thematic review

Catalysis of ACAT may be completed within the plane of the membrane: a working hypothesis

Ta Yuan Chang,[1] Catherine C. Y. Chang, Xiaohui Lu, and Song Lin

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755-3844

Abstract **Two ACAT sharing protein sequence homology near their C termini have been identified. Both proteins may span the endoplasmic reticulum (ER) membrane several times. There is good evidence implicating the role of ACAT1 in macrophage foam cell formation, and ACAT2 in intestinal cholesterol absorption. On the other hand, the functional roles of ACAT1 and ACAT2 in the VLDL or chylomicron assembly process are less clear. It is possible that both enzymes are able to form lipid droplets (which are present in the cytoplasm), and participate in lipoprotein assembly (which occurs in the ER lumen). To link the site of ACAT catalysis with its function, we propose that part of the ACAT catalytic site may reside within the lipid bilayer, allowing catalysis to be completed within the plane of the membrane. Cholesteryl esters (CE) produced in situ may burst into cytoplasmic lipid droplets, carrying phospholipid monolayers as their outer coats. In cells engaged in lipoprotein assembly and secretion, CE in the bilayer may be recognized by the specific protein microsomal triacylglycerol transfer protein (MTP), reaching out from the lumenal side of the membrane. MTP then lipidates the growing apolipoprotein B (apoB) chain with CE and TG during the early stages of apoB lipoprotein assembly.**—Chang, T. Y., C. C. Y. Chang, X. Lu, and S. Lin. **Catalysis of ACAT may be completed within the plane of the membrane: a working hypothesis.** ***J. Lipid Res.*** **2001.** 42: **1933–1938.**

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The current review contains a brief overview of certain key aspects of ACAT, and a working hypothesis. The hypothesis represents the authors' personal view and attempts to link the site of ACAT catalysis with its presumed dual functions in vivo. For detailed information about ACAT and ACAT inhibitors, the readers are encouraged to read several reviews and the references cited therein (1–9).

ACAT utilizes long-chain fatty acyl-CoA and cholesterol to catalyze the formation of cholesteryl esters (CE). In hepatocytes and intestinal enterocytes that specialize in lipoprotein assembly and secretion, CE produced by ACAT become part of the neutral lipid core of the lipoproteins (i.e., VLDL and chylomicrons). In most other tissues, the main function of ACAT is to produce CE as cytoplasmic lipid droplets. CE droplets serve as the major reservoir for free (unesterified) cholesterol, which is an important component of cell membranes. In steroidogenic tissues, CE are reservoirs for free cholesterol that serve as the precursor for biosynthesis of various steroid hormones. In early stages of the disease atherosclerosis, resident macrophages within the intimal layer of the artery take up denatured LDL through the actions of various scavenger receptors. Cholesterol liberated from denatured LDL is esterified by ACAT in a substrate-driven manner. This process may lead to CE-loaded foam cells within the arterial wall. On the basis of its physiological functions, ACAT has been considered as one of the major pharmaceutical targets for therapeutic intervention of hypercholesterolemia and atherosclerosis. In various animal models tested, ACAT inhibitors exhibit significant efficacy in reducing the size of foamy plaques. At present, the most effective drugs in treating hypercholesterolemia and atherosclerosis are statins, which are potent inhibitors of the rate-limiting enzyme HMG-CoA reductase. The roles of ACAT in cellular cholesterol homeostasis are distinct from those of HMG-CoA reductase. Therefore, it seems conceivable that ACAT inhibitor as a drug may complement the actions of the statins and further reduce the risk of coronary heart diseases in humans.

ACAT is a membrane-bound enzyme present in the microsomal fractions prepared from many different types of tissues and cells. Because the amount of ACAT protein present in most preparations is exceedingly small, conventional biochemical approaches failed to disclose its molecular identity. The first ACAT gene, *ACAT1*, was identified by a combination of somatic cell and molecular genetic approaches (10). Later, a different gene, *ACAT2*, was identified on the basis of protein sequence homology with

Abbreviations: CE, cholesteryl ester; CHO, Chinese hamster ovary; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; HA, hemagglutinin; MTP, microsomal triacylglycerol transfer protein; pCMBS, *p*-chloromercuribenzenesulfonic acid.

[1] To whom correspondence should be addressed.
e-mail: ta.yuan.chang@dartmouth.edu

ACAT1 near their C termini (11–13). Both ACAT1 and ACAT2 contain hydrophobic domains that may span the membrane multiple times. The human *ACAT1* gene is located on two different chromosomes (1 and 7), with the coding region and the proximal promoter located on chromosome 1. An optional 5′ noncoding region and distal promoter that control the expression of this noncoding region are located on chromosome 7 (14). ACAT1 is a homotetrameric protein (15). Depending on the techniques used for analysis, it may span the endoplasmic reticulum (ER) membrane seven times (16) or five times (17). In humans, ACAT1 is ubiquitously present in a variety of tissues and cell types that include hepatocytes, macrophages, adrenals, skin, and neurons (18, 19). The ACAT1 tissue distribution in mice and in monkeys is similar to that in humans, with the important exception that ACAT1 seems to be nearly absent in mouse liver (20–22) and in monkey hepatocytes (23). For ACAT1, the main mode of cholesterol-dependent regulation is by allosteric control of the substrate cholesterol (24). The *ACAT1* gene is not transcriptionally regulated by cholesterol; the sterol regulatory element (SRE), present within the promoters of many cholesterol-regulated genes (25), cannot be found within the *ACAT1* gene promoters. In cell culture studies, human *ACAT1* gene expression is significantly increased during the monocyte-macrophage differentiation process (26, 27). In vivo, ACAT1 detected by immunohistochemical staining is abundantly present in lipid-rich atherosclerotic lesions (27). Additional cell culture work showed that in the human monocytic cell line THP-1, *ACAT1* gene expression is upregulated by 1,25-dihydroxyvitamin D_3 (28). In human macrophages and macrophage-like cells, the *ACAT1* gene is upregulated by the cytokine interferon γ (29).

The mouse *ACAT2* gene is located on chromosome 12 (12); its genomic structure has been determined (30, 31). Similar to the *ACAT1* promoter, the sequence that conforms to the SRE-like element cannot be found within the human *ACAT2* promoter (30). Immunological quantitation and localization studies showed that in human small intestine, ACAT2 is mainly located at the apical region of the villi, whereas ACAT1 is uniformly distributed along the villus-crypt axis (32). ACAT2 is also present in adult human hepatocytes; however, on the basis of immunoblot analyses and immunodepletion studies in vitro, ACAT2 comprises only a minor portion (15% or less) of the total ACAT activity found in hepatocyte extracts, with the remainder of the activity attributed to ACAT1 (32). Consistent with this finding, by using a reporter gene construct in transient transfection studies the human *ACAT2* promoter was found to be expressed preferentially in differentiated CaCo-2 cells, which are intestinal enterocyte-like cells, than in HepG2 cells, which are hepatocyte-like cells (30). ACAT1 and ACAT2 display similar sigmoidal responses to cholesterol as their substrates, implying that for both enzymes, cholesterol serves as an activator as well as a substrate for reaction. On the other hand, subtle differences between the properties of ACAT1 and ACAT2 could be found; for example, a certain ACAT inhibitor (Dup128) inhibited ACAT2 more efficiently than ACAT1 (32).

Similar to humans, monkeys and mice express ACAT2 as the major isoenzyme in intestinal enterocytes. Unlike humans, mice and monkeys express ACAT2 (not ACAT1) as the major isoenzyme in hepatocytes (23, 33). Intestinal enterocytes and hepatocytes specialize in lipoprotein assembly and secretion. The results of ACAT1/ACAT2 mRNA and protein distribution studies in mice and in monkeys, as well as the phenotypes of mice lacking ACAT1 or ACAT2 (20, 21), led Rudel and colleagues (8, 11, 17) to put forth an interesting hypothesis regarding their distinct functions. The hypothesis proposes that ACAT1 may be involved in forming CE lipid droplets (in macrophages and other cell types), whereas ACAT2 may be involved in supplying the CE for lipoprotein assembly. It is known that CE lipid droplets are found in the cytoplasm, whereas the lipoprotein assembly process occurs within the lumen of ER. On the basis of their presumed physiological roles, Rudel and colleagues reasoned that the active sites of ACAT1 and ACAT2 may be located on opposite sides of the ER membrane, with the active site of ACAT1 facing the cytoplasm, and that of ACAT2 facing the lumenal side of the membrane. With regard to the ACAT active site, a conserved serine residue (Ser-269 in human ACAT1) is present in ACAT1, ACAT2, and a closely related enzyme called diacylglycerol acyltransferase (DGAT), whose gene was identified by Farese and colleagues (34). DGAT utilizes fatty acyl-CoA and diacylglycerol as substrates to produce TG. For ACAT1 or ACAT2, substituting leucine for this conserved serine caused the enzyme to lose activity when the altered enzyme was expressed in mammalian cells (17, 35), or in yeast cells (36). Thus, this conserved serine may be part of the ACAT active site (17). Membrane topographical studies of ACAT1 and ACAT2 led Joyce and colleagues (17) to propose that the putative active site serine for ACAT1 is located in the cytoplasmic side of the ER; whereas the equivalent serine for ACAT2 is located on the lumenal side of the ER. On the other hand, for the following reasons, the authors retain reservations about this hypothesis: *1*) In humans, ample ACAT1 proteins are found in adult hepatocytes (19), adult liver, and the hepatocyte-like HepG2 cells (32). Because both ACAT1 and ACAT2 are present in human hepatocytes and intestinal enterocytes, there is no a priori reason to assign the physiological function of these two isoenzymes in a mutually exclusive manner; and *2*) when expressed in a mammalian cell line that is not involved in the lipoprotein assembly and secretion process, both ACAT1 and ACAT2 are able to produce large amounts of CE as cytoplasmic lipid droplets (32).

In addition, in the authors' own opinion, the result implicating that the Ser-269 in ACAT1 (and the equivalent serine in ACAT2) is part of the ACAT active site can only be regarded as a tentative suggestion. It is possible that the serine-to-leucine mutation at position 269 causes gross structural alteration of the enzyme that leads to inactivation of the enzyme; it is also possible that this particular mutation causes the enzyme to be degraded in intact cells

much faster than the normal, wild-type enzyme. To distinguish among these possibilities, additional mutagenesis work that involves the change of serine to conservative amino acid(s) should be performed. A membrane-bound acyltransferase superfamily that includes ACAT, DGAT, and 20 other enzymes/proteins of diverse biological functions was identified; an invariant histidine residue (His-460 in human ACAT1) located within a long stretch of hydrophobic amino acids (more than 20) has been proposed to constitute part of the active site (37). Earlier, Lin and colleagues (16) found that inserting a short, antigenic tag [hemagglutinin (HA), nine amino acids] within this region (immediately before amino acid 459) completely inactivated the recombinant human ACAT1 enzyme activity. In addition, the HA tag inserted at this position could not be detected either by Western analysis or by immunofluorescence of permeabilized cells [result described on p. 23283 of ref. (16)]. These results suggested that the peptide region around amino acid 459 may be closely associated with the membrane lipid bilayer (16). In a separate study, Lu and colleagues (38) found that treating human ACAT1 with *p*-chloromercuribenzenesulfonic acid (pCMBS), a bulky sulfhydryl modification reagent, completely inactivated ACAT enzyme activity. Human ACAT1 contains nine cysteine residues. Lu and colleagues showed that none of the cysteine residues is needed for ACAT enzyme to be active; however, attaching the bulky mercuribenzenesulfonic acid group at Cys-467 completely inactivated the enzyme activity, suggesting that Cys-467 is near but not at the ACAT active site. The studies by Lin et al. (16) and by Lu et al. (38) support the idea that His-460 is part of the ACAT active site. The exact function of His-460 in ACAT catalysis is not known at present. On the basis of mutagenesis of conserved sequences, Guo and colleagues (36) proposed that several other residues may be involved in substrate binding of ACAT.

On the basis of the above discussions, we formulate the following hypothesis: we believe that it is possible that both enzymes are able to form lipid droplets that are present in the cytoplasm, and also that both enzymes participate in lipoprotein assembly, which occurs in the ER lumen. To link the site of ACAT catalysis with its function, we propose that part of the ACAT catalytic center may be located within the plane of the ER membrane, allowing the enzyme to produce CE in situ. We elaborate our hypothesis as follows. In general, an enzyme-catalyzed reaction consists of two steps: binding and catalysis. Usually, the binding site(s) for the substrate(s) and the catalytic center of the enzyme are in close proximity but do not overlap with each other. ACAT may prefer to utilize fatty acyl-CoA and cholesterol that are already bound to the ER membrane. Fatty acyl-CoA are not permeable to membranes (39); they may partition at the cytoplasmic side of the bilayer (40), while cholesterol may partition either at the cytoplasmic side or at the lumenal side of the bilayer. These two substrates laterally diffuse along the membrane, encounter ACAT, and cause binding between the enzyme and the two substrates to occur. The binding site of ACAT for fatty acyl-CoA may be located in close proximity to the cytoplasmic side of the membrane. The binding site of ACAT for cholesterol may involve certain transmembrane domains of the enzyme. Part of the ACAT catalytic center, particularly those amino acid residues involved in the transfer of the fatty acyl moiety from the fatty acyl-CoA to the 3β-OH of cholesterol, may reside within the membrane lipid bilayer. Binding between ACAT and the two substrates may cause conformational change of the enzyme, enabling the catalytic center of the enzyme to be exposed to the bound substrates, thus allowing the enzyme to complete the catalytic process within the plane of the membrane. The product CE formed in situ may encounter one of two fates: the accumulation of CE within the lipid bilayer (41) destabilizes the local membrane region, causing CE to burst into cytoplasmic lipid droplets, carrying a monolayer of phospholipid as their outer coats. This process may or may not be assisted by specific protein(s). Alternatively, in cells engaged in lipoprotein assembly, the specific protein called microsomal triacylglycerol transfer protein (MTP) recognizes CE present in the lipid bilayer. MTP can transfer both TG and CE between membranes and liposomes (42), and is mainly found in hepatocytes and intestinal enterocytes. It is generally agreed that MTP plays a key role in the lipidation of the growing apolipoprotein B (apoB) chain with neutral lipids during the early stage of the apoB lipoprotein assembly process (43). The functional form of MTP is as a heterodimeric complex with another protein, called protein disulfide isomerase (44). MTP is located exclusively within the lumen of the ER, but is capable of penetrating the inner leaflet of the lipid bilayer, reaching out for neutral lipids TG and CE (45). Binding of MTP to CE (and probably to TG) would allow MTP to lipidate the growing apoB chain with CE (and with TG) (46, 47). Binding of MTP to CE (and TG) may also lead to accumulation of CE (and TG) in the lumen of certain vesicles that are involved in later stages of the lipoprotein assembly process [for discussion of this possibility, see ref. (44)]. Irrespective of the difference in membrane topography between ACAT1 and ACAT2, our model would allow both ACAT1 and ACAT2 to participate in CE lipid droplet formation and in lipoprotein assembly. It may also explain why many of the potent ACAT inhibitors tend to be hydrophobic molecules that can partition in the lipid bilayer. At present, we do not know what type of enzyme-substrate intermediate may form during ACAT catalysis; however, the mechanism we propose would protect the reactive intermediate formed from coming in contact with water. The principles on which our model is based may also serve to explain in part how DGAT may be involved in cytoplasmic TG lipid droplet formation and in various stages of lipoprotein assembly. Our model is depicted in **Fig. 1**. To test the validity of this model, further experimentation at the biochemical and cell biological levels will be needed. Other examples of enzyme catalysis occurring within the plane of the membrane can be found in the review by Brown et al. (48).

If both ACAT1 and ACAT2 participate in cytoplasmic lipid droplet formation and in lumenal lipoprotein assembly, why should single cell types such as hepatocytes and intestinal enterocytes express both ACAT1 and ACAT2? The answer is not known, and we can offer only some

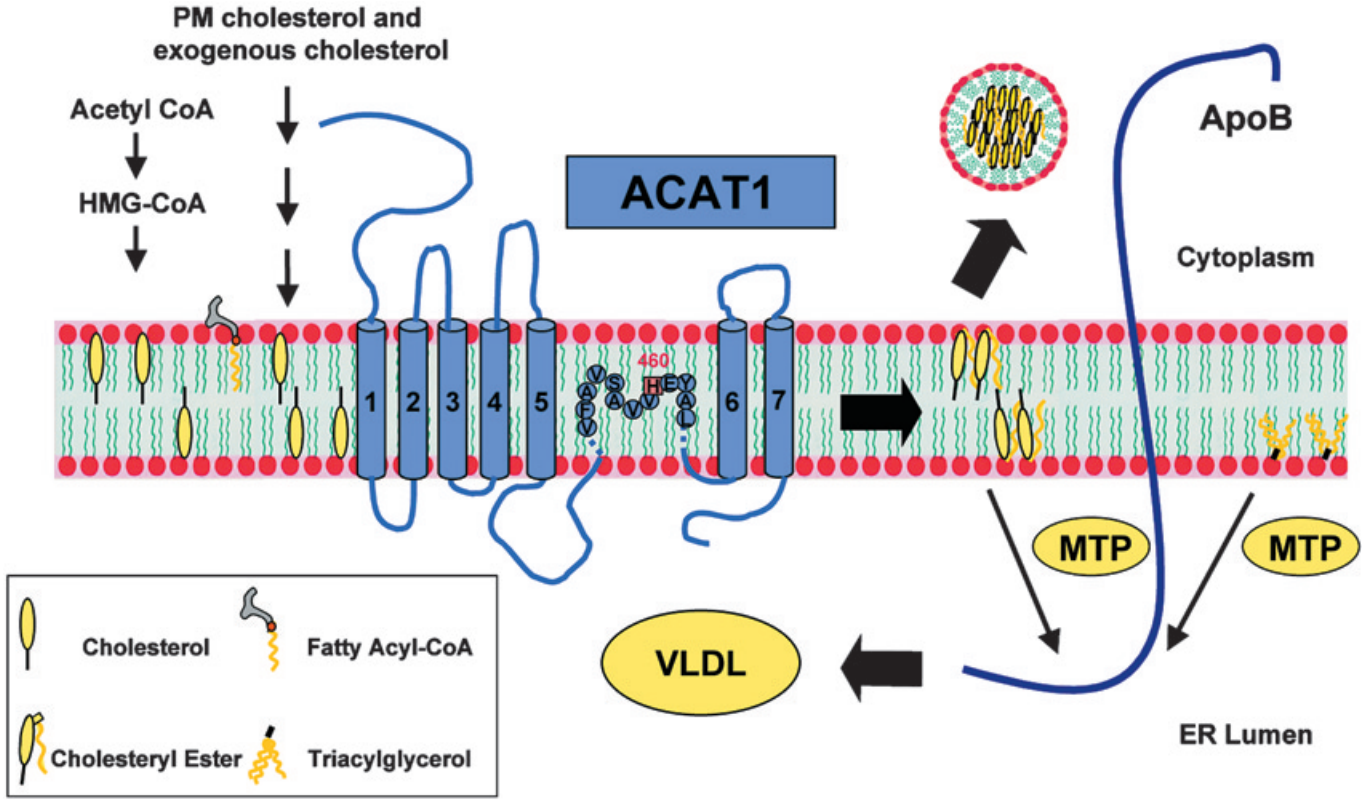

Fig. 1. A hypothetical model describing the involvement of ACAT in cytoplasmic CE lipid droplet formation and in lumenal lipoprotein assembly. The model proposes that part of the ACAT catalytic site, including His-460, may be located within the lipid bilayer, enabling the enzyme to produce CE in the same membrane. The extent of membrane insertion of the hydrophobic peptide, including the sequence $VFAVSAVVH_{460}EYAL$, is uncertain at present. For simplicity, only ACAT1, with the seven-transmembrane domain model [as proposed by Lin and colleagues (16)], is drawn. ACAT1 may contain only five transmembrane domains, as proposed by Joyce and colleagues (17). Irrespective of the difference in membrane topography between ACAT1 and ACAT2, the basic principle described in this model may apply to both ACAT1 and ACAT2, and may also apply to the chylomicron assembly process in intestinal enterocytes. As shown here, ACAT can use exogenous cholesterol, cholesterol internalized from the plasma membrane, or cholesterol synthesized endogenously as its substrate. Depending on the cell types, exogenous cholesterol includes cholesterol derived from LDL, HDL, chylomicron remnant, and/or from intestinal micelles, etc. See text for details. PM, Plasma membrane.

speculation at this point. These are polarized cells that express various lipoprotein receptors at their surfaces, including the LDL receptor, the chylomicron remnant receptor, the HDL receptor, etc. These receptors mediate the uptake of various lipoproteins. Exogenous cholesterol derived from these lipoproteins is then delivered to the cell interior for esterification at the ER membrane. In addition, cholesterol synthesized de novo (through the acetyl-CoA/HMG-CoA pathway), and cholesterol internalized from the plasma membrane, is also available to ACAT for esterification. For intestinal enterocytes, the apical sides of their plasma membranes are actively involved in the uptake of dietary cholesterol and biliary cholesterol, whereas the basolateral sides of their plasma membranes are involved in the uptake of LDL. ACAT is generally believed to reside in the ER; however, the "ER" membranes are composed of heterogeneous subcellular organelles; they can be further categorized as rough ER, smooth ER, and various ER-derived vesicles. It is tempting to speculate that ACAT1 and ACAT2 in these cells may be localized at different types of ER membranes and/or ER-derived vesicles, to provide optimal routes for cholesterol esterification. In addition, it is also possible that the modes of regulation of ACAT1 and ACAT2 in hepatocytes and in enterocytes by various nonsterol agents are distinct from each other. Future research focusing on using single cell types that express both isoenzymes may help to better define the roles of ACAT1 and ACAT2 in a tissue-specific manner.

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