Identification of putative active site residues of acyl-Coenzyme A:cholesterol acyltransferase enzymes

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Running Title: Catalytic triad in the active site of ACAT enzymes

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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 proteins was selected as the putative active site domain due to its high degree of sequence conservation from yeast to humans. As 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 towards serine modifying reagents whereas ACAT1 was not. Further studies indicated tyrosine residues may be important for ACAT activity. Mutational analysis showed the tyrosine residue of the highly conserved FYXDWWN motif was important for ACAT activity. Furthermore, Y518 was necessary for ACAT1 activity while 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.

Key words: ACAT, Cholesterol, Cholesteryl Ester, Catalytic Triad, Active site,
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). While 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 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 knock down of ACAT2 using antisense oligonucleotides resulted in significantly reduced hepatic cholesterol concentration, plasma LDL cholesterol oleate, and aortic atherosclerosis (15). Since 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).

Since ACAT enzymes are very 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 were performed in animal models, biochemical studies with ACAT enzymes are very limited, mainly due to lack of purified proteins. Using a histidine modifying reagent, Kinnunen et.al showed a histidine residue(s) is necessary for ACAT activity (17). T.Y. Chang and colleagues extended this observation and suggested one invariant histidine residue at the C-terminal end of ACAT enzymes as an active site residue (18;19). Recently, one report suggested that active site of ACAT enzymes are different (20). Using mutagenesis studies they showed the histidine residue required for ACAT1 activity is different from that required for ACAT2 activity. Since 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 with 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 the enzymes.

While ACAT1 and ACAT2 are highly homologous in their amino acid sequence, they do not have the same intron-exon structure suggesting that the two enzymes diverged quite early during evolution (1). The enzymes are structurally different and perform distinct intracellular functions (2). However, studies have shown 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, 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 like lipases and cholesteryl ester hydrolase use a catalytic triad comprised of serine, histidine and aspartic acid in their active site (23). The crystal structure of human pancreatic lipase has revealed the presence of a catalytic triad in its active site (24), while other studies using site directed mutagenesis have shown the presence of a catalytic triad in the acyltransferase enzyme from Aerimonas hydrophilia (25). Finally, using structural homology modeling along with mutagenesis of conserved residues, the presence of a ser-asp-his catalytic triad in the active site of the plasma enzyme lecithin:cholesterol acyltransferase (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-asp-his catalytic triad in their active site, the proposed mechanism would likely follow the classical charge relay mechanism in which the negative
charge of a carboxyl ion of aspartic acid will be transferred to histidine and then to a serine residue to enhance its nucleophilic power. This nucleophilic serine could then attack the thioester bond of acyl-CoA molecule forming an acyl-enzyme intermediate. In the final step, enzyme assisted (likely His and Asp mediated) hydrolytic attack of the 3β-OH moiety of 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, one cycle; 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 90 sec for 25 cycles, followed by one 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 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 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: About 3 x 10⁶ AC29 cells were transiently transfected with 6μg of cDNA encoding for either WT or mutant ACAT proteins using nucleofection technology (amaxa biosystems, MD) according to the manufacturer’s instructions. Suspended cells were divided into two aliquots after transfection; one aliquot was used to seed four 35mm dishes to be used for activity assay and the remaining aliquot of cells was plated into a 60mm dish to use for subsequent immunoblotting. 72 hrs post transfection, cells in 35 mm dishes were pulse labeled with 1μCi of [9,10-3H(N)]-oleic acid (Perkin Elmer Life Sciences,NET-289) for 2 hrs. Thereafter, cells were harvested and total cellular lipids were extracted by the Bligh and Dyer method (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
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, scraped and radioactivity was measured in a liquid scintillation spectrometer. Under this assay condition, the 2 hr time point falls within the linear range of the ACAT activity curve.

Preparation of post nuclear supernatant (PNS): At 72 hrs 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 phosphate buffered saline (PBS)] in the presence of 3 μl protease inhibitor cocktails (Sigma). Sonication followed and then removal of the nucleus and cell debris by centrifugation at 14,000 r.p.m. 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 PNS solution was measured by BCA assay (Pierce).

Preparation of microsomes: Cells grown in 150mm 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, 0.1M K2HPO4, 1mM 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 r.p.m., 4°C for 15min. Supernatant was collected and subjected to ultracentrifugation at 100,000 r.p.m, 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. 1 mg of BSA 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]oleyl-CoA (Amersham Biosciences) was added and incubated for 20 min. To stop the reaction, 6 ml of CHCl₃:methanol, 2:1 was added. 1.2 ml of 0.88% (w/v) KCl was then 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 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 PNS were suspended in an equal volume of protein solubilization buffer [120mM 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, 50mM iodoacetamide (IAA) was added and the mixture was 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 hr at 115V using a Western blot apparatus (Bio Rad). The membrane was blocked overnight in 5% non fat 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 (1µg/ml) were made as described before (5) and were incubated with the membrane for 2 hrs 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 horseradish peroxidase conjugated secondary antibody (Sigma) at 1:20,000 dilution at room temperature for 1 hr. After removing the secondary antibody, 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 comparable size 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 where 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 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.

Crosslinking 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 1M Tris at pH7.5 and protein solubilizing buffer at room temperature. All the 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. Amino acid sequence of this region of ACAT enzymes is highly conserved starting from yeast extending all the way to humans (Fig 1). Interestingly, this region contains two highly conserved motifs FYXDWNN and HEY. This region is also highly similar to the analogous region of AGM DGAT1 which is 57% similar to AGM ACAT1 and 53% similar to AGM ACAT2. All of the members of this gene family catalyze the transfer of a fatty acid molecule from an acyl-CoA to an acceptor alcohol (diacylglycerol or cholesterol) presumably 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: In order 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% activity of both the enzymes at 50 μM concentration, however, at lower concentrations, inhibition of ACAT1 activity was greater than ACAT2 (Fig 2A) as reported earlier (17). DEPC inhibition of the ACAT enzymes was covalent as the inhibition of ACAT activity was irreversible in the presence of 0.5M 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 as 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 where H364 and H403 were not required for activity of the enzyme whereas H438 (equivalent to H460 of ACAT1) was necessary for enzymatic activity of ACAT2 (Fig 2C) as shown earlier (19). The mutants have variable levels of expression compared to 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 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 residues, one of which, A1D406, is a part of the conserved FYXDWWN motif. This D residue of ACAT1 was not essential for activity of the enzyme, however 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 to its WT counterpart (Fig 3C). For ACAT2, residue D384, a part of the conserved FYXDWWN motif, also was not essential for activity of the enzyme (Fig 3B). The data we obtained for residue D378 were inconclusive, however, 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, while expression levels were low, the specific activity was normal to slightly higher than WT ACAT2 (Fig 3C). Of note, when aspartic acid residue of the conserved FYXDWWN motif was mutated to asparagine in both the enzymes, we got protein expression with very low enzymatic activity. Together, these data indicate 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 where ACAT activity was performed in the presence of phenylmethylsulfonylfluoride (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. Among these seven serine residues, four of them were located within the putative active site domain of the enzyme (for ACAT1 S410, S412, S414, S456 and for ACAT2 S388, S390, S392, S434), two of them were located at the N-terminal end of the enzyme (for ACAT1 S128, S194 and for ACAT2 S109, 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 mutant’s had varied levels of expression with A1S269A showing the highest protein mass (Fig 4D). Similar results were obtained for ACAT2 except 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 the ACAT2 serine mutants were approximated to their WT counterpart and A2S492L had the highest expression (Fig 4D). Interestingly, residues S269 for ACAT1 and S249 for ACAT2 which were once thought important for activity of the respective enzymes (3;29), were indeed not essential for the activity of the enzyme in agreement with Guo et.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: Since, our studies with mutations of serine residues showed only S456 to be required for ACAT1 activity while the analogous serine was not absolutely essential for activity of ACAT2, we sought to determine if 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 hexadecylsulfonfylfluoride (HDSF), a second
serine modifying reagent (an aliphatic analog of PMSF) to determine if any differential sensitivity of ACAT enzymes towards this reagent could be detected. Indeed, we saw that HDSF treatment inhibited ACAT1 activity in a dose dependent manner while 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 iodoacetamide (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 (Fig5B) 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 earlier (31). We found that activity of either of the ACAT enzymes was not inhibited by NP-LLL-VS treatment, 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 activities of both the ACAT enzymes were inhibited in a dose dependent manner with ICL treatment suggesting 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 the 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, Y433 and for ACAT2 Y382, Y411). Of note, Y404 in ACAT1 and Y382 in ACAT2 were part of conserved FYXFWNN motif. Among the seven ACAT1 tyrosine mutants Y404 had specific activity less than 20% 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 none were indeed required for activity of the enzyme. For ACAT2, only A2Y382F mutant had a specific activity less than 20% of its WT counterpart; however none of the remaining tyrosine residues in ACAT2 was indeed required for ACAT2 activity (Fig 6B). All the tyrosine mutants of both the enzymes were reasonably well expressed (Fig 6C). Altogether, these data suggest the tyrosine residue which is a part of the conserved FYXFWNN sequence is important for full enzymatic activity of both the enzymes.
Moreover, like serine mutants, there was a disconnect within ACAT tyrosine mutants, and while Y518 is 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 those residues were candidates as active site amino acids. It is also possible that, due to mutagenesis, an ACAT protein might have an altered structure that, in turn, resulted in an inactive enzyme. To investigate this possibility, we employed a crosslinking assay as an indirect measure to determine whether there were any changes in the overall protein folding due to mutation. We reasoned, if a catalytically inactive mutant had maintained a similar overall three dimensional structure like its WT counterpart, it should form an oligomeric structure similar to the WT enzyme upon crosslinking. For our experimental purpose we used DSG, a membrane permeable, homobifunctional, non-cleavable 7.7 Å arm length cross linker. The results suggest that the mutations indeed did not alter the overall oligomeric structure of the protein as 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 the mutants were apparently degraded rapidly causing loss of expression (data not shown) indicating D400 in ACAT1 is important for stability of the protein. Taken together, except for A1D400N, data from crosslinking assay suggest that the loss of enzyme activity is more likely a consequence of 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 since 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 FYXDWWN motif is required for full enzymatic activity of both the enzymes. In addition, Y518 was required for ACAT1 activity while 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 H460 in ACAT1 and H434 in ACAT2 are essential for ACAT activity. These results agree with the data published by T.Y.Chang and colleagues (18;19). In contrast, our data do not support 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 A1H386N mutant is catalytically active indicating this residue is not essential for ACAT1 activity. Of note, for assay they employed an in vitro ACAT assay using microsomes while we used whole cell based assay. Furthermore, they changed H386 of ACAT1 only to alanine while 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 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 is 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 the possibilities thus we are unable to definitely conclude whether indeed A1D400 can be considered an active site residue. In ACAT2, the corresponding residue D378 was very sensitive to mutation. All of its non conservative mutations did not show 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. All together, we concluded that this particular aspartic acid residue is important for structural stability of ACAT enzymes and may be for catalytic activity, at least for ACAT1, since 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 FYXDWNW 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. While 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 A2S434A mutant was 27% of its WT counterpart, the least of the seven conserved serine residues examined, but not low enough to be considered as essential for activity. The mutation
data suggesting the absence of a required serine in ACAT2 was supported by data from chemical modification. We found 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 concluded that S456 is important for ACAT1 activity while the identical serine residue in ACAT2 was 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 ACAT isozymes regarding requirement of a tyrosine residue(s) for enzyme activity. Catalytic activity of both the isozymes was dose dependently inhibited to the same extent with treatment of ICL. Further, mutation studies showed 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 while the corresponding tyrosine residue in ACAT2 was not required for activity. Of note, since it has been proposed that at neutral pH ICL can modify both 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.

Based on a previously published report on identification of the cholesterol binding site of proteins (33), Leon, et al, (34) suggested they had identified two tandem cholesterol binding motifs in ACAT enzymes. The proposed putative cholesterol binding site contains a conserved tyrosine residue which is supposed to interact with the polar 3’ OH group of cholesterol molecule (33). A1Y308, A1Y312 and A2Y286, A2Y290 are the 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 these residues are not essential for enzyme activity. Since, 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 enough to carry out the esterification reaction. Thus we made a double mutant in
ACAT isozymes where 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 there may be a Ser-His-Asp catalytic triad at the active site of ACAT enzymes. In accordance to our hypothesis, we have identified specific serine, histidine and a probable aspartic acid residue 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 to 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 non-conservative substitutions, suggesting that a mutant could result in an inactive enzyme due to its altered protein folding. Thus we employed a crosslinking assay (Fig. 7), in which we showed that the overall oligomeric state of the catalytically inactive mutants did not differ from the WT proteins except for A1D400N. Although indirect, this result suggests that mutation did not change the apparent overall folding of ACAT proteins, indicating loss of enzymatic activity was more likely 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 since 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, might 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).

Acknowledgement

We thank Charles W Joyce for performing initial chemical modifications in the early phases of these studies. We would also like to thank Prof. Sandra L. Hofmann for proving us HDSF, Ramesh Shah for giving us ICL solution, and Dr. Greg Shelnness for his critical input for several of these experiments. This work was supported by a grant from the National Institute of Health NIH-P01-HL49373 to L.L.R.
REFERENCES


FOOTNOTES

The official name for ACAT is sterol o-acyltransferase (SOAT)
FIGURE LEGENDS

**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 ClustalW program from EMBL-EBI web site. Two totally conserved motifs FYXDWWN and HEY within this putative active site domain and are represented by two boxes. X indicates any amino acid. ARE1 and ARE2 represent the yeast homolog of ACAT enzymes. AGM represents African green monkey.

**Fig 2.** Identification of essential histidines required for activity of the ACAT enzymes: A. After incubation for 30 min with ethanol solubilized DEPC at 37°C, pH 7.4, microsomes prepared from ACAT1 and ACAT2 stable cells were used for ACAT assay. ACAT1 (◊) and ACAT2 (■) activities at varying concentrations of DEPC are expressed as a percentage of ACAT1 or ACAT2 activity in the ethanol control. Data represent average of three replicates. B. AC29 cells were transiently transfected with the cDNA encoding WT and ACAT1 histidine mutants. 72hrs post transfection, cells were incubated with 1µCi ³H oleic acid for 2hrs. Incorporation of the radioactive oleic acid into the cellular CE 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. PNS obtained from transfections of WT and histidine mutants of ACAT proteins were subjected to immunoblot analysis. Affinity purified ACAT1 (1µg/ml) and ACAT2 (1µg/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: 25µg, A1H386N: 10µg, A1H425A: 10µg, A1H460A: 25µg, WTA2: 15µg, A2H364N: 25.5µg, A2H403N: 4.5µg, A2H438A: 1µg; 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.
Fig 3. **Evaluation of conserved aspartic acid modifications 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 figure 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 figure 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 immunoblotting as described for Fig. 2D. Amount of proteins loaded on the gel are as follows: WTA1: 0.5μg, A1D400N: 35μg, A1D406A: 2μg, WTA2: 5μg, A2D378E: 15μg, A2D384A: 5μg.

Fig 4. **Identification of the role of conserved serine residues in determination of activity of ACAT enzymes:** A. PMSF solubilized in DMSO was incubated with ACAT1 and ACAT2 containing microsomes at 37°C, pH 7.4 for 30min. The samples were then used for microsomal ACAT assay as outlined under the Experimental Procedures. ACAT1 (◊) and ACAT2 (■) activities at varying concentrations of PMSF are expressed as a percentage of ACAT1 or ACAT2 activity in the presence of DMSO control. Data represent 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: 15μg, A1S128A: 25μg, A1S194A: 20μg, A1S269A: 2.5μg, A1S410A: 25μg, A1S412A: 25μg, A1S414L: 25μg, A1S456A: 25μg, WTA2: 15μg, A2S109A: 10.5μg, A2S176A: 30μg, A2S249A: 9μg, A2S388A: 7μg, A2S390A: 19.5μg, A2S392L: 4.5μg, A2S434A: 9μg.

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 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 concentration of HDSF at 37°C, pH 7.4 for 30min. 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 IAA (Sigma, solubilized in water) at 37°C, pH 7.4 for 30min. The samples were then subjected to microsomal ACAT assay as described above. This experiment was repeated twice with similar results. Data represent mean ± SEM for n=2. C. Microsomes containing either ACAT1 or ACAT2 proteins were incubated with various concentrations of NP-LLL-VS (Calbiochem, solubilized in DMSO) at 37°C, pH 7.4 for 30min. The samples were then subjected to microsomal ACAT assay as described above. Data represent mean ± SEM for n=2. D. 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 30min. The samples were then subjected to microsomal ACAT assay as mentioned above. Data represent mean ± SEM for n=2.

**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 X axis. The activity assay was performed as mentioned in figure 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: 12μg, A1Y128F: 83μg, A1Y308F: 15μg, A1Y312F: 15μg, A1Y322F: 8μg, A1Y404F: 70μg, A1Y433F: 35μg, A1Y518F: 35μg, WTA2: 20μg, A2Y124F: 15μg, A2Y286F: 20μg, A2Y290F: 12μg, A2Y300F: 15μg, A2Y382F: 20μg, A2Y411F: 15μg, A2Y496F: 15μg.
Fig 7: *Amino acid mutation did not alter the crosslinking 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 DSG (solubilized in DMSO) for 30min at room temperature. The crosslinking reaction was quenched by adding 1M Tris, pH 7.5, along with Western blot loading buffer. The samples were then subjected to Western blot analysis. Apparent molecular weight of the oligomeric states is given on the right hand 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 crosslinking assay as described above.
Fig 2

A

% ACAT activity

DEPC Conc. (μM)

B

% Sp. Activity

ACAT1
ACAT2
A1H36N
A1H425A
A1H460A

C

% Sp. Activity

ACAT2
A2H364N
A2H403A
A2H438A

D

[Images of Western blot results for ACAT1 and ACAT2 variants with DEPC concentrations and protein concentrations]
Fig 3

**A**

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**B**

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ACAT1 A1D400N A1D406A WTA1
ACAT2 A2D378E A2D384A
Fig 5

**A** S Modification: HDSF

**B** C Modification: IAA

**C** T Modification: NP-LLL-VS

**D** Y Modification: ICL
Fig 6

A

% Sp. Activity

ACAT1  A1Y142F  A1Y308F
A1Y312F  A1Y322F  A1Y404F
A1Y433F  A1Y518F

B

% Sp. activity

ACAT2  A2Y124F  A2Y286F
A2Y290F  A2Y300F  A2Y382F
A2Y411F  A2Y496F

C

ACAT1  A1Y142F  A1Y308F
A1Y312F  A1Y322F  A1Y404F
A1Y433F  A1Y518F

1  0.2  1.1  1.2  3.8  0.14  0.4  0.3

ACAT2  A2Y124F  A2Y286F
A2Y290F  A2Y300F  A2Y382F
A2Y411F  A2Y496F

1  1.5  1  1.9  1.3  0.75  1.3  0.95
Fig 7

A

|-------------------|-------------|------------|----------------|---------------|----------------|---------------|----------------|---------------|----------------|---------------|----------------|---------------|

B

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Molecular Mass

- 50KDa
- 90KDa
- 150KDa
- 300KDa
- 50KDa