A COMPARATIVE ANALYSIS OF INTRACELLULAR CHOLESTEROL ESTERIFYING ENZYMES IN MAMMALS

BY

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A dissertation submitted to the graduate faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

in Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
Biochemistry and Molecular Biology

August 2009
Winston Salem, North Carolina

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"The whole of science is nothing more than a refinement of everyday thinking"

--Albert Einstein
ACKNOWLEDGEMENTS

This thesis would not have been possible without the support and encouragement of my advisor Dr. Larry Rudel. I am extremely grateful for his time and guidance for my scientific and personal development throughout the Ph.D. program. I especially valued his critical inputs and constant assistance during the preparatory phase of the next stage of my academic career. I am also thankful to my thesis committee members – Drs. Doug Lyles, Greg Shelness, Linda McPhail and Todd Lowther. Their constructive criticism and suggestions have immensely helped my thesis research. Additionally, I would like to offer my sincere thanks to Dr. Lyles for his time and suggestions during the preparation of my postdoctoral interviews. I am grateful to Dr. John Wilkinson for his time, suggestions and insightful discussions. I would also like to thank all the members of Rudel laboratory for their support and assistance, especially Matt Davis for teaching me fundamentals of biochemical techniques. I had a wonderful time working in this friendly and collaborative environment. I would also like to thank everybody from lipid sciences floor for making this section highly collaborative and congenial research environment. I am thankful to Dr. C. K. Dasgupta of University of Calcutta for helping me to pursue higher studies in Biology. I have really had a wonderful opportunity to study under some great teachers starting from my school days and I am grateful to all of them for their support, encouragement and confidence in me.

I would not come this far without the support and encouragement of my father Mr. Nimai Kumar Das, my mother Mrs. Smriti Das and my sister Ms. Lahari Das. My parents had made immense sacrifices to raise us and they always stood by me even at very odd times.
I wish to thank them for everything. I am deeply grateful to my uncle Dr. A.K.Mitra for his guidance. I would also like to thank all my friends from college. Their presence made my life wonderful.

Finally, I am deeply indebted to my confidant, Amrita, for helping me in all aspects throughout my Ph.D. career. I always enjoy our long telephonic conversations. It definitely helped me to be more critical about my own thinking.
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<tr>
<td>ACAT</td>
<td>Acyl CoA:cholesterol-O-acyl transferase</td>
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<tr>
<td>AGM</td>
<td>African green monkey</td>
</tr>
<tr>
<td>apoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>apoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl ester</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FC</td>
<td>Free cholesterol</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HDSF</td>
<td>Hexadecylsulfonyl fluoride</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyltransferase</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>MT</td>
<td>Mutant</td>
</tr>
<tr>
<td>PGH</td>
<td>Phenylglyoxal hydrate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PMSF</td>
<td>phenylmethysulfonyl fluoride</td>
</tr>
<tr>
<td>PPPA</td>
<td>pyripyropene A</td>
</tr>
<tr>
<td>PPT-1</td>
<td>palmitoyl protein thioesterase</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>UB</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoproteins</td>
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<td>WT</td>
<td>wild type</td>
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ABSTRACT

DAS, AKASH

A COMPARATIVE ANALYSIS OF MAMMALIAN INTRACELLULAR CHOLESTEROL ESTERIFYING ENZYMES

Dissertation under the direction of
Lawrence L. Rudel, PH.D., Professor of Pathology and Biochemistry

In higher eukaryotes, intracellular cholesterol esterification is catalyzed by two isoforms of acyl-CoA:cholesterol O-acyltransferase (ACAT) enzymes. Functional studies indicate that selective inhibition of ACAT2 prevents progression of atherosclerosis whereas deletion of ACAT1 is deleterious in mice. Due to high sequence similarity, it is difficult to find an isozyme specific inhibitor. Hence, detailed study is required to identify unique structural features of ACAT2 that can be utilized to make ACAT2-specific drugs. In this thesis, three independent studies are presented where the two ACAT enzymes were compared side by side to identify unique isozyme-specific properties. In the first study, putative active site residues of ACAT proteins were identified using chemical modification and site directed mutagenesis approaches. A conserved region within the protein was defined as the putative active site domain based on highly conserved sequence similarities from yeast to humans. Since ACAT enzymes have an intrinsic thioesterase activity, we hypothesized that by analogy with the thioesterase domain of fatty acid synthase, the active site of ACAT enzymes may comprise a catalytic triad of serine, histidine and aspartic acid residues. Results showed that in ACAT1, S456, H460 and D400 are essential for activity of the enzyme. In contrast, in ACAT2, only the analogous H438 was identified to be necessary for enzymatic activity suggesting that the
residues required for ACAT activity may be different between the two ACAT proteins. In the second project, the attributes of ACAT2 that lead to pyripyropene A (PPPA) inhibition was determined. PPPA is the only inhibitor as yet identified that has more than 2000 fold higher specificity for ACAT2 than ACAT1. By making chimeric proteins using both ACAT1 and 2 sequences, three residues, located within the fifth transmembrane domain of ACAT2, were identified as the PPPA interaction site. PPPA interaction is non-covalent and does not alter the oligomeric structure of the protein. In the final project, the effect of protein stability on ACAT activity was investigated. Results showed that in vitro ACAT2 activity is always higher than ACAT1 probably due to high abundance of ACAT2 enzyme protein in the assay system since it is intrinsically a more stable protein than ACAT1. Together all of these studies have greatly enhanced our understanding about structure/function relationships of the two distinct yet highly similar ACAT proteins.
CHAPTER I

INTRODUCTION

Akash Das prepared the following chapter. Dr. Lawrence Rudel acted in an advisory and editorial capacity.
Chapter I

**Cardiovascular disease.** Atherosclerosis is the underlying disease process leading to the clinical complications of coronary heart disease (CHD), a key component of cardiovascular disease, an epidemic that has cost more lives in this country over the past century than the next four causes combined. It is estimated that in 2007, cardiovascular diseases accounted for $431.8 billion in direct and indirect costs in the US alone, up from the $393.5 billion in 2005. These increases are occurring in spite of the fact that we have learned tremendous amounts about how to prevent and treat cardiovascular disease. For example, statin drugs, while enormously effective, have not solved CHD. Clearly, continued research on prevention is of paramount importance.

Atherosclerosis is a progressive and degenerative disease that causes blockage of blood vessels by deposition of cholesterol rich plaques. It begins early in life and remains undetected until the age of forty and above. The infiltration and accumulation of macrophages in the artery wall is a hallmark of the disease. Atherosclerotic plaques tend to occur in the branching areas of larger vessels and in the coronary arteries. The plaques start with fatty streaks and later progress into fibrous plaques. The coronary arteries provide heart muscle with oxygenated blood that is required for its functionality and viability. Once plaques are deposited in arteries, heart muscles can become deprived of oxygen and angina may occur. If the arteries are completely blocked, the region of the heart maintained by the arteries can die, eventually leading to heart failure.
**Lipoprotein metabolism and atherosclerosis.** High concentrations of low density lipoproteins (LDL) cholesterol and low concentrations of high density lipoproteins (HDL) cholesterol are major risk factors for premature complications of CHD. Less well recognized, but also of high importance, is the finding that cholesteryl oleate enrichment of LDL particles is consistently associated with increased atherosclerosis extent in experimental animals 5-8. In monkeys, enrichment of particle cores with cholesteryl oleate was strongly \( (r=0.8) \) and positively associated with more severe coronary artery atherosclerosis 5, 6, 9 and was additive to the contributions for LDL and HDL cholesterol concentrations. Similarities to these studies are found in the early work of several European groups 10-17 showing that plasma lipoproteins with a lower proportion of cholesteryl linoleate (and conversely a higher proportion of cholesteryl oleate) are typical of patients with complications of CHD compared to normal controls. In the large (2800 participants) Atherosclerosis Risk in Communities (ARIC) study, Ma et al. 18 found an inverse relationship between atherosclerosis (measured non-invasively as carotid artery wall intimal-media thickness (IMT)) and plasma cholesteryl linoleate, but found a positive correlation between cholesteryl oleate and IMT. Altogether these data demonstrate that in animal models and in comparatively understudied human subjects, cholesteryl ester composition is a major determinant of progression of CHD.

**Intracellular cholesterol esterifying enzymes in mammals.** There are two major forms of cholesterol in the cells, free or unesterified cholesterol (FC) and cholesteryl ester (CE). The latter has a long chain fatty acid covalently attached to the sterol moiety. FC provides fluidity and permeability to the cell membranes 19. When the
membrane is saturated with FC, CE is synthesized to prevent the cells from toxicity of excess FC. CE is poorly soluble in the membrane and thus accumulates in the cytoplasm as lipid droplets. The intracellular cholesterol esterification reaction in mammals is carried out by two isoforms of acyl-coA:cholesterol- O-acyltransferase (ACAT) enzymes also called sterol –O- acyltransferase using acyl-CoA as the fatty acid donor in the reaction \( \text{20-22} \). Both the ACAT proteins are localized in the ER membrane and span the membrane five times \( \text{23} \). Although both proteins share \( N_{\text{cyt}} \) and \( C_{\text{exo}} \) topology, significant variance exists in their topology towards the central region of the proteins. Apart from this, a separate topology map of ACAT proteins has been proposed where authors have reported that in spite of high degree of sequence similarity, ACAT1 has nine transmembrane domains \( \text{24} \) whereas ACAT2 has only two \( \text{25} \). ACAT1 expression is ubiquitous in all tissues including adrenal gland, ovary, preputial gland, skeletal muscle, adipose tissue, heart and brain \( \text{26} \). The cell types that express ACAT1 include Kupfer cells of the liver, goblet cells, paneth cells and macrophages of the intestine, cells of the adrenal cortex, and the distal tubular cells and podocytes of the kidney \( \text{26} \). In contrast, the distribution of ACAT2 is limited to cells that are known to secrete lipoprotein particles, namely hepatocytes of the liver and enterocytes of the small intestine in primates \( \text{26} \). In fact, ACAT2 is the major cholesterol esterifying enzyme in human liver \( \text{27} \).

**Functional studies with ACAT1.** The understanding of cholesterol esterification reaction was greatly enhanced by cloning ACAT1 from human macrophages using an expression cloning strategy \( \text{28} \). In this method, a chinese hamster ovary (CHO) cell line deficient in endogenous ACAT activity was transfected with cDNA fragments isolated
from human macrophage to complement the ACAT activity. The cDNA isolated from human macrophage predicts a 550 amino acid long ACAT1 protein. Expression of ACAT1 mRNA was found to be ubiquitous having highest expression level in adrenals and macrophages and the protein was found to be localized primarily in the ER membrane. To elucidate the role of ACAT1 in sterol homeostasis two separate gene knockout studies were performed in mice. In one study the 5’ end of the ACAT1 gene surrounding the exon 1 was deleted. These animals had a significant decrease in CE content in adrenal and peritoneal macrophages; however, they had normal hepatic CE concentration and cholesterol absorption in the intestine. To study the effects of ACAT1 knockout (KO) on plasma lipids, these mice were bred with hyperlipidemic LDL receptor (LDLr) KO or apolipoprotein E (apoE) KO mice. The double KO mice had lower plasma cholesterol concentrations than the wild type (WT) control mice. Although, the extent of atherosclerosis was not reported for these mice, they had a lower number of macrophages with neutral lipid deficiency in the atherosclerotic lesions. Moreover, dramatic accumulation of cholesterol in the skin and brain was observed in the double KO mice. Later, the same group showed that hyperlipidemic mice lacking ACAT1 only in macrophages develop atherosclerosis. In the second ACAT1 gene KO study a different region of the gene containing a portion of the first transmembrane domain was deleted. In these mice, there was no change in ACAT activity in the liver and intestine, however, ACAT activity was greatly reduced in the adrenals, ovary and testis. Furthermore, deletion of ACAT1 gene caused dry eye disease in these animals. To study the extent of atherosclerosis these mice were bred with hyperlipidemic mice models. The degree of atherosclerosis in the double KO mice was less compared to the control mice.
Plasma cholesterol concentration was less in ACAT1 +/- and apoE +/- double KO mice compared to the WT control mice fed a western type diet (a high fat diet containing 0.15% cholesterol (w/w) and 15% butter), however, there was no apparent change in the plasma cholesterol concentration in ACAT1/-/- LDLr/-/- double KO mice compared to the control mice fed a Paigen diet (a high fat diet containing 1.25% cholesterol (w/w), 15% cocoa butter and 0.5% cholic acid (w/w)). These mice had developed the cutaneous xanthomatosis like the ACAT1 KO mice reported by Accad et al. 32, however, existence of brain lesions was not reported for these mice. The ACAT1 gene disruption studies suggested two major conclusions: 1. ACAT1 is a housekeeping gene maintaining a proper balance between FC and CE in the cell and hence, its disruption may cause some adverse side effects, 2. A second ACAT enzyme is present in the liver and intestine since in ACAT1 KO mice the CE content in these two organs was unaltered. The later conclusion was supported by additional published reports. The identification of two sterol esterifying gene in yeast (ARE1 and ARE2), which were functional in absence of the other one, suggested the possibility of at least two ACAT genes in mammals 35, 36. In another study, ACAT isolated from rabbit aorta and liver showed a 40 fold difference in sensitivity to the same inhibitor 37. These studies led to the identification of the ACAT2 gene from human, monkey and mouse 38-40.

**Functional studies with ACAT2.** Expression of ACAT2 was found to be restricted only in the liver and intestine. Later, the ACAT2 KO mice were generated which did not have any detectable ACAT activity or CE in the liver and intestine. Furthermore, cholesterol content in the plasma and VLDL was greatly reduced compared
to the WT control mice fed a high fat, high cholesterol diet. In short, these mice were found to be resistant to diet induced hypercholesterolemia apparently in part due to the reduced absorption of cholesterol in the intestine \(^{41}\). To address the extent of atherosclerosis in ACAT2 KO animals, these mice were bred with apoE-/- mice \(^{42}\). The double KO mice had significant reduction in CE content in VLDL and IDL fraction; moreover, atherosclerosis was very low in these mice compared to the control WT mice. Interestingly, plasma HDL cholesterol level was increased in the double KO mice together with an increase in Lecithin Cholesterol Acyltransferase (LCAT) activity. This effect may have contributed to the anti atherogenic effect of the double KO mice. In summary, these studies indicated ACAT2 - mediated CE production is crucial for development of atherosclerosis.

From the gene KO studies of ACAT isozymes we can conclude the following: 1. Although the isozymes catalyze the same reaction, they play distinct roles in cholesterol homeostasis; ACAT1 primarily maintains the cholesterol homeostasis in the cell while ACAT2 delivers CE to the lipoprotein particles. 2. ACAT2 may be a suitable target for treatment of atherosclerosis. Further in mammals, in addition to ACAT’s, LCAT is another cholesterol esterifying enzyme that synthesizes CE in plasma. Although, LCAT deficiency in LDLr KO mice decreases LDL cholesterol concentrations, it also depletes the HDL cholesterol concentration and inhibits reverse cholesterol transport and hence leading to increased atherosclerosis \(^{43}\). Conversely, overexpression of LCAT in mice appears to have little or no effect on atherosclerosis \(^{44-46}\). Therefore, we can conclude, among the three cholesterol esterifying enzymes in mammals, ACAT2 may be a suitable
target for treatment of atherosclerosis but inhibition or KO of either ACAT1 or LCAT was associated with adverse side effects that appear to increase atherosclerosis.

**Biochemical studies with ACAT1.** Since the discovery of ACAT proteins, functional studies of these enzymes have been extensively done in mice. However, biochemical and structural studies were greatly limited mainly due to the difficulty to purify and solubilize the proteins in a suitable detergent. Although several attempts to purify these enzymes have been failed, only one report claimed to have purified human ACAT1 to near homogeneity from both tissue culture cells and baculovirus - mediated gene expression system in insect cells. These authors showed the substrate saturation curve of the partially purified enzyme was sigmoidal in the presence of an increasing amount of cholesterol, suggesting cholesterol may act as an allosteric activator of ACAT1 protein. Moreover, their study suggested that ACAT1 forms a homotetrameric protein \textit{in vitro} and in intact cells. Combining these two studies we can speculate a probable mechanism for cholesterol mediated activation of ACAT1. It is possible that binding of one cholesterol molecule to a monomer of a tetrameric ACAT1 complex increases its affinity for further substrate binding resulting in sharp cooperativity and thereby stimulating the enzyme activity. However, it is not clear that tetramer formation is necessary for activity of the protein. Apart from cholesterol, it has also been shown that adding oxysterol like 25-hydroxycholesterol to tissue culture cells stimulates cholesteryl ester formation. The supposition was that oxysterol activates ACAT1 in a similar fashion as cholesterol. More mechanistic studies are needed confirm these speculations.
Purification of ACAT2 has been tried numerous times but without success hence definition of its biochemistry is very limited (unpublished data).

**Conclusions.** ACAT1 and ACAT2 do not co-localize in the same cell type in any tissues of the body, indicating that possibly distinct cellular functions are performed by these two enzymes. However, when the isozymes are artificially expressed in a cell type where they do not express normally, they can carry out cell specific ACAT activity. For example, when ACAT2 is expressed in ACAT1 deficient CHO cells, formation of lipid droplets can be observed 26, indicating ACAT2 can take part in intracellular cholesterol homeostasis. Similarly, overexpression of ACAT1 in the liver of hamster leads to the elevation of plasma cholesterol concentration 52 suggesting that CE synthesized by ACAT1 can be packaged into apoB-containing lipoproteins. These studies indicate that functionally one enzyme can complement the other. Despite these similarities, functional differences exist between the two enzymes. At least three lines of evidence can be presented to support this point: 1. ACAT1 esterifies beta-sitosterol more effectively than ACAT2 53, 2. Pyropyrepene A (PPPA) inhibits ACAT2 with greater than 2000 fold higher specificity than ACAT1 54, 3. ACAT1 is more sensitive to histidine modifying reagent than ACAT2 37 (unpublished observation by Joyce .C). These studies indicate uniqueness among the ACAT isozymes. A possible similarity between the isozymes could be that both enzymes use similar residues during catalysis. Conversely, uniqueness among the enzymes may be a consequence of distinct sub-cellular localization of the active site of the enzyme. All these speculations will remain unanswered until structural studies on ACAT isozymes are completed. The hydrophobic nature of these membrane
bound enzymes makes them difficult to purify. Interestingly, uniqueness among these isozymes supports the possibility of making ACAT2 - specific drugs for the treatment of atherosclerosis although the molecular basis for uniqueness between these enzymes remains poorly understood. This thesis describes some fundamental observations of biochemical similarities and differences of individual ACAT proteins that may one day lead to ACAT2 - specific drug designing strategies.
REFERENCES


CHAPTER II

IDENTIFICATION OF PUTATIVE ACTIVE SITE RESIDUES OF ACAT ENZYMES

Akash Das, Matthew A. Davis, Lawrence L. Rudel

The following manuscript was published in the Journal of Lipid Research 2008 volume 49 pages 1770-1781 and is reprinted with permission. Stylistic variations are due to the requirements of the journal. A. Das conceived part of the project, performed the experiments, and wrote the manuscript. M. A. Davis performed the experiments. L. L. Rudel conceived the project and acted in an advisory and editorial capacity.
ABSTRACT

In this report, we sought to determine the putative active site residues of ACAT enzymes. For experimental purposes, a particular region of the C-terminal end of the ACAT protein was selected as the putative active site domain due to its high degree of sequence conservation from yeast to humans. Because ACAT enzymes have an intrinsic thioesterase activity, we hypothesized that by analogy with the thioesterase domain of fatty acid synthase, the active site of ACAT enzymes may comprise a catalytic triad of Ser-His-Asp (S-H-D) amino acid residues. Mutagenesis studies revealed that in ACAT1, S456, H460, and D400 were essential for activity. In ACAT2, H438 was required for enzymatic activity. However, mutation of D378 destabilized the enzyme. Surprisingly, we were unable to identify any S mutations of ACAT2 that abolished catalytic activity. Moreover, ACAT2 was insensitive to serine-modifying reagents, whereas ACAT1 was not. Further studies indicated that tyrosine residues may be important for ACAT activity. Mutational analysis showed that the tyrosine residue of the highly conserved FYXDWWN motif was important for ACAT activity. Furthermore, Y518 was necessary for ACAT1 activity, whereas the analogous residue in ACAT2, Y496, was not. The available data suggest that the amino acid requirement for ACAT activity may be different for the two ACAT isozymes.
INTRODUCTION

The intracellular cholesterol esterification reaction in vertebrates is carried out by two ACAT (EC 2.3.1.26) enzymes, ACAT1 and ACAT2 (1, 2). Both enzymes use two lipophilic substrates, cholesterol and acyl-CoA, during the esterification reaction. ACAT enzymes are localized in the endoplasmic reticulum (ER) membrane and span the membrane five times (3). Although expression of ACAT1 is ubiquitous, ACAT2 is localized only in the enterocytes of the intestine and the hepatocytes of the liver (4, 5). We have demonstrated previously that in nonhuman primates, hepatic ACAT activity is associated with cholesteryl oleate enrichment of LDL and increased coronary artery atherosclerosis (6–8). To define the relative roles of ACAT enzymes in the progression of atherosclerosis, functional studies were performed in hyperlipidemic mouse models. ACAT2 knockout mice were consistently protected from atherosclerosis (9–11). On the other hand, ACAT1 mice had only minor improvements in atherosclerosis, while at the same time showing adverse effects after accumulating excess free cholesterol in various tissues (12–14). Recently, we have demonstrated that liver-specific knockdown of ACAT2 using antisense oligonucleotides resulted in significantly reduced hepatic cholesterol concentration, plasma LDL cholesterol oleate, and aortic atherosclerosis (15). Because ACAT2 is also the major cholesterol-esterifying enzyme in the human liver specifically within the hepatocytes (4), prevention of hepatic ACAT2 activity could be beneficial and desirable for treatment of atherosclerosis in humans (16).

Because ACAT enzymes are similar in amino acid sequence, inhibitory molecules often interfere with both ACAT1 and ACAT2 activity. Thus, a detailed comparative biochemical analysis of these enzymes is needed. Although several functional studies
have been performed in animal models, biochemical studies with ACAT enzymes are limited, mainly owing to lack of purified proteins. Using a histidine-modifying reagent, Kinnunen, DeMichele, and Lange (17) showed that a histidine residue(s) is necessary for ACAT activity. Chang and colleagues (18, 19) extended this observation and suggested one invariant histidine residue at the C-terminal end of ACAT enzymes as an active site residue. Recently, one report suggested that active sites of ACAT enzymes are different (20). Using mutagenesis studies, they showed that the histidine residue required for ACAT1 activity is different from that required for ACAT2 activity. Because the cholesterol esterification reaction involves disruption of the high-energy thioester bond of an acyl-CoA molecule, we reasoned that more than one amino acid residue (potentially including histidine) may be involved in ACAT activity. Thus, we sought to identify the amino acid residues required for ACAT activity using a combination of studies with chemical modification, together with site-directed mutagenesis of both enzymes.

Although ACAT1 and ACAT2 are highly homologous in their amino acid sequences, they do not have the same intron-exon structure, suggesting that the two enzymes diverged quite early during evolution (1). The enzymes are different in their membrane topology and perform distinct intracellular functions (2). However, studies have shown that isozymes can functionally complement each other; i.e., cholesteryl ester (CE) synthesized by ACAT2 can be incorporated into the cytoplasmic lipid droplets, whereas CE synthesized by ACAT1 can participate in hepatic lipoprotein particle secretion (21), suggesting that the underlying reaction mechanism for cholesterol esterification may be similar for both enzymes. Accordingly, we hypothesized that the
amino acid residues required for catalysis of cholesterol esterification were similar and conserved in both isozymes.

The critical step in the cholesterol esterification reaction is the breaking of the thioester bond of an acyl-CoA molecule, inasmuch as it is a high-energy bond, and energy released during disruption of this bond is thought to drive the esterification reaction. The crystal structure of the thioesterase domain of human fatty acid synthase reveals a catalytic triad of serine, histidine, and aspartic acid residues as the active site of the enzyme (22). Furthermore, many lipid-modifying enzymes, such as lipases and CE hydrolase, use a catalytic triad comprised of serine, histidine, and aspartic acid in their active sites (23). The crystal structure of human pancreatic lipase has revealed the presence of a catalytic triad in its active site (24), and other studies, using site-directed mutagenesis, have shown the presence of a catalytic triad in an acyltransferase enzyme from Aerimonas hydrophilia (25). Finally, using structural homology modeling, along with mutagenesis of conserved residues, the presence of a Ser-His-Asp catalytic triad in the active site of the plasma enzyme LCAT has been proposed (26). By analogy, it is possible that ACAT enzymes may also use a similar catalytic triad to catalyze the formation of CE during transfer of a fatty acid from an acyl-CoA to a cholesterol molecule.

If indeed ACAT enzymes use a Ser-His-Asp catalytic triad in their active site, the proposed mechanism would probably follow the classic charge relay mechanism, in which a proton will transfer from the serine residue to the histidine ring. This transfer will facilitated by the presence of the neighboring aspartic acid residue. The transfer of the proton will make the serine residue a potent nucleophile. The nucleophilic serine could
then attack the thioester bond of the acyl-CoA molecule, forming an acyl-enzyme intermediate. In the final step, enzyme-assisted (probably His- and Asp-mediated) hydrolytic attack of the 3β-OH moiety of the cholesterol molecule to break the acyl-O-serine ester bond could result in net transfer of the fatty acid to the cholesterol molecule.
EXPERIMENTAL PROCEDURES

**Generation of mutants.** All mutants were generated by a site-directed mutagenesis approach using an overlap PCR method. African green monkey (AGM) ACAT1 and ACAT2 sequences were used as the templates. Using suitable primers (obtained from IDT DNA Technologies) a desired point mutation was introduced into the full-length DNA sequence. Proofstart DNA polymerase (Qiagen) was used during the PCR reaction, which was run with the following conditions: 95°C for 5 min, 1 cycle; 94°C for 30 s, 55°C for 30 s, 72°C for 90 s for 25 cycles, followed by 1 cycle at 72°C for 10 min. The full-length mutant DNA construct was gel extracted (Qiagen Gel Extraction Kit), followed by 5' Kpn1 and 3' Not1 restriction digestion (Promega) and ligation (Fast Link DNA ligase, Epicenter Biotechnologies) into a pre-digested pCDNA3 vector (Invitrogen). All the resulting sequences were confirmed by DNA sequencing. Confirmed sequences were then further purified using the Endo Free Maxi Kit (Qiagen) to get transfection-quality cDNA.

**Cell culture.** AC29 cells (a CHO-derived cell line), which lack any endogenous ACAT activity, mRNA, or proteins, were a gift from T. Y. Chang and were used for all experiments. Cells were maintained in the monolayer at 37°C in 5% CO₂ in Ham's F-12 medium supplemented with 1% Eagle's vitamins, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated FBS. For all experiments, cells were typically grown to 70–90% confluence.

**Cell-based ACAT assay.** Approximately 3 x 10⁶ AC29 cells were transiently transfected
with 6 µg of cDNA encoding for either wild-type (WT) or mutant ACAT proteins using nucleofection technology (Amara Biosystems) according to the manufacturer's instructions. Suspended cells were divided into two aliquots after transfection; one aliquot was used to seed four 35 mm dishes to be used for activity assay, and the remaining aliquot of cells was plated onto a 60 mm dish for subsequent immunoblotting. Seventy-two hours post transfection, cells in 35 mm dishes were pulse labeled with 1 µCi of [9,10-3H(N)]oleic acid (Perkin Elmer Life Sciences, NET-289) for 2 h. Thereafter, cells were harvested and total cellular lipids were extracted by the method of Bligh and Dyer (27). The lipid layer was isolated and CE standard was added. Samples were then dried down under nitrogen, redissolved in chloroform, and spotted, and lipid classes were separated by TLC using silica gel-60 plates in a solvent system containing hexane-diethyl ether-acetic acid (70:30:1). CE bands were visualized by exposure to iodine vapor, and scraped, and radioactivity was measured in a liquid scintillation spectrometer. Under this assay condition, the 2h time point falls within the linear range of the ACAT activity curve.

**Preparation of postnuclear supernatant.** At 72 h post transfection, cells from 60 mm dishes were washed twice with ice-cold balanced salt solution (BSS). Cells were harvested in 60 µl of RIPA buffer (0.1% SDS, 0.5% Na-deoxycholate, and 1% NP-40 in PBS) in the presence of 3 µl protease inhibitor cocktail (Sigma). Sonication followed, and then removal of the nucleus and cell debris by centrifugation at 14,000 rpm at 4°C for 15 min. Supernatant was isolated, and 3 µl of protease inhibitor cocktail was added and saved at −80°C until use. Protein concentration of the postnuclear supernatant (PNS)
solution was measured by BCA assay (Pierce).

**Preparation of microsomes.** Cells grown in 150 mm dishes were washed twice with ice-cold BSS and were scraped from the dish. Excess BSS was removed from the cells after centrifugation, and cells were solubilized in microsomal homogenization buffer (0.25 M sucrose, 0.1 M K2HPO4, 1 mM EDTA, pH 7.4). Protease inhibitor cocktail (5 µl) was added to the cells, and the cells were lysed by sonication. The nucleus and the cell debris were discarded after centrifugation at 14,000 rpm, 4°C for 15 min. Supernatant was collected and subjected to ultracentrifugation at 100,000 rpm, 4°C for 30 min. The pellet containing microsomes was collected and suspended in ice-cold 0.1 M K2HPO4 buffer at pH 7.4. Microsomal protein concentration was measured by BCA assay (Pierce).

**Microsomal ACAT assay.** Microsomes were thawed, and the desired amount of the inhibitor was added and incubated at 37°C in a water bath for 30 min. The incubation conditions and source of the inhibitors are described in the figure legends. BSA (1 mg) and 20 µl of a cholesterol-saturated solution of β-cyclodextrin were added to the microsomes, and the final volume was brought to 300 µl. The samples were equilibrated in a 37°C water bath for 30 min, and then [14C]oleoyl-CoA (Amersham Biosciences) was added, and the samples were incubated for 20 min. To stop the reaction, 6 ml of CHCl3-methanol (2:1) was added. Then 1.2 ml of 0.88% (w/v) KCl was added, and the samples were allowed to sit overnight at room temperature. A 3 ml aliquot of the organic phase (containing lipids) was removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 µl of chloroform containing CE standard and was then
applied to a silica gel-60 TLC plate. Subsequent separation of lipids was done in hexane-ethyl ether-acetic acid (70:30:1). The band on the TLC plate containing the CE was scraped and suspended in scintillation fluid, and radioactivity was determined in a liquid scintillation spectrometer.

**Western blotting.** Proteins from microsomes or the PNS were suspended in equal volumes of protein solubilization buffer (120 mM Tris, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromphenol blue] and 100 mM DTT. The samples were incubated at room temperature for 30 min. Then 50 mM iodoacetamide (IAA) was added, and the mixture was incubated at room temperature for another 30 min. Proteins were electrophoretically separated using 4–12% NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) and were transferred to a nitrocellulose membrane for 1 h at 115 V using a Western blot apparatus (Bio Rad). The membrane was blocked overnight in 5% nonfat dry milk in TBST buffer [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.1% (v/v) Tween-20] at 4°C. Affinity-purified ACAT antibodies (1 µg/ml) were made as described before (5) and were incubated with the membrane for 2h at room temperature. The primary antibody was then removed, and the membrane was washed three times (10 min each) with TBST. The membrane was then incubated with a goat anti-rabbit HRP-conjugated secondary antibody (Sigma) at 1:20,000 dilution at room temperature for 1 h. After the secondary antibody was removed, the membrane was washed three times with TBST (10 min each). The peroxidase signal was detected using Western illuminating reagents (Perkin Elmer), and signal was captured on film (Kodak BioMax light film). Specificity of the antibodies was consistently checked by running the PNS of empty vector-transfected cells on the gel
as negative control. No band of a size comparable to ACAT proteins was seen in negative control lanes (data not shown).

**Calculation of specific activity of the mutants.** Specific activity of the WT and the mutant proteins was measured by normalizing background-subtracted ACAT activity (in dpm) to its protein mass obtained by densitometric analysis from the immunoblots. During densitometric analysis, 10–15 µg of PNS proteins from transfected cells were used for Western blotting. Based on variability among the expression levels, a second immunoblotting was performed, in which proteins were loaded in amounts approximating equivalent signal strengths for the various ACAT proteins. A low exposure of this blot was used for desitometric measurement to maintain signal strengths within the linear range for the various mutant proteins. This densitometric value was then divided by the amount of protein loaded on the gel to estimate the ACAT protein mass. This normalized value was used for specific activity calculation for each mutant. Amount of protein loaded on the gel for all of the mutants is given in the respective figure legends. Relative levels of expression of each mutant against their WT counterparts are shown in the figures.

**Cross-linking experiment.** Microsomes were incubated with either DMSO or disuccinimidyl glutarate (DSG; Pierce) at room temperature for 30 min. The cross-linking reaction was quenched by adding 1 M Tris at pH7.5 and protein solubilizing buffer at room temperature. All samples were then subjected to Western blotting as described above.
RESULTS

Identification of the putative active site of ACAT enzymes. After the N-terminal 100 amino acid residues, where sequence similarity is only 2%, the ACAT1 and ACAT2 isozymes are 56% similar to each other. The area of highest sequence similarity of 83% is toward the C terminus, and includes amino acid residues 386–462 of AGM ACAT1 and amino acid residues 364–440 of AGM ACAT2. The amino acid sequence of this region of ACAT enzymes is highly conserved, starting from yeast and extending all the way to humans (Fig. 1). Interestingly, this region contains two highly conserved motifs, FYXDWWN and HEY. This region is also highly similar to the analogous region of AGM acyl-CoA:diacylglycerol acyltransferase 1, which is 57% similar to AGM ACAT1 and 53% similar to AGM ACAT2. All of the members of this gene family presumably catalyze the transfer of a fatty acid molecule from an acyl-CoA to an acceptor alcohol (diacylglycerol or cholesterol) via a similar reaction mechanism. Because this region has been so highly conserved in evolution, we have tested the hypothesis that this region contains the active site domain of the ACAT isozymes. A corollary to this hypothesis is that each isozyme shares the same active site residues.

H460 in ACAT1 and H434 in ACAT2 are essential for activity of the enzyme. To test whether a histidine residue is important for ACAT activity, we performed ACAT assay after treatment of the enzyme with diethylpyrocarbonate (DEPC), a histidine-modifying reagent. DEPC inhibits almost 100% of the activity of both the enzymes at 50 µM concentration. However, at lower concentrations, inhibition of ACAT1 activity was greater than that of ACAT2 activity (Fig. 2A), as reported previously (17). DEPC
Fig. 1. Amino acid sequences of the putative active site of ACAT enzymes. Sequence similarity within the putative active site domain of the ACAT enzymes across the species. The multiple sequence alignment was performed by the ClustalW program from the European Molecular Biology Laboratory-European Bioinformatics Institute website. Two totally conserved motifs, FYXDWNN and HEY, within this putative active site domain are represented by two boxes. ARE1 and ARE2 represent the yeast homolog of ACAT enzymes, AGM, African green monkey. Asterisks indicate sequence identity; colon indicates conservative substitution; single bullets indicate semiconservative substitution; no symbol means no match.
inhibition of the ACAT enzymes was covalent, inasmuch as the inhibition of ACAT activity was irreversible in the presence of 0.5 M hydroxylamine (data not shown). These data suggest that a histidine residue(s) is required for activity of the enzymes. To further extend this study, we identified three conserved histidine residues within the putative active site domain of the proteins and mutated each of these residues individually to investigate its requirement for activity of the enzyme. For ACAT1, H386 and H425 were not essential for activity of the enzyme, because both mutants were catalytically active, although with a somewhat lower specific activity than the WT counterpart (Fig. 2B). In all mutation studies, the initial amino acid substitutions were made to alanine, but if this mutant protein did not show expression, other amino acids were substituted until expression was observed, as for the H386N mutant. The A1H460A mutant was expressed but catalytically inactive, suggesting that this residue is required for activity of ACAT1, as has been reported earlier (18). A similar result was obtained for ACAT2, in which H364 and H403 were not required for activity of the enzyme but H438 (equivalent to H460 of ACAT1) was necessary for enzymatic activity of ACAT2 (Fig. 2C), as shown previously (19). The mutants have variable levels of expression compared with their WT counterparts (Fig. 2D). Taken together, these data show that the histidine residue of the conserved HEY motif (H460 in ACAT1 and H438 in ACAT2) is essential for ACAT activity.

**D400 is necessary for activity of ACAT1 enzyme.** Next, we investigated the requirement of aspartic acid residue(s) for activity of the ACAT enzymes. Within the putative active site domain of the enzymes, we identified two conserved aspartic acid
Fig. 2. Identification of essential histidines required for activity of the ACAT enzymes. A: After incubation for 30 min with ethanol-solubilized diethylpyrocarbonate (DEPC) at 37°C, pH 7.4, microsomes prepared from ACAT1 and ACAT2 stable cells were used for ACAT assay. ACAT1 (diamonds) and ACAT2 (squares) activities at varying concentrations of DEPC are expressed as a percentage of ACAT1 or ACAT2 activity in the ethanol control. Data represent the average of three replicates. B: AC29 cells were transiently transfected with the cDNA encoding wild-type (WT) and ACAT1 histidine mutants. Seventy-two hours post transfection, cells were incubated with 1 µCi 3H oleic acid for 2 h. Incorporation of the radioactive oleic acid into the cellular cholesteryl ester pool was measured as the determinant of the activity of the enzyme preparations. Background activity was obtained by a parallel kinetic assay where AC29 cells were transfected with empty vector. All the activity was corrected by background subtraction. Specific activity was measured as described in the Experimental Procedures section. This experiment was repeated twice with similar results. Data represent mean ± SEM for n = 4. C: Whole-cell-based kinetic assay for WT and specific histidine mutants of ACAT2 enzymes as indicated on the X axis. The assay was performed as described for B. This experiment was repeated three times with similar results. Data represent mean ± SEM for n = 4. D: Postnuclear supernatant (PNS) obtained from transfections of WT and histidine mutants of ACAT proteins were subjected to immunoblot analysis. Affinity-purified ACAT1 (1 µg/ml) and ACAT2 (1 µg/ml) antibodies (4) were used as the primary antibodies for Western blotting. Secondary antibody was used at 1:20,000 dilution. Proteins loaded on the gel are as follows: WTA1, 25 µg; A1H386N, 10 µg; A1H425A, 10 µg; A1H460A, 25 µg; WTA2, 15 µg; A2H364N, 25.5 µg; A2H403N, 4.5 µg; and A2H438A, 1 µg. The different amounts were used as the denominator to correct the densitometric value in order to estimate the ACAT protein mass during specific activity calculations.
residues, one of which, A1D406, is a part of the conserved FYXDWN motif. This D residue of ACAT1 was not essential for activity of the enzyme, but D400 was required for catalytic activity of the enzyme (Fig. 3A). It should be noted that the expression level of the A1D400N mutant was consistently low, although readily detectable, compared with its WT counterpart (Fig. 3C). For ACAT2, residue D384, a part of the conserved FYXDWN motif, also was not essential for activity of the enzyme (Fig. 3B). The data we obtained for residue D378 were inconclusive, however, inasmuch as we did not find an amino acid substitution other than glutamic acid that resulted in expression. We tried asparagine, alanine, and leucine without achieving detectable levels of expression. The data suggest that D378 is required for structural stability of ACAT2. When we substituted glutamic acid for D378, the mutant protein was catalytically active, and although expression levels were low, the specific activity was normal to slightly higher than that of WT ACAT2 (Fig. 3C). Of note, when the aspartic acid residue of the conserved FYXDWN motif was mutated to asparagine in both enzymes, results showed protein expression with very low enzymatic activity. Together, these data indicate that D400 is required for activity of ACAT1, but we could not establish with clarity that the analogous D378 residue in ACAT2 was required for activity. The available data do suggest that this particular aspartic acid residue is important for structural stability of the ACAT1 and ACAT2 proteins, because when we substituted for this residue, both enzymes consistently showed reduced expression levels.

**S456 is required for activity of ACAT1 enzyme.** To investigate whether any serine residue(s) is required for ACAT activity, we performed a chemical modification study in
Fig. 3. Evaluation of conserved aspartic acid residues in ACAT enzymes. A. Whole-cell-based activity assay for WT and two specific aspartic acid mutants of ACAT1 enzyme. The activity assay was performed as described in Fig. 2B. This experiment was repeated twice with similar results. Data represent mean ± SEM for n = 4. B: Whole-cell-based activity assays for WT and two specific aspartic acid mutants of ACAT2. The assay was performed as described in Fig. 2B. This experiment was repeated twice with similar results, and data represent mean ± SEM for n = 4. C: PNS, obtained from WT and the indicated aspartic acid mutants of ACAT proteins were used for immunobloting, as described for Fig. 2D. Amount of proteins loaded on the gel are as follows: WTA1, 0.5 µg; A1D400N, 35 µg; A1D406A, 2 µg; WTA2, 5 µg; A2D378E, 15 µg; and A2D384A, 5 µg.
which ACAT activity was performed in the presence of PMSF, a serine-modifying reagent. Surprisingly, PMSF did not inhibit activity of either isozyme, even at very high concentrations (Fig. 4A). Although these data suggest that serine residue(s) might not be involved with ACAT activity, we performed mutational analysis because of suggestions that even if there is a serine residue at the active site of the enzyme, the enzyme still can be insensitive to PMSF-mediated inhibition (28). We identified seven conserved serine residues within ACAT enzyme sequences. Of these seven serine residues, four were located within the putative active site domain of the enzyme (for ACAT1, S410, S412, S414, and S456, and for ACAT2, S388, S390, S392, and S434), two were located at the N-terminal end of the enzyme (for ACAT1, S128 and S194, and for ACAT2, S109 and S176), and the remaining residue was positioned on the opposite site of the membrane for the two enzymes (S269 for ACAT1 and S249 for ACAT2) as per our ACAT topology model (3). Mutational studies of the various serine residues showed only S456 to be required for activity of ACAT1 enzyme, whereas the remainder of the serine substitutions in ACAT1 resulted in catalytically active enzymes (Fig. 4B). The mutants had varied levels of expression, with A1S269A showing the highest protein mass (Fig. 4D). Similar results were obtained for ACAT2, except that residue S438, equivalent to residue S456 of ACAT1, while showing the lowest specific activity, still retained about 27% WT specific activity, i.e., it was not inactive (Fig. 4C). The expression levels of each of the ACAT2 serine mutants were approximated to their WT counterparts, and A2S492L had the highest expression (Fig. 4D). Interestingly, residues S269 for ACAT1 and S249 for ACAT2, which were once thought important for the activity of the respective enzymes (3, 29), were indeed not essential for the activity of the enzyme, in agreement with Guo et
Fig. 4. Identification of the role of conserved serine residues in determination of the activity of ACAT enzymes. A: PMSF solubilized in DMSO was incubated with ACAT1- and ACAT2-containing microsomes at 37°C, pH 7.4, for 30 min. The samples were then used for microsomal ACAT assay as outlined under Experimental Procedures. ACAT1 (diamonds) and ACAT2 (squares) activities at varying concentrations of PMSF are expressed as a percentage of ACAT1 or ACAT2 activity in the presence of DMSO control. Data represent the average of three replicates. B: Whole-cell-based activity assays performed as described in Fig. 2B for WT and specific serine mutants of the ACAT1 enzyme, as indicated on the X axis. Each experiment was repeated twice with similar results. Data represent mean ± SEM for n = 4. C: Activity assay data for WT and specific serine mutants of ACAT2 enzyme as indicated on the X axis and assayed as above. This experiment was repeated three times, and data represent mean ± SEM for n = 4. D: Immunoblot analysis of expression levels of the indicated serine mutants of ACAT1 (top) and ACAT2 (bottom). Protein amounts loaded on the gel are as follows: WTA1, 15 µg; A1S128A, 25 µg; A1S194A, 20 µg; A1S269A, 2.5 µg; A1S410A, 25 µg; A1S412A, 25 µg; A1S414L, 25 µg; A1S456A, 25 µg; WTA2, 15 µg; A2S109A, 10.5 µg; A2S176A, 30 µg; A2S249A, 9 µg; A2S388A, 7 µg; A2S390A, 19.5 µg; A2S392L, 4.5 µg; and A2S434A, 9 µg.
al. (18). When these particular serine residues were mutated to leucine, the proteins were not expressed in AC-29 cells. However, when these serine residues were changed to alanine, the mutants were expressed and were enzymatically active proteins, showing that these serine residues are not essential for ACAT catalytic activity.

**Effects of serine-, cysteine-, threonine-, and tyrosine-modifying reagents on ACAT activity.** Because our studies with mutations of serine residues showed only S456 to be required for ACAT1 activity, whereas the analogous serine was not absolutely essential for activity of ACAT2, we sought to determine whether ACAT2 had other amino acid residues containing an OH group that could act as a nucleophile within the context of a proposed active site catalytic triad. Thus, chemical modification studies were attempted first. We used hexadecylsulfonylfluoride (HDSF), a second serine-modifying reagent (an aliphatic analog of PMSF) to determine if any differential sensitivity of ACAT enzymes toward this reagent could be detected. Indeed, we saw that HDSF treatment inhibited ACAT1 activity in a dose-dependent manner, whereas ACAT2 activity was largely unaltered (Fig. 5A), a result that supports our earlier studies of serine mutations. Next, we modified cysteine residues of ACAT proteins using IAA, an alkylating reagent that modifies free sulfhydryl groups of the proteins. We found IAA treatment did not have a major effect on ACAT activity (Fig. 5B), a finding in agreement with previously reported data that cysteine-less ACAT1 mutant was catalytically active (30). We then modified threonine residues of both ACAT proteins using the 4-hydroxy-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (NP-LLL-VS) reagent as described previously (31). We found that the activity of neither of the ACAT enzymes was inhibited by NP-LLL-VS treatment,
Fig. 5. Effects of serine-, cysteine-, threonine-, and tyrosine-modifying reagents on the activities of ACAT enzymes. A: ACAT1 activity (left panel) and ACAT2 activity (right panel) after hexadecylsulfonylfluoride (HDSF) treatment. HDSF (generous gift from Prof. Sandra L. Hofmann) was solubilized in DMSO containing 0.1% Triton X-100. Microsomes prepared from either ACAT1 or ACAT2 stable cells were incubated with various concentrations of HDSF at 37°C, pH 7.4, for 30 min. The samples were then used for microsomal ACAT assay as outlined under Experimental Procedures. Background activity was obtained by performing ACAT assay with microsomes from untreated AC-29 cells. ACAT activity was corrected by background subtraction. The specific activity was calculated by normalizing ACAT activity with the microsomal protein mass and the assay run time. This experiment was performed three times with similar results, and data represent mean ± SEM for n = 2. B: Microsomes containing either ACAT1 or ACAT2 proteins were incubated with various concentrations of iodoacetamide (Sigma; solubilized in water) at 37°C, pH 7.4, for 30 min. The samples were then subjected to microsomal ACAT assay as described above. Data represent mean ± SEM for n = 2. C: Microsomes containing either ACAT1 or ACAT2 proteins were incubated with various concentrations of 4-hydroxy-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (Calbiochem; solubilized in DMSO) at 37°C, pH 7.4, for 30 min. The samples were then subjected to microsomal ACAT assay as described above. Data represent mean ± SEM for n = 2. D: Iodine monochloride (ICL) solution was prepared as described elsewhere (37). Microsomes containing either ACAT1 or ACAT2 proteins were incubated with various concentrations of ICL at 37°C, pH 7.4, for 30 min. The samples were then subjected to microsomal ACAT assay as mentioned above. Data represent mean ± SEM for n = 2.
although there was a trend toward decreased activity at the higher concentrations of the inhibitor (Fig. 5C). Finally, we used iodine monochloride (ICl), a reagent that covalently iodinates the tyrosine residues of the proteins. Surprisingly, we saw that activities of both of the ACAT enzymes were inhibited in a dose-dependent manner with ICl treatment, suggesting that a tyrosine residue(s) may be required for activity of ACAT isozymes.

**Requirement of tyrosine residue(s) for activity of ACAT enzymes.** Following up our chemical modification studies, we sought to identify tyrosine residue(s) required for activity of both of the enzymes using site-directed mutagenesis. We identified seven conserved tyrosine residues within ACAT enzymes, of which two were located within the putative active site domain of the enzymes (for ACAT1, Y404 and Y433, and for ACAT2, Y382 and Y411). Of note, Y404 in ACAT1 and Y382 in ACAT2 were part of the conserved FYXDWWN motif. Among the seven ACAT1 tyrosine mutants, Y404 had specific activity less than 20% that of the WT enzyme, and the A1Y518F mutant was completely inactive (Fig. 6A). The remaining tyrosine mutants show varied amounts of cholesterol esterification activity, suggesting that none were indeed required for activity of the enzyme. For ACAT2, only the A2Y382F mutant had a specific activity less than 20% that of its WT counterpart; however, none of the remaining tyrosine residues in ACAT2 were indeed required for ACAT2 activity (Fig. 6B). All of the tyrosine mutants of both of the enzymes were reasonably well expressed (Fig. 6C). Altogether, these data suggest that the tyrosine residue that is a part of the conserved FYXDWWN sequence is important for full enzymatic activity of both of the enzymes. Moreover, like serine mutants, there was a disconnect within ACAT tyrosine mutants, and although Y518 is
Fig. 6. Evaluation of the role of conserved tyrosine residue(s) as a requirement for the activity of ACAT enzymes. A: Whole-cell-based activity assay for WT and specific tyrosine mutants of ACAT1 enzyme as indicated on the X axis. The activity assay was performed as described in Fig. 2B. This experiment was repeated twice with similar results. Data represent mean ± SEM for n = 4. B. Whole-cell-based kinetic assay for WT and specific tyrosine mutants of ACAT2 enzyme as indicated. The kinetic assay was performed as described in Fig. 2B. This experiment was repeated twice with similar results. Data represent mean ± SEM for n = 4. C: Immunoblot analysis of ACAT1 (top) and ACAT2 (bottom) for each of the indicated tyrosine mutants. Amount of proteins loaded on the gel are as follows: WTA1, 12 µg; A1Y128F, 83 µg; A1Y308F, 15 µg; A1Y312F, 15 µg; A1Y322F, 8 µg; A1Y404F, 70 µg; A1Y433F, 35 µg; A1Y518F, 35 µg; WTA2, 20 µg; A2Y124F, 15 µg; A2Y286F, 20 µg; A2Y290F, 12 µg; A2Y300F, 15 µg; A2Y382F, 20 µg; A2Y411F, 15 µg; and A2Y496F, 15 µg.
essential for ACAT1 activity, the analogous Y496 residue is not required for enzymatic activity of ACAT2.

**Mutation did not alter the overall protein folding.** With our mutation studies, we identified several candidate residues that when mutated, resulted in an inactive enzyme, suggesting that those residues were candidates as active site amino acids. It is also possible that owing to mutagenesis, an ACAT protein might have had an altered structure that, in turn, resulted in an inactive enzyme. To investigate this possibility, we employed a cross-linking assay as an indirect measure to determine whether there were any changes in the overall protein folding that resulted from mutation. We reasoned that if a catalytically inactive mutant had maintained an overall three-dimensional structure similar to that of its WT counterpart, it should form an oligomeric structure similar to the WT enzyme upon cross-linking. For our experimental purpose, we used DSG, a membrane-permeable, homobifunctional, noncleavable 7.7 Å arm-length cross-linker. The results suggest that the mutations indeed did not alter the overall oligomeric structure of the protein, because both the WT and the mutant forms of ACAT1 (Fig. 7A) and ACAT2 (Fig. 7B) proteins formed similar cross-linked oligomeric forms, except for the A1D400N mutant. Although we made several attempts, we were unable to unequivocally identify the higher order oligomeric structure of A1D400N, suggesting that the mutant could have an altered structure. Furthermore, it should be noted that when A1D400 was mutated to alanine and glycine, respectively, both of the mutants were apparently degraded rapidly, causing loss of expression (data not shown) and indicating that D400 in ACAT1 is important for the stability of the protein. Taken together, except for A1D400N,
Fig. 7. Amino acid mutation did not alter the cross-linking patterns of ACAT enzymes. A: Microsomes, prepared from AC-29 cells transiently transfected with either WT or indicated ACAT1 mutants, were incubated with DMSO vehicle or disuccinimidyl glutarate (solubilized in DMSO) for 30 min at room temperature. The cross-linking reaction was quenched by adding 1 M Tris, pH 7.5, along with Western blot loading buffer. The samples were then subjected to Western blot analysis. Apparent molecular mass of the oligomeric states is given on the right side of the gel. B: AC-29 cells were transiently transfected with either WT or indicated ACAT2 mutants. Microsomes prepared from transfected cells were subjected to cross-linking assay as described above.
data from the cross-linking assay suggest that the loss of enzyme activity is more likely a consequence of the removal of a functional group rather than of grossly altered protein folding. More-sensitive analyses await the solubilization and purification to homogeneity of a functionally active enzyme, which has yet to be accomplished for ACAT enzymes.
DISCUSSION

After chemical modifications of intact ACAT proteins and site-directed mutagenesis of selected amino acids, we have identified several amino acid residues that are essential for ACAT activity and thus may be a part of the active sites of the two enzymes. We hypothesized that because ACAT enzymes have an intrinsic thioesterase activity, these proteins might also require serine, histidine, and aspartic acid residues for their catalytic activity as do other enzymes analogs with similar activities. Our results identified a putative catalytic triad, i.e., S456, H460, and D400, as necessary for ACAT1 activity. In addition, D400 also seemed essential for proper folding and structural stability of the protein. In ACAT2, only H438 was required for full enzymatic activity. We were not able to define whether A2D378 was essential for catalytic activity, although this residue was definitely important for structural stability of the enzyme. Finally, our results indicated that serine residues were apparently not absolutely required for ACAT2 activity, although the A2S434A mutant was reduced to only 27% of WT specific activity (Fig. 4C). Our tyrosine mutation studies show that the tyrosine residue of the conserved FYXDWNNW motif is required for full enzymatic activity of both of the enzymes. In addition, Y518 was required for ACAT1 activity, whereas the analogous residue was not important for ACAT2 activity. Taken together, our results suggest that the amino acid requirement for ACAT activity is similar but apparently not identical for the two ACAT isozymes.

Our data indicate that H460 in ACAT1 and H434 in ACAT2 are essential for ACAT activity. These results are in agreement with the data published by Chang and colleagues (18, 19). In contrast, our data do not support the recently published
mutagenesis data of An et al. (20). These authors reported that along with H460, H386 was also essential for ACAT1 activity, especially when cholesterol was used as substrate. Our data clearly indicate that the A1H386N mutant is catalytically active, indicating that this residue is not essential for ACAT1 activity. Of note, for an assay, they employed an in vitro ACAT assay using microsomes, whereas we used a whole-cell-based assay. Furthermore, they changed H386 of ACAT1 only to alanine, whereas we needed to mutate it to asparagine to show its nonessentiality. These experimental differences may explain some of the discrepancies between our results. For ACAT2, An et al. (20), reported that they did not see any protein expression when A2H434 (equivalent to A2H438 of AGM sequence) was mutated to alanine. However, we observed that this mutant had a significant level of protein expression and that this residue is essential for ACAT2 activity. They also reported that H360 and H399 (equivalent to H364 and H403 in AGM ACAT2 sequence) are essential for ACAT2 activity. When we mutated H364 to alanine we did not see any protein expression. We then substituted asparagine for the H364 residue, and this mutant was well expressed and catalytically active, showing that H364 is not essential for ACAT2 activity. Furthermore, the A2H403A mutant had 70% of the WT activity (data not shown), although its specific activity was only 20% of its WT counterpart (resulting from higher expression levels of A2H403A than WT ACAT2). In any case, in order for any one amino acid residue to be identified as an active site residue, we required zero activity when that particular amino acid was absent. Because A2H403A had a significant although low specific activity, we concluded that this residue can not be an active site residue of ACAT2.
In this report, for the first time, we show the possible association of an aspartic acid residue with ACAT activity. We found that A1D400N had zero catalytic activity (Fig. 3A). There may be two reasons for this: 1) loss of functional group of the mutated amino acid, and 2) possible alteration in the protein structure due to mutation (Fig. 7A). Our experimental data support both possibilities. Thus, we are unable to definitely conclude that A1D400 can indeed be considered an active site residue. In ACAT2, the corresponding residue, D378, was very sensitive to mutation. None of its non-conservative mutations showed any expression when transfected into AC-29 cells. Finally, when we substituted glutamic acid for D378, a conservative substitution, a functional protein resulted, although its expression level was low. Altogether, we conclude that this particular aspartic acid residue is important for the structural stability of ACAT enzymes and may be important for catalytic activity, at least for ACAT1, inasmuch as its mutant resulted in less than 1% WT specific activity. By contrast, the remaining conserved aspartic acid residue, which is a part of the conserved FYXDWNN sequence of ACAT enzymes, is not essential for catalytic activity.

The results of our studies of serine residue mutations of ACAT enzymes were quite surprising to us. Although we found that A1S456 is required for ACAT1 activity, the corresponding serine residue, S434 in ACAT2, was not absolutely required for catalytic activity. The specific activity of the A2S434A mutant was 27% of its WT counterpart, the least of the seven conserved serine residues examined, but not low enough to be considered essential for activity. The mutation data suggesting the absence of a required serine in ACAT2 was supported by data from chemical modification. We
found that ACAT1 activity was dose-dependently inhibited by the serine modifying reagent HDSF, whereas ACAT2 activity was relatively insensitive to this reagent (Fig. 5A). We then checked a serine residue that was not conserved between ACAT isozymes located within the putative active site domain of ACAT2 and substituted alanine for it. We did not see a considerable decrease in ACAT2-specific activity with this A2S408A mutation (data not shown). We conclude that S456 is important for ACAT1 activity, whereas the identical serine residue in ACAT2 is not absolutely required for catalysis of cholesterol esterification. The finding that none of the serine residues in ACAT2 are required for catalytic activity makes identification of the reaction mechanism difficult, and we are unable to resolve this issue with presently available data.

We also saw a discrepancy between the ACAT isozymes regarding the requirement of a tyrosine residue(s) for enzyme activity. Catalytic activity of both of the isozymes was dose-dependently inhibited to the same extent with treatment with ICl. Further, mutation studies showed that the tyrosine residue of the conserved FYXDWWN sequence is important for full enzymatic activity of both enzymes. Another tyrosine, Y518, was also found to be absolutely necessary for ACAT1 activity, whereas the corresponding tyrosine residue in ACAT2 was not required for activity. Of note, because it has been proposed that at neutral pH, ICl can modify both the tyrosine and histidine residues of proteins (32), we do not exclude the possibility that this may have occurred when ACAT activity was measured in the presence of ICl.
On the basis of a previously published report on the identification of the cholesterol binding site of proteins (33), Leon, Hill, and Wasan (34) have suggested that they identified two tandem cholesterol binding motifs in ACAT enzymes. The proposed putative cholesterol binding site contains a conserved tyrosine residue that is supposed to interact with the polar 3' OH group of the cholesterol molecule (33). A1Y308, A1Y312, A2Y286, and A2Y290 are part of the proposed cholesterol binding motifs of AGM ACAT enzyme sequences. When we mutated these residues to phenylalanine, all the mutants were catalytically active, suggesting that these residues are not essential for enzyme activity. Because there are two cholesterol binding sites and we mutated one residue at a time, we reasoned that in the absence of one substrate binding site, another motif was sufficient to carry out the esterification reaction. Thus, we made a double mutant in ACAT isozymes, in which both the tyrosine residues were mutated to phenylalanine. However, the double mutants were also catalytically active (data not shown), showing that the proposed tyrosine residues are not required for productive cholesterol binding to the enzymes.

We hypothesized that there may be a Ser-His-Asp catalytic triad at the active site of ACAT enzymes. In accordance with our hypothesis, we have identified specific serine, histidine, and (a probable) aspartic acid residues essential for ACAT1 activity. In general, it has been proposed that the Ser-His-Asp residues of a catalytic triad are located in three different regions of a protein (35). However, in ACAT1, the putative active-site Ser-His-Asp residues are located in close proximity to each other (the serine is four amino acids upstream of the histidine residue). It has been proposed that ACAT1 is a homotetrameric
protein in vitro and in intact cells (36). This oligomeric state may be required for activity of the enzyme. Thus, we speculate, if indeed there is a catalytic triad in ACAT1, the candidate active-site residues may be provided by different monomers of the ACAT1 oligomer.

Most of the mutations we examined were nonconservative substitutions, suggesting that a mutant could result in an inactive enzyme, owing to its altered protein folding. Thus, we employed a cross-linking assay (Fig. 7), in which we showed that the overall oligomeric state of the catalytically inactive mutants did not differ from that of the WT proteins, except for A1D400N. Although indirect, this result suggests that mutation did not change the apparent overall folding of ACAT proteins, indicating that the loss of enzymatic activity was more likely to have been caused by substitution of the functional group of the mutated amino acid rather than by alteration of the three-dimensional structure of the proteins. Loss of enzyme activity in the mutants may also be caused by poor substrate binding to the enzyme. It is always difficult to perform substrate binding assays with crude microsomal fractions, because there are many other proteins as well as lipids that can interact with cholesterol and acyl-CoA and give high signal-to-noise ratios. Thus, in the absence of a purified enzyme, it will always be difficult to interpret the signal specific to the various ACAT proteins. Hence, we do not exclude the possibility that loss of function of a mutant enzyme may be caused by poor substrate binding to the enzyme. A more detailed biochemical study, such as with X-ray crystallography, may be necessary to correctly ascribe the molecular basis of loss of catalytic activity among our mutants. Nevertheless, our comprehensive mutagenesis analyses of ACAT enzymes have
revealed a disparity between ACAT isozymes regarding the amino acids absolutely required for catalytic activity. This result may be of fundamental importance in designing ACAT2-specific inhibitory molecules, a treatment strategy believed to be potentially desirable for prevention of atherosclerosis in humans (16).
ACKNOWLEDGEMENTS

The authors thank Charles W. Joyce for performing initial chemical modifications in the early phases of these studies. The authors would also like to thank Sandra L. Hofmann for providing HDSF, Ramesh Shah for providing ICl solution, and Dr. Greg Sheliness for his critical input on several of these experiments.
REFERENCES


CHAPTER III

IDENTIFICATION OF THE INTERACTION SITE WITHIN ACYL-COA:CHOLESTEROL ACYLTRANSFERASE 2 FOR THE ISOFORM-SPECIFIC INHIBITOR PYRIPYROPENE A

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The following manuscript was published in the *Journal of Biological Chemistry* 2008 volume 283, issue 16, pages 10453-10460 and is reprinted with permission. Stylistic variations are due to the requirements of the journal. A. Das conceived part of the project, performed the experiments, and wrote the manuscript. M. A. Davis performed experiments. Dr. Tomoda and Dr. Ômura provided reagent. Dr. Rudel conceived the project and acted in an advisory and editorial capacity.
Targeted deletion of acyl-CoA:cholesterol-O-acyltransferase 2 (ACAT2) (A2), especially in the liver, protects hyperlipidemic mice from diet-induced hypercholesterolemia and atherosclerosis, whereas the deletion of ACAT1 (A1) is not as effective, suggesting ACAT2 may be the more appropriate target for treatment of atherosclerosis. Among the numerous ACAT inhibitors known, pyripyropene A (PPPA) is the only compound that has high selectivity (>2000-fold) for inhibition of ACAT2 compared with ACAT1. In the present study we sought to determine the PPPA interaction site of ACAT2. To achieve this goal we made several chimeric proteins where parts of ACAT2 were replaced by the analogous region of ACAT1. Differences in the amino acid sequence and the membrane topology were utilized to design the chimeras. Among chimeras, A2:1–428/A1:444–550 had 50% reduced PPPA selectivity, whereas C-terminal-truncated ACAT2 mutant A2:1–504 (C-terminal last 22 amino acids were deleted) remained selectively inhibited, indicating the PPPA-sensitive site is located within a region between amino acids 440 and 504. Three additional chimeras within this region helped narrow down the PPPA-sensitive site to a region containing amino acids 480–504, representing the fifth putative transmembrane domain of ACAT2. Subsequently, for this region we made single amino acid mutants where each amino acid in ACAT2 was individually changed to its ACAT1 counterpart. Mutation of Q492L, V493L, S494A resulted in only 30, 50, and 70% inhibition of the activity by PPPA, respectively (as opposed to greater than 95% with the wild type enzyme), suggesting these three residues are responsible for the selective inhibition by PPPA of ACAT2. Additionally, we found that PPPA non-covalently interacts with ACAT2 apparently...
without altering the oligomeric structure of the protein. The present study provides the first evidence for a unique motif in ACAT2 that can be utilized for making an ACAT2-specific drug.
INTRODUCTION

In mammals, intracellular cholesterol esterification is performed by two enzymes, ACAT1 and ACAT2, that use two lipophilic substrates, cholesterol and acyl-CoA. Both the enzymes are polytopic integral membrane proteins localized in the endoplasmic reticulum (ER) (1, 2). Expression of ACAT1 occurs in a wide variety of cell types, whereas ACAT2 is localized only to the enterocytes of the intestine and the hepatocytes of the liver (3). We have demonstrated that ACAT2 is an important cholesterol-esterifying enzyme in the human liver specifically within the hepatocytes (4). ACAT enzymes have long been associated with the pathogenesis of atherosclerosis. In particular, it has been shown in nonhuman primates that hepatic ACAT activity is associated with cholesteryl oleate enrichment of low density lipoprotein and increased coronary artery atherosclerosis (5–7).

To elucidate the role of ACAT enzymes in atherosclerosis, gene knock-out studies were performed in hyperlipidemic mouse models. ACAT1 knock-out (KO) mice were not well protected from atherosclerosis, whereas both apoE KO mice and low density lipoprotein receptor-deficient mice were dramatically protected by ACAT2 gene deletion (8–13). Furthermore, liver-specific knockdown of ACAT2 using antisense oligonucleotides significantly reduced hepatic cholesterol concentration, plasma low density lipoprotein cholesterol oleate, and aortic atherosclerosis (14). Together, the data of these studies suggest that inhibition of ACAT2 may be preferable to inhibition of ACAT1 for providing protection against atherosclerosis.
Many studies have been performed to identify the inhibitors for ACAT enzymes (15). Although many potent inhibitors of ACAT are available, most have been found to inhibit both isoforms with similar efficiencies (16) (S. Sturley, personal communication). One exception is pyripyropene A (PPPA, Appendix I Fig B.) that has more than a 2000-fold higher selectivity for inhibition of ACAT2 (16). PPPA-mediated ACAT2 inhibition can be achieved in a cell-free system as well as in intact cells (16), suggesting the possibility that PPPA may directly interact with ACAT2 and inhibit its activity. In this report we sought to identify the PPPA-sensitive site of ACAT2. As PPPA is specific for ACAT2, we hypothesized that if we replace the PPPA-sensitive site of ACAT2 with the analogous region of ACAT1, the chimeric protein would loose PPPA sensitivity. With this approach we succeeded in identifying the PPPA-sensitive residues of ACAT2.
EXPERIMENTAL PROCEDURES

**Generation of chimeras and mutants.** All the chimeras and single amino acid mutants were generated by an overlap PCR method. African green monkey (AGM) ACAT1 and ACAT2 sequences were used as the template for making all the chimeras and the mutants. For construction of chimeras, each of the fragments of the chimeras was amplified separately with suitable primers (obtained from IDT DNA Technologies). All the internal primers were designed with a forward primer with a 5' overhang sequence to its preceding fragment and a reverse primer with a 5' overhang sequence to a fragment immediately after it. In the first step, each of the fragments of a chimera was amplified using proofstart DNA polymerase (Qiagen). In the final step, all the fragments were mixed to perform an overlap PCR using two external primers having 5' Kpn1 and 3' Not1 restriction sites. The full-length DNA construct was gel-extracted (Qiagen gel extraction kit) followed by Kpn1 and Not1 digestion and ligation (Fast Link DNA ligase, Epicenter Biotechnologies) into a pre-digested pCDNA3 vector (Invitrogen). For making a C-terminal-truncated ACAT2 construct termed CT504, a 3’ reverse primer was designed that introduced a stop codon after residue 504 followed by Not1 restriction site. All single amino acid mutants were made by site-directed mutagenesis approach using suitable primers that introduce the desired mutation into the full-length DNA sequence. Full-length DNA sequences containing the point mutations were then digested and ligated into pCDNA3 in similar ways as for the chimeras. All the PCR reactions were run with the following conditions: 95 °C for 5 min, 1 cycle; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s for 25 cycles; followed by 1 cycle at 72 °C for 10 min. All modified sequences were confirmed by DNA sequencing. DNA with confirmed sequences was then further
purified using Endo free Maxi kit (Qiagen) to get transfection quality cDNA.

**Cell culture.** AC29 cells (a Chinese hamster ovary cell-derived cell line, which was a gift from T. Y. Chang) lack any endogenous ACAT activity, mRNA, or protein and were used for all of these experiments. Cells were maintained in monolayer at 37 °C in 5% CO₂ in Ham's F-12 medium supplemented with 1% Eagle's vitamins, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal bovine serum, and cells were typically grown to 70–90% confluence for all experiments.

**Cell-based ACAT assay.** 3 x 10⁶ AC29 cells were transiently transfected with 6 µg of various constructs of ACAT cDNA using nucleofection technology (Amaxa Biosystems) according to the manufacturer's instructions. Suspended cells were divided into two aliquots after transfection; one aliquot was used to seed four 35-mm dishes to be used for kinetic assay, and the remaining aliquot of cells was plated into a 60-mm dish to use for subsequent immunoblotting. 72h post-transfection the cells in 35-mm dishes were incubated for 30 min with either DMSO (2 dishes) as control or 5 µM PPPA (2 dishes). Thereafter cells were pulsed-labeled with 1µCi of [9,10-³H]oleic acid (PerkinElmer Life Sciences, NET-289) for 2h to stay within a linear response range. Cells were then removed by scraping and added directly to a glass tube containing 3.75 ml of 2:1 (v/v) chloroform:methanol. Extraction of total cellular lipids was completed by the addition of 1.25 ml of chloroform and 1.25 ml of water, respectively, to the tube followed by vigorous shaking. Finally, 3 ml of the lipid layer was isolated, and CE standards were added to samples. Samples were dried down under nitrogen, and lipid classes were
separated by thin layer chromatography (TLC) using Silica Gel 60 plates in a solvent system containing hexane:diethyl ether:acetic acid (70:30:1). CE bands were visualized by exposure to iodine vapor and scraped, and radioactivity was determined using a liquid scintillation counter.

**Preparation of postnuclear supernatant.** 72h post-transfection cells from 60-mm dishes were washed twice with ice-cold balance salt solution. Cells were solubilized in 60 µl of RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate and 1% Nonidet P-40 in phosphate-buffered saline) in the presence of 3 µl of protease inhibitor cocktails (Sigma). This was followed by sonication and removal of the nucleus and the cell debris by centrifugation at 14,000 rpm at 4 °C for 15 min. Supernatant was isolated, and 3-µl protease inhibitor cocktails were added to it and saved at −80 °C before use. Protein concentration of the PNS was measured by BCA assay (Pierce).

**Preparation of microsomes.** Cells were washed twice with ice-cold balanced salt solution and were scraped from the dish. Excess balanced salt solution was removed from the cells by centrifugation, and cells were suspended in homogenization buffer (0.25 M sucrose, 0.1 M K$_2$HPO$_4$, 1 mM EDTA, pH 7.4). Protease inhibitor mixture (Sigma) was added to the cells, and the cells were lysed by sonication. The nucleus and cellular debris were removed and discarded after centrifugation at 14,000 rpm at 4 °C. PNS was then subjected to ultracentrifugation at 100,000 rpm for 30 min. The pellet containing the microsomes was collected and subsequently suspended in ice-cold 0.1 M K$_2$HPO$_4$ buffer at pH 7.4. Protein concentration was measured by BCA assay (Pierce). Microsomes in
suspension were stored at –80 °C until assays were done.

**Microsomal ACAT assay.** Microsomes were thawed, and about 25–100 µg of proteins were mixed with 1 mg of bovine serum albumin and 20 µl of a cholesterol-saturated solution of β-cyclodextrin, and the final volume was brought to 300 µl. The samples were equilibrated in a 37 °C water bath for 30 min, and then [\(^{14}\)C]oleoyl-CoA (Amersham Biosciences) was added to the tubes and incubated for 20 min. To stop the reaction 6 ml of chloroform:methanol, 2:1, was added to the samples. 1.2 ml of KCl was then added, and the mixture was allowed to sit overnight at room temperature. An aliquot (3 ml) of the organic phase (containing lipids) was removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 µl of chloroform containing CE standard and then applied to a Silica Gel 60 TLC plate with subsequent separation in hexane:ethyl ether:acetic acid 70:30:1. The portion of the TLC plate containing the CE was scraped and suspended in scintillation fluid, and radioactivity was determined.

**Western blotting.** Proteins from microsomes or PNS were suspended in an equal volume of protein solubilization buffer, (120 mM Tris, pH 6.8, 20%(v/v) glycerol, 4%(w/v) SDS, 0.01% (w/v) bromphenol blue) 100 mM DTT, and incubated at room temperature for 30 min. 50 mM iodoacetamide was added to samples and incubated at room temperature for another 30 min. Proteins were electrophoretically separated using a 4–12% NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) and were transferred to a nitrocellulose membrane for 1 h at 115 V using a Western blot apparatus (Bio-Rad). The membrane was blocked overnight in 5% nonfat dry milk in TBST buffer (0.1 M Tris, pH 7.5, 0.15 M
NaCl, 0.1% (v/v) Tween 20) at 4 °C which was followed by its incubation with the purified ACAT primary antibody (1 μg/ml) for 2 h at room temperature. The primary antibody was then removed, and the membrane was washed 3 times (10 min each) with TBST. Thereafter, the membrane was incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma) at 1:20,000 dilution at room temperature for 1 h. After removing the secondary antibody, the membrane was washed 3 times with TBST (10 min each). The peroxidase signal was detected using Western illuminating reagents (PerkinElmer Life Sciences) and was captured on film (Eastman Kodak Co. BioMax light film). Affinity-purified ACAT antibodies were made as described before (3). Specificity of the antibodies was determined by running the PNS of empty vector-transfected cells on the gel as the negative control. We did not get any band on our negative control lane (data not shown).

**Cross-linking experiment.** Microsomes were incubated with either DMSO or 5 μM PPPA at room temperature for 30 min. Disuccinimidyl glutarate (solubilized in DMSO) was then added to the samples, and mixtures were incubated for 30 min at room temperature. The cross-linking reaction was stopped by adding 1 M Tris at pH 7.5. All the samples were then subjected to Western blotting.
RESULTS

PPPA-sensitive Site Is Located Outside the Putative Active Site Domain of ACAT2.

ACAT enzymes share more than 50% sequence similarity to each other after the first 100 amino acid residues, where the sequence similarity is only 2%. The area of highest sequence similarity among these proteins is toward the C-terminal end, including amino acid residues 386–462 of ACAT1 and amino acid residues 364–440 of ACAT2. Interestingly, this region contains two conserved motifs, FYXDWN and HEY. Because the sequence in this region is so highly conserved from yeast to human (Appendix I, Fig. C), we hypothesized that this region contains the active site domain of ACAT isozymes, and thus, both enzymes would have the same active site. Because PPPA is a highly selective inhibitor of ACAT2, we hypothesized that the PPPA-sensitive site of ACAT2 would be located outside of the putative active site domain of the enzyme. To test this hypothesis we made two chimeric proteins, A1:1–385 and A2:1–363, where the putative active site was swapped between the two enzymes (Fig. 1A). Kinetic assay result showed upon PPPA treatment ACAT1 activity was decreased 30% compared with the control cells, whereas this reduction was >95% for ACAT2-transfected cells, indicating PPPA is selective for ACAT2 (compare wild type enzymes of Fig. 1, B and C). Among the chimeras, A1:1–385 was inherently less catalytically efficient compared with its wild type (WT) counterpart since it had only 55% of the WT activity. With PPPA treatment, activity of this chimera in cells was lower by about the same 30% as seen when WT ACAT1 cells were treated with PPPA (Fig. 1B). This stands in direct contrast to the much greater decrease (>95%) induced by PPPA in activity of A2:1–363, a decrease that was comparable with WT ACAT2 enzyme (Fig. 1C). In each case the expression levels
FIGURE 1. PPPA-sensitive site of ACAT2 is located outside the putative active site domain of the enzyme. A, primary structure of the chimeric proteins termed A1:1–385 and A2:1–363 are indicated where sequences from ACAT1 are in open boxes and sequences from ACAT2 are in filled boxes. B, kinetic assay data for WTA1 and A1:1–385. AC29 cells were transfected with the cDNA encoding WTA1 and A1:1–385 proteins. 72h post-transfection cells were incubated with either vehicle (DMSO) or 5µM PPPA for 30 min at 37 °C. Thereafter, cells were pulse-labeled with 1µCi of [3H]oleic acid for 2h. The incorporation of [3H]oleic acid into cellular CE pool was measured as the determinant of the enzymatic activity of the respective proteins. Background activity was obtained by a parallel kinetic assay where cells were transfected with an empty vector. All activities were corrected by background subtraction and were normalized against the control (Ctrl) WT activity. Data represent the mean ± S.E. for n = 2. This experiment was repeated three times with similar results. C, kinetic assay data for of WTA2 and A2:1–363. The assay was performed essentially as described above, and data are presented as above. D, PNS made from cells transfected with WTA1 and A1:1–385 cDNAs were subjected to immunoblot using affinity-purified ACAT1 antibody as described under "Experimental Procedures." E, PNS, made from the cells transfected with WTA2 and A2:1–363 cDNAs, were subjected to Western blot analysis as described above.
of the chimeras were substantial and comparable with the respective WT proteins (Fig. 1, D and E). Taken together, these data support our hypothesis and suggest that the PPPA-sensitive site is located outside the highly conserved putative active site domain of ACAT2 (A2: 364–440).

**PPPA-sensitive Site Is Located between a Region Located within Amino Acids 428–504.** The sequence similarity among the first 100 amino acids of the ACAT enzymes is only 2%, suggesting this region might be a good candidate region for the PPPA-sensitive site. We made two chimeras, A1:1–71 and A1:1–123, to replace the entire N terminus of ACAT2 with that of ACAT1 (Fig. 2A). Both of these chimeras retained cholesterol esterification activity with normal expression levels when transfected into AC-29 cells (Fig. 2, B and C). However, in the presence of PPPA, activity of both the chimeras was fully inhibited, indicating the PPPA-sensitive site is not located within the N-terminal sequences of ACAT2.

Our experimentally obtained topology model of ACAT enzymes shows both have \( N_{cyto} C_{exo} \) orientation with five potential transmembrane domains (19). The computer-predicted transmembrane domain D was not utilized, whereas domain F was utilized in ACAT2; the opposite was true for ACAT1 (Appendix I Fig. A). Transmembrane domains with the intervening letters were likewise not utilized in either enzyme (19). Because of this difference in membrane topology, a large part of the ACAT1 sequence (amino acids 265–502) is predicted to be located on the cytoplasmic side of the membrane where the analogous sequence from amino acids 222–344 of ACAT2 is predicted to be on the luminal side of ER. To test whether the PPPA-sensitive site was located within this
FIGURE 2. PPPA-sensitive site of ACAT2 is located near the C-terminal end of the enzyme. A, amino acid sequences of the chimeras A1:1–71 and A1:1–123 are represented as in Fig. 1A. B, kinetic assay data for WTA2, A1:1–71, and A1:1–123 proteins. Assays were done as described in Fig. 1B. Data represent the mean ± S.E. for n = 2, and the experiment was repeated twice with similar results. C, PNS obtained from the cells transfected with WTA2, A1:1–71, and A1:1–123 cDNAs were subject to immunoblotting as described under “Experimental Procedures.” Primary antibodies used here were as follows. Affinity-purified ACAT2 antibody was used to detect WTA2 band, whereas affinity-purified ACAT1 antibody was used for A1:1–71 and A1:1–123 proteins. D, primary sequences of the chimeras A2:1–227 and A2:1–249 are indicated as Fig. 1A. E, amino acid sequence of the chimera A2:1–428 and ACAT2-C-terminal truncated mutant CT504. F, kinetic activity of WTA2, A2:1–428, and CT504. The enzymatic assay was performed as described in Fig. 1B. Data represent mean ± S.E. for n = 2. This experiment was repeated three times with similar outcomes. Ctrl, control. G, PNS obtained from the cells transiently transfected with WTA2, A2:1–428, and CT504 cDNAs were used for Western blotting.
region, we made two different chimeras. For A2:1–227 we swapped the entire sequence of this region of ACAT2 with the analogous region of ACAT1, and for A2:1–249 we swapped only the segment of this region whose subcellular localization was to be opposite among the enzymes (Fig. 2D). However, when we transfected these chimeras into AC-29 cells, they were not expressed perhaps due to the more gross structural changes in this region of the proteins.

Next, we made chimera A2:1–428 and CT 504, a C-terminal truncated version of ACAT2 where last 22 amino acids were removed from ACAT2 sequence (Fig. 2E). Transfection of the cDNAs encoding each of these two modified proteins shows both have cholesterol esterification activity (Fig. 2F), and their expression levels were comparable with the WT ACAT2 (Fig. 2G). Surprisingly, A2:1–428 showed only 50% inhibition of ACAT activity under PPPA treatment, suggesting the PPPA-sensitive site is located after amino acid 428. However, the activity of CT504 was nearly completely inhibited by PPPA in similar fashion to WT ACAT2. Thus, these two versions of modified ACAT2 helped narrow down the PPPA interaction site to be within the region located between amino acids 428 and 504.

**The PPPA-sensitive Site Is Located within the fifth transmembrane domain of ACAT2.** The region between amino acids 428 to 504 of ACAT2 contains part of the putative active site domain and the fifth transmembrane domain of the enzyme. We can divide this region into three segments as follows; 1) amino acids 428–440, the C-terminal boundary of the putative active site domain of the enzyme; 2) amino acids 480–504, which contain the fifth transmembrane domain; 3) amino acids 440–480, a region located
FIGURE 3. PPPA-sensitive site is located within the fifth transmembrane domain of ACAT2. A, amino acid sequence representation for ACAT2 within the region 428–526 indicating the 5th transmembrane domain with amino acids 480–498 (TM5) near the C-terminal end of the enzyme. B, amino acid sequence of the chimera Ch1 represented as Fig. 1A. C, the kinetic assay data WTA2 and Ch1. The assay was performed as described in Fig. 1A. Data represent the mean ± S.E. for n = 2. This experiment was repeated twice with similar results. D, PNS obtained from WTA2- and Ch1-transfected cells were used for Western blotting using the protocol as described under "Experimental Procedures." E, amino acid sequence of the chimera A2:1–440 indicated as Fig. 1A. F, the kinetic activity data for WTA2 and the chimera A2:1–440. The assay was performed as described for Fig. 1B. The assay was repeated twice with identical outcome. Data represent the mean ± S.E. for n = 2. G, Western blot analysis was performed using PNS obtained from WTA2- and A2:1–440-transfected AC29 cells. H, amino acid sequence representation of chimera A2:1–479 indicated as Fig. 1A. I, kinetic activity assay data for WTA2 and A2:1–479. The assay was performed as described in Fig. 1B. Data represent the mean ± S.E. for n = 4 of two independent experiments. J, PNS obtained from WTA2 and A2:1–479-transfected cells Western blotted as described under "Experimental Procedures."
in between the putative active site domain and the fifth transmembrane domain of the enzyme (Fig. 3A). In chimera Ch1 we replaced the 428–440 segment of ACAT2 with its ACAT1 counterpart (Fig. 3B). Ch1 was expressed as an active enzyme (Fig. 3C) with normal levels of expression (Fig. 3D); however, its response to PPPA was similar with that of the WT protein, suggesting the PPPA-sensitive site is not located within the sequence 428–440 of ACAT2. We then swapped the sequence between amino acids 440 and 480 of ACAT2 with the analogous sequence of ACAT1 in the chimera A2:1–440 (Fig. 3E). This chimera was active (Fig. 3F) and had normal protein expression level (Fig. 3G). PPPA also inhibits activity of this chimera, although the inhibition was 80% as oppose to 95% in the WT enzyme. Finally, we made chimera A2:1–479, where after residue 479 the remainder of the ACAT2 sequence was replaced by ACAT1 sequence (Fig. 3H). This chimera was enzymatically active (Fig. 3I), and its expression level was comparable with the WT protein (Fig. 3J). However, this chimera was completely insensitive to PPPA inhibition. Because we have already demonstrated that the sequence after residue 504 is not important for PPPA selective inhibition of ACAT2 (Fig. 2F), we concluded that the PPPA-sensitive site was located within a region between amino acid residues 480 and 504, which includes the putative fifth transmembrane domain.

**Residues 492, 493, and 494 Constitute the PPPA Interactive Site of ACAT2.** To further narrow down the PPPA-sensitive site of ACAT2, we performed sequence alignment of ACAT2 481–504 sequence with the analogous sequence of ACAT1 (Fig. 4A). Ten amino acids within this region were different between two enzymes. We speculated that because PPPA is an ACAT2-specific inhibitor, the PPPA-sensitive site
FIGURE 4. Identification of the residues responsible for PPPA-mediated inhibition of ACAT2. A, amino acid sequence alignment of the PPPA-sensitive region of ACAT2 with its ACAT1 counterpart. This region represents the fifth transmembrane domain of both enzymes. The asterisk represents sequence identity, the colon represents conserved substitutions, the dot represents semi-conserved substitutions, and no symbol indicates no match. The alignment was performed using ClustalW program from EMBL web site. B, enzymatic assay data of each of the single amino acid mutants where the amino acid in ACAT2 is replaced with the analogous amino acid in ACAT1 as indicated for the X axis. AC29 cells were transfected with the cDNA encoding either WTA2 or the various single amino acid mutants of ACAT2. Assays were done as indicated in Fig. 1B. Data represent the mean ± S.E. for n = 2. This experiment was repeated twice with similar results. C, Western blot analysis of the WT and each of various mutant ACAT2 proteins as indicated, performed as described under "Experimental Procedures."
must correspond to 1 or more of these 10 amino acids. Thus, we made the 10 single amino acid mutants of ACAT2 where each amino acid was mutated to its ACAT1 counterpart. All the mutants were active (Fig. 4B) and had comparable expression levels to the WT ACAT2 protein (Fig. 4C). Interestingly, among the 10 mutant proteins, A2Q492L, A2V493L, and A2S494C had only 30, 50 and 70% of the inhibition in enzymatic activity due to PPPA, respectively. More than 90% inhibition the activity was observed with PPPA for all the other mutants along with the WT enzyme. These results clearly suggest Gln-492 is required for PPPA selective inhibition of ACAT2. In addition, Val-493 and Ser-494 may also be required for PPPA-selective inhibition of ACAT2 perhaps by providing steric interactions around Gln-492 residue.

**Kinetic Properties of PPPA-insensitive Recombinants.** It is possible that the mutant and chimeric enzymes might have altered kinetic properties compared with the WT enzyme that contribute to the loss of PPPA sensitivity. To test this possibility, we determine the apparent $V_{\text{max}}$ and $K_{\text{m}}$ values for PPPA-insensitive recombinants and WT enzyme using the microsomal ACAT assay (Table 1). Our results indicate the $V_{\text{max}}$ value was highest for WT enzyme, whereas the various recombinants had lower but comparable maximum reaction velocities. Likewise, the apparent substrate affinity ($K_{\text{m}}$) of A2:1–428, A2:1–479, and A2S494C was comparable with that for the WT enzyme. Although the apparent $K_{\text{m}}$ of A2Q492L averaged about 2-fold higher than WT ACAT2 and the value for A2V493L was about 50% higher, these were not statistically significant differences, so that the substrate concentration requirement for each of the enzyme constructs remained similar. Furthermore, to determine whether CE synthesis rates in
### TABLE 1 Kinetic properties of PPPA-insensitive chimeras and mutants.

Microsomes were prepared from AC-29 cells that had been transiently transfected with either WT or various mutant and chimeric constructs of ACAT2 protein as indicated above. For the assay, 50 µg of microsomal protein was used for measurement of ACAT activity by the method described under "Experimental Procedures," which was run at four different [\(^{14}\)C]oleyl-CoA (5, 15, 30, and 50 nmol) concentrations. Using Western blotting, expression levels of the proteins were determined. Specific activity was calculated as the dpm of cholesteryl esters formed divided by the protein mass obtained from densitometric analysis of the Western blots. Substrate saturation curves of each of the recombinant constructs were made by plotting specific activity against its corresponding \(^{14}\)C]oleyl-CoA concentrations. Kinetics parameters were calculated using Hyperbolic Regression Analysis software. This program employs nonlinear regression to calculate kinetic parameters as shown earlier (17, 24). Each assay was run in duplicate. A.U represents arbitrary units.

<table>
<thead>
<tr>
<th>Clone</th>
<th>(V_{\text{max}}) (A.U)</th>
<th>(K_m) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT2</td>
<td>4237 ± 800</td>
<td>5.53 ± 4.44</td>
</tr>
<tr>
<td>A2:1-428</td>
<td>970.8 ± 198.3</td>
<td>3.33 ± 3.71</td>
</tr>
<tr>
<td>A2:1-479</td>
<td>707.1 ± 75.39</td>
<td>2.66 ± 1.75</td>
</tr>
<tr>
<td>A2Q492L</td>
<td>1268 ± 178.3</td>
<td>12.18 ± 5.14</td>
</tr>
<tr>
<td>A2V493L</td>
<td>1400 ± 198.2</td>
<td>8.094 ± 4.11</td>
</tr>
<tr>
<td>A2S494C</td>
<td>1371 ± 386.6</td>
<td>5.87 ± 6.85</td>
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whole cells of these recombinants may have been altered due to PPPA treatment, we performed whole cell-based ACAT assays that were terminated at different time intervals (1, 2, 4, and 8 h). For each recombinant, we then compare the plot of CE synthesis rate (Y axis CE synthesis versus X axis time) in the presence of either DMSO vehicle or PPPA. Our result showed that the shape of the curves remained similar for up to 4 h regardless of vehicle or inhibitor treatment (data not shown) so that the 2-h time point used for most comparisons was part of an apparent linear first order response. All together these results suggest that the kinetic characteristics of the recombinant enzymes were quite similar to those of the wild type enzyme and should not confound the interpretation of PPPA-induced inhibition.

**Nature of Interaction of PPPA with ACAT2.** Although PPPA inhibits ACAT2 activity, the mechanism of this was not known. Because PPPA can inhibit the activity of the enzyme in a cell-free system (microsomal assay), it is possible that it directly interacts with the enzyme to inhibit its activity. Because of the lack of a purified protein, we were not able to directly test this hypothesis; however, we performed two experiments to assess the nature of the protein-inhibitor interaction. In the first experiment, ACAT2 stable cells were incubated with the vehicle (DMSO) or 5 µM of PPPA in a 150-mm cell culture dish, and microsomes were then prepared from these cells. ACAT assay was performed thereafter with the microsomes, and only 35% inhibition was achieved with microsomes isolated from PPPA-treated cells (Fig. 5A). In another experiment, microsomes were prepared from ACAT2 stably transfected cells and were subsequently incubated with vehicle or 5 µM PPPA at 37 °C for 30 min. Microsomes were then
FIGURE 5. Nature of interaction of PPPA with ACAT2 and its effects on the oligomeric state of the protein. A, microsomes were prepared from ACAT2 stable cells incubated with either DMSO or 5 µM PPPA for 30 min at 37 °C. 50 µg of DMSO-treated microsomes and 25 µg of PPPA-treated microsomes were used to do a microsomal ACAT assay (described in detail under "Experimental Procedures"). Specific activity was calculated by normalizing activity as dpm of cholesteryl esters formed with the protein mass and the assay run time. Data represent the mean ± S.E. for n = 2. This experiment was repeated twice with similar results. B, 50 µg of ACAT2 stable cell microsomes were incubated with either DMSO or 5 µM PPPA at 37 °C. This was followed by reisolation of the microsomes by centrifuging at 100K for 15 min. Microsomal ACAT assay was then performed with the reisolated microsomes. Specific activity was calculated as above. Data represent the mean ± S.E. for n = 2. This experiment was repeated three times with similar results. C, 10 µg of protein in ACAT2 stable cell microsomes and 20 µg of protein in microsomes obtained from AC29 cells transiently transfected with A2Q492L mutant were treated with either DMSO or 5 µM PPPA at room temperature for 30 min. Samples were then treated with vehicle or disuccinimidyl glutarate (DSG) for another 30 min at room temperature. The cross-linking reaction was quenched by adding Western loading buffer containing 1 M Tris, pH 7.5, to the reaction, and all samples were subjected to Western blotting as described under "Experimental Procedures." As a control ACAT2 and A2Q492L was run on the gel without any treatment (1st and 4th lane on the gel). Apparent molecular weight of the bands is shown on the right side of the gel.
reisolated, and ACAT activity assay was performed again (Fig. 5B). There was almost no inhibition remaining for the activity of the PPPA-treated samples compared with the vehicle-treated microsomes. We concluded that PPPA interaction with the protein is likely non-covalent. Of note, the specific activity ACAT2 was lower in reisolated microsomes (Fig. 4B compared with Fig. 4A). We suspect that during reisolation of the microsomes by ultracentrifugation, we lost some proteins; thus, there was a reduction in ACAT activity.

It has been proposed that ACAT1 can form oligomers in vitro and in intact cells (18). It is possible that this oligomeric state is required for the activity of the enzyme. Because both the ACAT enzymes share a high sequence similarity and catalyze the same chemical reaction, it is possible that ACAT2 also forms a higher order oligomeric state during catalysis. We hypothesized that PPPA interaction may disrupt the oligomeric state of the protein, thereby inhibiting the activity of the enzyme. To test this hypothesis, we performed a cross-linking experiment using disuccinimidyl glutarate, a homobifunctional non-cleavable 7.7-Å arm-length cross-linker. Microsomes prepared from the WT or A2Q492L mutant were incubated with either DMSO or PPPA followed by another round of incubation with disuccinimidyl glutarate. The oligomeric state of the proteins was visualized by Western blotting (Fig. 5C). This result suggests that PPPA did not alter the overall oligomeric structure of the protein as both the WT and the mutant protein forms similar cross-linked oligomeric forms regardless of vehicle or inhibitor treatment.
DISCUSSION

PPPA is a selective inhibitor of ACAT2 showing 2000-fold higher specificity for inhibition of ACAT2 than ACAT1. In this report we sought to determine the PPPA interaction site within the ACAT2 enzyme protein. We employed a chimeric approach with the hypothesis that if the PPPA-sensitive site of ACAT2 is replaced by the analogous site of ACAT1, the chimeric enzyme will not be inhibited by PPPA. With this approach we identified an ACAT2 sequence, amino acids 480–504, the fifth transmembrane domain of the enzyme, which contains the amino acid sequence responsible for PPPA selectivity of ACAT2. Based on sequence alignment within the fifth transmembrane domain of the two ACAT enzymes, we performed single amino acid mutations where each of the 10 substituted amino acids in ACAT2 was individually changed to its ACAT1 counterpart. Substitution of glutamine 492 with leucine was the specific substitution that eliminated PPPA specificity in ACAT2 inhibition (Fig. 4B). Some loss of selectivity was also seen when the adjacent valine and serine were substituted with leucine and cysteine, respectively. We assume the latter substitutions may be related to steric hindrance surrounding the glutamine residue. In any case the data are clear that PPPA selectivity for ACAT2 inhibition requires Gln-492, which lies within the fifth putative transmembrane domain of ACAT2.

One major problem working with chimeric enzymes is that there might be an altered structure of the protein generated while making the chimera because of the significant changes in its amino acid sequences. Although we cannot completely rule out this possibility for all the chimeras we made, even if there were changes in the structure,
it did not affect the level of expression nor the activity or apparent $K_m$ for most of the chimeras. Thus, most of our chimeras were comparable in catalytic activity so that we were able to test their activity in the presence of PPPA. When we made A2:1–227 and A2:1–249, there was likely a significant alteration in the three-dimensional structure of the protein possibly as a result of alteration in transmembrane domain utilization. As a result, when attempts to express these two chimeras in AC-29 cells were made, no detectable expression resulted perhaps because misfolded proteins generated that were rapidly degraded within the cells. In the absence of crystal structure, the chimeric approach to study some of the structural features of ACAT isozymes has proven helpful. We believe this method could also be useful for other proteins where there is a need to document unique isozyme-specific characteristics.

To design the chimeras, we used the ACAT topology model generated by our laboratory (19). To assess membrane topology, a glycosylation reporter gene and FLAG epitope tag sequence was appended to a series of ACAT1 and ACAT2 cDNAs that had been truncated after each of the computer-predicted transmembrane domains. An in vitro translation system was used to express the truncated mutants. Based on glycosylation site utilization and accessibility to exogenous protease, we concluded that both the enzymes span the membrane bi-layer five times with the N-terminal end on the cytoplasmic side and the C-terminal end on the luminal side of the ER membrane. The strength of this approach was that both enzymes were analyzed in concert, so that differences could be detected. Using a different approach, T. Y. Chang and co-workers (20, 21) have predicted ACAT1 has seven transmembrane domains, whereas ACAT2 has only two. Using AC-29
cells they expressed modified human ACAT enzymes in which an epitope tag was inserted after each of the computer-predicted transmembrane domains of the enzyme. Recently, Guo et al. (22) have refined their previous model using a cysteine-scanning mutagenesis approach and reported that human ACAT1 spans the membrane bi-layer nine times. It is important to mention that all methods for membrane topology assessment rely upon modifications of the protein which can change utilization of particular transmembrane domains, depending upon charge density of the amino acid sequences immediately after the transmembrane domain. Thus, it is difficult to ascertain the correct model with certainty. However, when two enzymes are as similar in their amino acid sequence as ACAT1 and ACAT2 and both catalyze the same reaction, it seems unlikely that membrane topology will be so different as having nine transmembrane domains in ACAT1 and only two in ACAT2. The fact that the chimeric enzyme approach used here worked so well also supports the notion that a greater degree of similarity exists between the two enzymes than seven or nine versus two transmembrane domains.

Our evidence shows that Gln-492 is not an active site residue nor is it located within the substrate binding pocket of ACAT2 because when we mutate this residue to leucine, the enzyme is still active, indicating that this residue is not required for activity. This raises the important question, If Gln-492 is not essential for activity of the enzyme, why then does its presence together with the inhibitor affect the activity of the enzyme? According to our topology model, Gln-492 is located within the fifth transmembrane domain of the enzyme. Interestingly, Guo et al. (22) also show that the localization of Leu-514, the analogous residue of Gln-492 in human ACAT1, is localized within a
transmembrane domain. Assuming that localization of the Gln-492 and its surrounding residues is indeed within a transmembrane domain of the enzyme, we can speculate about a probable molecular mechanism of inhibition of ACAT2 in the presence of PPPA. Analogous to the proposal for ACAT1, it is possible that ACAT2 also forms a higher order oligomeric structure in vivo that is associated with activity of the enzyme. In fact our cross-linking experiments provide evidence that a higher order oligomeric structure of the WT ACAT2 may occur (Fig. 5C). Transmembrane domains of membrane-bound polytopic proteins often take part in the self-association of the proteins. We speculate that more than one transmembrane domain including the fifth transmembrane domain of ACAT2 is required for forming an oligomeric structure of the protein and such oligomerization is required for the activity of the enzyme. When PPPA interacts with the enzyme at the Gln-492 residue, the oligomeric structure of the protein may be disrupted to the extent that some of the associations among the transmembrane domain regions of the enzyme are not efficient. This disruption could have the effect of altering the cytoplasmic side conformation near the active site (located just upstream of the fifth transmembrane domain containing Gln-492 in the three-dimensional structure of the protein), resulting in enzyme inhibition. If PPPA interacts only with the fifth transmembrane domain of the enzyme, the oligomerization among the other transmembrane domains might well remain unaffected so that, in our cross-linking experiment, we still see a similarity in apparent higher order oligomeric structure of the WT ACAT2 protein in the presence of PPPA. The hydrophobic nature of the PPPA molecule seems consistent with the possibility that it fits into the membrane where it
exerts its effect. All of these speculations are currently under investigation in our laboratory.

In conclusion, in this report we have identified the putative fifth transmembrane domain as the PPPA-sensitive site of ACAT2. Furthermore, we have shown Gln-492 within this transmembrane domain is the primary residue responsible for PPPA interaction within the ACAT2 enzyme protein that results in inactivation of the enzyme. We have repeatedly shown through our animal model studies that liver-specific inhibition of ACAT2 may be beneficial for the treatment of atherosclerosis. Although the molecular mechanism of PPPA-mediated ACAT2 inhibition is not yet clearly understood, our present biochemical study definitely shows that by targeting the fifth transmembrane domain of ACAT2, especially in the region surrounding Gln-492, it is feasible to selectively inhibit ACAT2. These data suggest that specific drug molecules with similarities to PPPA might accomplish a similar selectivity, providing encouragement that selective ACAT2 inhibition is feasible as well as desirable (23).
REFERENCES


CHAPTER IV

ACAT1 PROTEIN TURNS OVER FASTER THAN ACAT2: IMPLICATION FOR IN-VITRO ENZYMATIC ACTIVITY

Akash Das, Matthew A. Davis, John C. Wilkinson, Lawrence L. Rudel

The following manuscript will be submitted to Journal of Biological Chemistry. Stylistic variations are due to the requirements of the journal. A. Das conceived the project, performed the experiments, and wrote the manuscript. M. A. Davis performed experiments. Dr. Wilkinson provided the reagents and acted in an advisory capacity. Dr. Rudel supervised the project and acted in an advisory and editorial capacity.
ABSTRACT

In higher eukaryotes, the intracellular cholesterol esterification reaction uses cholesterol and acyl-CoA as substrates and is catalyzed by two different isoforms of acyl-CoA:cholesterol- O -acyltransferase (ACAT). Both proteins are localized to the ER membrane and span the membrane five times. ACAT proteins play important roles in whole body cholesterol metabolism including hepatic lipoprotein particle packaging and intestinal cholesterol absorption. Furthermore, inhibition of ACAT2 in liver protects mice from development of atherosclerosis. Despite these varied and significant biological roles, the regulation of ACAT activity is poorly understood. In this report we present studies in which we have identified rather remarkable differences in protein stability between ACAT1 and ACAT2. To document protein stability we used stably transfected ACAT1 and ACAT2 cells to perform $^{35}$S–methionine pulse labeling experiments. The half-life of ACAT1 was 30 minutes while the half-life of ACAT2 was 15 hours. To identify candidate pathways leading to the difference in degradation specific inhibitors of the intracellular protein degradation machinery were tested. Both ACAT proteins undergo degradation via the proteasomal pathway requiring the covalent attachment of ubiquitin molecules. The data suggest that ACAT2 is intrinsically a more stable protein than ACAT1 perhaps accounting for the typical finding of higher amounts of ACAT2 activity than of ACAT1 activity in crude microsomal membranes from liver in spite of higher mRNA levels for ACAT1. We have yet to identify the molecular basis for the increased stability of ACAT2.
INTRODUCTION

Cholesterol is a major constituent of all biological cell membranes and its presence adjusts fluidity and permeability in the membrane (1). In mammalian cells, there are two major forms of cholesterol, free cholesterol and cholesteryl ester. When the membranes are saturated with free cholesterol, cholesteryl ester is synthesized by attachment of a fatty acid to the 3’ hydroxyl group of the cholesterol molecule. This conversion changes the physical property of cholesterol and it becomes poorly soluble in the membrane. The newly synthesized cholesteryl ester either accumulates in the cytoplasm within the lipid droplets or gets packaged into the lipoprotein particles and secreted from the cells (3). Conversion of cholesterol to its esterified form is often considered a protective mechanism employed by many types of cells to prevent the toxicity of excess free cholesterol where too much cholesterol can lead to membrane dysfunction.

In mammals, the intracellular cholesterol esterification reaction can be catalyzed by two isoforms of ACAT enzymes, using two lipophilic substrates, cholesterol and acyl-CoA (3,4). Both the enzymes are localized to the endoplasmic reticulum membrane and span the membrane for five times (5). Expression of ACAT1 occurs in a wide variety of cell types, whereas ACAT2 expression is restricted to the enterocytes of the intestine and the hepatocytes of the liver (6,7). ACAT enzymes have long been associated with the pathogenesis of atherosclerosis. In particular, it has been shown in animal models that hepatic ACAT activity is associated with cholesteryl oleate enrichment of low density lipoproteins and increased coronary artery atherosclerosis (8,9).
To elucidate the functional relevance of ACAT enzymes on whole body cholesterol metabolism, gene knockout studies were performed. The whole body ACAT1 knock out mice with a hyperlipidemic (i.e. apoE-/ or LDLr-/ ) background were characterized as having cytotoxic effects of accumulation of excess free cholesterol in various tissues like brain and skin (10,11). On the other hand, the whole body ACAT2 knockout mice had decreased cholesterol absorption, less cholesteryl ester packaging in the lipoprotein particles and resistance to diet induced hypercholesterolemia (12). When ACAT2 knock out mice were studied on hyperlipidemic background, the absence of ACAT2 provided beneficial effects against progression of atherosclerosis (13). In fact, inhibition of ACAT2 in the liver is sufficient to prevent atherosclerosis by reducing the concentration of cholesteryl oleate-rich low density lipoprotein particles in plasma (8,14).

Since ACAT proteins play an important role in whole body cholesterol metabolism it is important to study the regulation of enzymatic activity of these proteins. In our in-vitro assay system we have always found that ACAT2 activity is expressed at higher levels than ACAT1. In this report we wanted to identify molecular mechanisms responsible for higher ACAT2 activity. We hypothesized that ACAT2 is a more stable protein than ACAT1 and hence there is more ACAT2 enzyme protein in the assay system when ACAT protein is expressed. To study these possibilities, we compare the decay rate of two proteins by 35S amino acid metabolic pulse labeling experiments using ACAT stable cells. We extended these studies to compare the degradation pathways of the two ACAT proteins to determine the molecular basis for difference in protein stability.
EXPERIMENTAL PROCEDURES

Buffers. Buffer A contains 25mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS. Buffer B contains 8M urea, 300mM NaCl, 0.5% NP-40, 50mM Na₂HPO₄, 50mM Tris pH 8. All the buffers were supplemented with protease inhibitor cocktails (Sigma).

Cell culture. AC29 and ACAT stable cells (15) were maintained in Ham's F-12 medium. CHO-K1 and ts20 cells were maintained in 1:1 mixture of Ham’s F-12 and Dulbecco’s modified Eagle’s medium. All mediums were supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml). All cells were maintained at 37 °C in 5% CO₂ incubator except ts20 cells which were grown in 34°C incubator. All cells were grown to approximately 70% - 90% confluency before passaging. AC29 and ts20 cells were generous gifts from T.Y.Chang (16) and H.L.Ozer respectively (17).

Plasmids. Full length African green monkey ACAT1 and 2 cDNA’s were cloned into Kpn1 and Not1 restriction site of pCDNA3.1 vector by PCR. Recombinant ACAT plasmids were made by inserting a single Hemagglutinin (HA) tag at the N-terminus of ACAT cDNA by PCR. pMax plasmid encoding green fluorescence protein (GFP) was provided with the Nucleofector kit (Lonza). Full length biotin tagged human ubiquitin (b-Ub) was cloned in pEBB vector (a gift from Ezra Burstein) and wild type (WT) and mutant (MT, UbK48R) full length yeast ubiquitin were cloned into pCW7 vector (18,19).

Transfection. ACAT stable cells were transfected by Nucleofection technology (Lonza)
following manufacturer’s protocol. ts20 cells were transfected using calcium phosphate precipitation method and CHO-K1 cells were transfected using FuGene 6 (Roche).

**Quantitative Real time PCR.** Total RNA was extracted from monkey livers with Trizol (Invitrogen) reagent using the manufacturer’s protocol. Isolated RNA was dissolved in water and the concentration determined by spectroscopy. The cDNA was prepared from 1 µg RNA primed with 50 picomoles of random hexamers using Omniscript (Qiagen) following the manufacturer’s instructions. Upon completion of the reaction, the cDNA was diluted tenfold. The real-time PCR reaction was run in a final volume of 25 µl using 5 µl of the diluted cDNA, 5pM of forward and reverse primer and 12.5 µl of Sybr Green PCR master mix (Applied Biosystems). Real time PCR detection and analysis was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR parameters were an initial denaturation and taq activation step of 95°C for 10’ followed by 40 cycles of 95°C for 10” then 60°C for 1’. Message copy number was determined by comparison to the standard curve and dissociation analysis was performed to confirm amplification of a single product. The primers used to detect ACAT’s are as follows: for ACAT1 F: GAAACGGCTGTCAAGTCC, ACAT1 R: AATGGCTTC AATTCCCTCTGC, ACAT2 F: GACAGGATGTCTACCAGG, ACAT2 R: CGGT AGTAGTGGAGAGGAC, GAPDH F : CACCACTGCTTAGCACC, GAPDH R: TGGTCATGAGTCTCTCAG.

**Pulse chase analysis.** Exact experimental conditions are given in the individual Figure legends. On the day of experiment cells were washed with balance salt solution and
starved with cysteine and methionine free Dulbecco’s modified Eagle’s medium media supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). After chase, cells were fed with the same media containing $^{35}$S protein labeling mix (Perkin Elmer Life Sciences). Following labeling, media was removed, cells were washed twice with balance salt solution and chased for various time points with complete media. After each chase time point, media was removed from the cells, washed twice with ice cold balance salt solution and buffer A was added directly into the plates and incubated for 15 min on ice. Lysates were clarified by centrifuging at 15,000 X g for 15 min at $4^0$C. Equal amounts of lysates were incubated with specific antibody and rotated at $4^0$C overnight. Next morning, protein A agarose beads were added to the samples and rotated at room temperature for 1 hr. Beads were collected by centrifugation at 4000 X g for 5 min, washed three times with lysis buffer and resuspended in 4X SDS loading buffer containing dithiolthreitol and heated for 15 min at $60^0$C. Samples were centrifuged briefly and supernatants were collected and subjected to SDS-PAGE. Radiolabeled proteins were transferred to nitrocellulose membrane, dried and exposed to an imaging plate for 3-4 days at room temperature. After exposure, plate was scanned and band intensities were calculated by phosphoimaging device.

**Ubiquitination assay.** Exact experimental conditions are given in the figure legend. Cells were transfected with either control GFP or b-Ub plasmids. The day after transfection, cells were fed fresh media containing 4mM biotin. On the day of the experiment cells were treated with either vehicle or MG-132 (Calbiochem). After incubation cells were collected, washed twice with ice cold balance salt solution and 200
µl buffer B was added to each 60 mm dish. The cells were collected, sonicated and clarified by centrifuging at 15,000 X g for 15 min at 4°C. Protein concentration was measured by BCA assay (Pierce). Approximately, 250 – 500 µg proteins were taken into a fresh tube and 20 µl streptavidin beads (Pierce) were added to it and rotated overnight at 4°C. By adding buffer A the final concentration of urea was reduced below 2M. Next day, tubes were centrifuged at 4000 X g for 5 min at room temperature to collect the beads and supernatants were discarded. Beads were washed 7 times (5 min each) with the following buffers: twice with buffer B without any protease inhibitor cocktail, once with wash buffer 1 (8M urea, 200mM NaCl, 2% SDS, 50mM Tris HCl pH 8), once with wash buffer 2 (8M urea, 200mM NaCl, 50mM Tris HCl pH 8) and thrice with wash buffer 3 (50mM Tris HCl pH 8, 0.5 mM EDTA, 1mM DTT). After washing, beads were resuspended in 20µl of 4X SDS loading buffer containing beta-mercaptoethanol and heated for 20 min 75°C. Samples were spun briefly and supernatants were subjected to SDS PAGE. Ubiquitinated ACAT proteins were visualized by probing the immunoblot with ACAT specific antibodies.

**Microsome preparation, Immunoblotting, microsomal ACAT assay.** These experiments were performed as described earlier (20,21). For Immunoblotting 1µg/ml affinity purified ACAT antibodies (6), 1:1000 diltion of HA tag antibody (Cell signaling) were used.
RESULTS

*In-vitro ACAT2 activity is consistently higher than ACAT1.* To compare the ACAT activity between ACAT1 and ACAT2 in monkey livers we performed ACAT assay using microsomes isolated from the livers of two species of monkeys. As shown in Figure 1 liver from both African green (Fig 1A) and cynomolous (Fig 1C) monkeys had ACAT2 activity ten and eight fold higher than ACAT1 respectively. Surprisingly, the mRNA expression levels of ACAT proteins is completely opposite, i.e, in African green monkeys (Fig 1B) mRNA abundance of ACAT1 is four fold higher than ACAT2 whereas in cynomologous monkeys (Fig 1D) mRNA abundance was three fold higher for ACAT1 mRNA than ACAT2. Together, these data suggest there is disconnect between ACAT activity and mRNA transcript levels in monkey livers. As per our previously reported data, this conclusion is true as well for several human liver samples isolated from gallstone patients (7). To further test whether difference in ACAT activity of two isoforms can be manifested in cell culture system, we transfected ACAT deficient AC29 cells individually with the same concentration of either ACAT1 or 2 plasmids and subsequently measure ACAT activity in isolated microsomes. As shown in Fig 1E, consistent with the above observations, even in cell culture system ACAT2 activity is ~10 fold higher than ACAT1. Altogether, these data indicate in our *in-vitro* assay, ACAT2 is consistently more active than ACAT1.

**ACAT1 turns over faster than ACAT2.** There are at least two possible reasons for the higher ACAT2 activity: 1.) The specific activity of ACAT2 may be higher than ACAT1, or 2.) There may be more enzyme protein for ACAT2 than for ACAT1 in the microsomal
Fig 1: *In-vitro* ACAT2 activity is consistently higher than ACAT1. A. ACAT activity was performed using microsomes isolated from the liver of African green monkeys fed low cholesterol diet for 16 weeks. Assay was run in duplicates. B. Total RNA was isolated from the same monkey liver and subjected to quantitative RT-PCR using ACAT specific primers. C. Microsomes were prepared from the liver of cynomolgous monkeys fed low cholesterol diet for 16 weeks. ACAT assay was performed in duplicates for each animal. D. Total RNA was isolated from the same monkey livers and subjected to quantitative RT-PCR using ACAT specific primers. All monkey data represents mean ± SD for n = 6. Details of the diet composition and monkey housing protocol is given elsewhere (2). E. AC-29 cells were transiently transfected with the plasmids encoding for AGM ACAT1 and ACAT2 proteins. 72 hrs post transfection cells were harvested and microsomes were prepared. These microsomes were used to run an ACAT assay in duplicates. Data represent mean ± SD for five independent experiments.
assay system. In this report we investigated the second possibility. We reasoned either higher mRNA abundance or altered protein decay rate may result in different amounts of the two ACAT isoforms in the assay system. We directly investigated whether ACAT proteins have altered protein decay rate.

Accordingly, using an $^{35}$S-amino acid pulse chase labeling experiment, the turnover rate of the proteins was measured using cells stably expressing either ACAT1 or ACAT2. Indeed, as shown in Fig 2A, ACAT1 turns over faster than ACAT2. And when all the data from several experiments were combined, the half-life of ACAT1 was about 30 min where as it was about thirty fold slower for ACAT2 at a $t_{1/2}$ of about 15hrs. suggesting ACAT2 is more stable protein than ACAT1. To further probe the ACAT2 stability relative to ACAT1, recombinant ACAT proteins were expressed in AC29 cells and at different time points and protein mass was compared by immunoblotting. As shown in Fig 2C, at all the three time points we found more protein mass for ACAT2 than ACAT1, again demonstrating that ACAT2 is a more stable protein than ACAT1. In a control experiment, we found that recombinant ACAT proteins were catalytically active (data not shown).

**ACAT proteins undergo degradation by proteasome.** Next, we want to investigate the mechanism behind the altered stability of the ACAT1 and ACAT2 proteins. We reasoned that the degradation pathways of each of the ACAT proteins may be different and hence one turns over faster than other. In general, proteins undergo degradation mainly by two different mechanisms, the proteasomal and lysosomal pathways (22). To test which
Fig 2: ACAT1 turns over faster than ACAT2 A. On day 0, approximately $1 \times 10^6$ ACAT stable cells were plated into 60 mm dishes. On day 1, cells were starved for 30 min in cysteine, methionine free DMEM media followed by 30 min pulse labeling with 100 µci/ml S$^{35}$ protein labeling mix in same media. Thereafter cells were chased for indicated time points. After chase, cells were harvested and lysed in buffer A and subjected to immunoprecipitation with ACAT specific antibody. ACAT specific bands were captured by autoradiography. B. Quantification of S$^{35}$ radioactivity corresponding to ACAT specific band. Data represent mean ± SEM for at least four independent experiments. Half-life was calculated by fitting the data in an exponential decay curve using Prism software. C. On day 0, AC-29 cells were transiently transfected with the plasmid encoding for recombinant ACAT proteins. Post transfection cells were collected and lysed in buffer A after indicated time period. Equal amounts of cell lysates were loaded on the gels for western blotting. Recombinant protein bands were visualized by using HA-tag specific antibody.
pathway is important for ACAT protein degradation we incubated ACAT stable cells with specific inhibitors that will block either of the degradation pathways and subsequently measured the protein mass by immunoblotting. As shown in Figure 3, when the activity of proteasome is inhibited by two specific inhibitors, MG-132 and ALLN, both ACAT1 (Fig 3A) and ACAT2 (Fig 3B) proteins accumulated in the cells in a time dependent manner compare to the vehicle control treated cells. In addition when the lysosomal pathway was inhibited by ammonium chloride or leupeptin, both ACAT1 and ACAT2 protein mass remained relatively unaltered compared to control showing that degradation of either ACAT protein was not blocked, suggesting that they do not undergo degradation via the lysosomal pathway. Of note, since ACAT2 turnover rate is slower compared to ACAT1, we had to block degradation pathway in ACAT2 cells for longer time period in order to see its stabilization.

**ACAT protein degradation is ubiquitin dependent.** Proteasome mediated protein degradation often requires covalent attachment of ubiquitin molecules to the target protein in order for it to be routed to the proteasome (22). To determine whether ubiquitination is required for ACAT protein degradation, we performed ubiquitination assays using ACAT1 and ACAT2 stable cells. Cells were transfected with the b-Ub plasmid and approximately 72hrs. after transfection, cells were treated with proteasomal inhibitor MG-132 to stimulate accumulation of ubiquitinated proteins. Next, we made cell lysates and precipitated all biotinylated-ubiquitinated proteins using streptavidin beads. The precipitated complex was solubilized and probed with ACAT antibodies. A GFP expression plasmid was used as the negative control to test non-specific protein
Fig 3: ACAT proteins undergo degradation by proteasome A. On day 0, approximately 500,000 ACAT1 stable cells were plated into 35 mm dishes. Day 1, cells were treated with the specific inhibitors for indicated time points. The final concentration of inhibitors were kept as follows: MG-132 (20μM), ALLN (50μM), NH₄Cl (50μM) and Leupeptine (100 μg/ml). After incubation, cells were harvested and lysed in buffer A. Equal amounts of cell lysates were loaded on gel for western blotting. Protein bands were visualized by developing the blot using ACAT1 specific antibody.

B. On day 0, approximately 500,000 ACAT2 stable cells were plated into 35 mm dishes followed by inhibitor treatment and western blotting as described above.
Fig 4: ACAT protein degradation is ubiquitin dependent A. On day 0, approximately $4 \times 10^6$ ACAT1 stable cells were transfected with either GFP or b-Ub plasmids. After transfection cells were divided into two aliquots and plated into two 60 mm dishes. Day 1 cell were fed with fresh media supplemented with 4mM biotin. 72 hrs post transfection one of the plate was treated with DMSO and the other with 20µm MG-132 for 4hrs. After inhibitor treatment cells were harvested and lysed in buffer B and subjected to ubiquitination assay.

B. On day 0, approximately $4 \times 10^6$ ACAT2 stable cells were transfected with either GFP or b-Ub plasmids and later these cells were used for ubiquitination assay as described above. However, for ACAT2, which turns over more slowly, cells were treated with proteasomal inhibitor for 8 hrs.
binding to the beads. As we can see in Figure 4A, b-Ub transfected cells accumulate low amounts of ubiquitinated ACAT1 proteins when treated with DMSO and this was augmented when cells were treated with MG-132, suggesting that ACAT1 degradation is ubiquitin dependent. Similar results were obtained when the ubiquitination assay was performed with ACAT2 stable cells (Fig 4B), indicating ACAT2 protein degradation is also ubiquitin dependent.

**Functional ubiquitination pathway is required for ACAT protein degradation.** So far we have shown ACAT protein degradation is proteasome dependent and when proteasomal pathway is inhibited ubiquitinated ACAT proteins started to accumulate. Thus, finally we asked whether functional ubiquitination pathway is necessary for ACAT protein degradation. To investigate this possibility we performed two separate experiments. In the first experiment we used ts20 cells. These cells have a functional ubiquitination pathway when grown in permissive temperature of 34°C, however, the ubiquitination pathway becomes defective when these cells are grown at 39°C because the ubiquitin activating enzyme E1 is inactive at the higher temperature (23). We co-transfected both ACAT1 and ACAT2 plasmids into these cells and grew them at the permissive temperature for 48hrs. Cells in one dish then remained in 34°C and a second dish of cells was switched to 39°C for another 18hrs. At end of the incubations, cells were collected, lysed and aliquots were analyzed to compare protein mass. Indeed, when ubiquitination pathway was inhibited at 39°C both of the ACAT proteins started to accumulate in the cell (Fig 5A) suggesting a functional ubiquitination pathway is required for ACAT protein degradation.
Fig 5: Functional ubiquitination pathway is required for ACAT protein degradation. **A.** On day 0, approximately 750,000 ts20 cells were plated into two 35 mm dishes. Day 1, cells were fed with fresh media and co-transfected with 2µg of ACAT1, 2µg of ACAT2 and 0.5µg of GFP plasmids. 48 hrs. after transfection one dish remain in 34°C and the other was transferred to 39°C incubator. After incubation for 18hrs. cells from both dishes were collected separately and lysed in buffer A. Equal amounts of lysates were loaded on the gel and subjected to western blotting. Protein bands were visualized by using ACAT specific antibody. Data shown above represent result of two independent experiments. **B.** On day 0, approximately 750,000 CHO-K1 cells were plated in 60 mm dishes. On day 1, cells were co-transfected with ACAT1 or ACAT1 plasmids along with either wild type (WT) or mutant (MT) ubiquitin plasmids. 24 hrs. after transfection cells were harvested and lysed in buffer A. Equal amounts of cell lysates were loaded on the gel and samples were subjected to western blotting.
In a second experiment, we wanted inhibit ubiquitination pathway by over expressing the ubiquitin K48R mutant. During poly-ubiquitin chain formation in a target protein, one ubiquitin is covalently attached to another via isopeptide linkage through lysine 48 residue (18,24). Thus, expression of the mutant will generate a premature chain terminating dominant negative effect i.e normal protein degradation via ubiquitination will be inhibited. CHO-K1 cells were co-transfected with ACAT plasmids along with either wild type or mutant ubiquitin plasmid. As expected, compared to the wild type when mutant plasmid was transfected, ACAT protein accumulated in the cells due to defective ubiquitination pathway again supporting functional ubiquitination pathway is necessary for ACAT protein turn over. Of note, the interpretations of the above data along with Figure 3 remain tentative due to lack of a positive control, i.e, a western blot of a protein that undergo degradation via ubiquitin mediated proteasomal pathway. We fail to do those experiments due to lack of an antibody that recognize endogenous hamster protein since these experiments were done in hamster cells.
DISCUSSION

The two ACAT enzymes are more than 50% similar in their amino acid sequence and almost all of the available ACAT enzyme inhibitors fail to show any specificity towards either isozyme. Due to this difficulty, it has long been impossible to distinguish the contribution of individual ACAT’s from total ACAT activity measured from animal tissues. However, with the discovery of pyropyrepene A (25), which has 2000 fold, higher specificity towards ACAT2 (15), we can now separate the contributions of ACAT1 versus ACAT2 activities from total ACAT activity. Using this tool, we have consistently seen that ACAT2 provides more than 90% of the total ACAT activity obtained from monkey livers. On the other hand, the expression levels of ACAT1 mRNA were significantly higher than ACAT2 mRNA showing a disconnect between ACAT mRNA abundance and activity in monkey liver. This situation holds true for human livers as well (7). Moreover, when we measured the individual ACAT activity after expressing either ACAT1 or ACAT2 in ACAT deficient AC29 cells we always found ACAT2 activity was 2-4 fold higher than ACAT1. We hypothesized that more ACAT2 activity in our in-vitro system may be attributed by the fact that there in more ACAT2 enzyme protein. One possible mechanism by which we can explain this speculation is if ACAT2 is a more stable protein than ACAT1. Hence, we measured the decay rate of two proteins using ACAT1 and ACAT2 stable cell lines. We found that the decay rate of ACAT1 was at least 30 times faster than ACAT2. So, this huge difference in half - life may result in higher amounts of ACAT2 enzyme protein. Therefore, in our in-vitro microsomal assay system, ACAT2 activity is consistently higher than ACAT1. The difference in the specific activity and mRNA abundance of the two proteins could also result in
differential activity, however, our data supports, that the huge difference in the decay rate may make a major contribution towards the differential in activity between the two ACAT enzymes.

The half-life studies of ACAT proteins were performed in AC29 cells stably expressing ACAT1 and ACAT2 proteins. AC29 cells were originally derived from 25RA cells, which in turn were originally derived from CHO cells (26). There are two genetic defects in AC29 cells. Due to mutation in ER resident cholesterol sensor protein SCAP (SREBP cleavage activating protein) cholesterol synthesis in these cells remains unregulated i.e these cells constantly produce cholesterol. In addition, AC29 cells do not have a functional ACAT protein. As a result of these two defects, the ER membrane of AC29 cells is highly enriched with free cholesterol compared to its wild type control cells (27). It has been shown previously that sterol can regulate the post translational stability of membrane proteins (28). Currently, it is not known whether cholesterol regulates stability of ACAT proteins, however one of the previous reports from this laboratory has suggested that cynomolgus monkeys fed a cholesterol-enriched diet upregulate hepatic ACAT2 (2). Hence, we can not rule out the possibility that since the ER membrane of AC29 cells are enriched with free cholesterol it might alter the decay rate of ACAT proteins. If the presence of cholesterol in the membranes increases the half-life ACAT proteins, for example, the decay rates reported here could be an overestimation. Investigations into the possibility of sterol mediated regulation of ACAT protein stability are currently underway in this laboratory. More studies are needed to shed light on precise molecular mechanisms of ACAT protein stabilization.
To understand the molecular mechanisms behind the 30 fold difference in the half-life of two ACAT proteins, we hypothesized that these two proteins undergo degradation via two different mechanisms. Hence, we initiated studies to decipher the degradation pathway of ACAT proteins. Our data demonstrate for the first time that both the ACAT proteins undergo degradation via an ubiquitin dependent proteasomal pathway (Fig 3 and 4) and that a functional ubiquitin pathway is necessary for turnover of these proteins (Fig 5). Hence our hypothesis for different pathways of degradation was not correct. However, one notable difference between ACAT1 and ACAT2 ubiquitination pattern was observed such that between the monomer and dimer bands of ACAT1 (50 and 100 Kda) there are several ubiquitinated ACAT1 proteins, i.e mono ubiquitinated, di-ubiquitinated etc. (Fig 4A). In contrast, this laddering pattern of ubiquitinated proteins is completely absent for ACAT2 (Fig 4B). Why the pattern of ubiquitin chain formation is different in ACAT2 is currently unknown. But, it is possible that the kinetics of ubiquitin conjugation to the two ACAT proteins is different and this difference may contribute directly or indirectly to the altered decay rate.

ACAT1 and ACAT2 proteins are very similar in their protein sequence; both use the same substrates and catalyze the same biochemical reaction. Nevertheless, their decay rate is hugely different. The meaning of this discrepancy in the turnover rates of ACAT proteins is not known. However, based on several functional studies done in the mouse models, we can speculate that the following scenario may pertain. ACAT1 expression is ubiquitous throughout the body whereas ACAT2 expression is restricted and only occurs in hepatocytes and enterocytes, the two specialized cell types in the body that normally
process a high cholesterol load and secrete lipoproteins loaded with cholesterol. Converting free cholesterol to cholesteryl ester may be a protective mechanism against the build up of excess free cholesterol in the body. Thus, it is possible that, since lipoprotein-secreting cells are required to regularly process a lot of free cholesterol at a high rate, they need a highly efficient enzyme that can quickly convert abundant amounts of free cholesterol to its ester form. In this case, expressing a more stable and abundant cholesterol esterifying enzyme may be necessary. In other words, it may be that the ACAT2 enzyme protein is more stable than ACAT1 to assure that it successfully meets the physiological needs of hepatocytes and enterocytes. However, this is pure speculation and other possibilities cannot be ruled out.
REFERENCES


Akash Das prepared the following chapter. Dr. Lawrence Rudel acted in an advisory and editorial capacity.
Chapter V

Converting free cholesterol to cholesteryl ester is an important step in whole body cholesterol metabolism in higher eukaryotes. Formation of cholesteryl esters is necessary to serve three major functions in the body, 1. cholesterol absorption from diet, 2. storage of cholesterol in its neutral form within the cytosolic lipid droplets as a future storage, 3. transport of cholesterol to the peripheral tissues via lipoprotein particles. In mammals, there are two isoforms of intracellular cholesterol esterifying enzymes, namely acyl-coA: cholesterol-O-acyltransferase (ACAT), have identified. ACAT1 expression is ubiquitous whereas ACAT2 synthesized only in the lipoprotein secreting cells which are hepatocytes and enterocytes where ACAT1 signal is not be detected. Currently, it is not clearly understood why the body requires two cholesterol esterifying enzymes. However, based on several functional studies in animals, it is thought that the main function of ACAT1 is to maintain a proper balance of free and esterified cholesterol for normal cell function, whereas the major function of ACAT2 is to provide cholesteryl ester for lipoprotein particle assembly and secretion. Moreover, gene knockout studies in mice demonstrated that inhibition of ACAT2 protects animals from progression of atherosclerosis while ACAT1 gene deletion was deleterious for the animals. Hence, between the two enzymes, ACAT2 inhibition is necessary and desirable for treatment of coronary artery atherosclerosis, but ACAT1 inhibition is not. Since, ACAT proteins are more than 50% similar in their amino acid sequence, it is difficult to find an isozyme specific inhibitor. Thus, detailed study is required to identify the unique structural features in ACAT2 that can be utilized to make ACAT2 specific drugs in future. This thesis described three specific studies where the two enzymes were compared side by
side to identify isozyme specific uniqueness. Indeed, we identified several unique characteristics in ACAT2, and, based on these unique structural and functional properties, we hypothesized that in future it may be possible to make ACAT2 specific drugs without affecting ACAT1 activity.

In chapter II of a study was presented where we tried to identify and compare the active site residues of two ACAT proteins. Before this study was undertaken, there were a few reports that addressed the similar question, but in an inadequate fashion. Hence, a new and more comprehensive study was needed. To identify the active site residues, we used chemical modification and site directed mutagenesis approaches. We identified a conserved region in the protein as the putative active site domain based on high sequence similarity from yeast to humans. Since ACAT enzymes have an intrinsic thioesterase activity, we hypothesized that by analogy with the thioesterase domain of fatty acid synthase, the active site of ACAT enzymes may comprise a catalytic triad of serine, histidine and aspartic acid residues. Our results showed that in ACAT1, S456, H460 and D400 are essential for activity of the enzyme. In contrast, in ACAT2, only H438 is necessary for enzymatic activity. Based on these data, we proposed that the residues required for ACAT activity may be different between two ACAT proteins. It is however important to mention that site directed mutagenesis approach is an oversimplified method to determine active site residues of proteins. Structural study is necessary to understand the molecular basis for catalytic inactivation of the enzyme due to mutation. ACAT enzymes are membrane bound proteins and it is hard to purify them to homogeneity in order to perform structural studies. Hence, we don’t want our studies to be over
interpreted and claim that we identified the active site of ACAT enzymes, rather, we have identified candidate active site residues by undertaking a comprehensive mutational analysis. Hence, in the future, when purified enzymes are available, we propose that the catalytically inactive mutants identified in this study would be used to determine the molecular basis for loss of function in order to better understand the chemistry of cholesterol esterification reaction.

ACAT proteins are members of the MBOAT (Membrane-Bound O-Acyltransferases) family of proteins, which catalyze the acylation reactions. The active site of MBOAT family proteins have been defined by two highly conserved histidine and asparagine residues. Recently, it has been shown that GOAT (Ghrelin O-Acyltransferase), a newly identified member of this family, shares the similar active site residues. In our mutational analysis, we identified the conserved histidine in ACAT proteins, however, we never tried to mutate the conserved asparagine residue. In future, it will be interesting to see if mutation of a conserved asparagine residue is indeed inhibit the enzymatic function of ACAT proteins.

Inhibition of ACAT2 without affecting ACAT1 activity in animal liver is sufficient to prevent progression of atherosclerosis. Among numerous ACAT inhibitors, Pyropyrepene A (PPPA) is the only known compound that has greater than 2000 fold higher specificity for ACAT2 compared to ACAT1. In the studies presented in chapter III we sought to determine the PPPA interaction site in ACAT2. To achieve this goal we made several chimeric proteins where a part of ACAT2 was replaced by the analogous region of ACAT1 in order make an ACAT2 chimera that will be insensitive
towards PPPA. Our approach was very successful and we were able to identify three residues located within the fifth transmembrane domain of the protein as the PPPA target site that distinguishes ACAT2 from ACAT1 for PPPA interaction. Additionally we have shown that the PPPA interaction is non-covalent and does not alter the oligomeric structure of the protein. The result of this study is of paramount interest to us for several reasons. This study clearly demonstrates that despite high sequence similarity with ACAT1, ACAT2 has a unique structural feature that confers its selectivity towards PPPA. In future, more detailed structural studies of ACAT2 complexed with PPPA can help us understand this unique structural motif. It will also immensely aid our understanding the structure function relationship of two ACAT proteins and facilitate ACAT2 specific drug design strategies. Since ACAT2 is a target for treatment of atherosclerosis in the future, it may be possible to use PPPA as the lead compound to make ACAT2 specific drugs. The chemical properties of PPPA do not provide good biological availability for testing in mice. The founders of this compound are now able to synthesize several derivatives of PPPA. The new compounds inhibit ACAT2 much more effectively. And due to modification in their chemical properties, we hope, in future, these compounds can be used as a drug to treat atherosclerosis in animal models.

In the chapter IV of this thesis we presented a work where we compare the protein stability of two ACAT enzymes. Although this is not a study relating to structure function relationship of the proteins, however, the results of this study highlighted some novel and interesting findings about regulation of ACAT proteins. Here, we found that ACAT2 is intrinsically a more stable protein than ACAT1. Also, we identified that both proteins
undergo degradation via ubiquitin-mediated proteasomal pathway, although the ubiquitination pattern of two proteins was not similar. In its present form, this study lacks few important control experiments. First, these studies were performed in the cells where ACAT proteins were over-expressed ectopically. Thus, to rule out the effects due to protein over-expression, it will be necessary to evaluate whether we can achieve the same conclusions using cell lines that express endogenous ACAT proteins. Second, ACAT stability studies were performed using AC29 cells. These cells have unregulated cholesterol synthesis\textsuperscript{13}. Currently, it is not known whether cholesterol regulates stability of ACAT proteins. Hence, in future, it will be necessary to determine if indeed excess cholesterol has any effect on ACAT protein turnover. Our study indicates ACAT1 is a short-lived protein. Proteins with rapid turnover rates often undergo multiple levels of regulation. ACAT1 plays an important role during macrophage foam cell formation\textsuperscript{14}. In future, it will be interesting to see the regulation of ACAT1 turnover during cholesterol overloading in macrophages.

We studied the degradation pathway of ACAT proteins in order to understand the molecular mechanism behind the thirty fold difference in their decay rate. However, the data clearly suggest that the difference in the decay rate of ACAT proteins is independent of their degradation pathway. Hence to further explore the mechanism behind altered decay rate of ACAT proteins we speculate that there may be a chaperon protein like Insig which might regulates ACAT protein turnover. Insig plays a central role in cellular cholesterol metabolism by modulating its synthesis and uptake\textsuperscript{15}. Like ACAT’s, there are two isoforms of Insigs. The turnover rate of Insig-1 is 15 times faster than Insig-2 in
sterol depleted cells. It is possible that Insig-1 may play an important role to regulate ACAT1 stability. Conversely, Insig-2 may modulate ACAT2 turnover. And hence, the huge difference in the turnover rate of two ACAT proteins. Currently, we are testing this hypothesis using Insig deficient cells.

ACAT proteins have long been associated with atherosclerosis. Subsequently, several studies from our lab have shown inhibition of ACAT2 in animal livers is sufficient to prevent the progression of the disease. Recently, in a separate study, we observed that hepatic cholesteryl ester levels also regulate liver triglyceride mobilization. When the liver is depleted of cholesteryl ester, i.e under ACAT2 knock out background, it also starts to package more triglyceride into the VLDL particles and secrete into plasma. Non-alcoholic fatty liver disease (NAFLD) is characterized by accumulation of excess lipids in the livers (mainly triglyceride) and currently there is no clinical treatment available to treat the patients having NAFLD. Hence, our recent findings again substantiate the importance of cholesteryl ester metabolism in the liver and prompted us that ACAT2 can be used as a drug target to treat NAFLD. Our studies with ACAT2 are very strong in animal models to establish its role in whole body lipid metabolism. It is now necessary to evaluate does ACAT2 plays similar role in humans. There is conflicting result regarding expression of ACAT2 in human livers. Definitely, more studies required resolve this issue. If indeed it turns out that ACAT2 is important in humans, it can be used as an alternative to Statin therapy to treat coronary artery atherosclerosis and also can be used to treat NAFLD patients. Surely, more studies are necessary to better
understand the regulation and biochemical properties of ACAT2 if indeed it is a drug
target.
REFERENCES


A: **Topology model of ACAT isozymes.** The model was based on a computer predicted topology map of ACAT enzymes. Truncated mutants were made, where, after each computer predicted transmembrane domain, a glycosylation reporter site was added. Based on utilization of the glycosylation site and accessibility by the exogenous proteases, the topology maps of both ACAT enzymes were determined in parallel. Both the enzymes have N_CytO C_exo orientation with five potential transmembrane domains. Interestingly, transmembrane domain D is not utilized and domain F is utilized in ACAT2, whereas the opposite is true for ACAT1. Computer predicted domains E, G, and H are not utilized in either of the enzymes. 

B: **Chemical structure of PPPA molecule.**

C: **Sequence similarity within the putative active site domain of the ACAT enzymes across the species.** Two conserved motifs FYXDWNN and HEY within this region are represented by two boxes. ARE1 and ARE2 are the yeast homologs of ACAT enzymes; AGM represents African green monkey. DGAT1 is an enzyme that belongs to same gene family (MBOAT) with ACAT proteins.
CURRICULUM VITAE

EDUCATION:


Advanced Diploma in Bioinformatics, University of Calcutta, India, 08/03 – 07/04.

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RESEARCH INTEREST:

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TEACHING EXPERIENCE:

• Co-taught Cellular and Molecular Physiology (PSPR 701) course during fall 2008. This course is offered to the first year graduate students of the Physiology and Pharmacology department of Wake Forest University School of Medicine. Lectures include Membranes, Lipids and Metabolism of lipids, amino acids and glycogen.

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PUBLICATIONS:

• **Das A**, Davis MA, Wilkinson JC, Rudel LL. ACAT1 protein turns over faster than ACAT2: Implication to their in-vitro enzymatic activity. manuscript under preparation.


**PRESENTATIONS:**

- **Das A.** A comparative analysis of acyl – CoA cholesterol-O-acyltrasferase (ACAT) isozymes, Invited Talk, University of Michigan, Ann Arbor, Nov’08.

- **Das A.** A comparative analysis of acyl – CoA cholesterol-O-acyltrasferase (ACAT) isozymes, Invited Talk, University Texas Southwestern Medical Center, Dallas, Oct’08.


- **Das A.** Davis MA, Rudel LL. Appearance of the Ser-His-Asp catalytic triad at the putative active site domain of the acyl coenzyme A:cholesterol acyltransferase (ACAT) enzymes, Poster presented at the Experimental Biology Meeting, San Diego Apr ’08.

- **Das A.** Davis MA, Tomoda H, Omura S, Rudel LL. Identification of the interaction site within acyl-CoA:cholesterol acyltransferase 2 for the isoform-specific inhibitor pyripyropene A, Poster presented at the Graduate Research Day, Wake Forest University School of Medicine, Winston Salem Mar ’08.

- **Das A.** Davis MA, Rudel LL. Identification of the target site within acyl Co-enzyme A (CoA): cholesterol O-acyltransferase 2 (ACAT2) for the isoform-specific inhibitor pyripyropene A, Poster presented at the 16th South East lipid Conference, Callway Gardens, Oct ’07.

- **Das A.** Davis M.A., Rudel L.L, Identification of putative active site amino acids of acyl coenzyme A:cholesterol acyltransferase (ACAT) enzymes by site-directed mutageneis, poster presented at the Gordon Research Conference entitled Cellular and Molecular Biology of Lipids, July ’07.

- **Das A.** Davis MA, Rudel LL., PyripyropeneA (PPPA) specifically inhibits acyl coenzyme A (CoA):cholesterol acyltransferase-2(ACAT2) by interacting with the enzyme near its C-terminal end, poster presented at the Experimental Biology meeting, Apr ’07.
• **Das A.**, Identification of active site residue(s) and Pyripyropene A interaction site of ACAT enzymes: A biochemical approach, Invited Talk, University of Calcutta, Dept. of Biophysics, Molecular Biology and Genetics, Kolkata, India, Feb’07.

**PROFESSIONAL EXPERIENCE/ CERTIFICATE:**

• Summer internship at Center for Cellular and Molecular Biology, India, 2004.
  - Designed and developed a web based database with information of various metabolic and biochemical pathways in prokaryotes to the most evolved organism. This database serves as a bioinformatics tool for in-house students and researchers.
• Worked as a software developer at Infopro India Ltd., India, 10/02 – 04/03.
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• 2008 Herbert C. Cheung Award from Dept. of Biochemistry for Outstanding Graduate School Career.
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• 2007-2008 Alumni Student Travel Award from Wake Forest University School of Medicine.
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