Identification of putative active site residues of ACAT enzymes

Akash Das,* Matthew A. Davis,† and Lawrence L. Rudel[1,*,†]

Department of Biochemistry,* and Department of Pathology,† Section on Lipid Sciences, Wake Forest University School of Medicine, Winston Salem, NC 27157-1040

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.—Das, A., M. A. Davis, and L. L. Rudel. **Identification of putative active site residues of ACAT enzymes. *J. Lipid Res.* 2008.** 49: **1770–1781.**

Supplementary key words ACAT • cholesterol • cholesteryl ester • catalytic triad

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

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Abbreviations: AGM, African green monkey; BSS, balanced salt solution; CE, cholesteryl ester; DSG, disuccinimidyl glutarate; DEPC, diethylpyrocarbonate; HDSF, hexadecylsulfonylfluoride; ICL, iodine monochloride; IAA, iodoacetamide; NP-LLL-VS, 4-hydroxy-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone; PNS: postnuclear supernatant; WT, wild type.

[1] To whom correspondence should be addressed.
e-mail: lrudel@wfubmc.edu

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 structurally different 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 the negative charge of a carboxyl ion of aspartic acid is transferred to histidine and then to a serine residue to enhance its nucleophilic power. This 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′ *Kpn*1 and 3′ *Not*1 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% CO_2 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 3×10^6 AC29 cells were transiently transfected with 6 μg of cDNA encoding for either wild-type (WT) or mutant ACAT proteins 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 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 2 h 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 K_2HPO_4, 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 K_2HPO_4 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 [^{14}C]oleyl-CoA (Amersham Biosciences) was added, and the samples were incubated for 20 min. To stop the reaction, 6 ml of $CHCl_3$-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 2 h 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

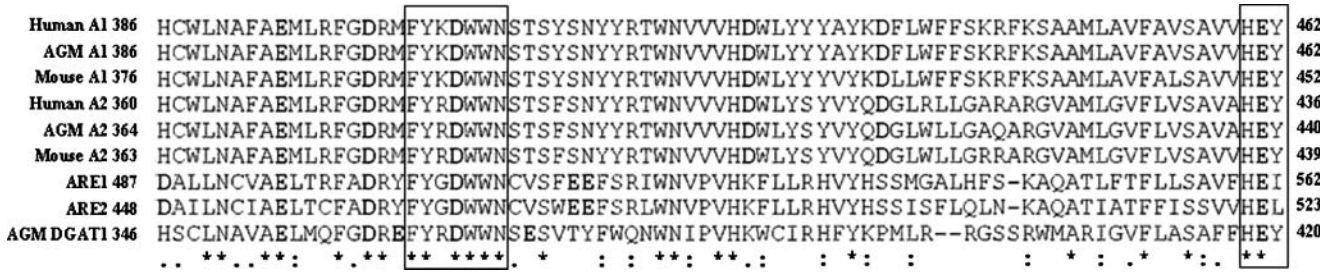

Fig. 1. Amino acid sequences of the putative active site of ACAT enzymes. Sequence similarity within the putative active site domain of the ACAT enzymes across the species. The multiple sequence alignment was performed by the ClustalW program from the European Molecular Biology Laboratory-European Bioinformatics Institute website. Two totally conserved motifs, FYXDWWN 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.

greater than that of ACAT2 activity (**Fig. 2A**), as reported previously (17). DEPC 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 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, 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 FYXDWWN 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 FYXDWWN 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 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

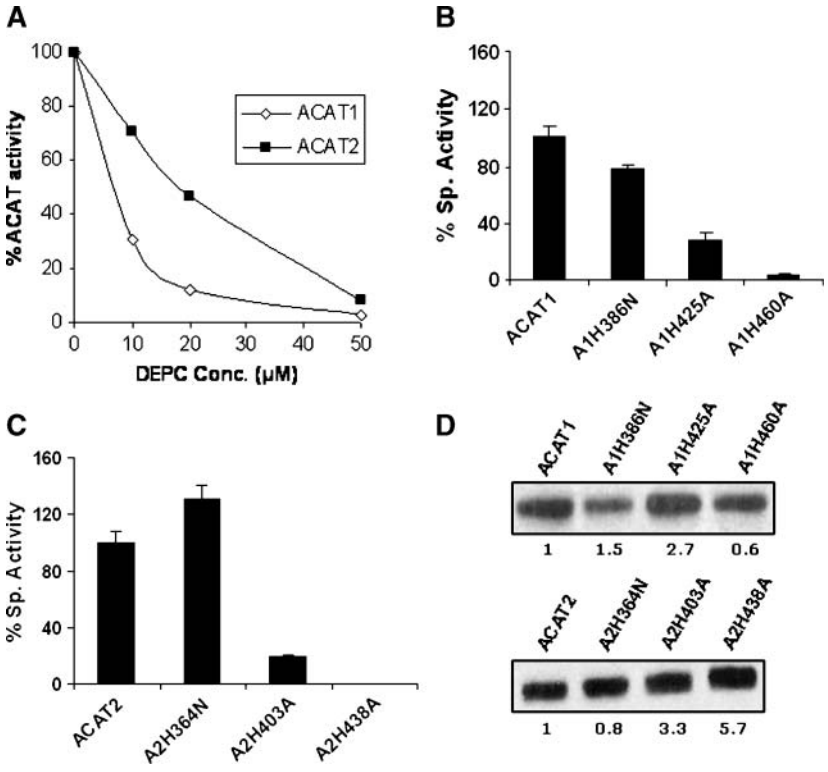

Fig. 2. Identification of essential histidines required for activity of the ACAT enzymes. A: After incubation for 30 min with ethanol-solubilized diethylpyrocarbonate (DEPC) at 37°C, pH 7.4, microsomes prepared from ACAT1 and ACAT2 stable cells were used for ACAT assay. ACAT1 (diamonds) and ACAT2 (squares) 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 ^{3}H 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.

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 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.

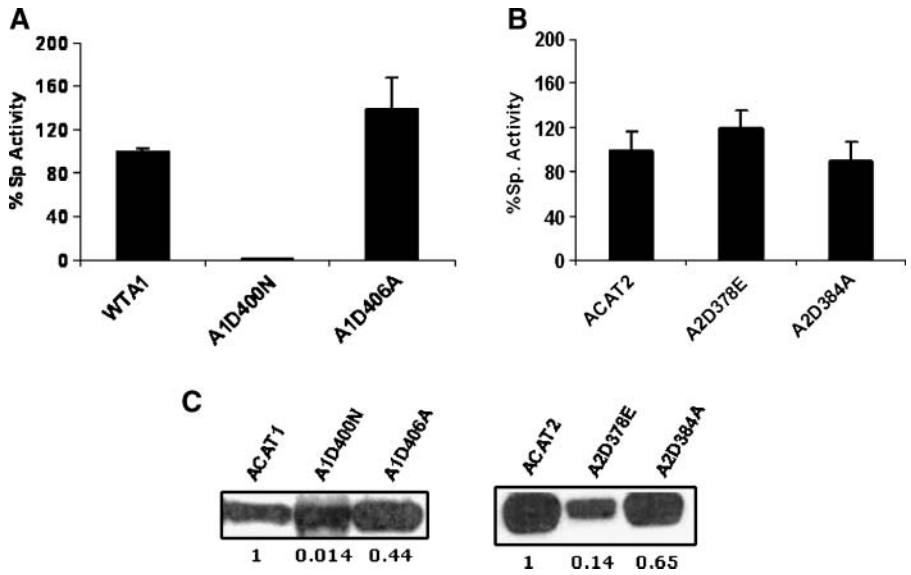

Fig. 3. Evaluation of conserved aspartic acid residues in ACAT enzymes. A. Whole-cell-based activity assay for WT and two specific aspartic acid mutants of ACAT1 enzyme. The activity assay was performed as described in Fig. 2B. This experiment was repeated twice with similar results. Data represent mean ± 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.

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, 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 impor-

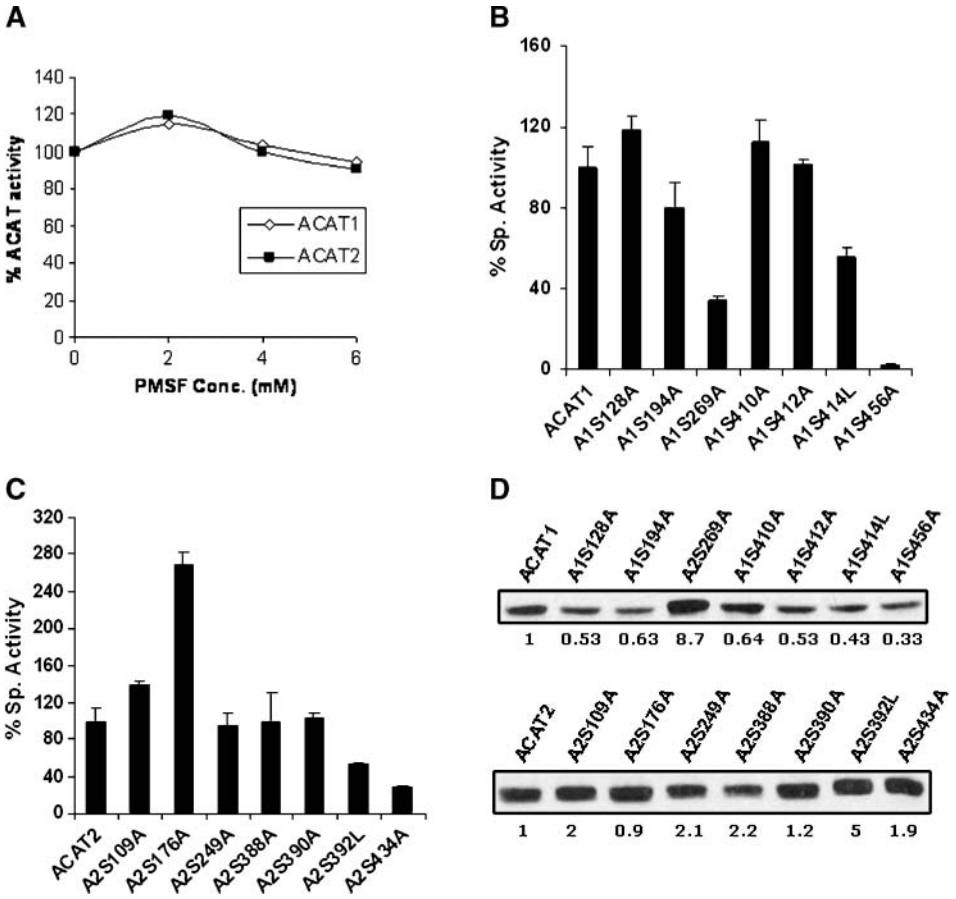

Fig. 4. Identification of the role of conserved serine residues in determination of the activity of ACAT enzymes. A: PMSF solubilized in DMSO was incubated with ACAT1- and ACAT2-containing microsomes at 37°C, pH 7.4, for 30 min. The samples were then used for microsomal ACAT assay as outlined under Experimental 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.

tant 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 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

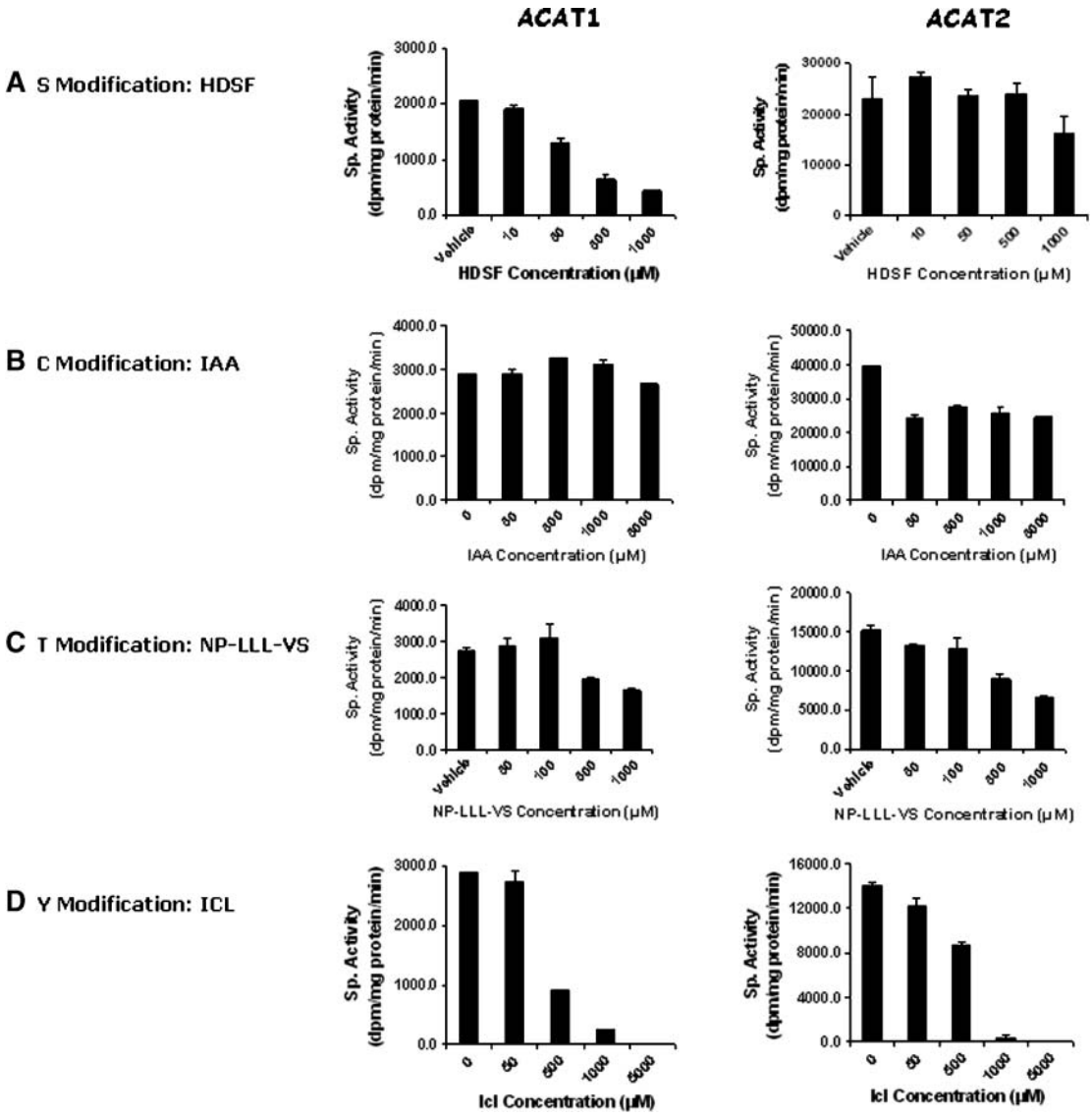

Fig. 5. Effects of serine-, cysteine-, threonine-, and tyrosine-modifying reagents on the activities of ACAT enzymes. A: ACAT1 activity (left panel) and ACAT2 activity (right panel) after hexadecylsulfonylfluoride (HDSF) treatment. HDSF (generous gift from Prof. Sandra L. Hofmann) was solubilized in 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. 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 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.

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, 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.

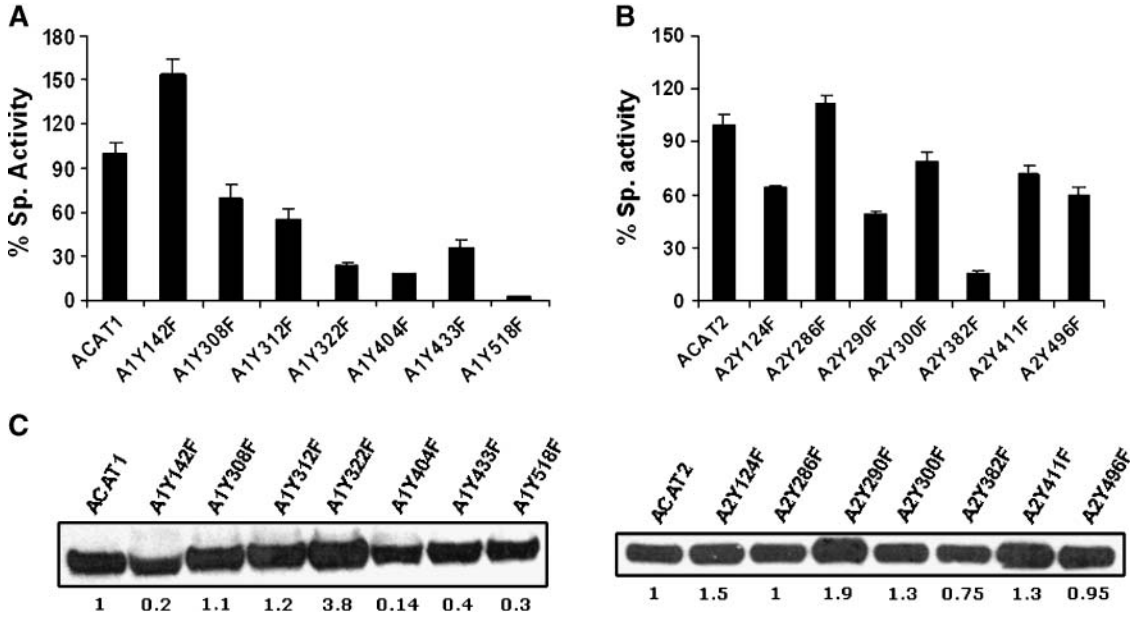

Fig. 6. Evaluation of the role of conserved tyrosine residue(s) as a requirement for the activity of ACAT enzymes. A: Whole-cell-based activity assay for WT and specific tyrosine mutants of ACAT1 enzyme as indicated on the X axis. The activity assay was performed as described in Fig. 2B. This experiment 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.

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 FYXDWWN 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),

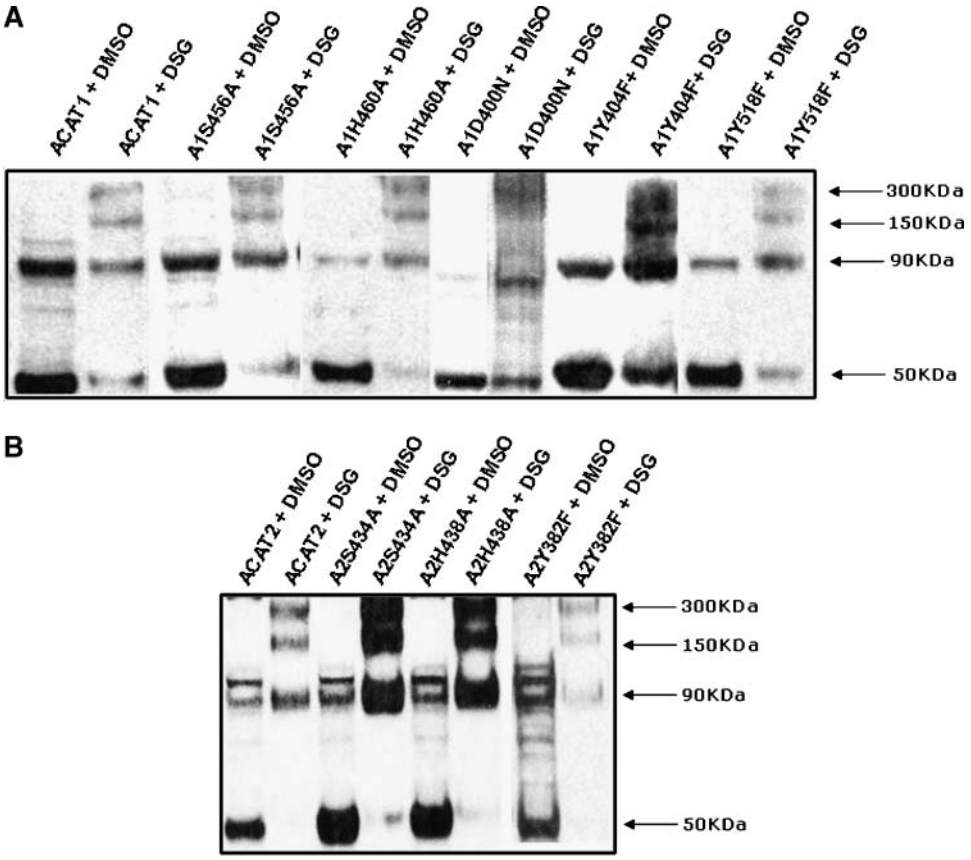

Fig. 7. Amino acid mutation did not alter the cross-linking patterns of ACAT enzymes. A: Microsomes, prepared from AC-29 cells transiently transfected with either WT or indicated ACAT1 mutants, were incubated with DMSO vehicle or disuccinimidyl glutarate (solubilized in DMSO) for 30 min at room temperature. 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.

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 cannot 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 nonconservative 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 FYXDWWN 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).

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