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REVIEW

Acyl-coenzyme A: cholesterol acyltransferase family

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Abstract The enzymes of the acyl-coenzyme A: cholesterol acyltransferase (ACAT) family are responsible for the *in vivo* synthesis of neutral lipids. They are potential drug targets for the intervention of atherosclerosis, hyperlipidemia, obesity, type II diabetes and even Alzheimer's disease. ACAT family enzymes are integral endoplasmic reticulum (ER) membrane proteins and can be divided into ACAT branch and acyl-coenzyme A: diacylglycerol acyltransferase 1 (DGAT1) branch according to their substrate specificity. The ACAT branch catalyzes synthesis of cholesteryl esters using long-chain fatty acyl-coenzyme A and cholesterol as substrates, while the DGAT1 branch catalyzes synthesis of triacylglycerols using fatty acyl-coenzyme A and diacylglycerol as substrates. In this review, we mainly focus on the recent progress in the structural research of ACAT family enzymes, including their disulfide linkage, membrane topology, subunit interaction and catalysis mechanism.

Keywords lipid, acyl-coenzyme A: cholesterol acyltransferase (ACAT), acyl-coenzyme A: diacylglycerol acyltransferase 1 (DGAT1), acyltransferase, catalysis

1 Introduction

The acyl-coenzyme A: cholesterol acyltransferase (ACAT) family is a small enzyme family comprising three homologous members, acyl-coenzyme A: cholesterol acyltransferase 1 and 2 (ACAT1 and ACAT2), and acyl-coenzyme A: diacylglycerol acyltransferase 1 (DGAT1). These enzymes are responsible for *in vivo* neutral lipid synthesis. Among them, ACAT1 and ACAT2 catalyze the synthesis of cholesteryl esters using long-chain fatty acyl-coenzyme A and free cholesterol as substrates, while DGAT1 catalyzes the synthesis of triacylglycerols using long-chain fatty acyl-coenzyme A and diacylglycerol as substrates. Both ACAT and DGAT1 have been extensively reviewed in past years (Chang et al., 1997, 2001a; Farese, 1998; Buhman et al., 2000; Chen and Farese, 2000, 2005; Rudel et al., 2001). Therefore, in this review we mainly focus on the recent progress in their structural research, including their disulfide linkage, membrane topology, subunit interaction and the catalysis mechanism.

ACAT family enzymes have important biological functions. ACAT1 and ACAT2 are critical for *in vivo* cholesterol homeostasis. At the single cell level, they prevent excess free cholesterol from building up in the cell membranes. At the physiological level, they contribute cholesteryl esters as part of neutral lipid cargo, to be packaged into the cores of very low density lipoproteins and chylomicrons. Under pathophysiological condition, they convert excess cholesterol into cholesteryl esters in cholesterol-loaded macrophages. The macrophages are gradually converted into foam cells, which is a hallmark of early lesions of atherosclerosis. Therefore, ACAT plays a critical role in the progress of atherosclerosis. For the intervention of atherosclerosis, various ACAT inhibitors have been designed and isolated (Miyazaki et al., 2005; Chang et al., 2006). Unfortunately, recent clinical trials of ACAT inhibitors have failed (Tardif et al., 2004; Nissen et al., 2006). For patients with coronary disease, treatment with an ACAT inhibitor had adverse effects rather than positive effects (Fazio and Linton, 2006). Moreover, recent studies show that ACAT1 is also relevant to Alzheimer's disease (Puglielli et al., 2001; Hutter-Paier et al., 2004; Huttunen et al., 2007). ACAT1-knockout/knockdown or ACAT inhibitors can reduce Aβ production in the mouse brain. A recent study also has shown that ACAT1 is related to long-term memory in mice (Matynia et al., 2008).

DGAT is responsible for *in vivo* synthesis of triacylglycerols. Two nonhomologous isozymes, DGAT1 and DGAT2, have been identified. Of these, DGAT1 belongs to the ACAT family, while DGAT2 belongs to the DGAT2 family (Cases et al., 2001; Turkish et al., 2005). Besides triacylglycerol, DGAT1 can also synthesize retinyl esters, diacylglycerols and waxes (Orland et al., 2005; Yen et al., 2005; Wongsiriroj et al., 2008). DGAT1-knockout mice

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have increased energy expenditure and insulin sensitivity, and therefore are protected against diet-induced obesity and glucose intolerance (Smith et al., 2000; Chen et al., 2002a, 2003b). Mice transplanted with white adipose tissue lacking DGAT1 also have similar phenotype (Chen et al., 2003a). Over-expression of DGAT1 in mice produced controversial results (Chen et al., 2002b, 2005). A DGAT1 inhibitor can also reproduce major phenotypical characteristics of DGAT1-knockout mice (Zhao et al., 2008), suggesting DGAT1 inhibitors could be used for the treatment of obesity and type II diabetes in future.

2 Cloning of ACAT family enzymes

The ACAT activity has been known as early as the 1970s (Drevon and Norum, 1975; Haugen and Norum, 1976; Norum et al., 1977; Beck and Drevon, 1978), but purification of the enzyme failed due to its minute quantity in various tissues. Using an ACAT-deficient Chinese hamster ovary (CHO) cell line (Cadigan et al., 1988), Chang's laboratory at Dartmouth College first cloned the full-length cDNA of human ACAT1, one of two ACAT isozymes, in 1993 (Cadigan et al., 1989; Chang et al., 1993). The cloning of human ACAT1 is a milestone of ACAT family research. It paves the way for cloning of other homologous enzymes; it also opens an era of ACAT research using the combined approach of molecular biology and biochemistry.

After the breakthrough of cloning human ACAT1, more ACAT1s and its homologues were cloned from different species in later years. In 1996, both Sturley's laboratory and Rothblatt's laboratory cloned two ACAT1 homologues from yeasts (Yang et al., 1996; Yu et al., 1996). In baker's yeast (*Saccharomyces cerevisiae*), two ACAT genes exist. They encode two homologous isozymes, ARE1 and ARE2, which can esterify yeast sterols, such as ergosterol. They can also esterify cholesterol, but with a lower preference. In 1995, Chan's laboratory cloned mouse ACAT1 (Uelmen et al., 1995). In the next year, Farese's laboratory obtained ACAT1-knockout mice (Meiner et al., 1996). ACAT1-knockout in mice results in decreased cholesterol esterification in fibroblasts and adrenal membranes, and markedly reduces cholesterol ester levels in adrenal glands and peritoneal macrophages, suggesting that ACAT1 plays a major role in these tissues. However, the liver of ACAT1-deficient mice contains substantial amounts of cholesterol esters and exhibits no reduction in cholesterol esterification activity, suggesting other unknown ACAT isozyme(s) is present in the liver. In 1998, three laboratories reported their cloning of ACAT2, a homologous isozyme of ACAT1 (Cases et al., 1998a; Oelkers et al., 1998; Anderson et al., 1998). In contrast to the ubiquitous expression of ACAT1, ACAT2 is expressed primarily in the liver and small intestine, suggesting that ACAT2 contributes to cholesterol esterification in these tissues (Chang et al., 1995, 2000; Lee et al., 1998, 2000; Miyazaki et al., 1998; Khelef et al., 1998; Sakashita et al., 2000).

Besides ACAT2, another homologous enzyme of ACAT1 was also cloned in 1998 by both Sturly's laboratory and Farese's laboratory (Cases et al., 1998b; Oelkers et al., 1998). Activity analysis shows that the enzyme had no ACAT activity, but it could esterify diacylglycerols using acyl-coenzyme A as acyl donor (Cases et al., 1998b). Thus, the enzyme was designated as DGAT1, which was a key enzyme in triacylglycerol biosynthesis *in vivo*.

3 Disulfide bond of ACAT family enzymes

All of the ACAT family enzymes contain several cysteine residues (human ACAT1: 9 cysteines; human ACAT2, 13 cysteines; human DGAT1, 8 cysteines) (Fig. 1). Cysteines in proteins can exist in two states: free cysteine or forming a disulfide bond between two cysteines. Formation of disulfide bond usually has a profound effect on protein structure and function. Therefore, experimental approaches have been designed to quantitate and map protein disulfide bonds. However, these conventional approaches cannot be used to analyze ACAT family enzymes in that all of them need a significant amount of purified proteins. Using an mPEG5000-maleimide (PEG-mal) modification approach, Guo et al. quantitated the disulfide of human ACAT1: human ACAT1 contains only one disulfide bond (Guo et al., 2005a). PEG-mal is a large cysteine-specific modification reagent whose attachment can significantly shift the modified protein band on SDS-PAGE (Lu and Deutsch, 2001; Katzen and Beckwith, 2003; Kosolapov and Deutsch, 2003). Using mutant ACAT1s with a single cysteine to alanine replacement, Guo et al. (2005a) also mapped the unique disulfide bond of human ACAT1 to C528 and C546. The C-terminal fragment of ACAT1 is located at the ER lumen where oxidative redox potential favors disulfide formation and oxidoreductases catalyze this process. Although the disulfide bond has no effect on the activity of ACAT1 (Lu et al., 2002), it has significant contribution to ACAT1 stability *in vivo* (Guo et al., 2005a). The two C-terminal cysteines (C528 and C546 in human ACAT1) are conserved in ACAT1s and ACAT2s from mammals and *Caenorhabditis elegans*. The loop length between the two cysteines is always 17 residues. Therefore, the C-terminal disulfide bond is probably conserved in all ACAT1s and ACAT2s. However, DGAT1s lack the responding C-terminal fragment, so there are probably no disulfide bonds in DGAT1s.

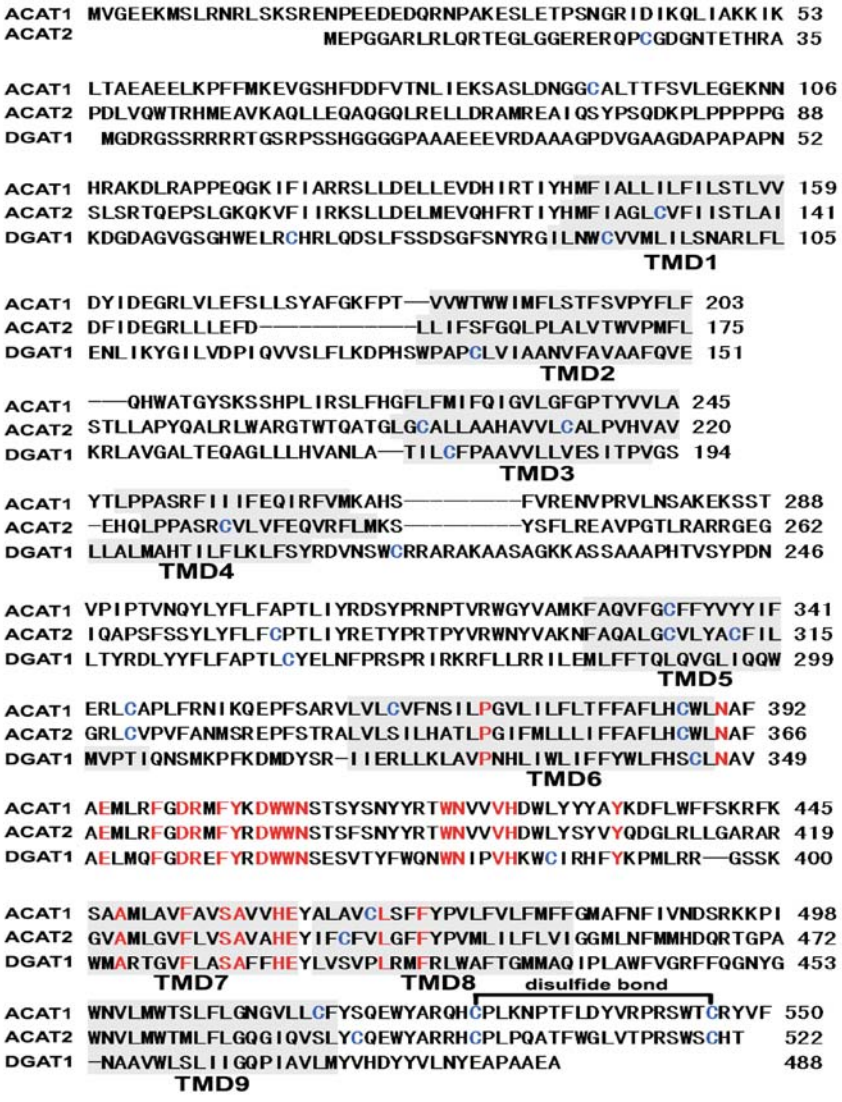

Fig. 1 Amino acid sequence alignment of human ACAT1, human ACAT2, and human DGAT1. The possible transmembrane domains (TMDs) are shaded. The conserved residues are shown in red, and the cysteine residues are shown in blue.

4 Membrane topology of ACAT family enzymes

The ACAT family enzymes are integral ER membrane proteins with multiple transmembrane domains (TMDs) as predicted by TMD algorithms. Membrane topology is important for understanding substrate-binding and catalysis of membrane enzymes. Therefore, various experimental methods have also been designed to investigate membrane topology. The membrane topology of ACAT1 has been experimentally studied using different approaches. In 1999, Lin et al. first proposed a 7-TMD model for ACAT1 based on the results of HA-tag insertion and subsequent immunofluorescence observation after selective permeabilization of the cell membrane and the ER membrane (Lin et al., 1999). In this model, two long hydrophobic polypeptide stretches and a long hydrophilic polypeptide stretch rich in conserved residues were located in the ER lumen. One year later, Joyce et al. proposed a 5-TMD model for ACAT1 based on their C-terminal truncation method (Joyce et al., 2000). In this model, three hydrophobic polypeptide stretches were located in the cytosol. In 2005, Guo et al. reported a 9-TMD model for ACAT1 based on their cysteine-scanning mutagenesis and subsequent cysteine-specific modification approach (Guo et al., 2005b). In the 9-TMD topology model (Fig. 2), all long hydrophobic polypeptide stretches are imbedded in the membrane bilayer. In this model, a long polypeptide stretch rich in conserved hydrophilic and hydrophobic residues between TMD6 and TMD7 is located in the cytosol. This peptide stretch may form the binding site of

acyl-coenzyme A that is synthesized in the cytosol and is impermeable to the ER membrane. In the 9-TMD model, the so-called active site His-460 of ACAT1 is located in a membrane sealed region at the luminal end of TMD7. This location seems relevant to its catalysis role as discussed later.

The membrane topology of ACAT2 has also been experimentally studied using two different approaches (Joyce et al., 2000; Lin et al., 2003). HA-tag insertion and subsequent immunofluorescence observation led to a 2-TMD model (Lin et al., 2003), while the C-terminal truncation approach led to a 5-TMD model (Joyce et al., 2000). However, sequence analysis shows that ACAT2 contains 9 long hydrophobic peptide stretches corresponding to the 9 TMDs of ACAT1 (Fig. 1). So ACAT2 probably also contains 9 TMDs, which needs to be demonstrated using new approaches in future. In the proposed 9-TMD model (Fig. 2), all long hydrophobic peptide stretches of ACAT2 are imbedded in the membrane bilayer; the probable acyl-coenzyme A-binding site between TMD6 and TMD7 is located at the cytosol; the so-called active site His-434 is located at the luminal end of TMD7 (Fig. 2).

The membrane topology of DGAT1 has not been experimentally studied so far. Sequence analysis shows that DGAT1 also has 9 long hydrophobic polypeptide stretches (Fig. 1). So DGAT1 probably also contains 9 TMDs. In the proposed model, the so-called active site His-415 is located at the luminal end of the TMD7 (Fig. 2).

For ACAT family enzymes, we proposed a general topology model with 9 TMDs (Fig. 2). Among these, TMD7 is crucial because it is probably involved in substrate-binding and catalysis. TMD7 is rich in conserved residues: the absolutely conserved His at the luminal end is proposed to be an active site. Other conserved residues are probably responsible for cholesterol/diacylglycerol-binding. Since cholesterol and diacylglycerol are insoluble in water, ACAT family enzymes may use the membrane-bound cholesterol and diacylglycerol as substrates. Two long cytosolic loops (between TMD6 and TMD7, and between TMD4 and TMD5) are rich in conserved hydrophobic and hydrophilic residues. They are probably involved in binding of acyl-coenzyme A that is synthesized in the cytosol and is impermeable to the ER membrane.

5 Subunit interactions of ACAT family enzymes

After cloning of its cDNA, ACAT1 was recombinantly expressed in CHO cells and insect cells and purified to essential homogeneity (Cheng et al., 1995; Chang et al., 1998). Using homogeneous ACAT1 as enzyme source, sigmoidal cholesterol saturation curves were observed in both lipid vesicles and mixed micelles (Cheng et al., 1995; Chang et al., 1998; Zhang et al., 2003). In contrast, the saturation curve of acyl-coenzyme A is hyperbolic. The sigmoidal saturation curve suggests that the activity of ACAT1 is allosterically activated by cholesterol, one of its substrates. The cholesterol activation effect was also observed in intact cells (Liu et al., 2005). Allosterically regulated enzymes are usually composed of several subunits, and the allosteric regulation is a result of conformational change transfer among subunits. The results of Yu et al. indeed show that ACAT1 formed homotetramers *in vitro* and in intact cells (Yu et al., 1999), and their further work shows that the cytosolic N-terminal domain was responsible for dimerization (Yu et al., 2002). Deletion of the N-terminal domain converts ACAT1 into a homodimer, but it is still allosterically activated by cholesterol. Using cysteine-scanning mutagenesis and a disulfide cross-linking approach, Guo et al. (2007) had shown that the TMD7 and TMD8 were also involved in ACAT1 subunit interaction. Therefore, ACAT1 contains two independent subunit interaction surfaces: one is located at the cytosolic N-terminal domain, and the other is located at the TMD7 and TMD8.

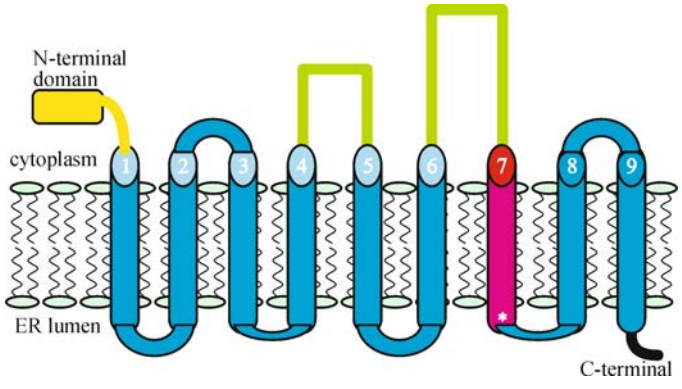

Fig. 2 A general ER membrane topology model for ACAT family enzymes. The possible cholesterol/diacyglycerol-binding region (TMD7) is shown in red, and other TMDs are shown in blue. The possible acyl-coenzyme A-binding regions (the loop between TMD6 and TMD7 and the loop between TMD4 and TMD5) are shown in green. The position of the active site His in TMD7 is indicated by a white star.

Activity assay and disulfide cross-linking show that the helix of TMD7 has two distinct functional sides: all residues critical for its activity (involving in cholesterol binding and catalysis) are located at one side of the helical wheel, while all residues involved in subunit interaction are located at the opposite side (Fig. 3A). This phenomenon seems relevant to its allosteric regulation. When cholesterol (a substrate but also an activator) binds to one subunit of ACAT1 (TMD7 seems to be a major binding site), a conformational change is induced by cholesterol-binding and the subunit is simultaneously activated by this induced conformational change. The conformational change is also transferred to the other subunit through subunit interaction mainly involving TMD7 and TMD8. Thus, the other subunit is also activated although it does not bind cholesterol.

Available data shows that the activity of ACAT2 is also allosterically activated by cholesterol (Liu et al., 2005). ACAT2 probably also forms homotetramers. Previous experiments show that DGAT1 forms homotetramers (Cheng et al., 2001), but detailed subunit interaction sites have not yet been identified. It is also unclear whether the activity of DGAT1 is allosterically regulated by diacylglycerol or not so far.

6 Catalysis of ACAT family enzymes

The catalytic mechanism of ACAT family enzymes has not yet been fully understood. When Ser-269 of human ACAT1 was replaced by a leucine residue, the mutant enzyme lost activity completely. Based on this observation, Joyce et al. proposed that Ser-269 was the catalysis center of ACAT1 (Joyce et al., 2000). But further research shows that the mutant enzyme could not be expressed in CHO cells at all (Guo et al., 2005b). When replaced by alanine or threonine, the mutant enzymes could be expressed and show almost normal activity (Guo et al., 2005b). Therefore, Ser-269 is not the active site of ACAT1. Hofmann analyzed the membrane-bound O-acyltransferases and proposed that an absolutely conserved His (His-460 in human ACAT1; His-434 in human ACAT2; His-415 in human DGAT1) was the active site (Hofmann, 2000). When the His of ACAT1 or ACAT2 was replaced by other residues, the mutant enzymes lost activity completely but could be expressed almost normally in CHO cells (Lin et al., 2003; Guo et al., 2005b). Besides, its critical role for enzyme activity, its location and local environment also suggest that the conserved His is the most possible candidate of the active site of ACAT family. To fulfill the catalysis role, the active site residue should have the following characteristics: (1) His, Ser, and Cys are all possible candidates for the active site of the ACAT family because their side-chains can, theoretically, fulfill the acyl-transfering function. (2) The active site residue is likely located near the edge of lipid bilayer. The ACAT family enzymes probably use membrane-bound cholesterol and diacylglycerol as substrates due to their insolubility in water (Chang et al., 2001b). Cholesterol and diacylglycerol are incorporated into the membrane bilayer with their hydroxyl group near the hydrophilic edge of the lipid bilayer. The active site residue is probably near the hydroxyl group of their substrates. Otherwise, the substrates must undergo drastic movement during catalysis. (3) The active site is probably sealed to small hydrophilic molecules, such as water, because water can hydrolyze acyl-coenzyme A once it enters into the active site cavity.

In the 9-TMD model (Guo et al., 2005b), the active site His-460 of human ACAT1 is located at the luminal end of TMD7 that has two distinct functions: subunit interaction and substrate-binding/catalysis (Guo et al., 2005). Using

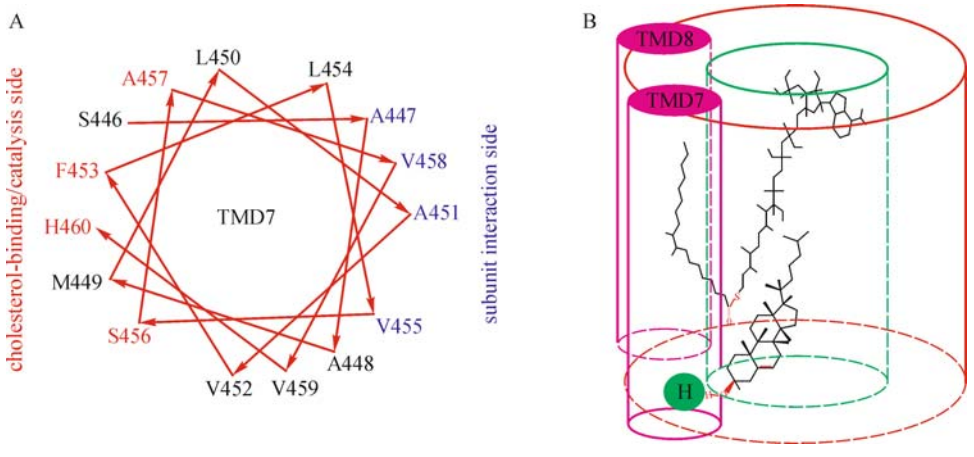

Fig. 3 Structure of ACAT family enzymes. A: two distinct functional sides of TMD7 of ACAT1. Residues critical for enzyme activity are shown in red; residues involved in subunit interaction are shown in blue. B: A proposed tunnel model for ACAT family enzymes. The TMDs form a tunnel structure in the center. Only TMD7 and TMD8 are shown. Other TMDs, the N-terminal domain and the loops are omitted.

small chemical probes with different hydrophobicities, Guo et al. studied the local environment of His-460 of human ACAT1 (Guo et al., 2005b). When His-460 was replaced by cysteine, the introduced cysteine was resistant to hydrophilic thiol-specific modification reagents, such as 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), while it was much more sensitive to hydrophobic modification reagents, such as N-phenylmaleimide (NPM). These results suggest that the active site His-460 is located at a membrane-sealed hydrophobic region where hydrophobic molecules, such as cholesterol, can enter, while hydrophilic molecules, such as water, cannot enter. So available experimental data support the hypothesis that His-460 is the active site of ACAT1.

The active site His-460 of ACAT1 is located at the luminal end of TMD7. So, ACAT1 probably only utilizes cholesterols that are incorporated into the inner leaflet of the lipid bilayer. While the other substrate, acyl-coenzyme A, is synthesized in the cytosol and is impermeable to the ER membrane. How do the two substrates, cholesterol and acyl-coenzyme A, approach each other during the catalysis? When cysteines are introduced into TMD7 and TMD8, some of them, such as V452C, F453C, S456C, F470C, and Y472C, are accessible to charged hydrophilic modification reagents, although they are located at the middle of TMDs (Guo et al., 2007). Thus, we deduce that there is an acyl-coenzyme A-binding tunnel in ACAT1 (Fig. 3B). Through this tunnel, the thiol-ester bond of acyl-coenzyme A can approach the hydroxyl group of cholesterol during catalysis.

Sequence alignment shows that His-434 is the active site of human ACAT2 (Fig. 1). When it was replaced, the mutant enzymes lost activity completely but could be expressed in CHO cells (Lin et al., 2003). Although previous experimental studies failed to locate His-434 in a transmembrane region, our above analysis shows this possibility. Sequence alignment shows that His-415 is the active site of human DGAT1 (Fig. 1). But no experiments have been carried out to demonstrate its importance for such activity so far. Our above analysis suggests that it is located at the luminal end of TMD7, which needs to be experimentally demonstrated in future.

The ACAT family enzymes probably share the same catalysis mechanism that is highly conserved during evolution. They all utilize a conserved His as active site, which probably serves as a general base to activate the hydroxyl group of cholesterol/diacylglycerol that will subsequently attack the thiol-ester bond of the acyl-coenzyme A. The active site His is likely sealed by a lipid bilayer at the luminal end of a TMD. Cholesterol and diacylglycerol are mainly bound by TMDs, such as TMD7. Whereas acyl-coenzyme A is mainly bound by cytosolic loops between TMD4 and TMD7. The two substrates can approach each other during catalysis through a tunnel structure.

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