Identification of a Sequence of Apolipoprotein A-I Associated with the Activation of Lecithin:Cholesterol Acyltransferase*

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We aimed to distinguish between the effects of mutations in apoA-I on the requirements for the secondary structure and a specific amino acid sequence for lecithin:cholesterol acyltransferase (LCAT) activation. Several mutants were constructed targeting region 140–150: (i) two mutations affecting α-helical structure, deletion of amino acids 140–150 and substitution of Ala143 for proline; (ii) two mutations not affecting α-helical structure, substitution of Val149 for arginine and substitution of amino acids 63–73 for sequence 140–150; and (iii) a mutation in a similar region away from the target area, deletion of amino acids 63–73. All mutations affecting region 140–150 resulted in a 4–42-fold reduction in LCAT activation. Three mutations, apoA-I(D140–150), apoA-I(P143A), and apoA-I(140–150 → 63–73), affected both the apparent Vmax and Km, whereas the mutation apoA-I(R149V) affected only the Vmax. The mutation apoA-I(D63–73) caused only a 5-fold increase in the Km. All mutants, except apoA-I(P143A) and apoA-I(D63–73), were active in phospholipid binding assay. All mutants, except apoA-I(P143A), formed normal discoidal complexes with phospholipid. The mutation apoA-I(D63–73) caused a significant reduction in the stability of apoA-I-phospholipid complexes in denaturation experiments. Combined, our results strongly suggest that although the correct conformation and orientation of apoA-I in the complex with lipids are crucial for activation of LCAT, when these conditions are fulfilled, activation also strongly depends on the sequence that includes amino acids 140–150.

ApoA-I is a key element of the reverse cholesterol transport pathway. This pathway removes excess cholesterol from extrahepatic cells and thus protects the artery wall against developing atherosclerosis (1). Most of the apoA-I in the plasma is associated with high density lipoprotein (HDL),1 although apoA-I may dissociate from the major HDL subfraction, α-HDL (2), and up to 13% of apoA-I is present in lipid-poor form as pre-β2-HDL (3). ApoA-I is essential for the correct assembly and overall stability of HDL (4), activates lecithin:cholesterol acyltransferase (LCAT) (5), is required for binding of phospholipid transfer protein to HDL (6), and mediates the interaction of HDL with cells (7, 8). Lipid-free and lipid-bound apoA-I are efficient acceptors of cholesterol released from the cell plasma membrane (9, 10). ApoA-I regulates the translocation of intracellular cholesterol to the plasma membrane (11, 12), promotes efflux of intracellular cholesterol (13–16), triggers signaling pathways that could be related to cholesterol efflux (17–19), and regulates expression of adhesion molecules (20). Many of these activities are related to the unique secondary structure of apoA-I: when bound to lipid, apoA-I consists of nine 22-mer and two 11-mer amphipathic α-helices spanning almost the entire length of apoA-I (21). Amphipathic α-helices are essential for the lipid binding properties of apoA-I and for those functions of apoA-I that rely on its interaction with lipids. This, however, creates a problem in analyzing the structure-function relationship of the protein: most mutations as well as monoclonal antibodies, which have been used to probe apoA-I, affect its secondary structure and lipid binding properties, masking the possible direct effect of a sequence alteration or the blocking of an active site. In this study, we describe a strategy to overcome this constraint. This approach involved designing a series of mutations, some that were predicted to affect or not the 22-mer α-helical repeat structure of apoA-I, and another mutation in which a selected region between amino acids 140 and 150 of apoA-I was substituted with another sequence of very similar structure. The sequence between amino acids 140 and 150 belongs to the central domain of apoA-I that is implicated in the ability of apoA-I to activate LCAT and that may also be involved in the stimulation of efflux of intracellular cholesterol (15). In this paper, we report the lipid binding and LCAT activation properties of these apoA-I mutants. We found that mutations within a segment of apoA-I between amino acids 140 and 150 reduce the ability of apoA-I to activate LCAT independently of their effect on the secondary structure of apoA-I. We also show that in addition to the carboxyl-terminal end sites, a lipid-binding domain of apoA-I might also reside between amino acids 63 and 73.

MATERIALS AND METHODS

Site-directed Mutagenesis and Expression of Recombinant ApoA-I—The construction, expression, and verification of the recombinant apoA-I mutants were as described in detail previously (22). Briefly, three mutations, apoA-I(P143A), apoA-I(R149V), and apoA-I(D140–150), were constructed utilizing the U.S.E. mutagenesis system (Amerham Pharmacia Biotech, Boronia, Victoria, Australia) and the pGEX-KN proapoA-I plasmid made previously (23). Mutated apoA-I fragments were cloned into the BaePak8 plasmid containing pDproapoA-I (BaePak8 proapoA-I) (24) using the restriction endonuclease sites MluI and EcoRI. For the deletion of apoA-I residues 63–73, the Stratagene QuickChange site-directed mutagenesis kit was utilized. For the apoA-I(140–150 → 63–73) substitution, a mutated DNA fragment of apoA-I was generated from pGEX-KN proapoA-I by polymerase chain reaction utilizing a 5′-mutagenic primer and a 3′-primer, both

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1 The abbreviations used are: HDL, high density lipoprotein; rHDL, reconstituted HDL; LCAT, lecithin:cholesterol acyltransferase; POPC, palmitoyloleoylphosphatidylcholine; DMPC, dimyrustoylphosphatidylcholine; GdnHCl, guanidine hydrochloride.
**A Sequence of ApoA-I Involved in LCAT Activation**

**Preparation of Reconstituted High Density Lipoprotein—**Reconstituted high density lipoprotein (rHDL) was prepared by the sodium cholate dialysis method according to Jonas et al. (27, 28) using palmitoyloleoylphosphatidylcholine (POPC) (Sigma, Castle Hill, New South Wales, Australia), apoA-I, and sodium cholate (Sigma) in a molar ratio of 80:1:80. After the removal of sodium cholate by dialysis, the rHDL preparations were examined by electrophoresis on 3–30% nondenaturing gradient polyacrylamide gels (Gradiopore, North Ryde, New South Wales, Australia) run at 2500 V·h. Following staining with Coomassie Blue, gels were scanned, and the size of the rHDL particles was calculated according to high molecular weight calibration standards (Amersham Pharmacia Biotech). The chemical composition of the particles was determined by the Bradford protein assay (26) and the enzymatic/fluorometric phospholipid assay (Roche Molecular Biochemicals, Castle Hill).

**Cross-linking Experiments—**To determine the number of apoA-I molecules in rHDL particles, rHDL preparations (final concentration of 15 μM protein) were incubated for 30 min at room temperature with bis(sulfosuccinimidyl) suberate (final concentration of 0.25 mM; Pierce). The reaction was stopped by addition of 50 mM Tris-HCl (pH 7.3) and incubated a further 15 min at room temperature. Samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by Western blotting.

**LCAT Purification—**LCAT was purified from human plasma by the method of Chen and Albers (29) with modifications. Briefly, the preparation procedure involved the following steps: (i) precipitation with dextan sulfate/MgCl₂ solution (final concentration of 1 g/liter); (ii) chromatography on a phenyl-Sepharose CL-4B column (Amersham Pharmacia Biotech); loading in buffer containing 10 mM Tris, 1 mM NaCl, and 1 mM EDTA (pH 8.0) and elution with H₂O; (iii) removal of albumin by chromatography on an Affi-Gel blue column (Bio-Rad, Regents Park, New South Wales); (iv) chromatography on a DEAE-Sepharose column (Amersham Pharmacia Biotech) eluting with a linear Tris/NaCl gradient (1 mM Tris and 25 mM NaCl to 10 mM Tris and 200 mM NaCl (pH 7.4)); (v) removal of contaminating apoA-I by chromatography on hydroxylapatite.

**LCAT Assay—**The substrate particles were prepared by adding apolipoproteins to a reaction containing egg phosphatidylcholine, cholesteryl (Sigma), and [³H]cholesterol (specific radioactivity 1.81 TBq/mmol; Amersham Pharmacia Biotech, Castle Hill) in 12 mM sodium cholate in Tris buffer (10 mM Tris, 140 mM NaCl, and 1 mM EDTA (pH 7.4)); sodium cholate was then removed by dialysis (28, 30). The final phosphatidylcholine/cholesterol/apoA-I ratio was 100:10:1 (mol/mol/mol). The complexes were analyzed by electrophoresis on 3–30% non-denaturing polyacrylamide gels as described above for rHDL. All complexes were of a similar size and represented by two populations of particles with the Stokes diameters of 10.1 and 8.4 nm.

The apoA-I-phosphatidylcholine-cholesteryl complexes were assayed in duplicate using 0–2 μM concentrations of each substrate in a final concentration of 10 mM Tris, 140 mM NaCl, 1 mM EDTA, and 0.6% (w/v) bovine serum albumin (essentially fatty-acid free; Sigma) at pH 7.4. After a 15-min preincubation at 37 °C, β-mercaptoethanol was added to a final concentration of 2 mM, and the reaction was initiated by addition of LCAT. The reaction was allowed to proceed for 30 min at 37 °C and was arrested by addition of 1 ml of absolute ethanol. Lipids were extracted, and the cholesterol and cholesteryl esters were separated by thin-layer chromatography (14). The conversion rate was kept below 15% to maintain first-order kinetics. The apparent V₅₀ and K₅₀ were determined from plots of cholesterol concentration ([S]) against rate of cholesteryl ester formation (V), and data were fitted to Michaelis-Menten kinetics of V = V₅₀[S]/(K₅₀ + [S]).

**Interaction of Apolipoproteins with Phospholipid Liposomes—**Dry dimyristoylphosphatidylcholine (DMPC; Sigma) was sonicated in Tris buffer (pH 8.0) to form multimembranous liposomes. Apolipoproteins (final concentration of 0.2 mg/ml) were preincubated for 10 min at 24.5 °C, and the reaction was initiated by adding DMPC liposomes (final DMPC concentration of 0.5 mg/ml). The reduction of absorption at 325 nm (light scattering) was monitored for 1.5 h at 2-min intervals at 24.5 °C to assess formation of apoA-I-DMPC complexes. For each recombinant apoA-I, rate constants (k) and half-times (t₅₀) were determined from plots of fractional absorption at 325 nm (A) against time (minutes), and data were fitted to second-order kinetics of A = 1/(1 + kt).

**Circular Dichroism Studies—**The stability of apoA-I rHDL was determined by measuring the ellipticity at 222 nm of rHDL in the presence of increasing concentrations of GdnHCl. Briefly, 60 μg of apoA-I rHDL was incubated with 0–6 M GdnHCl (final volume of 300 μl) for 50 h at 4 °C. The ellipticity of the samples and appropriate blanks was measured at 222 nm using a 0.5-mm quartz cell in an Aviv Model 62DS spectrometer. Twenty measurements of each sample were averaged, and the average ellipticity at 222 nm was determined. The ellipticity values (millidegrees) were converted to mean residue ellipticity after blank subtraction. The percentage of α-helical content of HDL was calculated by the equation of Chen et al. (31). The concentration of GdnHCl at which denaturation of apoA-I was 50% completed (D₅₀) and the standard free energy of denaturation (ΔG°) were calculated according to Sparks et al. (32).

**Prediction of the Structural Properties of the Mutants—**Predicted hydrophobicity (Kyte-Doolittle), average charge, and amphipathicity (Eisenberg) of the regions of apoA-I were calculated using Protein software (DNASTAR Inc.). Wheel diagrams and predicted orientations of α-helices were generated using Anheptor Version 4.0 (Microsoft).

**RESULTS**

The predicted effects of the various mutations on the structure of apoA-I are schematically shown in Fig. 1. Deletion of the target sequence, amino acids 140–150 (apoA-I(140–150)), slightly increases the overall hydrophobicity of the region. However, since the deleted area includes a proline residue (Pro¹⁴¹), which separates two α-helical repeats, this mutation is predicted to substitute two 22-mer α-helical repeats with one
longer α-helical region (Fig. 1, A and B). Substituting alanine for proline 143 (apoA-I(P143A)) also slightly increases hydrophobicity, but results in a fusion of two α-helical repeats, and this is predicted to have a significant effect on the structure of apoA-I (Fig. 1, A and C). Substitution of valine for arginine 149 (apoA-I(R149V)) adds an extra hydrophobic domain, increasing the overall hydrophobicity and reducing the average charge of the region. This mutation, however, is predicted to have little effect on the secondary structure of the region: Arg149 is positioned on the border between the hydrophilic and hydrophobic faces of the helix, and the type, length, and orientation of the α-helix should be only minimally affected (Fig. 1, A and D). The region between amino acids 63 and 73 is predicted to have a secondary structure very similar to the target sequence 140–150, but it is located at the amino-terminal region of apoA-I. It has one additional hydrophobic domain (Trp72); consequently, deletion of amino acids 63–73 (apoA-I(Δ63–73)) will reduce hydrophobicity of the region, but its effect on the overall structure of apoA-I is predicted to be similar to the deletion of amino acids 140–150 (Fig. 1, A, B, and E). The substitution of region 140–150 with region 63–73 (apoA-I(140–150 → 63–73)) is predicted to have little effect on the overall structure of apoA-I. It adds an extra hydrophobic amino acid to the target region (Trp149); however, the length, type, and orientation of the helix are not predicted to change (Fig. 1, A and F).

**LCAT Activation Properties of the ApoA-I Mutants**—To investigate the ability of apoA-I mutants to activate LCAT, kinetic studies were conducted. Human plasma apoA-I was used as a control in these and other experiments, as we did not find any difference in the properties examined in this study between human plasma apoA-I and recombinant mature apoA-I. The dependence of LCAT activity on the apoA-I concentration is presented in Fig. 2, and the apparent $K_m$, $V_{max}$, and $V_{max}/K_m$ are summarized in Table I. Two mutations that were predicted to cause a significant impact on the structure of the target region, apoA-I(Δ140–150) and apoA-I(P143A), caused a 15–20-fold reduction in the ability of apoA-I to activate LCAT ($V_{max}/K_m$). This was due to both a lower apparent $V_{max}$ and higher apparent $K_m$. Two other mutations predicted to have a limited effect on the structure of the target region, apoA-I(R149V) and apoA-I(140–150 → 63–73), caused 4- and 42-fold reductions, respectively, in the ability of apoA-I to activate LCAT. The effect of the mutation apoA-I(140–150 → 63–73) was due to both a lower $V_{max}$ and higher $K_m$, whereas the effect of the mutation apoA-I(R149V) was entirely due to a lower apparent $V_{max}$. The mutation apoA-I(Δ63–73) caused a 5-fold reduction in LCAT activation, an effect entirely due to a higher apparent $K_m$.

**Lipid Binding Properties of the ApoA-I Mutants**—The ability of apoA-I to activate LCAT may depend on its capacity to bind and to form proper complexes with phospholipid. Thus, the ability of apoA-I mutants to bind DMPC was analyzed in time course experiments (Fig. 3), and the rate constants and $t_{1⁄2}$ are presented in Table II. Wild-type human apoA-I, apoA-I(R149V), and apoA-I(140–150 → 63–73) showed very similar rates of lipid binding. The capacity of apoA-I(P143A) to bind DMPC was half that of human apoA-I ($p < 0.01$), and the rate was 30% slower. DMPC binding to apoA-I(140–150) was faster, with a $t_{1⁄2}$ almost 7-fold lower compared with its binding to human apoA-I. The capacity and the rate of DMPC binding to apoA-I(Δ63–73) were 4.5-fold lower than to human apoA-I.

**Properties of the ApoA-I Mutant: Phospholipid Complexes**—To study the properties of apoA-I-phospholipid complexes, we characterized reconstituted discoid HDL prepared from POPC and various mutants (initial POPC/apoA-I ratio of 80:1 (mol/mol)). Fig. 4 shows densitometric analysis of non-denaturing polyacrylamide gels, and Table III summarizes the size and composition of the particles. All particles contained two molecules of apoA-I/particle. Human apoA-I, apoA-I(Δ140–150), and apoA-I(R149V) formed single type particles with a diameter of ~10 nm and a POPC/apoA-I ratio of 70–80:1. apoA-I(Δ63–73) formed a single class of particles 0.5 nm larger compared with human apoA-I. apoA-I(140–150 → 63–73) formed two overlapping populations of rHDL, one with the usual size of 10 nm and another slightly larger, 10.6 nm. The mutant apoA-I(P143A) formed a very heterogeneous population of particles with a size of 6–10.5 nm; the POPC/apoA-I ratio was 92:1, suggesting that not all of apoA-I was incorporated into rHDL particles. This is consistent with the major impact of this mutation on the 22-mer α-helical repeat structure of apoA-I.

The stability of apoA-I on the surface of rHDL particles was analyzed by incubating HDL with increasing concentrations of GdnHCl and by monitoring the decrease in ellipticity at 222 nm. Denaturation curves are shown in Fig. 5, and parameters are presented in Table IV. Mutations apoA-I(P143A) and apoA-I(140–150 → 63–73) resulted in no change in the midpoint of

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**Table I**

| Mutant          | $V_{max}$ | $K_m$ | $V_{max}/K_m$ | $t_{1⁄2}$ |
|-----------------|-----------|-------|---------------|-----------|
| Human apoA-I    | 44 ± 1.5  | 0.14 ± 0.02 | 314         |          |
| apoA-I(Δ140–150)| 9.0 ± 1.0*| 0.6 ± 0.2*  | 15          |          |
| apoA-I(P143A)   | 13 ± 1.0* | 0.6 ± 0.2*  | 22          |          |
| apoA-I(R149V)   | 14 ± 1.0* | 0.16 ± 0.06 | 87.5        |          |
| apoA-I(Δ63–73)  | 41 ± 3.0  | 0.7 ± 0.1*  | 59          |          |
| apoA-I(140–150 → 63–73) | 6.0 ± 0.9* | 0.8 ± 0.3* | 7.5 | |

*p < 0.001 (versus human apoA-I).

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Multilamellar DMPC liposomes were added to the apoA-I preparations at a final ratio of 2.5:1 (phospholipid/protein, w/w), and the formation of particles was assessed by following reduction in absorbance at 325 nm (light scattering) measured at 24.5 °C. All points represent the average of two independent experiments. *hapoAI*, human apoA-I.

**Table II**

Interaction of DMPC with apoA-I mutants

Experiments were performed as described in the legend to Fig. 3. Rate constants (*k*) and halftime (*t*<sub>1/2</sub>) were determined from plots of fractional absorption at 325 nm against time (minutes), and data were fitted to second-order kinetics of 1/(1 - *k*<sub>t</sub>). Means ± S.D. are given.

| Mutant                     | Rate constant | *t*<sub>1/2</sub> |
|----------------------------|---------------|-------------------|
| Human apoA-I               | 0.09 ± 0.004  | 11                |
| ApoA-I(Δ140–150)           | 0.7 ± 0.02<sup>a</sup> | 14               |
| ApoA-I(P143A)              | 0.07 ± 0.004  | 14                |
| ApoA-I(R149V)              | 0.13 ± 0.003  | 7.7               |
| ApoA-I(Δ63–73)             | 0.02 ± 0.001<sup>a</sup> | 50               |
| ApoA-I(140–150 → 63–73)    | 0.15 ± 0.006  | 6.7               |

<sup>a</sup> *p* < 0.001 (versus human apoA-I).

**Discussion**

LCAT is an important element of the reverse cholesterol transport pathway. LCAT reacts with discoid and spherical HDLs, transferring the 2-acyl group of lecithin or phosphatidylethanolamine to the free hydroxyl residue of cholesterol (33). Esterified cholesterol is transferred to the core of the HDL particle, which precludes spontaneous cholesterol exchange with cells and other lipoproteins and vacates a space for more cellular cholesterol to be incorporated into the HDL particle (1). In blood, LCAT is mainly associated with HDL particles (1), and apoA-I is essential for the activation of LCAT (5). The exact mechanism of LCAT activation by apoA-I is not known and may include proper organization of lipid substrates, mediation of binding of LCAT to the substrate, as well as a direct allosteric effect on the LCAT activity.

Several reports published in recent years have examined the relationship between the structure of apoA-I and its ability to activate LCAT. Most data implicate the central region of apoA-I and include results of studies with monoclonal antibodies (34, 35), site-directed mutagenesis (36–41), natural apoA-I mutants (42, 43), and synthetic peptides (44). However, whereas these studies identify the region of apoA-I that is important for LCAT activation, they do not describe the sequence of apoA-I involved in the activation of LCAT or indicate a requirement for such a sequence. The most convincing results suggest a role for two α-helices, regions 143–164 and 165–186, as apoA-I “active sites” for LCAT activation (37, 40, 41, 45).
These data were obtained, however, by deleting or substituting one or both 22-mer repeats. Considering the importance of the number, length, hydrophobic properties, and orientation of the $\alpha$-helical repeats for the correct organization of the apoA-I-phosphatidylcholine complex, these types of mutations would almost certainly affect the structure of the apoA-I-phosphatidylcholine substrate, making it difficult to distinguish between the effects of mutations on the properties of the substrate and on the direct activation of LCAT. Sorci-Thomas et al. (45) have recently reported that inverting the sequence of domain 143–164 of apoA-I also reduces LCAT activation. The natural mutant apoA-I(P143R) is also defective in LCAT activation (46). Another mutation, apoA-I(D140–150), had an opposite effect on the structure of rHDL. This mutant formed the normal 9.9-nm rHDL particles and was more efficient than wild-type apoA-I in a DMPC binding assay, although the stability of apoA-I(D140–150) rHDL was slightly reduced. It is tempting to speculate that region 140–150 has a flexible and unstable conformation, a property consistent with its being a “receptor-binding” or an active site domain of an otherwise rigid molecule. However, this mutation can also affect the optimal alignment and flexibility of apoA-I on the surface of the particle, which may be an important determinant for the interaction of LCAT with the substrate. An interesting comparison for our data is that of the natural mutation apoA-I(D146–160). Although this mutant forms larger rHDL particles than human apoA-I, the alignment of the carboxyl-terminal end of apoA-I, responsible for the lipid binding, is altered, and LCAT activation by this mutant is significantly reduced (43).

Two mutations that potentially do not change the size and conformation of 22-mer $\alpha$-helical repeats around the target area, apoA-I(R149V) and apoA-I(P143–150 $\rightarrow$ 63–73), also reduced LCAT activation. The degree of reduction was disproportional to the effect of these mutations on the structure of the substrate particles; these mutants were similar to human apoA-I with respect to DMPC binding and formation of rHDL particles. The stability and $\alpha$-helical content of apoA-I(D149V) rHDL were slightly reduced, which could contribute to the reduction in LCAT activation. Overall, however, it is unlikely that the lipid binding properties of apoA-I or the structure of the substrate particle was responsible for the decrease in catalytic efficiency of these two mutants. Rather, the region between amino acids 140 and 150 could be a part of the site that is involved in the activation of LCAT. Since the mutation apoA-I(R149V) affected only the apparent $V_{\text{max, R}}$ we speculate that Arg$^{149}$ could be a part of an active site without affecting the binding of LCAT. Although this position may be important for LCAT activation, substitution of the whole region further reduced the $V_{\text{max}}$ which makes it more likely that other parts of the region are involved in the activation of LCAT.

The mutation at the amino-terminal half of apoA-I, apoA-I(D63–73), also reduced LCAT activation, but to a much lesser extent. Moreover, this reduction was entirely attributable to the higher apparent $K_{\text{cat}}$, suggesting that reduced binding affinity of LCAT for the substrate was responsible for the effect. This reduced $K_{\text{cat}}$ could be related to the lipid binding properties of the mutant (see below). Thus, two mutations that are likely to impose similar changes on the 22-mer $\alpha$-helical repeat structure of apoA-I, but that are located in different $\alpha$-helices, have very different impact on LCAT activation properties. This again suggests the requirement for a specific amino acid sequence in apoA-I for LCAT activation.

The mutant apoA-I(D63–73) had a severely impaired ability to bind DMPC, and although it formed rHDL particles of the usual size, the stability of apoA-I in these particles was significantly reduced. We have previously suggested that in addition to the strong lipid-binding region at the carboxyl-terminal end, the amino-terminal half of apoA-I may also possess a lipid-binding domain (47). The possibility of the presence of the second lipid-binding domain in this region of apoA-I was also indicated by Palgunachari et al. (48) and Mishra et al. (49) from the results of experiments with model synthetic peptides. We suggest that the region between amino acids 63 and 73 is a part...
of this second lipid-binding region of apoA-I. Combined, our results suggest that although the correct conformation and orientation of apoA-I in HDL are crucial for the binding of LCAT to the substrate and its activity, when this condition is fulfilled, the activation of LCAT may also depend on a specific sequence that includes amino acids 140–150.

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