Role of glutamic acid residues 154, 155, and 165 of lecithin:cholesterol acyltransferase in cholesterol esterification and phospholipase A_2 activities

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Abstract Previous studies have shown that cholesterol esterification activity by lecithin:cholesterol acyltransferase (LCAT) is progressively inhibited as up to three acidic acid residues are chemically modified. The purpose of this study was to determine whether three glutamic acid residues in LCAT (154, 155, and 165), that align exactly with three acidic acid residues (270, 271, and 281) in the amphipathic phospholipid binding region of apoE, were necessary for enzymatic activity. Site-directed mutagenesis was used to generate mutant constructs of LCAT in which glutamic acid residues 154, 155, and 165 were replaced with glutamine or lysine. Media harvested from transiently transfected COS cells was used as a source of LCAT for cholesterol esterification and phospholipase A_2 (PLA_2) assays. Cholesterol esterification for all mutant constructs (11–26 nmol CE/h/μg) was similar to or greater than that of wild type LCAT (16 nmol CE/h/μg), except for a triple mutant, in which glutamic acid residues 154, 155, and 165 were changed to lysines (5 nmol CE/h/μg). PLA_2 activity followed a similar trend. There was a significant decrease in the cholesterol esterification to PLA_2 activity ratio when residue 165 was mutated from its wild type negative charge (E) to an uncharged (Q) or positive (K) charged residue (10.2 vs. 6.0 vs. 4.3, respectively). We conclude that glutamic acid residues 154, 155, and 165 individually or collectively are not necessary for LCAT activity and that residue 165 may be in a region of LCAT that is involved with cholesterol binding or is sensitive to cholesterol binding at the active site of the enzyme.—**Wang, J., J. A. DeLozier, A. K. Gebre, P. J. Dolphin, and J. S. Parks.** Role of glutamic acid residues 154, 155, and 165 of lecithin:cholesterol acyltransferase in cholesterol esterification and phospholipase A_2 activities. *J. Lipid Res.* 1998. **39:** 51–58.

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Lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) is a 67 kD plasma enzyme that is responsible for the synthesis of nearly all cholesteryl esters (CE) in human plasma (1). It plays a major role in the maturation of nascent high density lipoprotein (HDL) particles, secreted from the liver or intestine or generated from redundant surface during lipolysis of triglyceride-rich lipoproteins, with the generation of a CE-rich core (2–4). In familial LCAT deficiency, LCAT activity in plasma is low and free cholesteryl-enriched, discoidal HDL particles accumulate (5). LCAT has also been postulated to have a pivotal role in reverse cholesterol transport, a process in which there is net movement of excess cholesterol from peripheral tissues back to the liver (1).

The LCAT reaction consists of two activities, a phospholipase A_2 and a transacylase activity (6, 7). First, LCAT cleaves the *sn*-2 fatty acyl group from phosphatidylcholine (PC) forming an acyl-enzyme intermediate and lysoPC, and then the fatty acyl group is transferred to the hydroxyl group of cholesterol forming CE. The reaction is activated by apolipoprotein A-I (apoA-I), the major apolipoprotein of HDL (8). Because the substrates for LCAT are water-insoluble, the reaction takes place at the interface of macromolecular substrate particles such as HDL. Thus, the initial step in the LCAT reaction is the binding of the enzyme to the surface of the HDL particle. However, the mechanisms of interfacial binding of LCAT and apoA-I activation are poorly

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; CE, cholesteryl esters; PLA_2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl PC (*sn*-1 16:0, *sn*-2 18:1 PC); PAPC, 1-palmitoyl-2-arachidonyl PC (*sn*-1 16:0, *sn*-2 20:4 PC); HDL, high density lipoproteins; rHDL, recombinant HDL; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; EDAC, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide; EDTA, ethylenediaminetetraacetic acid.

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understood. LCAT has a primary sequence between amino acid residues 151 and 174, which is predicted to form an amphipathic α-helix. This region of LCAT also has a high degree of sequence similarity to a region of apoE (amino acids 268–288), which has been shown to be involved in lipid binding (9, 10). Fielding and Fielding (11) suggested that the amphipathic helix between residues 151–175 of LCAT is involved in interfacial binding of the enzyme and may serve as a sterol binding domain, concentrating the cholesterol around the periphery of the discoidal HDL particle. Furthermore, the charge interaction of this helix of LCAT with an antiparallel helix of apoA-I (residues 144–163) has been proposed to stabilize the binding of LCAT to the HDL substrate particle (11).

As the three-dimensional structure of LCAT is unknown, the information about functional residues in LCAT is limited to the active serine 181 (12, 13), four N-linked glycosylation sites (13–15), and two cysteine residues close to the active site (16). Recently, a region of the enzyme was identified that determines fatty acyl specificity (17). It is well known that human LCAT prefers oleic or linoleic acid in the *sn*-2 position of PC for CE synthesis, whereas rat LCAT prefers arachidonic acid. A single amino acid substitution (E149A) was sufficient to alter the fatty acyl specificity of human LCAT to that of rat LCAT. The glutamic acid residue at 149 is immediately upstream of the amphipathic helical region hypothesized to be involved in interfacial binding of the enzyme to HDL particles.

Previous studies have demonstrated that the transacylase activity of LCAT was progressively and eventually totally inhibited after the chemical modification of up to three carboxyl residues by EDAC followed by glycine methyl ester treatment (18). In conjunction with earlier studies (7), these data were interpreted to support the hypothesis that a catalytic triad involving Ser-181, a histidine residue, and the carboxyl group of an acidic amino acid (Asp or Glu) was functional within the catalytic site of LCAT. Recently, Fielding and Fielding (11) identified a region in LCAT (residues 151–175) that exhibited a significant sequence homology with residues 268–288 of apoE and suggested that this amphipathic helical region of LCAT may be involved in the interfacial binding of the enzyme and may also serve as a sterol binding domain. The 151–175 region of LCAT contains three glutamic acid residues at positions 154, 155, and 165. Similar acidic residues are conserved in a homologous region of apoE (Glu-270, Asp-271, and Glu-281). Based on these data, we hypothesized that previous experiments with EDAC may have modified the carboxyl groups of glutamic acid residues at positions 154, 155, and 165 of LCAT, thereby reducing substrate binding and inhibiting activity. As we had also demonstrated that increasing the cholesterol content of the artificial proteoliposome substrate appeared to protect LCAT activity against carbodiimide treatment, we decided to test the possibility that glutamic acid residues 154, 155, and 165 were necessary for LCAT activity. As this region of LCAT may be involved in both cholesterol and phospholipid binding, we measured both the phospholipase A_2 and the cholesterol esterification activity of LCAT after substitution of these residues with either glutamine or lysine by site-directed mutagenesis.

METHODS

Polymerase chain reaction (PCR) site-directed mutagenesis

Site-directed mutagenesis of hLCAT cDNA was accomplished by a megaprimer PCR procedure similar to that described previously (17). The first round of PCR included a 5′ sense PCR primer (5′-CTTCT TCACC ATCTG GCTGG-3′) about 300 bp upstream of the mutation sites and a mutagenic antisense primer. The sequences of mutagenic primers were as follows: E154Q: 5′-GCTTG CGGTA GTACT CCTGC TGCTG GC-3′; E155Q: 5′-GCTTG CGGTA GTACT GCTCC TGCTG GC-3′; E165Q: 5′-GCGTG CATCT CCTGC ACCAG CCCTG C-3′; E154K: 5′-GCTTG CGGTA GTACT CCTTC TGCTG GC-3′; E155K: 5′-GCTTG CGGTA GTACT TCTCC TGCTG GC-3′; E165K: 5′-GCGTG CATCT CCTTC ACCAG CCCTG C-3′; E1-2Q: 5′-GCTTG CGGTA GTACT GCTGC TGCTG GC-3′; E1-2K: 5′-CTTGC GGTAG TACTT CTTCT GCTGG C-3′. The triple mutants E1-3Q, and E1-3K were obtained using the constructed double mutation as template, and the E165Q and E165K mutagenic primer, respectively. The PCR reactions (100 μl) consisted of 0.04 pmol of pUC19.LCAT plasmid (kindly provided by Dr. John McLean, Genentech Inc., San Francisco, CA) as template, 1 μM each of 5′ PCR sense and 3′ mutagenic primers, 0.2 mM dNTPs, 2 mM $MgSO_4$, 1 U Vent^R DNA polymerase (NE BioLabs, Beverly, MA), and reaction buffer. Amplification protocol involved heating to 94°C for 5 min, followed by 20 cycles of 94°C for 30 sec, 45–50°C (depending on primers) for 30 sec, and 72°C for 30 sec, and a final extension at 70°C for 10 min. For the second round of PCR, the ~300 bp product from the first round of PCR was agarose gel isolated and PCR Prep™ minicolumn (Promega Co., Madison, WI) purified before use as a megaprimer with a 3′ antisense PCR primer (5′-CTCGG TGCTG CGGGT CGCCA-3′) about 600 bp downstream of the mutation sites. The

thermocycling procedure was similar to the first round of PCR except the annealing temperature was raised to 70°C, and the time for the extension step was increased to 1 min. The 900 bp PCR products were purified by a PCR minicolumn, digested with Kpn I and Pst I restriction enzymes, and ligated into the pCMV5.LCAT expression vector. The entire coding sequence of the mutant constructs between the Kpn I and Pst I restriction sites was confirmed by dideoxy sequencing.

Cloning and in vitro expression of mutants

Two μg of pCMV.LCAT cDNA wild type or mutant constructs was transiently transfected, using a DEAE–dextran method (19), into COS-1 cells grown in 35-mm tissue culture dishes. After transfection, the cells were washed three times with phosphate-buffered saline, switched to serum-free Dulbecco's modified Eagle's medium, and incubated an additional 72 h at 37°C. The medium then was collected, centrifuged at 500 *g* for 10 min to remove cell debris, and immediately frozen at −70°C until assays were performed.

Recombinant HDL (rHDL) synthesis and characterization

rHDL were used as substrate particles for measurement of CE formation and PLA_2 activity by LCAT as described previously (17). For measurement of CE formation, rHDL were made with purified human plasma apoA-I, [^{3}H]cholesterol (50,000 dpm/μg), and 1-palmitoyl-2-oleoyl PC (POPC) in a starting molar ratio of 1:5:80. ApoA-I was purified from human plasma (20) and [7-^{3}H(N)]cholesterol was purchased from DuPont NEN (Boston, MA). Two PC species were used for rHDL synthesis for PLA_2 activity measurements: POPC and 1-palmitoyl-2-arachidonoyl PC (PAPC). For measurement of LCAT PLA_2 activity, rHDL were made with purified human plasma apoA-I and radiolabeled PC (12,000 dpm/μg) in a starting molar ratio of 1:80. Radiolabeled PC (1-palmitoyl-2-[^{3}H]oleoyl PC and 1-palmitoyl-2-[^{3}H]arachidonoyl PC) were synthesized from lysoPC and the corresponding radiolabeled fatty acid ([9,10-^{3}H(N)]18:1 or [5,6,8,9,11,12,14,15-^{3}H(N)] 20:4, DuPont NEN) as described previously (21).

Assays for CE formation and PLA_2 activity by LCAT

LCAT incubations were performed in triplicate in 0.5 ml buffer (10 mM Tris, 140 mM NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4) containing: rHDL (1.2 μg cholesterol for CE formation or 25 μg PC for PLA_2 activity), 0.6% bovine serum albumin (fatty acid-free; Sigma Chemical Co.), 2 mM β-mercaptoethanol, and 30 μl (CE formation) or 150 μl (PLA_2) media as a source of LCAT enzyme, as described previously (17). Control incubations included media from cells transfected with the cDNA for beta-galactosidase as well as an inactive construct of LCAT (S181A); the background activity was similar for both controls. The mean value (n = 3) for the beta-galactosidase controls for each transfection was subtracted from the individual activity values (nmol/h/ml) for each cDNA construct to report net activities. Specific activity (nmol/h/μg LCAT) was calculated by dividing the net activity values (nmol/h/ml) by the concentration of LCAT in the media, which was assayed in triplicate using an ELISA procedure as described previously (17).

Data and statistical analysis

DNA and protein analyses were obtained using the Genetics Computer Group (Madison, WI) and DNA Strider™ 1.2 (Christian Marck, Gif-Sur-Yvette, France) software. All data are presented as mean ± standard error of the mean. Analysis of variance and Fisher's least significant difference test were used for statistical analysis of the data.

RESULTS

Figure 1 shows a helical wheel projection of amino acids 152–169 of LCAT and amino acids 268–285 for apoE. Note the similarity in distribution of hydrophobic amino acids on the right face of the helical wheel and the charged amino acids on the left face for both proteins. There is exact alignment of glutamic acid residues 154, 155, and 165 in LCAT with glutamic acid residues 270 and 281 and aspartic acid residue 271 in apoE. There was also a similar distribution of positive charges on the left side of the helical faces for LCAT and apoE with both having a lysine and arginine residue.

We hypothesized that the three glutamic acid residues of LCAT in this region of the protein (amino acids 152–169) are necessary for LCAT activity, perhaps being involved in phospholipid and/or cholesterol binding, and in our previous study, were subjected to chemical modification by EDAC and glycine methyl esterification, resulting in LCAT enzyme inhibition (18). To test this hypothesis a series of LCAT mutants were generated and expressed in vitro, in which glutamic acid residues 154, 155, and 165 were systematically changed to neutral (glutamine) or positive charged (lysine) amino acid residues.

The results of the cholesterol esterification assay are shown in **Table 1**. Among the single mutations, glutamic acid to glutamine or lysine, only the mutation at E165K showed decreased activity (1.8 ± 0.1 nmol/h/ml) compared to wild type LCAT (8.7 ± 1.4 nmol/h/ml). Both E165Q and E165K had decreased secretion

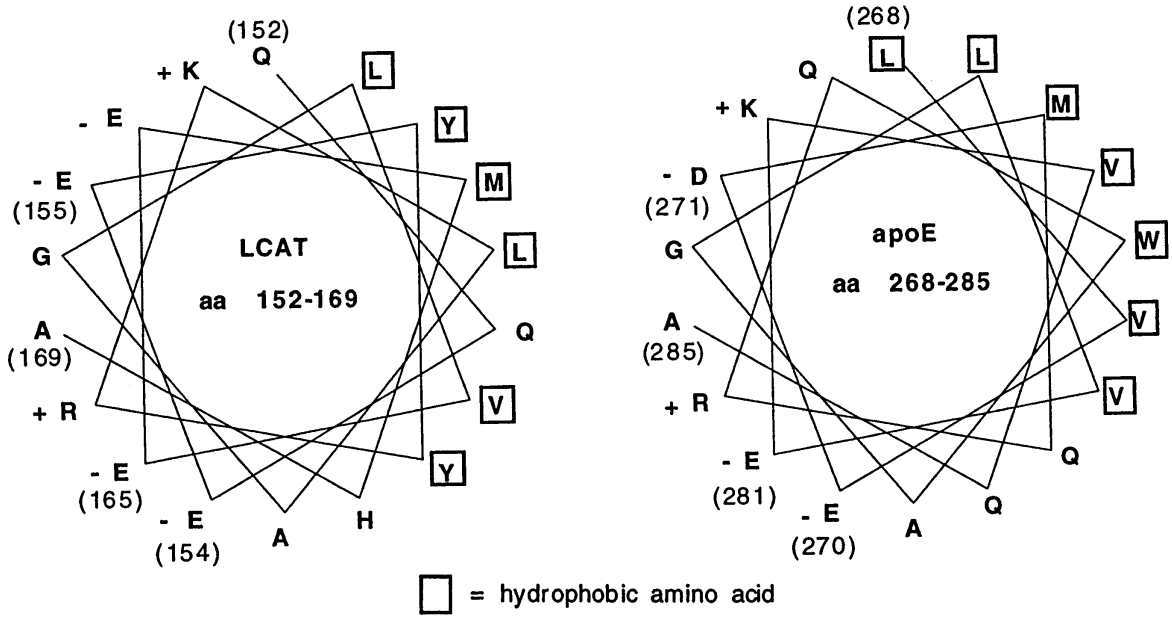

Fig. 1. Helical wheel alignment of homologous regions of human LCAT (residues 152–169) and apoE (residues 268–285). Amino acid residues are designated by one letter abbreviations. A, alanine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; Q, glutamine; R, arginine; V, valine; W, tryptophan. Hydrophobic residues are indicated by the boxes.

in the transfected COS cells (0.37 ± 0.01 and 0.17 ± 0.01 μg/ml, respectively) compared to that of human LCAT (0.55 ± 0.01 μg/ml). After normalization to the concentration of media LCAT, the specific activities (21.0 ± 0.7 and 10.8 ± 1.3 nmol/h/μg for E165Q and E165K, respectively) were not statistically different from that of human wild type LCAT (15.8 ± 2.7 nmol/h/μg). The substitution of glutamine or lysine for glutamic acid at residues 154 and 155 resulted in higher secretion and specific activities that were similar or higher on average than that of wild type LCAT. The double mutations E1-2Q and E1-2K contained point mutations of both glutamic acid residues 154 and 155 to glutamine and lysine, respectively. Both E1-2Q and E1-2K had higher average specific activities ($P < 0.05$ for E1-2Q) compared to human LCAT, but similar concentrations of LCAT mass in the media. E1-3Q and E1-3K contained triple mutations of glutamic acid residues 154, 155, and 165 to glutamine and lysine, respectively. Both showed decreased mass secretion (0.25 ± 0.02, and 0.15 ± 0.01 μg/ml, respectively) compared to wild type LCAT. The volume activity of E1-3K was dramatically decreased to 0.8 ± 0.03 nmol/h per ml compared to human LCAT as was the specific activity (5.4 ± 0.1 nmol/h/μg; $P < 0.05$), whereas the specific activity of E1-3Q was similar to wild type LCAT. In summary, all of the glutamic acid mutants except E1-3K had specific activities that were similar to or greater than that of wild type LCAT.

Table 2 shows the PLA_2 activities of LCAT with two rHDL substrates, containing POPC or PAPC, using media from the same transfections shown in Table 1. The specific activity values obtained using POPC rHDL were significantly higher than wild type LCAT for most of the mutant constructs except E1-3K, which was significantly lower than wild type. Our previous study showed that glutamic acid residue 149 was important in determining the fatty acyl specificity of LCAT (17). Because of its close proximity in the primary sequence to the glutamic acid residues that were mutated in this study, we also assayed PLA_2 activity using PAPC rHDL (Table 2). The specific activity values for PAPC rHDL exhibited trends that were similar to those observed for POPC rHDL. We have used a PAPC/POPC activity ratio in past studies to indicate fatty acyl preference of LCAT, with human LCAT exhibiting a ratio <1, whereas rat LCAT had a ratio >1. **Figure 2** illustrates the PAPC/POPC activity ratios for the LCAT constructs used in this study. All LCAT constructs had a ratio <1, typical of that of human wild type LCAT and there was no significant difference in the activity ratios among the various mutants.

To determine whether any of the LCAT mutations differentially affected cholesteryl ester formation versus PLA_2 activity, an activity ratio of CE formation to PLA_2 activity was calculated from the data in Tables 1 and 2 and plotted in **Fig. 3**. Although the absolute activity ratio is arbitrary because of the difference in substrate

TABLE 1. Cholesterol esterification assay of wild type and glutamic acid mutants of human LCAT

cDNA Construct	Concentration	CE Formation	Specific Activity
	μg/ml	*nmol/h/ml*	*nmol/h/μg*
humLCAT	0.55 ± 0.01	8.7 ± 1.4	15.8 ± 2.7
E154Q	0.85 ± 0.05	16.2 ± 1.8	19.3 ± 2.9
E155Q	1.43 ± 0.06	23.5 ± 3.9	16.2 ± 2.2
E165Q	0.37 ± 0.01	7.7 ± 0.1	21.0 ± 0.7
E154K	1.02 ± 0.03	19.2 ± 3.0	19.0 ± 3.5
E155K	0.88 ± 0.21	18.6 ± 4.2	24.1 ± 8.0
E165K	0.17 ± 0.01	1.8 ± 0.1	10.8 ± 1.3
E1-2Q	0.54 ± 0.03	14.0 ± 0.7	26.1 ± 1.1[a]
E1-2K	0.52 ± 0.03	11.8 ± 0.7	22.7 ± 0.9
E1-3Q	0.25 ± 0.02	4.5 ± 0.5	18.3 ± 0.9
E1-3K	0.15 ± 0.01	0.8 ± 0.0	5.4 ± 0.1[a]

Values are the mean ± SEM (n = 3) for cholesterol esterification using rHDL containing POPC, [^{3}H]cholesterol, and apoA-I (80:5:1 molar ratio). The cDNA designations indicate the glutamic acid residue followed by its mutant amino acid substitution. E1-2Q and E1-2K represent the double mutation at 154 and 155 to glutamine and lysine, respectively. E1-3Q and E1-3K represent the triple mutation at 154, 155, and 165 to glutamine and lysine, respectively. Media was harvested from COS-1 cells 72 h after transfection and frozen at −70°C until the assays were performed. The beta-galactosidase blank (0.9 ± 0.1 nmol CE formed/h/ml) has been subtracted from the values in the table; the activity of the S181A mutant was not detectable after blank subtraction.

[a]Significantly different from human wild type LCAT ($P < 0.05$); only the specific activity data were analyzed statistically.

particle compositions and reaction kinetics between the cholesterol esterification and PLA_2 assays, the relative values can be compared among the mutant constructs. For the single mutations there was a statistically lower ratio for E165 as it was mutated from a negative charged residue (E) to an uncharged (Q) or positive (K) charged residue (10.2 vs. 6.0 vs. 4.3, respectively). The double and triple mutations all had a significantly lower CE/PLA_2 activity ratio compared to wild type.

DISCUSSION

The purpose of this study was to determine whether glutamic acid residues 154, 155, and 165 of LCAT were essential for cholesterol esterification and PLA_2 activity. We tested the hypothesis that the amphipathic α-helix that contains these residues might be part of a substrate binding domain based on the high degree of sequence similarity with a lipid binding domain of apoE and on chemical inhibition studies with EDAC, which showed that cholesterol could protect three carboxyl residues from modification with EDAC and prevent enzyme inhibition (18). Our results show that mutating glutamic acid residues 154, 155, and 165 individually or collectively to glutamine or lysine did not significantly inhibit cholesteryl esterification or PLA_2 activity, except for the triple mutation E1-3K, suggesting that these residues are not critical for LCAT activity. However, changing the amino acid residue at 165 from glutamic acid to glutamine or lysine did result in a progressive decrease in cholesterol esterification relative to PLA_2 activity (Fig. 3). Although the substitution of these amino acids involved more than just a change in charge, it is likely that the charge differences among these three amino acid residues is the most perturbing feature. We believe that changing the amino acid charge at position 165 of LCAT from negative to uncharged to positive resulted in a selective decrease in cholesterol esterification of the

TABLE 2. PLA_2 activity of wild type and glutamic acid mutants of human LCAT

	POPC rHDL		PAPC rHDL	
cDNA Construct	nmol/h/ml	nmol/h/μg	nmol/h/ml	nmol/h/μg
humLCAT	0.85 ± 0.07	1.53 ± 0.13	0.77 ± 0.05	1.39 ± 0.09
E154Q	2.00 ± 0.11	2.38 ± 0.27	1.50 ± 0.08	1.78 ± 0.18
E155Q	2.60 ± 0.19	1.83 ± 0.19	1.83 ± 0.06	1.28 ± 0.07
E165Q	1.29 ± 0.02	3.51 ± 0.05[a]	1.08 ± 0.03	2.95 ± 0.05[a]
E154K	2.40 ± 0.02	2.35 ± 0.06	1.74 ± 0.08	1.71 ± 0.13
E155K	2.18 ± 0.09	2.78 ± 0.62[a]	1.61 ± 0.10	2.06 ± 0.49
E165K	0.42 ± 0.01	2.46 ± 0.13[a]	0.34 ± 0.05	1.97 ± 0.30
E1-2Q	2.36 ± 0.15	4.42 ± 0.48[a]	1.68 ± 0.08	3.13 ± 0.21[a]
E1-2K	1.86 ± 0.00	3.60 ± 0.20[a]	1.51 ± 0.04	2.92 ± 0.14[a]
E1-3Q	0.84 ± 0.06	3.45 ± 0.32[a]	0.77 ± 0.23	3.03 ± 0.63[a]
E1-3K	0.05 ± 0.06	0.34 ± 0.42[a]	0.11 ± 0.01	0.72 ± 0.05

Values are the mean ± SEM (n = 3) for PLA_2 activities using rHDL containing 1-16:0, 2-[^{3}H]18:1 PC (POPC), or 1-16:0, 2-[^{3}H]20:4 PC (PAPC) and apoA-I (80:1 molar ratio). The beta-galactosidase blank (0.89 ± 0.03 nmol FA released/h per ml for POPC and 0.29 ± 0.01 nmol FA released/h per ml for PAPC) has been subtracted from the values in the table; the activity of the S181A mutant after blank subtraction was 0.0 nmol FA released/h per ml for POPC rHDL and 0.15 ± 0.06 nmol FA released/h per ml for PAPC rHDL. See Table 1 for abbreviations of cDNA constructs.

[a]Significantly different from human wild type LCAT ($P < 0.05$); only the specific activity data were analyzed statistically.

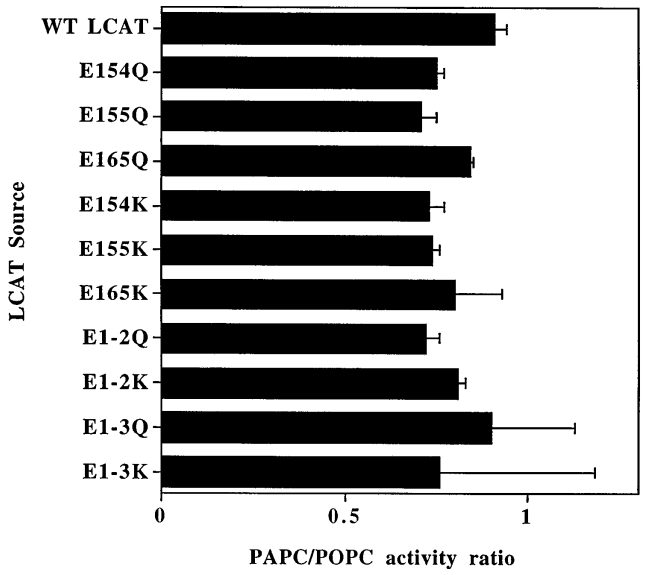

Fig. 2. The ratio of PLA_2 activity of wild type and mutant LCAT using rHDL substrates containing PAPC and POPC. The PLA_2 activity ratio was calculated from results in Table 2. Values are the mean ± SEM for three separate transfections. None of the values is significantly different from human wild type LCAT.

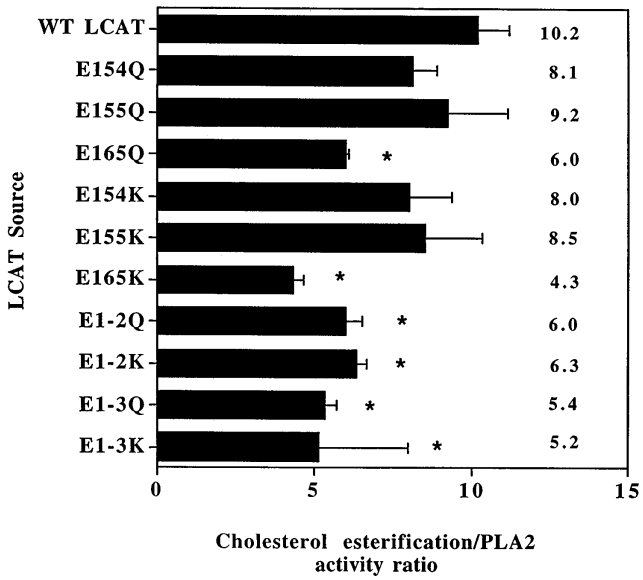

Fig. 3. CE/PLA_2 activity ratio using POPC rHDL substrate. The ratio was calculated from results in Tables 1 and 2. Values are the mean ± SEM for three separate transfections. Mean values are shown to the right of the bars. *, Significantly different from human wild type LCAT ($P < 0.05$).

enzyme. Thus, residue 165 may be in a region of LCAT that is involved with cholesterol binding or is sensitive to cholesterol binding at the active site of the enzyme.

There may be at least two explanations why the mutagenesis of glutamic acid residues 154, 155, and 165 did not result in the progressive and complete inhibition of cholesterol esterification. The most obvious is that these three glutamic acid residues are not the ones that were modified by EDAC and methyl glycine esterification in our previous study (18). There are 21 glutamic acid and 21 aspartic acid residues in human LCAT (22, 23), any of which could have been chemically modified by EDAC. Our rationale for focusing on glutamic acid residues 154, 155, and 165 was based on the identical alignment of these three glutamic acid residues with three acidic amino acid residues in an amphipathic region of apoE thought to be involved in lipid binding. However, the neutralization or reversal of charge of these three glutamic acids did not inhibit cholesterol esterification by LCAT. Another possible explanation for our results could be that chemical modification and derivatization by EDAC and methyl glycine ester may have a more perturbing effect on the structure of LCAT than the mutagenesis approach. Thus, the glutamic acid residues at position 154, 155, and 165 could have been the ones involved in EDAC derivatization in our previous study, but the inhibition of cholesterol esterification resulted because of some steric hindrance and structural perturbation of the enzyme.

The fact that many of the LCAT glutamic acid mutants demonstrated an increase in specific activity for cholesterol esterification and PLA_2 activity demonstrates that the loss of net negative charge at positions 154, 155, and 165 of LCAT does not compromise the specific activity of the enzyme. Fielding and Fielding (11) have proposed that the anti-parallel alignment of an amphipathic α-helix of apoA-I (residues 145–162) with the amphipathic helical region of LCAT (residues 152–169) is important in stabilizing the binding of LCAT to the lipid interface. In their model the binding of LCAT to the interface is stabilized by salt bridges between these two regions of LCAT and A-I. **Figure 4** shows a helical wheel projection of these two regions aligned so that the hydrophobic face of each helix is in the same plane and the potential for salt bridges between helices is maximized. Based on this projection there exists the potential for glutamic acid residues 154 and 165 of LCAT to salt bridge with arginine residues 149, 153, and 160 of apoA-I. However, neutralizing the negative charge of residues 154, 155, and 165 of LCAT did not significantly diminish cholesterol esterification or PLA_2 activity (Tables 1 and 2). These data suggest that salt bridge formation between positive charged amino acids of apoA-I and negative charged amino acids of LCAT in this region of the proteins is not critical in determining catalytic activity of the enzyme. A study by Jonas, Covinsky, and Sweeny (24) demonstrated that chemical modification of the positive charged lysine

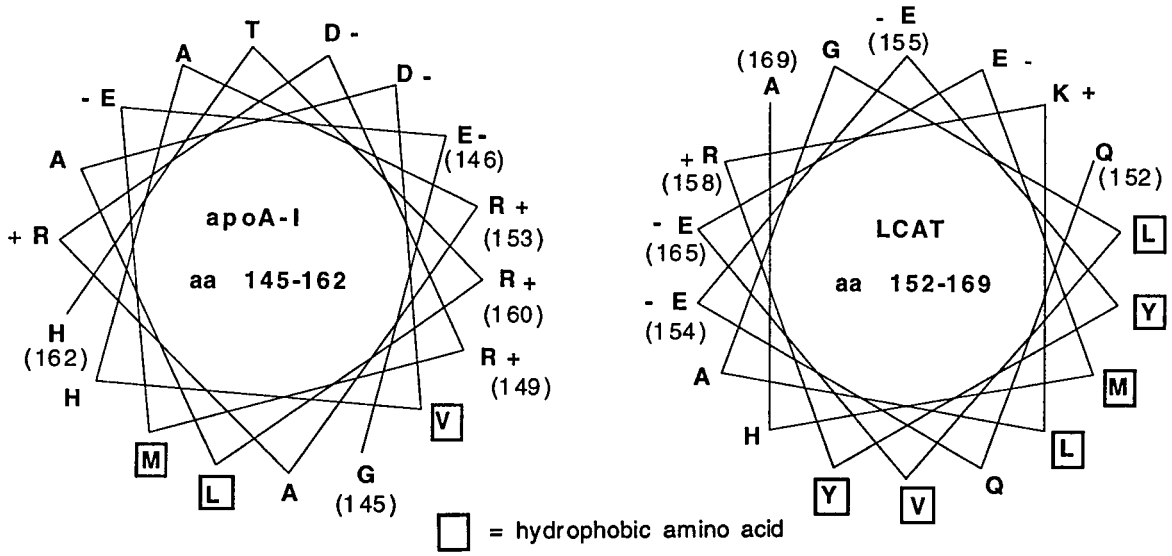

Fig. 4. Helical wheel alignment of human apoA-I (residues 145–162) and human LCAT (residues 152–169). The two helical wheel projections are anti-parallel to each other. The helical wheel projections were arranged so that the hydrophobic face of each helix is in the same plane and the potential for salt bridges between helices is maximized.

residues of apoA-I resulted in decreased LCAT activation, arguing for a role for salt bridge formation in the activation of the enzyme. However, the location of the modified residues was unknown and the decreased activation of LCAT by chemically modified apoA-I may have resulted from a conformational change in apoA-I, as documented by the investigators. Using a synthetic amphipathic peptide $(GALA)_n$ that contained no positive charged amino acids, Subbarao et al. (25) observed LCAT activation using DMPC and DPPC vesicles. Although these results suggest that the presence of positive charges on the hydrophilic face of the peptide were not necessary for LCAT activation, the $(GALA)_n$ peptide did not bind to substrate particles and did not activate LCAT when PC substrates containing unsaturated fatty acids were used, limiting the general conclusions of the study. However, it is possible that a salt bridge could form between positive charged amino acids in LCAT and negative charged amino acids in apoA-I. It is noteworthy that a naturally occurring mutation of LCAT (R158C), neutralizing the positive charge at residue 158, results in a 50% decrease in LCAT specific activity when the mutant construct is transfected into human embryonic kidney 293 cells (26). Arginine 158 of LCAT could potentially form a salt bridge with glutamic acid residue 146 of apoA-I (Fig. 4), stabilizing the interfacial binding of LCAT. Further studies will be required to determine the validity of the LCAT–apoA-I salt bridge hypothesis and the role of individual amino acids in salt bridge formation.

Our previous studies showed that glutamic acid residue 149 is important in determining the fatty acyl specificity of LCAT (17). Mutagenizing residue 149 from glutamic acid to alanine activated human LCAT towards PAPC rHDL compared to POPC rHDL, resulting in a PAPC/POPC activity ratio >1 compared to a value of <1 for human wild type LCAT for both cholesterol esterification and PLA_2 activity. Because glutamic acid residue 149 was close to the glutamic acid residues that were mutagenized in this study, we calculated a PAPC/POPC PLA_2 activity ratio for our mutants. There was no significant alteration in the ratios among the mutants used in the study (Fig. 2), suggesting that the charge distributions of glutamic acid residues downstream from 149 are not involved in determining fatty acyl specificity of the enzyme.

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