Mutant Acyl-coenzyme A:Cholesterol Acyltransferase 1 Devoid of Cysteine Residues Remains Catalytically Active*

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Acyl-coenzyme A:cholesterol acyltransferase (ACAT)¹ plays important roles in cellular cholesterol homeostasis and in the early stages of atherosclerosis. ACAT1 is an integral membrane protein with multiple transmembrane domains. Human ACAT1 contains nine cysteine residues; its activity is severely inhibited by various thiol-specific modification reagents including p-chloromercuribenzene sulfonic acid, suggesting that certain cysteine residue(s) might be near or at the active site. We constructed various ACAT1 mutants that contained either single cysteine to alanine substitution at various positions, contained a reduced number of cysteines, or contained no cysteine at all. Each of these mutants retained 20% or more of the wild-type ACAT activity. Therefore, cysteine is not essential for ACAT catalysis. For the cysteine-free enzyme, its basic kinetic properties and intracellular localization in Chinese hamster ovary cells were shown to be very similar to those of the wild-type enzyme. The availability of the cysteine-free ACAT1 will facilitate future ACAT structure function studies. Additional studies show that Cys⁴⁶⁷ is one of the major target sites that leads to p-chloromercuribenzene sulfonic acid-mediated ACAT1 inactivation, suggesting that Cys⁴⁶⁷ may be near the ACAT active site(s).

¹ This work was supported by National Institutes of Health Grant HL60306 (to T. Y. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol acyltransferase; ACP, acyl carrier protein; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio-1-propanesulfonate; CHO, Chinese hamster ovary; DTNB, 5,5'-dithiobis-2-nitrobenzoate; ER, endoplasmic reticulum; NEM, N-ethylmaleimide; PC, phosphatidylcholine; pCMBS, para-chloromercuribenzenesulfonic acid; WT, wild-type.

This paper is available on line at http://www.jbc.org
cysteine with an alanine and found that each of the single Cys to Ala mutants retained a significant amount of ACAT activity. We then constructed various ACAT1 mutants with reducing numbers of cysteines, as well as an ACAT1 mutant that completely lacks cysteine. We found that each of these additional mutants retained at least 20% of the activity found in the WT ACAT1; their Km values for oleoyl-CoA and the sigmoidal responses to cholesterol concentration were all similar to those of the WT enzyme. These results indicate that none of the cysteines is essential for ACAT1 catalysis. We also showed that Cys467 is one of the major pCMBS target sites that leads to ACAT1 inactivation. Together, our results suggest that pCMBS completely lacks cysteine. We found that each of these additional cysteines is essential for ACAT1 catalysis. We also showed that Cys467 as the template and appropriate primers, Cys345 and Cys365 were mutated to alanines to

| Mutations | Sense strand primer | Anti-sense strand primer |
|-----------|---------------------|-------------------------|
| C92A      | GATAATGTTGGGgccGCTTCTACACCC | GGTTGGAGAGGgccCACCATTATCC |
| C333A     | GACACATTTGGTGGtcctTTTTTCACTGTG | CACATAGAAAGggccACAAACGCTGTGC |
| C345A     | TTGTTGAAGTTGtcctGCCCCCTTTTGGG | CCGAAAAAGGGGGGgacAGCTTTCTCAAG |
| C365A     | GCTTCTGGCTCTCAgtctCCTAACTCTGC | GGAGTAAAAATCagcTGAAGCATCCGAC |
| C387A     | GCTTCTGGCTCTAGgtctGCTCTAGCTGC | CCTTGAAGAAATggcTGAAGCATCCGAC |
| C467A     | CCGTAAAGGCTGCTGCTGAGCTGCTGCTG | TATTTTCAAGAAggtGTCGGCTGGTCAATCC |
| C516A     | GCTTCTGGCTCTAGgtctGCTCTAGCTGC | CTTTTTCAAGAAggtGTCGGCTGGTCAATCC |
| C528A     | GCGTACGATGCGCCAGtctCTCTGAAAAATC | CTAAAACAGTGAAGgAcGTCGAGAAGCTGG |
| C546A     | CAGCGTCCAGGGCGTctGCTTGTGTTTGA | TATAAAGCAGTGAAGgAcGTCGAGAAGCTGG |

### EXPERIMENTAL PROCEDURES

#### Materials—
- CHAPS, taurocholate, fetal bovine serum, oleoyl-CoA
- N-ethylmaleimide (NEM), 5,5′-dithiothreitol (DTNB), and iodoacetamide
- Fatty acid-free bovine serum albumin, pCMBS, Oleoyl-CoA

#### Plasmid and Recombinant Baculovirus Construction—
- His-tagged human ACAT1 cDNA (HisACAT1) (14) was cloned in pGEM-7Zf(+) vector (Promega). Using this construct as a template, various ACAT1 mutants containing single Cys to Ala substitutions were generated by high fidelity PCR-based mutagenesis, using Stratagene’s QuickChange site-directed mutagenesis kit. Table I lists the pairs of primers used in the PCR mutagenesis experiments. The mutated ACAT1 cDNAs were released from pGEM-7Zf(+) vector by EcoRI and NcoI double digestion and inserted into EcoRI- and PolI-treated transfer vector pVL1393. The ACAT1-positive transfer vectors were co-transfected into insect SF9 cells with linearized BaculoGold DNA. Five days after transfection, recombinant viruses produced from transfected cells were harvested from the medium and then amplified using SF9 cells. To construct various additional cysteine-deficient mutants, including the one devoid of cysteines, multiple rounds of PCR mutagenesis and subcloning were performed. The construction procedures are briefly described as follows: 1) Using the mutant ACAT1 C333A construct as the template and appropriate primers, Cys345 and Cys365 were mutated to alanines to produce the construct ACAT1 C6, with 6 cysteines remaining (at positions 92, 387, 467, 516, 528, and 546). 2) Using the mutant ACAT1 C467A as the template and appropriate primers, Cys467 and Cys486 were mutated to alanines; the resultant construct was then cut by NdeI and HindIII. The released fragment that contained the region from residues 240 to the C terminus was directionally cloned into the truncated mutant ACAT1 construct C92A produced by cutting the construct by the same enzymes. This procedure produced the mutant construct ACAT1 C5, with 5 cysteines remaining (at positions 333, 345, 346, 387, and 528). 3) The C6 construct was sequentially digested with NcoI and StyI; the released region containing residues 223-464 was swapped with the corresponding region present in the C5 construct by restriction digestion and directional cloning. The resultant construct only retained Cys467 and Cys486 and is designated as C2 (387/528). 4) Using construct C2 (387/528) as the template and appropriate primers, a similar strategy was used to produce construct C1 (528), which has only one cysteine remaining (at position 528), and to produce construct C0, which has no cysteine remaining. 5) The construct C2 (528/546) was produced by reverting the Ala in position 546 back to Cys through mutagenesis, using construct C1 (528) as the template. The C1 (467) mutant and C1 (345) mutant were generated in a similar manner. After PCR mutagenesis, the authenticity of all of the mutations present in mutant cDNAs and in mutant viruses were verified by automatic DNA sequencing at regions flanking the mutations. Viral DNAs were isolated using a published procedure (21). The results verified that all of the mutant cDNAs and all of the mutant viruses contained the correct mutations as predicted.

#### Protein Expression and Purification—
The purification procedure was adopted with modifications from the one published earlier using CHO cells (as the enzyme source (14)). In brief, insect HS cells were cultured in 20–30 150 mm-size tissue culture plates with Grace’s medium plus 5% fetal bovine serum. Each plate was infected by a high titer recombinant ACAT1 baculovirus; all of the recombinant ACAT1s contain a His tag at their N termini. The cells were harvested by hypotonic shock and scraping after 48 h of infection. The protein concentration of the cell homogenates was kept at 2–4 mg/ml in 50 mM Tris at pH 7.8, with 0.5 µg/ml leupeptin, 2.5 µg/ml aprotinin, 0.1 µg/ml chymostatin, and 2 µg/ml aprotinin. The cell extracts were solubilized in 2% CHAPS and 1 mM KCl. After centrifugation at 100,000 × g at 4 °C for 45 min, the supernatants were loaded onto a 10-ml (1.6 cm) coil affinity column pre-equilibrated with buffer that consists of 50 mM Tris-HCl, pH 7.8, 0.5% CHAPS, and 1 mM KCl. The column was extensively washed with the column equilibration buffer and then eluted with a buffer of similar composition but containing 100 mM imidazole. The eluted fractions were dialyzed against buffer with higher imidazole concentration (50 mM). The active fractions were pooled and further purified by using a 5-m1 ACAT1 monoclonal antibody affinity column according to the procedure described previously (14).

#### ACAT Activity Assay—
The assays were carried out in bile salt/cholate/PC micelle mixture described before (14).

#### Thiold-specific Chemical Modification Experiments—
For each reaction, 20 µl of purified WT ACAT1 or various partially purified mutant ACAT1s in 50 mM Tris-HCl, pH 7.8, 0.5% CHAPS, and 1 mM KCl were incubated with 1 µl of a specific thiol modification reagent, prepared individually as stock solutions in Me2SO, at 4 °C for 30 min. Next, the mixtures were diluted 5-fold by adding 80 µl of taurocholate/PC/cholesterol-mixed micelles, and the ACAT activity was measured at 37 °C for 5 min after adding 20 µl of labeled oleoyl-CoAbovine serum albumin mixture (5 nmol). The activities of the enzyme treated with 1 µl of MeSO only were used as the 100% values.

#### Protein Assay—
The protein contents of purified ACAT1 were determined by using Coomassie Blue staining after SDS-PAGE, using bovine serum albumin as the standard for quantitation purposes. All other protein determinations were made using Peterson’s modification of the Lowry method (22).

#### SDS-PAGE and Immunoblotting Analysis—
Salt and detergent present in each sample were removed using the chlorpromazine/methanol extraction procedure (23). All samples were then resuspended in loading buffer containing 50 mM Tris, pH 6.8, 9% SDS, 50 mM dithiothreitol, 10% glycerol, and 0.05% bromphenol blue, incubated at 37 °C for 15 min, and then analyzed by SDS-PAGE. DM10, the affinity-purified...
polyclonal rabbit IgGs against ACAT1 amino acid residues 1–131, were used as the primary antibodies for immunoblotting (24). Densitometric analysis of immunoblot signals was performed using National Institutes of Health imaging software.

RESULTS

Procedure for Purifying ACAT1 from Infected Insect Cells—Previously, we had purified the recombinant HisACAT1 to homogeneity from a stable transfectant of CHO cells that over-express hACAT1 (14). The amount of purified ACAT1 protein from this source is very limited. To seek a better source as starting material for enzyme purification, we attempted to express HisACAT1 through a recombinant virus infection in insect H5 cells, using a procedure similar to the one previously developed in this laboratory (25). We found that the specific activity of ACAT1 present in homogenates of infected H5 cells was 5–10 times higher than that of the same enzyme stably expressed in CHO cells. We thus used this source as the starting material and developed a purification scheme as summarized in Fig. 1A. Using this scheme, we purified HisACAT1 protein to electrophoretic homogeneity, as shown in Fig. 1B (lanes 6 and 7). We can routinely obtain 5–10 μg of purified ACAT1 protein from 20 150-mm tissue culture plates of infected H5 cells. The specific activity of the final preparation is 3,500–4,000 nmol/min/mg, which is comparable with the specific activity of the final preparation obtained by using the CHO cells as the enzyme source (14).

Various Thiol Modification Reagents Inhibit ACAT Activity—Using purified HisACAT1 as the source, we tested its susceptibility to inactivation by various thiol modification reagents. Four different —SH modifying reagents were employed: pCMBS, DTNB, NEM, and iodoacetamide. We found that either pCMBS or DTNB, each containing a bulky group, inactivated ACAT1 at relatively low concentration (with IC50 at about 10 μM), whereas NEM, a reagent smaller in size, inactivated the enzyme at much higher concentrations. The IC50 for NEM-mediated inhibition is about 50-fold higher than that of pCMBS-mediated or DTNB-mediated inhibition. We also found that iodoacetamide, which is smaller than NEM, did not inactivate ACAT activity even at 1 mM concentration (Fig. 2). Therefore, the inhibitory efficiency of these modification reagents seems to correlate well with their sizes.

The above result supported the hypothesis that certain Cys residue(s) may be near or at the ACAT active site. We undertook the site-specific mutagenesis approach to further investigate this issue. Human ACAT1 contains 9 cysteines, with their locations indicated in Fig. 3A. Among them, Cys352, Cys365, Cys467, and Cys516 are conserved in ACAT1s from various species; Cys333, Cys340, Cys387, Cys528, and Cys546 are conserved in both ACAT1s and ACAT2s. On the other hand, none of these cysteines is conserved in DGAT1, an ACAT homolog that also uses long chain fatty acyl-CoA as a substrate. We converted each of these 9 cysteines to an alanine, then incorporated each mutant into the recombinant baculovirus genome, and expressed it in H5 cells. The ACAT protein expression levels were monitored by performing Western blotting using anti-ACAT1 IgGs (DM10) and by measuring ACAT activity in the crude cell extracts. The results showed that, similar to the WT ACAT1, each mutant ACAT1 was expressed as a single, untagged 50-kDa band (Fig. 3B, bands near the top). The ACAT enzyme specific activity determinations showed that each mutant ACAT1 was at least partially active when compared with the activity of the WT ACAT1 (Fig. 3B, bars near the bottom). The expression levels of individual ACAT1 mutants varied to some extent, as judged by the intensities of bands in Western blot analysis (Fig. 3B). When the activities of the WT and mutant ACAT1s present in the cell extracts were normalized by the ACAT protein content, the results showed that all of the Cys to Ala mutants contained 50% or more of the WT ACAT activity (Fig. 3C). The variations in activity in some of the ACAT1s with single Cys to Ala substitutions might be caused by subtle structural alteration(s) induced by the individual mutations.

Properties of the Cys-deficient ACAT1 Mutants—The above result suggested that cysteines might not be required for ACAT1 catalysis. We asked whether mutant ACAT1s with reducing numbers of cysteines still retain enzyme activity. To test this possibility, we produced the following mutant ACAT1 viruses: C6 (retaining cysteines at positions 92, 387, 467, 516, 528, and 546); C5 (retaining cysteines at positions 333, 345, 365, 387, and 528); C2 (387/528) (retaining cysteines at positions 387 and 528); C3 (528/546) (retaining cysteines at positions 528 and 546); C1 (528) (retaining cysteine at position 528); and C0 (retaining no cysteines). The enzyme activities of these mutant proteins expressed in infected cells were then measured. We found that the activity of the C0 mutant was essentially the same as that of the WT ACAT1. The enzymatic activities of the C5 and C2 mutants were close to each other. The specific activities of both mutants were increased by 30% when compared with the WT ACAT1. The specific activities of the C6 and C3 mutants were increased twofold over the WT ACAT1. The enzymatic activity of the C1 mutant was slightly increased over that of the WT ACAT1. These results clearly indicated that the 5 cysteines conserved in DGAT1 were not required for ACAT1 catalysis. The enzymatic activity of the C2 mutant was similar to that of the WT ACAT1. Therefore, the enzymatic activity of the C1 mutant was less than that of the WT ACAT1. These results clearly indicated that the 5 cysteines conserved in DGAT1 were not required for ACAT1 catalysis.
analyzed. The results showed that all of the Cys-deficient ACAT1s examined retained at least partial enzyme activity (based on specific activity measurement (Fig. 4A)). To compare the relative ACAT activity in a more precise manner, we loaded the same amount of ACAT activity found in each cell extract and then monitored their relative ACAT protein contents by Western analysis. When analyzed in this manner, one expects that those cell extracts containing higher intrinsic enzyme activity will give less intense ACAT1 protein signals. The results (Fig. 4B) showed that mutant ACAT1 C6 contained essentially the same activity as the WT ACAT1; other mutant ACAT1s (C5, C2 (387/528), and C1 (528)) contained −33–59% of the activity of WT ACAT1. The one completely devoid of cysteines (C0) still contained ∼40% of the activity of WT ACAT1. We next expressed several Cys-deficient mutant ACAT1s (the C2 (528/546), C1 (528), and C0) in ACAT-deficient CHO cells (AC29) by transient transfections. The relative enzyme activities of these mutants were evaluated by performing [3H]oleate incorporation in intact cells (Fig. 5A) and by immunoblot (Fig. 5B). The normalized ACAT activities were calculated by dividing the values in Fig. 5A by the values in Fig. 5B. The result showed that the C1 (528) mutant and the C0 mutant still contained a significant amount of residual enzyme activity, supporting the conclusion derived from insect cell expression studies. The expression levels of the C1 and C0 mutant ACAT1s in CHO cells were much lower than that of the WT ACAT1 (Fig. 5B), and it is difficult to precisely evaluate their relative contents within the same blot. Therefore, for ACAT-specific activity determinations, the results obtained in Fig. 5C may not be as precise as those obtained in Fig. 4B. To examine the intracellular location of the C0 mutant in CHO cells, we performed double immunofluorescence experiments using laser scanning confocal microscopy with differential interference contrast optics. We found that the mutant ACAT1 C0 extensively colocalized with the ER marker Bip (Fig. 5D). Its localization pattern is essentially the same as what we had previously reported for the WT ACAT1 expressed in CHO cells (12). The same localization patterns were also found for ACAT1 mutant C2 (528/546) and for mutant C1 (528) (results not shown). We had previously used chemical cross-linking in intact cells as one of three methods to estimate the oligomeric state of WT ACAT1 (11). We found that the amine-specific homo-bifunctional chemical cross-linking agent disuccinimimidyl suberate caused the formation of material two to four times the size of the monomeric enzyme when added to intact insect cells expressing either WT ACAT1 or mutant ACAT1 C0 (Fig. 6). This result suggested that mutant ACAT1 C0, similar to WT ACAT1, is also a homotetrameric enzyme.

We next compared the basic kinetic properties of the WT and the three Cys-deficient mutant ACAT1s (C2 (528/546), C1 (528), and C0) by performing substrate saturation curve studies, using partially purified preparations as the enzyme sources. The WT HisACAT1 exhibited the $K_m$ for oleoyl-coenzyme A of 7.1 μM, which is the same as the value obtained when the enzyme was expressed in CHO cells (14). For the various Cys-deficient mutant ACAT1s, the $K_m$ value for oleoyl-coenzyme A was approximately 2-fold higher than the value for the WT enzyme, ranging between 12 and 15 μM (Fig. 7). We also found that the WT and the three Cys-deficient ACAT1s all responded to cholesterol in a sigmoidal manner (Fig. 8A). For a given allosteric enzyme, the Hill coefficient has been used to quantitatively estimate the degree of cooperativity (26). The Hill plot analysis (Fig. 8B) revealed that the Hill coefficient value for the WT enzyme (2.6) is essentially the same as the value obtained when the enzyme was expressed in CHO cells (14). Also, the Hill coefficient did not change significantly between the WT enzyme (2.6) and the three Cys-deficient mutant ACAT1s (2.3–2.4).

**Cys$^{345}$ Is One of the Major Targets for pCMBS Inhibition**—Because none of the Cys residues is needed for ACAT catalysis, the pCMBS inhibition of ACAT1 activity (Fig. 2) may be due to the steric hindrance effect created by covalently attaching a bulky group (the mercuribenzenzene sulfonic acid moiety) to the Cys residue(s) near the active site. To elucidate the Cys residues responsible for mediating the pCMBS inhibition, we tested various ACAT1 mutants with single Cys to Ala substitutions for their sensitivity toward pCMBS inhibition. The results (Fig. 9A) showed that essentially all of the single Cys to Ala mutants remained sensitive to pCMBS inhibition. We also found that the C345A mutant and the C467A mutant retained higher residual enzyme activities than other Cys to Ala mutants, especially when pCMBS was used at higher concentrations (0.5 mM and higher). This observation persisted in three separate experiments, and raised the possibility that Cys$^{345}$ and/or Cys$^{467}$ may be one of the main target(s) for mediating the pCMBS inhibition effect. To test this possibility, we produced three more mutants: a double Cys to Ala mutant (C345A/C467A, with 7 other cysteines remaining), a C1 (345) mutant,
None of the nine cysteines is needed for ACAT1 activity. A, diagram indicating locations of the 9 cysteines (solid circles) in ACAT1. The membrane topographical arrangement of ACAT1 is according to Ref. 12. B, the expression of mutant ACAT1s with various Cys to Ala substitutions in H5 cells. H5 cells were infected with WT or with mutant ACAT1 virus as indicated for 48 h. The cell extracts were solubilized in 2% CHAPS and 1 M KCl; 20 μl was used to measure ACAT activity, and 1 μl was used for immunoblot analysis. The immunoblots are shown in B as insets. The ACAT assays and Western blots were described under “Experimental Procedures.” C, the relative ACAT activity of various Cys to Ala mutants. The values shown are ACAT activity divided by the relative ACAT protein content present in each cell extract; the value found in WT was defined as 1.0. The relative ACAT protein content was determined by densitometric analysis of results shown in the insets of B.

FIG. 4. Various Cys-deficient ACAT1 mutants retain catalytic activity. The six different Cys-deficient mutants were designated as C5 (with 5 cysteines at residues 333, 345, 356, 387, and 528), C6 (with 6 cysteines at residues 92, 387, 467, 516, 528, and 546), C2 (387/528) (with 2 cysteines at residues 387 and 528), C2 (528/546) (with 2 cysteines at residues 528 and 546), C1 (528) (with only one Cys at residue 528), and C0 (without any C). The WT and the Cys-deficient mutants were produced as recombinant viruses and were expressed in H5 cells. A, ACAT specific activity in solubilized crude cell extracts, measured as nmol of cholesteryl ester formed per min per mg protein. The Control lane shows ACAT activity of cells infected by an irrelevant gene. B, comparing the relative activities of the WT and various Cys-deficient mutant ACAT1s. After ACAT activity determinations as described in A, an equal amount of ACAT activity (2 pmol/min) derived from each cell extract was loaded per lane and was subjected to SDS-PAGE followed by Western analysis using anti-ACAT1 antibodies DM10. The values reported at the end of each lane represented the relative intensity of each ACAT1 band shown in the immunoblot, as estimated by densitometric analysis. The results were from one of two representative experiments.

FIG. 3. None of the nine cysteines is needed for ACAT1 activity. A, diagram indicating locations of the 9 cysteines (solid circles) in ACAT1. The membrane topographical arrangement of ACAT1 is according to Ref. 12. B, the expression of mutant ACAT1s with various Cys to Ala substitutions in H5 cells. H5 cells were infected with WT or with mutant ACAT1 virus as indicated for 48 h. The cell extracts were solubilized in 2% CHAPS and 1 M KCl; 20 μl was used to measure ACAT activity, and 1 μl was used for immunoblot analysis. The immunoblots are shown in B as insets. The ACAT assays and Western blots were described under “Experimental Procedures.” C, the relative ACAT activity of various Cys to Ala mutants. The values shown are ACAT activity divided by the relative ACAT protein content present in each cell extract; the value found in WT was defined as 1.0. The relative ACAT protein content was determined by densitometric analysis of results shown in the insets of B.

and a different C1 (467) mutant. We found that the double Cys to Ala (C345A/C467A) mutant, the C1 (345) mutant, and the C0 mutant were insensitive to pCMBS inhibition (Fig. 9B). These results indicate that Cys467 is one of the major pCMBS targets to inactivate ACAT activity. Consistent with this interpretation, we found that the ACAT1 C6 mutant was sensitive to pCMBS inhibition, whereas the two C2 mutants (C2 (387/528) and C2 (528/546)) and the C1 (528) mutant were resistant to pCMBS inhibition (results not shown). Our results suggest that Cys467 may be located near the ACAT active site(s).

DISCUSSION

In the current work, we first described a procedure to purify the recombinant ACAT1 expressed in insect cells to homogeneity. Through the current procedure we can obtain three to six times more pure ACAT1 protein than by using CHO cells as the expression system. The specific activity, the $K_m$ for oleoyl-coenzyme A, and the Hill coefficient for the cholesterol saturation curve of the purified enzyme are all comparable with the values obtained by using the enzyme purified from the CHO cells. We next used ACAT1 expressed in insect H5 cells as the source to perform chemical modification and to characterize site-specific ACAT1 mutants. Our results showed that none of the 9 cysteines present in ACAT1 is essential for enzymatic catalysis. In fact, the Cys-free mutant ACAT1 (C0) still maintained about 40% of the activity of WT ACAT1 and exhibited kinetic properties toward the substrates oleoyl-coenzyme A and cholesterol similar to the WT ACAT1. When expressed in CHO cells, the localization pattern of the C0 enzyme was essentially the same as the WT enzyme. For structure and function studies of membrane proteins, much valuable information can be obtained by exploiting the unique chemical reactivity of cysteine, as exemplified by the work of Kohrana, Hubell, and co-workers (27, 28) to study bacterial rhodopsin, and by the work of Kaback and co-workers (29, 30) to study lactose permease. In the future, the availability of the cysteine-free ACAT1 will facilitate ACAT1 structure function studies by allowing the investigators to engineer a single cysteine at certain defined position and to probe its local environment with various cysteine-specific biochemical reagents.

Potential Implication of Our Current Findings on ACAT Active Site and on the Dual Functions of ACAT—Our mutagenesis studies indicated that cysteines are not part of the active site of
ACAT1, although cysteine modification reagents, such as pCMBS, strongly inhibit ACAT1. Additional studies showed that inhibition by pCMBS depends on the presence of cysteine(s) located at certain positions. In addition, we found that the inhibitory efficiency of the various thiol modification reagents correlated with their sizes. In data not shown, we also found that p-chloromercuribenzoate, a hydrophobic derivative of pCMBS, inhibits ACAT1 in manner very similar to that of pCMBS. These results imply that steric hindrance generated near the active site by covalently attaching a bulky group to cysteine residue(s) may be the major cause that leads to inactivation of ACAT1. However, other possibilities, such as change(s) in local conformation near the enzyme active site, cannot be ruled out at present.

Further investigation showed that Cys467 is one of the major targets for pCMBS-mediated inactivation of ACAT1. These results allowed us to hypothesize that Cys467 might be near the active site. Earlier, a histidine residue near Cys 467 (460 in human ACAT1) was proposed to constitute part of the ACAT active site (17). This histidine is invariant within the membrane-bound acyltransferase superfamily that includes ACAT1, ACAT2, and DGAT. His460 is within a long hydrophobic region that comprises more than 20 amino acids, and function of the His460 is currently unknown (17). Our results sug-
gest that the para-mercuribenzenesulfonic acid group covalently attached to Cys 467 may create steric hindrance to block the action of His460, thus supporting the idea that His460 may constitute part of the ACAT active site.

At present, there is good evidence implicating the role of ACAT1 in macrophage foam cell formation and the role of ACAT2 in intestinal cholesterol absorption. In contrast, the roles of ACAT1 and ACAT2 in the very low density lipoprotein or chylomicron assembly process are less clear (5). It is possible that both enzymes are able to form lipid droplets (which are present in the cytoplasm) and participate in lipoprotein assembly (which occurs in the ER lumen). To link the site of ACAT catalysis with its presumed dual function, we have recently proposed that part of the ACAT catalytic site, including His460, may be located within the lipid bilayer, enabling the enzyme to complete its catalytic action within the plane of the membrane (31). Cholesteryl esters produced in situ may burst from the ER membranes and form cytoplasmic lipid droplets carrying phospholipid monolayers as their outer coats. In cells engaged in lipoprotein assembly and secretion, cholesteryl esters in the bilayer may be recognized by the specific protein microsomal triacylglycerol transfer protein, present in the luminal side of the membrane. The microsomal triacylglycerol transfer protein then lipidates the growing apo-B chain with cholesteryl ester and triacylglycerol and participates in the apo-B lipoprotein assembly. Although the membrane topography of ACAT1 and ACAT2 may be different, the basic principle described in this model may apply to both ACAT1 and ACAT2.

We also noted that each of the single Cys to Ala mutants, including the C467A mutant and the C345A mutant, was susceptible to inactivation by pCMBS (Fig. 9A), whereas the double Cys to Ala mutant (C345A/C467A) was resistant to inactivation by pCMBS (Fig. 9B). These results suggested that in the absence of C467, pCMBS could inactivate ACAT by a second mode of action: by attaching bulky groups to several other Cys residues. Among the eight Cys residues available, the presence of Cys345 seems essential for mediating the pCMBS action, because converting Cys345 to an Ala abolished the second mode of pCMBS-mediated inactivation. The single C345A mutant expressed higher residual enzyme activities at higher pCMBS concentrations.
concentrations (Fig. 9A). However, unlike Cys<sup>467</sup>, Cys<sup>345</sup> alone cannot serve as a major target for pCMBS-mediated inactivation of ACAT, because the C1 (345) mutant is resistant to inactivation by pCMBS (Fig. 9B). Thus, our current data do not allow us to determine whether Cys<sup>467</sup> or any other Cys residue is also near the ACAT active site.

**Possible Significance of the pCMBS Inhibition Studies on ACAT1 Membrane Topography**—In mammalian cells, it is known that most of the cysteine residues of proteins that reside in the lumen of the ER form disulfide bonds (through the action of the enzyme disulfide isomerase). The pCMBS inhibition studies reported in our current study implied that at least several cysteine residues present in ACAT1 probably maintained their sulfhydryl moieties at reduced state, i.e. they were not cross-linked to form disulfide bonds. It should be pointed out that the enzyme source employed for the pCMBS inhibition studies was prepared from infected insect H5 cells. Because the subcellular localization and the membrane topography of ACAT1 expressed in insect cells and in CHO cells may be different, we cannot assume that these same cysteine residues also maintain their sulfhydryl moieties at a reduced state when ACAT1 is expressed in CHO cells. In the future, the availability of the C0 mutant described in the current work can allow us to express ACAT1 mutants that contain single cysteine residues engineered at various positions along the ER membranes of CHO cells. This strategy will permit us to design various experiments to test the validity of the proposed ACAT1 membrane topography models in mammalian cells (12, 13).

**Possible Catalytic Mechanisms of ACAT1—Acyltransferases** are involved in a variety of biochemical pathways. A literature survey revealed that in general, acyltransferases catalyze the reactions via one of three types of catalytic mechanisms. The first possibility is exemplified by the GCN5-related N-acetyltransferase superfamily, catalyzing the transfer of acyl group from acetyl coenzyme A to an amine group of an acceptor molecule (32–35). The proposed catalytic mechanism involves the enzyme-assisted, deprotonated amino group present in the acceptor molecule attacking the carbonyl group of acetyl CoA, forming a tetrahedral intermediate. This is followed by the release of the coenzyme A from the intermediate, resulting in the net transfer of the acetyl group to the amine group of the acceptor molecule. If ACAT catalysis occurs in a similar manner, the amino group of the acceptor molecule would instead be the 3β-hydroxy group of cholesterol. The second possibility is exemplified by the serine protease superfamily, including the pancreatic lipase (36), cholesteryl esterase (37), and lecithin:cholesterol acyltransferase (38). These enzymes contain the Asp-His-Ser catalytic triad and catalyze the reactions by a two-step mechanism; the first step involves the formation of a covalent, acyl-enzyme intermediate that involves a serine residue at the active site. This is followed by the enzyme-assisted hydrolytic attack by the OH moiety of the water molecule, breaking the acyl-O-siline ester bond, releasing the acyl group as free fatty acid. The hydrolytic step is usually assisted by a His residue and an Asp residue within the enzyme. If ACAT catalysis occurs in a similar manner, the –OH moiety of the water molecule would instead be the 3β-hydroxy group of cholesterol. The third possibility is exemplified by various enzymes in the fatty acid biosynthesis and polyketide biosynthesis, including acetyl coenzyme A:acyl carrier protein (ACP) acyltransferase, β-ketoacyl-ACP synthase, chalcone synthase, etc. The catalysis involves the transfer of acyl moiety from coenzyme A to the –SH moiety of 4-phosphopantetheine of ACP moiety or of a cysteine residue (39, 40) of the enzyme. A covalent thioester intermediate is formed during catalysis. Our current work shows that cysteine is not required for ACAT catalysis, thus ruling out the possibility that a covalent thiolester intermediate may be formed. Future research in this laboratory is directed toward determining whether ACAT catalysis occurs by forming a tetrahedral intermediate or by forming a catalytic O-serine ester enzyme intermediate.

**Acknowledgments**—We thank members of our laboratory for stimulating discussions and Helina Morgan for careful editing of this manuscript.

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Mutant Acyl-coenzyme A:Cholesterol Acyltransferase 1 Devoid of Cysteine Residues Remains Catalytically Active
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J. Biol. Chem. 2002, 277:711-718.
doi: 10.1074/jbc.M109427200 originally published online October 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109427200

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