Amino Acid Residue 149 of Lecithin:Cholesterol Acyltransferase Determines Phospholipase A$_2$ and Transacylase Fatty Acyl Specificity*  

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Human LCAT prefers phosphatidylcholine (PC) with sn-1-palmitoyl-2-oleoyl PC (POPC) as substrate for cholesteryl ester synthesis, whereas rat LCAT (which is 92% similar in amino acid sequence) prefers sn-1-palmitoyl-2-arachidonoyl PC (PAPC). Six recombinant human LCAT cDNA clones were constructed with unique clusters of rat sequence substitutions in the human background spanning the region encoding amino acids 121–296. Media from transfected COS cells expressing each of the constructs was assayed for LCAT, cholesterol esterification (CE) or phospholipase A$_2$ (PLA$_2$) activity using substrate particles containing POPC or PAPC. The PAPC/POPC CE activity ratio of the cluster 1 construct (amino acids 149–158) was 1.3, resembling rat LCAT, whereas cluster 2–5 clones produced CE activity ratios <0.3, unchanged from human LCAT. The cluster 6 clone (Y292H/W294F) had an intermediate ratio (0.6). Similar results were observed for LCAT PLA$_2$ activity. In additional studies, position 149 of human LCAT was changed to the rat sequence (hE149A) and compared to a triple mutation containing the remainder of the cluster 1 changes (G151R/E154D/R158Q). CE and PLA$_2$ activity ratio for the hE149A construct was >1.7, similar to rat LCAT, whereas the triple mutation construct retained a ratio similar to human LCAT (<0.6). Thus, a single amino acid substitution (E149A) was sufficient to alter the fatty acyl specificity of human LCAT to that of rat LCAT, with an increase in activity toward PAPC. This is the first example of a point mutation in an enzyme with PLA$_2$ activity that results in an increase in activity toward arachidonic acid.

Lecithin:cholesterol acyltransferase (LCAT); EC 2.3.1.43 is a 67-kDa glycoprotein that is responsible for cholesterol esterification in plasma (1). The enzyme displays two activities: a phospholipase A$_2$ (PLA$_2$) activity, which hydrolyzes the fatty acyl group from the sn-2 position of phosphatidylcholine (PC); and a transacylase activity, which catalyzes the transfer of the fatty acyl group from the acyl-enzyme complex to the 3-hydroxyl group of cholesterol to form cholesteryl ester (CE) (2). The reaction is activated by apolipoprotein A-I (apoA-I), the major apolipoprotein of high density lipoproteins (HDL) (3).

LCAT has an important physiological role in the maturation of nascent, discoreal HDL to mature, spherical HDL, with the generation of a CE-enriched core (4, 5). The enzyme has also been implicated in the reverse cholesterol transport pathway, which results in the net transport of cholesterol from peripheral tissues back to the liver for excretion (1, 6). The central role of LCAT in these physiological processes is supported by the finding of plasma and tissue accumulation of free cholesterol and the presence of nascent HDL particles in plasma in LCAT-deficient states (7, 8).

The enzymatic activity of LCAT is affected by the fatty acyl composition of HDL PC. When plasma HDL PC become enriched in long chain polyunsaturated fatty acids (i.e. carbon chain length >18 and number of double bonds >2) by dietary fat modification, the reactivity of LCAT is reduced (9, 10). This effect can be reproduced using recombinant HDL (rHDL) as substrate particles for the LCAT reaction. rHDL are discoidal particles consisting of PC, cholesterol and apoA-I that are made by cholate dialysis (11, 12). rHDL with PC species containing long chain n-3 or n-6 polyunsaturated fatty acids in the sn-2 position are less reactive than those containing 18:1 or 18:2 (13). The observed decrease in activity may be related to the molecular size of the PC substrate molecule. Using a monolayer apparatus, Parks et al. (13) observed an inverse relationship between the PLA$_2$ activity of human plasma LCAT and the mean molecular area of the PC substrate molecules. Pownall et al. (14) also demonstrated that a bulky substrate such as diphytanoyl PC reacted poorly with human LCAT. These studies suggest that PC substrate molecular size, which can be influenced by fatty acyl composition, is an important determinant of enzyme activity.

There is also a phylogenetic difference in LCAT reactivity. Portman and Sugano (15) were the first to demonstrate that the fatty acyl preference of rat plasma LCAT was different from that of human plasma LCAT. Subsequent studies with purified enzyme showed that rat LCAT was more reactive with PC substrates containing arachidonic acid compared to human LCAT (16–19). This difference occurs in spite of a 87% identity between the rat and human LCAT protein sequence (20, 21). The molecular explanation for the difference in reactivity is
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unknown. However, a recent study by Subbaiah et al. (22) using chimeric constructs of human and mouse LCAT cDNA suggested that the middle third of the LCAT protein (amino acids 130–306) was responsible for conferring fatty acyl specificity. These authors have also reported that the positional specificity of human versus rodent LCAT is different; according to their data, human LCAT switches from using a sn-2 fatty acid to a sn-1 fatty acid when the PC substrate contains arachidonate in the sn-2 position (22–24). However, under the same experimental conditions, neither rat nor mouse LCAT display a change in positional specificity. Thus, little is known regarding the region of LCAT that is responsible for controlling fatty acyl substrate preference or positional specificity.

The purpose of the present study was to define the minimal region of LCAT necessary to confer fatty acyl specificity. We hypothesized that a discrete region of primary sequence is responsible for the fatty acyl specificity of LCAT. We used a comparative species approach that focused on the amino acid differences between human and rat LCAT over the middle third of the enzyme. Our results demonstrate that amino acid 149 is critical in determining the fatty acyl specificity of cholesteryl esterification and PL_2 activity by LCAT.

EXPERIMENTAL PROCEDURES

Construction of Human-Rat-Chimeric LCAT—Rat LCAT cDNA was produced by reverse transcription of total hepatic RNA using a kit from Promega Co. (Madison, WI) followed by PCR amplification of the LCAT cDNA. The PCR reactions (100 μl) consisted of 2 μl of reverse transcription products as template, 1 μM each of 5′ sense and 3′ anti-sense primers, 0.2 mM dNTPs, 10% Me_2SO, 2 mM MgSO_4, 1 unit of Vent polymerase (New England Biolabs, Beverly, MA), and reaction buffer. The primer oligonucleotides were designed from sequences published for rat LCAT (21) with the addition of an EcoRI site at the end of the 5′ sense primer (5′-TAGAA TTCTG GGCTG TAATG GGGAT CCGCT GGTTG TCACC TGAGG-3′) and a HindIII site at the end of the 3′ antisense primer (5′-CGTCA AGCTT AACAG TAAGT CTTTA TTC-3′). Amplification protocol involved heating to 94°C for 5 min, followed by 35 cycles of 1.6 mM NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4) containing: rHDL (0.2 μg/ml of purified human plasma apoA-I, [1^H]cholesterol (50,000 dpm/μg), and PC in a starting molar ratio of 1:80. Radiolabeled PC (1-palmitoyl-2-[3H]oleoyl PC and 1-palmitoyl-2-arachidonoyl PC (PAPC). PAPC was purchased from Sigma, and PAPC was synthesized and characterized as described previously (13). For measurement of LCAT activity, HDL were made with purified human plasma apoA-I and radiolabeled PC in a starting molar ratio of 1:80. Radiolabeled HDL were used as substrate particles for measurement of CE formation and PLA2 activity by LCAT.

Assays for CE Formation and PLA2 Activity by LCAT—LCAT incubations were performed in triplicate in 0.5 ml of buffer (10 mM Tris, 140 mM NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4) containing: HDL (0.2 μg/ml of cholesterol for CE formation or 25–50 μg of PC for PLA2 activity), 0.6% bovine serum albumin (fatty acid-free; Sigma), 2 mM β-mercaptoethanol, and 20–150 μl of media or 5 μl of plasma as a source of LCAT enzyme. Control incubations contained all constituents except the LCAT source. Tubes were gassed with N_2 and incubated at 37°C for 1 h for all PLA2 reactions and for the CE formation from media samples. In the case of plasma samples used for measurement of CE formation, an incubation time of 10 min was used. Control experiments showed that CE formation was linear up to 2 h using our experimental conditions, provided CE formation was <25%. The reaction was stopped by Bligh-Dyer extraction solution (31) consisting of 1:2 chloroform:methanol with 25 μg/ml added cholesterol and cholesteryl oleate (CE formation reactions) or 40 μg/ml oleic acid (PLA2 reactions). The phases were split by the addition of 0.29% NaCl (CE formation) or 0.5% H_2SO_4 (PLA2 activity). The radiolabeled products were separated by thin-layer chromatography and quantified by liquid scintillation counting (9).

HPLC was used to separate radiolabeled CE formed by LCAT using a C18 reverse phase column as described previously (32). Fractions were analyzed by liquid scintillation counting to monitor the elution profile of the radiolabeled CE.

ELISA for Quantification of LCAT Mass—Media samples were assayed in triplicate for LCAT mass using an ELISA procedure. A series of media volumes (2.5–10 μl) was adjusted to 5 μl by β-mercaptoethanol in Tris-buffered saline (200 μl final volume) and incubated at 37°C for
2 h in microtiter plates. Purified human plasma LCAT (33) was used as standard for the assay. The wells were washed with water, blocked for 2 h at room temperature with 0.05% Tween 20 and 0.25% bovine serum albumin in Tris-buffered saline, and washed again. A 1:1000 dilution of anti-human LCAT chicken IgY in blocking buffer was applied to each well and was incubated at room temperature for 2 h. The IgY was purified from the eggs of chickens immunized with purified human plasma LCAT (34). The plate was washed, incubated for 2 h at room temperature with rabbit anti-chicken IgY conjugated with alkaline phosphatase (1:1000 dilution; Sigma), developed with p-nitrophenyl phosphate substrate (Sigma) and read at 405 nm after color development.

Data Analysis—DNA and protein sequence comparisons were made using the Genetics Computer Group (Madison, WI) and DNA Strider™ 1.2 (Christian Mark, Gif-Sur-Yvette, France) software. All data are presented as mean ± standard error.

RESULTS

Our mutagenesis strategy was based on a comparative species approach that focused on differences in amino acid sequence between rat and human LCAT in the middle third of the protein. A comparison of the human and rat sequence is shown in Fig. 1. Overall, the two sequences share an 87% identity with 9 differences located between amino acids 1 and 120, 22 differences between amino acids 121 and 300, and 22 differences between 301 and 416. The differences in sequence from amino acids 121–296 could be roughly clustered into six regions with additional point differences as indicated in Fig. 1. Our experimental approach involved the mutation of all amino acids within these regions or clusters from the human sequence to that of the rat sequence (see "Results").

To determine whether the middle third of rat LCAT was responsible for conferring fatty acyl specificity as previously observed for mouse LCAT (22), we generated a HRH chimeric LCAT construct, in which the human sequence was exchanged for mouse LCAT (Fig. 1). The HRH construct was transiently expressed in COS cells and the medium was harvested 48 h later and assayed for LCAT esterification activity using rHDL containing POPC or PAPC, [3H]cholesterol, and apoA-I (73:3.7:1 and 71:4.1:1 mol ratio, respectively). LCAT mass was determined by ELISA as described under "Experimental Procedures." Values represent mean ± S.E. of three separate transfections. Human-rat-human (HRH) chimeric cDNA encoded a LCAT protein in which amino acids 121–296 of the human sequence were replaced by the rat sequence.

### Table I

| LCAT source | LCAT concentration (µg/ml) | CE formation (nmol CE/h/µg LCAT) | Activity ratio (PAPC/POPC) |
|-------------|---------------------------|---------------------------------|-----------------------------|
| Human LCAT  | 0.7 ± 0.1                 | 3.12 ± 0.19                     | 1.01 ± 0.02                 |
| cDNA        |                           |                                 |                             |
| Rat LCAT    | 0.9 ± 0.2                 | 1.93 ± 0.41                     | 1.92 ± 0.55                 |
| cDNA        |                           |                                 |                             |
| HRH chimeric LCAT cDNA | 2.3 ± 0.4 | 0.71 ± 0.10 | 0.86 ± 0.13 | 1.21 ± 0.06 |

The HRH chimeric LCAT displayed a reactivity profile more similar to that of rat recombinant LCAT than human recombinant LCAT, resulting in an activity ratio of 1.21. Thus, amino acids 121–296 of the rat LCAT sequence were sufficient to confer the fatty acyl preference of rat LCAT for arachidonate, similar to the previous findings for mouse LCAT (22).

To better define the region of LCAT responsible for conferring fatty acyl specificity, we generated and transiently expressed a series of cDNA constructs that contained 2–4 amino acid substitutions of the rat sequence for the human sequence at six different sites or clusters over the middle region of the LCAT protein (see Fig. 1). The results of the cholesterol esterification assay are shown in Table II. Several controls are shown including a recombinant LCAT in which the active site serine has been mutated to alanine (S181A), human and rat wild-type LCAT, and human and rat plasma as sources of the enzyme. The activity of all of the recombinant LCAT proteins with rHDL containing POPC was similar; however, on average the values for CL6, HRH, and rat LCAT were lower than the others. The values for human and rat plasma were also similar to each other and, as expected, the S181A mutation eliminated enzymatic activity. The results with rHDL containing PAPC were somewhat different. CL1 and rat LCAT had activities that

### Table II

| LCAT source | LCAT concentration (µg/ml) | CE formation (nmol CE/h/µg LCAT) | Activity ratio (PAPC/POPC) |
|-------------|---------------------------|---------------------------------|-----------------------------|
| Human LCAT  | 0.7 ± 0.1                 | 3.12 ± 0.19                     | 1.01 ± 0.02                 |
| cDNA        |                           |                                 |                             |
| Rat LCAT    | 0.9 ± 0.2                 | 1.93 ± 0.41                     | 1.92 ± 0.55                 |
| cDNA        |                           |                                 |                             |
| HRH chimeric LCAT cDNA | 2.3 ± 0.4 | 0.71 ± 0.10 | 0.86 ± 0.13 | 1.21 ± 0.06 |

The HRH chimeric LCAT displayed a reactivity profile more similar to that of rat recombinant LCAT than human recombinant LCAT, resulting in an activity ratio of 1.21. Thus, amino acids 121–296 of the rat LCAT sequence were sufficient to confer the fatty acyl preference of rat LCAT for arachidonate, similar to the previous findings for mouse LCAT (22).
were severalfold higher than those of the other recombinant LCAT proteins. In addition, the activity with rat plasma was 4-fold higher than that of human plasma.

The PAPC/POPC activity ratio is also shown in Table II. The CL1 activity ratio (1.29) is similar to that of HRH (1.20) and rat LCAT (0.97), whereas CL2–CL5 and the point mutation H168Y have activity ratios similar to that of human LCAT (0.32) and human plasma (0.51). Note that CL6 had an activity ratio (0.61) that was 2-fold greater than CL2–CL5, but less than half that of CL1. The activity ratio for human and rat plasma showed the expected 3-fold difference. These data demonstrate that CL1 region was critical in determining fatty acyl specificity for cholesterol esterification of LCAT.

The results of the PLA₂ assay are shown in Table III. The PLA₂ values for all of the recombinant LCAT proteins ranged from 1.6 to 3 nmol/h/µg when assayed with POPC rHDL except for HRH, which was noticeably lower. When POPC rHDL was used as substrate, the PLA₂ activity for CL1, CL6, and rat LCAT, on average, was higher than that of the other recombinant constructs. The PLA₂ activity in rat plasma was 9-fold greater than that of human plasma.

The PAPC/POPC activity ratio for PLA₂ hydrolysis is also shown in Table III. The ratio for CL1 (1.45) was nearly 5-fold greater than that of CL2–CL5 (0.28–0.35). CL6 had an activity ratio that was 2-fold greater than CL2–CL5 and H168Y. The values for HRH and rat LCAT were similar to that of CL1. The activity ratios for recombinant human LCAT and human plasma were low (0.27–0.33) and similar to CL2–CL5. Thus, the CL1 region of LCAT is involved in determining the fatty acyl specificity of the PLA₂ step of the LCAT reaction.

Having identified CL1 as a critical region in determining fatty acyl specificity, we wished to determine the minimal sequence change necessary in CL1 to confer fatty acyl specificity. We performed a multiple sequence alignment of the CL1 region of LCAT using two primate sequences (human, baboon) and two rodent sequences (rat, mouse). The results are shown in Fig. 2. Our strategy was to identify amino acid residues that were conserved in the rat and mouse but were different in the baboon and human, which might give rise to the fatty acyl specificity difference between rodents and primates. Amino acid 158 was not conserved among the four species, whereas amino acid 154 was glutamic acid in the primates and aspartic acid in the rodents. Position 151 was a glycine in the primates but was not conserved in the rodents. However, position 149 was glutamic acid in the primates and alanine in the rodents.

Thus, the four amino acid variations in the CL1 region of LCAT, position 149 showed the greatest divergence in the primate versus rodent comparison, yet it did not change within the primate and rodent species analyzed. Based on this analysis, we generated a E149A mutation, in which position 149 of the human LCAT sequence was mutated to alanine. We also made a triple mutation, designated CL1_3, in which the amino acids at positions 151, 154, and 158 of the human sequence were changed to the rat sequence (i.e. G151R, E154D, and R158Q). The mutations were generated by megaprimer PCR as described for the cluster mutants and transiently expressed in COS cells.

Table IV shows the results of the cholesteryl ester formation assay of the CL1 mutants. With rHDL containing POPC, the activity of rat and eH149A LCAT averaged less than that of human and CL1_3 LCAT. However, with rHDL containing PAPC, the activities of rat and eH149A LCAT were greater than human and CL1_3 LCAT.

Fig. 3 shows the PAPC/POPC activity ratio for cholesteryl ester formation derived from the data in Table IV. The ratio for human LCAT (0.51 ± 0.02) agrees closely with that of CL1_3 (0.58 ± 0.03), whereas the ratio for rat LCAT (1.55 ± 0.03) agrees closely with the value for eH149A (1.78 ± 0.02). Thus, a single mutation at position 149 of the human enzyme was sufficient to change the fatty acyl specificity of LCAT cholesterol esterification from the human to the rat pattern.

PLA₂ activity was also assayed from the same mutant constructs (Table V). As observed for the cholesterol esterification assay in Table IV, the PLA₂ activities for rat and eH149A
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See Table I legend for details. hE149A represents a point mutation in which amino acid 149 of the human sequence was changed from Glu to Ala. CL1–3 represents a triple mutation in which the following changes to the human LCAT amino acid sequence were made: G151R, E154D, and R158Q. Values are mean ± S.E. for three separate transfections.

### Table IV
Cholesteryl ester formation activity in media of COS cells expressing wild-type or CL1 mutants

| Source LCAT concentration | POPC | PAPC |
|---------------------------|------|------|
| µg/ml                     |      |      |
| Human LCAT                | 0.80 ± 0.16 | 4.46 ± 0.53 | 2.27 ± 0.38 |
| Rat LCAT                  | 1.00 ± 0.20 | 2.28 ± 0.59 | 3.54 ± 0.95 |
| hE149A                    | 0.88 ± 0.18 | 3.90 ± 1.21 | 6.90 ± 2.10 |
| CL1–3                     | 0.93 ± 0.10 | 4.68 ± 0.61 | 2.71 ± 0.34 |

### Table V
PLA2 activity in media of COS cells expressing wild-type or CL1 mutants

| Source LCAT concentration | POPC | PAPC |
|---------------------------|------|------|
| µg/ml                     |      |      |
| Human LCAT                | 0.80 ± 0.16 | 1.06 ± 0.23 | 0.51 ± 0.17 |
| Rat LCAT                  | 1.00 ± 0.20 | 0.52 ± 0.21 | 1.53 ± 0.45 |
| hE149A                    | 0.88 ± 0.18 | 0.95 ± 0.31 | 3.00 ± 1.07 |
| CL1–3                     | 0.93 ± 0.10 | 1.43 ± 0.06 | 0.65 ± 0.18 |

The PLA2 activity ratio was calculated from results in Table V. Values are the mean ± S.E. for three separate transfections. Mean values are shown to the right of the bars. The sequence of each mutant construct from amino acids 148–159 is also shown. See Table III legend and “Experimental Procedures” for details of the experiment.

### DISCUSSION
The species-specific fatty acyl preference of LCAT was first reported by Portman and Sugano (15) in 1964. Later reports confirmed that human LCAT has a higher reactivity with PC species containing oleic or linoleic acid in the sn-2 position compared to arachidonic acid, whereas rat LCAT displayed the opposite preference profile (16–19). However, the molecular explanation for this well documented fatty acyl specificity difference between rat and human LCAT was not established. We hypothesized that a discrete region of primary sequence was responsible for the fatty acyl specificity of the enzyme, and the present study was undertaken to define the minimal region of LCAT necessary to confer fatty acyl preference. Using the previous observation of Subbaiah et al. (22), indicating that amino acid residues 130–306 were involved in determining the fatty acyl preference of LCAT and a comparative species approach that focused on the amino acid differences between human and rat LCAT over this region, we identified a single amino acid residue in the human enzyme, the glutamic acid at position 149, that is responsible for conferring fatty acyl specificity. When E149 of human LCAT was mutated to an alanine, which is present in the rat sequence at position 149, the fatty...
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Fig. 5. HPLC separation of cholesterol ester products synthesized by LCAT from rHDL containing POPC or PAPC, [3H]cholesterol, and apoA-I. The LCAT reaction was performed and CE products were separated using a Microsorb C18 reverse phase column equilibrated with (1:1) isopropanol:acetonitrile (1 ml/min; 45 °C). One-ml fractions were collected except from 20 to 25 min, when 0.5-ml fractions were collected for increased resolution of peaks. Each fraction was analyzed by liquid scintillation counting to monitor the elution profile of the radiolabeled CE. Authentic standards were monitored at 210 nm to identify the elution position of each CE species (FC, free cholesterol; C20:4, cholesterol arachidonate; C18:1, cholesteryl oleate; C16:0, cholesteryl palmitate). The source of LCAT enzyme is shown at the top of each panel. hE149A represents a point mutation in which amino acid 149 of the human sequence was changed from Glu to Ala. CLI_3 represents a triple mutation in which the following changes to the human LCAT amino acid sequence were made: G151R, E154D, and R158Q.

The acyl specificity of the human enzyme was converted to that of rat LCAT, with no loss in reactivity toward rHDL containing POPC and an increased reactivity toward rHDL containing PAPC. The change in fatty acyl specificity for hE149A recombinant LCAT was observed for both PLAs and cholesterol esterification activities and reflected an increased reactivity with PAPC substrate particles compared to those containing POPC. To our knowledge this is the first example of a point mutation of an enzyme that results in such a striking change in fatty acyl specificity.

The molecular explanation for the increased reactivity of hE149A with PAPC rHDL is unknown, but may be related to the size of the active site pocket of the enzyme. Crystallographic studies have shown that pancreatic lipase (35) and secretory PLA2 molecules (36, 37) contain a hydrophobic pocket in which the PC molecule binds prior to hydrolysis of the sn-2 fatty acid. While LCAT shares a lipase family active site motif (GXGXXG) at the active site serine 181 and amino acid residues 151–174 form a putative interfacial binding region (6), few structural features of LCAT are known and a hydrophobic pocket for substrate binding has not been identified. A previous study has described an inverse relationship between the molecular surface area of the PC substrate molecules and the PLA2 hydrolysis rate of PC monolayers by human plasma LCAT (13). These results suggested that the PC molecules with a greater molecular surface area, such as those containing long chain polyunsaturated fatty acids (38, 39), might not fit well into the active site pocket of the enzyme. A similar conclusion was reached by Pownall et al. (14), who showed that 1,2-dipalmitoyl, which has a larger molecular surface area than POPC, is a competitive inhibitor of human plasma LCAT, presumably because it has difficulty fitting into the active site of the enzyme. We hypothesize that the hE149A mutation results in a larger active site pocket that can more easily accommodate the larger PC molecules, such as PAPC, leading to increased activity with these substrates. Since crystallographic data of secretory PLA2 molecules demonstrate hydrophobic amino acid residues in the interior of the binding pocket (40), it seems unlikely that the hE149 residue is part of the active site pocket. Rather, it seems more likely that the hE149A mutation results in a conformational change in the enzyme, which increases the size of the binding pocket. A systematic study of the hE149A and human wild-type LCAT enzymes using PC substrates with varying molecular surface areas is currently under way to test whether the size of the active site pocket plays an important role in determining enzyme reactivity.

The importance of size versus charge of the amino acid residue at position 149 of LCAT in conferring fatty acyl specificity is presently unknown. The hE149A mutation, which leads to increased reactivity with PAPC substrate, substitutes a smaller, hydrophobic, uncharged amino acid for glutamic acid in a region of the protein that is predicted to be hydrophilic based on Kyte-Doolittle analysis (41). Of the known LCAT sequences in the GenBank™ data base, the ones with glutamic acid at position 149 (human, baboon, rabbit, and pig) do not show a preference for arachidonic acid (42) compared to those that contain alanine at position 149 (rat and mouse). However, chicken LCAT, which has a fatty acyl specificity similar to human LCAT (42), contains a glycine at amino acid 149. Since glycine is more similar to alanine in size and hydrophobicity than glutamic acid (26), there is no obvious relationship between size and hydrophobicity of the amino acid at position 149 of LCAT and fatty acyl specificity. The effect of substituting different amino acid residues at position 149 on the fatty acyl specificity of LCAT is under active investigation.

An alternative explanation for the increased reactivity of hE149A toward PAPC rHDL could be an increased interfacial binding affinity or capacity compared to the wild-type human LCAT. LCAT must bind to the interface of its lipoprotein substrate particle and be activated by apoA-I before it hydrolyzes the sn-2 fatty acid of PC. Using a solid-phase binding assay or an activity inhibition assay, Bolin and Jonas (43) have shown that the apparent Km for cholesterol esterification of LCAT reflects interfacial binding affinity for the HDL particle surface, whereas the apparent Vmax for esterification reflects a preference of the molecular substrates (PC and cholesterol) for the active site of the enzyme. Furthermore, they showed that changing the phospholipid head group composition of the substrate HDL influenced the binding affinity of LCAT and the apparent Km and Vmax for cholesterol esterification. The substitution of alanine for glutamic acid at amino acid 149 could result in a conformational change in LCAT that could increase...
its binding to PC. A potential interfacial binding region has been identified (amino acids 151–174; Ref. 6) that is close to residue 149 in the primary sequence. Direct binding studies of human wild-type and hE149A LCAT to POPC and PAPC will be necessary to test this possibility.

Our studies revealed that both PLA₂ and cholesterol esterification activities were increased with hE149A LCAT when incubated with PAPC compared to POPC rHDL. However, the PAPC/POPC activity ratio for hE149A and rat LCAT was 2-fold greater for the PLA₂ assay (Fig. 4) compared to the cholesterol esterification activity (Fig. 3). These results suggest that the fatty acyl specificity of LCAT is determined at the PLA₂ step of the reaction and not at the transacylase step. After hydrolysis of the sn-2 fatty acyl group of PC by LCAT, an acyl-enzyme intermediate is formed with the active site serine 181 (2). The preferred acceptor of the fatty acyl group in the subsequent transacylation step is cholesterol, but water, lysoPC, diacylglycerol, and monoacylglycerol can also act as acceptors of the fatty acyl group (44). Thus, the transacylase step appears to be rather nonspecific with regard to acceptor molecules compared to the high specificity of the PC PLA₂ step, which discriminates not only the position of the fatty acyl group in PC (sn-2 versus sn-1) but also the type of fatty acyl group in the sn-2 position.

There are reports that human LCAT switches from using a sn-2 fatty acid to a sn-1 fatty acid when the PC substrate contains arachidonic acid or another long chain polyunsaturated fatty acid in the sn-2 position, whereas the rat enzyme does not (22–24). We investigated this possibility with our mutants by separating the LCAT synthesized CE species by snake venom PLA₂ digestion studies (9). Thus, the release of radiolabeled fatty acid in the LCAT PLA₂ assays (Tables III and V) reflected hydrolysis at the sn-2 position and represented sn-2 positional specificity for LCAT. In addition, we have found no evidence for accumulation of radiolabeled lysoPC during our LCAT PLA₂ assays, which would occur if the enzyme were to have a positional switch from sn-2 to sn-1.² Therefore, taken together, our data do not support a positional switch of human LCAT or any of the mutant recombinant LCAT proteins generated in this study.

In summary, we have shown that a single amino acid substitution (E149A) is sufficient to alter the fatty acyl specificity of human LCAT to that of rat LCAT, with an increase in activity toward PC substrate containing arachidonic acid. Ar-
