

Synthetic substrates of lecithin:cholesterol acyltransferase

Ana Jonas

University of Illinois at Urbana-Champaign, 506 South Mathews Avenue, Urbana, IL 61801

Abstract Investigation of the substrate specificity of lecithin:cholesterol acyltransferase has been greatly aided by the use of synthetic particles containing the molecular lipid substrates and the apolipoprotein activators of the enzyme. These synthetic particles, in vesicle or disc-like micelle form, are described in some detail noting their preparation, properties, advantages, and limitations as substrates for lecithin:cholesterol acyltransferase. The reactions of the enzyme with the synthetic particles are reviewed in terms of acyl donor and acceptor specificity, activation by apolipoproteins, effects of various inhibitors, and the kinetics of the reaction. — Jonas, A. Synthetic substrates of lecithin:cholesterol acyltransferase. *J. Lipid Res.* 1986. 27: 689–698.

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INTRODUCTION

Lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for cholesterol esterification in plasma, catalyzes the removal and transfer of the *sn*-2 acyl chain from phosphatidylcholine (PC) to cholesterol (1). This reaction occurs preferentially on the surface of the smaller, spherical high density lipoprotein (HDL) subclasses and nascent, discoidal HDL (1, 2) probably as a result of LCAT activation by apolipoprotein A-I (apoA-I) and other HDL apolipoproteins (3–5). Although the enzymatic reaction takes place on HDL particles, the reactants (PC and cholesterol) and the products (lyso-PC and cholesteryl esters) of the LCAT reaction are widely distributed. This distribution is the result of spontaneous or catalyzed transfers of phospholipids and unesterified cholesterol from other lipoproteins [chylomicrons, very low (VLDL) and low density lipoproteins (LDL)] and from cell membranes into HDL, and the transfer of cholesteryl esters, in exchange for triglycerides, from HDL to VLDL and LDL (6). The lyso-PC products of the LCAT reaction are mostly removed by spontaneous transfer to serum albumin.

The physiological role of LCAT is intimately connected with the metabolism and functions of HDL and other

lipoproteins in plasma. LCAT participates in the efflux of cholesterol from cells into HDL; it reduces the levels of unesterified cholesterol in plasma, participates in the maturation and transformation of HDL and other lipoproteins, and modulates the action of plasma lipid transfer proteins, which equilibrate phospholipids, cholesteryl esters, and triglycerides among lipoproteins (1, 7).

The physiological importance of LCAT, together with the general interest in the mode of action of interfacial enzymes, stimulated work on the purification, antibody development, physical and chemical characterization, and the investigation of the substrate requirements and specificity of this enzyme. Human LCAT is a 60,000 molecular weight, single polypeptide chain glycoprotein, containing 25% by weight carbohydrate (8, 9). Very little is known about its structure aside from the amino acid composition (9), the content of α -helical structure (21%) (10, 11), and overall asymmetrical shape (11). In terms of the macromolecular interactions of LCAT, fractionation of plasma and analysis of LCAT activity and mass have indicated variable levels of enzyme binding to all HDL subclasses but also the presence of lipoprotein-free LCAT (1, 12, 13). Although it is very important to understand the different binding and enzymatic reactivity of LCAT with the various HDL subclasses and to elucidate the molecular events on the lipoprotein surface, such information is difficult, if not impossible, to obtain with the chemically and physically heterogeneous natural HDL particles. Therefore, it has been necessary to rely on synthetic, chemically and physically defined substrates to investigate the molecular properties of the LCAT reaction.

The first synthetic substrates to be introduced were unilamellar vesicles of PC and cholesterol with added

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoproteins; PC, phosphatidylcholine; apoA-I, apolipoprotein A-I; VLDL, very low density lipoproteins; LDL, low density lipoproteins; DPPC, dipalmitoyl-PC; DMPC, dimyristoyl-PC; apoA-II, apolipoprotein A-II; apoC-I, apolipoprotein C-I; PPOPC, palmitoyl palmitoleoyl-PC; apoD, apolipoprotein D; apoE, apolipoprotein E; POPC, palmitoyl oleoyl-PC; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; PS, phosphatidylserine.

apolipoproteins (14). They were particularly useful in defining the preferential activation of LCAT by apoA-I and the selectivity of the enzyme for acyl acceptors. More recently, discoidal complexes of PC, cholesterol, and apolipoproteins have been prepared by the spontaneous reaction of PC dispersions with apolipoproteins or by the sodium cholate dialysis method (15–17). These particles resemble very closely the discoidal nascent HDL in physical properties, and have defined chemical compositions. They are remarkably stable entities, and some have been useful in the investigation of the substrate specificity of the enzyme, its activation by HDL apolipoproteins, and its kinetic properties.

This short review will concentrate on the properties of the LCAT reaction as revealed by studies with synthetic substrates, vesicles and discoidal micelles, and will include a brief description of the properties, advantages, and limitations of these synthetic substrates (Fig. 1).

VESICLE SUBSTRATES OF LCAT

Vesicle substrate properties

Ho and Nichols in 1971 (14) were the first to report the use of PC/cholesterol vesicles, prepared by sonication of the lipids, as substrates for LCAT. Three different vesicle preparation methods have been used since then: sonication of aqueous lipid dispersions, injection of ethanolic solutions of lipids into an aqueous buffer, and French press extrusion of lipid dispersions in aqueous medium (18–20). In some instances uniform size vesicles have been isolated by fractionation of these preparations on large-pore gel-filtration columns. The unilamellar vesicles obtained by these methods exceed 200 Å in diameter and contain over 2000 PC molecules, organized in a bilayer shell separating

an inner aqueous compartment from the bulk solution (18). Unilamellar vesicles, particularly those of small diameters, are metastable structures. Their stability depends on the nature of the PC component and on other factors such as temperature and solvent composition. For example, egg-PC vesicles may be stored for several days at 4°C, whereas dipalmitoyl-PC (DPPC) vesicles aggregate and fuse readily at temperatures below 41°C.

When used as LCAT substrates, vesicles of PC and cholesterol are generally exposed to apolipoproteins. In studies with apoA-I and vesicles containing egg-PC, with or without cholesterol, reversible and saturable apolipoprotein binding has been demonstrated. Vesicles prepared with egg-PC alone bind a maximum of 5 apoA-I molecules, while vesicles containing a molar ratio of 4/1, egg-PC/cholesterol, bind 8–10 apoA-I molecules per particle (~2700 PC/particle) with an increased affinity relative to the vesicles of egg-PC alone (8, 21). The extent of LCAT activation by apoA-I increases in proportion to the apolipoprotein bound to the vesicle surface (8, 21). Such detailed information about vesicle/apolipoprotein interactions does not exist for other stable vesicle systems and other apolipoproteins. Furthermore, it is now well known that some PC vesicles may be irreversibly disrupted by water-soluble apolipoproteins (17, 22). The breakdown of the vesicles is favored by apolipoprotein and lipid properties that may destabilize the vesicle structure and favor bilayer penetration by the apolipoproteins. Thus, relatively unstable unilamellar vesicles prepared with dilauroyl-PC or dimyristoyl-PC (DMPC) are most easily disrupted by apoA-I at the phase transition temperature of the PC (24°C for DMPC) when the vesicles contain under 20 mole % of cholesterol. The products of the reaction are PC bilayer discs stabilized by apolipoprotein organized into a peripheral ring. These particles, and the identical counterparts prepared by the sodium cholate dialysis method, will be discussed in more detail in a subsequent section. Similarly, small unilamellar vesicles prepared with DPPC may be broken down into discs by reaction with apoA-I at 41°C, but at considerably slower rates than DMPC vesicles at their own transition temperature (23). In contrast to these examples, small vesicles prepared with egg-PC remain intact in the presence of apoA-I over a broad temperature range (8, 21).

Therefore, in evaluating the results obtained with vesicle substrates of LCAT, it is essential to understand the behavior of the substrate particles, which may affect the reactivity of the enzyme either by altered apolipoprotein binding or by structural changes in the system. For the most part, studies performed with egg-PC vesicles, in the presence of apoA-I, are interpretable because the particles remain stable. The effects of other apolipoproteins on egg-PC vesicles are not as well defined as those of apoA-I; however, apoA-II and apoC-I are capable of displacing apoA-I from egg-PC vesicles without apparent changes in

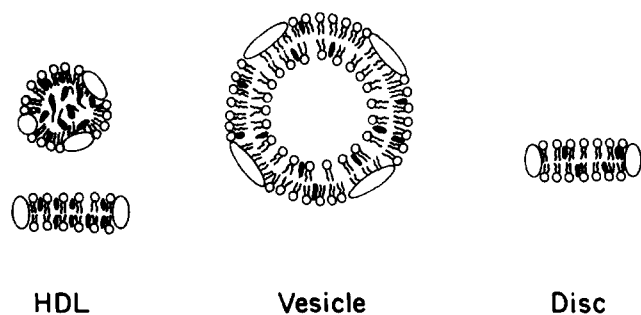


Fig. 1. Diagrams of LCAT substrates in cross-section. The natural LCAT substrates include the smaller spherical HDL subclasses and nascent HDL discs. Synthetic vesicles contain the lipid substrates of the enzyme and reversibly bound apolipoproteins. Vesicles enclose an inner water compartment and may become disrupted under certain experimental conditions. Synthetic discoidal particles are very similar to nascent HDL, usually contain tightly bound apolipoproteins, and are quite stable.

the particle size, suggesting a retention of vesicle structure (8). On the other hand, a synthetic 37 residue amphipathic peptide has recently been reported to disrupt egg-PC vesicle structure (24).

Studies of PC reactivity with LCAT, using vesicle substrates prepared with a variety of PCs and apolipoproteins and performed at varied temperatures, are particularly susceptible to the pitfalls of vesicle disruption and changing LCAT interactions with different interfaces. Because of the considerations described above, the following review of the LCAT reactions using vesicle substrates will be limited to those reports where vesicle morphology was probably maintained throughout the study. These studies include work on apolipoprotein activation of LCAT, the description of acyl acceptors of LCAT, and work with various effectors of LCAT activity.

Apolipoprotein activation of LCAT

Using partially purified LCAT and isolated HDL apolipoproteins, Fielding, Shore, and Fielding (3) were the first to demonstrate the marked activation of LCAT by apoA-I cosonicated with a mixture of egg-PC, cholesterol, and cholesteryl oleate. Although the nature of the substrate particles was not defined, it is likely that it consisted of a heterogeneous mixture of vesicles and micelles of various sizes. Addition of apoA-II inhibited, in part, the activation of LCAT by apoA-I. Subsequent work by Soutar et al. (25) demonstrated that partially purified LCAT, in the presence of palmitoyl palmitoleoyl-PC (PPOPC) and cholesterol vesicles, was preferentially activated by apoA-I, but also exhibited a significant activation by purified apoC-I. The two largest cyanogen bromide cleavage fragments of apoA-I were also activating. The relative order of activation of LCAT by apoA-I and apoC-I, observed with PPOPC vesicles, was reversed when DMPC vesicles were used; apoC-I appeared to be a more effective activator with DMPC than apoA-I. In the latter system, however, the extent of DMPC vesicle breakdown is difficult to evaluate. Other apolipoproteins had little or no activating capacity in both of these vesicle systems, but apoA-II and C apolipoproteins (C-II and C-III) inhibited the effect of apoA-I, as described by Fielding et al. (3). Albers, Lin, and Roberts (26) using pure LCAT and vesicles of egg-PC and cholesterol confirmed the preceding observations made with partially purified LCAT and showed, in addition, that apoD was not an activator of the enzyme.

The activating effect of apoA-I on lipid substrate vesicles was shown, in several studies, to be proportional to the surface saturation with apoA-I (8, 21, 27), and the inhibiting effect of added apoA-II and C apolipoproteins was attributed to the displacement of apoA-I from the vesicle surface by competing apolipoproteins. In fact, a quantitative measure of the dissociation constant of apoA-II from egg-PC/cholesterol vesicles indicated that apoA-II

has a greater affinity for the vesicle surface than does apoA-I (8).

Recent work with pure LCAT, egg-PC/cholesterol vesicles, and apoA-I variants established that two mutant apoA-I molecules, one with a Pro¹⁴³ → Arg substitution and another with a deletion (Lys¹⁰⁷ → 0), demonstrate a diminished activation of LCAT: 40–70% relative to apoA-I (28, 29). In part, this effect may be due to an altered binding of the apoA-I variants to the lipid vesicles. Other variants and pro-apoA-I are similar to apoA-I in their activating capacity (30).

A variety of synthetic amphipathic peptides have been prepared and tested in their ability to bind lipid and to activate LCAT. The lipid systems have included DMPC vesicles, which, as noted above, may break down in the presence of apolipoproteins, and more stable egg-PC vesicles. Even the latter are susceptible to breakdown by some peptides as described by Chung et al. (24). Nevertheless, it is clear that certain synthetic amphipathic peptides may activate LCAT significantly.

Acyl acceptors

Another area of investigation where vesicle substrates have provided useful information is in the selectivity of LCAT for acyl acceptors. Piran and Nishida (31) and Kitabatake et al. (32) used pure LCAT and vesicles of egg-PC containing radiolabeled DPPC as the acyl donor, and 20 mole % of sterols or long chain alcohols as acyl acceptors. They showed that a 3- β -hydroxyl group and a *trans*-configuration of the A/B rings are essential for LCAT transfer activity to sterols. Side chain modification of cholesterol had variable effects on activity, and side chain removal apparently increased activity (31). Alcohols with chains longer than 12-carbons may act as acyl acceptors, but less effectively than water (32). The relative rates of reaction when cholesterol, water, and alcohols are the acyl acceptors are 12.2/1.8/0.9, respectively, in comparable systems (31, 32). This and earlier work by Piran and Nishida (33) and by Aron, Jones, and Fielding (34) demonstrated conclusively that LCAT can exhibit phospholipase activity, in addition to its more physiological transferase function.

Modulators of LCAT activity

The effects of various sulfhydryl reagents, serine reagents, detergents, and metal ions on the LCAT reaction had already been described with HDL substrates using plasma or partially purified enzyme (1). Studies with purified LCAT, using vesicle substrates and reagents such as 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), diethyl *p*-nitrophenyl phosphate (E-600), and Ag⁺ established, in addition, that the transferase and phospholipase activities of LCAT are equally inhibited by these reagents, suggesting the involvement of the same active site residues in both activities (34), and demonstrated that, unlike the A₂

phospholipases, LCAT does not require Ca^{2+} for activity. For optimal activity, the reaction mixtures with vesicle substrates require serum albumin in order to remove the lyso-PC products of the reaction (34, 35).

Furukawa and Nishida (36) examined the stability of pure LCAT employing substrate vesicles and buffers of varying ionic strength. They determined that the enzyme is most stable at low salt concentrations (0.001 ionic strength) but is most reactive and most susceptible to denaturation at 0.1 ionic strength (or 40 mM sodium phosphate buffer, pH 7.4). Presumably, at the higher ionic strength, a conformational change exposes the active site of the enzyme resulting in a more effective interaction with substrates but also with the air/water interface.

Work by Chajek, Aron, and Fielding (37), using vesicle substrates of egg-PC and cholesterol, indicated that cholesteryl ester accumulation reaches a maximum at 3 mole % of the total vesicle lipid, and inhibits the LCAT reaction when cholesteryl esters are not removed by lipid transfer protein. Although product inhibition is the explanation offered by the authors, changes in the interface properties affecting the apolipoprotein and/or enzyme binding or structure could also be involved.

MICELLAR, DISCOIDAL SUBSTRATES OF LCAT

Discoidal substrate properties

Discoidal analogs of HDL were initially prepared by the spontaneous reaction of HDL apolipoproteins and DMPC vesicles in 1976 (38). In subsequent studies with pure apoA-I, it was established that the spontaneous reaction with PC vesicles is kinetically controlled by various factors: the nature of the PC component and the stability of the vesicle bilayer, the temperature of the reaction relative to the main transition temperature of the lipid, the curvature and size of the vesicle, and the content of cholesterol in the bilayer (17, 22). Therefore, the spontaneous reaction is limited to only a few, mostly unphysiological PCs (e.g., DMPC, DPPC) under a restricted set of conditions. In 1981 (15) our laboratory introduced a general method based on the dispersion of lipids in cholate, followed by the removal of detergent in the presence of apolipoprotein (16). This method can be used with all water-soluble apolipoproteins, all types of PCs, sphingomyelin, ether PC analogs, glycerophospholipid mixtures, and cholesterol (up to 30 mole %), to produce discoidal particles, containing a bilayer disc surrounded by a peripheral ring of protein which is mostly present in α -helical structure. A complete description of the preparation and characterization of the particles, particularly those containing apoA-I, can be found in recent reviews (17, 22).

By varying the proportion of PC to apoA-I in the re-

action mixture, particles of various sizes can be prepared and can be isolated by gel-filtration. Gradient gel-electrophoresis is best suited for the analysis of the sizes and distributions of these particles (39). In the size and density range similar to HDL, particles containing egg-PC, cholesterol, and apoA-I have the properties summarized in **Table 1**. The size distributions and densities vary somewhat from one PC to another. Thus, particles prepared with DPPC instead of egg-PC may attain larger disc sizes, with lower densities, when starting with similar reagent mixtures (40). For reaction mixtures containing 90 mole % PC, 9 mole % cholesterol, and 0.9 mole % apoA-I, dipolyunsaturated PCs give rise to particles with higher protein contents than mixed chain PCs or disaturated PCs. All the PCs, however, give particles of comparable diameters (unpublished results, N. Zorich and A. Jonas, 1985) (see **Table 2**).

Chen and Albers (41) reported the preparation of "proteoliposomes" employing a sodium cholate method very similar to that used by our laboratory (15, 16, 17, 22) and by others (39, 42). According to Chen and Albers (41), the product particles were vesicles with stably bound apoA-I. Employing comparable reaction conditions (high PC to apoA-I molar ratios: 300/1) we were only able to prepare discoidal particles with diameters around 200 Å, rather similar to the reported dimensions of the "proteoliposomes." The origin of this discrepancy is not evident, but it is quite clear that at PC/apoA-I molar ratios of 100/1, only discoidal, micellar particles are formed.

When discoidal particles are compared with vesicles of similar composition, the former are 4- to 5-fold better substrates for LCAT. In addition, the discoidal substrates, unlike vesicles, are capable of storing large amounts of cholesteryl esters formed by LCAT: at least 20 mole % of the particle lipid, instead of 3 mole % of the vesicle lipid (43). In extensive reactions with LCAT, leading to substantial accumulation of cholesteryl ester products, we have shown changes in the physical properties of the original flat discs to less asymmetrical, ellipsoidal particles (43). In this case the transformation did not proceed further, because of the exhaustion of the free cholesterol reactant; however, Nichols and coworkers (44) have recently reported the transformation of synthetic discoidal substrates to spherical HDL-like particles in the presence of VLDL and LDL (as free cholesterol donors) and LCAT.

Whereas apolipoproteins equilibrate with vesicles with dissociation constants around 10^{-6} M (21), there are no measurable amounts of free apolipoproteins (A-I, A-II, E, and C-III) in the presence of their discoidal complexes with lipids (4, 5). Nevertheless, added apolipoproteins can exchange for bound apolipoproteins in discoidal particles (4, 5).

Over the last several years, discoidal particles have become the most widely used synthetic substrates for LCAT, and have permitted a more detailed investigation of the

TABLE 1. Properties of apoA-I · egg-PC · cholesterol discoidal complexes prepared by the sodium cholate dialysis method and isolated by gel-filtration (16, 40)^a

	Small	Large
PC/apoA-I (mol/mol)	68:1	132:1
PC/cholesterol (mol/mol)	11:1	10:1
Diameter (× width) (Å)	105 (× 42)	190 (× 44)
Mol. wt.	2 × 10 ⁵	7 × 10 ⁵
ApoA-I/particle	2-3	3-4
ApoA-I properties		
α-Helix (%)	72	73
Max. fl. λ (nm)	330	330
Fl. polarization (at 25°C)	0.120	0.120

^aIn the preparation of the small and large complexes, the reaction mixtures contained 80:1 and 150:1 egg-PC/apoA-I molar ratios, respectively.

apolipoprotein activation, acyl donor specificity, and kinetics of the LCAT reaction.

Apolipoprotein activators

Discoidal substrates, prepared with human HDL apolipoproteins (A-I, A-II, C-I, C-II, C-III-1, C-III-2, and E), egg-PC, and cholesterol have been used as LCAT substrates at equal apolipoprotein concentrations in the bulk solution (4, 5). As previously observed with vesicle substrates, apoA-I is the best activator of LCAT and apoC-I is a secondary one; however, all other apolipoproteins, particularly apoE are activators of LCAT in this more reactive substrate system using highly pure and active LCAT. Table 3 shows the relative activating

capacity of the apolipoproteins using discoidal substrates, and includes data with vesicle substrates as well. In the discoidal systems, apoA-II is capable of displacing apoA-I and apoC-I from their complexes with lipids, thus inhibiting the LCAT activation relative to pure apoA-I complexes (4). On the other hand, apoA-I is capable of displacing apoE from its complexes, resulting in a relative activation (5). Steinmetz and Utermann (45), Steinmetz, Kaffarnik, and Utermann (46), and Chen and Albers (47) have also reported a significant activation of LCAT by apoA-IV and apoE (E-2 and E-3) in complexes with egg-PC prepared by the sodium cholate method and termed "proteoliposomes." Interestingly, apoA-IV may be a better activator for LCAT in discs prepared with DMPC than apoA-I, but apoA-IV is only 13-38% as effective as apoA-I in activating LCAT when egg-PC-containing particles are used (45). It appears, therefore, that the interaction of apolipoproteins with different PCs produces interfaces which are recognized differently by the enzyme. That the apolipoprotein conformation and lipid-apolipoprotein boundary are important factors in the LCAT activation is suggested by three lines of evidence. First, the different levels of LCAT activation obtained with the various HDL apolipoproteins, present in physically similar complexes and containing similar secondary structures, indicates the existence of an optimal LCAT activating structure in apoA-I (4, 5). Second, chemical modification of apoA-I, in discoidal complexes with egg-PC, with amino group reagents has no effect on LCAT activation as long as the overall apolipoprotein structure is not affected, even upon 90% modification of Lys residues to N-dimethyl Lys

TABLE 2. Chemical composition and relative reactivity with LCAT of discoidal apoA-I · PC · cholesterol complexes^a

Phosphatidylcholine	Fatty Acid Chains <i>sn</i> -1, <i>sn</i> -2 Positions	PC/apoA-I ^b (mol/mol)	App $V_{max}/App K_m^c$ (nmol CE/hr · M) (× 10 ⁵)	E_a^d (Kcal/mol)
Palmitoyl linoleoyl-PC	16:0, 18:2	87/1	19.2	17.6
Palmitoyl oleoyl-PC	16:0, 18:1	109/1	18.2	18.8
Oleoyl palmitoyl-PC	18:1, 16:0	112/1	10.8	20.0
Palmitoyl arachidonyl-PC	16:0, 20:4	51/1	14.0	13.4
Di-oleoyl-PC	18:1, 18:1	73/1	9.8	18.2
Stearoyl oleoyl-PC	18:0, 18:1	111/1	8.2	24.2
Oleoyl stearoyl-PC	18:1, 18:0	107/1	7.3	21.4
Dipalmitoyl-PC	16:0, 16:0	82/1	1.6	34.8
Linoleoyl stearoyl-PC	18:2, 18:0	90/1	2.6	20.9
Stearoyl palmitoyl-PC	18:0, 16:0	96/1	2.8	39.9
Dilinoleoyl-PC	18:2, 18:2	40/1	2.6	12.6
Stearoyl arachidonyl-PC	18:0, 20:4	70/1	3.3	13.9
Diarachidonyl-PC	20:4, 20:4	34/1	0.2	7.7
Distearoyl-PC	18:0, 18:0	75/1	0.02	32.5

^aUnpublished results, Zorich and Jonas, 1985. Uniform particles were prepared by the sodium cholate dialysis method (15, 16). The diameters were approximately 110 Å.

^bThe PC/apoA-I molar ratios were determined by chemical analysis of complexes isolated by gel-filtration.

^cInitial reaction velocities were measured at 37°C in 10 mM Tris buffer, pH 8, plus 0.15 M NaCl. All samples contained equal apoA-I concentrations. The apparent kinetic constants were obtained from linear Lineweaver-Burk plots by varying substrate particle concentrations (in terms of bulk PC concentrations); CE, cholesterol ester.

^dActivation energies were measured between 15 and 38°C under the conditions given in footnote c.

TABLE 3. Apolipoprotein activators of human LCAT

Apolipoprotein	Relative LCAT Activation ^a %	App $V_{max}/App K_m^b$ (nmol CE · ml/hr · mg) ($\times 10^{-1}$)	Substrate Particles ^c	References
A-I	100	37.6	Discs, 100-200 Å in diameter.	4, 5
E	18			
C-I	12.1	7.65		
C-III-1	5.4	1.06		
C-III-2	4.0	0.37		
C-II	3.0			
A-II	1.5			
A-I	100		Prepared by the cholate dialysis method. ^d	45, 46
A-IV	38			
A-II	3.5			
E	17			
A-I	100		Prepared by the cholate dialysis method. ^d	47
A-IV	25			
E-2	40			
E-3	30			
A-I	100		Vesicles.	28-30
A-I (Pro ¹⁴³ -Arg)	60-70			
A-I (Lys ¹⁰⁷ -0)	40-60			
Pro-apoA-I	100			

^aBased on relative initial velocity measurements at equivalent bulk PC or apolipoprotein (mg/ml) concentrations, and equal LCAT concentrations (4).

^bFrom linear Lineweaver-Burk plots; CE, cholesteryl ester.

^cEgg-PC was used in preparation of substrate particles.

^dAlthough the references indicate that "proteoliposomes" were prepared by the sodium cholate method, it is very likely that the products were discoidal. Other laboratories have observed discs under similar preparation conditions (39, 40).

groups. However, extensive charge modification with concomitant structural changes in apoA-I does inhibit LCAT activation, supporting the hypothesis that an apoA-I structural domain(s) determines its optimal activation of LCAT (48). Finally, discoidal complexes of various sizes (approximately 100, 200, and 380 Å diameters) prepared with DPPC, cholesterol, and apoA-I, have variable apoA-I structures depending on the size of the particles. The structural variability is detected in the spectral (fluorescence) properties of apoA-I in these particles. The intermediate size, 200-Å particles, are most unreactive whereas the smallest, 100-Å particles, are the most reactive with LCAT (40). Here again, altered apoA-I structure and/or altered apolipoprotein boundary with lipid affect the activation of LCAT.

Acyl donors

As indicated earlier, problems with vesicle stability and variable apolipoprotein binding to vesicles of different PC compositions and cholesterol contents have precluded their effective use in studies of acyl donor specificity. Discoidal particles, in contrast, are stable and, in most cases, contain irreversibly bound apolipoproteins. However, even physically similar discoidal particles prepared with various PCs present different interfaces to the enzyme in

terms of apolipoprotein structure, boundary between apolipoprotein and lipid, fluidity of the bilayer, and lipid polar head group separation. Thus, the apparent kinetic constants shown in Table 2 reflect the molecular and interfacial selectivity of LCAT for the series of particles with different PCs; in general, mixed chain PC particles are the preferred substrates of LCAT, followed by disaturated PCs of increasing chain length, and by long chain polyunsaturated-PC particles.

Pownall, Pao, and Massey (49) were able to separate the molecular and interfacial selectivity of LCAT by using discoidal particles containing mostly an ether PC analog matrix (89 mole %) and including apoA-I (1 mole %), test lipid (10 mole %), and a trace of radiolabeled cholesterol reagent. The enzymatically unreactive matrix lipids, ether analogs of DMPC, DPPC, and POPC, and sphingomyelin, were shown to affect markedly the absolute reactivity of test glycerophospholipids. The best matrix was provided by the POPC ether analog, followed by the DMPC, DPPC analogs, and then by sphingomyelin. The relative reactivity of test lipids within a common matrix (i.e., in an equivalent interface) indicate that phosphatidylethanolamine (PE) is a somewhat better substrate for LCAT than PC, and that dimethyl N-PE, PG, PA, and PS are also reactive with the enzyme. Di- and tripalmitin are not reactive with LCAT. In terms of acyl chain com-

position of PC substrates, LCAT prefers shorter chain disaturated PCs, and the diunsaturated 18-carbon PC series have improved reactivity over distearoyl-PC (DPPC). The results of Pownall et al. (49) are depicted in Fig. 2. It can be concluded from these observations that the LCAT binding site for the acyl donor recognizes the phosphate glycerol backbone, has some selectivity for a basic head group, but does not exclude other glycerophospholipid head groups. There must be a large, hydrophobic region for the recognition of acyl chains, which favors the binding of shorter, saturated, less bulky chains.

LCAT reaction kinetics

A few reports on the kinetics of the LCAT reaction with vesicles (43) and with HDL (50) substrates have appeared in the literature, but systematic studies have only been performed with discoidal particles. In all the LCAT reactions investigated so far, apparent Michaelis-Menten kinetics have been observed, giving apparent K_m and V_{max} values, which do not have the common meaning found in simple enzyme kinetics. Various kinetic models have been proposed for interfacial enzymes: the one introduced by Verger, Mieras, and de Haas (51) is applicable to the reaction of LCAT with discoidal substrates. In this model $K_m = K_d (K_m^* S)/(S + K_m^*)$ and $V_{max} = k_{cat} E_o \cdot S/(S + K_m^*)$ where K_d is the dissociation constant of the enzyme-

interface complex, K_m^* is the intrinsic Michaelis-Menten constant, S the two-dimensional concentration of the molecular substrate in the interface, k_{cat} the catalytic rate constant, and E_o the total enzyme concentration.

Although the apparent kinetic constants are readily obtained for a variety of substrates, they cannot give directly the intrinsic constants or provide a mechanistic interpretation for observed differences in reactivity. This would require the independent determination of K_d , not an easy task with an enzyme that can only be prepared in very small amounts. Nevertheless, until equilibrium binding results become available, apparent V_{max} over apparent K_m ratios ($k_{cat} E_o/K_d K_m^*$) may be used as a very convenient, quantitative measure for the efficiency of LCAT with different substrates. Tables 2 and 3 illustrate the use of apparent V_{max} /apparent K_m ratios in ranking various LCAT substrates. Table 2 also includes activation energy values from the determination of the temperature dependence of reaction rates.

Additional kinetic information may be obtained by diluting molecular substrates in the interface with an enzymatically unreactive amphiphile. In studies with phospholipases, detergents have been used in different proportions with lipid substrates in the form of small micelles (52). We used the DPPC ether analog to dilute DPPC and egg-PC substrates in the interface of discoidal

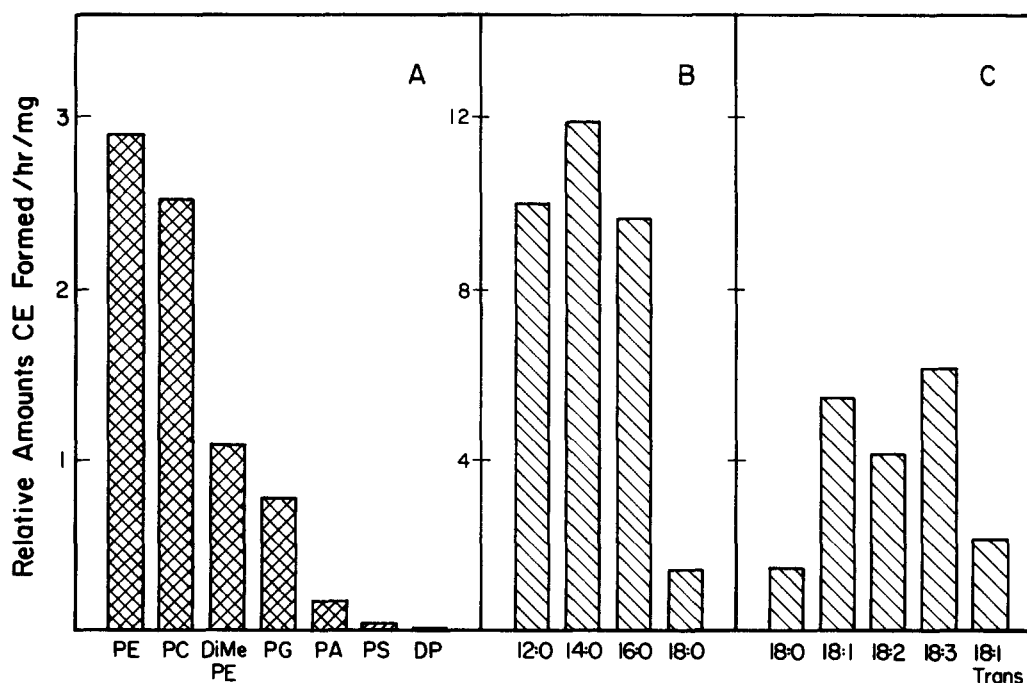


Fig. 2. Molecular specificity of human LCAT for phospholipids. The test phospholipids represent 10 mole %, radiolabeled cholesterol (trace), apolipoprotein A-I 1 mole %, and the matrix lipid (an ether PC analog) 89 mole % of discoidal particles prepared by the sodium cholate method. Relative reaction velocities were obtained under conditions approaching enzyme saturation with substrates. Panel A represents the headgroup selectivity of LCAT using palmitoyl oleoyl-test phospholipids in a DMPC ether matrix. Dipalmitin (DP) and tripalmitin (not shown) were essentially unreactive. Panel B shows the chain length effects using diacyl-PC test lipids in a palmitoyl oleoyl-PC ether matrix. Panel C represents the effects of unsaturation with distearoyl-PC (18:0), dioleoyl-PC (18:1), dilinoleoyl-PC (18:2), dilinolenoyl-PC (18:3), and the *trans*-isomer of dioleoyl-PC (18:1, *trans*) all present in a palmitoyl oleoyl-PC ether matrix. Adapted with permission from reference 49 by Pownall et al.

particles with apoA-I (53). The kinetics indicated inhibition of LCAT by added ether PC, not only as a diluent of the interfacial PC, but also as a competitive inhibitor at the active site.

Modulators of LCAT activity

Since most chemical modulators of LCAT activity, such as sulfhydryl, serine reagents, and heavy metal ions, act at the level of the enzyme, reactions of LCAT with discoidal substrates and vesicle substrates are equally susceptible to these agents. Product inhibition by lyso-PC and cholesteryl esters and detergent effects may occur directly on the enzyme or on the substrate particles; therefore, they may affect the LCAT reaction differently, depending on the nature of the substrate particle. Systematic studies of the latter effectors have not been performed with discoidal substrates, but inclusion of serum albumin in the reaction mixtures increases LCAT activity similarly to the vesicle system. On the other hand, cholesteryl ester accumulation inhibits reaction in vesicles much more than it does in discoidal particles (37, 43).

Using vesicle substrates, Furukawa and Nishida (36) attributed the increase in LCAT activity, and the decrease in its stability at an ionic strength of 0.1, to a conformational change in the enzyme. We recently investigated neutral salt effects on the LCAT reaction with discoidal substrates of apoA-I with various PCs, and established that the kinetic effects of salts were due to modifications of the enzyme rather than the substrate particles (54). The salt effects can be attributed to the monovalent anions (rather than to the cations in the system) following the lyotropic series in their inhibiting capacity. The order of increasing inhibition is: F^- , Cl^- , Br^- , NO_3^- , I^- , and SCN^- . LCAT reactivity with different PC particles is affected in different ways by these ions. Thus discoidal egg-PC-containing particles react optimally in 0.15 M NaCl and show marked inhibition at a 1 M NaCl concentration. Particles containing DPPC and DMPC have very low reactivities in the absence of NaCl (in the background, 10 mM Tris buffer, pH 8.0). The DPPC particles reach maximum reactivity at 0.15 M NaCl but are only slightly inhibited by 1 M NaCl; DMPC particles, on the other hand, increase monotonically in reactivity with increasing NaCl concentration, becoming more reactive than either egg-PC or DPPC particles in 1 M NaCl. Although a mechanistic explanation is not yet available for these results, it is clear that modulators may change the order of LCAT reactivity with substrate particles containing different PCs.

CONCLUSIONS

The studies of the LCAT reaction using synthetic substrates have contributed significantly to our understanding of the LCAT reaction in plasma. It is clear now

that apoA-I, by virtue of its structure, is the best activator of LCAT, but apoE, apoA-IV, and apoC-I are good activators of LCAT to the extent that, in the absence of apoA-I in vivo, they may promote significant cholesterol esterification in plasma.

Regarding the acyl donor specificity of LCAT, the enzyme may play a role in the turnover in plasma of glycerophospholipids other than PC, especially PE. In terms of abundance, cholesteryl linoleate is the main product of the LCAT reaction in plasma, but the synthesis of cholesteryl palmitate is favored kinetically, accounting for the relatively elevated amounts of saturated cholesteryl esters compared to the availability of *sn*-2 saturated chains in the glycerophospholipid acyl donors. The reducing medium of plasma, the presence of serum albumin, and the ionic composition near 0.15 M NaCl of plasma are optimal for the activity of LCAT. Thus the modulation of LCAT activity in vivo depends, most likely, on changes in substrate composition and structure.

In closing, it should be mentioned that synthetic spherical analogs of HDL, to be used as LCAT substrates, are currently under development in our laboratory. One of the approaches to their preparation is to transform discoidal particles, enriched with free cholesterol, into spherical particles by the enzymatic action of LCAT; another approach is to cosonicate all the lipid components (PC, cholesterol, and cholesteryl ester) into a microemulsion, followed by the addition of denatured apoA-I and further sonic dispersion (personal communication, Dr. M. Walsh, Boston University, and ref. 55). In addition to discoidal and spherical HDL analogs, lipids dispersed in detergent micelles, pure lipid micelles prepared with short chain PCs, and monomeric PC substrates may be useful alternatives to vesicles and discoidal micelles in the continuing investigation of the mechanism of action of LCAT. ■■

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