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Chemical Modification of Acyl-CoA:Cholesterol *O*-Acyltransferase. 1. Identification of Acyl-CoA:Cholesterol *O*-Acyltransferase Subtypes by Differential Diethyl Pyrocarbonate Sensitivity†

Paula M. Kinnunen, Angela DeMichele, and Louis G. Lange*
Cardiology Division, Jewish Hospital of St. Louis at the Washington University Medical Center, St. Louis, Missouri 63110
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ABSTRACT: Acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26) (ACAT) catalyzes the intracellular synthesis of cholesteryl esters from cholesterol and fatty acyl-CoA at neutral pH. Despite the probable pathophysiologic role of ACAT in vascular cholesteryl ester accumulation during atherogenesis, its mechanism of action and its regulation remain to be elucidated because the enzyme polypeptide has never been identified or purified. Present chemical modification results identify two distinct tissue types of ACAT, based on marked differences in reactivity of an active-site histidine residue toward diethyl pyrocarbonate (DEP) and acetic anhydride. The apparent K_i of the DEP-sensitive ACAT subtype, typified by aortic ACAT, was 40 μM, but the apparent K_i of the DEP-resistant ACAT subtype, typified by liver ACAT, was 1500 μM, indicating a 38-fold difference in sensitivity to DEP. Apparent K_i's of aortic and liver ACAT for inhibition by acetic anhydride were also discordant (less than 500 μM and greater than 5 mM, respectively). On the basis of the reversibility of inhibition by hydroxylamine, a neutral pK_a for maximal modification, and acetic anhydride protection against DEP inactivation, DEP and acetic anhydride appear to modify a common histidine residue. Oleoyl-CoA provided partial protection against inactivation by DEP and acetic anhydride, suggesting that the modified histidine is at or near the active site of ACAT. Systematic investigation of ACAT activity from 14 different organs confirmed the existence of 2 subtypes of ACAT on the basis of their different reactivities toward DEP and acetic anhydride. These studies not only implicate a histidine in the catalytic mechanism of ACAT but also provide the first structural basis for differentiating ACAT subtypes and are potentially useful for identifying the ACAT polypeptide.

Acyl-CoA:cholesterol *O*-acyltransferase (ACAT)[1] (EC 2.3.1.26) catalyzes the synthesis of cholesteryl esters from fatty acyl-CoA and cholesterol at neutral pH and is the major cholesterol esterifying activity in vascular tissue under physiological conditions (Spector et al., 1979). Up to 50-fold increases and decreases in ACAT activity parallel induction and regression, respectively, of experimental atherosclerotic lesions (St. Clair et al., 1970; St. Clair, 1983), indicating that ACAT activity regulation is pathophysiologically related to cholesteryl ester accumulation during atherogenesis. Thus, ACAT activity represents a critical control point for vascular intracellular cholesteryl ester metabolism potentially subject to regulation by atherogenic or protective influences.

In other organ systems, ACAT activity is also important for intracellular cholesterol esterification. For example, intestinal ACAT is postulated to partly mediate cholesterol absorption (Suckling & Stange, 1985; Heider et al., 1983), and hepatic ACAT may modulate the cholesteryl ester composition of VLDL (Drevon et al., 1980). In adrenal gland and ovary, cholesteryl esters formed by ACAT are substrates stored for ultimate use in the synthesis of steroid hormones, and ACAT activity is regulated reciprocally with steroid hormone synthesis (Civen et al., 1984; Tavani et al., 1982). Thus, ACAT activity regulation, known to be important in atherogenesis, may also have pathophysiological relevance in cholesterol metabolism in other organs.

Despite its recognized importance, little knowledge exists about the structure or regulation of ACAT. This deficit primarily reflects the lack of identification of the ACAT polypeptide, an integral and very hydrophobic microsomal membrane protein which has proved extremely difficulty to purify. Major efforts in this area have employed exacting preparative methods (Doolittle & Chang, 1982a) which have failed to yield significantly purified enzyme. Postulated mechanisms for the marked increases and decreases in enzyme activity in vascular tissue and intestine during cholesterol feeding and deprivation include variations in cellular free

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* Address correspondence to this author at Cardiology Research, Yalem 305, Jewish Hospital of St. Louis, 216 S. Kingshighway, St. Louis, MO 63110.

[1] Abbreviations: ACAT, acyl-CoA:Cholesterol *O*-acyltransferase; DEP, diethyl pyrocarbonate; BME, 2-mercaptoethanol; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; BHT, butylated hydroxytoluene.

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cholesterol content and/or availability (Doolittle & Chang, 1982b), increased or decreased quantity of enzyme protein (Hashimoto et al., 1974), enzyme phosphorylation and dephosphorylation (Gavey et al., 1983), and, most recently, the presence or absence of a short-lived peptide inhibitor (Chang et al., 1986). However, the ACAT activity changes produced in vitro by any of these factors are much smaller in magnitude than the in vivo changes produced by cholesterol feeding, and none of these has been demonstrated to enhance ACAT activity in vascular tissue in vivo during atherogenesis.

The catalytic mechanism and structure–function relationships of ACAT also remain unknown. Many in vitro hydrophobic inhibitors of ACAT have been described (Ross et al., 1984; Morin et al., 1974; Heider et al., 1983; Bell, 1981, 1984), but the mechanisms of action of these agents have not been related to the structure of ACAT. No systematic chemical modification study of ACAT has been reported, though such a strategy for covalent protein labeling is ideal for understanding the mechanism of action of such an elusive, membrane-bound enzyme. Therefore, to identify the residue(s) critical for expression of ACAT activity and to provide potential means for identifying the ACAT polypeptide, we have undertaken a detailed, systematic investigation of the effects of chemical modification on ACAT activity. Results of this chemical modification survey differentiate two subtypes of ACAT which exhibit a 38-fold difference in their susceptibilities to inhibition by diethyl pyrocarbonate (DEP) and acetic anhydride and relate not only to the mechanism of action of acyltransferases but also to the design of strategies for identification of this particular transferase.

Materials and Methods

Materials. All reagents used were of the highest commercially available grade.

[1-^{14}C]Oleic acid (50 mCi/mmol) and [^{3}H]oleic acid (2 Ci/mmol) were obtained from Amersham. Aquasol was purchased from New England Nuclear.

The protein modification reagents phenylmethanesulfonyl fluoride, water-soluble carbodiimide, methyl acetimidate, acetic anhydride, phenylglyoxal, diethyl pyrocarbonate, tetranitromethane, and *p*-(hydroxymercuri)benzoate were obtained from Sigma Chemical Co., St. Louis, MO.

Coenzyme A, ethyl oleate, ATP, dithiothreitol, BME, and oxalyl chloride were also obtained from Sigma. Fatty acid poor BSA was purchased from Calbiochem. Methanol, chloroform, punctilious ethanol, tetrahydrofuran, DMSO, and diethyl ether were obtained from Sigma and used without further purification.

Synthesis of Ethyl Oleate. Ethyl [^{3}H]oleate was synthesized by acid-catalyzed esterification of [^{3}H]oleic acid in ethanol (Lange et al., 1981). A total of 5 mCi of [^{3}H]oleic acid was dissolved in 100 mL of ethanol, and the solution was bubbled with HCl gas for 20 min. The ethanol was evaporated under reduced pressure, and residual lipids were dissolved in acetone. Ethyl [^{3}H]oleate was then isolated by preparative thin-layer chromatography on silica gel H plates developed in petroleum ether/diethyl ether/acetic acid (75:5:1) (Lange et al., 1981). Prior to its use as an internal standard, the [^{3}H]ethyl oleate was diluted with ethyl oleate to a final specific radioactivity of 40 dpm/nmol.

Synthesis of Oleoyl-CoA and [^{14}C]Oleoyl-CoA. Oleoyl-CoA and [^{14}C]oleoyl-CoA were synthesized from oleic acid and [^{14}C]oleic acid via their acid chlorides (Bishop & Hajra, 1980). For example, 2.5 μmol of [^{14}C]oleic acid and 2.5 μmol of BHT in 0.5 mL of dry benzene were incubated with 2 mL of oxalyl chloride at 22 °C for 2 h under nitrogen. After evaporation of excess oxalyl chloride, a 5-fold molar excess of coenzyme A in 3 mL of 2.2:1 tetrahydrofuran/150 mM sodium bicarbonate, pH 8.8, was immediately added, and the reaction mixture was incubated at room temperature for 90 min. After evaporation of THF with nitrogen, cold perchloric acid was added to a final concentration of 1%, and unreacted oleic acid was removed by extraction with diethyl ether. The precipitated [^{14}C]oleoyl-CoA was pelleted by centrifugation at 40000*g* for 20 min and dissolved in 50 mM potassium phosphate, pH 6.0, after being washed with cold 10% perchloric acid and dry acetone. The concentration of synthesized oleoyl-CoA was determined after exhaustive base hydrolysis (Ellman, 1959).

Purification of Oleoyl-CoA by DEAE Chromatography. Because of contamination of commercially available fatty acyl-CoA by free CoA and our demonstration of a thiol-containing CoA inhibitory site on ACAT (Kinnunen et al., 1988), synthesized oleoyl-CoA was purified by chromatography over DEAE before use (Steinman & Hill, 1973). For example, 25 μmol of oleoyl-CoA was applied to DEAE-cellulose (2.5 × 10 cm) equilibrated with 5 mM lithium acetate, pH 5.3. The resin was washed with 2 volumes of lithium acetate buffer and then developed with a 0.1–1 M lithium chloride gradient. The A_{232} and A_{260} of collected fractions were determined, and fractions containing oleoyl-CoA (identified as those with the highest A_{232}:A_{260} ratio) were pooled. Purified oleoyl-CoA was precipitated by adding perchloric acid to a final concentration of 1%, pelleted by centrifugation, and redissolved in 50 mM potassium phosphate, pH 6.0. The concentration of purified oleoyl-CoA was determined by Ellman's assay after exhaustive base hydrolysis. Commercially obtained oleoyl-CoA subject to such purification was shown to be up to 50% contaminated by compounds absorbing at 260 nm, presumably CoA or its derivatives.

Microsome Preparations. Microsomes were prepared from the organs of normal and cholesterol-fed white New Zealand male rabbits (fed 2% cholesterol chow for 2 months). After sacrifice by cervical dislocation, tissues were excised rapidly into ice-cold 0.1 M potassium phosphate buffer at pH 7.4, then cleaned, weighed, minced, and homogenized in 0.1 M potassium phosphate (10%, w/v) using a Polytron (Brinkmann) at half-maximal power for 60 s. The tissue homogenates were then centrifuged sequentially at 800*g* for 10 min, 13300*g* for 10 min, and 100000*g* for 60 min to isolate microsomal pellets, which were reconstituted in 0.1 M potassium phosphate, pH 7.4, using a Duall tissue homogenizer to final protein concentrations of 2–30 mg/mL. Microsomal protein was determined by the method of Lowry et al. (1951), using BSA as a standard.

ACAT Assay. Microsomal ACAT activity was measured according to a modification of the method of Brown et al. (1975). Standard ACAT assays included 0.8 mg/mL BSA, 5 mM ATP, 5 mM magnesium chloride, 10 μM [^{14}C]oleoyl-CoA with a specific radioactivity of 107 dpm/pmol, and 30–400 μg of microsomal protein in a total volume of 0.5 mL of 0.1 M potassium phosphate, pH 7.4, unless otherwise stated. Product formation was linear with respect to time for 30 min; reactions were then quenched with a lipid extraction medium (Bligh & Dyer, 1959) containing 1 μmol of cholesteryl oleate and 1 μmol of [^{3}H]ethyl oleate (40 dpm/nmol) added as cold carrier and internal standard, respectively. The extracted lipids were separated by thin-layer chromatography on silica gel in an apolar solvent system (petroleum ether/diethyl ether/acetic acid, 75:5:1), and the cholesteryl esters and fatty acid ethyl esters were localized by the exposure of the plates to iodine

vapor. After removal of iodine by gentle heating, the silica gel containing cholesteryl esters and fatty acid ethyl esters were scraped separately into scintillation vials, 10 mL of Aquasol was added, and incorporated radioactivity was quantitated by liquid scintillation counting. Quenching was monitored by using carbon-14 as reference. ACAT activity was measured as the ^{14}C radioactivity incorporated into cholesteryl ester and was expressed as picomoles of cholesteryl [^{14}C]oleate formed per milligram of microsomal protein per minute. Yield was calculated by measuring the recovery of [^{3}H]ethyl oleate, and blanks were calculated from parallel assays containing no microsomes.

Protein Modification Experiments. *p*-Mercuribenzoate was dissolved in 2 mM NaOH, and phenylmethanesulfonyl fluoride was dissolved in DMSO. Water-soluble carbodiimide, methyl acetimidate, and phenylglyoxal were dissolved in 0.1 M potassium phosphate, pH 7.4. Acetic anhydride was dissolved in diethyl ether, and diethyl pyrocarbonate and tetranitromethane were dissolved in ethanol. Five microliters of the specified reagent was mixed with 95 μL of microsomes and incubated at room temperature for the stated time interval. Twenty microliters of modified microsomes was then added to ACAT assay mixtures (total volume 0.5 mL) containing BSA, ATP, $MgCl_2$, and [^{14}C]oleoyl-CoA as detailed above, and ACAT activity was determined after incubation at 37 °C for 30 min. Controls for each reagent were done by incubating microsomes with the appropriate solvent, and blanks were determined by using incubations containing no microsomes.

Experiments assessing protection by oleoyl-CoA against inhibition by protein modification reagents were performed by incubating aortic or liver microsomes (5–7 mg of protein/mL) with 0 or 200 μM oleoyl-CoA (purified by DEAE chromatography before use) for 1 min at 4 °C followed by addition of the indicated concentration of DEP or acetic anhydride. After further incubation for 1 min at 4 °C 20-μL aliquots were diluted into standard ACAT assay mixtures which were then incubated at 37 °C for 30 min. ACAT activity was measured as picomoles of cholesteryl [^{14}C]oleate formed per minute per milligram of microsomal protein after lipid extraction and thin-layer chromatography. Control ACAT activity was measured in microsomes incubated with oleoyl-CoA to which aliquots of ethanol or ether only were added. Volumes of ethanol or ether added did not exceed 5% of total volume and did not significantly inhibit ACAT activity.

In experiments using hydroxylamine to assess the reversibility of ACAT modification by DEP or acetic anhydride, aortic or liver microsomes (5 mg/mL) were incubated for 1 min at 22 °C with the indicated concentrations of DEP or acetic anhydride and then diluted 1:1 with 1 M hydroxylamine, pH 7, or 0.1 M potassium phosphate, pH 7.4. After incubation at 22 °C for 30 min, activity was assayed by adding 40-μL aliquots to standard ACAT assay mixtures incubated at 37 °C for 30 min. Control ACAT activity was measured in parallel incubations without acetic anhydride or DEP, but including the appropriate volume of solvent and hydroxylamine or phosphate buffer. Hydroxylamine treatment alone did not change ACAT activity.

RESULTS

Chemical Modification Survey. Because no survey of the efefcts of chemical modification reagents on ACAT has been reported, numerous reagents were screened for their ability to inhibit ACAT activity in microsomes from normal rabbit liver. Incubation of liver microsomes (2.4 mg of protein/mL) in 0.1 M potassium phosphate, pH 7.4, with different protein modification reagents at concentrations up to 5 mM inhibited

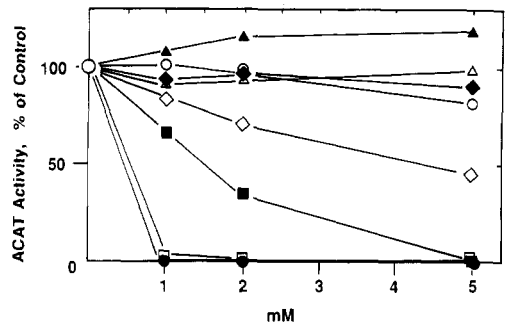

FIGURE 1: Liver microsomal ACAT activity: inhibition by protein modification reagents. Liver microsomes (2.4 mg of protein/mL) were incubated with phenylmethanesulfonyl fluoride (▲), water-soluble carbodiimide (△), methyl acetimidates (♦), acetic anhydride (○), phenylglyoxal (◇), diethyl pyrocarbonate (■), tetranitromethane (□), or *p*-mercuribenzoate (●) at 22 °C for 30 min; then 20-μL aliquots were added to standard ACAT assay mixtures (total volume 0.5 mL). After incubation at 37 °C for 30 min, assays were quenched, and the cholesteryl [^{14}C]oleate synthesized was quantitated after lipid extraction and TLC as described under Materials and Methods. Results are expressed as percentage of control ACAT activity, determined in microsomes incubated with the same volume of the appropriate solvent or buffer. Blanks were determined as incorporated radioactivity in parallel incubations in the absence of added microsomes. Each point represents the average of three assays, which varied less than 10%.

liver ACAT to varying degrees of (Figure 1). Liver ACAT activity was not significantly inhibited by phenylmethanesulfonyl fluoride, water-soluble carbodiimide, or methyl acetimidate, agents typically expected to modify serine, aspartic and glutamic acid, and lysine, respectively. Acetic anhydride, phenylglyoxal, and diethyl pyrocarbonate resulted in concentration-dependent ACAT inhibition with 20, 65, and 100% inhibition produced by 5 mM reagent, respectively. Tetranitromethane and *p*-mercuribenzoate (1 mM) produced 100% inhibition of liver ACAT activity. These pilot results implicated sulfhydryl, histidyl, arginyl, and possibly tyrosyl residues as important for liver ACAT activity and suggested the lack of essential modifiable serine, lysine, and carboxyl groups.

Susceptibility of aortic ACAT activity to inactivation by chemical modification was next determined and compared to that of liver ACAT, using a 1 mM concentration of each reagent to screen for aortic ACAT inhibition. Aortic and liver microsomes (2 mg of protein/mL) were incubated with a 1 mM concentration of each reagent at 22 °C for 30 min, followed by addition of 20-μL aliquots to standard ACAT assays. As found for liver ACAT, the activity of aortic ACAT was 100% inhibited by 1 mM *p*-mercuribenzoate and tetranitromethane and not significantly inhibited by 1 mM methyl acetimidate, water-soluble carbodiimide, phenylmethanesulfonyl fluoride, or phenylgyloxal (1%, 0%, 21%, and 20%, respectively, for aortic ACAT and 4%, 0%, 22%, and 10%, respectively, for liver ACAT). However, incubation of microsomes with 1 mM acetic anhydride resulted in significantly more inhibition of aortic ACAT than of liver ACAT (70% vs 19% inhibition, respectively). Diethyl pyrocarbonate (1 mM) also inhibited aortic ACAT more than liver ACAT (100 vs 73% inhibition, respectively).

ACAT Subtypes Differentiated by DEP and Acetic Anhydride Reactivity. To further explore these potential differences between liver and aortic ACAT with respect to DEP and acetic anhydride inhibition, liver and aortic microsomes were incubated with varying concentrations of these two reagents, followed by measurement of ACAT activity. Indeed, aortic ACAT was markedly more sensitive than liver ACAT to both DEP and acetic anhydride (Figure 2). For example, 500 μM

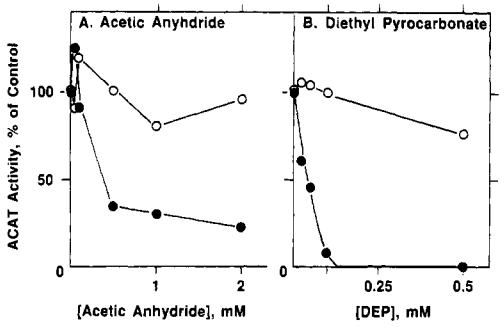

FIGURE 2: Liver versus aortic ACAT: inhibition by acetic anhydride and DEP. Liver (○) and aortic (●) microsomes (2.2 mg of protein/mL) were incubated with the indicated concentrations of DEP or acetic anhydride for 30 min at 22 °C, and then 20-μL aliquots were added to standard ACAT assay mixtures (total volume 0.5 mL). After incubation at 37 °C for 30 min, the assays were quenched, and cholesteryl [^{14}C]oleate was extracted and quantitated as described under Materials and Methods. Acetic anhydride was dissolved in diethyl ether and DEP in ethanol. Results are expressed as percent of control ACAT activity, which was determined as ACAT activity in microsomes incubated with ethanol or ether alone for 30 min.

acetic anhydride inhibited aortic ACAT to 35% of activity in control (unmodified) microsomes but did not measurably inhibit liver ACAT. Measurable inhibition of liver ACAT required acetic anhydride concentrations above 2 mM (Figure 1). A pronounced difference between liver and aortic ACAT was also noted with respect to diethyl pyrocarbonate inhibition: aortic ACAT was fully inhibited by 100 μM DEP, but liver ACAT was unaffected by up to 500 μM DEP. Full inhibition of liver ACAT required 5 mM DEP (see also Figure 1). Thus, aortic and liver ACAT activities exhibit a 38-fold difference in the apparent K_i for DEP (40 μM for aorta vs 1500 μM for liver) and a greater than 50-fold difference in the apparent K_i for acetic anhydride (250 μM for aorta vs greater than 5 mM for liver). These data suggested an essential structural difference between aortic and liver ACATs, identifying the existence of two subtypes of ACAT activity.

Structural Basis for ACAT Subtype Differentiation. Inhibition of aortic and liver ACAT by these reagents was further characterized. First, the time course of the DEP-induced inactivation was studied. ACAT inhibition by DEP was extremely rapid since both aortic liver ACAT modification by 10–50 μM DEP at 37, 25, 4, or −20 °C (in 50% glycerol) was instantaneous, i.e., less than mixing time (data not shown). Because of the unusually low K_i and rapidity of DEP inhibition of aortic ACAT, experiments were performed to determine whether inhibition of aortic ACAT by DEP was covalent or noncovalent as assessed by its reversibility after separation by centrifugation of unbound DEP from microsomes. Aortic microsomes (2 mg of protein/mL) were incubated with 100 μM DEP at pH 6.7 in 0.1 M potassium phosphate buffer for 2 min and then centrifuged at 100000*g* for 1 h to separate microsomes from unreacted DEP. After removal of the 100000*g* supernatant and resuspension of the microsomal pellet in 0.1 M phosphate at pH 7.4, ACAT activity was measured. Prior to centrifugation, 100 μM DEP inhibited ACAT activity 90% compared to microsomes incubated without DEP. After centrifugation, ACAT activity in the resuspended pellet was 99% inhibited compared to ACAT activity in untreated microsomes centrifuged and resuspended under identical conditions. These results indicate that ACAT inhibition by DEP is not reversible by removal of unreacted reagent after modification and thus is likely covalent.

Localization of the amino acid(s) modified by DEP or acetic anhydride to the active site of ACAT was not strictly possible through substrate protection experiments because exogenous cholesterol is poorly utilized and high concentrations of oleoyl-CoA, a potent detergent, irreverisibly denature ACAT. Nevertheless, studies employing aortic microsomes treated with DEP demonstrate that partial protection against ACAT inhibition was afforded by the presence of excess oleoyl-CoA during DEP modification. In the absence of oleoyl-CoA, modification of aortic microsomes by 10 μM DEP resulted in 36% inhibition of ACAT activity compared to control (untreated) microsomes. Inclusion of 200 μM oleoyl-CoA (a concentration equal to 20 times the K_m under our assay conditions) during DEP modification reduced ACAT inhibition to 7% compared to activity in control microsomes incubated with 200 μM oleoyl-CoA in the absence of DEP. Liver ACAT was similarly protected against DEP inhibition by substrate; that is, 1 mM DEP resulted in 35% inhibition of ACAT activity, whereas the presence of 200 μM oleoyl-CoA reduced inhibition to 8%. The presence of oleoyl-CoA during modification also partially protected against ACAT inhibition by acetic anhydride. Thus, in the presence of 200 μM oleoyl-CoA, aortic ACAT inhibition by 1 mM acetic anhydride was reduced from 69% to 45% compared to control microsomes, a small but measurable change. The results suggest that the DEP-modified residue either is in or near the oleoyl-CoA binding site or is in a site influenced by oleoyl-CoA-induced conformational changes.

Identification of the DEP-sensitive and acetic anhydride sensitive amino acid residue was approached by assessing the reversibility of inhibition by hydroxylamine. Aortic ACAT inhibition by acetic anhydride was rapid, being complete after 2 min. It was also *fully* reversible by hydroxylamine. Thus, addition of 0.5 M hydroxylamine at pH 7 to aortic ACAT inactivated to 12% of control activity by 1 mM acetic anhydride resulted in return of 100% native activity in 30 min. These results indicated that the residue modified by acetic anhydride was likely a histidine, tyrosine, or cysteine, rather than a lysine. ACAT inhibition by DEP was only slightly reversible by hydroxylamine. Exposure of aortic microsomes to 100 μM DEP at pH 7.4 resulted in 88% inhibition of ACAT activity compared to control microsomes not treated with DEP. After incubation of DEP-treated microsomes with 0.5 M hydroxylamine at pH 7 for 30 min, ACAT inhibition was reduced to 73% compared to microsomes treated with hydroxylamine only. Liver ACAT behaved similarly: after exposure to 2 mM DEP, ACAT activity was 90% inhibited, which was reduced to 77% inhibition after incubation with hydroxylamine. This slight but measurable reversibility of DEP inhibition by hydroxylamine at neutral pH is also most consistent with modification of tyrosine or histidine residues rather than lysine in aortic and liver ACAT by DEP.

Modification of the Same Essential Histidine by DEP and Acetic Anhydride. Although acetic anhydride inhibition was fully reversible by hydroxylamine whereas DEP inhibition was only slightly reversible, the different sensitivities of aortic and liver ACAT to *both* reagents, and the reactivity of both reagents toward tyrosine, histidine, or cysteine (Means & Feeney, 1971), suggested that ACAT inhibition by these two reagents might be occurring through modification of a common amino acid residue. Thus, sequential modification of aortic microsomes with acetic anhydride and then DEP was performed to assess whether prior acetic anhydride treatment would protect aortic ACAT from (largely irreversible) DEP inhibition. First, aortic microsomes were incubated with 1 mM acetic anhydride, resulting in inhibition of ACAT activity to 12% of control ACAT activity (Figure 3). Subsequent addition of 10 μM DEP did not further inhibit ACAT activity

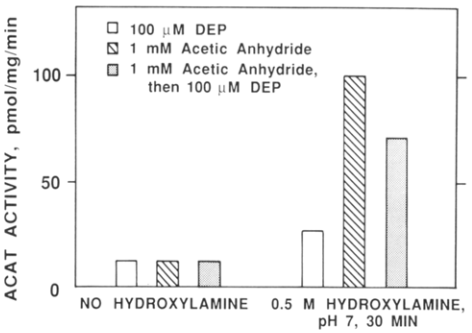

FIGURE 3: Reversibility of aortic ACAT inhibition by DEP and acetic anhydride: effect of sequential modification. Aortic microsomes (6 mg/mL) in 0.1 M potassium phosphate, pH 7.4, were incubated with 1 mM acetic anhydride, or 100 μM DEP at 22 °C for 2 min, and then diluted 1:1 with 0.1 M potassium phosphate, pH 7.4, or 1 M hydroxylamine, pH 7. After 30 min at 22 °C, 20-μL aliquots were added to standard ACAT assay mixtures (total volume 0.5 mL), which were incubated at 37 °C for 30 min. After lipid extraction and TLC, ACAT activity was calculated as described under Materials and Methods. Parallel samples were treated first with 1 mM acetic anhydride for 1 min; then 100 μM DEP was added for 1 min more at 22 °C before dilution with buffer or hydroxylamine.

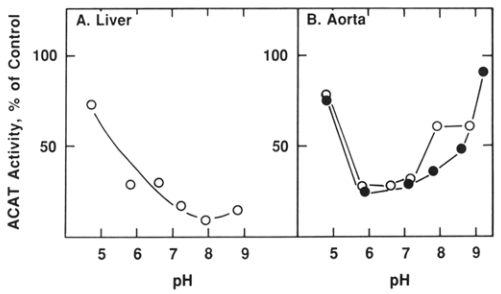

FIGURE 4: Aortic ACAT inhibition by DEP and acetic anhydride: pH dependence. Aortic (4 mg/mL) and liver (4 mg/mL) microsomes in 0.1 M potassium phosphate, pH 7.4, were diluted 1:1 with 0.1 M sodium acetate (pHs 4 and 5), 0.1 M potassium phosphate (pHs 6 and 7), 0.1 M Tris (pHs 8 and 9), and 0.1 M glycine (pH 10) to give final measured pH values of 4.6–9.4. After 1 min at 22 °C, the indicated concentrations of DEP (○) or acetic anhydride (●) were added, and the mixtures were incubated further at 22 °C for 5 min. ACAT activity was assayed by adding 20 μL of modified microsomes to standard ACAT assay mixtures (0.5 mL total volume), in 0.1 M potassium phosphate at pH 7.4, and incubated for 30 min at 37 °C. ACAT activity is expressed as percent of control activity, which was determined in parallel incubations at each pH value in the absence of DEP or acetic anhydride, but including the same volume of ethanol or ether, as appropriate.

in the sequentially modified microsomes. However, hydroxylamine treatment reactivated ACAT activity in these microsomes to 71% of control. In contrast, ACAT activity was regenerated to only 27% of control activity by hydroxylamine in a parallel sample treated with DEP without prior acetic anhydride treatment. Thus, acetic anhydride prevented the largely irreversible ACAT inactivation by DEP, indicating that acetic anhydride and DEP most likely inhibit ACAT through modification of the same or a closely proximate active-site residue. Prior modification of microsomes with the sulfhydryl reagent PMB in a similar experiment did not protect against irreversible inactivation by DEP, and DEP inhibition was not reversed by 2-mercaptoethanol, data indicating that the group modified in common by acetic anhydride and DEP is not a cysteine.

pH Effects. To distinguish between tyrosine and histidine as the active-site residue modified by DEP and acetic anhydride, investigation of the pH dependence of DEP modification was conducted for liver and aortic ACAT from pH 4.7 to pH 8.8. Modification of aortic ACAT by DEP and acetic anhydride and modification of liver ACAT by DEP (Figure 4) at different pH values with subsequent ACAT assays performed at pH 7.4 resulted in maximal ACAT inhibition at neutral pH, results most consistent with the presence of an essential histidine.

Because of the rapidity of ACAT inhibition by DEP which precluded a more detailed analysis of the pH dependence of inhibition (Cousineau & Meighen, 1976), we additionally measured the pH dependency of the rate of ACAT-catalyzed esterification of cholesterol for both liver and aorta microsomes (Figure 5). Both liver and aortic ACAT activities were undetectable below pH 6 but increased sharply between pH 6.5 and 7.5, with maximal activity seen at pH 7–8. Both liver and aortic ACAT activities decreased significantly above pH 8.5. Thus, the overall pH vs activity profiles of liver and aortic ACAT were most consistent with the presence of a histidyl residue rather than a tyrosyl residue at the active site of both enzymes.

Classification of ACAT Subtypes in Multiple Organs. The markedly different sensitivities of liver and aortic ACAT to histidine modification by DEP and acetic anhydride indicated the existence of at least two subtypes of ACAT, based on the reactivity of an active-site histidine residue. To identify po-

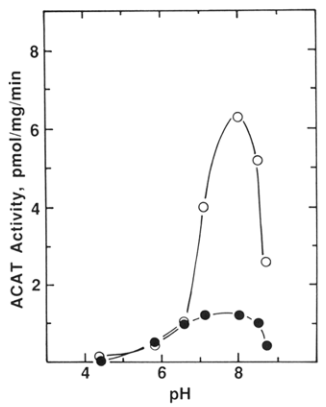

FIGURE 5: pH dependence of aortic and liver ACAT activities. Thirty microliters of liver or aortic microsomes (both 5.9 mg/mL) was added to ACAT assay mixtures (total volume 0.5 mL) containing 0.04 M potassium phosphate plus 0.06 M sodium acetate, potassium phosphate, Tris, or glycine buffers so that the final pHs of the assay mixtures were 4.6, 5.6 (acetate), 6.7, 7.2 (phosphate), 8, 8.5 (Tris), and 8.7 (glycine); buffer ion effects were not present. The assays also contained 5 mM ATP, 5 mM $MgCl_2$, 0.8 mg/mL BSA, and 10 μM [^{14}C]-oleoyl-CoA (107 dpm/pmol) and were incubated at 37 °C for 30 min followed by lipid extraction and TLC as described under Materials and Methods. ACAT activity in liver (○) and aortic (●) microsomes is expressed as picomoles of cholesteryl [^{14}C]oleate formed per minute per milligram of protein.

tential similarities or differences in cholesterol metabolism in different organs, microsomal ACAT activity from 14 normal rabbit organs was screened for DEP sensitivity. Microsomes were incubated with 100 μM DEP at pH 7.4, conditions chosen because 100 μM DEP resulted in complete inactivation of aortic ACAT but no loss of liver ACAT activity. In fact, two types of response were again found (Table I). Intestine, pancreas, and liver ACATs were uninhibited by 100 μM DEP, whereas all other organs tested were 60–100% inhibited by 100 μM DEP. Further analysis (not shown) of the concentration dependence of inhibition by DEP in some of these organs, e.g., normal kidney and adrenal, confirmed that both were DEP sensitive, with apparent K_i's of 100 and 250 μM, respectively. Thus, at least two subtypes of ACAT based on DEP sensitivity were identified. The gastrointestinal organs of liver, pancreas, and intestine contained a resistant subtype, and other organs including aorta, adrenal, spleen, testis, and

Table I: DEP Subtypes of Microsomal ACAT Activity[a]

	ACAT act. (pmol mg^{-1} min^{-1})	induction (x-fold)	inhibn by 100 μM DEP (%)	K_i for DEP (μM)
	(A) Normal Organs			
intestine	2		0	1000
pancreas	2		0	1000
liver	10		9	1500
adrenal	932		60	250
kidney	30		62	100
lung	15		63	–[b]
testis	47		68	500
fat	15		69	–
heart	1		83	–
spleen	41		89	–
brain	3		93	–
aorta	3		98	40
stomach	35		98	–
	(B) Cholesterol-Fed Organs			
intestine	46	23	68	200
pancreas	49	25	94	50
liver	273	27	6	1000
adrenal	1576	1.7	41	800
kidney	37	1.0	35	–
lung	42	3.0	40	–
testis	84	1.8	0	1000
fat	20	1.3	45	–
heart	6	6	86	–
spleen	269	7	72	–
brain	5	1.6	91	–
aorta	124	41	98	40
stomach	32	1	99	–

[a] Microsomes were prepared from organs of a normal rabbit and a rabbit fed 2% cholesterol chow for 2 months, resuspended in 0.1 M potassium phosphate, pH 7.4, and diluted to final protein concentrations of 2 mg/mL. ACAT activity was determined by addition of 20 μL of microsomes to standard ACAT assay mixtures (total volume 0.5 mL) which were then incubated at 37 °C for 30 min. ACAT activity was determined in parallel samples treated with 100 μM DEP at 22 °C for 30 min prior to ACAT assay. ACAT activity in untreated microsomes is expressed as picomoles of cholesteryl [^{14}C]oleate formed per milligram of microsomal protein per minute, and percent inhibition by DEP was calculated by comparing ACAT activity in treated and untreated microsomes from the same tissue source. [b] Not determined.

kidney contained a sensitive subtype.

To ascertain whether the DEP subtype of ACAT present in a given organ correlated with ACAT induction during experimental cholesterol feeding, we also assayed microsomal ACAT activity and DEP sensitivity in rabbit organs after 60 days of cholesterol feeding (Table IB). Varying degrees of ACAT induction with cholesterol feeding were noted in the different organs assayed. In skeletal muscle, kidney, stomach, fat, brain adrenal, or testis, ACAT specific activity did not change significantly with cholesterol feeding (less than 2-fold induction). There were relatively small increases (3–7-fold) in ACAT specific activity in microsomes from lung, heart, and spleen. Marked increases in ACAT specific activity with cholesterol feeding occurred in intestine, pancreas, liver, and aorta (23-, 25-, 27-, and 41-fold increases, respectively). Thus, cholesterol feeding resulted in selective inductions of ACAT activity, with large increases occurring in aorta and in the gastrointestinal organs pancreas, intestine, and liver.

Determination of DEP sensitivity after cholesterol feeding indicated that either the sensitive or the resistant ACAT subtype could be induced in a given organ during cholesterol feeding and that the induced subtype did not necessarily correspond to the ACAT subtype normally present. For example, liver, pancreas, and intestine, all DEP resistant in the normal state (0–9% inhibited by 100 μM DEP), had marked inductions of ACAT activity with cholesterol feeding, but the type of ACAT induced was DEP sensitive in pancreas and intestine (94 and 68% inhibited by 100 μM DEP, respectively, after cholesterol feeding) and DEP resistant in liver (6% inhibited by 100 μM DEP after cholesterol feeding). Other organs with smaller degrees of ACAT induction showed little change in ACAT sensitivity to inhibition by DEP, with the exception of testicular ACAT (1.8-fold induced), which was 68% inhibited by 100 μM DEP in the normal state but virtually uninhibited by 100 μM DEP after cholesterol feeding. Thus, both resistant and sensitive ACATs were induced by cholesterol feeding, and the ACAT subtype normally present did not necessarily correspond to the subtype induced with cholesterol feeding, indicating that the capability to manifest either type of ACAT activity exists in many organs.

Discussion

A survey of the effect of protein modification reagents on ACAT activity in liver and aorta revealed susceptibility of both enzymes to inhibition by sulfhydryl- and histidine-modifying reagents and resistance to inhibition by reagents expected to modify serine, lysine, and carboxyl groups. Further analysis indicated that liver and aortic ACATs were strikingly different with respect to their sensitivities to modification by DEP and acetic anhydride. This was manifested by a 38-fold difference in sensitivity to DEP and clearly differentiated two types of ACAT: one sensitive with a K_i of 40 μM (aortic type) and one relatively resistant with a K_i of 1500 μM (liver type).

The identity of the DEP-modified and acetic anhydride modified amino acid was assigned as histidine, on the basis of the pH dependence of inhibition by DEP, the activity versus pH profile of ACAT activity, and the reversibility of inhibition by hydroxylamine. However, acetic anhydride and DEP can reversibly modify cysteine and tyrosine as well as histidine (Means & Feeny, 1971; Miles, 1977), and the presence of either of these residues with an abnormally low pK_a has not been entirely excluded. Lack of full reactivation of the DEP-treated enzyme by hydroxylamine is consistent with disubstitution of the histidine ring by this reagent, with subsequent ring cleavage during reversal with hydroxylamine (Miles, 1977). Our sequential inactivation studies confirmed that acetic anhydride protects against irreversible inactivation by DEP; one mechanism of such protection could be through acylation of one nitrogen of the histidine ring, which would alter the nucleophilicity of the second nitrogen and thus prevent disubstitution on subsequent exposure to DEP.

Other enzymes known to contain active-site histidine residues usually require millimolar concentrations of DEP for modification (Miles, 1977), and, in this regard, ACAT from aortic microsomes is atypical: half-maximal inactivation by DEP occurred at 40 μM DEP. ACAT inactivation by DEP was also extremely rapid compared to inactivation of other enzymes (Cousineau & Meighen, 1976; Topham & Dalziel, 1986), occurring within seconds. This marked reactivity of the aortic enzyme is likely due to specific physicochemical environmental factors around the reactive histidine, which may be important in the transferase mechanisms of action. Protective effects of oleoyl-CoA against inhibition by DEP suggest that the modified histidine is located at or near the active site of ACAT and may be important in its catalytic mechanism. We have also found that ACAT activity in aortic microsomes solubilized with 2% deoxycholate (Cadigan, 1988) is 96% inhibited by 200 μM DEP and 94% inhibited by 1 mM PMB, indicating that ACAT inhibition by these reagents likely occurs by specific modification of histidine and sulfhydryl residues on ACAT or an essential cofactor, rather than by nonspecific modifications of other membrane components. However,

definitive identification and characterization of these critical residues will have to await purification and characterization of the ACAT polypeptide.

Inhibition of aortic ACAT by histidine- and sulfhydryl-specific reagents suggests potential similarities to another cholesterol-metabolizing enzyme, namely, lecithin–cholesterol acyltransferase (LCAT), which catalyzes the synthesis of cholesteryl esters in plasma from lecithin and HDL-derived cholesterol. LCAT has been shown to contain essential cysteine, serine, and histidine residues (Jauhiainen & Dolphin, 1986). Serine is essential for the phospholipase activity of LCAT, but not for cholesterol esterification. Thus, LCAT modified with PMSF, though 30% inhibited compared to unmodified LCAT, is capable of synthesizing cholesteryl esters utilizing fatty acyl-CoA rather than lecithin as a substrate. We have identified essential histidine and sulfhydryl groups on ACAT, indicating that these two enzymes may partially share a common mechanism of action with respect to cholesterol esterification. This mechanism might involve an acyl-enzyme intermediate which is a thioester and histidine as a base catalyst as postulated for the sulfhydryl proteases (Storer & Carey, 1985). Our data indicate, however, that ACAT is not inhibited by serine modification, and these results differentiate it from LCAT.

The different DEP sensitivities of liver and aortic ACAT may reflect different active-site configurations of the enzyme itself, or differences in extrinsic factors governing enzyme activity. Regardless of their structural basis, however, different DEP sensitivities can be used to classify ACAT from different organs as DEP sensitive or DEP resistant. DEP-resistant ACAT was found in the normal state only in organs intimately involved in the processing of dietary cholesterol, implying that these enzymes may have common features possibly relating to ACAT activity regulation. However, the heterogeneity of the responses to cholesterol feeding in different organs with respect to ACAT activity and DEP subtype indicates that ACAT regulation in response to cholesterol feeding is a complex event(s). Other factors involved in ACAT regulation and changes in DEP sensitivity in response to cholesterol feeding potentially include altered tissue morphology and composition, resulting in membrane fluidity changes which may affect the ACAT polypeptide itself or its sensitivity to DEP. Thus, once the ACAT polypeptide is identified and characterized, the basis for these heterogeneous responses, whether intrinsic or extrinsic to the ACAT polypeptide, can be further elucidated.

In summary, present data represent the first identification of ACAT subtypes, possibly reflecting important structural variations at the active site of this enzyme. While the mechanisms of ACAT activity induction during cholesterol feeding has not been elucidated nor the ACAT polypeptide identified, our findings are relevant to both issues. A strategy of probing the mechanism of action of ACAT by using highly reactive, potentially radioactive reagents to selectively modify and/or label the enzyme has not been previously applied to ACAT, a membrane-bound enzyme which to date has eluded primary structural investigations. Our results indicate that differential labeling experiments using protein modification reagents may allow identification of the ACAT polypeptide. Additionally, further assessment of the effects of these reagents on ACAT activity may provide clues to mechanisms of enzyme activation and/or inhibition under physiological conditions.

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