Recombinant Acyl-CoA:cholesterol Acyltransferase-1 (ACAT-1) Purified to Essential Homogeneity Utilizes Cholesterol in Mixed Micelles or in Vesicles in a Highly Cooperative Manner*

(Received for publication, July 24, 1998, and in revised form, September 18, 1998)

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Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is an integral membrane protein located in the endoplasmic reticulum. It catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acyl coenzyme A. The first gene encoding the enzyme, designated as ACAT-1, was identified in 1993 through an expression cloning approach. We isolated a Chinese hamster ovary cell line that stably expresses the recombinant human ACAT-1 protein bearing an N-terminal hexahistidine tag. We purified this enzyme approximately 7000-fold from crude cell extracts by first solubilizing the cell membranes with the zwitterionic detergent 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, then proceeding with an ACAT-1 monoclonal antibody affinity column and an immobilized metal affinity column. The final preparation is enzymologically active and migrates as a single band at 54 kDa on SDS-polyacrylamide gel electrophoresis. Pure ACAT-1 dispersed in mixed micelles containing sodium taurocholate, phosphatidylcholine, and cholesterol remains catalytically active. The cholesterol substrate saturation curves of the enzyme assayed either in mixed micelles or in reconstituted vesicles are both highly sigmoidal. The oleoyl-coenzyme A substrate saturation curves of the enzyme assayed under the same conditions are both hyperbolic. These results support the hypothesis that ACAT is an allosteric enzyme regulated by cholesterol.

80% of total measurable ACAT activity is resistant to immunodepletion, suggesting that ACAT activity in this particular tissue may be largely due to the presence of a different ACAT protein (22). Judging from results currently available, it is possible that the physiological functions of ACAT-1 and ACAT-2 are different in different species. Whether ACAT-2 is responsible for most of the observed ACAT activity in human intestines is not clear at present.

The ACAT protein has never before been purified to homogeneity. The difficulty in doing so was largely due to its minute quantity and the lack of a suitable detergent for solubilizing the protein from the endoplasmic reticulum membrane with retention of enzyme activity (reviewed in Ref. 5). As described in this article, we have isolated a CHO cell line (HisACAT-1 cells) that stably expresses the human ACAT-1 (hACAT-1) protein bearing a hexahistidine tag at its N terminus. We report the use of this cell line as the starting material to develop a purification scheme that enables us to purify the enzyme to essential homogeneity. We also present some kinetic properties of the purified enzyme in mixed micelles made of bile salt, cholesterol, and phosphatidylcholine (PC), or in cholesteryl/PC vesicles. Collectively, our results support the hypothesis...
that the sigmoidal dependence of ACAT activity upon cholesterol concentration (1, 5) is an intrinsic property of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Methods**

**HisACAT-1 Cells**—A fragment was generated containing an ATG translation initiation codon, a 6-histidine tag that functions as a metal-binding domain, a T7 tag that serves as a transcript stabilizing sequence from gene 10 of phage T7, and an enterokinase cleavage recognition sequence (a total of 40 amino acids). This construct (originally designed by Invitrogen for baculovirus transfer vector pBlueBacHis) was ligated into the N terminus of hACAT-1 DNA encoding the mature sequences 1397–3046, a total of 550 amino acids, with the first methionine (ATG) converted to leucine (CTA) by site-specific mutagenesis. This entire fragment was then ligated into the expression vector pcDNA3 (from Invitrogen). Using a modified calcium phosphate-DNA co-puriﬁcation method (23), the plasmid was transfected into an ACAT-deﬁcient cell line, AC29, that lacks endogenous ACAT message and protein (19). One day after transfection, cells were selected for G-418 resistance by including 500 μg/ml G-418 in growth medium for 1 week. The G-418 resistant colonies were examined for their cytoplasmic cholesteryl ester lipid droplet content by microscopic examination according to procedures described earlier (13). The clones that scored positive for cytoplasmic lipid droplets were approximately 5% of the total G-418 resistant clones; they were isolated with cloning rings and recloned by dilution. In this manner, a stable clone expressing high ACAT activity in vitro and in hACAT protein content was isolated and named HisACAT-1. To maintain clonal purity, cloned HisACAT-1 cells were stored at 2–4 mg/ml in Buffer A (50 mM Tris, 1 mM EDTA at pH 7.8). The ACAT activity was expressed in intact cells and whole cell extract. The activity between whole cell extract and microsome. The activity

**Isolation of ACAT Monoclonal Antibodies against GST-ACAT-1** (Amino Acid Residues 1–131)—The recombinant fusion protein GST-ACAT-1 (amino acid residues 1–131) (19) was used to immunize three male BALB/c mice. Each mouse was injected subcutaneously with 20 μg of GST-ACAT-1 emulsified in the presence of complete Freund’s adjuvant and phosphate-buffered saline (50/50, v/v). Two to three days later, the cultured supernatants were removed for screening of antibody secretion with enzyme-linked immunosorbent assay using GST-ACAT-1 or GST protein alone coated microwells. Only two hybrid cell lines, designated as ACAT-1a and ACAT-1c, were found to secrete antibodies to ACAT-1 protein. Additional results showed that ACAT-1a IgG and ACAT-1c IgG recognized the same epitope within hACAT-1 amino acid residues 1–40. Antibody-containing ascites ﬂuid of each cell line was generated by intraperitoneally injecting pristane-primed BALB/c mice with about 1–3 × 10^6 hybridoma cells. The antibody was puriﬁed by protein A-Sepharose column. Monoclonal ACAT-1a IgG was coupled with Affi-Gel 10 (Bio-Rad) according to instructions given in the manufacturer’s manual.

**Protein Assays**—All protein determinations were made using Peterson’s modiﬁcations (28) of the Lowry method. Trichloroacetic acid precipitation was per-formed when samples contained interfering substances.

**SDS-PAGE and Immunoblotting Analysis**—Salt and detergent were removed from samples by chloroform/methanol extraction (29). All samples were then resuspended in loading buffer containing 50 mM Tris (pH 6.8), 10% glycerol, 10% sodium dodecyl sulfate, 0.1% sodium dodecyl sulfate, and 0.025% bromophenol blue. Samples were incubated at 37 °C for 15 min before loading onto SDS-PAGE. DM10, the affinity-puriﬁed polyclonal rabbit IgGs against GST-ACAT-1 (amino acid residues 1–131), was used as the primary antibody for immunoblotting. For silver stain gels, Bio-Rad’s Silver Plus Kit was used according to instructions.

**Materials**

CHAPS, taurocholate, oleoyl-coenzyme A, egg PC, cholesteryl oleate, cholesteryl fatty acid-free bovine serum albumin were all obtained from Sigma. The software program PRISM was purchased from Sigma. All reagent-grade solvents were obtained from Fisher. Triton X-100 was from CalBiochem. Charcoal-puriﬁed cholic acid and deoxycholate were generous gifts from Dr. Bernard Trumpower (Dartmouth Medical School). 1,2-D(N)-[3H]Cholesterol was from American Radiolabeled Chemicals, 50 Ci/mmol. [3H]-Oleoyl-coenzyme A was synthesized as described (30). The monoclonal antibodies against hACAT-1 are available through Vancouver Biotech Ltd.

**RESULTS**

**Purification of HisACAT-1**—Through various insertional and deletion mutagenesis experiments with cloned human ACAT-1 cDNA, we found that the region comprising the ﬁrst 63 amino acids of hACAT-1 protein is not essential for enzyme catalysis (results not shown). We therefore placed a 40-amino acid sequence tag as the N-terminal extension of the hACAT-1 cDNA. This tag contains a methionine codon followed by hexahistidine and T7-antigenic sequences. The fragment was ligated with an expression vector pcDNA3 and then transfected with ACAT-deﬁcient CHO cell mutant AC29, which lacks endogenous ACAT message and protein (19). A stable transfectant clone designated as HisACAT-1, expressing ample human ACAT activity in intact cells and in vitro, was isolated (see “Experimental Procedures”). According to SDS-PAGE analyses, the HisACAT-1 protein expressed in this cell line is a single 54-kDa protein without detectable intracellular degradation (results not shown). This cell line was chosen as the starting material to develop a scheme for purifying the enzyme to homogeneity.

We prepared cell homogenates from HisACAT-1 cells and isolated the membrane fraction by sequential centrifugation. The subcellular fractionation procedure provided an average of 3-fold purification as measured by comparing ACAT speciﬁc activity between whole cell extract and microsome. The activity recovery averaged approximately 50% (Fig. 1, panel A). Immunoblotting analysis showed a similar degree of enrichment in the HisACAT-1 protein; a typical result is shown in Fig. 1, panel B. We next searched for an appropriate detergent to
solubilize the HisACAT-1 protein, yet leave the solubilized enzyme amenable to various purification procedures. We found that the zwitterionic detergent CHAPS at 2–2.5% could effectively solubilize the HisACAT-1 protein with retention of enzyme activity; a typical result is shown in Fig. 2. We also found that the inclusion of 1M KCl during the solubilization procedure helped increase the ACAT activity recovery in the 100,000 g supernatant fraction. Immunoblotting analysis showed that CHAPS along with 1M KCl could solubilize at least 70% of total detectable hACAT-1 protein present in either whole cell extracts or in the microsome fraction (results not shown). The hACAT-1 protein solubilized by this procedure is not entirely stable. At 4 °C, the solubilized enzyme loses 30–50% of its original activity within 4–10 h. The loss of enzyme activity can be partially prevented by including certain protease inhibitors in the buffer solutions (results not shown). For this reason, we have routinely added various protease inhibitors (see “Experimental Procedures”) up to the solubilization step of the purification scheme (Fig. 1).

We next attempted to purify the solubilized enzyme using ACAT monoclonal antibody affinity column chromatography. The column was pre-equilibrated at 4 °C with 50 mM KH₂PO₄ at pH 7.4 containing 1M KCl and 0.5% CHAPS (Buffer B). Upon loading the solubilized enzyme solution through the column four times by gravity flow, we found that around 10–15% of the enzyme did not bind to the column. After extensively washing the column with 12 column volumes of 10 mM KH₂PO₄ at pH 7.0 containing 1M KCl and 0.5% CHAPS, the bound enzyme was then detached from the column by eluting with 50 mM citrate buffer at pH 3.5 containing 1M KCl and 0.5% CHAPS. A typical result is shown in Fig. 3. The pH of the eluted protein fractions was immediately neutralized by including 2M KH₂PO₄ at pH 9.0 in the collection vessels. The eluted enzyme was found to remain enzymologically active. In three separate experiments, the recovery of ACAT activity in the pH 3.5 eluant averaged 10–30% of the applied activity. Due to the low protein concentration in the pH 3.5 eluant, we could only roughly estimate that the fold purification provided by the monoclonal antibody column was around 100. Still, this method did not provide us with a homogeneous enzyme. This is demonstrated in Panel C (silver stain) of Fig. 3, which shows the ACAT protein band (located at 54 kDa) sandwiched between two major impurity bands in SDS-PAGE. As evaluated by silver staining after SDS-PAGE, the purity of the enzyme at this stage varied from 5 to 10%. We next used nickel column chromatography for the final purification step. After the ACAT monoclonal antibody column purification step, the purified HisACAT-1 protein firmly bound to the nickel beads. With extensive buffer washes, HisACAT-1 was able to be eluted off the column with buffer containing 250–500 mM imidazole in Buffer B. In several experiments, we found that nickel column chromatography consistently provided a very good recovery of both ACAT activity and protein (averaging around 60% of total ACAT activity and HisACAT-1 protein loaded onto the column). It also serves the purpose of concentrating the enzyme solution. A typical result is shown in Fig. 4. Nickel column chromatography used alone with the solubilized enzyme could not provide us with a homogeneous pure HisACAT-1 protein. However, when it was added after the monoclonal antibody column step, the resultant enzyme eluted off the column at high imidazole concentration (500 mM) exhibited essentially a single protein band at 54 kDa as analyzed by SDS-PAGE with silver staining (Fig. 4, panel C, lanes 13–15). A parallel set of samples were analyzed by immuno blot using the specific anti-ACAT-1 antibodies; the results indicate a single immunoreactive band at the same molecular weight (54 kDa) (Fig. 4, panel
Furthermore, the variation in intensity of the immunoreactive band correlated well with the variation in intensity of the silver staining band, indicating that the single 54-kDa protein band demonstrated in panel B is ACAT-1.

As shown in lanes 13–15 of panel C, Fig. 4, in addition to the 54-kDa HisACAT-1 protein band, there are zonal material(s) present at a higher molecular weight range (90–120 kDa) that can be stained positively with silver. We do not know the nature of this material, but we have noted that similar material can sometimes be detected even in empty lanes that contain no protein samples. Also, this material was not always found in the final ACAT preparations. We repeated the purification procedure four times, then assessed the purities of the final preparations by SDS-PAGE followed by silver staining. In

**Fig. 2.** Solubilization of HisACAT-1 enzyme by detergents. HisACAT-1 cells (or AC29 cells, as control) were seeded at a density of 2 × 10^6 cells per 25-cm² flask for 48 h in Ham’s F-12 supplemented with 10% fetal bovine serum. Fresh medium was replaced 2 h before harvesting. Cells were subjected to hypotonic shock for 3 min, then thoroughly homogenized in Buffer A at a protein concentration of ~3 mg/ml (see “Experimental Procedures”). Cell extract (15 µl) was solubilized by either deoxycholate (DOC) or CHAPS (with or without 1 M KCl) at the indicated detergent concentrations, with a final volume of 40 µl. Cholesterol/PC vesicles were added to dilute the detergent and reach a final detergent/PC molar ratio of 0.4. ACAT assay was carried out at 37 °C for 5 min.

**Fig. 3.** hACAT-1 monoclonal antibody affinity column chromatography. Solubilized enzyme (6 ml) prepared as described in Fig. 1 (with 2.5% CHAPS and 1 M KCl) was loaded through an hACAT antibody affinity column (5 ml) four times by gravity flow. 60 ml of buffer (see text) was used to wash off unbound proteins. Citrate buffer at pH 3.5 (50 mM) containing 1 M KCl and 0.5% CHAPS was used to elute the bound protein off the column. 1-ml fractions were collected. Panel A shows the ACAT activity measurement by cholesterol/PC vesicle assay using aliquots of 20 µl/fraction. Panel B shows the immunoblot using rabbit anti-ACAT-1 IgGs DM10, with each lane containing 30 µl (except lane 1, which contained 8 µl). The numbers in parentheses shown in Panel A correspond to the fraction numbers in Panels B and C. Panel C shows the silver stain of each of the fractions from the column; aliquots of 120 µl/fraction were loaded onto each lane (except lane 1, which contained 30 µl). The gel was stained using the Silver Stain Plus Kit from Bio-Rad.
each of these four preparations, we found that only a single protein band at 54 kDa was detectable (lanes 10–13 in Fig. 5; designated as HisACAT-1). Based on this criterion, we judge that the ACAT-1 protein after the nickel column chromatography has been purified to essential homogeneity.

It has been difficult to estimate the quantity of HisACAT-1 protein present in the last two steps, mainly due to its low concentration. To semi-quantitate the protein at low levels, we first extracted ACAT-1 samples and various protein standards prepared in parallel with chloroform/methanol (29) (to remove the detergents and salts and to concentrate the protein samples), then used silver staining after SDS-PAGE to compare the intensity of HisACAT-1 protein with intensities of protein standards. A representative result is shown in Fig. 5 (see Fig. 5, legend, that describes the details of this method). Based on this method for quantitating the ACAT-1 protein content, in several experiments, the specific activity of HisACAT-1 in the final preparation ranged from 2,000 to 4,000 pmol/min/mg, representing approximately 7000-fold purification in specific activity from the crude cell extracts. The large variation in specific activity determinations is mainly due to uncertainty in determining the ACAT-1 protein content in the final preparations.

In a representative experiment, using HisACAT-1 cells grown in 30 150-mm tissue culture dishes (providing around 125 mg of total cellular protein) as the starting material, we recovered approximately 1–2 mg of pure HisACAT-1 protein in the final preparation, with 8% recovery in ACAT activity. The ACAT enzyme in the final preparation stored at −20 °C in 50 mM KH2PO4 with 1 M KCl, 0.5% CHAPS, and 250–500 mM imidazole (pH 7.4) in siliconized tubes remained soluble and enzymatically active for at least 1 month.

**Kinetics of Purified HisACAT-1**—In preliminary observations, we found that the hACAT-1 enzyme present in both crude extracts and in purified form was able to utilize cholesterol as its substrate in either unilamellar PC vesicles or bile salt-PC mixed micelles. In fact, the enzyme was much more active (by 2–5-fold) in cholesterol/PC/bile salt mixed micelles.
than in cholesterol/PC vesicles. To further pursue these observations, we used pure HisACAT-1 protein as the enzyme source to investigate the effects on ACAT activity of various bile salts used to form mixed micelles. We maintained the cholesterol/PC concentrations at a fixed ratio (1.86 mM cholesterol, 9.3 mM PC; molar ratio of cholesterol:PC = 0.2) and varied the concentrations of bile salts from 2.4 to 36 mM (0.15–1.5%). We found that taurocholate provided the highest ACAT activity, followed by cholate and deoxycholate (Fig. 6; the symbol \( E \) denotes the ACAT activity in cholesterol/PC vesicles). Similar results were obtained when partially purified HisACAT-1 was used as the enzyme source (results not shown). Additional results in this figure also showed that if the non-ionic detergent Triton X-100 was used to form the mixed micelles, ACAT activity was completely inhibited.

Since the cholesterol content in PC vesicles and in taurocholate/PC mixed micelles could be varied with ease, we used pure HisACAT-1 protein as the enzyme source to determine the cholesterol substrate saturation curves in either taurocholate/cholesterol/PC mixed micelles or cholesterol/PC vesicles. The curves were highly sigmoidal (i.e. cooperative) under both assay conditions. A typical result is shown in Fig. 7. Similar results were obtained when partially purified His-ACAT-1 enzyme was used as the enzyme source (results not shown). For
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Fig. 7. Cholesterol substrate saturation curves of purified HisACAT-1 in either (A) cholesterol/PC vesicles or (B) taurocholate/cholesterol/PC mixed micelles. Cholesterol content present in each assay tube was expressed as molar ratios of cholesterol versus cholesterol + PC. In Panel B, the cholesterol content was also expressed in terms of millimolar. Two separate purified HisACAT-1 protein preparations were used for these experiments. Panel A, for each assay point, 20 μl of pure HisACAT-1 protein was added to 100 μl of PC vesicles containing PC at a final concentration of 13 mM and cholesterol at increasing concentrations. 20 μl of assay mixture containing 10 nmol of oleoyl-coenzyme A pre-mixed with 10 nmol of fatty acid-free bovine serum albumin was then added to initiate the reaction (final concentration of oleoyl-CoA was around 70 μM). Panel B, for each assay point, 20 μl of pure HisACAT-1 protein was added to 100 μl of taurocholate-cholesterol-PC micelles containing taurocholate at 18.6 mM, PC at a concentration of 9.3 mM, and cholesterol at increasing concentrations. 20 μl of assay mixture (as above) was used to initiate the reaction. Assay was carried out at 37 °C for 30 min. Both Panels A and B show results from one of three experiments. For hACAT-1 in cholesterol/PC vesicles, the S₀₉/S₀₄ ratio for cholesterol was found to be 7.4 ± 2.8 (n = 3); in taurocholate/cholesterol/PC micelles, the S₀₉/S₀₄ ratio for cholesterol was found to be 5.8 ± 1.9 (n = 3).

DISCUSSION

As the starting material for the purification work described in this article, we used a transfectant cell line that stably expresses the recombinant hACAT-1 protein. This transfectant cell line was derived from a mutant CHO cell line (AC 29) that does not contain any detectable endogenous ACAT-1 protein (19). By using AC 29 cells we thus avoided potential complications caused by endogenous ACAT present in other mammalian cells. We have developed a scheme to purify the HisACAT-1 protein by approximately 7000-fold from crude cell extracts. The final preparation is essentially a single protein species and is enzymatically active. The key steps for the purification include: 1) solubilization of the microsome using the zwitterionic detergent CHAPS in the presence of 1 M KCl; 2) use of an hACAT-1 monoclonal antibody affinity column; and 3) use of nickel column chromatography. We previously found that the detergent deoxycholate is able to solubilize the ACAT protein with retention of catalytic activity (32, 33). However, it has been difficult to develop effective purification procedures with deoxycholate as detergent, mainly due to its anionic property. In other experiments reported elsewhere, we have found that 2–2.5% CHAPS along with 1 M KCl can effectively solubilize ACAT-1 protein with retention of catalytic activity in homogenates prepared from various native human tissues (22). We included a high salt concentration (1 M KCl) in all the buffers used during purification and during storage. The presence of 1 M KCl in the buffer significantly stabilizes ACAT enzyme activity; why this occurs is unknown at present and is under investigation in this laboratory. For the purification scheme, the overall ACAT activity recovery is approximately 4–8%. The scheme reported here has been found to be reproducible in our laboratory throughout the course of at least 1 year. The greatest single loss in enzyme activity occurs at the hACAT-1 monoclonal antibody affinity column chromatography step; to elute...
the enzyme off the column, a pH of 3.5 has been found to be necessary. In the future, it may be worthwhile to attempt eluting the ACAT protein off the column with specific peptide(s) that can compete with the enzyme for binding to the monoclonal antibody.

In this article, we have demonstrated that pure HisACAT-1 is enzymologically active in bile salt/cholesterol/PC mixed micelles. Among the three different bile salts examined, taurocholate provides the highest ACAT activity at all concentrations tested. Irrespective of what bile salt was used, the result in Fig. 6 shows that the dependence of ACAT activity on bile salt concentration is bell-shaped, with optimal activity occurring when the molar ratio of PC to bile salt is about 1. At this molar ratio, most of the bile salt combines with the PC to form mixed micelles; a certain amount of supersaturated PC also exists in the form of bilayer vesicles. Beyond this molar ratio, the supersaturated PC vesicle disappears and the bile salt begins to form pure micelles without the PC molecules (34, 35). It is possible that the ascending portion of the curve in Fig. 6 is due to an increase in the concentration of the bile salt/cholesterol/PC mixed micelles, while the descending portion of the curve is due to an increase in the concentration of the pure bile salt micelles without cholesterol/PC; i.e. the pure bile salt micelles may inhibit the ACAT activity. Other explanations cannot be ruled out at present. To conduct the ACAT enzyme kinetics in mixed micelles, we chose taurocholate as the detergent. Unlike cholate or deoxycholate, taurocholate at neutral pH values is in a completely ionized form. We used 18 mM sodium taurocholate and 9 mM egg PC to prepare micelles (total lipid concentration, 1.7%). Under these conditions, the bile salt-PC mixed micelles coexist with the pure bile salt micelles; supersaturated PC in bilayer vesicles is not present. (Supersaturated PC begins to form bilayer vesicles only when the PC/bile salt molar ratio exceeds 0.6 (Refs. 34 and 35)). With 18 mM taurocholate and 9 mM egg PC, the maximum micellar solubility of cholesterol is about 15 mol % of PC

We previously developed a rapid procedure for incorporating detergent-solubilized ACAT molecules into unilamellar PC vesicles (32, 33, 36). The sterol content of these vesicles can be controlled during their preparation. Following this procedure, we have used extracts prepared from CHO cells, human fibroblast cells, or Sf9 cells infected with the recombinant hACAT-1 virus, and have consistently shown that the cholesterol substrate saturation curve is highly sigmoidal. Cholesterol causes the largest increase in enzyme activity at approximately 10 mol

![Diagram](http://www.jbc.org/)

**FIG. 8.** Oleoyl-CoA substrate saturation curves of purified HisACAT-1 in either (A) cholesterol/PC vesicles or (B) taurocholate/cholesterol/PC mixed micelles. Each tube contained 20 μl of pure HisACAT-1 protein and 100 μl of vesicles or taurocholate-mixed micelles containing cholesterol (1.9 mM with 3H labeling, at 0.2 × 10^6 dpm/reaction) and PC (9.3 mM). Taurocholate was used at 18.6 mM. The reaction was initiated by adding 20 μl of assay mixture with increasing amounts of oleoyl-coenzyme A pre-mixed with fatty acid-free bovine serum albumin at an equal molar ratio. Assay was carried out at 37 °C for 30 min. Panel A shows results from one of two experiments; Panel B shows results from one of four experiments. For hACAT-1 determined in taurocholate/cholesterol/PC micelles, the S_{0.9}/S_{0.1} ratio for oleoyl-CoA was 84 ± 5 (n = 3).
% and reaches a saturation level at approximately 20 mol % (reviewed in Ref. 1). Based on these and other results, we postulate that ACAT may be an allosteric enzyme regulated by cholesterol. The physiological advantages of ACAT being an allosteric enzyme in extra-hepatic and hepatic cells have been thoroughly discussed (reviewed in Ref. 1). Alternative interpretations of the sigmoidal shape of the ACAT-cholesterol response curve were also possible. For example, it was possible that cholesterol stimulates other protein(s) to act on ACAT in some unknown fashion; or cholesterol might modulate membrane microviscosity, and/or might form microdomain(s) within the vesicles (for an example, see Ref. 37). A critical point was to determine whether the sigmoidal curve is the result of a lipid-specific event, a protein-specific event, or both. As described in this article, we compared the cholesterol substrate saturation curves of the purified enzyme assayed in mixed micelles and in reconstituted vesicles, and found that both are highly sigmoidal. The "ultrasensitivity" value, \( S_{0.9}/S_{0.1} \), can be used to measure the degree of sigmoidicity (31). We found that the \( S_{0.9}/S_{0.1} \) values for HisACAT-1, using cholesterol as the variable substrate in vesicles and in taurocholate/cholesterol/PC mixed micelles were very similar (between 6 and 7). Thus, although other possibilities can not be ruled out, our current results support the hypothesis that the sigmoidal dependence of ACAT activity upon cholesterol concentration (1, 5) is an intrinsic property of the enzyme.

To study the kinetic properties of lipid-dependent enzymes in mixed micelles, the concept of surface dilution kinetics has been formulated (reviewed in Ref. 40). It is assumed that the enzyme solubilized in mixed micelles is available at the micellar surface and interacts with its lipophilic substrate/effectors also present at the surface of the mixed micelles; the concentration of that particular substrate/effectors in mixed micelles is thus expressed in surface concentration terms, such as mole fraction within the mixed micelles, instead of their absolute concentrations in water. Surface dilution kinetics has been applied successfully to more than 10 different lipid-dependent enzymes dispersed in mixed micelles. Under these conditions, essentially all of the enzymes examined thus far have exhibited classical Michaelis-Menten (hyperbolic) kinetics with respect to the surface concentrations of their lipid substrates or effectors (reviewed in Ref. 40). The only enzyme that exhibits sigmoidal kinetics in mixed micelles is protein kinase C, Newton and colleagues (41–43) showed that the activation of protein kinase C by phosphatidylserine sequestered within the binding domain of the enzyme. To the authors’ knowledge, the results described in this article are the first reported results that demonstrate a striking allosteric effect of cholesterol on a purified enzyme molecule.

The molecular mechanism that causes ACAT to be an allosteric enzyme is not known at present. The original theory of allosterism is based on the idea that cooperative binding of substrates may arise in proteins with two or more structures in equilibrium (reviewed in Ref. 44). ACAT in membranes is believed to consist of three or four subunits (45, 46). We do not yet know the number of monomers per pure hACAT-1 enzyme in mixed micelles or vesicles. If pure hACAT-1 enzyme in mixed micelles or vesicles is an oligomeric protein, then it is possible that binding of cholesterol in one ACAT monomer may cause conformational change(s) in its neighboring subunits, enabling them to bind cholesterol with a higher affinity. It is also possible that ACAT may bind more than one cholesterol molecule per monomer; i.e. ACAT may contain a sterol catalytic site as well as a sterol regulatory site that are distinct from one another. The two-site model and the subunit interaction model are not necessarily mutually exclusive. It is also possible that cholesterol at certain concentrations may alter the oligomerization state of the ACAT protein. In addition, ACAT is believed to span the phospholipid bilayer multiple times (13), and its enzyme activity is known to be influenced by phospholipid head groups as well as by the fatty acid composition of the lipid vesicles (47). It is therefore possible that the allosteric interaction between the sterol and ACAT protein may involve phospholipid as a third participant. Future investigations are needed to identify the molecular basis of the cholesterol-dependent ACAT allostery described in this paper.

Acknowledgments—We thank Drs. Gustav Lienhard, William Winkler, Charles Barlowe, and Donald Trumpower for helpful advice throughout the course of this work. We are grateful to Dr. Donald Small of Boston University Medical School for stimulating discussions, help in analyzing the results of Fig. 6, and advice in predicting the solubility behavior of PC and cholesterol under the conditions described in the legends to Figs. 6 and 7. We also thank Drs. Gary Doolittle, Christopher Giza, Chung-Yu Yu, Edmond Leung, Jonathan Jun, Xiaohui Lu, Dong Cheng, Jun Chen, Song Lin, Akira Miyazaki, Onell Lee, and Jay Liu from this lab for invaluable discussions or participation in various stages of this work. We thank Dr. Bob Gross for providing excellent biomedical computing facilities at Dartmouth.

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Highly Cooperative Manner

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J. Biol. Chem. 1998, 273:35132-35141.
doi: 10.1074/jbc.273.52.35132

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Recombinant acyl-CoA:cholesterol acyltransferase-1 (ACAT-1) purified to essential homogeneity utilizes cholesterol in mixed micelles or in vesicles in a highly cooperative manner.

Catherine C. Y. Chang, Chi-Yu Gregory Lee, Ellen T. Chang, Jonathan C. Cruz, Marc C. Levesque, and Tu-Yuan Chang

Page 35136, left-hand column: The last three lines should read: “...the specific activity of HisACAT-1 in the final preparation ranges from 2,000 to 4,000 nmol/min/mg, representing approximately 7,000-fold purification in specific activity from the crude cell extracts.”