. [4-^{14}C]Cholesterol (60 mCi/mmol), 1-16:0-2-[^{3}H]-16:0 PC (42 mCi/mmol), and 1-16:0-2-[^{14}C]-20:4 PC (52 mCi/mmol) were purchased from Dupont-NEN. Egg PC and 16:0−18:1 (n-9) PC were purchased from Sigma Chemical Co. 16:0−18:2(n-6) PC, 16:0−14:0 PC, 14:0−16:0 PC, 16:0−18:0 PC, and 18:0−16:0 PC were all products of Avanti Polar Lipids. 16:0−20:4(n-6) PC, 20:4(n-6)−16:0 PC, 16:0−phytanoyl PC, phytanoyl−16:0 PC, 16:0−22:6(n-3) PC, 22:6(n-3)−16:0 PC, 16:0−20:5(n-3) PC, 20:5(n-3)−16:0 PC, 16:0−20:3(n-6) PC, and POPC diether were synthesized in our laboratories by the procedures described earlier (Paltauf & Hermetter, 1991; Hermetter et al., 1989).

The positional purity of all PCs was tested by the fatty acid analysis of FFA and lyso PC generated by snake venom phospholipase A_2 treatment (Subbaiah et al., 1992). All PCs were incorporated into the proteoliposome substrate by the cholate-dialysis procedure (Chen & Albers, 1982). The initial molar ratio of PC:^{14}C free cholesterol:apo A-I was 250:12.5:0.8. When 16:0−16:0 PC, 16:0−18:0 PC, and 16:0-phytanoyl PC were used as substrates, a 50:50 mixture of the test PC and POPC diether was used instead of pure test PC, because these PCs did not yield clear proteoliposomes when used alone. As shown earlier (Subbaiah et al., 1992), the analysis of all proteoliposomes by electrophoresis on 2-16% polyacrylamide gels revealed one major band of 240 Å diameter for all the substrates, with varying amounts of minor bands of 160, 120, and 107 Å.

Enzyme Assays. LCAT activity was estimated by the conversion of labeled cholesterol to cholesteryl ester (CE) in the presence of the proteoliposome substrate, as described earlier (Liu & Subbaiah, 1993b). The incubations were generally carried out for 1 h at 37 °C, except in the case of the substrates containing POPC diether, where the incubations were carried out for 6 h at 37 °C, because of lower enzyme activities, due to competitive inhibition by POPC diether (Subbaiah & Liu, 1993). All the enzyme reactions were linear under the conditions of assay, and hence the values obtained represent the initial rates. Each experiment included a control tube containing egg PC substrate which was used to normalize the activities assayed on different days. The activities were first calculated as nmol of cholesterol esterified/h and then expressed as percent of activity obtained with egg PC substrate assayed at the same time. In experiments where mixtures of two positional isomers of same PC were used, the activities were expressed as percent of activity with 100% pure *sn*-1-16:0 isomer, which was derived from the left *Y* intercept of the graph.

Positional Specificity. The positional specificity of LCAT was determined by the percentage of labeled CE formed from each position of the PC substrate. The reactions were carried out for 4 h instead of 1 h to get sufficient label incorporated into CE. Samples containing POPC diether were incubated for 12 h instead of 6 h for the same purpose. The total lipid extract from the reaction mixture (Bligh & Dyer, 1959) was injected on to a C-18 column, after filtration through a 0.45 μm filter. The CE species were separated from each other by employing the solvent system of acetonitrile:tetrahydrofuran:water (65:35:1.5) at a flow rate of 2 mL/min. Fractions of 1 mL each were collected into scintillation vials, and the solvent was evaporated off by placing the vials in a fume hood overnight. The radioactivity in each vial was determined in a liquid scintillation counter after adding 5 mL of a scintillation fluid (Cytoscint, ICN Biomedical). From the percentage of radioactivity in each of the two expected CE peaks, the percent contribution of *sn*-1 and *sn*-2 acyl groups of PC was calculated.

When 1-16:0-2-[^{3}H]-16:0 PC was used as substrate, unlabeled free cholesterol was incorporated into the proteoliposomes, instead of labeled cholesterol. The molar ratio of total PC:cholesterol in the substrate was fixed at 4:1 instead of the 20:1 ratio used routinely, in order to minimize the phospholipase A activity of the enzyme. After the reaction, the lipid extract was 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). After the plate was developed half way (10 cm) in this solvent, it was air-dried briefly and developed to the top in the second solvent (hexane:diethyl ether:acetic acid, 70:30:1, v/v). The spots of lyso PC, PC, FFA, and CE were identified with the help of authentic standards which were separated on the same plate, and their radioactivity was determined in a liquid scintillation counter. The radioactivity in lyso PC represented the utilization of the *sn*-1 acyl group by the enzyme, whereas the radioactivity in FFA and CE measured the hydrolysis or transfer of *sn*-2 acyl group.

The positional specificity of phospholipase A activity was determined in the presence of 1-16:0-2-[^{14}C]-20:4 PC. Proteoliposomes were prepared in the absence of free cholesterol, and after incubation with the enzyme (37 °C, 60 min) the reaction was stopped with methanol containing 50 μg each of unlabeled 1-palmitoyl lyso PC and oleic acid. The total lipids were extracted (Bligh & Dyer, 1959) and separated on silica gel TLC with the solvent system of chloroform:methanol:water (65:25:4, v/v). The spots corresponding to lyso PC, PC, and FFA were scraped off and their radioactivities determined. The label present in lyso PC represents the phospholipase A_1 activity, whereas the label present in FFA measures the phospholipase A_2 activity.

RESULTS

Effect of sn-2 Acyl Group on Enzyme Activity. Table 1 shows the activities of human and rat LCATs in the presence of proteoliposome substrates, all of which contained 16:0 at *sn*-1 position but different acyl groups at *sn*-2 position. In order to correct for the day to day variations in enzyme activity, as well as to normalize the activities obtained with different enzyme preparations, the activities are expressed as a percent of the activity obtained with egg PC substrate, assayed under identical conditions. All activities were linear

Table 1: Effect of the *sn*-2 Acyl Group on LCAT Activity[a]

PC substrate	relative activity (% of egg PC)	
	human LCAT	rat LCAT
16:0−14:0	15.10	31.22
16:0−16:0[b]	26.52	46.84
16:0−18:0[b]	22.96	39.34
16:0−18:1(n-9)	121.50	102.26
16:0−18:2(n-6)	131.23	134.91
16:0−18:3(n-3)	113.58	149.91
16:0−phytanoyl[b]	2.58	4.66
16:0−20:3(n-6)	47.40	123.85
16:0−20:4(n-6)	59.69	190.36
16:0−20:5(n-3)	65.93	227.46
16:0−22:6(n-3)	31.82	91.95

[a] Various *sn*-1-16:0 PCs were incorporated into proteoliposome substrates by cholate dialysis (Chen & Albers, 1982). All reactions contained 0.6−1.0 μg pure human LCAT, or 2−4 μg of partially purified rat LCAT. The incubations were carried out for 1 h at 37 °C, except when the substrate contained POPC diether, in which case the incubation time was extended to 6 h. The enzyme activities were linear under these conditions. All values are expressed as percent of the activity obtained with egg PC substrate which was prepared by the same procedure, and was assayed at the same time. The values shown are averages of at least two separate experiments. [b] Also contained 50 mol % POPC diether.

under the conditions of assay. Human LCAT showed the highest activity with 16:0−18:2 PC in agreement with our earlier results on native plasma (Subbaiah & Monshizadegan, 1988). Rat LCAT showed the highest activity with 16:0−20:5 PC, followed by 16:0−20:4 PC. The lowest activity for both the enzymes was obtained with 16:0−phytanoyl PC, which has a branched chain 20 carbon fatty acid. The activities of 16:0−16:0 PC, 16:0−18:0 PC, and 16:0−phytanoyl PC were determined in the presence of 50% POPC diether and were therefore compared with egg PC substrate which also contained 50% POPC diether. Although we did not make any attempts to separate the macromolecular (matrix) effects of PC from its molecular interaction with the active site, the relative order of activities was in general agreement with published data (Jonas, 1986).

Effect of sn-2 Acyl Group on Positional Specificity. The positional specificities of human and rat LCATs were determined in the presence of various 1-16:0-2-acyl PCs by estimating the percentage contribution of *sn*-1 and *sn*-2 positions for the CE formation. As shown in Table 2, human LCAT transferred predominantly the *sn*-2 acyl group from most of the PCs tested except 16:0−18:0, 16:0−phytanoyl, 16:0−20:3, 16:0−20:4, and 16:0−22:6 PCs. In accordance with our earlier data (Subbaiah et al., 1992), a significant percent of the acyl groups for CE formation were derived from the *sn*-1 position of 16:0−20:4 PC as well as 16:0−22:6 PC, suggesting altered positional specificity. The specificity was also altered in the presence of another *sn*-2-20 carbon PC, namely 16:0−20:3 PC. However, in the presence of 16:0−20:5 PC, most of the acyl groups were derived from *sn*-2 position. Rat LCAT transferred acyl groups from *sn*-2 position of most of the PCs tested excepting 16:0−18:0 and 16:0−phytanoyl PCs, from which it utilized mostly *sn*-1 acyl groups. In addition, it transferred a significant percentage of acyl groups from the *sn*-1 position of 16:0−20:3 PC and 16:0−22:6 PC, although not as much as human LCAT did. These results confirm the differences between human and rat LCATs not only in their substrate specificities but also in their positional specificities.

Table 2: Effect of the *sn*-2 Acyl Group on the Positional Specificity of LCAT[a]

PC substrate	positional purity[b]	16:0 CE as % of total CE formed	
		human LCAT	rat LCAT
16:0−14:0	85.32	22.92	10.58
16:0−[^{3}H]16:0	99.36	0.74[c]	0.00[c]
16:0−18:0	82.73	80.80	87.54
16:0−18:1(n-9)	97.58	9.68	7.92
16:0−18:2(n-6)	92.25	6.21	9.67
16:0−18:3(n-3)	75.25	32.38	23.55
16:0−phytanoyl	82.47	96.10	97.97
16:0−20:3(n-6)	95.05	47.80	32.72
16:0−20:4(n-6)	85.81	62.09	14.13
16:0−20:5(n-3)	93.02	20.01	3.82
16:0−22:6(n-3)	96.63	56.21	42.76

[a] The labeled CE species formed after incubation with each substrate were separated by HPLC, and their radioactivity was determined. The percent of total CE radioactivity in 16:0 CE peak is shown, except in the case of 16:0−[^{3}H]16:0 PC, where the percent of radioactivity released as lyso PC is shown. All values are averages of at least two experiments. [b] As determined from the composition of free fatty acids released by treatment with snake venom phospholipase A_2 treatment. [c] Percent radioactivity in lyso PC product.

Effect of Positional Impurities on the Activity and Positional Specificity. In most of the *sn*-1-16:0 PC preparations tested, either obtained from commercial sources or prepared in our laboratories, there was some contamination with positional isomers in which 16:0 was present at *sn*-2 position (Table 2). Since the standard proteoliposome substrate contains PC:free cholesterol ratio at 20:1, even a 100% esterification of cholesterol would only consume 5% of total PC. It is therefore theoretically possible that the formation of 16:0 CE from 16:0−20:4 PC was because of a preferential utilization of the contaminating 20:4−16:0 PC. Since in practice it is difficult to synthesize 100% positionally pure PC, we addressed this question by performing a series of recombination experiments with positional isomers of known purity. We synthesized various PCs containing 16:0 at *sn*-2 position, mixed them with the corresponding *sn*-1-16:0 isomers at various percentages, and determined the CE species formed from each mixture by LCAT. By plotting the percentage of 16:0 CE formed against the percentage of 16:0 present at *sn*-2 position in the mixture, we obtained regression lines with the help of the best-fit formula (SlideWrite Plus, Advanced Graphics Software). Extrapolating the regression line to 0% 16:0 at *sn*-2 (pure 16:0−20:4 PC, for example), one can obtain the true positional specificity of the enzyme with the natural isomer. Similar regression lines were obtained for the total enzyme activity to determine whether *sn*-2-16:0 isomers are preferred as substrates over the *sn*-1-16:0 isomers. The results obtained for mixtures of 16:0−20:4 PC and 20:4−16:0 PC are shown in Figure 1. The activity of human LCAT increased as the percentage of 16:0 at *sn*-2 position is increased (Figure 1A). Pure 20:4−16:0 PC was utilized about 2.5 times better than pure 16:0−20:4 PC. On the other hand rat LCAT was inhibited by the presence of 16:0 at *sn*-2 position, and its activity with pure 20:4−16:0 PC was only 25% of that observed with pure 16:0−20:4 PC. These results show that the contaminating isomer is indeed used preferably by human LCAT and therefore may have affected the positional specificity calculations reported earlier (Subbaiah et al., 1992). However, as shown in Figure 1B, the percent of 16:0 CE synthesized by human LCAT in the presence of pure 16:0−20:4 PC (left

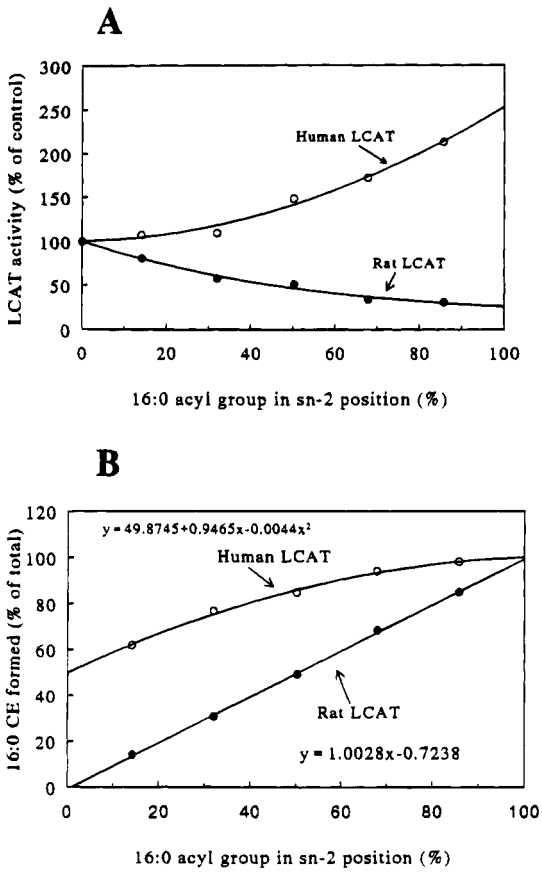

FIGURE 1: Effect of positional isomers of 16:0−20:4 PC on the activities and positional specificities of human and rat LCATs. (A) Synthetic 16:0−20:4 PC (positional purity, 85.8%) and 20:4−16:0 PC (positional purity, 85.8%) were mixed at various ratios to give the indicated percent of 16:0 at the *sn*-2 position. The PC was then incorporated into proteoliposome substrate (Chen & Albers, 1982), with the molar ratios of PC:free cholesterol:apo A-I at 250: 12.5:0.8. The LCAT activities were plotted against the concentration of 16:0 at *sn*-2, and curve fitting was performed with SlideWrite Plus software. The activity of the enzyme with pure 16:0−20:4 PC was obtained by extrapolating the curve to 0% 16:0 at *sn*-2. For ease of comparison of rat and LCAT enzymes, all activities are expressed as percent of the activity obtained with pure 16:0−20:4 PC. (B) The composition of labeled CE formed in the presence of each PC mixture in the above experiment was determined by HPLC, and the percent of 16:0 CE was plotted against the percent of 16:0 at the *sn*-2 position of PC. Curve fitting was performed with Slide Write Plus program, using least square regression. Extrapolation of the curve to 0% 16:0 at *sn*-2 position yields the percent of 16:0 CE formed from pure 16:0−20:4 PC, whereas the right *Y* intercept shows the percent of 16:0 CE formed from pure 20:4−16:0 PC.

Y intercept) is 49.9%, showing that the enzyme utilized the two positions of this PC equally. This value agrees closely with the results we obtained previously with *sn*-2 acyl-labeled 16:0−20:4 PC (Subbaiah et al., 1992). Rat LCAT on the other hand derived 100% of the acyl groups from the *sn*-2 position of 16:0−20:4 PC. From pure 20:4−16:0 PC, both enzymes derived almost 100% of the acyl groups from the *sn*-2 position (right *Y* intercept). It may be calculated from Figure 1B that when the substrate is composed of equal amounts of the two isomers, human LCAT formed 86% 16:0 CE and 14% 20:4 CE, whereas rat LCAT synthesized equal amounts of 16:0 CE and 20:4 CE.

Figure 2 shows the regression lines for mixtures of 16:0−20:5 PC and 20:5−16:0 PC. In contrast to the 16:0−20:4 PC mixtures, both human and rat LCATs utilized

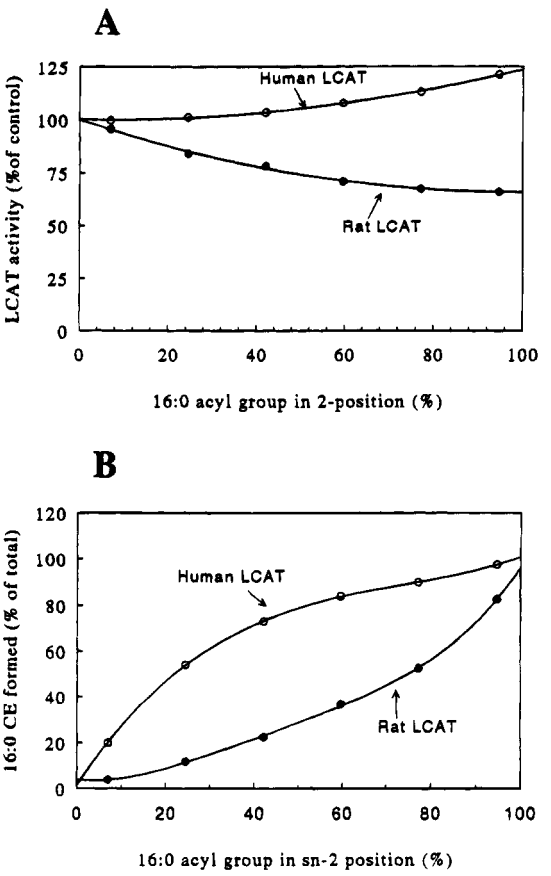

FIGURE 2: Effect of positional isomers of 16:0−20:5 PC on the activities (A) and positional specificities (B) of human and rat LCATs. The substrates were prepared with mixtures of 16:0−20:5 PC (positional purity, 93.0%) and 20:5−16:0 PC (positional purity, 94.7%). The activities and positional specificities were calculated as described in Figure 1.

preferentially *sn*-2 group from pure 16:0−20:5 PC (Figure 2B). Furthermore, the activity of human LCAT was stimulated only modestly by the presence of an increasing percentage of 20:5−16:0 PC (Figure 2A). The activity of rat LCAT was inhibited by the presence of 20:5−16:0 PC, but the effect was again less dramatic when compared to that of 20:4−16:0 PC. These results show that the activity and positional specificity of human LCAT are altered significantly by the presence of an extra double bond at the *sn*-2 position, although it is possible that the position of the initial double bond (n-6 vs n-3) may also play some role.

The results with another n-3 fatty acid-containing PC, namely 22:6(n-3) are shown in Figure 3. Both human and rat LCATs showed an increase in activity with the increasing presence of 16:0 at *sn*-2, in contrast to the experiment with 16:0−20:4 PC and 16:0−20:5 PC, where only human LCAT showed such an increase. Furthermore, the formation of 16:0 CE from the *sn*-1 position was exhibited by rat LCAT also. With 100% pure 16:0−22:6 PC, the *sn*-1 position contributed about 55% and 42% of the acyl groups for CE synthesis in the presence of human and rat LCATs, respectively. These results show that not all n-3 fatty acids at the *sn*-2 position have same effect on the positional specificity.

The results obtained with mixtures of 16:0−18:0 PC and 18:0−16:0 PC were essentially similar to those for 16:0−22:6 PC, although both LCATs were activated more by the presence of 16:0 at *sn*-2. The human enzyme showed nearly 5 times higher activity, and the rat enzyme showed about 3

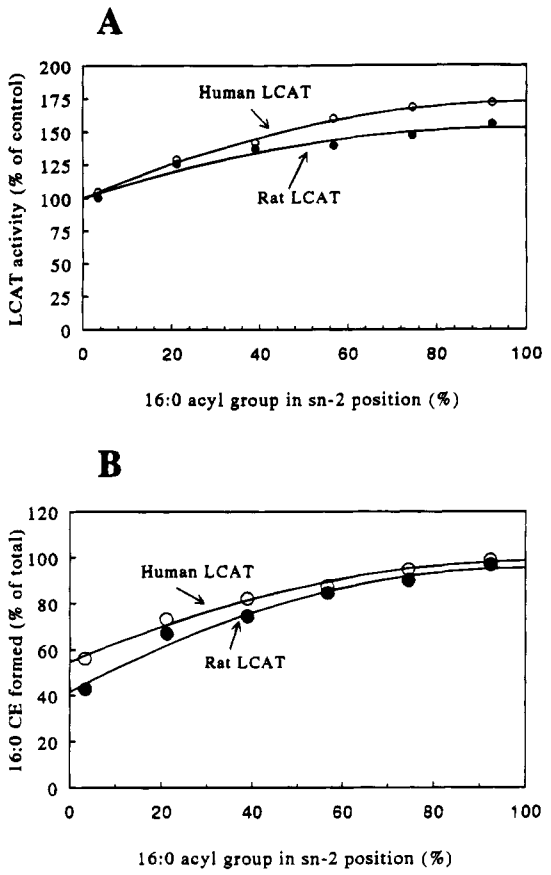

FIGURE 3: Enzyme activities (A) and positional specificities (B) of human and rat LCATs in the presence of mixtures of 16:0–22:6 PC and 22:6–16:0 PC. 16:0–22:6 PC (96.6% positional purity) and 22:6–16:0 PC (92.4% positional purity) were mixed in various proportions to give the indicated percent of 16:0 at the *sn*-2 position. The enzyme activities and positional specificities were determined as described in Figure 1.

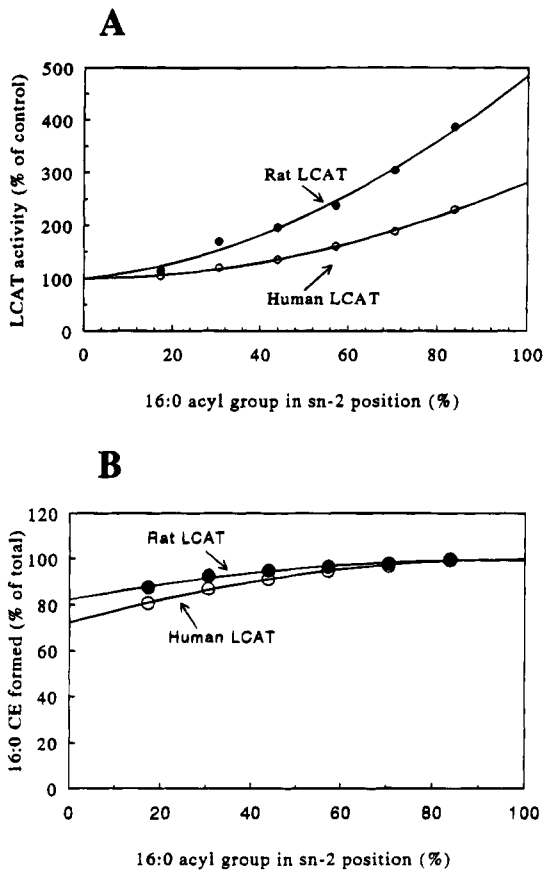

FIGURE 4: Effects of positional isomers of 16:0–18:0 PC on the activities (A) and positional specificities (B) of human and rat LCATs. Mixtures of 16:0–18:0 PC (positional purity, 82.8%) and 18:0–16:0 PC (positional purity, 83.8%) were incorporated into proteoliposome substrate which also contained 50 mol % POPC diether to aid in solubilization. The enzyme activities and positional specificities with pure isomers were calculated by extrapolation as described in Figure 1.

times higher activity in the presence of pure 18:0–16:0 PC, when compared to pure 16:0–18:0 PC (Figure 4A). The positional specificity of both human and rat LCATs was altered dramatically in the presence of pure 16:0–18:0 PC, where more than 70% of the total acyl groups for CE formation were derived from the *sn*-1 position (Figure 4B).

In contrast to 18:0, the presence of 14:0 at the *sn*-2 position resulted in only modest changes in enzyme activity and positional specificity of the two LCATs. Human LCAT was stimulated by a maximum of 10% by the presence of 16:0–14:0 PC, compared to pure 14:0–16:0 PC, whereas the rat enzyme was inhibited by 10% (results not shown). Both enzymes also transferred predominantly the *sn*-2 acyl group, regardless of the isomer composition of the mixture. The left *Y* intercepts of the regression lines showed that human LCAT derived only 8.5%, and rat LCAT derived only 6%, of the total acyl groups from the *sn*-2 position of 16:0–14:0 PC (results not shown).

When mixtures of 16:0–phytanoyl PC and phytanoyl–16:0 PC were tested similarly (in the presence of 50% POPC diether), both rat and human enzymes were found to synthesize only 16:0 CE from all isomer mixtures. The enzyme activities were directly proportional to the percent of 16:0 at *sn*-2 position (results not shown).

Positional Specificity in the Presence of Dipalmitoyl PC. Since one cannot use the composition of labeled CE formed to study the positional specificity of symmetric PCs, we employed dipalmitoyl PC which was labeled at *sn*-2 with [^{3}H]palmitate and analyzed its products. Snake venom phospholipase A_2 treatment of this compound revealed that more than 99.5% of the labeled palmitate was at the *sn*-2 position. The substrate preparation contained 50% POPC diether and 50% dipalmitoyl PC, and the total phospholipid:free cholesterol ratio was 4:1 instead of 20:1 to minimize the phospholipase A activity (Aron et al., 1978; Subbaiah et al., 1992). After the reaction with LCAT, the radioactivity in lyso PC, PC, FFA, and CE was determined as described in Materials and Methods. The presence of radioactivity in lyso PC indicates the hydrolysis or transfer of *sn*-1 palmitoyl residue, whereas the presence of label in FFA and CE indicates the utilization of *sn*-2 palmitoyl residue. As shown in Table 3, almost all the radioactivity released from PC was in FFA and CE in the presence of both LCATs, indicating that both enzymes specifically attacked *sn*-2 ester bond in dipalmitoyl PC.

Positional Specificity of Phospholipase Activities. The LCAT reaction is believed to take place via the formation of an acyl–enzyme intermediate (Subbaiah et al., 1980; Dolphin & Jauhiainen, 1987). In the absence of an acyl acceptor the enzyme acts as a phospholipase A, releasing the acyl group as FFA. In order to determine whether the positional specificity observed here is due to a selectivity in the transfer of the acyl group to cholesterol rather than in the initial step of the acyl–enzyme intermediate formation,

Table 3: Positional Specificity of Human and Rat LCATs in the Presence of *sn*-1−16:0−2−[^{3}H]16:0 PC[a]

	cpm in the product[b]	
product	human LCAT	rat LCAT
lyso PC	60 (1.01)	57 (1.25)
FFA	333 (5.63)	280 (6.16)
CE	5526 (93.36)	4206 (92.58)

[a] Dipalmitoyl PC, labeled at the *sn*-2 acyl group, was mixed with POPC diether at 1:1 molar ratio and was incorporated into proteoliposomes with total phospholipid:free cholesterol:apo A-I ratio at 250:62.5:0.8. Each reaction contained 50 000 cpm of substrate and either 1 μg of purified human LCAT or 2 μg of partially purified rat LCAT. Incubations were carried out for 1 h at 37 °C, and the radioactivity in the labeled products was determined as described in the text. Counts in lyso PC represent the utilization of *sn*-1 acyl group, whereas the counts in FFA and CE represent the utilization of *sn*-2 acyl group. The results presented are from one typical experiment (average of duplicate samples). [b] Values in parentheses represent percent of the total counts (in labeled products).

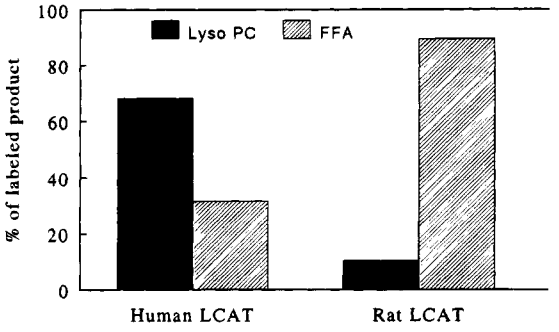

FIGURE 5: Positional specificity of phospholipase activities. 1-16:0-2-[^{14}C]-20:4 PC (positional purity 98.5%) was incorporated into proteoliposomes in the absence of free cholesterol and was incubated with either 1 μg of human LCAT or 2 μg of partially purified rat LCAT for 60 min at 37 °C. The lipids were extracted after adding 50 μg each of unlabeled lyso PC and oleic acid as carriers and were separated on silica gel TLC. Spots corresponding to lyso PC, PC, and FFA were scraped off and counted in a liquid scintillation counter. The radioactivity in lyso PC and FFA was calculated as percent of the sum of the two products. The label in lyso PC measures the hydrolysis of *sn*-1 ester linkage (phospholipase A_1), whereas the label in FFA measures the hydrolysis of *sn*-2 ester bond (phospholipase A_2). The values presented are averages of duplicate samples.

we studied the phospholipase activity of human and rat LCATs in the presence of 1-16:0-2-[^{14}C]-20:4 PC. The labeled PC used here contained >98% of the label at sn-2 position, as determined with snake venom phospholipase A_2, and all the label was in 20:4 fatty acid. Proteoliposomes containing labeled PC were prepared in the absence of free cholesterol, and incubated either with human or rat LCAT for 60 min. Lyso PC, PC, and FFA were separated on silica gel TLC, and their radioactivities were determined. The label present in lyso PC represents the hydrolysis of *sn*-1 acyl ester linkage, whereas the label present in FFA shows the extent of hydrolysis at the *sn*-2 position. As shown Figure 5, 68% of the label released by human LCAT was present in lyso PC, whereas 90% of the label released by rat LCAT was in FFA. These results are similar to the results obtained with labeled CE formation in the presence of labeled cholesterol and unlabeled PC (Figure 1) and therefore show that the positional specificity of the two LCATs is determined at the step of acyl−enzyme intermediate formation. Free cholesterol does not appear to have a significant effect on the positional specificity.

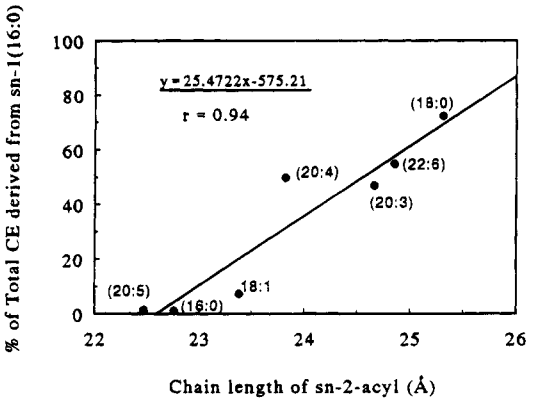

FIGURE 6: Correlation of *sn*-2 acyl chain length with the utilization of *sn*-1 16:0 by human LCAT. The percent of 16:0 CE formed in the presence of various *sn*-1-16:0-*sn*-2-acyl PC substrates was calculated from the data in Figures 1−4. The values for 16:0−18:1 PC and 16:0−20:3 PC were obtained from similar plots (data not shown). The value for 16:0−16:0 PC was from the experiment with *sn*-2 acyl-labeled PC (Table 3). The chain lengths for the various *sn*-2 acyl groups were taken from published data (Applegate & Glomset, 1991).

Correlation of sn-2 Acyl Chain Length with Positional Specificity. In order to identify the structural parameter of the *sn*-2 acyl group which influences the positional specificity of LCAT, we plotted the formation of 16:0 CE from the *sn*-1 position (left *Y* intercept) against the published values for the calculated chain length of the *sn*-2 group (Applegate & Glomset, 1991). The value for 20:5(n-3) was calculated from the computer model kindly provided by Dr. K. Applegate, University of Washington. In addition to the PC isomers discussed above, we have included the results from isomers of 16:0−20:3 PC and 16:0−18:1 PC, the regression lines of which are not shown. With human LCAT, a linear relationship was observed between *sn*-1-16:0 utilization and the *sn*-2 acyl chain length (r = 0.94), when only the physiologically relevant PCs are included in the graph (Figure 6). When 16:0−14:0 PC was also included, the correlation coefficient was 0.80 with linear curve fit, and 0.94 with second-order polynomial fit ($Y = 2384 - 222X + 5.16X^2$). No correlation was observed when either the number of carbons or the number of double bonds at *sn*-2 was plotted against 16:0 CE formation (results not shown). In the case of rat LCAT, the relationship between the *sn*-2 acyl chain length and 16:0 CE formation was exponential, rather than linear ($\ln Y = 1.2804X - 28.157$; $r = 0.95$) (Figure 7). These results therefore suggest that a critical determinant in the positional specificity of LCAT is the effective chain length of the *sn*-2 acyl group. They also show the possible differences between the active-site architectures of human and rat LCATs, with the rat enzyme having a higher "tolerance" for *sn*-2 acyl chain length. For both enzymes, the value for 20:4 deviated significantly from the projected trend suggesting that other factors in addition to the chain length may be operative.

DISCUSSION

The substrate specificity of LCAT for various molecular species of PC and the species differences in the substrate have been reported by several laboratories (Subbaiah & Monshizadegan, 1988; Jonas, 1986; Pownall et al., 1985b; Subbaiah et al., 1992; Sgoutas, 1972; Grove & Pownall, 1991). Most of these studies showed that human LCAT

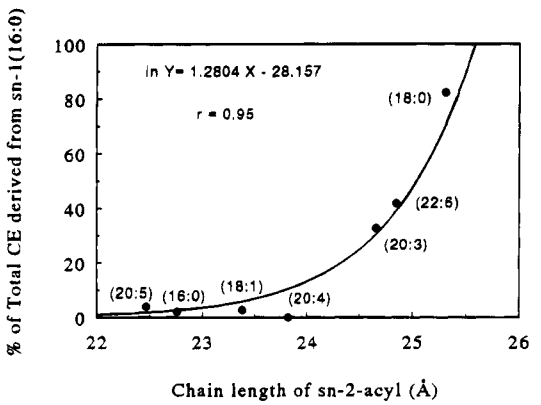

FIGURE 7: Correlation of *sn*-2 acyl chain length and utilization of *sn*-1-16:0 by rat LCAT. The values for *sn*-1-16:0 utilization were obtained from the data in Figures 1-4, Table 3 (for 16:0−16:0 PC), and from data not shown (for 16:0−18:1 PC and 16:0−20:3 PC). The values for *sn*-2 acyl chain length were obtained from published data (Applegate & Glomset, 1991).

prefers PC species containing 18:2 at *sn*-2, whereas rat LCAT prefers PC species containing 20:4 at *sn*-2. In addition, various PC species affect the macromolecular structure of the substrate particles and thereby indirectly affect the LCAT activity, at least *in vitro* (Pownall et al., 1985a; Jonas et al., 1987). However, the possible influence of the acyl group composition of PC on the positional specificity of LCAT has not received much attention. Most of the known phospholipases A have rigid positional specificities which are independent of the fatty acid composition of PC. Thus the phospholipases of snake and bee venoms and of pancreas are all specific for the *sn*-2 position of PC, whereas the lipases from pancreas, Rhizopus, and post-heparin plasma, are all specific for the *sn*-1 position of PC, irrespective of the acyl group composition (van Deenen & de Haas, 1964; Slotboom et al., 1970). In contrast, LCAT appears to belong to a unique class of phospholipases whose positional specificity is dependent on the fatty acid composition of the substrate.

Although it was previously reported that human LCAT can derive up to 40% of the acyl groups for CE synthesis from the *sn*-1 position of dilinoleoyl PC (Assmann et al., 1978), this study found that the asymmetric PCs donated only the *sn*-2 acyl group. Another study (Aron et al., 1978) suggested that the positional specificity is altered in the presence of 16:0−18:1 PC also. However, our recent results with several asymmetric PCs showed that the positional specificity of human LCAT is altered only when the *sn*-2 position is occupied by 20:4 or 22:6 (Subbaiah et al., 1992). One reason for the disparate findings in literature may be the presence of varying amounts of positional isomers in the synthetic PCs tested, because a preferential utilization of *sn*-2-16:0 isomers would result in increased formation of 16:0 CE even without a change in the positional specificity of the enzyme. The present studies not only extended the specificity studies to several naturally occurring *sn*-1-16:0 PCs, but also corrected for any effect of the contaminating positional isomers. Our previous studies showed that the fluidity of the substrate matrix does not affect the positional specificity of human LCAT, although it may influence the overall enzyme activity (Subbaiah et al., 1992). Similarly the free cholesterol/PC ratio does not affect the positional specificity. Therefore, the structure of the PC molecule and its molecular interaction with the active site of the enzyme probably determine the positional specificity. Based on our present results, the following general conclusions can be drawn regarding the effect of PC structure on the positional specificity of human LCAT: (1) The first preference of the enzyme is for the *sn*-2 position of PC, as evident from the studies with dipalmitoyl PC, where both positions are occupied by 16:0 but only the *sn*-2 acyl group was transferred. (2) This preference for the *sn*-2 position is maintained to varying degrees, depending on the structure of the *sn*-2 acyl group. (3) The number of carbon atoms in the *sn*-2 acyl group does not by itself determine the specificity because 18:0 was not transferred whereas 18:1, 18:2, and 18:3 were transferred efficiently. Similarly 20:4 was not transferred efficiently while 20:5 was a good substrate. (4) The number of double bonds in the *sn*-2 acyl group is also not critical because fatty acids with three (20:3), four (20:4), and six (22:6) double bonds are not preferred substrates, whereas a fatty acid with five double bonds (20:5) is a good substrate. (5) The position of the first double bond from the methyl end (n-6 vs n-3) is also not important since two of the n-3 fatty acids (20:5 and 22:6) were handled differently as were two of n-6 fatty acids (18:2 and 20:4). (6) A factor which appears to have the most influence on the positional specificity is the effective chain length of the *sn*-2 acyl group (Figures 6 and 7), which depends not only on the number of carbons but also on the number and type (cis vs trans) of double bonds. It should be pointed out that the chain length of the *sn*-1 acyl group may have an additional role in determining the positional specificity, and therefore these conclusions may be applicable only to the *sn*-1-16:0 PCs.

It appears from the available evidence that the active site of LCAT contains the triad of serine-histidine-aspartate, in common with several lipolytic enzymes (Francone & Fielding, 1991; Jonas, 1991). The hydrophobic "pocket" which accommodates the PC molecule may have a finite capacity, as suggested by other studies (Pownall et al., 1987). We speculate that the PC species which fit into this pocket properly orient only their *sn*-2 ester linkages for the nucleophilic attack by serine-histidine in the active site (Jauhiainen et al., 1987), whereas those PCs which are too large to fit completely in the pocket are bound in an altered configuration, enabling the enzyme to attack the *sn*-1-ester linkage as well.

The results of the present study also show that the over all enzyme activity is higher when the *sn*-2 acyl group is utilized efficiently. Thus in general for human LCAT, the positional isomers containing 16:0 at *sn*-2 are better substrates than the corresponding isomers containing 16:0 at *sn*-1. Previous studies (Pownall et al., 1987; Parks et al., 1992) showed an inverse correlation between the molecular surface area of the PC and its efficiency as substrate for LCAT. Since the two positional isomers of a PC with given fatty acid composition should have the same molecular surface area (Demel et al., 1972), our results indicate that the *sn*-2 acyl group has an independent effect on the enzyme activity, which is not related to the surface area of the whole PC molecule. It is possible that the *sn*-2 acyl group has direct interaction with the hydrophobic groups in the active-site pocket.

Since the enzyme activities were measured in most cases by the amount of labeled CE formed from labeled free cholesterol, the possibility should be considered that the

observed positional specificity reflects the selectivity of the enzyme in the transfer of the acyl group to cholesterol, rather than in the initial cleavage of the acyl ester bond of PC. However, the results presented in Figure 5 show that the phospholipase A activities of the two LCATs exhibited same specificity as their cholesterol−esterifying activities. Recently we found that the transfer of acyl group from unlabeled 16:0−20:4 PC to labeled lyso PC (the lysolecithin acyltransferase reaction) showed similar results (M. Liu and P. V. Subbaiah, unpublished results). These results show that at least in the case of 16:0−20:4 PC, the role of acyl acceptor in influencing the positional specificity is minimal.

An interesting finding in our study is that while the positional specificity of human LCAT is altered in the presence of 16:0−20:4 PC and 16:0−22:6 PC, it is not altered in the presence of 16:0−20:5 PC. This supports the hypothesis that the chain length of the *sn*-2 acyl group has significant effect on the positional specificity of the enzyme, because 20:5(n-3) (22.47 Å) is shorter than both 20:4(n-6) (23.82 Å) and 22:6(n-3) (24.85 Å) (Applegate & Glomset, 1991). This observation is also consistent with the *in vivo* findings. Thus, feeding fish oil to humans (Holub et al., 1987; Rapp et al., 1991; Subbaiah et al., 1993) or to nonhuman primates (Parks et al., 1989; Soltys et al., 1989) results in an increased formation of 20:5 CE, which is commensurate with an increase in 20:5-containing PC species, but the increase in 22:6 CE is much lower than expected from the PC composition. At the same time, there is a paradoxical increase in the formation of 16:0 CE although the percentage of 16:0 in PC is not increased. These results can be explained by the present findings that the reaction of LCAT with 16:0−22:6 PC leads primarily to the formation of 16:0 CE, whereas its reaction with 16:0−20:5 PC leads to the formation of 20:5 CE. Our recent studies show that the primate (baboon) LCAT behaves similar to human LCAT with respect to its positional specificity in the presence of 16:0−20:4, 16:0−20:5, and 16:0−22:6 PCs (Liu & Subbaiah, 1993a). A practical consequence of this specificity by LCAT is that the ingestion of fish oils containing high 22:6/20:5 ratios may actually increase the saturated CE content of plasma.

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